

## Cobalt limitation and uptake in *Prochlorococcus*

Mak A. Saito<sup>1</sup>

MIT/Woods Hole Oceanographic Institution Joint Program in Chemical Oceanography, Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02534

James W. Moffett

Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

Sallie W. Chisholm

Department of Civil and Environmental Engineering and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

John B. Waterbury

Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

### Abstract

Processes that enable marine phytoplankton to acquire trace metals are fundamental to our understanding of primary productivity and global carbon biogeochemical cycling. Here we show that the abundant marine cyanobacterium, *Prochlorococcus* strain MED4-Ax, has an absolute cobalt requirement and that zinc cannot substitute for cobalt in the growth medium, as is the case in some other phytoplankton species. When resuspended into fresh medium, uptake of cobalt into the cell occurs as free cobalt ( $\text{Co}^{2+}$ ). In contrast, cultures augmented with conditioned medium assimilated cobalt significantly faster than those in fresh medium, leading to the hypothesis that *Prochlorococcus* produced organic cobalt ligands in the conditioned medium. This work suggests that the availability of cobalt might influence the composition of phytoplankton assemblages in the open ocean.

Analytical methods for measuring trace metals in seawater have improved significantly over the past two decades, contributing to important advances in our understanding of oceanic biogeochemistry. The findings that major regions of the oceans are iron limited (Martin and Fitzwater 1988) and that iron can have nutrient-like profiles (Martin and Gordon 1988) have illustrated the importance of trace metals in global carbon cycling. The development of electrochemical iron speciation methods (Rue and Bruland 1995; Wu and Luther 1995) has led to the idea that biogenic ligands may in fact be controlling the residence time of iron in the surface ocean (Johnson et al. 1997). With the discovery that zinc, cobalt, and cadmium can each occupy the active site of carbonic anhydrase enzymes in phytoplankton (Price and Morel 1990; Morel et al. 1994; Roberts et al. 1997) and that potentially growth limiting levels of Co and Zn have been measured in oceanic surface waters (Martin et al. 1989; Sunda and Hunts-

man 1995), researchers have hypothesized that these metals can result in metal-carbon colimitation and hence influence the size and composition of the biological carbon pump (Morel et al. 1994).

Organic complexation can dominate the chemical speciation of many transition metals in seawater. While we know little about the chemical structure of these ligands, it is expected that they significantly alter metal bioavailability. For example, the addition of the siderophore desferrioxamine to bottle incubations can significantly decrease iron uptake rates into eukaryotic phytoplankton (Hutchins et al. 1999). Evidence of strong organic complexes that dominate the speciation of cobalt in oceanic waters has recently been reported using a new electrochemical technique (Saito and Moffett 2001a). The importance of these ligands in cobalt acquisition by phytoplankton and the sources of the ligands themselves are uncertain. However, production of significant amounts of strong organic cobalt ligand was observed in cobalt enrichment experiments using waters from a *Synechococcus* dominated bloom (Saito and Moffett 2001b), which suggests that this cyanobacterium is a potential source of these cobalt ligands.

*Prochlorococcus marinus* is a marine cyanobacterium (oxygenic photoautotroph) whose global distribution and abundance (Partensky et al. 1999) suggest that it has a major role in marine biogeochemical cycling. However, due to the difficulty in developing axenic cultures, little is known about the trace metal requirements of this organism. Sunda and Huntsman (1995) have shown that another marine cyano-

<sup>1</sup> To whom correspondence should be addressed. Current address: Princeton University, Department of Geosciences, Guyot Hall, Princeton, New Jersey 08544-1003 (mak@whoi.edu).

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bacterium, *Synechococcus bacillaris*, has an absolute cobalt requirement for which zinc cannot substitute. Concentrations of cobalt in oceanic surface waters are extremely low (4–20 pM, Martin et al. 1989; Saito and Moffett 2002), yet Sunda and Huntsman (1995) reported high cellular Co quotas for *Synechococcus bacillaris* (0.08 to 1.43  $\mu\text{mol mol}^{-1}$  Co:C). Particulate cobalt measurements in the upper water column of the Sargasso Sea (1.5  $\mu\text{mol Co mol}^{-1}$  C; Sherrell and Boyle 1992) are comparable to these *Synechococcus* laboratory quotas and may be reflective of *Prochlorococcus* and *Synechococcus* cobalt quotas in nature since these organisms are major contributors to total primary productivity in the Sargasso Sea (Goericke and Welschmeyer 1993).

Here we present evidence that *Prochlorococcus* has an absolute cobalt requirement and that the uptake of cobalt may be facilitated by cobalt ligands produced by the cells.

## Materials and methods

**Cultures**—All cultures were grown in polycarbonate 28-ml tubes or glass borosilicate tubes. All labware was rigorously washed in detergent overnight, rinsed in Milli-Q water, soaked in 10% HCl (Baker Instra-Analyzed), and rinsed and soaked in pH 2 HCl prior to use. Culture media was a modified K-medium (Chisholm et al. 1992) and consisted of filtered Sargasso seawater amended with major nutrients (10  $\mu\text{M}$   $\text{H}_3\text{PO}_4$ , 50  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , 100  $\mu\text{M}$  urea) treated with chelex (Price et al. 1988/1989) and an EDTA (ethylenediamine-tetraacetic acid) (Sigma Ultra) trace metal mix (11.7  $\mu\text{M}$  EDTA, 90 nM  $\text{MnCl}_2$ , 3 nM  $\text{Na}_2\text{MoO}_4$ , 10 nM  $\text{Na}_2\text{SeO}_3$ , 1.17  $\mu\text{M}$   $\text{FeCl}_3$ , final concentrations) prepared without copper, cobalt, nickel, and zinc. Nickel (10 nM  $\text{NiCl}_2$ ) and zinc (0 to 8 nM  $\text{ZnSO}_4$ ) were added independently of the trace metal mix. Copper was omitted from the medium, and cobalt (as  $\text{CoCl}_2$ ) was added to medium as described in individual experiments. The filtered Sargasso seawater was microwave sterilized (Keller et al. 1988) and all amendments were sterile filtered. Culture experiments with NTA (nitrilotriacetic acid) consisted of the same medium as above with the exception that 100  $\mu\text{M}$  NTA replaced EDTA.

The EDTA medium had a small cobalt blank of  $140 \pm 13$  pM as determined by adsorptive cathodic stripping voltammetry (see Saito and Moffett 2001a for analytical protocol). The blank of the NTA medium was not determined because the experiments were completed prior to the development of our analytical method. Thus we assumed a medium blank of 100 pM, as have previous workers (Sunda and Huntsman 1995). This seems a reasonable assumption since it is the EDTA blank that would need to be much higher than 140 pM rather than the NTA blank to alter the interpretation of the experiments (see below).

*Prochlorococcus* (strain MED4) isolated from the Mediterranean Sea was used for all experiments. Subsequent to completion of the NTA culturing experiments, we isolated an axenic strain of MED4 (described as MED4-Ax; Saito and Waterbury pers. comm.). All of the uptake experiments and growth experiments using EDTA were conducted with this axenic strain.

Cultures were grown in duplicate with constant light at

Table 1. Cobalt uptake rates in *Prochlorococcus* strain MED4-Ax.

Media conditions	Calculated log [ $^{57}\text{Co}^{2+}$ ] (M)	$^{57}\text{Co}$ uptake rate (atoms cell $^{-1}$ h $^{-1}$ )	Calculated [Co $^{2+}$ ] (cold* M)	Co uptake rate (cold atoms cell $^{-1}$ h $^{-1}$ )
$10^{-5}$ M DTPA 94 fM $^{57}\text{Co}$	-18.4	n.d.	-15.2	n.d.
$10^{-5}$ M DTPA 290 fM $^{57}\text{Co}$	-17.8	n.d.	-15.2	n.d.
$10^{-5}$ M DTPA 940 fM $^{57}\text{Co}$	-17.4	0.0032	-15.2	0.476
$10^{-5}$ M EDTA 94 fM $^{57}\text{Co}$	-16.0	0.0079	-12.8	11.8
$10^{-5}$ M EDTA 290 fM $^{57}\text{Co}$	-15.5	0.040	-12.8	19.3
$10^{-5}$ M EDTA 940 fM $^{57}\text{Co}$	-15.0	0.080	-12.8	11.9
$10^{-5}$ M NTA 94 fM $^{57}\text{Co}$	-14.8	0.20	-11.6	300
$10^{-5}$ M NTA 290 fM $^{57}\text{Co}$	-14.3	0.56	-11.6	270
$10^{-5}$ M NTA 940 fM $^{57}\text{Co}$	-13.8	1.7	-11.6	250

\* Cold refers to nonradioactive cobalt, n.d. = not detectable.

17  $\mu\text{E m}^{-2} \text{s}^{-1}$  at 23°C. Growth was monitored by in vivo fluorescence of both replicates and by intermittent flow cytometric measurements of one of each duplicate pair (using the unopened culture as an uncontaminated reference). Experiments in which opened and unopened treatments deviated, as determined by in vivo fluorescence, were considered metal contaminated, discarded, and repeated. Growth rates were calculated from the exponential portion of the growth curve. In the long-term culture studies error bars were calculated from the standard deviation of successive replicate transfers ( $n = 4$  to 12 depending on cobalt concentration). Growth rates were normalized to the maximum growth rate in an experiment to allow comparison between experiments run in different years under slightly different culture conditions. In the metal substitution studies, all treatments were inoculated from a single 300 pM total cobalt treatment (1% volume) to allow comparisons between treatments.

**$^{57}\text{Co}$  uptake experiments**— $^{57}\text{Co}$  free ion experiment: In order to examine the chemical form of cobalt assimilated, cells were exposed to three metal ion buffers that resulted in distinct  $\text{Co}^{2+}$  concentrations while maintaining an equal total cobalt concentration across the buffer systems. Tracer concentrations of  $^{57}\text{Co}$  were used to measure uptake in each metal buffer system. Axenic *Prochlorococcus* (MED4-Ax) was grown in medium with 11.7  $\mu\text{M}$  EDTA and 0.8 nM total cobalt added, centrifuged at 10,000 rpm ( $11,180 \times g$ ) in a Biofuge 22R (Heraeus) at 20°C for 47 min, decanted with sterile and trace metal clean technique, resuspended in fresh medium without added cobalt, and returned to the constant light incubator for 48 h at 23°C (Anderson and Morel 1982). The cells were then washed three times by centrifugation and resuspended into fresh medium prepared without added cobalt. Aliquots of cell concentrates ( $2.8 \times 10^7$  cells  $\text{ml}^{-1}$ ) were added to preequilibrated (overnight) experimental treatments of sterile cobalt depleted medium with  $10^{-5}$  M NTA, EDTA, or DTPA (diethylenetriamine-pentaacetic acid) metal ion buffers and 940, 290, or 94 fM  $^{57}\text{Co}$  (carrier-free  $^{57}\text{CoCl}_2$ , 7,000 Ci  $\text{g}^{-1}$  specific activity, Isotope Products Laboratories, Table 1), 50 nM  $\text{FeCl}_3$ , and chelex treated major nutrients (as described above). These cultures were

checked for heterotrophic bacterial contamination using a marine purity broth (20 g NaCl, 8 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.5 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 17 g AC Difco broth per liter of distilled water as developed by F. Valois).

Cells collected for radioactive counting were filtered onto 0.2- $\mu\text{m}$  polycarbonate filters and rinsed with filtered seawater. Filters were counted with a germanium gamma counter detector (Canberra) to  $\pm 5\%$  counting error. Uptake rates were calculated by multiplying the initial slope of the uptake curve by the ratio of total cobalt media blank to added  $^{57}\text{Co}$ . Error bars were calculated from the standard deviation of the three uptake rates in each metal ion buffer, except for DTPA treatments in which the 290 fM and 94 fM  $^{57}\text{Co}$  treatments were below the detection limit of the gamma detector.

**Cobalt ligand experiment:** To study the potential influence of natural cobalt ligands on cobalt uptake, an experiment was performed using 0.8 nM total cobalt medium that had been previously conditioned by axenic *Prochlorococcus*. This experiment used the same inoculum as the  $^{57}\text{Co}$  free ion experiment described above and was run concurrently to make the results comparable. The first supernatant was collected and filtered through a trace metal clean acid-rinsed 0.2- $\mu\text{m}$  syringe filter (all-plastic syringe). This filtrate (described as conditioned medium) was also checked for sterility using the marine broth purity test and was added as 10% of the volume to fresh media with  $10^{-5}$  M EDTA, 290 fM  $^{57}\text{Co}$ , 50 nM  $\text{FeCl}_3$ , and the chelex treated major nutrients (as described above) and allowed to equilibrate overnight prior to inoculation. The 10% conditioned medium addition was chosen to minimize the influence of nutrient depletion in the conditioned media on the receiving culture. Two controls for this experiment were used, one without conditioned media and one with glutaraldehyde (0.75%) as a dead control. An additional treatment with conditioned medium from nonaxenic MED4 cultures showed the same enhanced response as the axenic conditioned medium, which suggests that bacteria were not responsible for the enhancement of cobalt uptake.

**Free metal calculations**—Free metal ion concentrations were calculated using stability constants from Martell and Smith (1993) for calcium and magnesium interactions with NTA, EDTA, and DTPA. Inorganic complexes with cobalt were also taken into account with the  $\text{CoCl}^+$  as the major inorganic species. For the cobalt limiting medium buffered with 11.7  $\mu\text{M}$  EDTA, the ratio of  $\text{Co}^{2+}$  to total cobalt was calculated to be  $10^{-2.94}$ . The ratio of  $\text{Zn}^{2+}$  to total zinc is calculated to be  $10^{-2.88}$ , using the empirically derived conditional stability constant of Sunda and Huntsman (1995) and correcting for the difference in EDTA concentration.

## Results and discussion

Understanding the geochemical distribution of transition elements in seawater will depend, at least in part, on an understanding of their biological utility. For example, it has been known for some time that cadmium correlates strongly with phosphate and zinc with silica throughout the oceans (Boyle et al. 1976), which suggests a use for these trace elements in oceanic microbes. But only recently has the use

and function of these trace elements been documented in marine organisms (Price and Morel 1990; Lane and Morel 2000). Seawater concentrations of cobalt are extremely low (Saito and Moffett 2002) and can display a nutrient-like depth profile (Martin and Gordon 1988; Saito and Moffett 2001a,b). Moreover, cobalt has a significantly shorter oceanic residence time and a smaller oceanic inventory (Saito and Moffett 2002) than that of zinc and cadmium (Taylor and McLennen 1985). This is due to scavenging of cobalt and the resultant low deep water concentrations. Cobalt has generally not been thought of as a dynamic micronutrient; however, some marine phytoplankton species have been shown to substitute cobalt for zinc (Morel et al. 1994) and hence could contribute to its depletion in oceanic surface waters. The dichotomy between the different geochemistries of cobalt, cadmium, and zinc and their potential overlapping biological function in marine phytoplankton is intriguing.

Here we show that *Prochlorococcus*, the dominant phytoplankton species in the subtropical and tropical oceans (Partensky et al. 1999), has an *absolute* requirement for cobalt, as has been shown for another marine cyanobacterium, *Synechococcus bacillaris* (Sunda and Huntsman 1995). We further hypothesized that the production of cobalt ligands by *Prochlorococcus* may facilitate cobalt uptake.

**Growth studies**—*Prochlorococcus* was found to have an absolute requirement for cobalt: when grown in batch cultures, decreased cobalt concentrations resulted in lower growth rates (Fig. 1), entry into stationary phase at lower biomass, and a failure to grow upon transferring a log phase culture to fresh media with no added cobalt. To determine whether zinc could relieve the cobalt requirement of *Prochlorococcus* through substitution, cultures were grown in EDTA medium with varying concentrations of cobalt and zinc. Increasing zinc concentration did not relieve cobalt limitation in this organism (Fig. 2A). We note, however, that the growth rate of *Prochlorococcus* was slightly reduced at low zinc concentrations, which was observed (Sunda pers. comm.) but not reported for the related cyanobacterium *Synechococcus bacillaris* (Sunda and Huntsman 1995; data replotted in Fig. 2B). The effect of cobalt on the growth rate of *Prochlorococcus* was related to the total cobalt added instead of the free cobalt ion ( $\text{Co}^{2+}$ ) concentrations calculated using NTA and EDTA stability constants (compare Fig. 1A,B). This is in contrast to the free ion model for phytoplankton metal uptake, in which uptake is proportional to the free ion of the metal rather than organically complexed forms (e.g.,  $\text{CoEDTA}^{2-}$ ). This concept that marine phytoplankton can only acquire the free ion chemical species has a long history in the trace metal oceanography literature (Sunda and Guillard 1976; Anderson and Morel 1982). Exceptions are thought to be limited to organisms that produce siderophores (Murphy et al. 1976) or that acquire iron via cell surface iron reductases (Maldonado and Price 2001). The free ion model contends that the metal ion buffer holds the majority of the metal, as  $\text{CoEDTA}^{2-}$  complexes for example, and that the trace amount of  $\text{Co}^{2+}$  present is replenished by reequilibration with  $\text{CoEDTA}^{2-}$  as described by the equations

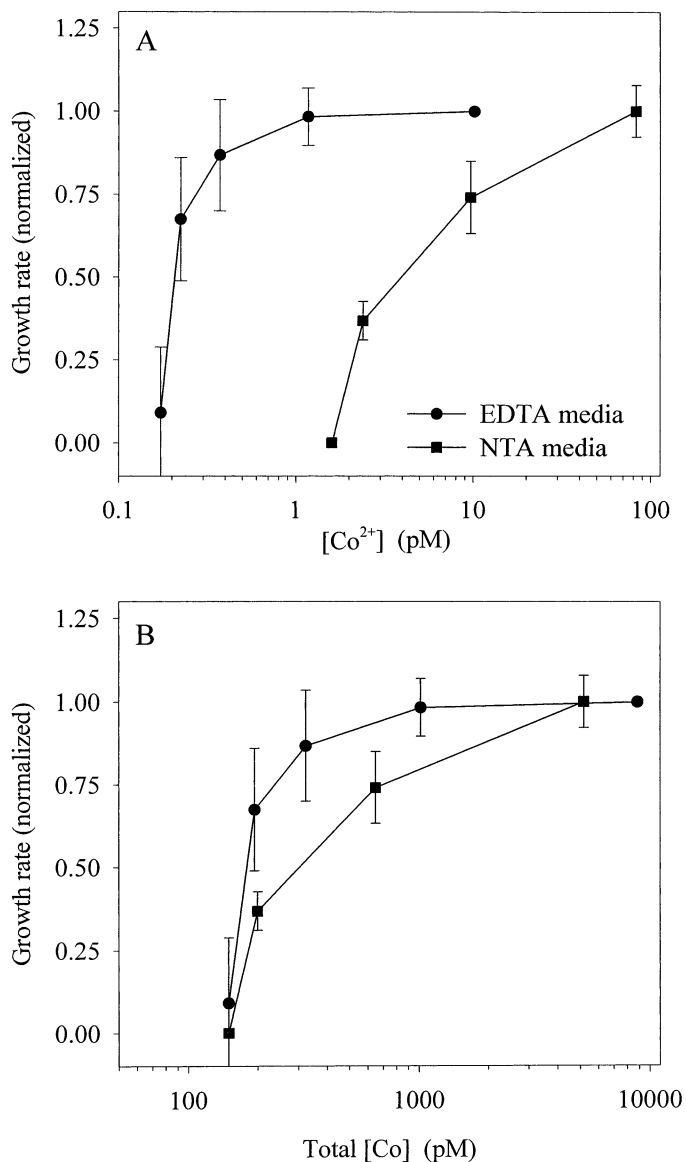


Fig. 1. Relative growth rates of *Prochlorococcus* MED4 as a function of (A)  $Co^{2+}$  and (B) total cobalt in two different metal ion buffers: one with EDTA and one with NTA.



$$K = \frac{[CoEDTA^{2-}]}{[Co^{2+}][EDTA^{4-}]} = 10^{16.45} \quad (2)$$



$$\frac{dCo^{2+}}{dt} = k_b[CoEDTA^{2-}] - k_{cell}[Cells][Co^{2+}] \quad (4)$$

where  $k_b$  refers to the dissociation rate of the  $CoEDTA^{2-}$  complex and  $k_{cell}$  refers to a second-order rate constant for uptake of  $Co^{2+}$  ions into the cell. At steady state, the  $Co^{2+}$  concentration is constant, where any  $Co^{2+}$  assimilated by the cells is replaced by the dissociation of  $CoEDTA^{2-}$  complex-

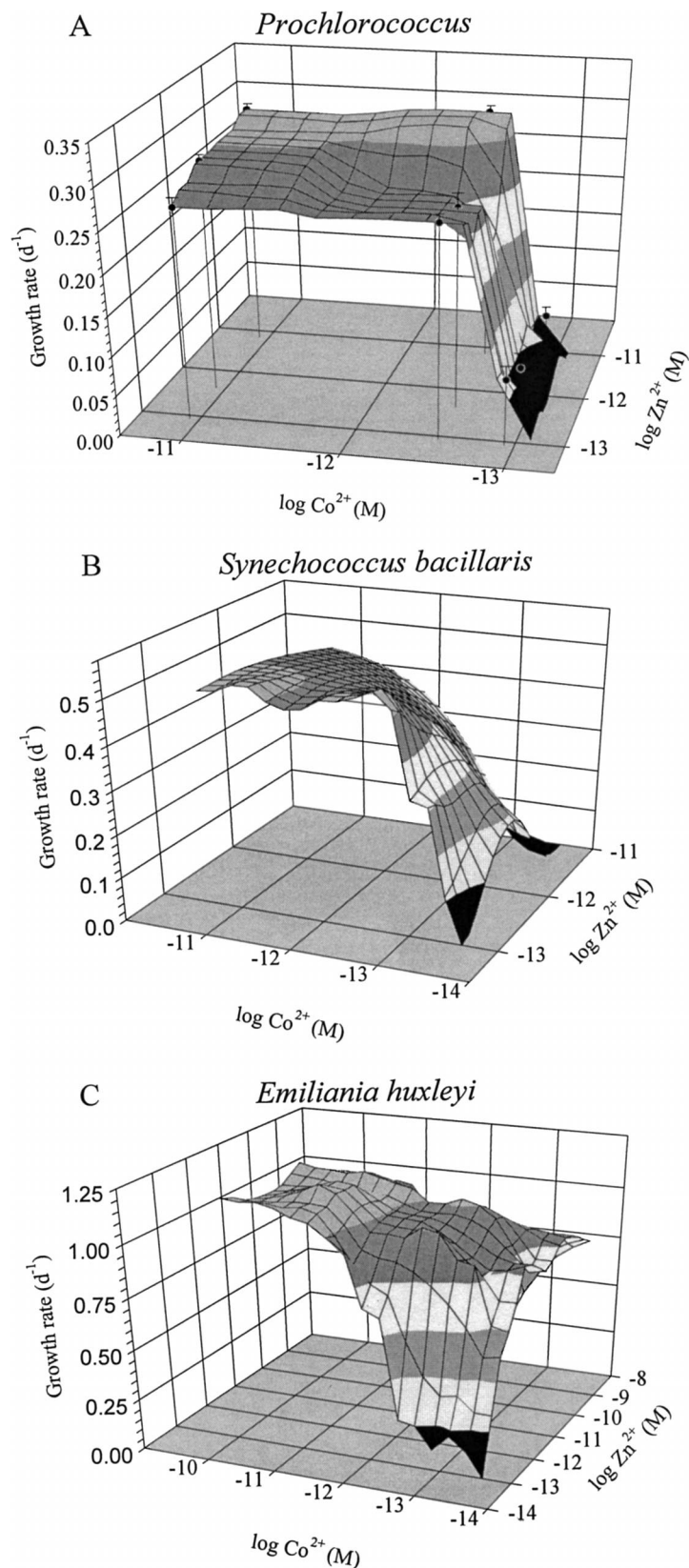


Fig. 2. The effect of varying cobalt and zinc concentrations on growth rate in (A) *Prochlorococcus* (MED4-Ax, this study, filled circles indicate data points used to create the surface plot), compared with (B) *Synechococcus bacillaris* and (C) *Emiliana huxleyi* (data replotted from Sunda and Huntsman 1995).

es. Growth should then be proportional to the steady state  $\text{Co}^{2+}$  if all other nutrients are in excess. Cultures are generally grown with sufficiently low cell densities such that the rate of cobalt uptake is not greater than the rate of  $\text{CoEDTA}^{2-}$  dissociation ( $k_b[\text{CoEDTA}^{2-}] \ll k_{\text{cell}}[\text{Cells}][\text{Co}^{2+}]$ ). Our data deviates from this free ion model for phytoplankton metal uptake (Eqs. 1–4) by showing a correspondence between growth rate and total cobalt rather than to  $\text{Co}^{2+}$  (Fig. 1B). A plausible explanation for this is that *Prochlorococcus* produces cobalt ligands that are perturbing the equilibrium of the metal buffered medium, and that *Prochlorococcus* has the ability to take up these ligands, analogous to siderophore production and uptake:



where  $L^{n-}$  refers to the natural cobalt ligands.

**Uptake studies**—To explore the mechanism of cobalt uptake by *Prochlorococcus*, we first conducted an experiment similar to the classic free ion model experiment of Anderson and Morel (1982). Three metal ion buffers and three total  $^{57}\text{Co}$  concentrations were used to measure free ion uptake rates (Fig. 3A,B,C). The different buffers resulted in dramatically different uptake rates, with higher uptake rates in the NTA medium due to the lower affinity of cobalt for NTA as compared to EDTA and DTPA. Uptake rates decreased with time in the NTA treatments (Fig. 3A) due to the depletion of the  $\text{CoNTA}^-$  reservoir. At the uptake rates measured in NTA, cobalt assimilated by the cells would be expected to exceed the total cobalt added given sufficient time (~220% of the total cobalt would be needed to supply an estimated cobalt quota). Hence, the combination of relatively fast dissociation kinetics for  $\text{CoNTA}^-$  and the very high cell number in this experiment result in the depletion of the  $\text{CoNTA}^-$  in only a few hours (as described in Eq. 4). In contrast, in the EDTA and DTPA  $^{57}\text{Co}$  experiments, the same number of cells use only a fraction of the total cobalt reservoir (<15%), and hence  $^{57}\text{Co}$  uptake is linear with time (Fig. 3B,C).  $^{57}\text{Co}$  uptake in the 94 fM and 290 fM  $^{57}\text{Co}$  treatments in the DTPA experiment were below detection limit of the gamma counter, and the 940 fM  $^{57}\text{CoDTPA}^{2-}$  uptake rate may be underestimated if cellular uptake rate exceeds the rate of  $\text{CoDTPA}^{2-}$  dissociation (since calculations show 70-fold greater cell number than  $\text{Co}^{2+}$  atoms per milliliter of media, Table 1).

When plotted together, the uptake rates from Fig. 3 are not proportional to total cobalt in the three types of metal ion buffers (Fig. 4A). Instead, cobalt uptake is proportional to the free ion form of cobalt ( $\text{Co}^{2+}$ , Fig. 4B). If this uptake were proportional to total cobalt the strength of the metal ion buffer ligand would not affect the uptake rate in Fig. 4A. Yet because the metal ion buffers have different affinities for cobalt ( $K_{\text{NTA}} = 10.38$ ,  $K_{\text{EDTA}} = 16.45$ ,  $K_{\text{DTPA}} = 19.15$ ; Martell and Smith 1993), these buffers result in different  $\text{Co}^{2+}$  concentrations at equivalent total cobalt concentrations. This experiment suggests the presence of a  $\text{Co}^{2+}$  transporter and an inability to use  $\text{CoNTA}^-$ ,  $\text{CoEDTA}^{2-}$ , and  $\text{CoDTPA}^{2-}$  as cobalt sources.

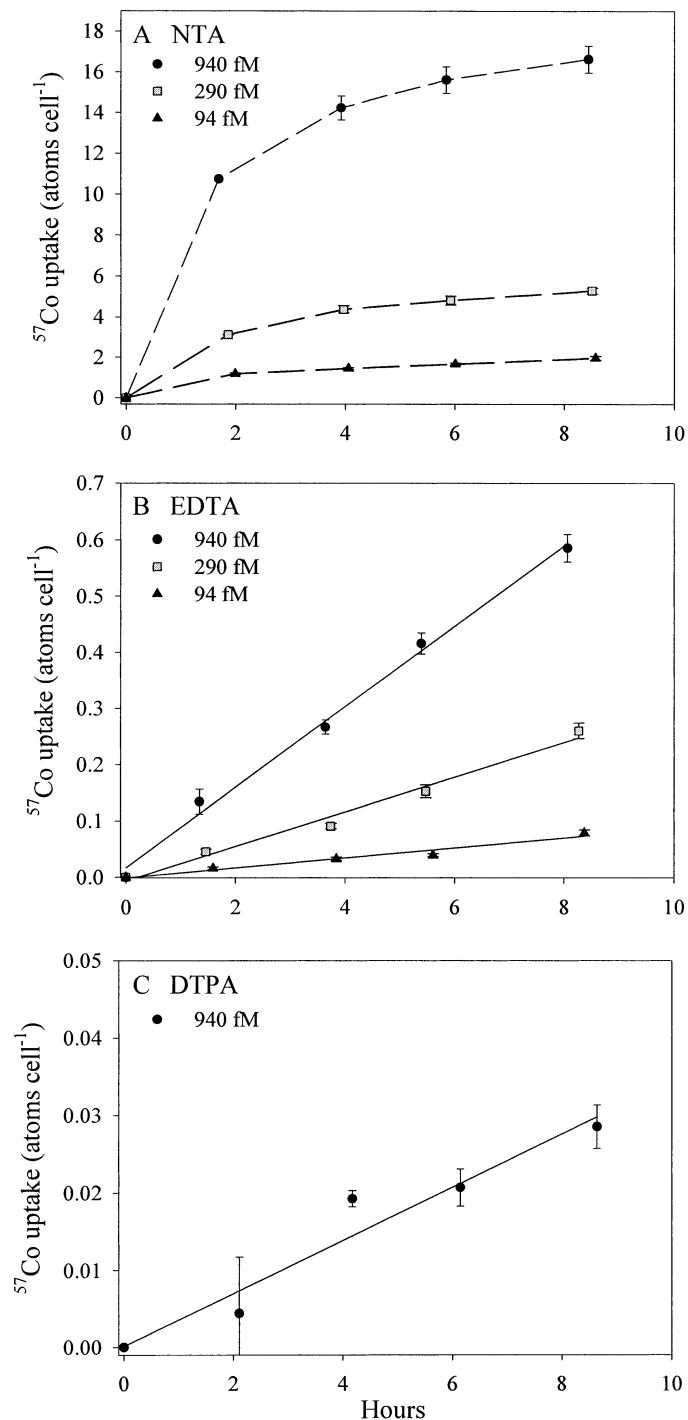


Fig. 3. Time courses of  $^{57}\text{Co}$  uptake by *Prochlorococcus* (MED4-Ax) using (A) NTA, (B) EDTA, and (C) DTPA as metal buffers, and varying the  $^{57}\text{Co}$  concentrations in the presence of 140 pM total cobalt (media blank).

We next explored the effect of adding conditioned media to the uptake experiments to see if this would have an influence on uptake rates. The uptake rate of  $^{57}\text{Co}$  in a culture with a 10% addition of conditioned medium (medium from a late log phase culture with 0.8 nM and sterile filtered under trace metal clean conditions) was more than double that of

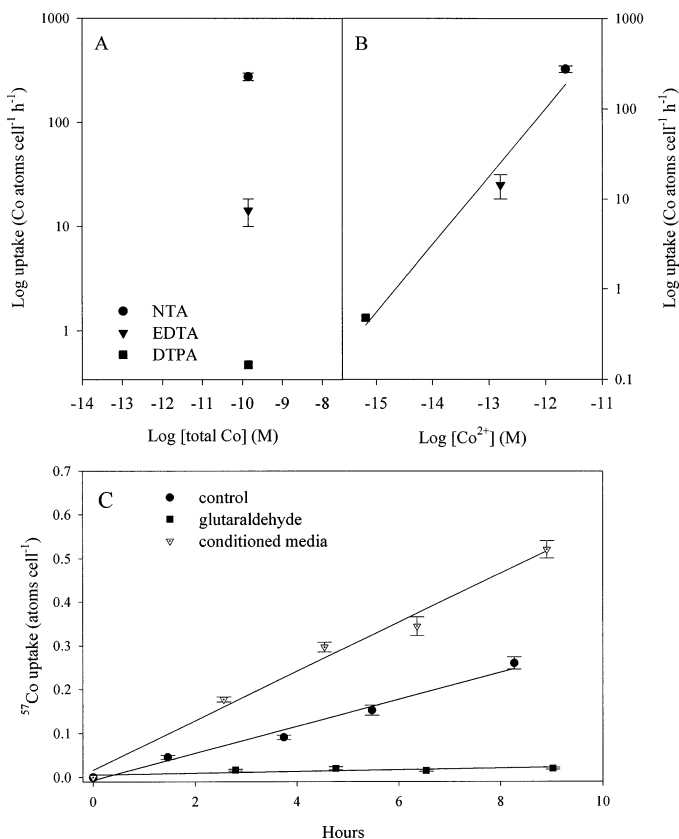


Fig. 4. Analysis of the uptake of cobalt by *Prochlorococcus* (MED4-Ax) as a function of total cobalt and Co<sup>2+</sup> concentration, comparing fresh and conditioned media. (A) For cells rinsed and placed in fresh medium, uptake rates in media with a single total cobalt level and different metal ion buffers (NTA, EDTA, and DTPA) were wide ranging. (B) When the binding affinities of those the metal ion buffers are taken into account, uptake rates correlated with the resulting Co<sup>2+</sup> concentrations. (C) Adding conditioned medium to the fresh medium resulted in an increased cobalt uptake rate, relative to a control treatment that was resuspended in fresh media without conditioned medium, and a glutaraldehyde killed control.

<sup>57</sup>Co uptake rates in fresh medium (Fig. 4C). These results suggest that the conditioned medium may have contained cobalt complexes produced by *Prochlorococcus* and that the cells have a mechanism to acquire cobalt from these compounds. The observed increase in cobalt uptake rates (Fig. 4C) can be explained by a cobalt ligand that has coordinated some of the cobalt away from the CoEDTA<sup>2-</sup> buffer, creating a bioavailable cobalt ligand fraction. In contrast, cells resuspended in fresh medium would not have the ability to accumulate biogenic ligands in the medium and would be expected to (and do) follow the free ion model by using Co<sup>2+</sup> (Fig. 4B).

Based on these experiments, we cannot determine whether the hypothesized cobalt ligands were transported into the cells or whether the metal was removed from the ligand at the cell surface.

**Biogeochemical implications**—If proved to be true, the production of Cobinding ligands by *Prochlorococcus* could

have significant implications for the biogeochemistry of cobalt. As proposed for iron in seawater (Johnson et al. 1997), the solubility of cobalt may be enhanced by complexation with strong and specific organic cobalt ligands. These ligands could serve to protect cobalt from scavenging by particles and from oxidation and precipitation as insoluble Co(III) oxides by oxidants like hydrogen peroxide. Increases in cobalt ligand concentrations have been observed in cobalt enriched bottle incubations with a *Synechococcus* dominated natural phytoplankton community (Saito and Moffett 2001b). The cobalt ligands measured in seawater may be apo-cobalamin (B<sub>12</sub>) or its degradation products. Vitamin B<sub>12</sub> has been explored as a potentially limiting micronutrient for marine phytoplankton (Guillard and Cassie 1963; Swift 1992). Yet calculations of B<sub>12</sub> quotas and cobalt quotas in *Synechococcus* suggest that B<sub>12</sub> is only a minor component of the cobalt requirement (~4%, Sunda and Huntsman 1995; Wilhelm and Trick 1995).

The absolute requirement of cobalt in *Prochlorococcus* shown in this study and the low concentrations of dissolved cobalt present in the Sargasso Sea (Saito and Moffett 2002) suggest that cobalt could be important in determining the growth rate and species composition of phytoplankton in the open ocean. Assuming that the particulate cobalt values measured by Sherrell and Boyle (1992) are reflective of *Prochlorococcus*, which tends to dominate this environment (Goericke and Welschmeyer 1993), we can estimate the turnover of cobalt in the natural environment. Under steady state conditions, the concentration of cobalt per cell,  $Q$ , is equal to the uptake rate  $\rho$ , divided by the specific growth rate,  $\mu$ . With the Sherrell and Boyle particulate cobalt value measured in surface waters (1.5  $\mu\text{mol Co mol}^{-1} \text{C}$ ), a population of  $10^5$  cells ml<sup>-1</sup> *Prochlorococcus* growing at 0.4 d<sup>-1</sup> in the Sargasso Sea (Mann 2000) with 53 fg C cell<sup>-1</sup> (Campbell et al. 1994) would incorporate 0.3 pM d<sup>-1</sup> of cobalt in new cells, and an equivalent population of *Prochlorococcus* cells growing at 1.1 d<sup>-1</sup> in an iron fertilized patch in the Equatorial Pacific (Mann and Chisholm 2000) would incorporate 1.3 pM d<sup>-1</sup> of cobalt. Cobalt incorporation at these levels would represent the use of a significant fraction of the total dissolved cobalt reservoir: total cobalt near Bermuda in the Sargasso has an annual average of  $20 \pm 10$  pM (Saito and Moffett 2002). This suggests the need for rapid recycling to avoid inducing cobalt limitation in *Prochlorococcus*. The high biological demand for Co implied by these calculations and the evidence that Co is complexed by strong organic ligands in the open ocean (Saito and Moffett 2001a) suggest that marine cyanobacteria may have developed high affinity uptake mechanisms for cobalt that presumably allow them to access organically complexed species in a manner analogous to the systems hypothesized for iron acquisition.

The physiological data suggesting the presence of a Co<sup>2+</sup> transporter in *Prochlorococcus* is also intriguing given the speciation data from the open ocean indicating that virtually all of the cobalt there is bound by strong organic ligands (Saito and Moffett 2001a). There are three genes thought to be involved in Co<sup>2+</sup> transport in other organisms: a high affinity transporter known as *nhlF* from *Rhodococcus rhodochrous* (Pogorelova et al. 1996; Komeda et al. 1996), a cobalt toxicity suppressor in *Saccharomyces cerevisiae*

called *cotI*, and a  $Mg^{2+}$ ,  $Ni^{2+}$ , and  $Co^{2+}$  transport system called *corA* in *Salmonella typhimurium* and *Escherichia coli* (Kobayashi and Shimizu 1999). However, BLAST searches of the MED4 genome did not reveal sequences on the amino acid level with any significant homology to these three transporters (<http://jgi.doe.gov/JGLmicrobial/html/index.html>), which suggests that the  $Co^{2+}$  transport we have observed in this study was performed by an unknown transporter.

While the biological function for cobalt in *Prochlorococcus* is unknown, the MED4 genome has genes for the synthesis of the cobalt containing vitamin  $B_{12}$  (*cobN*, *cobO*, and *cobW* genes). Furthermore, given the high carbonic anhydrase activity observed in *Synechococcus* (Tchernov et al. 1997) and low zinc requirement in *Prochlorococcus* (Fig. 2A) and *Synechococcus* (Fig. 2B, data replotted from Sunda and Huntsman 1995), it is plausible that a large fraction of the cobalt requirement is for this enzyme. This assumes that the high carbonic anhydrase activity is due to a high concentration of cellular carbonic anhydrase with stoichiometric amounts of metal (cobalt) in the active site. Zinc, cadmium, and cobalt limitation have been shown to decrease carbon uptake in marine phytoplankton by limiting the synthesis of the enzyme carbonic anhydrase (Morel et al. 1994; Yee and Morel 1996). DCA1, a carbonic anhydrase enzyme isolated from *T. weissflogii*, is known to substitute cobalt for zinc at the active site (Roberts et al. 1997). While neither the MED4 nor MIT9313 *Prochlorococcus* genomes contain genes that have homology to the three well-known classes of carbonic anhydrases ( $\alpha$ ,  $\beta$ , and  $\gamma$ ; Smith and Ferry 2000; Hess et al. 2001), two new carbonic anhydrases have been found in marine diatoms that cannot be classified as  $\alpha$ ,  $\beta$ , or  $\gamma$  types (Roberts et al. 1997; Lane and Morel 2000). Hence, we cannot rule out the possibility that *Prochlorococcus* has a cobalt requiring carbonic anhydrase. The production of cobalt ligands may be important in affecting the ability of marine phytoplankton to obtain  $CO_2$  via carbonic anhydrase mediated uptake mechanisms, either by reducing the availability of cobalt to eukaryotic phytoplankton or by increasing the availability of cobalt to cyanobacteria.

The cobalt and zinc independence in *Prochlorococcus* and *Synechococcus* suggests that the "trace metal trio" relationship that has been observed in eukaryotic marine phytoplankton (Cd, Co, and Zn, based on their biochemical substitution in carbonic anhydrases) may be decoupled in the marine cyanobacteria. For example, the diatoms *Thalassiosira pseudonana* and *Thalassiosira oceanica* were able to substitute cobalt and zinc, although both strains grew significantly better with high zinc concentrations than equivalent cobalt concentrations (Sunda and Huntsman 1995). Cobalt and zinc were also observed to substitute in the coccolithophore *Emiliania huxleyi*, but in contrast to these two diatoms species, optimal growth occurred with high cobalt rather than high zinc (Fig. 2C, Sunda and Huntsman 1995). Interestingly, *E. huxleyi* does not have a  $B_{12}$  requirement (Menzel and Spaeth 1962). If *E. huxleyi* synthesizes its own  $B_{12}$ , as *Prochlorococcus* and *Synechococcus* appear to, it would not explain the substitution of Co for Zn in Fig. 2C since there is no evidence for Zn substitution in  $B_{12}$ .

Since the cyanobacteria evolved earlier than eukaryotic algae, it is conceivable that their absolute requirement for

cobalt is vestigial from a time period when the Earth's atmosphere and oceans were still anoxic, making cobalt abundant and zinc scarce (Sunda and Huntsman 1995). By analogy to iron and siderophores, these hypothesized cobalt ligands may have evolved as a means to obtain cobalt in a newly oxic environment where cobalt became scarce by scavenging and oxidation. Given this scarcity of cobalt, eukaryotic organisms evolving later in an oxic ocean may have evolved to use zinc as a substitute for cobalt (Sunda and Huntsman 1995). Similar arguments exist for the evolution of the cobalt center in  $B_{12}$  (Eschenmoser 1988; Scott 1990). The inability of zinc to substitute for cobalt that we observed in Fig. 2A is consistent with this ancient ocean hypothesis. Besides increasing cobalt solubility, another possible function of the cobalt ligands may be to restrict other species' access to a limiting resource as hypothesized with iron (Murphy et al 1976; Wilhelm and Trick 1994). In this sense, the cyanobacteria (e.g., *Prochlorococcus* and *Synechococcus*), which dominate the oligotrophic regions, may have evolved the ability to produce cobalt ligands and siderophores that increase the availability of cobalt and iron while restricting access of these metals to nonsiderophore/cobalt ligand synthesizing organisms.

The absolute requirement for cobalt by *Prochlorococcus*, which cannot be relieved by zinc, helps explain the observations of the nutrient-like behavior (i.e., surface depletion) of cobalt in the oligotrophic oceans. Furthermore, the presence of a  $Co^{2+}$  uptake system, as evidenced by experiments with different metal buffered media, and the possible presence of cobalt ligands that are involved in cobalt uptake suggest a duality for cobalt acquisition that may improve the competitiveness of *Prochlorococcus* in regions of very scarce cobalt. Conclusive evidence of this hypothesis will require isolation of the ligand and an analysis of its chemical structure, as well as a mechanistic understanding of its production and regulation by the cells. Given the differing cobalt and zinc requirements in *Prochlorococcus* and *Synechococcus* relative to eukaryotic phytoplankton like *T. pseudonana* and *E. huxleyi*, the potential role of cobalt in regulating phytoplankton species composition in the open oceans is a promising area of future study.

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