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Polar and non-polar species of the protozoan ciliate *Euplotes* behave differently in response to environmental oxidative stress

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Abstract
Polar coastal seawaters are saturated by high oxygen concentrations, which impose very effective adaptive strategies to strive against a continuous environmental oxidative stress. We studied these strategies in *Euplotes nobilii* (Ciliophora: Spirotrichea), a ciliate species dwelling in Antarctic and Arctic coastal seawaters, in comparison with *Euplotes raikovi*, a sister species living in temperate seawaters. Cell samples of the two species were exposed to hydrogen peroxide (H_2O_2)-induced oxidative stress and analyzed for their survival rates and levels of expression of the genes encoding the enzyme methionine-sulfoxide reductase (Msr) A, which restores oxidized methionines (in their S form) of damaged proteins to the status of functional methionines. While 6 h of exposure to a 750-μM concentration of H_2O_2 did not affect *E. nobilii* viability, these conditions were lethal to *E. raikovi*. In correlation with this inter-specific difference in the cell survival to oxidative stress, the MsrA-coding genes of the two species showed different mechanisms of expression: constitutive in *E. nobilii*, elicited by induction in *E. raikovi*.

Keywords: *Polar ciliates, oxidative stress,* Euplotes-*environment interactions, methionine sulfoxide reductases, MsrA*

Introduction

Microbial life thrives in polar coastal waters, notwithstanding the detrimental effects of various adverse environmental factors, in primis ionizing and UV radiation, high oxygen concentrations and pressure. These factors cause substantial increases in the intracellular levels of reactive oxygen species (ROS) that are particularly deleterious to biological macromolecules (Abele & Puntarulo 2004). One of the major ROS targets is methionines of the polypeptide chains, particularly those methionine residues which lie exposed on the molecular surfaces (Vogt 1995; Stadtman et al. 2003; Friguet 2006; Alimenti et al. 2012). The modification of substantially hydrophobic methionines into hydrophilic sulfoxides usually changes the polarity of a protein, with dramatic effects on its stability and bioactivity. Organisms living exposed to high oxygen concentrations and pressure in stably cold waters appear to be particularly vulnerable to protein damage by ROS because of their lower rates of protein turnover that greatly favor accumulation of oxidized proteins (Philipp et al. 2005; Fraser et al. 2007). However, extreme environmental conditions usually create stronger selective pressures that, over long timescales, drive the evolutionary process to endow organisms with very effective adaptive strategies.

We studied these adaptive strategies in a model system represented by a free-living marine species of the ciliate *Euplotes* (Ciliophora: Spirotrichea), *E. nobilii* Valbonesi and Luporini, 1990, which is a common inhabitant of Antarctic and Arctic coastal waters (Valbonesi & Luporini 1990; Di Giuseppe et al. 2011, 2013a, 2013b). Having observed that this polar species faces UV radiation and oxidative stress with much more effective mechanisms than its closest relative, *E. raikovi* Agamaliev, 1966, which dwells in mid-latitude sea waters (Di Giuseppe et al. 2012), we sought to identify a genetic basis for these mechanisms. Primary interest was focused on characterizing the role played by the methionine sulfoxide reductase (Msr) genes which are basic components of the genome of every aerobic organism (Moskovitz 2005; Delaye et al. 2007), since Msr enzymes repair oxidized methionines of damaged

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proteins restoring them to the status of functional methionines.

First, we characterized the structure and function of *E. nobilii* and *E. raikovi* genes, which in the transcriptionally active genome of the cell macronucleus encode Msrs of type B, which are committed to repair the methionine-sulfoxide R-forms (Dobri et al. 2013). Next we examined the activity of the *E. nobilii* and *E. raikovi* macronuclear genes encoding Msrs of type A, which are specific for the reduction of the methionine-sulfoxide S-forms. While the MsrA gene expression in *E. raikovi* appeared to be an inducible phenomenon, we obtained evidence that in *E. nobilii* this expression is constitutive, thus suggesting that constant synthetic activity of Msr genes may be decisive in helping polar ciliates to face environmental oxidative damage more efficiently.

Materials and methods

Chemicals

For the analysis of cell viability, the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay from Promega (Madison, WI, USA) was used following the manufacturer's instruction. Rabbit polyclonal antibodies raised against a recombinant bovine MsrA and Enhanced Chemi-Luminescence (ECL) system were purchased from LabFrontiers (Seoul, Korea) and GE Healthcare (Milan, Italy), respectively. Other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell cultures

The Antarctic strain AC-4 of *E. nobilii* (Di Giuseppe et al. 2013a) and the Mediterranean *E. raikovi* strain 13 (deposited at the American Type Colture Collection (ATCC) centre with the catalog #PRA-327) were used. Cultures were maintained under a cycle of 8 h of moderate exposure to light and 16 h of darkness at 22°C (*E. raikovi*), or 4–6°C (*E. nobilii*). They were grown in filtered and pasteurized natural seawater (salinity adjusted to 32–33‰; pH 8.1–8.2), using the green alga *Dunaliella tertiolecta* as food. Cells were maintained for at least 2 days without food before being used in experiments.

Cell viability assays

In the motility-based assay, cells at a density of approximately 2×10^3/mL were suspended, at 4°C (*E. nobilii*), or 22°C (*E. raikovi*), in fresh sea water with increasing hydrogen peroxide (H_2O_2) concentrations (from 0 to 2 mM), and aliquots (100 μL) were then isolated from each cell suspension at intervals from 30 min to 6 h to count the final ratios between motile (viable) and motionless (dead) cells.

In the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay, cells (10,000/well) were exposed for 2 h, at 4°C (*E. nobilii*), or 22°C (*E. raikovi*), to increasing concentrations (from 0 to 2 mM) of H_2O_2, and incubated with MTS for additional 6 h. The absorbance of the formazan produced by reduction of tetrazolium salts was then measured at 490 nm, using a Microplate Reader 680 XR Spectrophotometer (Bio-Rad, Hercules, CA, USA).

The statistical significance of data was evaluated using analysis of variance (ANOVA) followed by Tukey's highly significant difference (HSD) test (Lane 2010).

Western blot analysis

Total cell lysates were obtained from cell pellets suspended in 2× Laemmli sample buffer (10% β-mercaptoethanol, 0.125 M tris-[hydroxymethyl]aminomethane hydrochloride (Tris-HCl), pH 6.8, 4% Sodium Dodecyl Sulfate (SDS), 20% glycerol and 0.004% blu-bromophenol) and boiled for 5 min. Aliquots corresponding to approximately 10^4 cells were separated by electrophoresis on 15% SDS-polyacrylamide gels, and electro-transferred onto Polyvinylidene fluoride (PVDF) membranes, at 240 mA for 35 min, in transfer buffer containing 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 11, 10% methanol. Membranes were blocked with 5% non-fat dried milk in Tris-Buffer Saline (TBS) (20 mM Tris-HCl, pH 7.6, 130 mM NaCl), and incubated overnight, at 4°C, with rabbit anti-MsrA polyclonal antibodies used at the dilution of 1:1000 in TBS containing 1% non-fat dried milk. After washing with TBS and 0.1% Tween-20, blots were incubated, at room temperature for 1 h and in constant agitation, with Horseradish Peroxidase (HRP)-conjugated anti-rabbit secondary antibodies at 1:5000 dilution. Blots were stained with ECL, and chemiluminescent signals were revealed by exposing blots to Hyperfilm ECL.

Results

Cell viability under oxidative stress

A motility-based assay, as described by Madoni and Romeo (2006), was first used to analyze the viability of cells exposed to oxidative stress. Increasing H_2O_2 concentrations were added to cell suspensions as single doses, and the cell viability was measured at

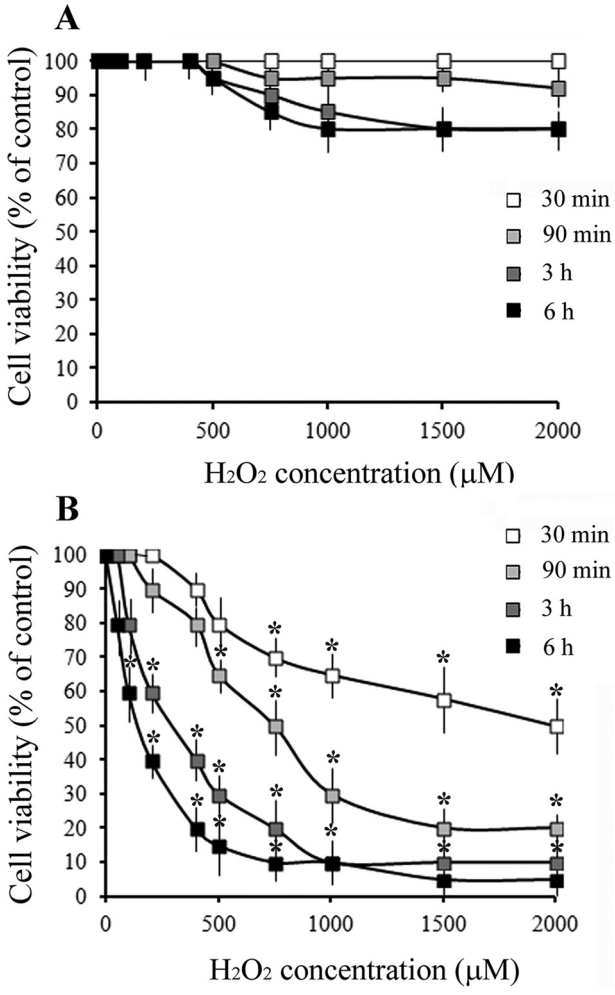

Figure 1. Hydrogen peroxide (H_2O_2)-induced oxidative stress on (A) *Euplotes nobilii* and (B) *E. raikovi*. Cell viability is measured according to the motility-based assay (see text) in cell samples exposed to increasing H_2O_2 concentrations for the indicated times. Values are means (± standard deviation, SD) of two independent experiments carried out in duplicate, and are expressed as percents, taking as 100% the viability of not-exposed cell samples (control). * $p < 0.01$ vs control.

increasing intervals after H_2O_2 addition. Short intervals were taken to analyze the immediate toxic effects of H_2O_2 on cells, while long intervals were taken to assess the H_2O_2 cumulative damage on the cell metabolic activity.

As shown in Figure 1, the resistance of *E. nobilii* cells to the oxidative stress was much stronger than that of *E. raikovi* cells. Whereas more than 80% of *E. nobilii* cells were fully viable even 6 h after the addition of the maximal H_2O_2 concentration (2 mM), cytotoxic effects in *E. raikovi* increased in parallel with increased H_2O_2 concentrations and increased intervals of cell exposure to H_2O_2. They were already evident after 30 min of exposure to the maximal H_2O_2 concentration (2 mM) and the great majority of *E. raikovi* cells were no longer viable 6 h after the addition of 750 μM H_2O_2.

Subsequently, the viability of cells exposed to H_2O_2 was assessed with a colorimetric microplate procedure (Mosmann 1983; Bernas & Dobrucki 2002). This procedure correlates the cell viability with the cell metabolic ability to convert MTS (i.e. a modified version of tetrazolium salts) into water-soluble formazan. Only fully viable cells are able to carry out this enzymatic reaction, which results in the production of brown-colored formazan; cells damaged by oxidative stress lose metabolic energy and are no longer able to reduce the yellow-colored MTS.

As shown in Figure 2, the *E. raikovi* cell viability decreased with increasing H_2O_2 concentrations, whereas *E. nobilii* cells appeared to be totally unaffected by the oxidative stress. Their viability always remained close to 100%.

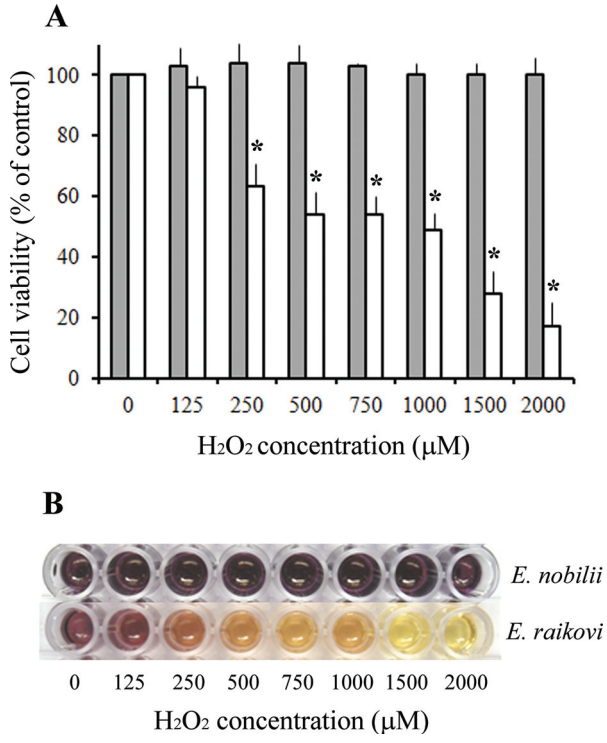

Figure 2. Hydrogen peroxide (H_2O_2)-induced oxidative stress on *Euplotes nobilii* and *E. raikovi*. Cell viability is measured as formazan production (see text) on cell samples exposed at increasing H_2O_2 concentrations before 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay. A, Values of cell viability in *E. nobilii* (gray columns) and *E. raikovi* (light columns), taking as 100% the viability of not-exposed cell samples (control). Data are means (± standard deviation, SD) of two independent experiments carried out in triplicate, and are expressed as percents, taking as 100% the viability of not-exposed cell samples (control). *p < 0.01 vs control. B, Microplate lines showing the production of purple formazan by equivalent *E. nobilii* and *E. raikovi* cell samples exposed to increasing H_2O_2 concentrations after 6 h of incubation with MTS.

Analysis of MsrA expression

The analysis of the MsrA gene expression was based on Western blots carried out with polyclonal antibodies specific to Msrs of type A, and unable to cross-react with Msrs of type B which differ significantly in their amino acid sequences.

Cells at a density of approximately 5×10^3/mL were exposed (for 1 or 2 h) to increasing H_2O_2 concentrations (from 0 to 1 mM), collected by centrifugation, directly lysed in Laemmli sample buffer, and cell lysates were eventually analyzed in Western blot with the anti-MsrA antibodies. As shown in Figure 3, *E. raikovi* cell lysates revealed multiple bands (two major ones of approximately 24 and 38 kDa, plus a minor one of approximately 30 kDa), implying that these cells produce at least two, or three MsrA isoforms. The intensities of these bands (the minor one of 30 kDa excepted, as it tends to vanish in cells exposed at higher H_2O_2 doses) increased in precise correlation with increased H_2O_2 concentrations and increased times of cell exposure to H_2O_2, thus providing evidence that *E. raikovi* raises its MsrA synthesis.

Differently from *E. raikovi*, *E. nobilii* cell lysates produced a single band of 30 kDa, and the intensity of this band revealed no appreciable variations between stressed and not-stressed cells (Figure 4), implying that *E. nobilii* synthesizes MsrA in constant concentrations, independently of the stress conditions to which it is exposed.

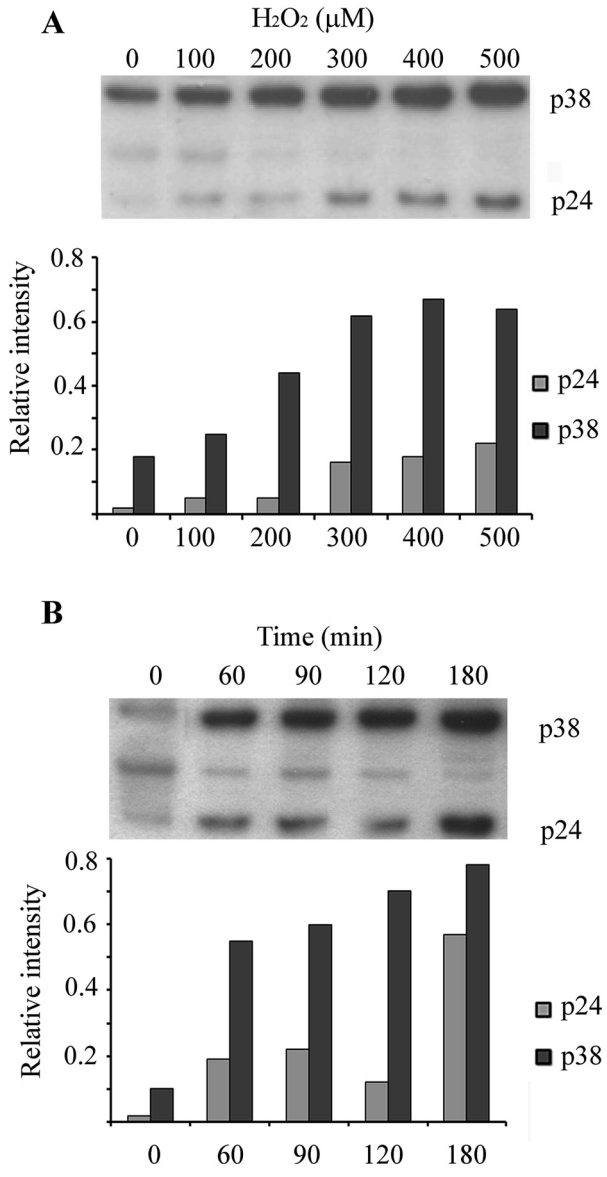

Figure 3. MsrA expression in *Euplotes raikovi* cells subjected to H_2O_2-induced oxidative stress. Cells were suspended with (A) increasing H_2O_2 concentrations for 1 h, or (B) 200 mM of H_2O_2 for increasing times, before being lysed and analyzed in Western blot with anti-MsrA antibodies. In each lane, lysate preparations from equivalent cell numbers were loaded. The 38- and 24-kDa immuno-recognized bands are indicated by p38 and p24, respectively, and their relative intensities were measured with the ImageJ software taking an immune-recognized tubulin band from the same cell samples as value 1. The experiment was repeated twice, with equivalent results.

Discussion

The data described above provide evidence that H_2O_2-induced oxidative stress causes markedly different responses from polar and non-polar *Euplotes* species. While non-polar *E. raikovi* cells were shown to be particularly sensitive to oxidative stress and destined to die soon after exposure to exogenously added H_2O_2, polar *E. nobilii* cells survived well in harsh oxidative conditions. In apparent causal relationship with these observations, immune-recognition reactions revealed that *E. nobilii* cells do not undergo any appreciable variation in their MsrA concentrations in response to oxidative stress, in contrast with *E. raikovi* cells which markedly varied their MsrA concentrations. These observations are limited to immune-recognized MsrA isoforms and do not exclude the presence of additional MsrA forms in *E. nobilii* and *E. raikovi*. Nevertheless, the observed inter-species differences in MsrA synthesis clearly imply that the MsrA gene expression in *E. raikovi* requires a specific induction by oxidative stress, whereas in *E. nobilii* this expression is continuous and constitutive analogously to what was previously observed in relation to the *E. nobilii* MsrB genes (Dobri et al. 2013).

This constitutive activity of *E. nobilii* cells in synthesizing MsrA and MsrB enzymes likely reflects a very effective ecological adaptation of this microorganism

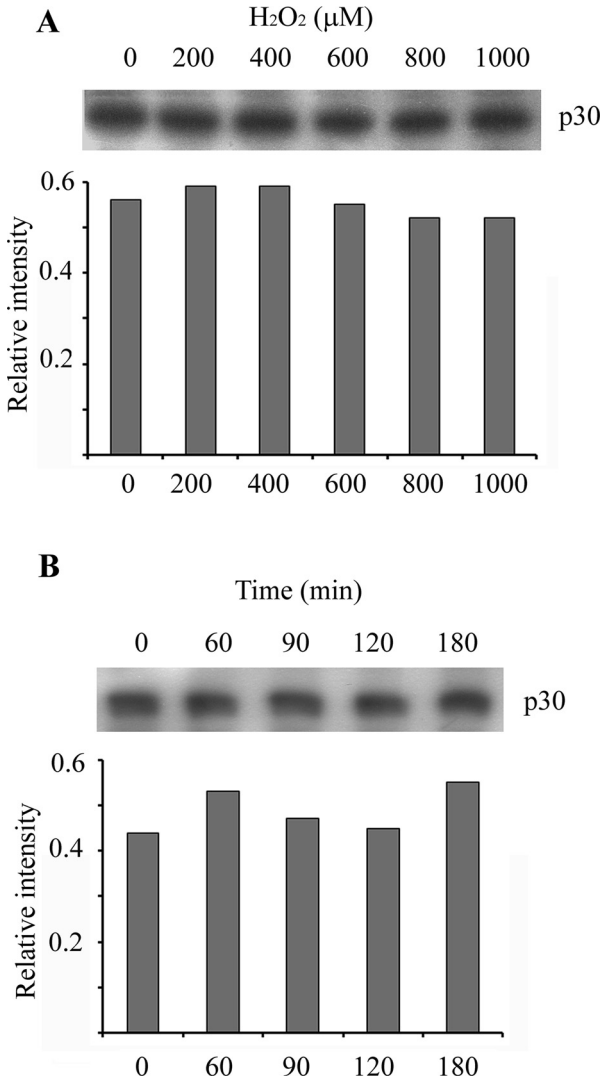

Figure 4. MsrA expression in *Euplotes nobilii* cells subjected to H_2O_2-induced oxidative stress. Cells were suspended with (A) increasing concentrations of H_2O_2 for 2 h, or (B) 400 mM of H_2O_2 for increasing times, before being lysed and analyzed in Western blot with anti-MsrA antibodies. In each lane, lysate preparations from equivalent cell numbers were loaded. The 30 kDa immune-recognized band is indicated by p30, and its relative intensity is measured with the ImageJ software taking an immune-recognized tubulin band from the same cell samples as value 1. The experiment was repeated twice, with equivalent results.

to the strong oxidative forces of the marine polar environment. Furthermore, it appears to be consistent with the hypothesis (Levine et al. 2000) that the oxidation of surface-exposed methionine is a general strategy adopted by polar organisms to cope with the deleterious effects of these forces. By causing a constitutive expression of the Msr genes, it would appear that methionine oxidation plays a central role in increasing the scavenging efficiency of this genetic mechanism and, hence, in protecting proteins from the oxidative damage of other functionally essential amino acid residues of their chains.

This concept is supported by transcriptome analyses in polar species of fishes, invertebrates and microalgae. The Antarctic notothenioid fish *Dissostichus mawsoni* and the Antarctic krill *Euphausia superba* express their Msr genes, as well as other genes encoding stress proteins, to a much greater extent than their allied species living in more temperate waters (Chen et al. 2008; Clark et al. 2011). Genomes of polar species of diatoms contain nearly twice the number of Msr-gene homologues than genomes of temperate species, 14 Msr-gene homologues having been identified in the genome of the polar species *Fragilariopsis cylindrus*, in comparison with seven or 10 identified in the genomes of the temperate species *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, respectively (Lyon et al. 2014).

Overall, these observations thus offer forceful indications that marine polar life relies strongly on the continuous expression of Msr genes to strive against the oxidative damage that is imposed by the unusually high oxygen concentration and pressure of their environment.

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