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Inhibition of nitrogenase by oxygen in marine cyanobacteria controls the global nitrogen and oxygen cycles

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Abstract

Cyanobacterial N_2-fixation supplies the vast majority of biologically accessible inorganic nitrogen to nutrient-poor aquatic ecosystems. The process, catalyzed by the heterodimeric protein complex, nitrogenase, is thought to predate that of oxygenic photosynthesis. Remarkably, while the enzyme plays such a critical role in Earth's biogeochemical cycles, the activity of nitrogenase in cyanobacteria is markedly inhibited *in vivo* at a post-translational level by the concentration of O_2 in the contemporary atmosphere leading to metabolic and biogeochemical inefficiency in N_2 fixation. We illustrate this crippling effect with data from *Trichodesmium* spp. an important contributor of "new nitrogen" to the world's subtropical and tropical oceans. The enzymatic inefficiency of nitrogenase imposes a major elemental taxation on diazotrophic cyanobacteria both in the costs of protein synthesis and for scarce trace elements, such as iron. This restriction has, in turn, led to a global limitation of fixed nitrogen in the contemporary oceans and provides a strong biological control on the upper bound of oxygen concentration in Earth's atmosphere.

1. Introduction

Only a small fraction of prokaryotic organisms from the bacterial and archaeal domains can procure and utilize atmospheric nitrogen by reducing it to ammonia. This process is critical in supplying nitrogen for further biological activity in N-limited environments such as many regions of the modern oceans. Investment in nitrogen fixation is an expensive metabolic process which demands large inputs and maintenance requirements of energy, reducing power, antioxidant enzymes and metal co-factors such as iron and Mo (Raven, 1988; Kustka et al., 2003). Moreover, biological nitrogen fixation, and specifically the nitrogenase enzyme, is notorious for its sensitivity to molecular oxygen. Nitrogenase consists of two proteins, the iron protein containing a single Fe_4S_4 cluster bound between subunits, and the iron-molybdenum protein composed of two types of

clusters, the P cluster and the FeMoco center (sometimes called the M cluster) which is the site of N_2 reduction. Whereas both the Fe_4S_4 and P clusters are inactivated by O_2, the Fe_4S_4 cluster is much more susceptible and irreversibly damaged *in vitro* (Burgess and Lowe, 1996). Moreover, high oxygen stress causes proteolysis of nitrogenase subunits (Durner et al., 1996), suppresses nitrogenase synthesis, and leads to a shortage of respiratory substrates and reductants necessary for nitrogen fixation and assimilation (Gallon, 1992). Inhibitory effects of moderate levels of oxygen, or short exposure times, *in vivo* may be reversed, leading to an increase in nitrogen fixation rates (Prosperi, 1994; Ludden and Roberts, 1995; Pan and Vessey, 2001; Yakunin et al., 2001) and, in some diazotrophs, post-translational modification of the Fe protein from an inactive to active form (Sweet and Burris, 1982; Jouanneau et al., 1983; Ernst et al., 1990; Ohki et al., 1991; Zehr et al., 1993). The level of resistance to oxygen stress and the mechanisms involved vary between diazotrophs (Gallon, 1992; Tuli et al., 1996; Berman-Frank et al., 2003). Furthermore, diazotrophic cyanobacteria, which provide the bulk of fixed nitrogen to the surface oceans, are the only diazotrophs that actively produce oxygen via photosynthesis and must contend with further restrictions on the nitrogen (Gallon, 1992; Tuli et al., 1996; Bergman et al., 1997; Berman-Frank et al., 2003). Thus, nitrogenase in the real-world operates at only a fraction of its potential activity, yet is a major elemental taxation on diazotrophic cyanobacteria both for scarce trace elements, such as iron, and in the costs of protein synthesis. These taxes have, in turn, led to a global limitation of fixed nitrogen in the oceans (Falkowski, 1997). We examine these constraints with data from the bloom-forming marine cyanobacterium *Trichodesmium* IMS101 which contributes significantly to nitrogen fixation in the tropical and sub-tropical oceans.

2. Materials and methods

2.1. Culture growth conditions

Trichodesmium IMS101 cultures were grown and maintained as described previously (Berman-Frank et al., 2001b). Oxygen concentrations were adjusted according to requirement, supplemented with ambient CO_2 concentrations (0.035%) and a balance of nitrogen gas and bubbled into the cultures at a saturating rate for the necessary experimental duration.

2.2. Nitrogenase activity

Nitrogen fixation rates for *Trichodesmium* were measured as described previously (Berman-Frank et al., 2001a) using the acetylene reduction method according to Capone (Capone, 1993). Ethylene production was measured on a SRI 310 gas chromatograph with a flame ionization detector and quantified relative to an ethylene standard. Results were normalized to carbon and chlorophyll.

2.3. Western blots

Proteins were extracted according to Chen et al. (1998), and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 8–16% gradient gels (Gradipore iGels). After transfer to a polyvinylidend difluoride membrane (PVDF – Millipore), the proteins were challenged with antisera raised against the Fe protein of nitrogenase (generously provided by P. Ludden, U.California Berkley) conjugated to IgG HRP and visualized using SuperSignal® chemiluminescent substrate for HRP detection (Pierce).

2.4. RNA analysis

RNA extraction and northern analysis were carried out based on methods described previously (Chen et al., 1999). Briefly, equal volume of from each treatment were fil-

tered and immediately lysed in RNA extraction buffer (5% Triton X-100, 10% sucrose, 20 mM EDTA, 50 mM Tris, 100 mM DTT). The subsequent total RNA processing was performed following commonly used phenol/chloroform extraction protocols. Northern blot analyses were carried out following widely used protocols with some modifications (Chen et al., 1999). Total RNA extracted from equal amounts of biomass was fractionated by electrophoresis on a 1% agarose gel with 1 M formaldehyde. The RNA was then transferred to a charged nylon membrane (Nytran, Schleicher & Schuell) using a TurboBlottor System (S & S). All Northern blots were initially hybridized to a specific *nifH* probe. Probes were labeled with α^{32}P-dATP using Promega Prime-A-Gene Labeling System. All hybridization was performed using Ambion PerfectHyb system. The blots were washed twice with 0.2×SSC and 0.1% SDS, followed by 30 min incubation at 42°C before being exposed to Kodak BioMax MR/MS films.

3. Results and discussion

To highlight the effect of O_2 concentration on the cost of nitrogen fixation in cyanobacteria, we illustrate these restrictions with data from the bloom-forming marine cyanobacterium *Trichodesmium* IMS101. *Trichodesmium* is a colonial, non-heterocystous diazotroph which separates oxygenic photosynthesis and nitrogen fixation spatially and temporally within the photoperiod (Berman-Frank et al., 2001b). Sequence and structural analyses of its nitrogenase are similar to those of other organisms (Zehr et al., 1997a). Previous work showed that nitrogenase inhibition by oxygen may be reversed when cells are exposed for short (~30 min) periods to oxygen levels of 40% or lower (Zehr et al., 1993). This quick response to both activation and inhibition indicates that new transcription and synthesis of the nitrogenase protein are not essential for enhanced activity and that constitutive protein levels are present that can be "turned-on" when oxygen concentrations are lowered.

We verified this by assaying the abundance of *nifHDK* transcripts and the corresponding gene product in *Trichodesmium* cultures incubated for 5 h with 0, 5, 21

(present atmospheric level – PAL), and 50% O_2 (Fig. 1). Incubations were performed after circadian- controlled daily transcription began. The results indicate that extracellular O_2 does not significantly influence gene transcription or protein synthesis of nitrogenase on this time scale except at very high (50%) oxygen concentrations (Figs. 1a and 1b). In contrast, nitrogenase activity was strongly depressed by PAL and higher levels of O_2; at 50% O_2>90% of the nitrogenase activity was inhibited within 1 h (Fig. 1c).

The limited sensitivity of *nifHDK* transcription, and nitrogenase protein to oxygen concentrations was previously observed in *Trichodesmium* (Dominic et al., 1998) and other cyanobacterial diazotrophs which exhibit a similar behavior. In *Anabaena* sp. PCC 7120 oxygen concentration required to repress *nifHDK* transcription was greater than that required to destroy nitrogenase activity (Elhai and Wolk, 1991). In *Gloeothece* sp. and *Oscillatoria limosa* high oxygen stress did not repress protein synthesis of nitrogenase (Gallon, 1992).

In *Trichodesmium*, as in other diazotrophs (Sweet and Burris, 1982; Jouanneau et al., 1983; Hallenbeck, 1992), a modification of nitrogenase Fe-protein, distinguishes an active and an inactive form of the protein (Ohki et al., 1991; Zehr, 1993; Chen, 1998). Our results concur with other studies that the total amount of nitrogenase Fe-protein does not change rapidly in response to reduced oxygen and only when oxygen levels were very high (50%) did the protein abundance decrease. Addition of the protein-synthesis inhibitor, chloramphenicol (CAMP), for 4 h resulted in demonstratively little change in protein levels under all oxygen concentrations (results not shown),

Post-translational modification of activity appears relatively common in diazotrophic cyanobacteria and operates at much lower concentrations of oxygen then those required for repression of *nif* genes (Tuli et al., 1996; Dominic et al., 1998). A compilation of published data on the response of nitrogen fixation to varying ambient O_2 concentrations suggests a general relationship that is consistent with both the short term response of the enzyme to O_2 and the organisms' longer evolutionary adaptations where O_2 is used as sink for electrons in respiration (Fig. 1d). In *Trichodesmium* maximum nitrogen fixation occurs at oxygen concentrations of 2 to 5%. In an anaerobic or mi-

croaerobic milieu, respiratory requirements are not met and substrates essential for nitrogen fixation are not produced, causing a decline in the enzyme's performance. As oxygen concentrations increase above 5%, the enzyme activity is inhibited, with only ~70% activity measured at atmospheric oxygen concentrations of 21%. At oxygen concentrations greater than 30%, a sharp drop in activity reduces fixation efficiency (potential) to <10% within minutes. This pattern of a "chronically crippled" nitrogenase is corroborated in data from other aerobic cyanobacteria such as *Anabaena* and *Gloethece* (Fig. 1d). In these cyanobacteria, lowering the oxygen below atmospheric concentrations also enhances relative nitrogenase activity at short durations (as in Fig. 1c). However, at very low oxygen concentrations, respiratory performance and the production of energy and substrates for nitrogen fixation is affected, thus lowering nitrogen-fixation rates and the overall metabolic performance of *Trichodesmium* as we observed with the enhanced mortality and biomass crash under the prolonged exposure to the reduced oxygen concentrations (Fig. 2).

Nitrogenase may account from 10% (Thorneley and Ashby, 1989) to 40% (Jouanneau et al., 1985) of total cellular protein and is a significant sink for Fe in diazotrophs. Accordingly, the "chronic crippling" of the enzyme at PAL levels of O_2 imposes a metabolic inefficiency that reflects an extraordinarily slow tempo of evolution for this critical biogeochemical process. The high conservation within the primary sequence of *nif* genes (Zehr et al., 1997b; Zehr et al., 2003; Raymond et al., 2004) indicates that the evolutionary risks associated with modifying nitrogenase outweigh the costs of its production. The inefficiency in nitrogenase is analogous to other core metabolic proteins, such as the reaction center protein of photosystem II, D1, and ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco), which also evolved under anaerobic conditions; all three either operate at a fraction of their capacities under ambient atmospheric conditions or undergo extremely high rates of turnover as a result of post-translational damage. The inhibition of all of these proteins, either directly by elevated O_2, or indirectly through reactive oxygen species, potentially exerts a strong biological control on the upper bound of the concentration of the gas in Earth's atmosphere.

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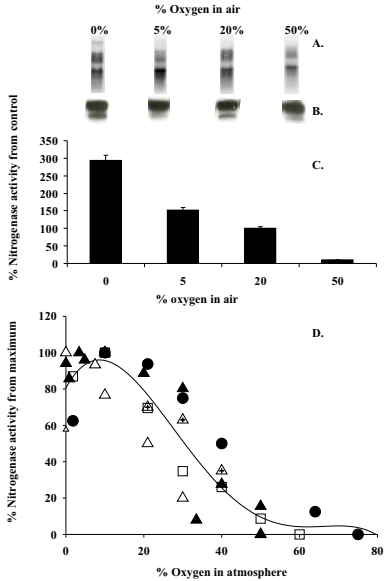

Fig. 1. (a) *nifHDK* transcript abundance for *Trichodesmium* IMS101 cultures bubbled with nitrogen (0% oxygen), 5%, 20%, and 50% O_2 for 5 h. **(b)** Western blots of nitrogenase protein abundance (challenged with universal Fe-protein polyclonal antibodies) for the above experiment **(c)**. Nitrogenase activity (presented as % of air-control) measured by acetylene reduction for the above experiment 1 h after induction of bubbling. **(d)** Relationship between O_2 concentrations and nitrogen fixation (% from maximum rates) for *Trichodesmium* IMS101 and other diazotrophs. Filled triangles: experimental data from our cultures of *Trichodesmium* IMS101; empty triangles with cross – *Trichodesmium* NIBB1067 (Ohki and Fujita, 1988), empty triangles – *Trichodesmium* spp. field populations (Mague et al., 1977; Saino and Hattori, 1982), black circles – *Anabaena cylindrical* Gallon et al., 1993), black squares – *Gloeothece* (Nageli) (Gallon et al., 1993). Short term (1–2 h) anaerobic incubation yields maximum nitrogenase activity for aerobic diazotrophs (Fig. 1c). Under prolonged exposure, respiratory requirements are essential and yield maximum nitrogenase activity (*in vivo*) at microaerobic oxygen concentrations.

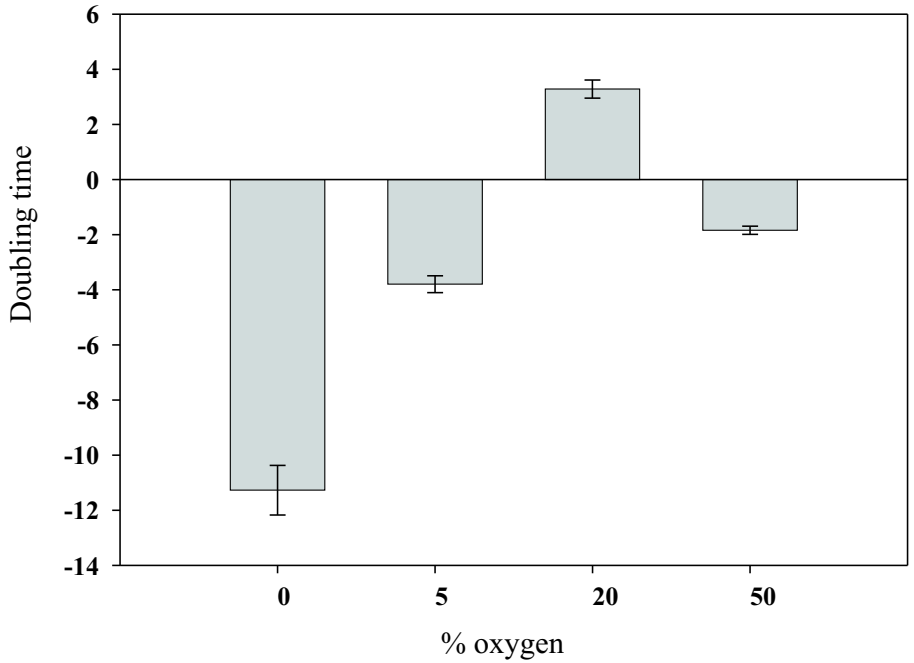

Fig. 2. Effects of varying oxygen concentration on *Trichodesmium* IMS101 growth. Cultures grown under air were transferred to different oxygen concentrations (+0.035% CO_2 + balance N_2) and incubated for 72 h under these conditions. Carbon specific growth or mortality rates (doubling times – days) were determined for cultures after 3 days. Negative growth or biomass decline was measured for all cultures except those under air. Error bars are standard deviations from the average of 3 replicate bottles per treatment.