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INTRASPECIFIC VARIATION IN STRESS-INDUCED HYDROGEN PEROXIDE SCAVENGING BY THE ULVOID MACROALGA *ULVA LACTUCA*[1]

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We investigated the presence and kinetics of the oxidative stress response in intertidal and subtidal individuals of the ulvoid macroalga *Ulva lactuca* L. Stress responses, as measured with both enzymatic and fluorescent-based antioxidant assays, differed between individuals collected from a subtidal and an intertidal habitat. Subtidal individuals secreted significantly more hydrogen peroxide (H_2O_2) than intertidal individuals when subjected to osmotic stress or desiccation. The activity of reactive-oxygen-scavenging enzymes and the ability to scavenge exogenous H_2O_2 were lower in subtidal than in intertidal individuals, suggesting that subtidal individuals are less stress tolerant. In vitro experimentation demonstrated that millimolar concentrations of dimethylsulfoniopropionate (DMSP) and its breakdown products could efficiently scavenge H_2O_2, with DMSP being a less-effective scavenger than dimethyl sulfide (DMS), acrylic acid, and acrylate. The addition of H_2O_2 at concentrations of 2.5 mM or greater induced the cleavage of DMSP into DMS and acrylic acid in subtidal individuals. Intertidal individuals were affected in the same manner with the addition of 5 mM H_2O_2. There were no differences in the amounts of DMSP cleavage in subtidal and intertidal algae when the algae were subjected to hyposaline conditions. Our data suggest that the oxidative-stress-induced cleavage of DMSP affords products with efficient H_2O_2-scavenging abilities. In addition, *U. lactuca* individuals growing in intertidal habitats are better acclimatized to changing environments and thus have a higher threshold for oxidative stress than conspecifics in subtidal habitats.

Key index words: **acrylate; acrylic acid; antioxidants; DMS; DMSP; hydrogen peroxide; macroalgae; oxidative stress**

Abbreviations: **APX, ascorbate peroxidase; CAT, catalase; DCFH-DA, dichlorodihydrofluorescein diacetate; DMS, dimethyl sulfide; DMSP, dimethylsulfoniopropionate; FM, fresh mass; GR, glutathione reductase; IC_{50}, inhibitory concentration required to consume 50% of H_2O_2; ROS, reactive oxygen species; SOD, superoxide dismutase; TSP, total soluble protein**

Marine organisms are susceptible to a variety of dynamic environmental stresses that influence survivorship and distribution (Raffaelli and Hawkins 1996, Sousa 2001). In intertidal habitats, extremes in small-scale chemical stresses and physical disturbances are generally larger and more frequent than those in subtidal habitats (Davison and Pearson 1996). Because intertidal individuals are more likely to routinely experience these extremes and rapid shifts in environmental conditions, it would be anticipated that they would be better adapted physiologically to deal with environmental stresses than subtidal individuals of the same species.

Under normal metabolic conditions, plants and marine algae accumulate reactive oxygen species (ROS) as by-products of the electron transport systems (i.e., during photosynthesis and mitochondrial respiration). While the production of ROS is generally low in unstressed organisms, an increased production of ROS occurs in response to a diverse array of environmental stresses that includes excess UV irradiation, temperature fluctuation, nutrient deficiency, desiccation, and herbivory (Dring 2006, Lesser 2006). If the accumulation of ROS exceeds the capacity of enzymatic and nonenzymatic antioxidant systems to remove them, photosystems may be damaged because of the destruction of lipids, proteins, and nucleic acids (Fridovich 1978, Asada and Takahashi 1987, Halliwell and Gutteridge 1989, Dummermuth et al. 2003). To prevent oxidative damage, ROS may be detoxified enzymatically (Mallick and Mohn 2000) or via the interaction with low-molecular-weight antioxidant compounds (Apel and Hirt 2004).

Species living in environments where they experience oxidative stresses appear to produce lower concentrations of ROS and eliminate them more efficiently. Following environmental stress, ROS

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levels accumulate much more rapidly in nonacclimated (i.e., less-stress-tolerant) species (Pastori and Trippi 1993, Collén and Davison 1999a,b) than in conditioned (stress-tolerant) species (Butow et al. 1994, Tottempudi et al. 1994). The activities of antioxidant enzymes can be significantly higher in more-stress-tolerant organisms (Murthy and Sharma 1989, Dummermuth et al. 2003, Choo et al. 2004). Thus, enzymatic activity reflecting the status of an organelle's oxidative machinery may be used as an indicator of cell acclimation and stress (Downs et al. 2000, Miller-Morey and Van Dolah 2004).

Much less is known about within-species variation in the production and elimination rates of ROS; however, limited data exist suggesting that seaweeds may physiologically acclimate to changing environmental conditions by altering rates of ROS production and elimination. For example, long-term hyposaline and hypersaline stresses produced distinct antioxidant responses in the marine chlorophyte *Dunaliella tertiolecta* Butcher (Jahnke and White 2003). Individual specimens of *Fucus vesiculosus* L. that were grown in low-temperature conditions in the laboratory produced fewer ROS and had higher activities of antioxidant enzymes after a severe freezing stress than individuals grown at higher temperatures (Collén and Davison 2001). The magnitudes of the changes in these laboratory studies corresponded well with seasonal fluctuations in reactive oxygen metabolism in *F. vesiculosus* (Collén and Davison 2001).

In the current study, we examined the production and elimination of ROS in *Ulva lactuca*, a widely distributed green macroalga whose vertical zonation ranges from the upper intertidal zone to the subtidal zone (Abbott and Hollenberg 1976). Intertidal and subtidal *U. lactuca* were subjected to abiotic stresses to examine their relative production of ROS. We measured the ability of intertidal and subtidal individuals to eliminate ROS and compared the activities of several known antioxidant enzymes from individuals collected at the two sites. Finally, we evaluated the effect of exogenous H_2O_2 on dimethylsulfoniopropionate (DMSP) cleavage and examined the use of DMSP and its cleavage products, dimethyl sulfide (DMS) and acrylic acid, as ROS scavengers.

MATERIALS AND METHODS

Algal collections. Intertidal *Ulva lactuca* were collected at low tide from the Fort Pierce Jetty (27°28.248′ N, 80°17.403″ W), and subtidal *U. lactuca* were collected from the Stan Blum Memorial Boat Launch, Fort Pierce, Florida (27°28.727′ N 80°18.487′ W), in April 2005. Algae were transported to the Smithsonian Marine Station (SMS) at Fort Pierce for immediate analysis after collection.

Hydrogen peroxide release following stress. Ulva lactuca was subjected to several stressors to qualitatively and quantitatively evaluate hydrogen peroxide (H_2O_2) release. To visualize the ROS-induced effects of desiccation, subtidal samples of *U. lactuca* were air-dried for 3 h and subsequently placed in petri dishes containing 10 mL of seawater and 10 μL of dichlorodihydrofluorescein diacetate (DCFH-DA, 5 μM final concentration; Invitrogen, Carlsbad, CA, USA). Samples were incubated on a rotary shaker in the dark for 15 min and washed in filtered seawater to remove any unbound probe (Collén and Davison 1997, Rijstenbil et al. 2000). Samples were transferred to petri dishes containing fresh seawater and appropriate concentrations of catalase (10–200 $U \cdot mL^{-1}$; Sigma, St. Louis, MO, USA) or DMS (1–20 mM; Sigma) to evaluate the ROS-scavenging ability of these antioxidants on *U. lactuca.* Samples were incubated in the dark for an additional 10 min. Confocal laser scanning microscopy with a Nikon Eclipse E800 compound microscope (Nikon Instruments, Kanagawa, Japan) equipped with a Bio-Rad Radiance 2000 laser system (Bio-Rad, Hercules, CA, USA) was used to examine H_2O_2 emission from the desiccation-stressed algae. The laser power was set at 20% with an excitation of 488 nm and an emission of 525 nm (channel 1) or 580 nm (channel 2). A series of 0.45 μm optical sections with maximum intensity projection along the *z*-axis were made into one two-dimensional image with greater focal depth.

To quantitatively examine the effects of osmotic stresses on the release of H_2O_2, *U. lactuca* individuals (0.5 g, $n = 3$–4 per treatment) were placed in beakers containing 50 mL of either full-strength seawater (35‰) or seawater diluted to 50% or 10% with deionized water and continuously mixed. At 10 min intervals, for 120 min, 1 mL of media surrounding the alga from each replicate was collected and added to a cuvette containing 0.40 $U \cdot mL^{-1}$ esterase and 25 μM of DCFH-DA. The total volume was then brought up to 2000 μL in filtered seawater. Hydrogen peroxide was quantified fluorometrically on a Bio-Rad VersaFluor fluorometer (excitation: 488 nm; emission: 525 nm) using the methods of Ross et al. (2005). Known volumes of commercially obtained H_2O_2 (Sigma) were used as standards. Data were analyzed with repeated-measures analysis of variance (ANOVA; SPSS 11.0) with algal source (intertidal versus subtidal) and salinity as factors. Because there were insufficient degrees of freedom to conduct the analysis with all of the data, only the data from 0, 20, 40, 60, 80, 120, 140, 160, 180, and 200 min readings were used for the analysis.

To examine the effects of desiccation, pieces of algae (0.5 g) were air-dried for 3 h and placed in 50 mL of seawater. Aliquots (1 mL) of the seawater were removed from each replicate at 10 min intervals, and H_2O_2 was measured as described above. The data from 0, 40, 80, 120, 160, and 200 min readings were analyzed with repeated-measures ANOVA (SPSS 11.0), with algal source and desiccation treatment as factors.

In vivo scavenging of hydrogen peroxide. The in vivo scavenging capabilities of intertidal and subtidal *U. lactuca* were compared using a protocol adapted from Collén and Davison (1999c). Subtidal and intertidal algae (0.5 g per replicate; 3–4 replicates per source) were placed in 50 mL seawater with 100 μM exogenous H_2O_2. Aliquots (1 mL) of the surrounding media were collected every 10 min for an hour and assayed for H_2O_2 as described above. The data from the 20, 40, and 60 min readings were analyzed with repeated-measures ANOVA (SPSS 11.0), with algal source as the factor.

Enzyme assays. To evaluate any variation in the enzymatic elimination of ROS between habitats of *U. lactuca*, ascorbate peroxidase (APX, EC 1.11.1.11), superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and glutathione reductase (GR, EC 1.8.1.7) activities were measured in both intertidal and subtidal specimens. To measure APX, SOD, and CAT activities, freshly collected algae (2.5 g per replicate) were ground in liquid nitrogen, extracted, and assayed according to the methods described by Choo et al. (2004).

The GR activity was assayed with a method briefly modified from that of Collén and Davison (1999c). Fifteen microliters of supernatant was added to 600 μL of 100 mM Tris–HCl buffer

(pH 7.8) containing 1 mM EDTA, 100 μM of NADPH, and 0.5 mM oxidized glutathione. The oxidation of NADPH was detected by monitoring the absorbance at 340 nm at 25°C. The GR activity was calculated by subtracting nonenzymatic reaction controls.

Total soluble protein (TSP) was quantified with the Quick Start ™ Bradford Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions. Data were analyzed after logarithmic transformation to ensure normality and homoscedasticity with a two-way ANOVA. Enzyme type and algal source were factors in the analysis.

Hydrogen-peroxide-scavenging activities of DMSP and its cleavage products. Because DMSP and its cleavage products have been implicated in antioxidant activities (Sunda et al. 2002), we examined the effects of these compounds on H_2O_2 scavenging. The in vitro scavenging potential of DMSP, DMS, acrylate, acrylic acid, and the known H_2O_2 scavenger ascorbic acid were determined by incubating the compounds at a range of concentrations with 100 μM H_2O_2, 0.82 U esterase, and 25 μM DCFH-DA, for a total reaction volume of 2000 μL. The concentration of H_2O_2 was determined fluorometrically as described above.

To determine if the addition of H_2O_2 could cause the cleavage of DMSP into the more potent scavengers DMS and acrylic acid, intertidal and subtidal algae (~0.2 g pieces; $n = 10$ per treatment) were incubated for 3 h in 8 mL of seawater with H_2O_2 at concentrations from 0 to 5000 μmol. After 3 h, the algae were collected, weighed, and dried at 60°C overnight. The dried algae were reweighed and placed in gas-tight vials containing 4 mL of 2 N NaOH. The vials were stored overnight at 4°C in darkness. The next day the concentration of DMS in the headspace was measured by direct injection onto an SRI gas chromatograph (Chromasil 330 column: Supelco 11496, Bellefonte, PA, USA; flame-photometric detector: SRI Instruments 8690-1080, Torrance, CA, USA) as described in Van Alstyne et al. (2001). Vials containing known amounts of DMSP were used to generate a standard curve. The data were analyzed independently for each site with a one-way ANOVA. A Tukey's post hoc test was used to test for differences in the mean DMSP concentrations across the range of H_2O_2 concentrations.

As an additional method of confirming that H_2O_2 could cause DMSP cleavage, both intertidal and subtidal *U. lactuca* were incubated with exogenous H_2O_2 within gas-tight containers, and the evolution of DMS in the headspace of the containers was quantified. Algae (~0.2 g) were placed in gas-tight vials with 8 mL of seawater with H_2O_2 at concentrations from 0 to 5000 μmol. After 0.5 h ($n = 2$), 1.5 h ($n = 4$), and 3 h ($n = 4$), DMS concentrations were measured in the headspace of the containers using solid phase microextraction with 75 μm carboxen-polydimethylsiloxane (PDMS) (Supelco 57318; absorption: 10–30 s, desorption: 10 s, 250°C) on a SRI gas chromatograph (Chromasil 330 column, flame-photometric detector). Data were analyzed separately for the intertidal and subtidal algae after angular transformation to ensure normality and homoscedasticity with a two-way ANOVA (SPSS 11.0). Time and H_2O_2 concentrations were factors. A Tukey's post hoc analysis ($\alpha = 0.05$) was used to compare differences among H_2O_2 treatments.

The effects of hyposaline conditions, an environmental stress known to cause the release of H_2O_2, on DMSP cleavage was examined by incubating both intertidal and subtidal *U. lactuca* in concentrations of 10, 50, and 100% seawater. Disks of *U. lactuca* (~0.2 g; $n = 10$ per treatment) were placed in ~50 mL of either 10, 50, or 100% naturally collected seawater (35‰) that had been produced by mixing full-strength seawater with the appropriate amount of deionized water. After 3 h, the disks were removed from the media, blotted dry, weighed, and dried in a drying oven at 60°C overnight. The following day, the algae were reweighed, and DMSP concentrations were measured as described above. A two-way ANOVA was used to examine the data, with the factors being the algal source and the salinity of the media.

RESULTS

In vivo hydrogen peroxide release and scavenging. Hydrogen peroxide was released by *U. lactuca* following osmotic stress and desiccation. Subtidal specimens that were air-dried for 3 h showed a pronounced release of ROS localized to the apoplastic regions between cells (Fig. 1a). The exogenous addition of catalase (final concentration of 20 $U \cdot mL^{-1}$) was capable of significantly inhibiting ROS detection, implicating H_2O_2 as the major oxidative species (Fig. 1b). The exogenous addition of DMS (final concentration of 5 mM) resulted in a notable decrease in H_2O_2 detection, providing evidence that this DMSP cleavage product can efficiently scavenge H_2O_2 (Fig. 1c).

Hydrogen peroxide was also released when the algae were subjected to hyposaline conditions, but the patterns of release rates differed between intertidal and subtidal individuals (Fig. 2). Very low concentrations of H_2O_2 [<0.2 $\mu mol \cdot g^{-1}$ fresh mass (FM)] were emitted by intertidal algae in half-strength seawater, whereas H_2O_2 production in subtidal algae was nearly an order of magnitude higher after 200 min (Fig. 2). In 10% seawater, maximum H_2O_2 levels were >50% higher in subtidal thalli than in intertidal thalli. Desiccation triggered an increased release of H_2O_2 in both intertidal and subtidal algae when compared with nondesiccated controls (Fig. 3). Subtidal individuals released approximately 25 times more H_2O_2 than intertidal individuals over a 200 min period when desiccated. Intertidal algae were, on average, about twice as effective as subtidal algae at scavenging exogenous H_2O_2 over a 60 min period (Fig. 4). In controls that lacked algae, there was no loss of H_2O_2.

In vitro removal of hydrogen peroxide by algal constituents. Overall, the activities of antioxidant enzymes were significantly higher in intertidal algae when compared with the subtidal conspecifics (Fig. 5). There was no significant interaction between enzyme type and source (two-way ANOVA: $P = 0.101$). The APX, CAT, and GR activities were, on average, approximately 15.5, 3, and 10 times more efficient, respectively, in intertidal than subtidal individuals. However, SOD activities did not significantly differ between the two sample populations (Fig. 5).

Both DMS and acrylic acid were approximately an order of magnitude more effective at scavenging H_2O_2 than their precursor DMSP (Table 1). The antioxidant capabilities of acrylate and DMSP were similar. The antioxidant capability of ascorbic acid exceeded the antioxidant capability of DMSP and all of its breakdown products.

Induction of DMSP cleavage. Tissue DMSP concentrations decreased significantly in both subtidal and

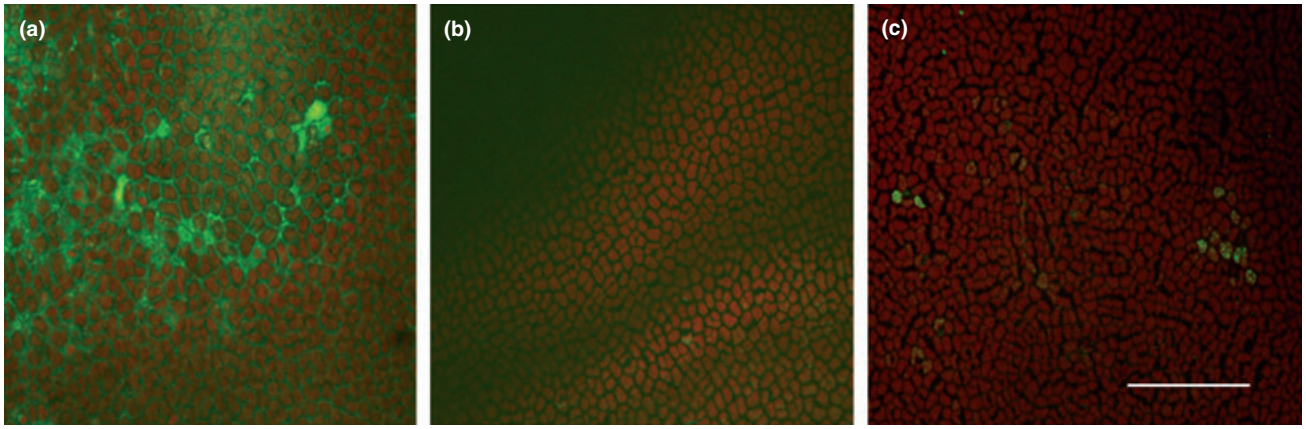

FIG. 1. Detection of reactive oxygen species (ROS) production in *Ulva lactuca* with dichlorodihydrofluorescein diacetate (DCFH-DA) using confocal laser scanning microscopy. Samples were air-dried for 3 h and subsequently analyzed for the production of ROS. (a) Desiccated specimen with strong visualization of chloroplasts shown in red, and ROS production in green. (b) The exogenous addition of catalase (20 U · mL^{-1}, 10 min incubation period postdesiccation treatment) rapidly diminishes the ROS signal. (c) The exogenous addition of dimethyl sulfide (DMS; 5 mM, 10 min incubation period postdesiccation treatment) effectively reduces the ROS signal. The green fluorescence of DCFH-DA was monitored on channel 1 concomitant with the red fluorescence of the chloroplasts on channel 2. The two channels were merged to illustrate the localized H_2O_2 response. Scale bar, 135 μm.

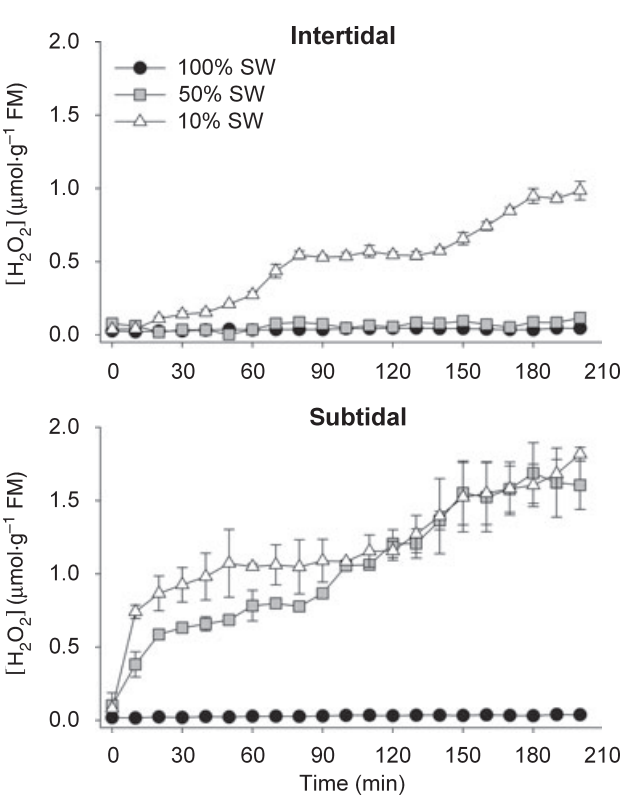

FIG. 2. Mean (±1 SE) concentration of H_2O_2 (as μmol H_2O_2 · g^{-1} fresh mass; n = 10) emitted from intertidal and subtidal *Ulva lactuca* following incubation in 100, 50, and 10% seawater. Repeated measures ANOVA: time × source effect (Pillai's trace = 10815, df = 9, error df = 4, $P < 0.001$); time × salinity effect (Pillai's trace = 33.375, df = 18, error df = 10, $P < 0.001$); time × source × salinity effect (Pillai's trace = 87.426, df = 18, error df = 10, $P < 0.001$). FM, fresh mass; SW, seawater.

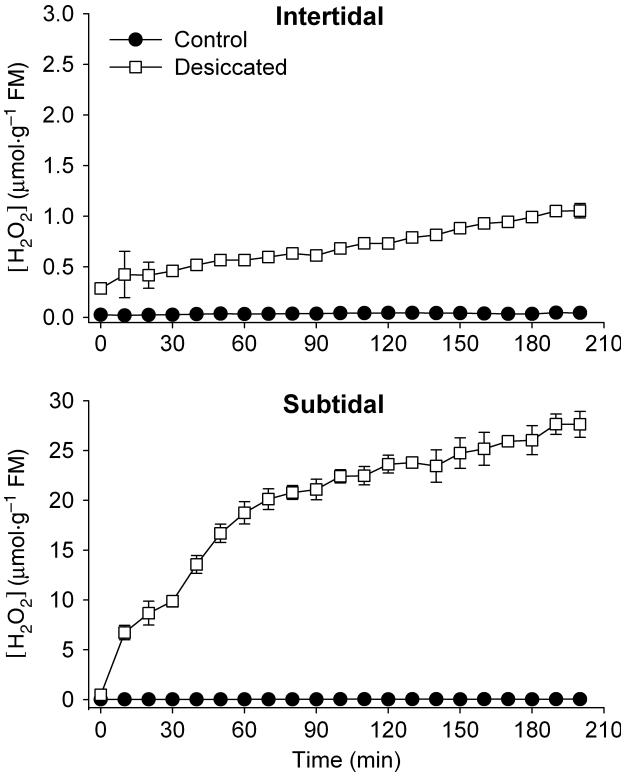

FIG. 3. Mean (±1 SE) concentration of H_2O_2 (as μmol H_2O_2 · g^{-1} fresh mass; n = 10) emitted from intertidal and subtidal *Ulva lactuca* after being air-dried for 3 h and then placed in seawater, and from undesiccated controls that remained in seawater. Repeated measures ANOVA: time × source effect (Pillai's trace = 1,278,740, df = 5, error df = 4, $P < 0.001$); time × desiccation effect (Pillai's trace = 1,420,554, df = 5, error df = 4, $P < 0.001$); time × source × desiccation effect (Pillai's trace = 1,278,734, df = 5, error df = 4, $P < 0.001$). FM, fresh mass.

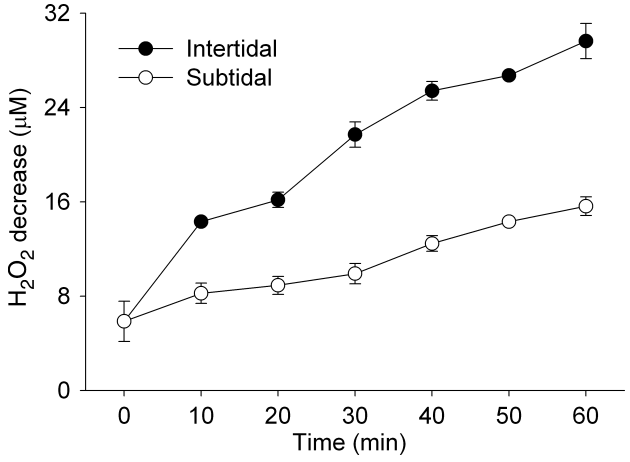

FIG. 4. Mean (±1 SE) H_2O_2-scavenging ability (expressed as the decrease in H_2O_2 as a function of time) of intertidal and subtidal *Ulva lactuca*. Algae were supplemented with 100 μM H_2O_2. As a function of time the surrounding media was sampled to measure the concentration of exogenous H_2O_2 remaining in solution. Repeated measures ANOVA: time × source effect (Pillai's trace = 0.998, df = 3, error df = 2, $P = 0.015$).

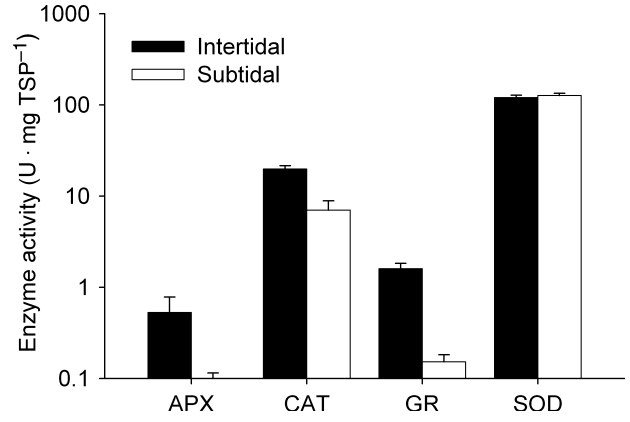

FIG. 5. Mean (±1 SE, $n = 3$) activities of ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD) from intertidal and subtidal *Ulva lactuca*. Activities are significantly different among enzymes (two-way ANOVA, enzyme effect: $P < 0.001$) and are higher in intertidal than subtidal algae (two-way ANOVA, source effect: $P < 0.001$). There was no significant interaction between enzyme type and source (two-way ANOVA: $P = 0.101$). TSP, total soluble protein.

intertidal *U. lactuca* upon exposure to exogenous H_2O_2 (Fig. 6). Significant decreases (Tukey's test: $P < 0.05$) occurred at lower concentrations (2500 μmol) in subtidal individuals than in intertidal individuals (5000 μmol). The relative decrease in DMSP concentrations was lower in intertidal algae.

The amount of DMS released by intertidal individuals increased over time, with the rates of DMS generation being affected by the amount of H_2O_2 to which the algae were exposed (Fig. 7). The pattern was similar but less clear for the subtidal algae. No released DMS was detected at low H_2O_2 concentrations (0 to 100 μmol). Dimethyl sulfide was present in the headspaces of vials containing algae incubated with higher concentrations of H_2O_2, but there was no clear relationship between the concentration of H_2O_2 to which the algae were exposed and the amount of DMS they produced. The DMSP concentrations also decreased significantly in response to decreases in salinity (Fig. 8). However, there was no significant difference in DMSP concentrations between intertidal and subtidal individuals.

TABLE 1. In vitro analysis of the H_2O_2-scavenging ability of metabolites found in *Ulva lactuca*.

Compound	IC_{50} (mM)
DMSP	10
DMS	1.8
Acrylic acid	0.62
Acrylate	10.7
Ascorbic acid	0.01

All concentrations of compounds were incubated with 100 μM H_2O_2 for 10 min. Data are shown for a range of biologically relevant concentrations of dimethylsulfoniopropionate (DMSP), its cleavage products, and ascorbic acid. The concentration of H_2O_2 remaining in solution was quantified as described in the Materials and Methods. Data are the mean inhibitory concentration (IC_{50}) required to consume 50% of the H_2O_2. DMS, dimethyl sulfide.

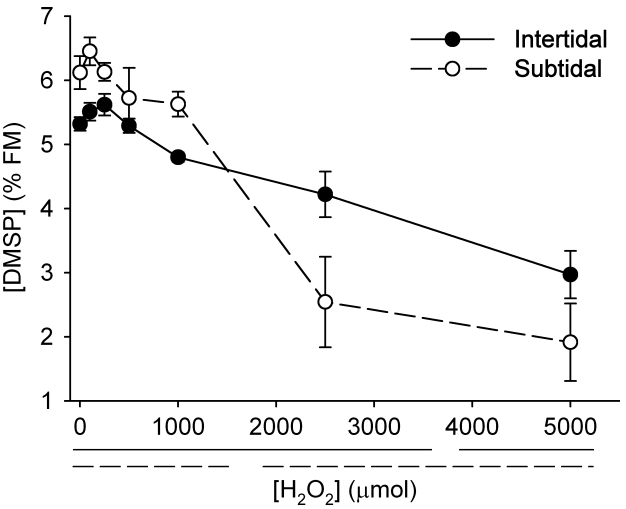

FIG. 6. Mean (±1 SE) dimethylsulfoniopropionate (DMSP) concentration (as % algal fresh mass) 3 h after the introduction of H_2O_2. Lines beneath the graph (solid: intertidal, dashed: subtidal) indicate mean concentrations that are not significantly different ($\alpha = 0.05$) with a Tukey's post hoc test (Intertidal: one-way ANOVA, $P < 0.001$; Subtidal: one-way ANOVA, $P < 0.001$). FM, fresh mass.

DISCUSSION

Differences in hydrogen peroxide production and scavenging between intertidal and subtidal individuals. Our data support the hypothesis that intertidal *U. lactuca*

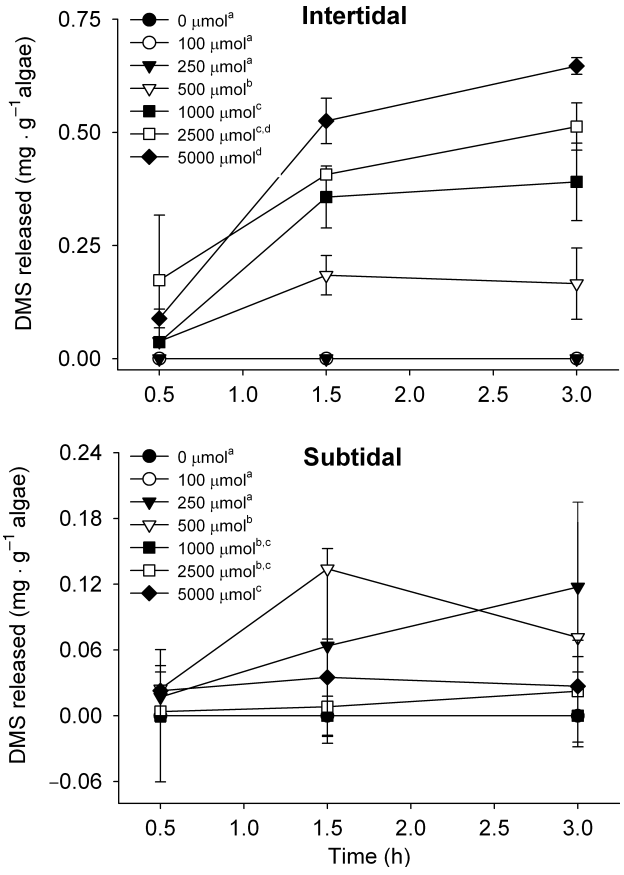

FIG. 7. Mean (±1 SE) amount of dimethyl sulfide (DMS) released (as mg · g^{-1} of algae) over 3 h after the addition of H_2O_2 at a range of concentrations (Intertidal: two-way ANOVA, time effect, $P < 0.001$; H_2O_2 effect, $P < 0.001$; time × H_2O_2 effect, $P < 0.001$. Subtidal: two-way ANOVA, time effect, $P = 0.210$; H_2O_2 effect, $P < 0.001$; time × H_2O_2 effect, $P = 0.130$). Amounts of DMS released denoted with the same superscripts ([a, b, c]) are not significantly different (Tukey's post hoc test: $\alpha = 0.05$).

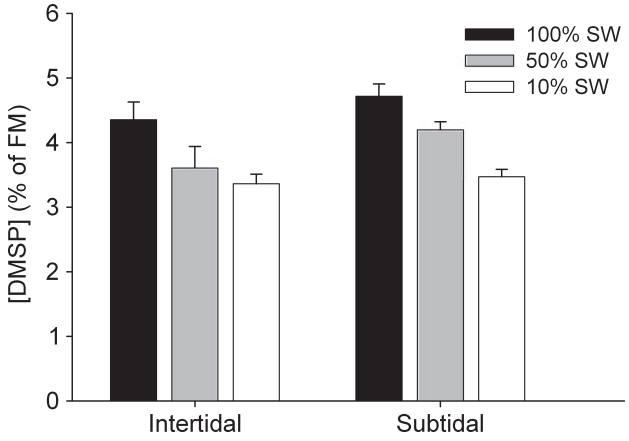

FIG. 8. Effects of immersion for 3 h in different salinity media on mean (±1 SE) *Ulva lactuca* dimethylsulfoniopropionate (DMSP) concentrations (as % fresh mass) in intertidal and subtidal individuals. Salinity had a significant effect on DMSP concentrations, but the source of the algae did not (two-way ANOVA salinity effect: $P < 0.001$; source effect: $P = 0.836$; interaction effect: $P = 0.331$). FM, fresh mass; SW, seawater.

plants, which should encounter environmental stresses more frequently than conspecific subtidal individuals, produce lower amounts of H_2O_2 when stressed (Figs. 2 and 3) and are more efficient at removing it (Fig. 4). It should be noted that these conclusions are drawn from a comparison of only two sites, and that studies across multiple pairs of sites will be needed to determine whether this is a general pattern. However, the data are consistent with the results of other studies that have examined ROS production and the activities of antioxidant enzymes in individuals subjected to environmental stresses (Lu et al. 2006).

Intraspecific comparisons of ROS production in marine macroalgae have found that ROS production is lower following stress in algae acclimated to stressful environments. *Fucus vesiculosus* that was acclimated to 0°C continuously or to 0°C for 21 h per day produced less reactive oxygen after being exposed to temperatures below the freezing point for 6 h than conspecifics maintained at 20°C (Collén and Davison 2001). However, a contradictory pattern was observed in interspecific comparisons of intertidal algae from the Baltic Sea. Hydrogen peroxide excretion following temperature stress was higher in *Cladophora glomerata* (L.) Kütz., an annual and presumably more-stress-tolerant macroalga, than in the ephemeral and presumably less-stress-tolerant alga *Enteromorpha ahlneriana* Bliding (Choo et al. 2004).

The reasons why ROS, such as H_2O_2, are produced when algae are stressed are not fully understood. Regardless of why exogenous ROS are produced, their continued presence near the thallus will have detrimental effects on the algae if they are not rapidly removed (Collén and Pedersén 1996, Kim et al. 1999, Dummermuth et al. 2003, Pinto et al. 2003). In this study, *U. lactuca* from an intertidal habitat that were regularly exposed to physiological stresses removed H_2O_2 more efficiently than subtidal conspecifics that would encounter these stresses much less frequently (Fig. 4). Similar trends have been noted in interspecific comparisons. For example, the North Atlantic red alga *Mastocarpus stellatus* (Stackh.) Guiry scavenges exogenous H_2O_2 more rapidly than *Chondrus crispus*, which occurs lower in the intertidal zone than *M. stellatus* (Collén and Davison 1999c).

We determined that intertidal *U. lactuca* plants were able to dissociate exogenous H_2O_2 more efficiently, in part, because they had higher activities of several antioxidant enzymes (Fig. 5). The two enzymes that showed the largest relative difference between the two sites were APX and GR. Catalase had a proportionately smaller difference, and there was little difference in SOD activity. Previous studies have shown that APX scavenges more H_2O_2 than CAT in *Ulva rigida* C. Agardh (Collén and Pedersén 1996). This difference in scavenging rates may account for the large difference in APX and the

relatively smaller difference in CAT activities at the two sites in our study. Similar trends in antioxidant activities were found in *M. stellatus* populations, with the exception that SOD activities were significantly lower in individuals collected from lower in the intertidal zone and APX activities were similar (Collén and Davison 1999c).

The differences in enzyme activities between the two habitats suggest that enzyme activity is phenotypically plastic and is being modified in response to environmental cues, and/or that localized selection is occurring. Localized selection would occur if there was a cost of producing antioxidant enzymes and individuals were selected against for producing inadequate levels of enzyme activity for their environment. Phenotypic plasticity in antioxidant enzyme activities in response to a variety of stressors has been documented in many higher plants (Apel and Hirt 2004). There is also evidence that antioxidant enzyme activities in marine algae are altered in response to changes in environmental conditions (Collén and Davison 2001).

Tissues of algae from the more stressful intertidal habitat contained slightly but consistently lower amounts of DMSP, which suggests that individuals living in stressful environments do not increase their tissue concentrations of DMSP in response to the types of stresses encountered in the intertidal zone. The lack of higher amounts of DMSP in intertidal *U. lactuca* suggests that concentrations of this compound are not altered in response to changes in environmental conditions. Small ROS-scavenging molecules, such as DMSP may be less expensive to produce and store than antioxidant enzymes. In other algae, differences in the amounts of antioxidant compounds among high and low intertidal populations were mixed, with some compounds being in greater concentrations in the higher intertidal algae, some being lower in concentrations, and others showing no differences (Collén and Davison 1999c). If the costs of producing these molecules are minimal, then the concentrations of the compounds may be constitutive. The DMSP concentrations in *U. lactuca* from the northeastern Pacific vary among sites but tend not to change in response to changes in environmental conditions (Van Alstyne et al. 2001, Van Alstyne et al. 2007). However, DMSP concentrations in other ulvoid algae do change in response to changes in light (Karsten et al. 1990, 1992) and salinity (Edwards et al. 1987, 1988, Karsten et al. 1992), suggesting that the degree of plasticity may be species specific or different for stimuli of different types or magnitudes.

DMSP as an antioxidant. While the biological roles of DMSP and its cleavage products are not completely understood, some of their proposed functions in algae have included osmoregulation (Dickson et al. 1980, Reed 1983a,b, Edwards et al. 1987, 1988), sulfur detoxification (Havill et al. 1985), and cryoprotection (Kirst et al. 1991, Karsten et al. 1992, 1996). The DMSP cleavage reaction may also be involved in an antiherbivore activated defense in *U. lactuca* (=*U. fenestrata*; Hayden and Waaland 2004) from the northeastern Pacific (Van Alstyne et al. 2001, Van Alstyne and Houser 2003). In the current study, we provide evidence that the cleavage of DMSP has an additional function in macroalgae (i.e., serving as a ROS scavenger to prevent oxidative damage). Given the diversity of the functions suggested for DMSP and the geographic and taxonomic breadth of species it occurs in, it is likely that DMSP has different functions in different species or locations, and that it may have multiple functions in many, if not most, species.

As in phytoplankton (Sunda et al. 2002), the use of DMSP as an antioxidant involves the cleavage of DMSP. The DMSP is a weak antioxidant relative to DMS and acrylic acid (Table 1). Enzymatic cleavage of DMSP can rapidly produce stronger antioxidants; however, this may come at a cost as both DMS and acrylic acid are bioactive (Sieburth 1960, Van Alstyne et al. 2001, Van Alstyne and Houser 2003) and could, potentially, be harmful to the alga producing them.

The cleavage products DMS and acrylate work as radical scavengers via different mechanisms. In controlled in vitro experiments, we found that DMSP is capable of acting as an H_2O_2 scavenger with an IC_{50} (inhibitory concentration required to consume 50% of the H_2O_2) of 10 mM using a fluorometric hydrogen peroxide assay (Table 1). The cleavage product DMS was an order of magnitude more potent (Fig. 1C; Table 1). The increased activity of DMS may be attributed to the fact that DMS is an electron-rich sulfide, which can be oxidized to the corresponding sulfoxide and eventually to sulfone (Librando et al. 2004). Hydrogen peroxide is routinely utilized to oxidize sulfides to sulfoxides and sulfones in organic chemical synthesis reactions (Chu and Trout 2004). As DMSP is an electron-deficient species, it will most likely not be readily oxidized to the corresponding sulfoxide.

Acrylic acid (IC_{50} 0.62 mM) was substantially more potent than its conjugate base acrylate (IC_{50} 10.7 mM). Acrylic acid's scavenging ability is based on its ability to donate a hydrogen radical to the peroxide radical to form water as a by-product, leaving a resonance-stabilized peroxy acid radical. As acrylate lacks a proton, it cannot undergo this mechanism and is therefore a less-efficient free-radical scavenger. It should be noted that DMSP and its cleavage products were shown to be one to two orders of magnitude less potent than the well-studied antioxidant ascorbic acid. However, given the abundant availability of DMSP (occurring at ~35 mM in *U. lactuca*) and its cleavage products, significant antioxidant levels can certainly be achieved.

It is notable that DMSP concentrations did not significantly decrease in the algae until they were exposed to relatively high concentrations of H_2O_2

(Fig. 6), indicating that high amounts of ROS were required to initiate DMSP cleavage. Desiccation of subtidal samples induced high concentrations of H_2O_2 (Figs. 1a and 3), and it is possible that these localized high concentrations are sufficient to initiate DMSP cleavage in cells immediately adjacent to the sites of H_2O_2 production. Stress susceptibility (characterized by increased H_2O_2 levels) could cause more DMSP to be cleaved to DMS and acrylate, which in turn would act as efficient antioxidants. *Ulva lactuca* may use DMSP cleavage as a maintenance method to minimize oxidative damage only when ROS levels are so high that they cannot be sufficiently lowered by antioxidant enzymes or other antioxidant compounds. This could be less time consuming and more energetically favorable than up-regulating a suite of antioxidant enzymatic machinery.

Subtidal individuals began cleaving DMSP at lower concentrations of H_2O_2 than intertidal ones (Fig. 6). This finding again suggests that intertidal individuals may be better adapted physiologically to coping with environmental stresses than subtidal ones. Assuming that the primary function of DMSP and its cleavage products in these populations is the elimination of ROS, intertidal individuals may need less DMSP and may cleave it less readily because they produce lower amounts of H_2O_2 when stressed (Figs. 2 and 3) and because their antioxidant enzymes are more active (Fig. 5).

The results presented in this study strongly support the hypothesis that *U. lactuca* is capable of regulating its antioxidant machinery to deal with the demanding environmental challenges commonly associated with intertidal habitats, and that DMSP and its enzymatic cleavage products are capable of contributing to the ROS elimination by serving as antioxidants. These results contribute to the understanding of the functional roles of DMSP as well as the ecophysiology of *U. lactuca* in marine environments.

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