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The main nitrate transporter of the dinoflagellate *Lingulodinium polyedrum* is constitutively expressed and not responsible for daily variations in nitrate uptake rates

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ABSTRACT

Dinoflagellates are unicellular eukaryotes capable of forming spectacular harmful algal blooms (HABs). Eutrophication of coastal waters by fertilizer runoff, nitrate in particular, has contributed to recent increases in the frequency, magnitude and geographic extent of HABs. Although physiological nitrate uptake and assimilation in dinoflagellates have often been measured in the field and in the laboratory, no molecular components involved in nitrate transport have yet been reported. This study reports the first identification and characterization of dinoflagellate nitrate transporters, found in the transcriptome of the bloom-forming *Lingulodinium polyedrum*. Of the 23 putative transporters found by BLAST searches, only members of the nitrate transporter 2 (NRT2) family contained all key amino acids known to be essential for nitrate transport. The dinoflagellate NRT2 sequences have 12 predicted transmembrane domains, as do the NRT2 sequences of bacteria, plants and fungi. The NRT2 sequences in *Lingulodinium* appear to have two different evolutionary origins, as determined by phylogenetic analyses. The most expressed transcript of all putative nitrate transporters was determined by RNA-Seq to be LpNRT2.1. An antibody raised against this transporter showed that the same amount of protein was found at different times over the light dark cycle and with different sources of N. Finally, global nitrate uptake was assessed using a ^{15}N tracer, which showed that the process was not under circadian-control as previously suggested, but simply light-regulated.

1. Introduction

Dinoflagellates are unicellular eukaryotes found in most marine and freshwater ecosystems. While members of this group contains important primary producers and, in the case of the genus *Symbiodinium*, are essential for the survival of tropical reef corals (Davy et al., 2012), dinoflagellates are also infamous because some species can form harmful algal blooms (HABs). These blooms can cause illness or death to aquatic wildlife, damaging ecosystems and negatively impacting tourism and fish industries. Public health is also threatened by toxic blooms, because human consumption of toxin-contaminated seafood can result in intoxication or even death in extreme cases. Some HAB species, such as *Karenia brevis* in the Gulf of Mexico, secrete neurotoxins that have an airborne component and can mix with marine aerosols (Pierce et al., 2003). When driven by winds, people onshore inhaling the toxic sea spray can suffer severe respiratory irritations. Thus, it is a growing concern that HAB events have globally increased in frequency, magnitude and geographic extent over the last 40 years (Anderson et al., 2012). It also justifies the heightened attention scientific and governmental authorities are giving to research on bloom dynamics and the causes leading to HAB expansions (Anderson et al., 2012).

While bloom formation and its persistence involve a complex interplay of biotic and abiotic factors (Anderson et al., 2012), increases in nutrients, particularly phosphorus (P) and nitrogen (N), has often been positively correlated with HABs in various coastal regions of the world (Anderson et al., 2008; Bodeanu and Ruta, 1998; Okaichi, 1997). A recent example is the Changjiang River in China where agricultural runoffs have contributed to a dramatic ~400% increase in nitrate concentration between 1960s and 2004, while phosphate concentration increased by ~30% between 1980s and 2004 (Zhou et al., 2008). As a result, in a 20 year-period there was a ~4-fold augmentation in phytoplankton standing stocks. Also during the same period, the initially diatom-dominated communities started to shift toward dinoflagellates, which is consistent with

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the observation that diatoms are poor competitors to flagellates when the N:P ratio is high (Egge, 1998). The most striking consequence of the Changjiang eutrophication was the difference in number of reported HABs in the adjacent coastal waters of the East China Sea, that passed from 2 events before 1980s to ~30–80 HABs per year between 2001 and 2005 (Zhou et al., 2008). Because these recent HABs are mainly caused by photosynthetic dinoflagellates (Zhou et al., 2008), these organisms must have very efficient ways in utilizing nitrate for rapid proliferation.

Since nitrate is the most abundant form of bioavailable N in most aerobic soils and marine environments (Crawford and Forde, 2002; Gruber, 2008), its uptake is expected to control the majority of N that can be assimilated for most organisms. This explains the intensive effort over the past several decades to isolate and characterize the genes responsible for nitrate transport in many phylogenetic groups, including bacteria (Moir and Wood, 2001), fungi (Unkles et al., 1991; Unkles et al., 2001), and green plants (Krapp et al., 2014; Tsay et al., 2007). The alveolates, among which are found the dinoflagellates, are a noteworthy exception. The rationale for the molecular characterizations is that nutrient uptake results from the combined action of multiple transporters each with particular enzymatic kinetics, regulation patterns and crosstalk, and that once transporter properties are defined, an overall system can be developed to help predict the behaviour of an organism under fluctuating nutrient conditions. Thus, characterization of dinoflagellate nitrate transporters could complement physiological and field studies in understanding how N uptake influences HABs.

Four gene families have been shown to possess nitrate transport activity in eukaryotes: the nitrate transporter 1/ peptide transporter (NPF) (previously NRT1/PTR), the nitrate transporter 2 (NRT2) of the major facilitator superfamily (MFS), the chloride channels (CLCs) and the slow anion channel-associated 1 homologues (SLAC1/SLAH) (Krapp et al., 2014). Although not working as a transporter alone, nitrate assimilation related (NAR2) is a small protein that directly interacts with several NRT2 (Kotur et al., 2012; Orsel et al., 2006). The NAR2 protein stimulates nitrate uptake of all seven *Arabidopsis* NRT2 (Kotur et al., 2012), and interaction is mandatory for NRT2.1 of the green alga *Chlamydomonas reinhardtii* where nitrate-elicited currents are only detected when both proteins are co-expressed in *Xenopus* oocytes (Zhou et al., 2000a). In contrast, a well characterized NRT2 from the fungus *Aspergillus nidulans* (NrtA, previously CrnA), functions without NAR2 (Zhou et al., 2000b). Members of the NPF and NRT2 families can be found in all kingdoms of life (Leran et al., 2014), except for animals that lack NRT2 proteins (Slot et al., 2007). The CLCs are also ubiquitous in nature (Jentsch, 2008), but nitrate transport has only been demonstrated for two members, both found in *Arabidopsis* (De Angeli et al., 2006; von der Fecht-Bartenbach et al., 2010). The AtCLC-a protein was shown to be a nitrate/proton antiporter involved in the accumulation of nitrate into the plant tonoplast (De Angeli et al., 2006), while AtCLC-b showed a similar activity after heterologous expression in *Xenopus* oocytes (von der Fecht-Bartenbach et al., 2010). The proteins AtSLAC1 and AtSLAH3 also displayed nitrate transport activity when expressed in oocytes (Geiger et al., 2011; Geiger et al., 2009), but they are the only members of the SLAC1/SLAH family that possess this function. These observations suggest that dinoflagellate nitrate transporters are more likely to belong to the NPF and NRT2 families than to the CLCs or SLAC1/SLAH.

The non-toxic marine dinoflagellate *Lingulodinium polyedrum* forms HABs along the Southern California coast (Kudela and Cochlan, 2000; Lewis and Hallet, 1997). Success of this organism during the upwelling season was attributed in part to its ability to migrate into nitrate-rich subsurface waters at night and assimilate nitrate in the dark (Harrison, 1976). Curiously, nitrate uptake was found to have a strong diel rhythmicity, with the peak observed in the middle of the day (Harrison, 1976). Furthermore, a circadian peak in nitrate reductase (NR) activity was also found during the day (Ramalho et al., 1995). It has been suggested that nitrate uptake could be controlled by an endogenous clock in *Lingulodinium* (Roenneberg and Rehman, 1996), but experimental data supporting this hypothesis has not yet been reported. Thus, the first aim of this study was to test for circadian nitrate uptake for *Lingulodinium* grown in constant light. The second aim was to identify putative dinoflagellate nitrate transporters from a recently published *Lingulodinium* transcriptome database (Beauchemin et al., 2012) and to characterize the most promising candidates. The final aim was to test the hypothesis that changes in nitrate uptake rates resulted from changes in transporter levels.

2. Materials and methods

2.1. Cell culture

2.1.1. Initial conditions

Unialgal but not axenic *Lingulodinium polyedrum* (CCMP 1936, previously *Gonyaulax polyedra*) was obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota (East Boothbay, ME, USA). For all experiments, cell cultures were initially grown in normal f/2 medium prepared using Instant Ocean under 12 h light (40 μmol photons m^{-2} s^{-1} cool white fluorescent light) and 12 h darkness at a temperature of 18 ± 1 °C. Cells were harvested by filtration on Whatman 541 paper and stored at −80 °C until use.

2.1.2. Daily and circadian nitrate uptake measurements

Nitrate uptake was monitored using a stable isotope analysis. Culture aliquots were spiked with ^{15}N-labeled $NaNO_3^-$ (98 atom % ^{15}N, Sigma-Aldrich) at different times during a light dark cycle (ZT; Zeitgeber times) or during constant light (CT; Circadian times) for 1 h durations. In the first experiment (ZT), cultures were grown under a 12:12 light/dark regime, with ZT0 corresponding to lights on and ZT12 to lights off. In the second experiment (CT), cultures were grown in continuous light for two days, with $Na^{15}NO_3^-$ incubations and cell harvests made on the second day. For both experiments, cell cultures were filtered on Whatman 541 filters and transferred to an f/2 medium supplemented with half the amount of $NaNO_3^-$ normally included in this medium (normal concentration = 880 μM). These ~1.5-L cultures were divided into multiple 150 ml aliquots and incubated under light dark or constant light conditions. $Na^{15}NO_3^-$ was added to the cultures to a final concentration of 440 μM at different times and cells were harvested 1 h later. Data are reported as δ ^{15}N, which represent the ^{15}N:^{14}N ratio in cells after one hour of ^{15}N accumulation relative to the same ratio in atmospheric air. In these experiments, specific nitrate uptake rates (V) were not calculated because the amount of ^{15}N used as a source was higher than levels accepted by the commercial facility (see below) performing the isotopic analysis. The δ ^{15}N is nonetheless a good approximation of nitrate uptake rates, since all variables except the time of sampling under the LD cycle were held constant. Samples without added ^{15}N have a δ ^{15}N of −5.6 ± 0.1.

2.1.3. Expression of LpNRT2.1 protein

Cell cultures were grown in one of four different media: (1) f/2, which is normally supplemented with 880 μM $NaNO_3$ (f/2 + NO_3^-); (2) f/2-N (or N-depleted), which is f/2 lacking added $NaNO_3$; (3) f/2 + NO_2^-, which is f/2-N supplemented with 10 μM $NaNO_2$; and (4) f/2 + NH_4, which is f/2-N supplemented with 40 μM NH_4Cl. To compare cell growth in media with different N, normal f/2 cultures were filtered on Whatman 541 paper, washed