Biometals (2009) 22:573–581
DOI 10.1007/s10534-009-9222-7

Multiple roles of siderophores in free-living nitrogen-fixing bacteria

A. M. L. Kraepiel · J. P. Bellenger · T. Wichard · F. M. M. Morel

Received: 18 November 2008 / Accepted: 11 February 2009 / Published online: 10 March 2009

Abstract Free-living nitrogen-fixing bacteria in soils need to tightly regulate their uptake of metals in order to acquire essential metals (such as the nitrogenase metal cofactors Fe, Mo and V) while excluding toxic ones (such as W). They need to do this in a soil environment where metal speciation, and thus metal bioavailability, is dependent on a variety of factors such as organic matter content, mineralogical composition, and pH. *Azotobacter vinelandii*, a ubiquitous gram-negative soil diazotroph, excretes in its external medium catechol compounds, previously identified as siderophores, that bind a variety of metals in addition to iron. At low concentrations, complexes of essential metals (Fe, Mo, V) with siderophores are taken up by the bacteria through specialized transport systems. The specificity and regulation of these transport systems are such that siderophore binding of excess Mo, V or W effectively detoxifies these metals at high concentrations. In the topsoil (leaf litter layer), where metals are primarily bound to plant-derived organic matter, siderophores extract essential metals from natural ligands and deliver them to the bacteria. This process appears to be a key component of a mutualistic relationship between trees and soil diazotrophs, where tree-produced leaf litter provides a living environment rich in organic matter and micronutrients for nitrogen-fixing bacteria, which in turn supply new nitrogen to the ecosystem.

Keywords *Azotobacter vinelandii* · Metalophore · Metal uptake · Molybdenum · Vanadium · Tungsten

A. M. L. Kraepiel (✉)
Chemistry Department, Princeton Environmental Institute, Guyot Hall, Princeton University, Princeton, NJ 08544, USA
e-mail: kraepiel@princeton.edu

J. P. Bellenger · T. Wichard · F. M. M. Morel
Department of Geosciences, Princeton Environmental Institute, Guyot Hall, Princeton University, Princeton, NJ 08544, USA

Introduction

In many of the Earth's ecosystems, plant growth is limited by the amount of available nitrogen (Perakis and Hedin 2002). The stock of nitrogen is determined in part by the rate of nitrogen fixation, the conversion by a few bacteria (diazotrophs) of atmospheric N_2 gas into plant-available ammonium (NH_4^+). The enzyme nitrogenase that catalyzes N_2 fixation is a highly conserved metalloenzyme containing three different types of Fe–S clusters (Burgess and Lowe 1996). One of them, the FeMo cofactor, contains a molybdenum (Mo) atom in addition to iron (Fe) and sulfur (S) and binds N_2 at the active site. The Mo-nitrogenase appears to be the most common and efficient form of the enzyme. Under low Mo availability, however, some N_2-fixing bacteria express the V-nitrogenase or

the Fe-only nitrogenase, called "alternative nitrogenases", which use V or Fe, respectively, at their active site instead of Mo (Lawson and Smith 2002). The metal cofactors Fe, Mo and/or V are thus required for nitrogen fixation. Another trace metal, tungsten (W), which has a chemistry similar to that of Mo, is toxic to diazotrophs, as it is incorporated into nitrogenase in place of Mo, resulting in an inactive form of the enzyme (Siemann et al. 2003; Wichard et al. 2008b).

While the importance of trace metals, and particularly Fe, for N_2 fixation in the world's oceans has received some attention (e.g., Kustka et al. 2003), the limitation of N_2 fixation by trace metals in natural terrestrial ecosystems has been little studied (Silvester 1989). Recently, however, a few studies (Barron et al. 2009; Hungate et al. 2004) indicate that Mo can be a limiting nutrient for symbiotic and free-living N_2-fixing bacteria.

Fe concentrations in soils are usually high (i.e., several percents), reflecting its abundance in the continental crust, where it is the fourth most common element after O, Si and Al (Wedepohl 1995). In contrast, Mo is extremely scarce. Its average soil concentration, 1–2 ppm, is the lowest of all the transition metals used in biology (Alloway 1995). Interestingly, V is more abundant, with average concentrations close to 100 ppm, indicating that V may be plentiful even in Mo-depleted soils. Average soil W concentrations are around 1 ppm, close to those of Mo, suggesting that diazotrophs need a Mo uptake system that discriminates efficiently between the two metals.

While free metals, or metals bound to inorganic ligands are usually considered available for uptake, metals bound to strong organic ligands, or bound to particles, are not, with important exceptions such as the bacterial uptake of iron bound to siderophores (Hudson and Morel 1992). In spite of its abundance, Fe is little available in oxic soils. Fe(III) precipitates as iron oxides and hydroxides, resulting in very low concentrations in solution and its availability can be further reduced by binding to natural organic matter (Werber et al. 2006). In contrast, the oxoanions molybdate (MoO_4^{2-}), vanadate ($H_2VO_4^-/HVO_4^{2-}$) and tungstate (WO_4^{2-}), which are the stable forms of Mo, V and W, respectively, in oxic environments, are highly soluble. Because of their strong association with oxygen atoms, these metals, unlike cationic metals, adsorb on particle surfaces only at low pH and form only weak complexes with most organic ligands, with the notable exception of catechols (Goldberg et al. 1996; Gustafsson 2003; Bellenger et al. 2007). The main form of Mo has thus often been assumed to be either free molybdate in circumneutral soils, or molybdate adsorbed on oxides in acidic soils. A recent study, however, shows that, in the highly organic topsoil layer, Mo is bound to the catechol groups of natural organic matter (NOM) over a wide pH range (pH = 4–8, Wichard et al. 2008a). The association of Mo with NOM suggests that Mo may not be readily bioavailable as free molybdate in neutral and basic soils. Though little information is available on V or W, it seems likely that these metals may also associate with the same organic binding groups in soils (Serrat and Morell 1994). As discussed later, the complexation of Mo, V and W by NOM, while decreasing their bioavailability, may help retain these metals in the topsoil layer.

In the soil environment, where metal speciation depends on a variety of parameters such as mineral composition, organic matter content and pH, N_2-fixing bacteria must regulate their metal uptake to acquire the metals they need and keep out the ones that are toxic. A series of recent studies by our group has shed new light on the metal (Fe, Mo, V, and W) requirements and uptake strategies by the gram-negative bacterium *Azotobacter vinelandii*, an ubiquitous organism in soils, which has been widely studied as a model diazotroph (Bishop et al. 1986; Stiefel and Watt 1979).

Metal (Fe, Mo, V, W) requirements of *A. vinelandii* in diazotrophic cultures

Iron

Diazotrophic cultures of *A. vinelandii* require large concentrations of Fe for growth. The iron-replete Burk medium, which is typically used to grow *A. vinelandii*, contains about $[Fe] = 2–7 \times 10^{-5}$ M added as Fe(II) (e.g., $FeSO_4$, Cornish and Page 1995), or Fe(III) (e.g., ferric sulfate (Duhme et al. 1998) or ferric citrate (Cornish and Page 1998)). At circumneutral pH, Fe(II) quickly oxidizes to Fe(III) (Stumm and Morgan 1995). In the absence of a strong organic ligand, Fe(III) precipitates rapidly as a hydrous ferric oxide. Citrate, which is sometimes added at micromolar concentrations, is too weak to

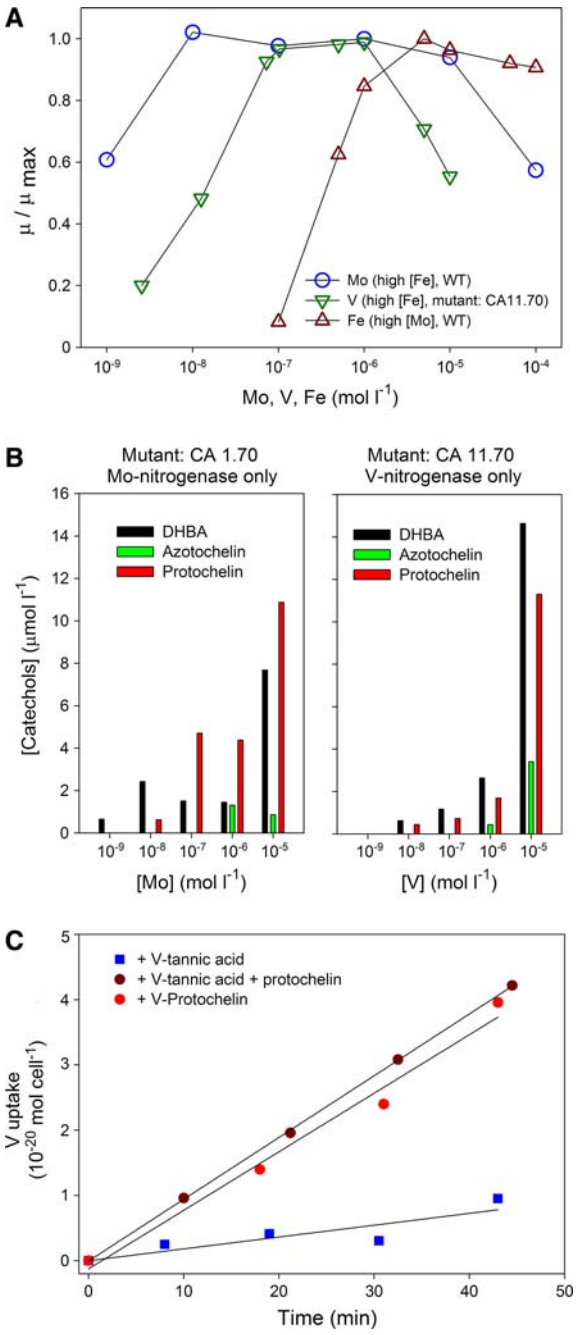

Fig. 1 Metal requirements, siderophore production and kinetics of metal uptake by *A. vinelandii* in diazotrophic cultures. **a** Relative growth rates (μ/μ_{max}) of strain OP (wildtype, WT) and of the double mutant strain CA11.70, which expresses only the alternative V-nitrogenase (Joerger et al. 1989), as a function of metal concentrations. *Circles* [Fe] $= 5 \times 10^{-6}$ M, no V added; *downward triangles* [Fe] $= 5 \times 10^{-6}$ M, no Mo added; *upward triangles* [Mo] $= 1 \times 10^{-6}$ M, no V added. **b** Effect of Mo and V on the production of catechol siderophores by the double mutant strains CA1.70 and CA11.70. Strain CA1.70 expresses only the Mo-nitrogenase (Joerger et al. 1989). **c** Uptake by *A. vinelandii* cells (mutant strain CA11.70) of V bound to tannic acid ($C_{76}O_{46}H_{52}$, Sigma, USA, *blue squares*) or protochelin (*red circles*). In one experiment (*brown circles*) protochelin was added to a solution of V and tannic acid and left to equilibrate for 5 days ([V] $= 4.1 \times 10^{-8}$ M, [tannic acid] $= 7.0 \times 10^{-7}$ M, [protochelin] $= 5.2 \times 10^{-6}$ M). Bacterial cells were then added to the mixture and their V uptake rate was measured. The fast uptake rate is evidence of the formation of V-protochelin in the presence of tannic acid. Bacteria were conditioned at low [V]

bind iron and prevent Fe(III) precipitation (Konigsberger et al. 2000). Fe precipitation results in low Fe availability, which may account for the very low growth rates measured under these conditions in diazotrophic cultures of *A. vinelandii* (Bellenger unpublished results). Indeed, in most studies reported in the literature, *A. vinelandii* is grown with a source of fixed nitrogen (nitrate or ammonia), which reduces the Fe requirements of the organism. To avoid Fe precipitation, we designed a modified Burk medium containing ethylenediaminetetraacetic acid (EDTA), a strong organic ligand that binds the bulk of the iron. In effect, Fe is thus provided to *A. vinelandii* in the form of Fe-EDTA. Diazotrophic cultures grow rapidly in this modified medium, suggesting that *A. vinelandii* may be better adapted at capturing Fe from organic sources than from iron oxides, possibly through siderophore production (see below). In this medium, a minimum of [Fe(III)] $= 5 \times 10^{-6}$ M is required to sustain maximum diazotrophic growth rates ($\mu = 0.22$ h^{-1} at 25°C, Fig. 1a). No evidence of iron toxicity was found, even at [Fe] $= 1 \times 10^{-4}$ M, which is the highest Fe concentration we tested.

Molybdenum and vanadium

Unlike Fe, Mo and V do not bind to EDTA in our growth medium (Kula and Rabenstein 1966; Przyborowski et al. 1965), so the oxoanions are initially supplied as free molybdate and free vanadate to the bacteria. When *A. vinelandii* is grown under iron-replete conditions, and low [Mo], the growth curve shows an initial fast growth phase and a second phase corresponding to slower growth. The second slower growth phase starts when the bacteria have consumed all Mo initially present in the medium, and they have to dilute their internal stock of Mo as they divide. As little as [Mo] $= 10^{-8}$ M is enough to yield maximum

growth rates initially, and toxicity appears for [Mo] $> 10^{-5}$ M (Fig. 1a). Interestingly, Mo is less toxic at high Fe concentrations, suggesting that Mo may be toxic by interfering with iron uptake (Bellenger et al. unpublished results; Cornish and Page 2000).

When Mo is not available but V is present in the growth medium, *A. vinelandii* expresses the V-nitrogenase. Growth curves of a mutant strain that expresses only the V-nitrogenase (CA11.70, Joerger et al. 1989) show also two distinct growth phases when the bacteria are grown on low [V] (Bellenger et al. 2008a). A concentration of [V] $= 5 \times 10^{-8}$ M is required for maximum initial growth rates (Fig. 1a). V is toxic at a lower concentration than Mo, at [V] $= 1 \times 10^{-6}$ M. Like Mo, V is less toxic at high [Fe].

Tungsten

As expected, W is not required for growth of *A. vinelandii*, and is toxic at concentrations as low as 1×10^{-7} M, depending on the Mo concentration in the medium (Keeler and Varner 1957; Premakumar et al. 1996; Wichard et al. 2008b). In spite of the high chemical similarity between Mo and W, the bacteria discriminate efficiently between the metals, since W is toxic only at high W:Mo concentration ratios (Wichard et al. 2008b).

The dependence of growth rates on metal concentrations shows that *A. vinelandii* has devised uptake mechanisms to acquire essential metals at very low concentrations and discriminate efficiently between essential and toxic metals. As detailed below, the production and excretion in the external medium of high affinity ligands plays a key role in the uptake or exclusion of metals by *A. vinelandii*.

Production of siderophores

In response to Fe

Like other bacteria, *A. vinelandii* excretes siderophores under low Fe concentrations (Andrews et al. 2003). These siderophores bind iron in strong complexes which are then transported inside the cell through highly specific transporters. Consistent with previous results (Cornish and Page 1995), we found that *A. vinelandii* produces at least five different siderophores: the monocatechols 2,3-dihydroxybenzoic acid (DHBA) and aminochelin, the bis(catechol) azotochelin, the tris(catechol) protochelin and the pyoverdine-like azotobactin (Fig. 2). Total siderophore concentrations respond strongly to changes in Fe concentrations and increase sharply at low [Fe] in diazotrophic cultures (Bellenger et al. 2008b). Even in Fe-replete cultures, siderophore concentrations are a few micromolar in the growth medium, showing that siderophore production, although reduced, is not suppressed at high Fe (Fig. 1b). The mono(catechol) DHBA is often the most abundant siderophore in the medium but it has a low metal affinity and plays no or little role in the complexation of metals (Fig. 1b). The tris(catechol) protochelin is usually the most abundant of the bis(catechol) and tris(catechol) siderophores, and often dominates the speciation of Fe, Mo, V, W in the medium. At extremely low Fe concentrations, aminochelin and the pyoverdine-like siderophore azotobactin, which has a catechol, a hydroxamate and a α-hydroxy acid group become dominant in the medium (Tindale et al. 2000; Page and von-Tigerstrom 1988, Wichard et al., unpublished results).

In response to other metals

While there is a sharp increase in siderophore concentrations at low Fe concentrations, there is also a small, but significant increase at very low Mo or V concentrations under Fe-replete conditions (Bellenger et al. 2008b). This is intriguing and suggests that *A. vinelandii* may produce siderophores to acquire Mo and V when these metals are limiting. At high oxoanion concentrations, just below the toxic threshold, siderophore excretion increases dramatically (Cornish and Page 2000) such that Mo, V or W are effectively titrated by the siderophores (Fig. 1b, Bellenger et al. 2008a; Wichard et al. 2008b). Siderophore production clearly appears to play a detoxifying role at high oxoanion concentrations. These observations suggest that siderophore production may in part be regulated by oxoanion requirements and toxicity. However, it is also possible that the changes in siderophore production that are observed reflect indirect effects of Mo, V and W on Fe nutrition: (1) Fe requirements may increase at low [Mo] and low [V] as the bacteria express the Fe-only nitrogenase. This hypothesis is consistent with

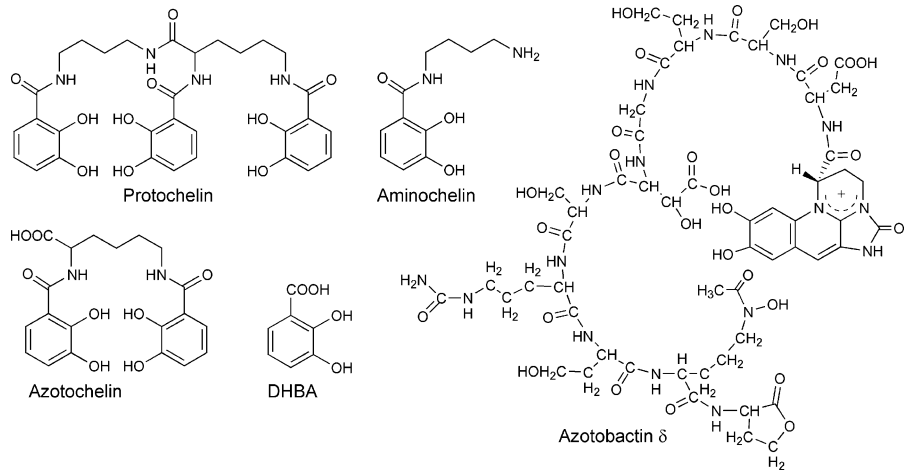

Fig. 2 Catechol siderophores of *Azotobacter vinelandii*

the fact that the increase in siderophore concentrations is not seen in mutant cultures that do not make the Fe-only nitrogenase (Fig. 1b). The very low growth rate of these cultures at low [Mo] and [V] precludes any definite conclusion, however; and (2) Fe uptake rate is inhibited at high Mo, V or W concentrations (Cornish and Page 2000), which could affect the Fe status of the cells and induce siderophore production. Ultimately, molecular studies will be needed to determine whether siderophore production is under the control of low and/or high oxoanion concentrations as well as low [Fe].

Binding of metals by siderophores

Siderophore concentrations in the medium increase over time as the culture grows, changing dramatically metal speciation in the medium. Siderophores have a high affinity for Fe(III), but also for molybdate, vanadate and tungstate (Bellenger et al. 2007). Interestingly, the production of siderophores normalized to the cell number peaks at the end of the lag phase and beginning of the exponential phase. At the end of the lag phase, the siderophore concentration is typically enough to titrate the free metals (i.e., in our experiments, the oxoanions, since the other metals are bound to EDTA). The bacteria thus appear to grow exponentially only after they have achieved partial control of metal speciation by binding the available metals with their own excreted ligands. For example, in a typical experiment under Fe-replete conditions, protochelin binds essentially all the oxoanions (Mo, V and/or W) and a sizeable fraction (typically about 10%) of the Fe in the growth medium (Bellenger et al. 2008b). The remaining of Fe is bound to EDTA.

The metal complexes measured in the growth medium likely reflect kinetics of reaction rather than equilibrium. According to the published constants (Cornish and Page 1998), protochelin has a much higher affinity for Fe than EDTA and should bind all the Fe in our growth medium at equilibrium. But the formation of Fe-protochelin requires metal transfer from Fe-EDTA, which is the initial form of Fe, to protochelin, which is produced during the growth. This is a slow reaction. In contrast, molybdate, vanadate and/or tungstate are free to react quickly with the produced protochelin, and the bulk of these oxoanions are found as protochelin complexes in the medium. Similar situations should obtain in soils where the bulk of the iron is precipitated as slow dissolving oxides or complexed to natural ligands (which are presumably, by analogy with EDTA, slow to exchange iron) while an important fraction of oxoanions is bound to natural organic matter in complexes that rapidly exchange metals with catechol siderophores (see below).

Availability of metal-siderophore complexes

As discussed above, all the metal cofactors of nitrogenase are bound to siderophores in the medium

during diazotrophic growth of *A. vinelandii*. These siderophore complexes must thus be available for uptake. As expected, we observed that Fe-siderophore complexes are taken up. The Fe complexes with catechol siderophores (aminochelin, azotochelin, protochelin) are all taken up at the same rate, suggesting that the same limiting step controls their uptake. Fe-azotobactin is taken up at a slower rate (Wichard et al., unpublished results).

The uptake of siderophore complexes is not limited to Fe, however. Cells also take up the siderophore complexes of Mo and V, but only under conditions where the metal is required for growth (Bellenger et al. 2008a, b). The transport systems for the oxoanion complexes shut down within minutes when the cells are exposed to high (toxic) Mo or V concentrations. The complexes of W, which is not needed for growth, are never taken up.

The molecular details of siderophore-mediated iron uptake in *A. vinelandii* remain to be investigated, but other gram-negative bacteria have been extensively studied. In a first step, the Fe-siderophore complex is transported into the periplasm by a highly specific transporter in the outer membrane. In a second step, either the entire Fe-siderophore complex is transported into the cytoplasm by an ABC transporter, as it is the case for Fe-enterobactin in *Escherichia coli* (Sprencel et al. 2000), or the complex dissociates in the periplasm [possibly after reduction of Fe(III) to Fe(II)], and the metal alone is transported into the cytoplasm, as is the case for Fe-pyoverdin in *Pseudomonas aeruginosa* (Schalk 2008).

The uptake of molybdate by *A. vinelandii* has been well studied. After entering the periplasm, presumably through porins, molybdate is transported by the high affinity ABC transporter encoded by the *mod* operon, with binding of molybdate to the periplasmic ModA protein, followed by transport into the cytoplasm by the ModB and ModC proteins sitting at the cytoplasmic membrane (Grunden and Shanmugan 1997; Self et al. 2001; Pau and Lawson 2002). A similar system was recently identified for vanadate in a N_2-fixing cyanobacterium, but evidence for its existence in *A. vinelandii* is lacking (Pratte and Thiel 2006). We have no molecular data on the uptake of the siderophore complexes of Mo and V. By analogy with the Fe transport system, we hypothesize that the Mo and V siderophore complexes are transported into the periplasm where the metal is exchanged with the ModA protein or its V equivalent before transport into the cytoplasm. The relative affinities of catechol siderophores and ModA for Mo are consistent with such a model. The exclusion of tungsten from the cells could be explained in such a mechanism by an exchange of the W-catechol complex with ModA that is either thermodynamically unfavorable or kinetically hindered.

Regardless of the underlying molecular mechanisms, the transport systems for the siderophore complexes with Fe, Mo and V are highly specific and tightly regulated. The bacterium conditions its external medium by producing siderophores and acquires the metals it needs by expressing the corresponding uptake system (Fig. 3). At high metal (Mo or V) concentrations, the increase in siderophore concentrations to match that of the metal (see above) and the shutting down of the uptake system ensures efficient detoxification. Toxic metals such as W, whose siderophore complexes are not taken up, are also detoxified by complexation with siderophores (Wichard et al. 2008b) .

Effect of siderophores on metal speciation in soils

According to our data, siderophores are produced by *A. vinelandii* at all metal concentrations. Once in the

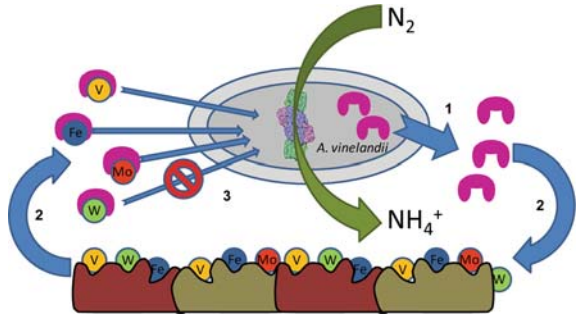

Fig. 3 Excretion of metallophores by *A. vinelandii* for metal uptake or detoxification. (1) Metallophores are produced and excreted into the soil. (2) Metallophores compete with natural organic matter and mineral phases for metal binding. (3) The metal-metallophore complex is unavailable for bacterial uptake unless specific transport systems are expressed at the cell membrane, resulting in the detoxification of toxic metals (such as W) and increased bioavailability of essential metals (such as Fe, Mo and/or V)

external medium, siderophores bind free metals and compete for metals bound to existing organic ligands and particle surfaces. In soils, Fe is bound to natural organic matter or precipitated as oxides, resulting in slow complexation kinetics with siderophores (Kraemer 2004). The production of DHBA might increase iron oxide dissolution rates by shuttling Fe from iron oxides to protochelin, in a process similar to that observed for citrate or oxalate in previous studies (Reichard et al. 2007; Cheah et al. 2003). The oxoanions of Mo, V and W are also bound to organic matter or adsorbed to soil particles. Our laboratory data show that catechol siderophores can capture Mo or V from naturally occurring ligands such as the tris(hydroxamate) desferrioxamine B (DFB), tannic acid or organic matter extracted from senescent leaves, over a time scale ranging from a few minutes to a few days (Fig. 1c, Bellenger et al. 2008b). We presume that a similar ligand exchange occurs in natural environments. As in the laboratory, differential complexation kinetics should thus allow the formation in the soil of relatively weak Mo-, V- and W-siderophore complexes in the presence of an excess of Fe that is organically complexed or precipitated. Overall, siderophore excretion results in a reduction of free metal concentrations and the formation of metal-siderophore complexes, which leads to an increased control of metal speciation by the bacteria. Noxious metals are detoxified as free metal concentrations decrease and rates of metal uptake are reduced. Metals needed for growth are extracted from unavailable forms and taken up as metal-siderophore complexes through the activation of specific uptake systems. Further, metal-siderophore complexes may also be unavailable for uptake by other bacteria, thereby allowing the control of potentially valuable metal resources.

It is still uncertain how the findings for *A. vinelandii* can be generalized to other N_2-fixing bacteria. Unlike *A. vinelandii*, many soil bacteria produce hydroxamate siderophores, which have a lower affinity for oxoanions than catechol siderophores. DFB, for example, does not bind Mo at neutral pH (Farkas et al. 2003). It is possible that the production of siderophores with different oxoanion affinities affect the fitness of N_2-fixing bacteria in soils with different concentrations of Mo, V and W. In turn, the variability in metal requirements among microorganisms (e.g., high Mo requirement in N_2-fixing bacteria) and in metal concentrations and speciation in various locals (e.g., Fe oxides vs. Fe-NOM complexes) may explain the variety in siderophore structures found in bacteria.

Mo cycling in soils

The regulated uptake of the metal complexes formed with excreted siderophores provides N_2-fixing soil bacteria with a mechanism to control the availability of nitrogenase metal cofactors in their external medium. This mechanism may also be one component in a larger machinery with which terrestrial ecosystems conserve and obtain scarce resources. Trees and other plants extract essential metals from soils and accumulate them in their leaves. As observed for Mo, these metals are then retained as complexes with catechol and other functional moieties in the topsoil organic matter produced by decaying leaves (Lang and Kaupenjohann 2000; Wichard et al. 2008a). They are then extracted and taken up by bacteria through the production of siderophores as discussed in this paper. In a classic mutualistic relationship, these metals are used by free-living diazotrophs to fix nitrogen that is utilized by the plants. This relationship presumably provides non-nodulating plants and their associated bacteria much of the benefits that nodulated plants and their symbionts derive from each other.

Acknowledgments This work was supported by a grant from the NSF (CHE-0221978, Center for Environmental Bioinorganic Chemistry) and by a fellowship from the Camille and Henry Dreyfus Postdoctoral Program in Environmental Chemistry to T.W.

References

Alloway BJ (1995) Heavy metals in soils. Blackie Academic and Professional, Glasgow

Andrews SC, Robinson AK, Rodriguez-Quinones F (2003) Bacterial iron homeostasis. FEMS Microbiol Rev 27:215–237. doi:10.1016/S0168-6445(03)00055-X

Barron AR, Wurzberger N, Bellenger JP, Wright SJ, Kraepiel AML, Hedin LO (2009) Molybdenum limits nitrogen fixation in tropical forest soils. Nat Geosci 2:42–45. doi:10.1038/NGEO366

Bellenger J-P, Arnaud-Neu F, Asfari Z, Myneni SCB, Stiefel EI, Kraepiel AML (2007) Complexation of oxoanions and cationic metals by the biscatecholate siderophore azotochelin.

J Biol Inorg Chem 12:367–376. doi:10.1007/s00775-006-0194-6

Bellenger J-P, Wichard T, Kraepiel AML (2008a) Vanadium requirements and uptake kinetics in the dinitrogen-fixing bacterium *Azotobacter vinelandii*. Appl Environ Microbiol 74:1478–1484. doi:10.1128/AEM.02236-07

Bellenger J-P, Wichard T, Kustka AB, Kraepiel AML (2008b) Nitrogen fixing soil bacterium uses catechol siderophores for molybdenum and vanadium acquisition. Nat Geosci 1:243–246. doi:10.1038/ngeo161

Bishop PE, Premakumar R, Dean DR, Jacobson MR, Chnisnell JR, Rizzo TM, Kopczynski J (1986) Nitrogen fixation by *Azotobacter vinelandii* strains having deletions in structural genes for nitrogenase. Science 232:92–94

Burgess BK, Lowe DJ (1996) Mechanism of molybdenum nitrogenase. Chem Rev 96:2983–3011. doi:10.1021/cr950055x

Cheah SF, Kraemer SM, Cervini-Silva J, Sposito G (2003) Steady-state dissolution kinetics of goethite in the presence of desferrioxamine B and oxalate ligands: implications for the microbial acquisition of iron. Chem Geol 198:63–75. doi:10.1016/S0009-2541(02)00421-7

Cornish AS, Page WJ (1995) Production of the tricatecholate siderophore protochelin by *Azotobacter vinelandii*. Biometals 8:332–338. doi:10.1007/BF00141607

Cornish AS, Page WJ (1998) The catecholate siderophores of *Azotobacter vinelandii*: their affinity for iron and role in oxygen stress management. Microbiology 144:1747–1754

Cornish AS, Page WJ (2000) Role of molybdate and other transition metals in the accumulation of protochelin by *Azotobacter vinelandii*. Appl Environ Microbiol 66:1580–1586. doi:10.1128/AEM.66.4.1580-1586.2000

Duhme AK, Hider RC, Naldrett MJ, Pau RN (1998) The stability of the molybdenum-azotochelin complex and its effect on siderophore production in *Azotobacter vinelandii*. J Biol Inorg Chem 3:520–526. doi:10.1007/s007750050263

Farkas E, Csoka H, Toth I (2003) New insights into the solution equilibrium of molybdenum(VI)–hydroxamate systems: ^{1}H and ^{17}O NMR spectroscopic study of Mo(VI)-desferrioxamine B and Mo(VI)–monohydroxamic acid systems. Dalton Trans 8:1645–1652. doi:10.1039/b300431g

Goldberg S, Forster HS, Godfrey CL (1996) Molybdenum adsorption on oxydes, clay minerals, and soils. Soil Sci Soc Am J 60:425–432

Grunden AM, Shanmugan KT (1997) Molybdate transport and regulation in bacteria. Arch Microbiol 168:345–354. doi:10.1007/s002030050508

Gustafsson JP (2003) Modelling molybdate and tungstate adsorption to ferrihydrite. Chem Geol 200:105–115. doi:10.1016/S0009-2541(03)00161-X

Hudson RJM, Morel FMM (1992) Trace metal transport by marine microorganisms: implications of metal coordination kinetics. Deep Sea Res Part I Oceanogr Res Pap 40:129–150. doi:10.1016/0967-0637(93)90057-A

Hungate BA, Stiling PD, Dijkstra P, Johnson DW, Ketterer ME, Hymus GJ, Hinkle CR, Drake BG (2004) CO_2 elicits long-term decline in nitrogen fixation. Science 304:1291. doi:10.1126/science.1095549

Joerger RD, Jacobson MR, Premakumar R, Wolfinger ED, Bishop PE (1989) Nucleotide sequence and mutational analysis of the structural genes (*anf*HDGK) for the second alternative nitrogenase from *Azotobacter vinelandii*. J Bacteriol 171:1075–1086

Keeler RF, Varner JE (1957) Tungstate as an antagonist of molybdate in *Azotobacter vinelandii*. Arch Biochem Biophys 70:585–590. doi:10.1016/0003-9861(57)90146-7

Konigsberger LC, Konigsberger E, May PM, Hefter GT (2000) Complexation of iron(III) and iron(II) by citrate. Implications for iron speciation in blood plasma. J Inorg Biochem 78:175–184. doi:10.1016/S0162-0134(99)00222-6

Kraemer SM (2004) Iron oxide dissolution and solubility in the presence of siderophores. Aquat Sci 66:3–18. doi:10.1007/s00027-003-0690-5

Kula RJ, Rabenstein DL (1966) Solution equilibria and structures of Molybdenum(VI) complexes chelates. (Ethylenedinitrilo)tetraacetic acid. Anal Chem 38:1581–1584. doi:10.1021/ac60243a032

Kustka AB, Sanudo-Wilhelmy SE, Carpenter EJ, Capone D, Burns J, Sunda WG (2003) Iron requirements for Dinitrogen- and Ammonium-supported growth in cultures of *Trichodesmium* (IMS 101): comparison with nitrogen fixation rates and iron: carbon ratios of field populations. Limnol Oceanogr 48:1869–1884

Lang F, Kaupenjohann M (2000) Molybdenum at German Norway spruce sites: contents and mobility. Can J Res 30:1034–1040. doi:10.1139/cjfr-30-7-1034

Page WJ, von-Tigerstrom M (1988) Aminochelin, a catecholamine siderophore produced by *Azotobacter vinelandii*. J Gen Microbiol 134:453–460

Pau RN, Lawson DM (2002) Transport, homeostasis, regulation, and binding of molybdate and tungstate to proteins. In: Sigel A, Sigel H (eds) Metal ions in biological systems: molybdenum and tungsten: their roles in biological processes. Dekker Inc., Basel

Perakis SS, Hedin LO (2002) Nitrogen loss from unpolluted South American forests mainly via dissolved organic compounds. Nature 415:416–419. doi:10.1038/415416a

Pratte BS, Thiel T (2006) High-affinity vanadate transport system in the cyanobacterium *Anabaena variabilis* ATCC 29413. J Bacteriol 188:464–468. doi:10.1128/JB.188.2.464-468.2006

Premakumar R, Jacobitz S, Ricke SC, Bishop PE (1996) Phenotypic characterization of a tungsten-tolerant mutant of *Azotobacter vinelandii*. J Bacteriol 178:691–696

Przyborowski VL, Schwarenbach G, Zimmermann T (1965) Komplexe XXVII. Die EDTA-Konplexe des Vanadiums (V). Helv Chim Acta 48:1556–1565. doi:10.1002/hlca.19650480716

Reichard PU, Kretzschmar R, Kraemer SM (2007) Dissolution mechanisms of goethite in the presence of siderophores and organic acids. Geochim Cosmochim Acta 71:5635–5650. doi:10.1016/j.gca.2006.12.022

Schalk IJ (2008) Metal trafficking via siderophores in gram-negative bacteria: specificities and characteristics of the pyoverdine pathway. J Inorg Biochem 102:1159–1169. doi:10.1016/j.jinorgbio.2007.11.017

Self WT, Grunden AM, Hasona A, Shanmugam KT (2001) Molybdate transport. Res Microbiol 152:311–321. doi:10.1016/S0923-2508(01)01202-5

Serrat FB, Morell GB (1994) Colorimetric method for the determination of vanadium with tannic acid in water and oils. Fresenius J Anal Chem 349:717. doi:10.1007/BF00325645

Siemann S, Schneider K, Oley M, Muller A (2003) Characterization of a tungsten-substituted nitrogenase isolated from *Rhodobacter capsulatus*. Biochemistry 42:3846–3857. doi:10.1021/bi0270790

Silvester WB (1989) Molybdenum limitation of asymbiotic nitrogen fixation in forests of Pacific Northwest America. Soil Biol Biochem 21:283–289. doi:10.1016/0038-0717(89)90106-5

Sprencel C, Cao Z, Qi Z, Scott DC, Montague MA, Ivanoff N, Xu J, Raymond KM, Newton SMC, Klebba PE (2000) Binding of ferric enterobactin by the *Escherichia coli* periplasmic protein FepB. J Bacteriol 182:5359–5364. doi:10.1128/JB.182.19.5359-5364.2000

Stiefel EI, Watt GD (1979) *Azotobacter* cytochrome b_{557} is a bacterioferritin. Nature 279:81–83. doi:10.1038/279081a0

Stumm W, Morgan JJ (1995) Aquatic chemistry. Wiley-Interscience Publication, New York

Tindale AE, Mehrotra M, Ottem D, Page WJ (2000) Dual regulation of catecholate siderophore biosynthesis in *Azotobacter vinelandii* by iron and oxidative stress. Microbiology 146:1617–1626

Wedepohl KH (1995) The composition of the continental crust. Geochim Cosmochim Acta 59:1217–1232. doi:10.1016/0016-7037(95)00038-2

Werber T, Allard T, Tipping E, Benedetti MF (2006) Modeling Iron bonding to organic matter. Environ Sci Technol 40:7488–7493. doi:10.1021/es0607077

Wichard T, Mishra B, Kraepiel AML, Myneni SCB (2008a) Molybdenum speciation and bioavailability in soils. Geochim Cosmochim Acta 72:A1019–A1019

Wichard T, Bellenger J-P, Loison A, Kraepiel AML (2008b) Catechols siderophores control tungsten uptake and toxicity in the nitrogen-fixer bacterium *Azotobacter vinelandii*. Environ Sci Technol 42:2408–2413. doi:10.1021/es702651f