Uptake of molybdenum and vanadium by a nitrogen-fixing soil bacterium using siderophores

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Nitrogen fixation, the reaction that transforms atmospheric nitrogen into bioavailable ammonia and is responsible for the supply of nitrogen to Earth's ecosystems, is mediated by the enzyme nitrogenase. This reaction requires molybdenum (Mo) or vanadium (V) in addition to iron (Fe) (refs 1,2). Therefore, the availability of these trace metals may control the Earth's nitrogen cycle[3,4]. Many bacteria release strong iron-binding compounds (siderophores) for iron acquisition[5,6], but the effect of these compounds on Mo and V availability to nitrogen-fixing organisms is not well understood. Here, we show that the siderophores produced in cultures of *Azotobacter vinelandii* while fixing atmospheric nitrogen under limitation by Mo or V form strong complexes with molybdate and vanadate, and that these complexes are available for uptake. We also show that addition of these siderophores rapidly reverses the effect of other natural binding compounds that make Mo and V unavailable for uptake. Our results resolve the long-standing debate regarding the existence of bacterial 'molybdophores'[7–9], as well as the corollary question regarding 'vanadophores'. We conclude that the production of strong binding compounds may be a widespread strategy for metal acquisition by bacteria, implying that the availability of Mo and V may be critical for the nitrogen cycle of terrestrial ecosystems.

In metal-replete diazotrophic cultures, the gram-negative soil bacterium *Azotobacter vinelandii* expresses the Mo nitrogenase, which is most efficient, preferentially to the V nitrogenase or the Fe-only nitrogenase[1] and its growth can be limited by Fe, Mo or V (Fig. 1a). Our growth medium contains 10^{-4} M EDTA to avoid the precipitation of Fe as colloidal iron hydroxides, the dissolution kinetics of which decrease over time, changing Fe bioavailability[10,11]. At an iron concentration ([Fe]) of 5×10^{-6} M, the wild type (strain OP) reaches a maximum growth rate of $0.25\,h^{-1}$ (faster than in media without EDTA; see the Methods section). Fe-sufficient cultures require a molybdate concentration ([Mo]) of 1×10^{-8} M for maximum growth in the absence of added V, but high [Mo] ($>10^{-4}$ M) is toxic. Diazotrophic cultures of strain CA11.70, a double-deletion mutant that expresses only the V nitrogenase[12], require a minimum vanadate concentration ([V]) of 10^{-7} M for maximum growth (Fig. 1a) but V is toxic above 10^{-6} M.

Under our culture conditions, *A. vinelandii* produces various types of siderophore. The monocatechol 2,3-dihydroxybenzoic acid (DHBA) and the tris(catechol) protochelin are produced in higher concentrations than the bis(catechol) azotochelin (Fig. 1b, Supplementary Information, Fig. S1). (The pyoverdin-like siderophore azotobactin[8] could not be detected.) The concentration of siderophores in the medium is significant ($>10^{-7}$ M) even at very high [Fe], and increases at low Fe concentrations. The production of protochelin and azotochelin also increases at low [Mo] and low [V], possibly reflecting the use of the Fe-only nitrogenase for bacterial growth. Here, we examine how these catechols affect the availability of Mo and V.

Whereas the metal affinity of DHBA is relatively poor[13], protochelin and azotochelin are strong complexing agents for Fe(III), molybdate and vanadate. For example, azotochelin (LH_5) reacts with molybdate to form a 1:1 complex with Mo(VI) ($LH_4^- + MoO_4^{2-} \rightarrow MoO_2L^{3-} + 2H_2O$, $\log K_{Mo}^{Az} = 7.6$) (ref. 14). As revealed by mass spectrometry, the reaction of molybdate with protochelin also yields a 1:1 complex (Mo–protochelin), with a structure probably similar to that of Mo–azotochelin[15] (Fig. 2a, inset). The oxoanion vanadate, which is the dominant form of vanadium in oxic environments, also forms strong complexes with protochelin (Fig. 2b) and azotochelin[14], although the exact structures of these complexes have not been elucidated.

To determine whether protochelin and azotochelin actually complex Mo and V in the culture medium, we used a high-performance liquid chromatograhy (HPLC) separation coupled to inductively coupled plasma mass spectrometry (ICP-MS) analysis of collected fractions to quantify the catechols and catechol–metal (Fe, Mo, V) complexes. In cultures of the wild type grown with Mo at high [Fe] ([Mo] = 7×10^{-7} M and [Fe] = 5×10^{-6} M), we accounted for 80% of the Mo originally present in the medium in the form of the Mo–protochelin complex (Fig. 2a). Similarly, we accounted for 84% of the V in the protochelin complex in V-containing cultures of the wild type (no Mo added, Fig. 2b). Such high recoveries after the concentration and separation steps demonstrate for the first time that the bulk of Mo and V are complexed by the catechols excreted by *A. vinelandii* in its growth medium. They also demonstrate

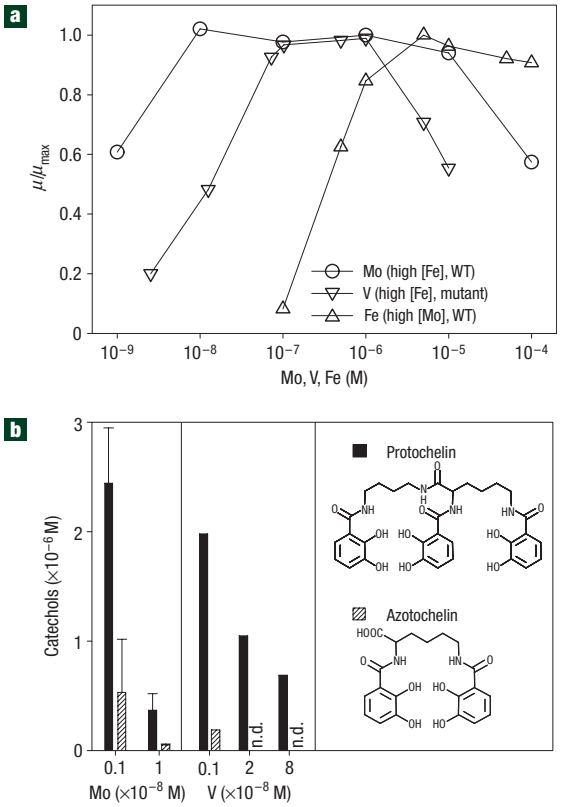

Figure 1 Effect of Mo, Fe and V on the growth and production of catechol siderophores by *A. vinelandii* under diazotrophic conditions. a, Relative growth rates (μ / μ_{max}) of strain OP (wild type) and mutant strain CA11.70 as a function of metal concentrations. Circles: [Fe] = 5×10^{-6} M, no V added; downward triangles: [Fe] = 5×10^{-6} M, no Mo added; upward triangles: [Mo] = 1×10^{-6} M, no V added. **b**, Total concentrations of protochelin and azotochelin released into the growth medium by strain OP under various concentrations of Mo or V ([Fe] = 5×10^{-6} M). Values represent means ±s.d. ($n = 3$) for Mo and are from a single experiment for V. n.d. = not detected.

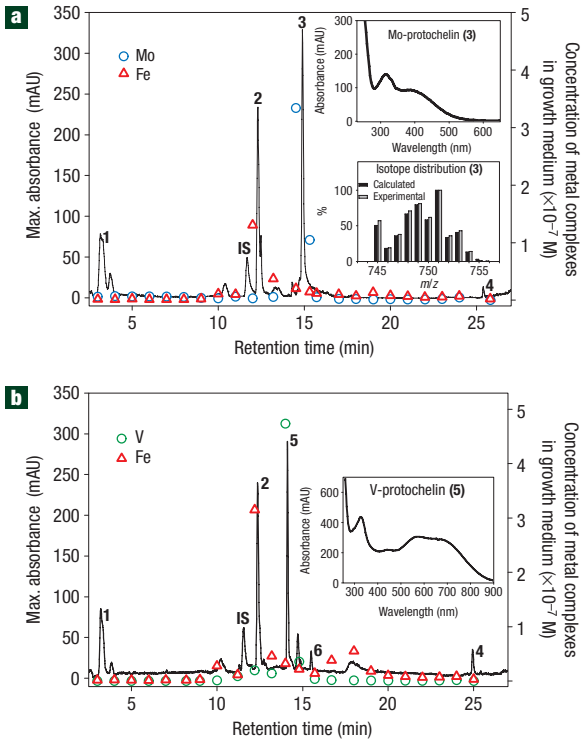

Figure 2 Complexation of Mo, V and Fe by catechol siderophores in cultures of *A. vinelandii* (strain OP). HPLC chromatograms of growth medium extracts (lines) and concentrations of metal complexes in the growth medium calculated on the basis of measurements of collected HPLC fractions (circles and triangles): **1** = 2,3-dihydroxy-benzoic acid, **2** = Fe–protochelin, **3** = Mo–protochelin, **4** = protochelin, **5** = V–protochelin, **6** = azotochelin, **IS** = internal standard. **a**, Bacteria grown at [Mo] = 7.0×10^{-7} M and [Fe] = 5×10^{-6} M. Top inset: Ultraviolet–visible spectrum of an HPLC fraction containing Mo–protochelin. Bottom inset: Selected part of the electrospray ionization mass spectrometry spectrum of the Mo–protochelin complex showing the characteristic isotopic pattern of the Mo-containing peak at m/z 751 corresponding to $[C_{31}H_{32}N_4O_{12}Mo]^{2-} + H^+$. **b**, Bacteria grown at [V] = 6.9×10^{-7} M and [Fe] = 5×10^{-6} M (no Mo added). Inset: Ultraviolet–visible spectrum of an HPLC fraction containing V–protochelin.

that the monocatechol siderophores of *A. vinelandii*, DHBA and aminochelin do not affect Fe, Mo and V speciation either because of their low metal affinity[16] or because they are not present in the medium under our culture conditions. In spite of the higher affinity of protochelin for Fe compared with EDTA, only 3–8% of the Fe is in the form of the protochelin complex in this EDTA-buffered medium. This reflects the slow formation of Fe–protochelin in the presence of EDTA[17]. Similarly slow complexation kinetics of Fe with siderophores are observed in the presence of iron hydroxides[11,18]. Complexes of catechol siderophores with Mo and V in the presence of an excess of Fe should thus form in soils as they do in laboratory cultures.

Unless the organisms have a specific uptake system for the Mo–and V–catechol complexes, the presence of protochelin in their external medium, while facilitating iron acquisition, could prevent the uptake of Mo and V. To examine the availability of the Mo–catechol complexes, wild-type *A. vinelandii* cells preconditioned with limiting concentrations of ^{98}Mo (2×10^{-9} M) were resuspended in fresh medium containing ^{95}Mo complexed to an excess of protochelin or azotochelin ([^{95}Mo] = 2×10^{-9} M and [ligand] = 10^{-6} M). (All Mo in the resuspension medium is bound to the ligand[14].) The rapid uptake of Mo bound to either protochelin or azotochelin shows that the complexes are available for *A. vinelandii* at low [Mo] (Fig. 3a, Supplementary Information, Fig. S2). As the uptake rate is unaffected by increasing the catechol concentration (see Supplementary Information, Fig. S3), it is the complex itself, not the free oxoanion at equilibrium with it, that is taken up. The measured short-term uptake rate of Mo–protochelin (8.3×10^{-21} mol Mo cell^{-1} min^{-1}) is very close to the steady-state uptake rate calculated from the cellular Mo quota and the growth rate of low-[Mo] cultures (1.8×10^{-18} mol Mo cell^{-1} × 4.9×10^{-3} min^{-1} = 8.8×10^{-21} mol Mo cell^{-1} min^{-1}) demonstrating that Mo–protochelin, which is the main form of Mo in the growth medium, is also the main source of Mo to the bacteria. The uptake shuts down in cells preconditioned at high [Mo] (=10^{-4} M), suggesting the existence of a regulated uptake system for Mo–protochelin and Mo–azotochelin, perhaps similar to that of Fe–siderophores. An outer membrane transporter, such as the 44 kDa protein that is hyperproduced at the outer membrane of *A. vinelandii* in response to Mo limitation[9], may serve to transport the complex (or the metal alone) inside the periplasm. There, Mo might be taken up through the high-affinity, ABC-type, molybdate transporter encoded by the mod operon[19].

To investigate the specificity of Mo transport, we studied the uptake of the oxoanion tungstate (WO_4^{2-}). Tungstate's structure and chemistry are analogous to those of molybdate, and it also forms strong complexes with protochelin and azotochelin[14]. Tungstate, however, is toxic to *A. vinelandii*, as it can be incorporated into nitrogenase, resulting in an inactive form of the enzyme[20]. In a resuspension medium with an excess of either protochelin or azotochelin, the uptake rate of tungstate by Mo-limited cells of *A. vinelandii* is below detection, even at high W concentrations ($v^{W}_{prot} \leq 6 \times 10^{-22}$ mol cell^{-1} min^{-1} at [W] = 10^{-7} M). The uptake system that takes up Mo–protochelin and Mo–azotochelin at low [Mo] is thus able to differentiate between the complexes of Mo (which is essential for growth) and the complexes of W (which is toxic). This is particularly interesting because the periplasmic protein ModA encoded by the mod operon does not discriminate efficiently between molybdate and tungstate[19].

We also investigated whether the V–protochelin and V–azotochelin complexes are available for uptake. V-requiring mutant cultures were grown under limiting V concentrations ([V] = 2×10^{-8} M), harvested and resuspended in a fresh medium containing 2×10^{-8} M of ^{49}V complexed to an excess (10^{-6} M) of either protochelin or azotochelin. The rapid uptake of V bound to either protochelin or azotochelin at a rate independent of the excess catechol concentration demonstrates that the V–protochelin and V–azotochelin complexes are taken up by bacteria grown at low [V] (Fig. 3b, Supplementary Information, Fig. S3). In contrast, bacteria grown at high [V] do not take up the protochelin or azotochelin complexes of V, indicating that, like for molybdate, the uptake system for the complexes shuts down at high V concentrations. V-limited bacteria do not take up V bound to the tris(hydroxamate) siderophore desferrioxamine B (DFB) (ref. 21), demonstrating again the specificity of the uptake system(s) (Fig. 3b).

Because in the wild-type strain, the expression of the V nitrogenase is repressed at high [Mo] (ref. 2), we expect the uptake system for V–catechol complexes to be regulated by Mo concentrations. Indeed, the wild type takes up V–azotochelin only when cells are preconditioned at low [Mo] ($2 \pm 0.5 \times 10^{-9}$ M, Fig. 3c). The uptake rates of the V complex are comparable in the wild type and the mutant strains ($v_{WT} = 1.9 \times 10^{-21}$ mol cell^{-1} min^{-1} and $v_{mutant} = 2.6 \times 10^{-21}$ mol cell^{-1} min^{-1}, Fig. 3). Very little is known of V uptake in *A. vinelandii*, but it is possible that, like other oxoanions, vanadate is transported by an ABC-type system. Indeed, an ABC transporter for vanadate was recently identified in the nitrogen-fixing cyanobacterium *Anabaena variabilis*[22].

Our results resolve the long-standing issue of whether catechol siderophores, and particularly azotochelin, are used to deliver molybdate to *A. vinelandii* cells[7–9]. Molybdenum and vanadium are the first oxoanions, and the only transition metals with iron and copper[23] shown to have an uptake system involving the extracellular formation of a high-affinity complex. But are azotochelin and protochelin truly 'molybdophores' and 'vanadophores' in addition to being siderophores, in the sense that they actually help in acquiring Mo and V from otherwise unavailable forms in their external medium?

In soils, particularly at low pH, the solubility of the scarce molybdate and vanadate is reduced by adsorption on Fe, Mn and Al oxides and clays[24]. In the organic-rich O horizon of soils, where heterotrophic N_2-fixing bacteria are most active[25], the dissolved fraction of oxoanions is apparently complexed with organic matter[26], further decreasing their availability to microorganisms. Indeed, the uptake by *A. vinelandii* of V previously equilibrated with natural organic matter is slow and nearly doubles when azotochelin is present in the medium (Fig. 4a). Although the organic ligands that bind V in natural organic matter have not been identified, hydroxamate siderophores similar to DFB are ubiquitous in soils[27]. As shown above, DFB binds strongly to vanadate and low-[V] cells resuspended in a medium with excess DFB do not take up V (Fig. 3b). Azotochelin is a stronger ligand than DFB[14,19] ($\log K^{V-Az}_{app} = 8.8$, versus $\log K^{V-DFB}_{app} = 7.0$) and on azotochelin addition, V is rapidly transferred from DFB to azotochelin, becoming available for bacterial uptake (Fig. 4b). The rapid acceleration of V uptake on addition of azotochelin, which decreases the free-vanadate concentration, provides a direct and marked demonstration that it is the complex itself that it is taken up. The same experiment could not be repeated with molybdate, which does not form strong complexes with DFB (or other commercially available ligands) at circumneutral pH. Nonetheless, the experiments of Fig. 4 demonstrate that catechols excreted by soil microorganisms are able to increase the availability of essential oxoanions.

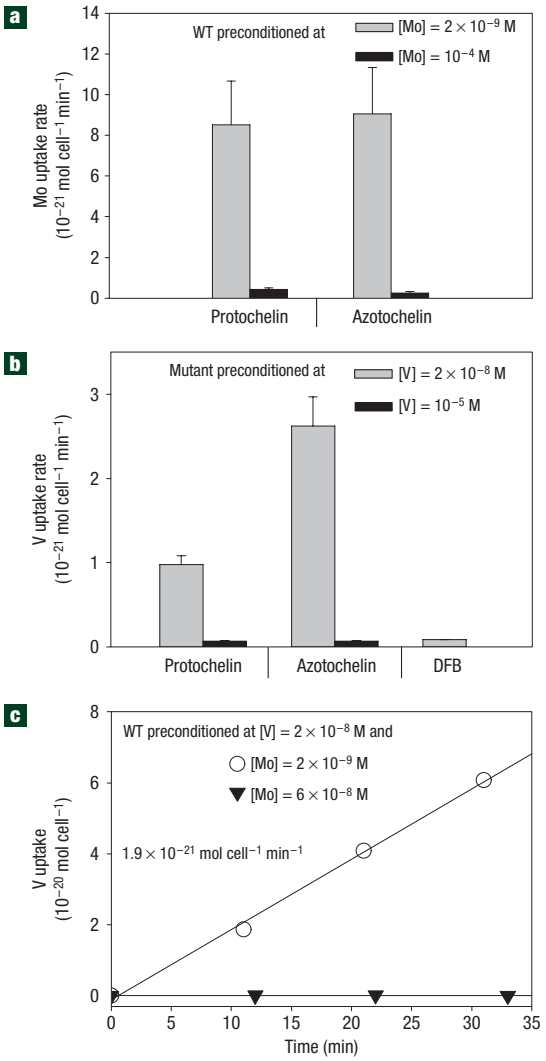

Figure 3 Short-term uptake of the Mo and V complexes with protochelin, azotochelin and DFB by *A. vinelandii*. **a**, Mo uptake rates by strain OP (wild type) in a medium with [Mo] = 2×10^{-9} M and [ligand] = 10^{-6} M. **b**, V uptake rates by mutant strain CA11.70 in a medium with [V] = 2×10^{-8} M and [ligand] = 10^{-6} M. Ligand=protochelin, azotochelin or DFB. Values represent means ±s.d. (n = 2–5). **c**, V uptake by strain OP in a medium with [V] = 2×10^{-8} M and [azotochelin] = 10^{-4} M.

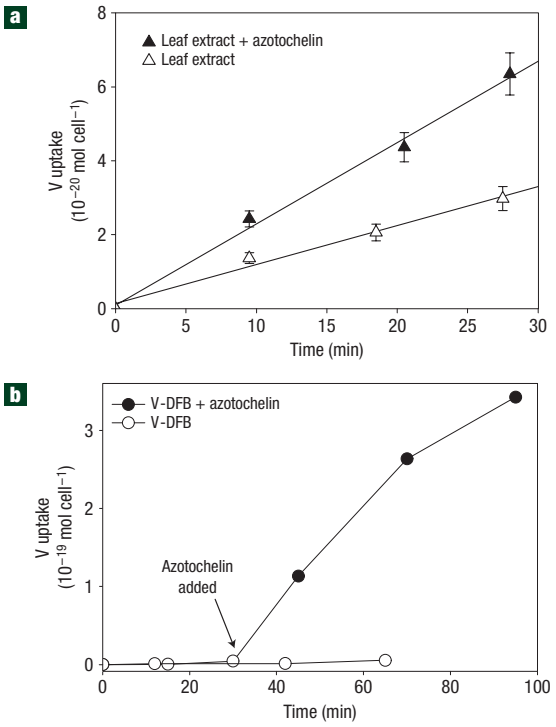

Figure 4 Effect of azotochelin on V uptake in the presence of other vanadium ligands by *A. vinelandii* (mutant strain CA11.70). a, V uptake in a medium with $[V] = 2 \times 10^{-8}$ M and leaf extract, with or without azotochelin (10^{-7} M); bacteria preconditioned at $[V] = 2 \times 10^{-8}$ M. **b**, V uptake in a medium with $[V] = 4 \times 10^{-8}$ M and $[DFB] = 3 \times 10^{-4}$ M; in one experiment, 10^{-4} M of azotochelin was added to the medium at $t = 31$ min; bacteria preconditioned at $[V] = 4 \times 10^{-8}$ M.

There is evidence that Mo limits N_2 fixation by heterotrophic N_2-fixing bacteria in various temperate and tropical ecosystems[28,29]. The role of the V nitrogenase may thus deserve more attention. It is also known that the reaction of siderophores with mineral iron phases is slow[18]. Our results imply that Mo and V will be complexed by catechol siderophores in soils where N_2-fixing bacteria such as *A. vinelandii* are active, and that the extent of this complexation will determine their bioavailability. Catechol compounds are thus likely to play a key role in the fixation of N_2 and the productivity of terrestrial ecosystems[4] by serving the multiple purposes of increasing the solubility of iron oxides and, particularly at low pH, capturing Mo and V from unavailable forms.

METHODS

Complexation of Mo and V by catechol siderophores was determined by reverse-phase HPLC and ICP-MS. Strain OP (wild type) and mutant strain CA11.70 (expressing only the V nitrogenase) of *A. vinelandii* were cultured under diazotrophic conditions in clean liquid minimal medium following a metal recipe adapted from Fraquil[10]. Cell-free supernatants were concentrated on hydrophilic–lipophilic balance (HLB-Oasis, Waters) solid-phase cartridges and eluted with methanol. Total amounts of catechol siderophores in the methanolic fraction were analysed by reverse-phase C_{18}-HPLC at pH 2.2 and their metal complexes were determined at pH 6.6. Siderophores and their metal complexes were identified by co-injections of synthesized and purified standards. The identities of the protochelin standard, which was purified from cultures of *A. vinelandii*, and of the Mo–protochelin complex standard, were verified by electrospray ionization mass spectrometry. To quantify the total amount of oxoanions bound by catechol siderophores in the growth medium, HPLC fractions were collected and their Mo, V and Fe content was measured by ICP-MS.

Short-term uptake rates of Mo or V were measured in the presence of various ligands (protochelin, azotochelin, desferrioxamine B, cold water extracts of leaf litter). Mo uptake was measured using the stable isotopes ^{95}Mo and ^{98}Mo (see Supplementary Information, Fig. S2). *A. vinelandii* (strain OP) was grown in a medium with $^{98}MoO_4^{2-}$. After several transfers, cells were collected and resuspended in a medium with $^{95}MoO_4^{2-}$ for short-term uptake experiments. Aliquots were collected at regular intervals for determination of intracellular ^{95}Mo and ^{98}Mo by ICP-MS. V uptake was studied using the radiotracer ^{49}V. *A. vinelandii* cells (OP or CA11.70) were preconditioned in a medium containing cold vanadate, collected and resuspended in a medium spiked with ^{49}V. Intracellular ^{49}V was measured by liquid scintillation counting.

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