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Siderophore production in *Azotobacter vinelandii* in response to Fe-, Mo- and V-limitation

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Summary

***Azotobacter vinelandii* is a terrestrial diazotroph well studied for its siderophore production capacity and its role as a model nitrogen fixer. In addition to Fe, *A. vinelandii* siderophores are used for the acquisition of the nitrogenase co-factors Mo and V. However, regulation of siderophore production by Mo- and V-limitation has been difficult to confirm and knowledge of the full suite of siderophores synthesized by this organism has only recently become available. Using this new information, we conducted an extensive study of siderophore production in N_2-fixing *A. vinelandii* under a variety of trace metal conditions. Our results show that under Fe-limitation the production of all siderophores increases, while under Mo-limitation only catechol siderophore production is increased, with the strongest response seen in protochelin. We also find that the newly discovered *A. vinelandii* siderophore vibrioferrin is almost completely repressed under Mo- and V-limitation. An examination of the potential nitrogen 'cost' of siderophore production reveals that investments in siderophore N can represent as much as 35% of fixed N, with substantial differences between cultures using the Mo- as opposed to the less efficient V-nitrogenase.**

Introduction

The availability of trace metals, particularly Fe, limits microbial growth in many environments. One common strategy for dealing with this limitation is the production of siderophores, small molecules that help to deliver iron to the producing organism (Hider and Kong, 2010). In addition to their role in Fe-acquisition, siderophores are also involved in the uptake of other trace metals (Johnstone and Nolan, 2015; Kraemer *et al.*, 2015). The regulation of siderophore production by Fe has been well established but much less is known about regulation by other trace metals. Although most bacteria produce multiple, structurally distinct siderophores, the utility of this diversity remains mostly unexplored. The possible use of siderophores or 'metallophores' in the acquisition of non-Fe metals is therefore a fertile area for the investigation of siderophore function and regulation with implications for trace metal dependent biogeochemical processes.

Nitrogen fixation is catalysed by nitrogenase, a metalloenzyme encoded by a small group of bacteria and archaea called diazotrophs. Nitrogenase requires several trace metals to perform this reaction; typically 38 iron and 2 molybdenum atoms per enzyme (Seefeldt *et al.*, 2009). In 'alternative' nitrogenase enzymes, molybdenum (Mo) can be replaced by vanadium (V) or iron (Fe) (Bishop *et al.*, 1986; Robson *et al.*, 1986). Nitrogen fixation therefore creates a demand for Fe as well as Mo and/or V and the availability of these trace metals to diazotrophs has the potential to constrain nitrogen fixation rates.

Azotobacter vinelandii is a soil-dwelling diazotroph that encodes Mo-, V- and Fe-only nitrogenases (Bishop *et al.*, 1986; Chisnell *et al.*, 1988). 'Siderophores' produced by this organism are used to acquire Fe as well as Mo and V needed for nitrogen fixation (Kraepiel *et al.*, 2009). *A. vinelandii* synthesizes a variety of catechol siderophores; aminochelin (Cornish and Page, 1998), azotochelin (Corbin and Bulen, 1969), protochelin (Cornish and Page, 1995) and 2,3-dihydroxybenzoic acid (DHBA) (Corbin and Bulen, 1969), as well as the large fluorescent siderophore azotobactin (Bulen and LeComte, 1962; Page *et al.*, 1991). We recently reported that *A. vinelandii* also makes vibrioferrin, an α-hydroxycarboxylate siderophore that was not previously known to be synthesized by this organism (Baars *et al.*, 2015). With the exception of vibrioferrin, *A. vinelandii* siderophores have been studied by numerous authors (for example, Page and Huyer, 1984; Duhme *et al.*, 1998; Liermann *et al.*, 2005; Kraepiel *et al.*, 2009; Yoneyama *et al.*, 2011). However, these investigations have typically focused on only one

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or a few siderophores and often used different culturing and trace metal conditions, making comparisons between studies difficult. Synergistic siderophore interactions have been invoked as one of the key explanations for multiple siderophore production (Challis and Hopwood, 2003; Cheah *et al*., 2003; Reichard *et al*., 2007), necessitating studies that track an organism's entire suite of siderophores.

Azotochelin, protochelin and azotobactin form stable complexes with Mo and V (Duhme *et al*., 1998; Bellenger *et al*., 2007; 2008b; Wichard *et al*., 2009a). *A. vinelandii* not only takes up Mo and V bound to these siderophores, it also distinguishes between different siderophore-metal complexes (Bellenger *et al*., 2008a,b; Wichard *et al*., 2008; Kraepiel *et al*., 2009). The specificity of this siderophore uptake system allows *A. vinelandii* to acquire trace metals as needed for growth and to avoid toxic metals such as tungsten (W) which have no biological utility in this organism (Wichard *et al*., 2008). At high concentrations, Mo and V can also be toxic, and indeed, a number of studies have shown that at concentrations >1 μM *A. vinelandii* increases siderophore production in order to detoxify these metals (Cornish and Page, 1995; Duhme *et al*., 1998; Bellenger *et al*., 2008b, 2011; Yoneyama *et al*., 2011). In contrast to the clear regulation of *A. vinelandii* siderophore production by high Mo and V concentrations, the control of siderophore production by low concentrations of these metals has been difficult to demonstrate. There is evidence that catechol siderophores are favoured at low Mo (Yoneyama *et al*., 2011) and that *A. vinelandii* increases catechol production under Mo- and V-limitation (Bellenger *et al*., 2008b) but these experiments are often complicated by differences in cell growth under limiting trace metal conditions, and have not always been consistent across experiments (Kraepiel *et al*., 2009). Consequently, while the use of siderophores for the uptake and detoxification of Mo and V is now well established, it is still unclear if *A. vinelandii* siderophore production is regulated by Mo- and V-limitation.

Here, we conduct a comprehensive study of siderophore production in nitrogen-fixing *A. vinelandii* under trace metal limitation. This work takes advantage of recent advances in both genome-mining and analytical chemistry which allow for the identification and quantification of the entire suite of siderophores (the 'chelome') made by *A. vinelandii* (Baars *et al*., 2015). Mutant strains that encode only the Mo- or V-nitrogenase were used in order to examine changes in siderophore production when *A. vinelandii* fixes nitrogen using different nitrogenase isozymes. Our results show that *A. vinelandii* increases catechol siderophore production when limited for Mo and that protochelin is the siderophore produced at the highest concentrations under these conditions. In contrast, production of the α-hydroxycarboxylate siderophore vibrioferrin is repressed under limitation for Mo and V. We also observed a consistent offset in the quantity of siderophores produced by cultures using the Mo-nitrogenase as opposed to the less efficient V-nitrogenase. The potential nitrogen 'cost' associated with siderophore production when using different isozymes was explored as an additional control on siderophore production.

Results

Growth, alternative nitrogenase use and Fe-limitation

Mutant *A. vinelandii* strains that encode only the Mo- or V-nitrogenase were cultured in nitrogen-free medium with varying Fe additions (see Experimental procedures). Wild-type cultures supplied only with Mo were also grown. As expected, Mo-only mutant and Mo-grown wild-type cultures (herein Mo-grown cells) showed similar growth rates (Fig. 1). At 2.5 μM Fe and 5 μM Fe, Mo-grown cells had higher growth rates than the V-only mutant. This offset in growth rate is attributed to the fact that the V-nitrogenase is less efficient than the Mo-nitrogenase (Eady and Robson, 1984; Bellenger *et al*., 2011). Fe had a clear effect on all strains with low concentrations leading to decreased growth rates (Fig. 1A–C) although the V-only mutant did not show much change between 5 μM Fe and 2.5 μM Fe. At low Fe, the growth rates of all cultures were indistinguishable (Fig. 1A), likely reflecting limitation of other Fe-requiring cellular processes, such as respiration, which may mask differences in the nitrogenase isozyme efficiencies.

Trace metal use

Fe, Mo and V concentrations remaining in culture medium were measured at several time points during the experiment (Fig. 2). Predictably, all trace metals were drawn down throughout growth. While high Fe treatments (2.5 μM and 5 μM Fe) generally had slightly higher final Fe concentrations ($\leq$ 200 nM) than low (0.5 μM) Fe treatments ($\leq$ 50 nM, Fig. 2A), there was no clear effect of nitrogenase isozyme on final Fe concentration in the growth medium. Mo and V were almost entirely exhausted in high Fe treatments. In low-Fe treatments, V was not entirely depleted and Mo concentrations often increased at the end of growth (Fig. 2B).

Siderophore production in response to Fe

Siderophore production was quantified in the extracellular medium of each experimental treatment (Fig. 3). All siderophore production data are plotted as a function of optical density (620 nm) to account for differences in growth rates (Fig. 1; siderophore production as a function of time is shown in Supporting Information Fig. S1). Vibrioferrin was

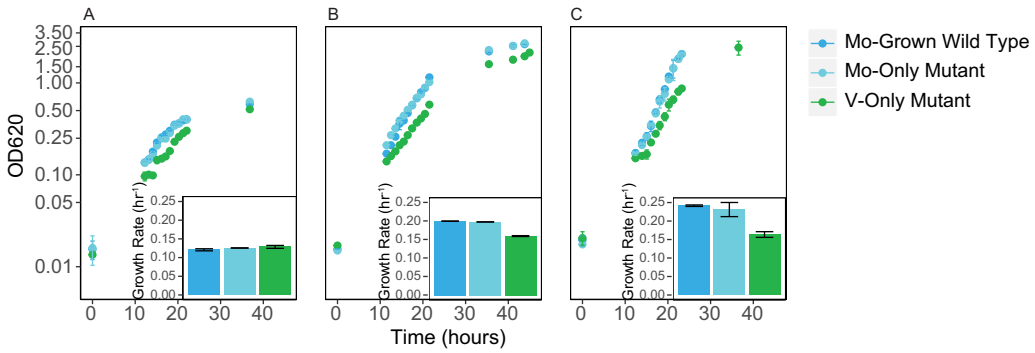

Fig. 1. Growth of *A. vinelandii* with A. 0.5 μM Fe, B. 2.5 μM Fe and C. 5 μM Fe. Growth was tracked using the optical density at 620 nm (OD620). Changes in optical density and calculated growth rates for the Mo-grown wild type, Mo-only mutant and V-only mutant are shown. Error bars represent standard deviations for two biological replicates.

the siderophore produced at the highest concentrations across experimental treatments (Fig. 3E, note the differences in scale) followed by protochelin and azotochelin (Fig. 3C and D). The production of vibrioferrin as well as all catechol siderophores increased at lower Fe concentrations. Despite clear Fe-limitation (Figs 1 and 2), there was no detectable azotobactin production in any of our experiments, consistent with previous studies conducted under similar conditions (Baars *et al.*, 2015).

Nitrogen investments in siderophores

Both siderophore quantity and type varied with the nitrogenase used. Concentrations of all siderophores were lower when the V-nitrogenase was used, with the exception of aminochelin and azotochelin, which were produced at higher concentrations by the V- as opposed to Mo-grown cells in the 0.5 μM Fe treatment (Fig. 3B and C). Known siderophore structures and measured siderophore concentrations were used to calculate the total siderophore nitrogen in each treatment (Fig. 3F). This analysis showed that cells using the V-nitrogenase produced less siderophore nitrogen than those using the Mo-nitrogenase.

To further understand fixed nitrogen allocations, cellular nitrogen quotas were determined for the 0.5 μM and 5 μM treatments. Quotas (Supporting Information Fig. S2) were uniform across Fe-treatments but were significantly lower in V- as opposed to Mo-grown cells ($1.8 \times 10^{-14} \pm 3.7 \times 10^{-15}$ versus $2.1 \times 10^{-14} \pm 3.3 \times 10^{-15}$ mole N cell^{-1}). The percentage of total nitrogen (biomass nitrogen + siderophore nitrogen) invested in siderophores clearly scaled with Fe limitation (note the difference in *y*-axis between Fig. 4A–C) and generally increased over time, implying that siderophore production rates increase as trace metal concentrations in the medium decrease. Nonetheless, the percentage of nitrogen invested in siderophores as opposed to biomass was consistently higher in cultures using the Mo- as opposed to V-nitrogenase (Fig. 4).

Short-term responses to Mo and V

In order to explore the possibility of Mo and V-based regulation of siderophore production, we conducted short-term experiments using 0 nM, 50 nM and 200 nM Mo or V under iron-replete (5 μM) and iron-limited (0.5 μM) conditions. Cells were starved for Mo or V overnight, inoculated into experimental treatments, and incubated for 5 h. Growth during the experiment was higher in 50 nM and 200 nM Mo or V treatments than in treatments with 0 nM Mo or V (for example, $\mu = 0.20$ versus 0.13 h^{-1} for Fe-replete Mo-grown cells, Supporting Information Table S1), confirming that cells were limited for these trace metals. Under Fe-replete conditions, production of the catechols protochelin, azotochelin, aminochelin and DHBA was greater in treatments with no added Mo than in those with 50 or 200 nM Mo (Fig. 5, Supporting Information Fig. S3). However, protochelin showed the greatest response, almost doubling in concentration at 0 nM Mo. We did not observe a clear difference between cultures grown with 0 nM as opposed to 50 or 200 nM V (Fig. 5, Supporting Information Fig. S3). V-limited cultures also appeared to experience a short lag phase during which siderophores were produced but no growth occurred. Under Fe-limited conditions, differences in catechol production across Mo/V treatments were much more muted and were sometimes below detection (Supporting Information Fig. S4). Vibrioferrin showed very high production at high concentrations of Mo and V and almost no production at low Mo and V, a trend that was seen across Fe-treatments (Fig. 5, Supporting Information Figs S3 and S4). The production of all siderophores increased in low as opposed to high Fe experiments. However, Mo-grown cells showed an almost 10-fold increase in siderophore production whereas V-grown cells showed more

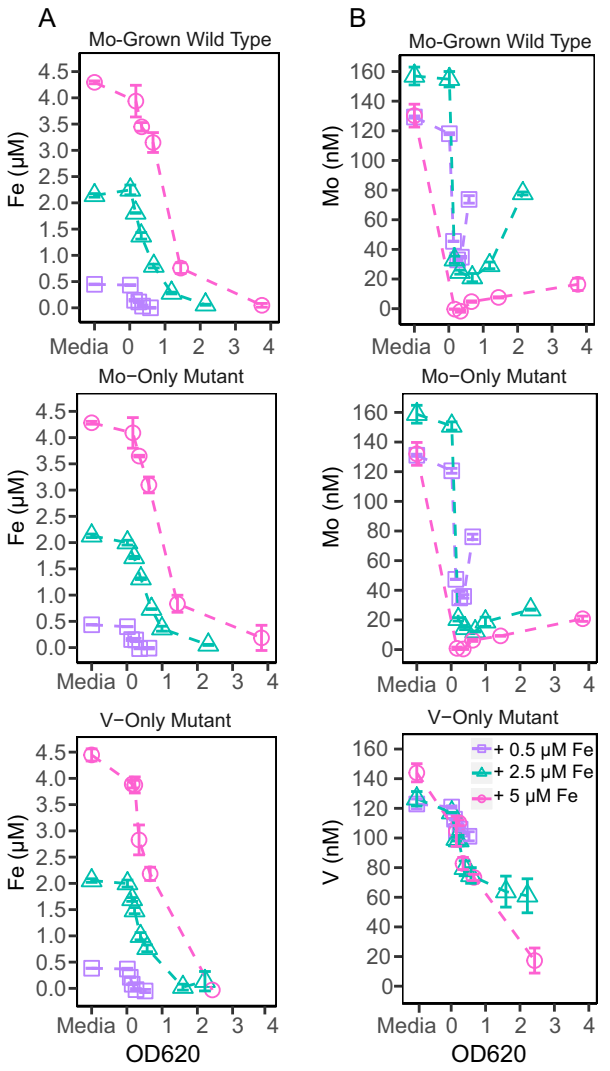

Fig. 2. Trace metal consumption by *A. vinelandii*.
A. Iron consumption.
B. Molybdenum (Mo-grown wild type and Mo-only mutant) and vanadium (V-only mutant) consumption. Fe-treatments are represented as 0.5 μM Fe (purple squares), 2.5 μM Fe (teal triangles) and 5 μM Fe (pink circles). Media indicates concentrations measured in uninoculated culture medium. Error bars represent the standard deviation for two biological replicates.

modest changes in siderophore production (compare Supporting Information Figs S3 and S4).

Discussion

Our results show that siderophore production in *A. vinelandii* is regulated not just by Fe-limitation, as is well established, but also by Mo- and V-limitation. The impact of metal limitation is most clearly seen in the production of vibrioferrin as well as the catechols. Azotobactin was not detected under our growth conditions, consistent with previous observations that this siderophore is only produced under extreme Fe-limitation (Kraepiel *et al.*, 2009; Yoneyama *et al.*, 2011; Baars *et al.*, 2015). Among the catechols, protochelin was usually present in the highest concentrations and had the most consistent response to Fe-, and Mo-limitation. Vibrioferrin production increased under Fe-limitation and decreased under Mo- and V-limitation.

Trace metal use

Fe was uniformly exhausted across cultures, regardless of its initial starting concentration (Fig. 2A). However, the extent of Mo and V consumption tracked the initial Fe concentration (Fig. 2B). Notably, Mo was drawn down rapidly at the start of experiments but increased at the end of growth. This pattern may be attributed to Mo-hyperaccumulation (Pienkos and Brill, 1981; Delarbre *et al.*, 2001; Fenske *et al.*, 2005) and subsequent release at the onset of stationary phase. V consumption was slower that of Mo consumption, and did not show the same pattern of release. However, final Mo and V concentrations were remarkably similar and likely reflect the 'unused' Mo/V remaining at the end of growth. Our initial experimental treatments ensure Fe limitation with Fe to Mo/V ratios ranging from ~3 to 31 (*A. vinelandii* requires Fe and Mo/V in a ratio of ~33, Bellenger *et al.*, 2011). However, the limiting nutrient may have changed at the end of growth – low Fe treatments likely remained Fe-limited, but high Fe treatments may have become gradually limited for Mo and or V.

Siderophore production in response to Mo- and V-limitation

Vibrioferrin is a relatively weak siderophore (Amin *et al.*, 2009) and its role in *A. vinelandii* Fe acquisition is not fully understood (Baars *et al.*, 2015). In our study vibrioferrin is strongly repressed at low concentrations of Mo and V and produced at high concentrations of these metals (Figs 3 and 5). One possible explanation is that under severe Mo- or V-limitation *A. vinelandii* devotes cellular resources to the synthesis of catechol siderophores which complex these metals (Hider and Kong, 2010), instead of vibrioferrin, which does not. Vibrioferrin biosynthesis is independent from that of the catechols and employs an unusual nonribosomal peptide synthetase (NRPS)-independent pathway (Challis, 2005), allowing for differential regulation. Repression of vibrioferrin may also represent a more general stress response that is induced by Mo- and V-limitation. Regardless of the ultimate trigger, our results show that vibrioferrin production in this organism is regulated at low Mo and V concentrations.

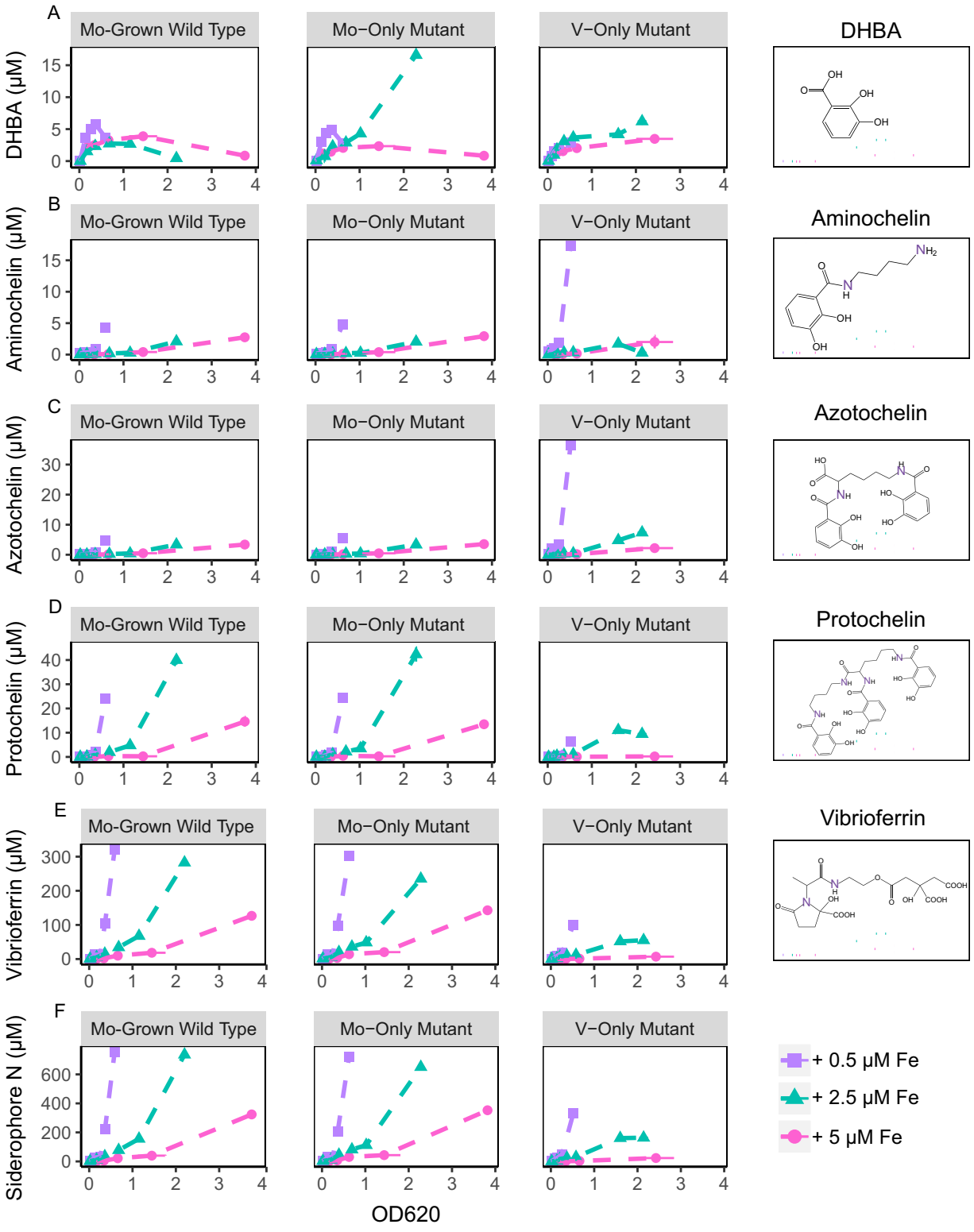

Fig. 3. *A. vinelandii* siderophore production. Concentrations of A. DHBA, B. aminochelin, C. azotochelin, D. protochelin, E. vibrioferrin and F. total siderophore nitrogen. The total amount of siderophore nitrogen was calculated using known siderophore structures (shown in far right panel). Different iron treatments are represented as follows: 0.5 μM Fe (purple squares), 2.5 μM Fe (teal triangles) and 5 μM Fe (pink circles). Error bars represent the standard deviation for two biological replicates.

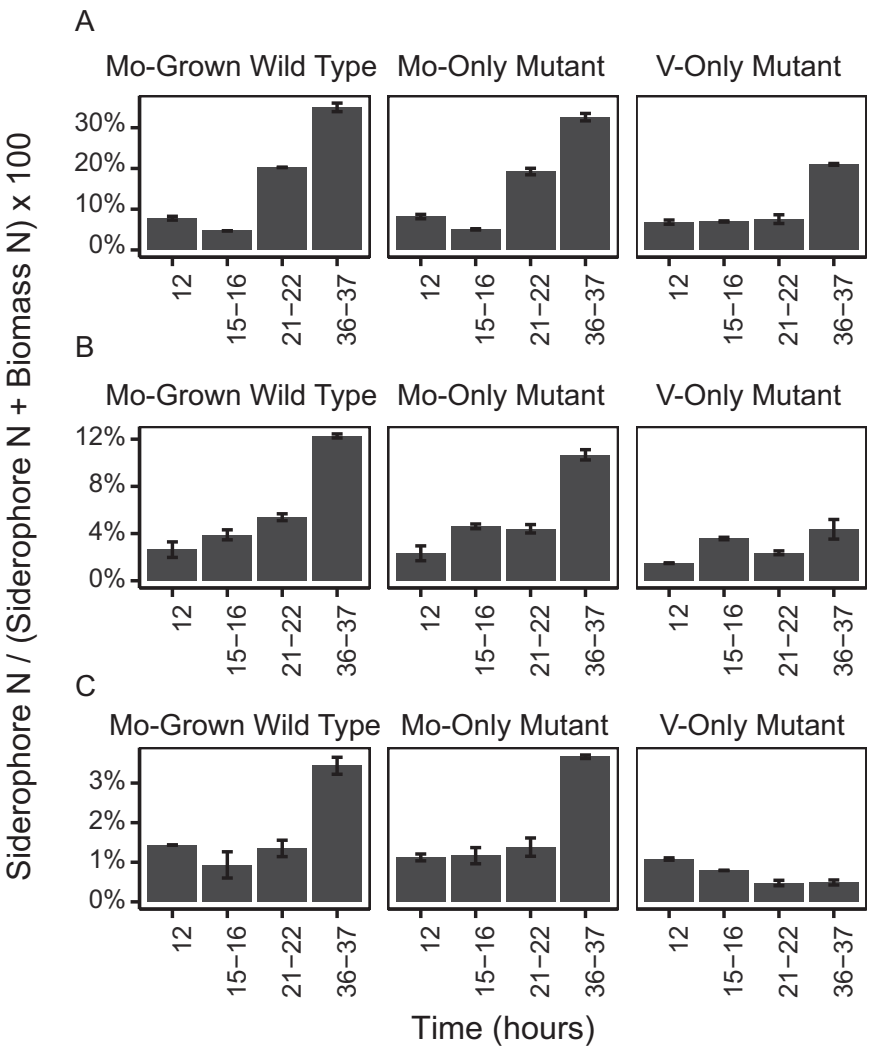

Fig. 4. Siderophore nitrogen allocations in *A. vinelandii* grown at A. 0.5 μM Fe, B. 2.5 μM Fe and C. 5 μM Fe. Siderophore nitrogen is shown in Fig. 3F. Biomass nitrogen was calculated using N per cell quotas of: $1.8 \times 10^{-14} \pm 3.7 \times 10^{-15}$ and $2.1 \times 10^{-14} \pm 3.3 \times 10^{-15}$ mole N cell^{-1} for V- and Mo-grown cells, respectively (see Supporting Information Fig. S2). Error bars represent the standard deviation for two biological replicates.

Production of the catechol siderophores azotochelin, aminochelin and DHBA was higher at 0 nM Mo as opposed to 50 nM or 200 nM Mo (Fig. 5, Supporting Information Figs S3 and S4). This trend was very systematic and was especially pronounced at high Fe, but it was at the limit of statistically significant ($p \geq 0.05$, one-way ANOVA with Tukey's post hoc HSD test), leaving the strength of this response unresolved. In contrast, protochelin showed very significant differences between 0 nM and 50 nM as well as 200 nM Mo ($p < 0.001$, one-way ANOVA with Tukey's post hoc HSD test) at high Fe. Nonetheless, the approximately twofold increase in protochelin production under Mo-limitation (Fig. 5) is much smaller than the ~10-fold increase seen under Fe-limitation (Fig. 3, Supporting Information Figs S3 and S4). Thus, while both Mo and Fe regulate siderophore production, Fe may exert a stronger influence than Mo. This type of asymmetric co-regulation is consistent with *A. vinelandii*'s much greater requirements for Fe as opposed to Mo (Fe:Mo ratios are ~33). Accordingly, at low Fe, the large response to Fe-limitation effectively masks any response to limitation for the other metals. In contrast, Mo-limitation experiments conducted in a high Fe background may reflect the combined effects of repression from high Fe and stimulation from Mo-limitation.

Previous research has shown that (i) protochelin complexes Mo, (ii) protochelin-bound Mo is available to *A. vinelandii* and (iii) cells discriminate between different metal-protochelin complexes (Bellenger *et al.*, 2008b, 2011; Wichard *et al.*, 2008). However, the regulation of protochelin by Mo has remained difficult to establish. Unlike Fe, which can be controlled by the addition of chelators [such as ethylenediaminetetraacetic acid (EDTA), added in our medium], Mo is typically present in the growth medium as the free oxoanion molybdate, such that limitation occurs only when Mo becomes exhausted. Additionally, large differences in growth between Mo-limited and Mo-replete cultures often make it difficult to compare

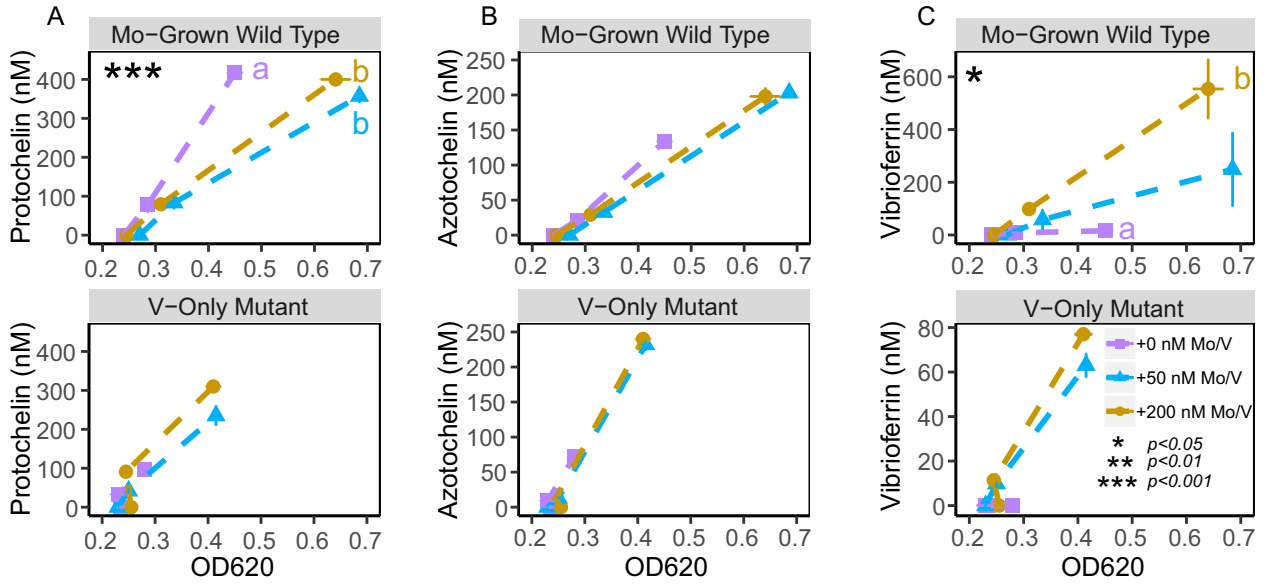

Fig. 5. *A. vinelandii* siderophore production under Mo- and V-limitation at high (5 μM) Fe. Concentrations of A. protochelin B. azotochelin, C. vibrioferrin. The figure shows nanomoles of siderophore produced at 2 and 5 h. Initial siderophore concentrations (0 h) are set to 0 as preliminary experiments showed that siderophore carryover was below detection (see Experimental procedures). Different Mo/V treatments are represented as follows: 0 nM Mo/V (purple squares), 50 nM Mo/V (blue triangles) and 200 nM Mo/V (mustard circles). Statistical significance was determined using a one-way ANOVA with Tukey's post hoc HSD test. Different letters denote statistically significant differences, the level of significance is indicated by asterisks (*) in the upper left-hand corner of each graph. V-only mutant experiments were not tested for statistical significance. Error bars represent the standard deviation for two biological replicates.

experimental treatments. As a result, previous studies have reported both increased and neutral protochelin production under Mo-limitation (Bellenger *et al.*, 2008b, 2011; Kraepiel *et al.*, 2009, 2011). Our short-term experiments eliminate the problem of Mo exhaustion and minimize differences in growth, providing a clear demonstration that *A. vinelandii* does indeed increase protochelin production in response to Mo-limitation (Fig. 5). Protochelin production has also been shown to increase at high Mo as a means of detoxification (Cornish and Page, 1995; Duhme *et al.*, 1998; Kraepiel *et al.*, 2009; Bellenger *et al.*, 2011). When taken together, these studies demonstrate that protochelin is a metallophore that is produced in order to acquire Mo when it is limiting and to detoxify it at high concentrations.

Azotochelin also complexes Mo, and its regulation as well as that of the other catechols by Mo- (and V-) limitation has been proposed (Bellenger *et al.*, 2008b). However in our experiments, azotochelin, aminochelin and DHBA showed an unclear response to Mo-limitation (Fig. 5, Supporting Information Figs S3 and S4). These patterns of catechol production are compatible with our current understanding of the biosynthetic pathway (Yoneyama *et al.*, 2011; Baars *et al.*, 2015). Azotochelin, aminochelin and DHBA are synthesized by the same NRPS and the pathway appears to be diversity oriented, favouring the production of multiple closely related structures (Baars *et al.*, 2015). However, protochelin production requires a second NRPS, making differential regulation possible.

We did not observe a clear response of siderophore production to V-limitation. *A. vinelandii* encodes three nitrogenases, but exhibits a preference for their use in the order Mo > V > Fe (Jacobson *et al.*, 1986; Walmsley and Kennedy, 1991; Bellenger *et al.*, 2011; Hamilton *et al.*, 2011). The V-only mutant used in our experiments has deletions in the structural genes for the Mo- (and Fe-only) nitrogenase. However, the regulation of trace metal use in this organism is un-altered, meaning that the mutant 'doesn't know' that it lacks the Mo-nitrogenase. Consequently, even when supplied with V, cells may continue to produce siderophores in an attempt to acquire Mo, the 'preferred' nitrogenase co-factor. In fitting with this idea, the V-only mutant often showed faster siderophore production than the Mo-grown wild type (although final concentrations were still lower, Fig. 5, Supporting Information Figs S3 and S4). Differences in growth between the Mo-grown wild-type and the V-only mutant during initial overnight starvation could have also resulted in less severe V-limitation and a more muted response. Based on our results, it remains unclear whether *A. vinelandii* increases catechol production in response to V-limitation.

Nitrogen expenditures on siderophores

According to our results, *A. vinelandii* can invest up to 35% of fixed nitrogen in siderophore production with a smaller percentage in the V-nitrogenase cultures (Figs 3F and 4).

On account of their slow growth rates, cultures using the V-nitrogenase can take up Fe more slowly and may simply need lower concentrations of siderophores. Differences in siderophore production between Mo-grown cells and V-grown cells could also help offset the increased cost of V-based nitrogen fixation. The V-nitrogenase requires six electrons for each molecule of NH_4^+ compared to only four for the Mo-nitrogenase (this difference is due to extra hydrogen production by the V-nitrogenase, Eady, 1996). A calculation of electron expenditures required to produce the biomass and siderophore nitrogen present for example, at 37 h in the low Fe treatment, reveals very similar values for V- and Mo-grown cells: ranging from ~8400 to 8900 μM electrons. While biomass nitrogen clearly accounts for the majority of electron expenditures (accounting for anywhere from 65 to 99%, depending on the treatment), the gap between the V- and Mo-nitrogenase is closed by the adjustment in siderophore production.

Large allocations of fixed nitrogen to siderophore production may have implications for the transfer of nitrogen from diazotrophs into ecosystems, as the fate of siderophore nitrogen is probably very different from that of the biomass. Siderophores might be more readily taken up by other microbes or plants as a source of nitrogen (Villa *et al.*, 2014) or stolen and traded with other microbes (Granger and Price, 1999; Traxler *et al.*, 2013). While it is unknown how these types of interactions play out in the environment, the large siderophore nitrogen investments seen in this study suggest the outcome may impact nitrogen cycling.

Implications for trace metal acquisition and nitrogen fixation in the environment

In our experiments Fe as well as Mo and/or V limit nitrogen fixers. The extent to which this occurs in soil environments remains poorly understood. Fe-limitation of nitrogen fixation has been established in marine systems, but has received less attention in terrestrial environments. Although Fe is the fourth most abundant element in the Earth's crust (Wedepohl, 1995), it is typically found as Fe-oxides, which can be difficult for microbes to access without the use of siderophores. In soils, Mo and V are often present as molybdate (MoO_4^{2-}) and vanadate ($H_2VO_4^-$). While these oxoanions are highly soluble they appear to be bound to natural organic matter (NOM) via phenolate and catecholate groups (Wichard *et al.*, 2009b). In this situation siderophores likely serve as a shuttle, capturing bound molybdate and vanadate and delivering them to the cells for uptake (Kraepiel *et al.*, 2009; Wichard *et al.*, 2009b; Noumsi *et al.*, 2016). Mo-limitation of nitrogen fixation in terrestrial environments has been observed by several studies (Silvester, 1989; Hungate *et al.*, 2004; Barron *et al.*, 2009; Wurzburger *et al.*, 2012; Rousk *et al.*, 2017). Under these conditions, a doubling of protochelin production (as seen in our study, Fig. 4) should allow faster uptake of Mo. Indeed, protochelin has been shown to facilitate V-uptake from tannic acid (Kraepiel *et al.*, 2009) and the potential effect of increased metallophore concentration on the release of metals from NOM has been documented in studies of the chalkophore methanobactin (Pesch *et al.*, 2013).

Conclusion

Our study provides evidence for the controlled use of 'siderophores' to acquire metals other than Fe. We show that Mo-limitation can induce the production of protochelin (and possibly other catechols), while both Mo- and V-limitation can suppress the production of vibrioferrin. We also confirm the well-established finding that Fe is an important regulator of all *A. vinelandii* siderophores, including the newly discovered vibrioferrin. Finally, our quantification of the high nitrogen 'cost' associated with siderophore production calls attention to the special challenge that this method of trace metal acquisition presents for nitrogen fixers.

Experimental procedures

Bacterial strains and growth conditions

The capsule-negative *Azotobacter vinelandii* wild-type strain OP as well as mutants encoding only the Mo-nitrogenase (CA1.70, Δ*vnf*DGK::spc, Δ*anf*HD70::kan) and only the V-nitrogenase (CA11.70, Δ*nif*HDK, Δ*anf*HD70::kan) were used in this study. *A. vinelandii* cultures were shaken (100 r.p.m.) at 30°C in a nitrogen-free chemically defined medium (Bellenger *et al.*, 2011) amended with 0.5 μM, 2.5 μM or 5 μM $FeCl_3$ and 0.16 μM Mo or V as well as 50–100 μM EDTA. Growth was tracked using absorbance at 620 nm and instantaneous growth rates (μ) were calculated as the slope of the curve during exponential phase. Samples were taken throughout the growth curve for siderophore and trace metal quantification. Briefly: 10–15 ml of culture was pelleted at 3200 × *g* for 10 min at room temperature to remove cells, the supernatant was then filtered (0.22 μm) and frozen at −20°C for further analysis. Tubes and syringes used in this process were acid washed (10% v/v hydrochloric acid) in order to remove any contaminating trace metals. All experiments were conducted using biological duplicates.

Short-term Mo- and V-limitation experiments

For short-term trace metal limitation experiments, wild-type and V-only mutant cultures were grown for 24 h in medium containing 0.16 μM Mo or V and either 5 μM or 0.5 μM Fe in order to partially exhaust trace metals (see characteristic trace metal use in Fig. 2). Cultures were then inoculated (OD = 0.1) into medium without added Mo or V and grown for 13–14 h in order to induce Mo- and V-limitation. At the start of experiments cells were pelleted via centrifugation (16,000 × *g* for 6 min at room temperature), the supernatant was discarded and

cells were resuspended in ~10–15 ml medium lacking both Mo and V. This centrifugation step was repeated a second time and a small volume (< 1 ml) of the resulting concentrated cell solution was inoculated into 25 ml of experimental medium (+0 nM Mo/V, +50 nM Mo/V, +200 nM Mo/V) at a starting OD of 0.2 (Supporting Information Table S1). Preliminary experiments showed that siderophore concentrations after the washing steps described above were below detection. Cultures were grown for 5 h and samples for siderophore quantification were collected as described above. Background Mo and V concentrations in 0 nM treatments were determined to be ≤ 5 nM (data not shown). For statistical analyses, the slope of the linear regression of siderophore concentration on optical density (with the intercept set to zero) was calculated for each replicate. Differences in the slopes of Mo-treatments were then assessed using a one-way ANOVA analysis with post-hoc Tukey's HSD test. V-limitation experiments were not tested as the data were not always linear.

Quantification of siderophores

Siderophore quantification was performed on a single quadrupole LC-MS system (Agilent 6120, Agilent, Santa Clara, CA, USA), equipped with a UV-vis diode array detector as described in Baars *et al.* (2015). Sample aliquots were syringe filtered (0.22 μm), acidified (0.1% acetic acid and 0.1% formic acid) and 100 μl was directly injected onto a C18 column (Agilent Eclipse Plus C18, 3.5 μm, 4.6 × 100 mm) without further sample preparation. The separation proceeded with a gradient of A and B solutions (solution A: water + 0.1% formic acid + 0.1% acetic acid; solution B: acetonitrile + 0.1% formic acid + 0.1% acetic acid) over 30 min, at a flow rate of 0.8 ml min^{-1}. Desalting of samples was achieved online by flushing the column for 5.25 min with a mixture of 98% A and 2% B. The flow was then diverted to the mass spectrometer operated in single ion monitoring (SIM) mode. Siderophore peaks were identified by comparison of retention time, mass and UV-vis spectrum to previously isolated standards (Baars *et al.*, 2015). For quantification, LC-MS and UV-vis peak areas were determined using MassHunter (Agilent). Peak areas for vibrioferrin were converted to concentrations by calibration with an isolated standard. For the catechol siderophores, UV chromatograms were extracted at 315 nm and concentrations were determined using published extinction coefficients for DHBA (3262.9 M^{-1} cm^{-1}), aminochelin (3262.9 M^{-1} cm^{-1}), azotochelin (6525.8 M^{-1} cm^{-1}) and protochelin (9788.7 M^{-1} cm^{-1}) (Cornish and Page, 1998). These extinction coefficients were in good agreement with values we determined independently with isolated standards for each siderophore. A quality control sample (a 'standard' *A. vinelandii* supernatant sample) was injected periodically to confirm that retention times and sensitivities remained stable between runs.

Trace metal measurements

Supernatant samples were collected as described above, notably, our sampling methods do not allow us to distinguish between trace metals bound to the cell surface as opposed to those assimilated for growth. For trace metal measurements, filtered cell supernatants and medium samples were diluted ~10 × in 5% trace metal grade HNO_3 (Optima, Douglas, GA, USA) and measured using on an inductively coupled plasma mass spectrometer (ICP-MS, Element 2; Thermo Finnigan, Somerset, NJ, USA) run at medium resolution. Element counts were normalized to internal (Indium) standards and Mo, Fe and V concentrations were determined using standard curves for each element.

Cellular nitrogen quotas

Samples for cellular nitrogen quotas were collected on precombusted 25 mm glass microfiber filters (GF/F, Whatman, Maidstone, UK). Sample volumes were adjusted at each time point in order to achieve ~ 4 nmol of nitrogen [based on previously published estimates of cellular nitrogen (Bellenger *et al.*, 2011) per filter]. Filters were then lyophilized and combusted in an elemental analyzer (vario ISOTOPE cube, Elementar, Mt Laurel, NJ, USA). Peak areas were converted to moles of nitrogen using comparisons to two chemically defined standards: amino caprionic acid and L-glutamic acid. Nitrogen per cell was calculated using the measured optical density (OD620) and a conversion of 1.16×10^8 cell ml^{-1} OD^{-1} from (Bellenger *et al.*, 2011). ANOVA and post hoc Tukey analyses were conducted in R (RCoreTeam, 2012).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. *A. vinelandii* siderophore production as a function of time. Concentrations of A. DHBA, B. aminochelin, C. azotochelin, D. protochelin, E. vibrioferrin and F. total siderophore nitrogen are shown. The total amount of siderophore nitrogen was calculated using known siderophore structures (shown in far right panel). Different iron treatments are represented as follows: 0.5 μM Fe (purple squares), 2.5 μM Fe (teal triangles) and 5 μM Fe (pink circles).

Fig. S2. *A. vinelandii* cellular nitrogen quotas. Data for two technical and two biological replicates per treatment are shown. Fe concentrations are represented as 0.5 μM Fe (squares) and 5 μM Fe (circles). The different nitrogenases are depicted by colour. Quotas were significantly different for V- and Mo-only mutant ($p < 0.05$) as well as the V-only mutant and Mo-grown wild type ($p < 0.01$) based on two-way ANOVA with post hoc Tukey's multiple comparisons test.

Fig. S3. *A. vinelandii* siderophore production under Mo- and V-limitation at high (5 μM) Fe. Concentrations of A. DHBA, B. aminochelin, C. azotochelin, D. protochelin and E. vibrioferrin. Figure shows nanomoles of siderophore produced at 2 and 5 h. Initial siderophore concentrations (0 h) are set to 0 as preliminary experiments showed siderophore carryover was below detection (see Experimental procedures). Different Mo/V treatments are represented as follows: 0 nM Mo/V (purple squares), 50 nM Mo/V (blue triangles) and 200 nM Mo/V (mustard circles). Statistical significance was determined using a one-way ANOVA with Tukey's post hoc HSD test. Different letters denote statistically significant differences, the level of significance is indicated by asterisks (*) in the upper left-hand corner of each graph. V-only mutant experiments were not tested for statistical significance.

Fig. S4. *A. vinelandii* siderophore production under Mo- and V-limitation at low (0.5 μM) Fe. Concentrations of A. DHBA, B. aminochelin, C. azotochelin, D. protochelin and E. vibrioferrin. Figure shows nanomoles of siderophore produced at 2 and 5 h. Initial siderophore concentrations (0 h) are set to 0 as preliminary experiments showed siderophore carryover was below detection (see Experimental procedures). Different Mo/V treatments are represented as follows: 0 nM Mo/V (purple squares), 50 nM Mo/V (blue triangles) and 200 nM Mo/V (mustard circles). Statistical significance was determined using a one-way ANOVA with Tukey's post hoc HSD test. Different letters denote statistically significant differences, the level of significance is indicated by asterisks (*) in the upper left-hand corner of each graph. V-only mutant experiments were not tested for statistical significance.

Table S1. Cell growth during short-term Mo/V-limitation experiments. Optical density (OD) at 620 nm for three time points throughout the experiment as well as the calculated instantaneous growth rate (μ) are shown.