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Antioxidant Capacity of Novel Pigments from an Antarctic Bacterium[§]

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In Antarctica microorganisms are exposed to several conditions that trigger the generation of reactive oxygen species, such as high UV radiation. Under these conditions they must have an important antioxidant defense system in order to prevent oxidative damage. One of these defenses are pigments which are part of the non-enzymatic antioxidant mechanisms. In this work we focused on the antioxidant capacity of pigments from an Antarctic microorganism belonging to *Pedobacter* genus. This microorganism produces different types of pigments which belong to the carotenoids group. The antioxidant capacity of a mix of pigments was analyzed by three different methods: 1,1-diphenyl-2-picrylhydrazyl, ROS detection and oxygen electrode. The results obtained from these approaches indicate that the mix of pigments has a strong antioxidant capacity. The oxidative damage induced by UVB exposure to liposomes was also analyzed. Intercalated pigments within the liposomes improved its resistance to lipid peroxidation. Based on the analysis carried out along this research we conclude that the antioxidant properties of the mix of pigments protect this bacterium against oxidative damage. These properties make this mix of pigments a powerful antioxidant mixture with potential biotechnological applications.

Keywords: ROS, antioxidant capacity assays, liposomes, Antarctica, pigments

Introduction

Psychrophilic microorganisms have successfully colonized cold environments from deep sea to mountain and polar regions (Suresh *et al.*, 2011). Some of these organisms, depending on their optimal growth temperature, are also known as psychrotolerant (Morita, 1975).

Antarctic psychrophiles are true extremophiles as they are adapted not only to low temperatures, but frequently also to further environmental constraints, such as, levels of nutrient, water activity, salinity and UV exposure (Friedmann, 1982; Carpenter *et al.*, 2000; Feller and Gerday, 2003). UV exposure trigger the generation of Reactive Oxygen Species (ROS), which could lead to an oxidative stress if the antioxidant mechanisms of the cell are overcome by pro-oxidant agents. The toxic effect induced by ROS includes metabolic malfunctioning, oxidative damage in DNA, lipids and proteins (Briviba *et al.*, 1997; Imlay, 2003; Wu *et al.*, 2005; Estevez *et al.*, 2001). Most common ROS species are anion superoxide (O^{2-}), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radicals (OH). Superoxide and hydroxyl radicals are the most reactive ROS species, but unlike hydrogen peroxide, are not diffusible through the membrane (Imlay, 2003; Halliwell, 2006). Despite the detrimental conditions caused by the oxidative damage, some microorganisms are able to adapt to moderate UV-B radiation by reversing the oxidative stress and efficiently repairing the damage, through enzymatic and non enzymatic defense mechanisms. Among the components of the non enzymatic antioxidant defence are glutathione, vitamins and pigments such as carotenoids. Pigments are one of the most important antioxidants systems that neutralize free radicals of the cells. The mechanisms involved in the pigments antioxidant reaction are various. The antioxidant behavior of carotenoids depends on their structures and on the nature of the oxidizing species. The interaction of carotenoids with singlet oxygen ($^1O_2$) for example, occurs by a transfer of the excitation energy to the carotenoid or by a chemical quenching of $^1O_2$; the results in the irreversible destruction of the carotenoid molecule. Carotenoids may interact with oxygen radicals by three main ways: electron transfer, hydrogen abstraction, and addition of a radical species. Another fact that may have a profound effect on the antioxidant capacity of pigments is microenvironment where the carotenoid molecule is located. Therefore, differences in the antioxidant actions of various carotenoids can be attributed to differences in their location within the lipid bilayer.

In this work, we focused on the non enzymatic mechanisms of one pigmented bacterium isolated from Antarctica, to understand how its pigments confer resistance to oxidative stress, in particular to UV radiation, and classical oxidative agents.

Materials and Methods

Microorganism and preparation of pigmented extract

A psychrotolerant aerobic red pigmented bacterium (denominated as yelcho2) was isolated from samples taken from Doumer Island, Antarctica during the Chilean Antarctic Scientific Expedition ECA45. The bacterium was grown in a medium containing peptone (0.5 w/v) and yeast extract

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(0.2 w/v). Gram staining was performed using the standard gram reaction as described in Bergey *et al.* (1994). Pigmented colonies were obtained after plating on solid media during 7 days (2.0% w/v agar). Colonies were collected and resuspended in isopropyl alcohol, at a ratio 1:1 of sample volume to solvent. The solution containing the extract was rotaevaporated until the total volume was reduced down to 10 ml. The pigmented extract was scanned between 330 and 750 nm using UV-Vis Scan Spectrophotometer (Shimadzu, UV-VIS). Reagents used were acquired from Merck (Germany).

DNA extraction and PCR amplification

Genomic DNA was extracted using chloroform-isoamyl alcohol method (Johnson, 1991). Total DNA was used to amplify the 16S rRNA gene by PCR using the bacteria specific primer 27F (Reysenbach, 2001) and the universal primer 1492R (Tanner *et al.*, 2000), as described previously (Tanner *et al.*, 2000). PCR product was observed on agarose gel (1.5%) with 1× TAE buffer (40 mM Tris-HCl, 40 mM acetate, 10 mM ethylene diamine tetraacetic acid (EDTA), and ethidium bromide (0.5 µg/ml) under UV light. PCR product was sequenced using the set of primers described above. Sequences obtained were analyzed, and manually edited using ChromasPro software (Technelysium Pty Ltd. Australia) for a final sequence extension of 870 bp. Analysis of amplified sequence was performed using the BlastN tool. Reagents used were acquired from Invitrogen.

Nucleotide sequence accession number

The partial 16S rRNA gene sequence obtained from the PCR amplification has been deposited in GenBank nucleotide sequences databank under the accession numbers JQ781659.

Characterization of the pigmented extract

Qualitative analysis of pigments in the experimental sample was carried out using thin layer chromatography (TLC) over a silica gel 60 plate (Merck) for TLC grade. 10% (v/v) of methanol in toluene was used as a solvent. Due to the coloration of the sample, the chromatogram could be visualized without other techniques. However UV exposure and staining with iodine vapour were also used. Developed spots were seen and taken out and marked. A purification step using silica gel 60 (Merck) minicolumn (10×1 cm) was performed according to Rodriguez-Amaya (1976). From the total mix of pigments obtained from cells, 35.71% of pigment was recovered after the minicolumn and developed in TLC plate in order to purify four different pigments. UV absorption spectrum between 330 and 750 nm were performed over the individual obtained pigments and later some of them were analyzed by mass spectrometry. Solvents used were acquired from Merck.

Electrospray ionization mass spectrometry

Samples were analyzed in a LC-MS system composed by an HPLC Agilent 1100 (Agilent Technologies Inc., USA) coupled to an electrospray-ion trap mass spectrometer ESI-IT Esquire 4000 (Bruker Daltonik GmbH). Programs ChemStation for LC 3D Rev. A.10.02 (Agilent Technologies Inc.) and Esquire-Control 5.2 (Bruker Daltonik GmbH) were used to control HPLC system and mass spectrometer respectively. Samples were prepared in methanol (Merck) at a final concentration of 3 mg/ml. HPLC separation was performed at room temperature according to the method of De Rosso and Mercadante (2007) using the column Kromasil C18 250×4.6 mm (Eka Chemicals AB, Sweden). The ionization process was performed at 3,000 V using nitrogen as nebulizer gas at 325°C, 30 psi and a flow rate of 10 L/min.

Diphenylpicrylhydrazyl (DPPH) method

In order to assess the antioxidant capacity of the pigments, the pigmented mix from yelcho2 sample was resuspended in methanol, added to a 0.5 mM DPPH (1,1-diphenyl-2-picrylhydrazyl) (Sigma, USA) EtOH solution and left standing in darkness at room temperature for 30 min. The absorbance of the resulting solution was spectrophotometrically measured at 517 nm. Control sample containing only DPPH was used. The scavenging effect on DPPH was determined as follows: initial OD – final OD/ extinction molar coefficient of DPPH (1.24×10^4 M-1 cm-1) (Brand-Williams *et al.*, 1995; Bondet *et al.*, 1997). The total DPPH moles scavenged per mg/ml of pigment was estimated using a standard curve, where the slope of the linear equation has direct relation to the amount of DPPH moles that react with the pigments (Molyneux, 2004). Results presented are the means of at least three experiments.

Detection of reactive oxygen species (ROS)

In order to determine the work range of paraquat (Sigma) concentration in relation to oxidative damage, the minimum inhibitory concentration (MIC) was calculated. Stock sterile solutions of 50 mM paraquat were serially diluted in 200 µl of LB medium per well in culture plates of 96 wells. Each well was inoculated with a concentration of 1% v/v of *Escherichia coli* culture, grown at 37°C in LB medium until a OD_{600} ~0.4. Culture plates were incubated at 37°C for 16 h. MIC determination under anaerobic conditions was identically performed. 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) (Sigma) probe was used to determine the intracellular ROS levels, specifically H_2O_2, in treated cells with paraquat (24 mM). Cells grown aerobically in LB medium (DO_{600} ~0.4) were individually subjected for 30 min of exposure with paraquat, washed with 10 mM phosphate potassium buffer pH 7.0 and incubated for 30 with the same buffer containing 10 mM $DCFH_2$-DA. Then, cells were washed and lysed by sonication (Branson sonifier 450). Cell extracts (100 µl) were mixed with 1 ml of phosphate buffer and fluorescence intensity was determined with a fluorimeter Biotek series FLX 800 TBI (excitation, 490 nm, emission, 519 nm). Emission values were normalized per mg of cells (wet weight).

Preparation of liposomes

In order to prepare liposomes, egg yolk phosphatidylcholine and asolectin (both from Sigma) were dissolved in chloroform:methanol (1:2). The organic solvent was removed by evaporation on a rotoevaporator. The thin lipids film obtained were dried under vacuum. Lipids were hydrated in

Table 1. Mass spectrometry analysis and pigment characterization (Supplementary data Figs. S2 and S3)

Pigment color	Fraction number	Absorption maximum[a]	Principal mass	Fraction mass	Putative compound dentification	Molecular formula
Red	1	460; 492	702.51	-----	Nostoxanthine 3-sulfate	$C_{40}H_{55}O_7SNa$
Orange	2	428; 487; 459	614.56	654; 612	Pyrrhoxanthin	$C_{39}H_{48}O_6$
Yellow	3	478; 452	658.57	622; 580	Fucoxanthin	$C_{42}H_{58}O_6$
Orange	7	440; 470	604.62	520; 440; 352; 222	Violaxanthin	$C_{40}H_{56}O_4$

[a] measured in petroleum ether

10 mM Tris-HCl buffer, pH 7.5. Then, the suspension was subjected to 10 cycles of freezing and thawing in order to obtain multilaminars vesicles (MLVs) and then suspension was extruded through a polycarbonate filter (pore size 400 nm) ten times in order to obtain unilaminars vesicles (LUVs). Size and appearance of liposomes were determined by transmission electron microscopy. Liposomes were negatively stained and visualizad at 80 kV in an electronic microscope Philips Tecnai 12.

Antioxidant capacity measured with an oxygen electrode using 2.2′-azobis(2-aminopropane)-dihydrochloride (ABAP) compound

To measure the antioxidant capacity, a slightly modified version of Cubillos *et al.* (2000) method was used. Autoxidation experiments were carried out at 37°C. The rate of the process was followed by measuring the oxygen concentration as a function of time using an oxygen electrode Oxygraph (Hansatech Instruments, England). Solutions containing the oxidable compound (phosphatidylcholine and asolectin liposomes with and without the mix of pigments from yelcho2) were poured into the reaction vessel. After thermal equilibration was reached, the oxidation was started by addition of an aliquot of a concentrated solution of 2.2′-azobis(2-aminopropane)-dihydrochloride (ABAP) (Sigma) in buffer. In the absence of ABAP no oxygen consumption was observed. The solubility of oxygen in all the working solutions was taken as 2 mM (Chappell, 1964).

Determination of Thiobarbituric Acid Reactive Substances (TBARS)

The oxidative damage to liposomes membranes induced by UVB exposure was analyzed detecting the TBARS levels as a measure of loss of membrane integrity. A liposomes solution (1 mg/ml) containing the pigmented extract from yelcho2 in their structure, at different percentages (1–10%), was exposed to variable UVB radiation doses in cultures plates of 96 wells. Samples were taken at different times and malondialdehyde levels present were detected with the TBARS Assay Kit (ZeptoMetrix, USA). A standard curve was made to express the TBARS levels in terms of malondialdehyde (MDA) equivalents.

Results

Microorganism characterization

Cells of this obligate aerobic microorganism were rod shape, non-motile and Gram-negative. Colonies on solid medium after 7 days, were red and round (2–3 mm in diameter), convex and smooth and with entire margins. Growth occurs between 4 and 37°C and pH 6 and 8; optimal temperature and pH for growth were 27°C and pH 7, respectively. Grows well without NaCl. Analysis of 16S rRNA sequence from BlastN showed 94% of identity with the sequence of the psychrotolerant bacterium *Pedobacter terrae*.

Pigmented extract characterization

The pigmented mix obtained from yelcho2 microorganism was red and showed an absorption maximum at 480 nm, indicating the presence of carotenoids compounds in the sample (Zhang *et al.*, 1997). The requirements of the bacterium yelcho2 to produce the maximum amount of all the pigments were: pH 7.4, 4°C and 0% (w/v) NaCl. TLC analysis of yelcho2 pigmented mix showed the presence of 9 bands of different colours (Supplementary data Fig. S1). Additionally, TLC plates of different individual pigments were exposed to hydrochloric acid vapors showing the presence of epoxides in the pigmented mix, which correspond to xantophylls. Based on their absorption maximum (400 to 500 nm), only four pigments were selected to further analysis by mass spectrometry. Table 1 shows the most probably identification of the four selected pigments which corresponded to different kind of xantophylls.

Antioxidant capacity measured by the DPPH method

The scavenging effect of the pigmented mix from yelcho2, β-carotene, and α-tocopherol on DPPH was examined. Table II shows the stoichiometric ratio between the pigmented mix, β-carotene, α-tocopherol, and DPPH. The pigmented

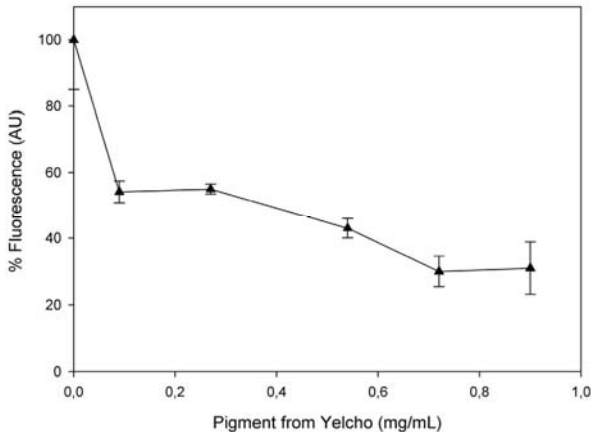

Fig. 1. Intracellular levels of H_2O_2 using fluorescent probe $DCFH_2$-DA. Fluorescence percentages obtained after treatment of *E. coli* with paraquat 24 mM.

Table 2. Scavenging ratio of the pigmented extract from yelcho2, β-carotene, and α-tocopherol

Compound	DPPH activity (mol of DPPH per mg/ml of pigments) (±SD)
Pigments from yelcho2	0.72 (±0.08)
β-Carotene	3.5 (±0.7)
α-Tocopherol	1.5 (±0.4)

mix from yelcho2 showed a stronger effect than that of β-carotene and α-tocopherol, since its reacts faster with DPPH. These results suggest that the pigmented extract is a strong antioxidant.

Intracelular levels of H_2O_2 measured with $DCFH_2$-DA probe

The capacity of the pigments to reduce the intracellular concentration of H_2O_2 was evaluated in the presence of the oxidative damage elicitor paraquat, showed by the fluorescent probe $DCFH_2$-DA. Figure 1 indicates a decrease in the fluorescence intensity in samples containing different pigments concentration in relation to the sample without pigments. This behaviour is associated with a decrease in the amount of free radicals in *E. coli* which is due to the antioxidant capacity conferred by the pigments.

Oxygen level using oxygen electrode and ABAP compound

The antioxidant capacity measured with an oxygen electrode and the ABAP [2.2′-azobis(2-aminopropane)-dihydrochloride] compound is shown in Fig. 2. The oxygen level decreased as higher pigment concentration was used. This behaviour was observed in Egg yolk phosphatidylcholine and asolectin liposomes containing yelcho2 mix of pigments and also in liposomes containing the standard β-carotene. These results suggest that mix of pigments and the standard were able to decrease the oxidation of lipids. No significant differences in the response to oxygen consumption were observed between the mix of pigments and the standard.

Lipid peroxidation induced by UVB radiation

The oxidative damage to liposomes membranes induced by UVB exposure was analyzed. Figure 3 shows a decrease in the MDA (malondialdehyde) concentration in egg yolk phosphatidylcholine and asolectin liposomes with yelcho2 mix of pigments. The same behaviour was observed in liposomes with β-carotene. These results show that both pigments confer resistance to UVB radiation by decreasing lipid peroxidation.

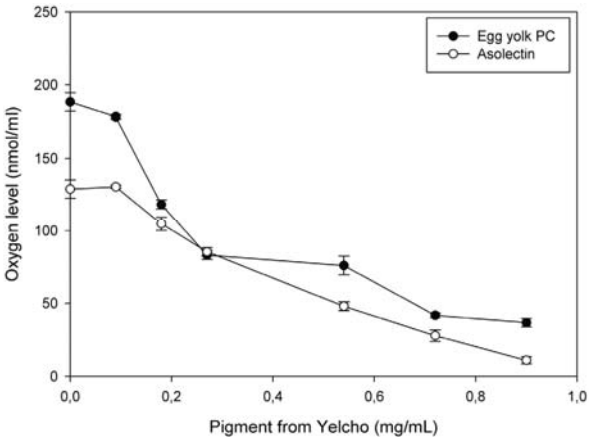

Fig. 2. Measurement of oxygen level by an oxygen electrode and the ABAP compound. The studies were carried out in egg yolk phosphatidylcholine and asolectin liposomes (PC, phosphatidylcholine).

Discussion

Antarctic microorganisms have to withstand not only cold temperatures but also other stress conditions such as high UV radiation (Smith *et al.*, 1992). To thrive in this extreme environment, these microorganisms have developed different antioxidant systems to avoid the oxidative stress caused by ROS. One of these is the non-enzymatic system which includes pigments. In this work we focused on one Antarctic microorganism isolated from Doumer Island, to study the antioxidant capacity of its pigments. The characterization of the mix of pigments produced by this microorganism, indicate that it is composed by nine pigments that belong to the carotenoids group, showing a maximum absorption at 480 nm (Zhang *et al.*, 1997). The ionic masses from the principal ion and its fragmentation, obtained from the mass spectrometry analysis, were used to identify the most probably structure of each pigment using the Lipid Bank database, indicating the presence of xantophylls in the mix of pigments (Table 1). The optimal growth conditions for

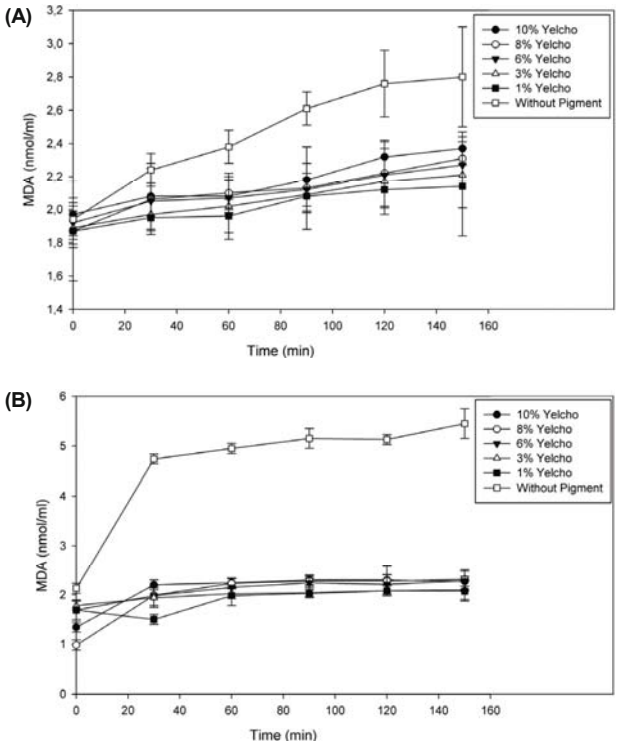

Fig. 3. Lipid peroxidation induced by UVB radiation. Levels of MDA generated in liposomes containing the pigmented extract from yelcho2 in their structure, at different percentages (1–10%). The studies were carried out in egg yolk phosphatidylcholine (A) and asolectine (B) liposomes.

the production of pigments were determined (pH 7.4; 4°C and 0% NaCl). Unlike other microorganisms that produce pigments at high NaCl concentration (Dundas and Larsen, 1962; Gochnauer *et al.*, 1972; Asker and Ohta, 1999), this bacterium produces higher amount of pigments in the absence of NaCl. Pigment production is also affected by temperature, having a better production at low temperatures as observed and described in other psychrophiles (Jagannadham *et al.*, 1991; Chattopadhyay *et al.*, 1997; Fong *et al.*, 2001).

DPPH method was preliminary used to determine the antioxidant capacity of the mix of pigments. As shown in Table 2 the antioxidant capacity of the mix of pigments is higher to that of β-carotene and α-tocopherol. This could be related to the chemical nature of the pigments because some of them have been classified as xantophylls based on chemical analysis, showing terminal hydroxyl groups on their structure that make them more reactive to DPPH radicals. Due to the possible interference of pigments with the determinations in this colorimetric method, two additional approaches (ROS detection and oxygen electrode) were used to evaluate the antioxidant capacity of the mix of pigments. Results of the intracellular levels of H_2O_2 in treated cells with paraquat suggested that the mix of pigments protects *in vivo* the cells against the oxidative damage by decreasing ROS levels. The antioxidant capacity observed by this method showed a different response in comparison with the behavior observed in solution (determined by DPPH method), since the pigments and β-carotene showed a similar antioxidant capacity and not higher as with the DPPH method. The other determination of antioxidant capacity was performed using an oxygen electrode in liposomes with and without pigments incorporated into the structure. The decrease in the rate of oxygen consumption observed (Fig. 2) is due to the antioxidant capacity of the mix of pigments. These pigments could be stabilized to ABAP compound, preventing its interaction with oxygen and therefore diminishing the production of radicals or could be stabilizing the free radicals produced by the ABAP preventing their later interaction with oxygen. In both cases less oxygen is consumed and therefore less lipid oxidation occurred. As in the ROS detection method, a similar antioxidant capacity between the mix of pigments and the standard was observed with this more sensitive technique. These results could be due to the different antioxidant capacity of the pigments when they are in solution (as in the DPPH method) versus to their capacity when they are inserted in membranes. Moreover, the antioxidant capacities of β-carotene and xantophylls are dependant on their different location in the bilayer. As β-carotene is a non-polar carotenoid it was able to quench radicals in the hydrophobic part of the membrane. However due to the characteristics of xantophylls they were effective as antioxidant in the polar region, exposed to an aqueous environment (Sujak *et al.*, 1999). Moreover since it is a mix of pigments composed by several carotenoids, it is probably that the insertion of them into the liposome membranes could be more effective for some of them or their orientations in the membrane may not be the optimal, which could produce a lower antioxidant capacity. As lipids are major targets during oxidative stress we also assess the protection that the mix of pigments confers against lipid peroxidation produced by UVB. The behaviour observed in Fig. 3 shows that the presence of pigments intercalated within the liposomes confers resistance to UVB radiation by decreasing lipid peroxidation. This result suggests that these pigments have an important role against oxidative damage protecting the cell membranes. Besides this protecting role, these pigments could also affect the membrane fluidity allowing their metabolic functions even at low temperatures (Chintalapati *et al.*, 2004). It is probable that antioxidant properties presented by the mix of pigments from yelcho2, are due to their biological function allowing the presence of this microorganism in Antarctica. Therefore this mix of pigments could be considered as a powerful antioxidant mix that protects the bacteria against oxidative damage, caused in this continent mainly by high levels of UVB radiation. Due to the antioxidant properties, this mix of pigments could be very useful for biotechnological applications, demonstrated by its potent antioxidant capacity and the conferred resistance to liposomes, interesting properties in the process of drug delivery.

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