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Vitamin B_{12} excretion by cultures of the marine cyanobacteria *Crocosphaera* and *Synechococcus*

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Abstract

Axenic cultures of the N_2-fixing marine cyanobacterium *Crocosphaera watsonii* exhibit very high B_{12} excretion rates (up to 40 times higher per cell), compared to the smaller non–N_2-fixing strain of marine *Synechococcus*. The effect of N availability on vitamin synthesis is also evident in the non-diazotrophic strain, as they release five times more B_{12} under N-replete conditions relative to N-limiting conditions, although this value is still an order of magnitude smaller than the amount produced by the diazotroph. The cyanobacterial contribution to the oceanic B_{12} supply may be $\sim$ 50 times higher than the contribution of heterotrophic bacteria. Oceanic cyanobacteria do not compete for exogenous B_{12} with other prokaryotes and eukaryotes, but instead are obligate producers, thereby defining a unique dual ecological role for N_2-fixing cyanobacteria in the oligotrophic ocean. They provide both a source of "new" bioavailable nitrogen and the B_{12} needed to support the growth of larger auxotrophic eukaryotic phytoplankton important for the biological carbon pump.

Vitamin B_{12} (cobalamin; B_{12} hereafter) is an essential growth factor for most marine eukaryotic phytoplankton (Provasoli and Carlucci 1974; Droop 2007) that is required for the activity of several vital enzymes in central metabolism (e.g., methylmalonyl-CoA mutase, methionine synthase, and type II ribonucleotide reductase; Raux et al. 2000; Martens et al. 2002). Many of the eukaryotic phytoplankton (over half of all species surveyed to date) lack the biosynthetic pathway for this nitrogen (N)-containing B vitamin ($C_{63}H_{88}CoN_{14}O_{14}P$), and thus must depend on an exogenous pool of dissolved B_{12} (Croft et al. 2005, 2006). Recent studies suggest this ambient pool may be insufficient to support maximum productivity in both coastal and open ocean environments, as phytoplankton growth is enhanced by picomolar B_{12} amendments (Panzeca et al. 2006; Sañudo-Wilhelmy et al. 2006; Bertrand et al. 2007). Genomic data indicate that many prokaryotes (phototrophs and heterotrophs) could be significant sources of B_{12} to the oceans (Palenik et al. 2003; Rocap et al. 2003). However, there is currently little information translating this genomic potential into documented B_{12} production rates or defining the chemical factors (e.g., N availability) controlling its in situ production.

Cyanobacteria (including *Crocosphaera*, *Prochlorococcus*, *Synechococcus*, and *Trichodesmium*) are ubiquitous in the world's oceans, where they often contribute significantly to primary production (e.g., Partensky et al. 1999; Capone et al. 2005; Bonnet et al. 2009). In addition to this well-described role, representatives of each cyanobacterial genus contain the genes required to synthesize B_{12} (Palenik et al. 2003; Rocap et al. 2003; E. A. Webb unpubl. data); however, their role in B_{12} production has not been assessed so far. In this study, we provide for the first time B_{12} production rates of two globally distributed oceanic cyanobacterial groups with different N acquisition capabilities: the unicellular, free-living N_2-fixing (diazotrophic) cyanobacterium *Crocosphaera watsonii* WH 8501, and the ubiquitous non–N_2-fixing cyanobacterium *Synechococcus* sp. WH 7803.

Methods

Axenic strains of *C. watsonii* WH 8501 and *Synechococcus* sp. WH 7803 were grown in sterile YBCII medium (Chen et al. 1996) in a temperature-controlled incubator (27°C) with light supplied on a 12 : 12 light : dark (LD) cycle (80 μmol quanta m^{-2} s^{-2}). *C. watsonii* were grown under three initial B_{12} conditions (0, 400, and 800 pmol L^{-1}) and N depletion, except the treatment with no added vitamin B_{12}, in which *Crocosphaera* were grown under both N-deplete and N-replete (500 μmol L^{-1} NH_4^+) conditions. *Synechococcus* sp. were grown under two B_{12} conditions (0 and 400 pmol L^{-1}) and two N conditions (10 and 500 μmol L^{-1} NH_4^+). Each nutrient condition was performed in triplicate. For all experiments, the final dissolved cobalt concentrations used were 50 pmol L^{-1} (+ 2 μmol L^{-1} ethylenediaminetetraacetic acid; i.e., a lower concentration than the one traditionally used in YBCII medium in order to avoid any cobalt toxicity and excess cobalt release through vitamin B_{12} release). These cobalt concentrations are about 3 times higher than those considered limiting for B_{12} synthesis in the field (Panzeca et al. 2008). Prior to experiments, the cells were acclimated to each nutrient condition for at least three generations. To check that cultures were axenic, the medium was systematically examined for the presence of contaminating bacteria using 4′-6-diamidino-2-phenylindole (DAPI) staining during the course of the experiments. Growth rates

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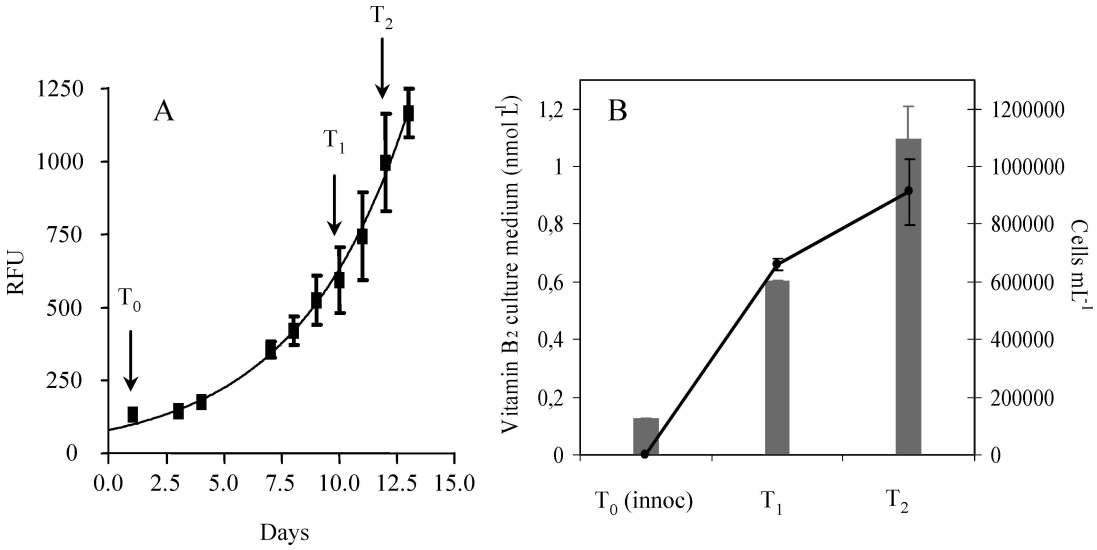

Fig. 1. Nanomolar levels of B_{12} released by *C. watsonii*. (A) Growth curve of *C. watsonii* cultured under N- and B_{12}-deplete conditions. Arrows indicate the time points of sampling and B_{12} measurements. (B) Coevolution of B_{12} concentrations and *C. watsonii* cell density in the culture during the exponential growth phase. Black line denotes mean ($n = 3$) B_{12} concentrations (nmol L^{-1}; error bars $\pm$ SD). Grey bars denote mean ($n = 3$) *C. watsonii* WH 8501 density (cells mL^{-1}; error bars $\pm$ SD). T_0: start of experiment (inoculation); T_1 and T_2 correspond to time points sampled during the exponential phase (growth rate: 0.3 d^{-1}).

were determined daily (for *Synechococcus*) and every other day (for *Crocosphaera*) using relative fluorescence units.

At 1 (for *Synechococcus*) or 2 (for *Crocosphaera*) selected time points during the exponential growth phase, an aliquot of 20–30 mL was extracted from triplicate cultures and filtered by hand through a 0.2-μm polycarbonate membrane using extremely low vacuum pressure (< 40 mm Hg) to avoid any cell breakage. Dissolved vitamin B_{12} concentrations were determined in the filtrate using the protocol described in Okbamichael and Sañudo-Wilhelmy (2004). Cells were systematically counted at the same time points using flow cytometry (MoFlo cell sorter, Dako) in order to determine vitamin B_{12} production rates per cell. At the same time points, microscopic analysis were performed under DAPI long-path filters in order to confirm that 100% of cells were in a good physiological state.

In order to confirm that the molecule released by the cells was actually vitamin B_{12}, we tested whether it was sufficient to allow the growth of a phytoplankton strain known to be auxotrophic for B_{12}, *Synechococcus* PCC7002. Briefly, the indicator cells were grown in SN media (Chen et al. 1996) to mid–log phase, incubated in soft agar SN media (1% agar without B_{12}), and allowed to grow for ~13 d at 27°C with a 14 : 10 LD cycle at 80 μmol quanta m^{-2} s^{-2}. After this time, a B_{12} standard dilution was spotted on the plate as well as the unknown compound from *Synechococcus* and *Crocosphaera* cultures. After 3 d of additional incubation, there was clear growth above the background level in the B_{12} dilution series as well as the putative purified B_{12} (data not shown).

Results

Our results show that the synthesis and release rates of vitamin B_{12} vary substantially between the representatives of two cyanobacterial groups. The diazotrophic cyanobacterium *C. watsonii* grown under N- and vitamin B_{12}-deplete conditions was able to produce and excrete nanomolar levels of the vitamin to the surrounding environment after 12 d of growth (Fig. 1). This corresponds to an average production of 1×10^{-18} ($\pm$ 1.4×10^{-19}) mol B_{12} $cell^{-1}$ over the exponential phase of cell growth, and a daily production rate of 1×10^{-19} mol B_{12} d^{-1} $cell^{-1}$. The same organism grown under a range of initial B_{12} concentrations indicates that external B_{12} availability can repress the vitamin synthesis pathway, as *C. watsonii* produced on average two times less B_{12} per cell when grown under 400 pmol L^{-1} initial B_{12} concentrations and stopped production and began to consume the external pool when grown under 800 pmol L^{-1} initial B_{12} conditions (Fig. 2).

Similar experiments conducted with the non-diazotrophic cyanobacterium *Synechococcus* (WH 7803) indicate that over the exponential phase this species produces 40 times less B_{12} per cell compared to the diazotroph when grown under N-limited conditions ($2.5 \times 10^{-20} \pm 1.1 \times 10^{-20}$ vs. $1 \times 10^{-18} \pm 1.4 \times 10^{-19}$ mol B_{12} $cell^{-1}$; Fig. 3) and eight times less B_{12} when grown under N-replete conditions ($1.2 \times 10^{-19} \pm 3.7 \times 10^{-20}$ vs. $1 \times 10^{-18} \pm 1.4 \times 10^{-19}$ mol B_{12} $cell^{-1}$). *Synechococcus* produced five times less B_{12} per cell when grown in low-N compared to N-replete conditions, whereas in the diazotroph *C. watsonii*, this effect was not observed (Fig. 3), probably because *Crocosphaera* is able to satisfy its N requirements through N_2 fixation.

Discussion

N availability—Vitamin B_{12} synthesis—Our study indicates that the two cyanobacteria tested are possibly important contributors to the supply of dissolved B_{12} in

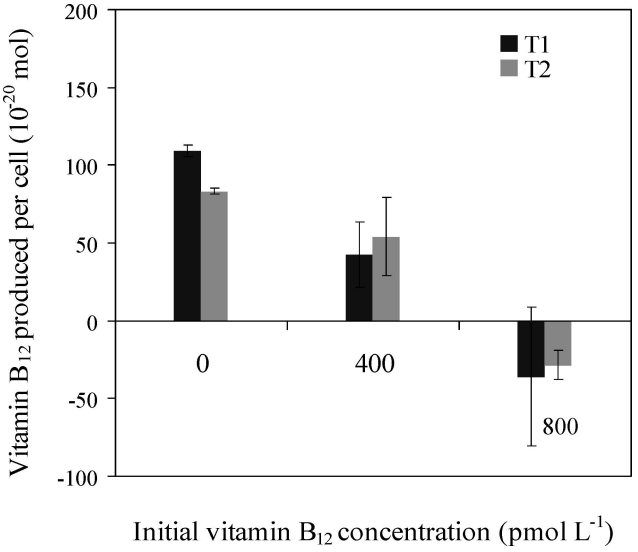

Fig. 2. Apparent regulation of B_{12} production by *C. watsonii* WH8501 by ambient B_{12}. Black and grey bars represent the mean (n = 3, error bars ± SD) quantity of B_{12} produced per cell during the exponential growth phase (growth rate: 0.3 d^{-1}), respectively after 10 and 12 d of growth. The x-axis represents initial B_{12} concentrations (pmol L^{-1}).

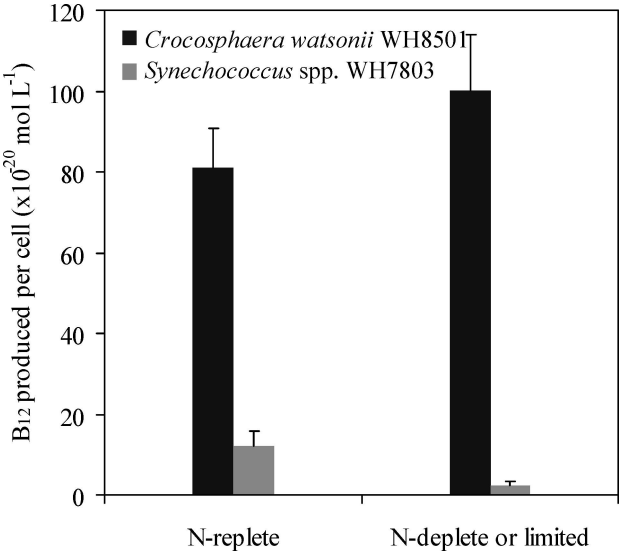

Fig. 3. Comparison of B_{12} production capacity by a diazotrophic and a non-diazotrophic cyanobacterium. The bars represent the mean quantity of B_{12} produced per cell by *C. watsonii* WH 8501 (black, n = 3, error bars ± SD) and *Synechococcus* WH 7803 (grey, n = 2, error bars ± SD), under N-replete conditions (500 μmol L^{-1} NH_4^+) and N-deplete (for *C. watsonii*) and N-limited (10 μmol L^{-1} NH_4^+ for *Synechococcus*) conditions.

the euphotic zone of the world ocean. However, their relative importance in B_{12} supply may depend on the degree of oligotrophy and N limitation, which in turn determine the cyanobacteria community structure and the B_{12} synthesis capacity. For example, although the amount of vitamin synthesized by the non–N_2-fixing *Synechococcus* was significantly lower than that produced by the N_2-fixing *Crocosphaera*, the former cyanobacterium is ubiquitous in temperate mesotrophic waters, reaching average abundances between 10^2 and 10^5 cells mL^{-1} (Partensky et al. 1999). At those abundances, and with a B_{12} synthesis capacity of 1.2×10^{-19} mol $cell^{-1}$ over the exponential growth phase (in N-replete conditions), their contribution to the dissolved B_{12} pool is potentially significant, ranging from 0.01 to a maximum of 12 pmol L^{-1} assuming that vitamin uptake was not occurring (Table 1). However, in oligotrophic N-depleted waters, the abundance of *Synechococcus* and their concomitant B_{12} synthesis capacity decrease. In those oligotrophic environments, unicellular diazotrophs can form significant blooms (Campbell et al. 2005; Le Moal and Biegala 2009), reaching up to 5×10^3 cells mL^{-1}. Although not as dense as the *Synechococcus* blooms, the B_{12} synthesis capacity of unicellular diazotrophs such as *C. watsonii* is 40 times higher and therefore their contribution to the ambient B_{12} pool could be significant in these N-depleted environments, potentially reaching up to 5 pmol L^{-1} of B_{12} released to the surrounding environment over the exponential growth period (Table 1). Our calculations on the effects of cyanobacterial blooms on the dissolved B_{12} pool are consistent with the range of B_{12} values measured in different marine systems (0.2–4 pmol L^{-1}; Panzeca et al. 2006; Sañudo-Wilhelmy et al. 2006). It seems that in all cases, the maximal cyanobacterial contribution to the oceanic B_{12} supply is higher (~ 50 times) than the currently estimated contribution of heterotrophic bacteria (Table 1). Because of the scarcity of published B_{12} production rates in marine prokaryotes, the values presented herein are an important first step for predicting the contribution of autotrophic prokaryotes to B_{12} flux in situ. Additional future data on B_{12} production by other cultured representatives and natural marine bacterial assemblages will further increase the accuracy of such estimates. In particular, it will be important to focus on *Prochlorococcus*, the most abundant marine cyanobacterium in the ocean (Partensky et al. 1999) that also possesses the biosynthetic pathway for B_{12} production.

Why do cyanobacteria produce such high amounts of B_{12}?—Our laboratory results suggest that cyanobacteria could influence the amount of available B_{12} in surface waters of the world ocean. These results are consistent with the aforementioned genomic data showing that marine cyanobacteria contain the genes coding for B_{12} synthesis (Palenik et al. 2003; Rocap et al. 2003).

Botanists have long suspected that cyanobacteria play a dual beneficial role in rice fields, by providing both N (through N_2 fixation) and B_{12}. Some authors have shown, for instance, that an algal inoculation on rice plants increased the yield of plants in the presence or absence of inorganic fertilizer, suggesting that algae played an auxiliary role besides fixing atmospheric N (Sundara Rao et al. 1963). Shah and Vaidya (1977) later showed in axenic cultures that N_2-fixing cyanobacteria associated with rice plants were able to produce extremely high quantities of B_{12} (12–35 nmol L^{-1} depending on the culture age).

Table 1. Oceanic B_{12} supply contribution by cyanobacteria (diazotrophic and non-diazotrophic) and heterotrophic bacteria (calculation based on B_{12} production over the exponential phase of cell growth).

	B_{12} synthesis capacity (pmol cell^{-1} over exp. phase)	Oceanic density (cells mL^{-1})	B_{12} supply (pmol L^{-1})
Cyanobacteria (non-diazotrophic)	$2.5–12\times10^{-20}$ (this study)	100–100,000*	0.002–12
Cyanobacteria (diazotrophic)	1×10^{-18} (this study)	0–5000†	0–5
Heterotrophic bacteria	$0.1–1.7\times10^{-22}$‡	500,000–1,000,000§	0.005–0.17

* Partensky et al. 1999.
† Le Moal and Biegala 2009.
‡ Croft et al. 2005.
§ Azam et al. 1983.

One potential explanation for such high levels of B_{12} production is that in several cyanobacteria, the enzymes ribonucleotide reductase (which forms the deoxyribonucleotide precursors for deoxyribonucleic acid synthesis) and methionine synthase are B_{12}-dependent (Gleason and Wood 1976; E. A. Webb pers. comm.), and actively growing cultures of such algae are required to synthesize B_{12} in substantial amounts. Moreover, our data seem to indicate that the cells have incomplete control over biosynthesis of this vitamin that could lead to the excessive synthesis and ultimate release into the external medium. Our data are consistent with this supposition, as 800 pmol L^{-1} exogenous B_{12} (i.e., two orders of magnitude higher concentrations than those measured in the oceans; Panzeca et al. 2006; Sañudo-Wilhelmy et al. 2006) was required to eliminate significant B_{12} excretion. Similarly to B_{12}, diazotrophic cyanobacteria such as *Trichodesmium* are known to release up to 80–90% of the N fixed from the atmosphere, in the form of dissolved organic N and ammonium (Gilbert and Bronk 1994), but the explanation for this is still unclear. Similarly, no conclusive explanation is currently available to explain why cyanobacteria excrete such high amounts of N and B_{12}. Our excretion results are not completely unexpected, as similar large corrinoid ring molecules have been shown to be excreted by some heterotrophic prokaryotes, such as *Pseudomonas aeruginosa*, *Pseudomonas putida*, or *Pseudomonas fluorescens*, when intracellular levels are in excess (Harris et al. 1993), and the concentration of the excreted B_{12} is an order of magnitude higher than those measured in our experiments (μmol L^{-1}; Mazumder et al. 1987). Our results show that the synthesis of B_{12} by the diazotrophic cyanobacterium *Crocosphaera* is disproportionately high when compared with the non-diazotrophic cyanobacterium *Synechococcus*; however, we must consider that *Crocosphaera* has a higher (5–70 times) biovolume compared to *Synechococcus* (Waterbury et al. 1986; Webb et al. 2009), which could partly explain those differences, as nutrient requirements are known to be size-dependent (Hein et al. 1995).

Potential biogeochemical implications of cyanobacterial B_{12} synthesis and excretion—This study focuses specifically on cyanobacteria that produce and excrete B_{12}, and thus illustrates the dependency existing in the ocean between prokaryotes and eukaryotes for primary production. Until recently, the oceanic supply of B_{12} in the ocean was considered to be associated mainly with heterotrophic prokaryotes (Croft et al. 2005). These authors have shown that many eukaryotes may satisfy their B_{12} requirements through direct symbiosis with heterotrophic prokaryotes (Croft et al. 2005), in which the algae supply fixed carbon in return for B_{12}, or through direct phagotrophy on B_{12}-producing bacterial prey (Croft et al. 2006). We show that the maximal amount produced by studied heterotrophic bacteria ($0.1–1.7 \times 10^{-22}$ mol B_{12} cell^{-1}; Croft et al. 2005) is between 150 and 6000 times less than the average B_{12} produced by the cyanobacteria tested here. This suggests that the cyanobacterial compartment now needs to be taken into account when studying the biogeochemical cycle of B_{12} in the surface ocean, although a larger number of marine prokaryotes need to be tested for B_{12} synthesis and excretion to draw stronger conclusions.

In particular, diazotrophic cyanobacteria constitute key phytoplanktonic species in N-depleted environments, as they provide a source of "new" N to fuel further primary production (Capone et al. 2005). This culture study, even if performed at higher cell density compared to the field, shows that diazotrophs are also able to provide B_{12}. If we consider an accumulation of *Crocosphaera* ($1–5 \times 10^3$ cells L^{-1}) fixing N_2 at a rate of 0.02 pmol N cell^{-1} d^{-1} with an atomic C : N ratio of 7, the "new" N provided by such a bloom could sustain a carbon fixation rate of 0.14–0.7 μmol C L^{-1} d^{-1}. If we apply a mean cell quota of 4.1 nmol B_{12} : mol C (Carlucci and Bowes 1972), the "new" dissolved B_{12} pool produced by these diazotrophs could support a phytoplankton carbon fixation rate of 0.5 μmol C L^{-1} d^{-1} (about the same amount of carbon fixation supported by "new" N). This dual ecological function illustrates the importance of diazotrophs for potentially increasing phytoplankton biomass by providing both "new" N and coenzymes that can facilitate the growth of large auxotrophic eukaryotic species such as diatoms, and thus possibly affecting increased export production in the ocean. As an example, the ecological success of diatom–diazotroph associations, which sequester high quantities of atmospheric carbon (Subramaniam et al. 2008), may be potentially explained by a dual supply of N and B_{12} from the cyanobacterium to its host (Karl 2002). In contrast, high sporadic B_{12} inputs in the coastal environment from runoff have been hypothesized to cause phytoplanktonic succes-

sions (Yu and Harrison 2000), thus potentially resulting in the formation of harmful algal blooms.

Further laboratory and field studies quantifying the internal quotas of B vitamins in eukaryotic phytoplankton as well as the extracellular release rates of vitamins in representative autotrophic and heterotrophic bacterial cultures are required to develop a robust quantitative model for the ecological importance of these important cofactors. It would also be interesting to study in the field the interrelations existing between phytoplanktonic successions and B_{12} cycling, in order to determine the role of B_{12} production in the so-called "biological pump."

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