BioMetals **14:** 59–66, 2001.

Alleviation of aluminum toxicity to *Rhizobium leguminosarum* bv. *viciae* by the hydroxamate siderophore vicibactin

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Received 21 December 2000; accepted 3 January 2001

Key words: aluminum, root nodule bacteria, siderophores

Abstract

Acid rain solubilises aluminum which can exert toxic effects on soil bacteria. The root nodule bacterium *Rhizobium leguminosarum* biovar *viciae* synthesises the hydroxamate siderophore vicibactin in response to iron limitation. We report the effect of vicibactin on the toxicity of aluminum(III) to *R. leguminosarum* and kinetic studies on the reaction of vicibactin with Al(III) and Fe(III). Aluminum (added as the nitrate) completely inhibited bacterial growth at 25 μM final concentration, whereas the preformed Al-vicibactin complex had no effect. When aluminum and vicibactin solutions were added separately to growing cultures, growth was partly inhibited at 25 μM final concentration of each, but fully inhibited at 50 μM final concentration of each. Growth was not inhibited at 50 μM Al and 100 μM vicibactin, probably reflecting the slow reaction between Al and vicibactin; this results in some aluminum remaining uncomplexed long enough to exert toxic effects on growth, partly at 25 μM Al and vicibactin and fully at 50 μM Al and vicibactin. At 100 μM vicibactin and 50 μM Al, Al was complexed more effectively and there was no toxic effect. It was anticipated that vicibactin might enhance the toxicity of Al by transporting it into the cell, but the Al-vicibactin complex was not toxic. Several explanations are possible: the Al-vicibactin complex is not taken up by the cell; the complex is taken up but Al is not released from vicibactin; Al is released in the cell but is precipitated immediately. However, vicibactin reduces the toxicity of Al by complexing it outside the cell.

Introduction

Aluminum, the most abundant metal on earth, is now recognised as a serious hazard in the biosphere. Many naturally acid soils have high levels of soluble aluminum, particularly in subsoil horizons (Ritchie 1995). Increases in soil acidity resulting from acid rain also lead to increases in soluble aluminum, with consequent declines in crop yields, either as a direct effect on the crop plant or through toxic effects on soil microorganisms. The biological mechanisms behind the toxicity of aluminum are largely unknown, but it may be taken up into the cell by pathways for other essential elements.

Many bacteria have specialised mechanisms for obtaining iron under aerobic, neutral conditions, where iron solubility is very low, including secretion of ligands (siderophores) for the solubilisation and specific transport of Fe(III) into the cell. Root nodule bacteria produce hydroxamate siderophores

under conditions of iron-stress (Carson *et al.* 1992a,b, 1994, 2000). *Rhizobium leguminosarum* biovar *viciae* WSM710 synthesizes a trihydroxamate siderophore, vicibactin, a cyclic molecule containing three residues each of (R)-2,5,diamino-N^2-acetyl-N^5-hydroxypentanoic acid (N^2-acetyl-N^5-hydroxy-D-ornithine) and (R)-3-hydroxybutanoic acid, arranged alternately, with alternating ester and peptide bonds (Dilworth *et al.* 1998). The synthesis of vicibactin by *R. leguminosarum* WSM710 at pH 6.8 is repressed under high-iron conditions (20 μM added iron) and derepressed under low-iron conditions (0.5 μM added iron) (Carson *et al.* 1992a, 2000). Cells of *R. leguminosarum* WSM710, previously grown under low-iron conditions, transport ^{55}Fe complexed to other di- or tri-hydroxamates and citrate, as well as to vicibactin (Carson *et al.* 1994). However, cells grown with 20 μM added Fe(III) failed to transport ^{55}Fe-labelled vicibactin but did transport ^{55}Fe-citrate at a very low rate. Vicibactin from *R. leguminosarum* WSM710 therefore fits the definition of a siderophore. Iron-repressible outer membrane proteins (IROMPs) are usually involved with recognition and transport of Fe(III)-siderophore complexes into bacterial cells (Hughes & Poole 1989). *R. leguminosarum* WSM710 has three such proteins, which may be involved with the recognition and uptake of iron-vicibactin.

Siderophores bind other metal(III) ions such as gallium, chromium and aluminum (Emery 1971). The question arises therefore as to whether the similarity in chemistry of Fe(III) and Al(III) may allow aluminum complexed to siderophores to enter microbial cells. If concentrations of Fe(III) are low, excess siderophore may be available to bind Al(III). Watteau & Berthelin (1994) showed that hydroxamate siderophores, produced by an ectomycorrhizal fungus, and Desferal (desferrioxamine B) were effective at solubilising aluminum from soil minerals. The binding of aluminum to Desferal has been investigated using ^{27}Al NMR by Garrison & Crumbliss (1986). The complex of ferrioxamine B with Al(III) appears to be significantly weaker than the complex with Fe(III) (Evers *et al.* 1989), as is generally the case for the binding of ligands by these two metals.

Aluminum is taken up into cells by some iron uptake pathways. For example Al^{3+}- and Ga^{3+}-substituted ferrichromes are transported by the fungus *Ustilago sphaerogena* (Emery and Hoffer 1980). Davis *et al.* (1971) found that the production of the siderophore schizokinen by *Bacillus megaterium* ATCC 19213 increased in response to aluminum (and chromium) toxicity. Further, a hydroxamate-negative mutant of this strain was more sensitive to aluminum than was the wild type, but this sensitivity was reversed by addition of iron or Desferal to the growth medium. Both observations suggest that the binding of aluminum to the siderophore obviated its toxic effects. Hu & Boyer (1996) found that, while in *B. megaterium* the siderophore transport receptor for shizokinen was involved in aluminum uptake at low aluminum concentrations (up to 37 μM), aluminum uptake occurred by passive transport at higher concentrations (370 μM). The toxicity of aluminum that accumulated via siderophore transport was not determined but, for *B. megaterium*, 370 μM Al had little or no effect on growth (Hu & Boyer 1996).

This paper reports the formation of an aluminum-vicibactin complex and the ability of this siderophore to protect cells of *R. leguminosarum* WSM 710 from aluminum toxicity.

Methods

Rhizobium leguminosarum biovar *viciae* WSM710 was isolated in 1985 from a pea nodule from a Japanese field by Dr JG Howieson (Centre for Rhizobium Studies, Murdoch University, Western Australia) (Carson *et al.* 1992b). Cultures were grown in the minimal salts medium of Brown & Dilworth (1975) containing (g l^{-1}): 2-[N-morpholino]ethanesulfonic acid (MES), 3.9; NH_4Cl, 0.5; mannitol, 3.64; $MgSO_4 \cdot 7H_2O$, 0.25; NaCl, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02. Trace elements were provided by adding 0.5 ml l^{-1} of a solution containing (g l^{-1}): $Na_2MoO_4 \cdot 2H_2O$, 0.05; $B(OH)_3$, 2.5; $MnCl_2 \cdot 4H_2O$, 2.0; $CuSO_4 \cdot 5H_2O$, 0.24; $CoCl_2 \cdot 6H_2O$, 0.005; and $ZnCl_2 \cdot 7H_2O$, 0.32. Vitamins were provided by adding 0.5 ml l^{-1} of a solution containing (g l^{-1}): thiamine, 1.0; calcium pantothenate, 2.0; and biotin, 0.01. After autoclaving the medium and cooling to below 50 °C, D-glycerophosphate (16.5% w/v, 1 ml l^{-1}) was added aseptically.

To obtain iron-limited mineral salts medium, a solution containing MES buffer, NH_4Cl and mannitol was passed at a flow rate of 2–3 ml min^{-1} through a column (20 cm × 3 cm diameter) of Chelex-100 ion exchange resin which had been well washed with water. A stock solution (∼2000× final concentration) of the vitamin solution was also passed through the Chelex column. Alternatively, concentrated stock solutions of the buffer (0.5 M MES),

carbon source (0.5 M mannitol) and mineral salts were passed through a CPG/8-hydroxyquinoline column (Pierce Chemical Co., Rockford, Ill.) for iron removal (Smith & Neilands 1984). $FeCl_3$ from a 20 mM stock solution in 20 mM HCl was added to the medium to give a final concentration of 0.5 μM Fe. Additions of aluminum were from a freshly prepared and filter-sterilised (Millipore 0.45 μm pore size) 50 mM solution of aluminum nitrate, $Al(NO_3)_3$.

Cultures of *R. leguminosarum* were grown in 50 ml medium in 250 ml conical flasks, shaken at 200 rpm and at 30 °C. A starter culture was grown for 72 h to the start of the stationary phase and then aseptically centrifuged at 5500 rpm for 30 min. The cells were washed once with minimal salts medium, resuspended, and used to inoculate fresh medium, giving a starting OD_{600} of 0.05. Growth was monitored by measuring the apparent absorbance of cultures (OD_{600}) using Pye-Unicam SP6-450 or Beckman DU-64 spectrophotometers, with dilutions such that OD_{600} was less than 0.5. Experiments carried out for Tables 1 and 2 were each carried out three times and each experiment involved studies on duplicate cultures grown from different starter cultures. Therefore the data for each set of growth experiments are means of six individual experiments.

Iron-free and ferrated vicibactin and vicibactin 7101 (the non-acetylated form of vicibactin) were purified as described by Dilworth *et al.* (1998). The availability of pure vicibactin allowed small volumes of vicibactin solution to be added in growth experiments and avoided the possibility of complications from adding culture supernatant solutions. The aluminum-vicibactin complex was formed by addition of excess aluminum [6-fold molar ratio of $Al(NO_3)_3$] to the culture supernate under iron-free conditions. After 12 h to allow for its formation, the aluminum-vicibactin complex was purified by benzyl alcohol extraction (Neilands 1952), as for the iron complex (Dilworth *et al.* 1998).

The concentrations of vicibactin in culture supernatants were determined using the ferric perchlorate method (Carson *et al.* 1992a) and a molar absorbance of 1510 M^{-1} cm^{-1} at 450 nm (Carson *et al.* 1994). The binding of Fe(III) to vicibactin or vicibactin 7101 was studied by adding 5 μmol $Fe(ClO_4)_3$ and 100 μmol $HClO_4$ to 1 μmol siderophore in a final volume of 1.5 ml and following the increase in absorbance at 450 nm with time at 25 °C. Final concentrations of Fe(III) and vicibactin were 3.33 and 0.67 mM, respectively, and the pH was about 1.5.

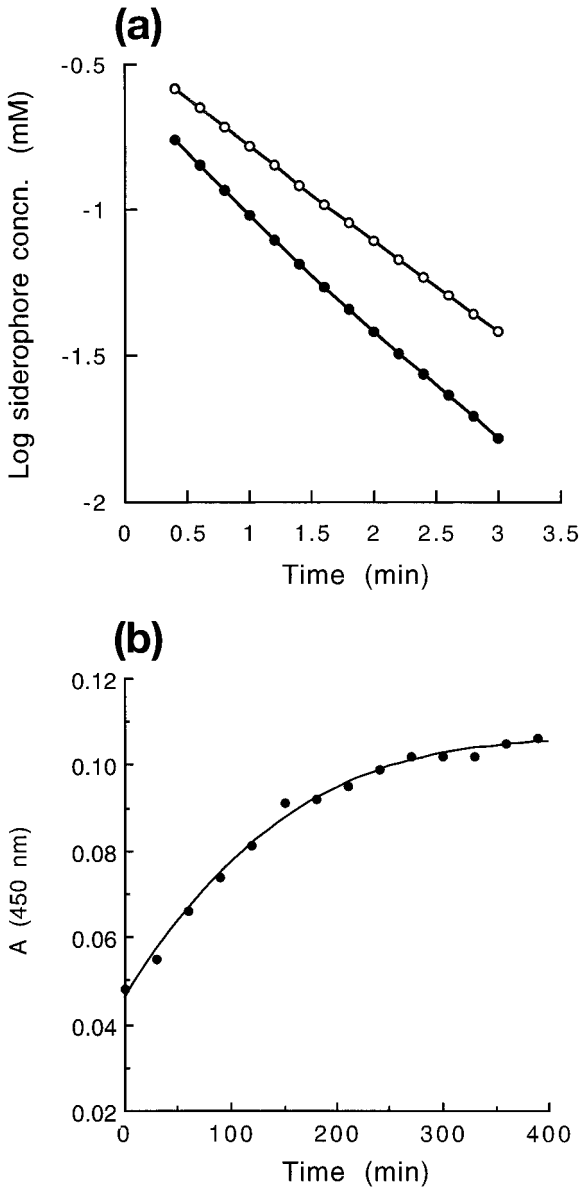

Fig. 1. Formation of the Fe(III) complexes of vicibactins and displacement of aluminum from an aluminum-vicibactin complex by iron. In (a) is shown the decrease in concentrations of vicibactin (•) and vicibactin 7101 (○) with time after addition of ferric perchlorate at 25 °C. In (b) is shown the time course of increase of absorbance at 450 nm resulting from displacement of aluminum from an aluminum-vicibactin complex by iron (3.3 mM) at 25 °C.

Table 1. Effect of vicibactin on aluminum toxicity to *R. leguminosarum* bv. *viciae* WSM710

Conditions	25 μM Al, vicibactin or Al-vicibactin		5 0 μM Al, vicibactin or Alvicibactin	
	Doubling time (h)	Final cell density (OD_{600})	Doubling time (h)	Final cell density (OD_{600})
Control	5.8	0.69	5.5	0.72
Al alone	no growth	0.04	no growth	0.05
Al-vicibactin complex	5.8	0.70	5.5	0.70
Vicibactin	5.8	0.76	5.5	0.73
Al *plus* vicibactin, added separately	9.8	0.36	no growth	0.04

Doubling times are given for the exponential phases of growth; growth yields are those measured after 80–100 h growth.

Table 2. Alleviation by 100 μM vicibactin of the toxicity to *R. leguminosarum* bv. *viciae* WSM710 of 50 μM aluminum

Conditions	Culture doubling time (h)	Final cell density (OD_{600})
Control	7.0	0.42
50 μM Al	no growth	0.033
50 μM Al *plus* 50 μM vicibactin	no growth	0.042
50 μM Al *plus* 100 μM vicibactin	11.8	0.40

Doubling times are given for the exponential phases of growth; growth yields are those measured after 80–100 h growth.

The binding of Al(III) to vicibactin was followed by measuring the concentration of the uncomplexed vicibactin. Because the reaction of vicibactin with Al(III) is much slower than with Fe(III), the concentration of free vicibactin can be measured using the ferric-perchlorate reagent (10 mM, in 0.2 M perchloric acid) which reacts rapidly with the uncomplexed vicibactin. Aluminum was added to a volume of culture supernate containing a known concentration of vicibactin. Samples were withdrawn at regular time intervals, the ferric perchlorate reagent added, and the absorbance measured at 450 nm after a further 15 min. The kinetics of binding of aluminum to pure vicibactin was followed in the same way: for example, 60 μmol $Al(NO_3)_3$ was added to 10 μmol vicibactin and 150 μmol glutamate buffer (pH 4.0) in a total volume of 30 ml at 25 °C. Final concentrations of Al and vicibactin were 2 mM and 0.33 mM, respectively. Samples were taken at 15 min intervals and analysed for free vicibactin as described above. Other runs were carried out at 25 °C between pH values 2.82 and 4.90, in acetate buffers, with concentrations of Al(III) between 1.5 and 6.0 mM, in order to establish the rate equation. In all kinetic experiments (Figure 1), the error was of the order of 2%.

Results

The rates of formation of the Fe(III) complexes of vicibactin and the non-acetylated vicibactin 7101 were studied to determine if the lack of acetylation on N^2 of N^5-hydroxyornithine affected the rate of reaction. The formation of the Fe(III) vicibactin complex from 3.33 mM Fe(III) and 0.2 mM vicibactin is rapid, reaching completion in about 3 min at pH about 1.5 and 25 °C. Plots of log [siderophore] against time were linear for both vicibactin and vicibactin 7101 (Figure 1a). The values of the first order rate constant k_1 were similar (0.901 min^{-1} for vicibactin and 0.741 min^{-1} for the unacetylated vicibactin 7101).

The formation of the aluminum-vicibactin complex was studied at 25 °C, under pseudo first-order conditions, with [Al(III)] in large excess, in culture supernate, glutamate buffer (pH 4.0) and in acetate buffers covering the pH range 2.0 to 4.9. Similar results were obtained in all cases, confirming that there are no complications from possible speciation changes of Al(III). In glutamate buffer at 2 mM [Al] and pH 4.0, $k_1 = 2.58 \times 10^{-2}$ min^{-1} and $k_2 = 12.9$ min^{-1} M^{-1}, where the second order rate constant $k_2 = k_1/[Al]$. Two series of kinetic runs were

carried out, at 0.6 mM vicibactin and either 3 mM or 6 mM [Al], in acetate buffers at various pH values in the range 2.0 to 4.9. Again, the reaction was first order with respect to [vicibactin], with excellent straight line plots of $\log(D_t - D_{\text{infty}})$ against time. Values of the first order rate constant were generally independent of pH from pH 2 to pH 4.6, over which range the speciation of Al is largely unchanged, and the mean value of the second order rate constant $k_2 = 13.0\ \text{min}^{-1}\ \text{M}^{-1}$, in excellent agreement with the result in glutamate buffers. Values of k_1 of increased very rapidly as the pH is decreased below 2. Complexes of mono- and divalent metal ions usually form rapidly in aqueous solution in a dissociative process, with the rate-determining step being the dissociation of a water molecule from the aqua complex. This step is much slower for aquaM(III) ions, in view of the much greater polarising power of the cation, and so reaction of these cations with ligands may occur associatively, with second order kinetics, as observed for the reaction between vicibactin and Al(III). At lower pH values the reaction is acid-catalysed.

In experiments with the aluminum complex of purified vicibactin and 3.3 mM [Fe(III)] perchlorate at pH about 1.2, Al(III) was only slowly displaced by Fe(III), with $k_1 = 4.3 \times 10^{-3}\ \text{min}^{-1}$ (Figure 1b). This reaction is too slow to compromise the measurements of free vicibactin with ferric perchlorate during measurements of Al(III) binding.

The effects of aluminum, the aluminum-vicibactin complex and equimolar mixtures of aluminum and vicibactin on the growth of *R. leguminosarum* WSM710 were studied. Five different treatments were used in each experiment: (i) a control, with no added aluminum or vicibactin; (ii) aluminum alone (25 or 50 μM $Al(NO_3)_3$); (iii) the aluminum-vicibactin complex (25 or 50 μM); (iv) vicibactin alone (25 or 50 μM) and (v) simultaneous addition of equimolar concentrations of aluminum and vicibactin (25 μM or 50 μM final concentration of each reactant). To ensure reproducibility across cultures of the effects observed, each experiment for each specific set of conditions was carried out using duplicate cultures established from separate starters and the whole set of treatments was repeated three times. In addition, separate preparations of vicibactin and the aluminum-vicibactin complex were used for each experiment.

The concentration of the aluminum-vicibactin complex was estimated from the difference between $A_{450\ \text{nm}}$ measured 15 h after the addition of iron and $A_{450\ \text{nm}}$ measured 15 min after the addition of iron, using the known molar absorptivity of 1510 $\text{M}^{-1}\ \text{cm}^{-1}$ for ferric vicibactin (Carson *et al.* 1994). The absorbance at 450 nm after 15 min was taken to be the 'free' siderophore concentration. Total vicibactin concentration was estimated from the absorbance at 450 nm after the samples had stood overnight, when all the aluminum had been displaced by iron. The difference between the 15 min and 15 h measurements was used to calculate the approximate concentration of bound aluminum. Replicates from individual ex-

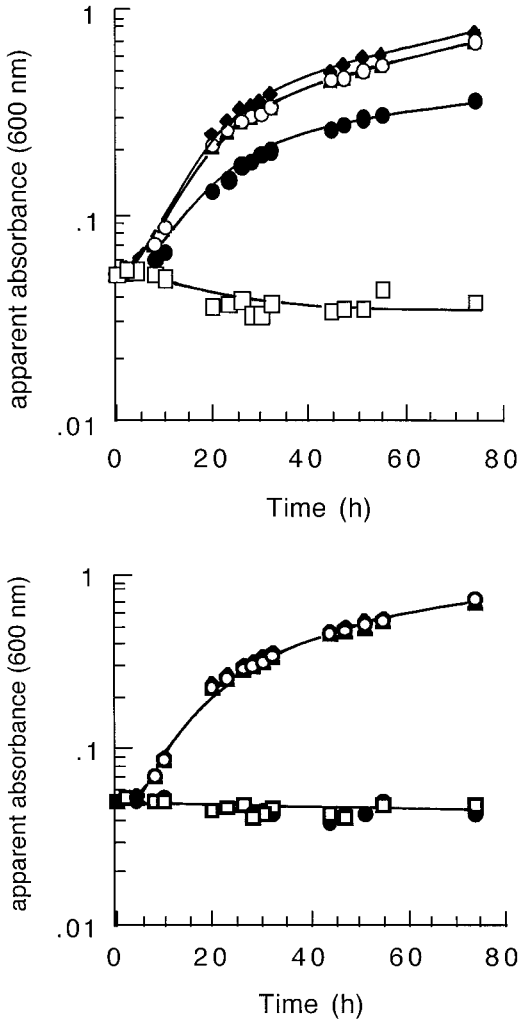

Fig. 2. Growth curves for *R. leguminosarum* WSM710 cultures with aluminum, vicibactin and Al-vicibactin complexes. In (a), cultures contained: (○) no aluminum or vicibactin; (□) 25 μM Al; (▲) 25 μM Al-vicibactin complex; (♦) 25 μM vicibactin; (●) 25 μM vicibactin + 25 μM Al, added separately. In (b), cultures contained: (○) no aluminum or vicibactin; (□) 50 μM Al; (▲), 50 μM Al-vicibactin complex; (♦, upper line) 50 μM vicibactin; (●) 50 μM vicibactin + 50 μM Al.

periments were in excellent agreement. From growth curves such as those shown in Figures 2a and 2b, the final OD_{600} and mean generation times calculated from the log phase of growth are given in Table 1 for one such experiment. The amount of aluminum added as vicibactin complex was estimated as the amount of vicibactin immediately unreactive towards Fe(III). The addition of the purified metal-free vicibactin alone did not significantly alter the growth rate or final cell yield of the cultures. Cells treated with the aluminum-vicibactin complex at 25 or 50 μM grew at the same rate and reached similar final apparent absorbance values as did control cultures. Added aluminum (25 or 50 μM) completely inhibited growth in all cultures. The effects of adding vicibactin and aluminum separately at the start of the experiment depended on the concentrations used. With 25 μM aluminum, the addition of 25 μM vicibactin significantly alleviated its toxic effect (Table 1), restoring final cell yield (OD_{600}) to approximately half that of the controls and giving a doubling time of 9.8 h compared to one of 5.8 h for the control. By contrast, addition of vicibactin (50 μM final concentration) to cultures treated with 50 μM aluminum did not alleviate the toxic effects even though the overall vicibactin:aluminum ratio was the same as for the experiment with 25 μM each of aluminum and siderophore.

The effects of adding separately an excess of vicibactin (100 μM final concentration) to the cultures inhibited by 50 μM aluminum are shown in Figure 3 and Table 2. The toxicity of 50 μM aluminum was clearly decreased by adding 100 μM vicibactin.

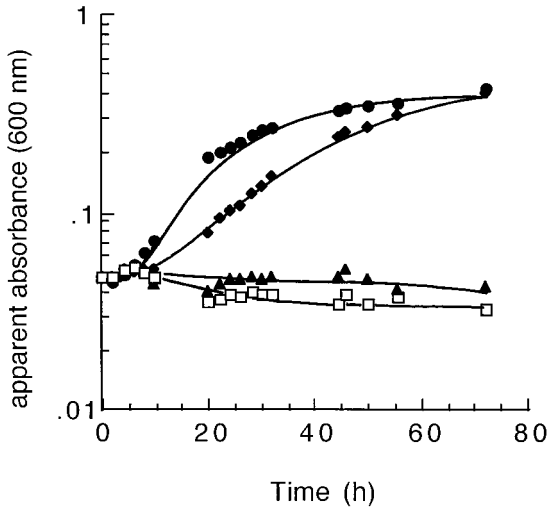

Fig. 3. Growth curves for *R. leguminosarum* WSM710 cultures containing: (●) no aluminum or vicibactin; (□) 50 μM Al; (▲) 50 μM Al + 50 μM vicibactin; and (♦) 50 μM Al + 100 μM vicibactin.

Discussion

The aims of this work were firstly to determine if the siderophore vicibactin, produced by *R. leguminosarum* BV. *viciae* WSM710 under conditions of iron deficiency, could bind aluminum, and secondly, whether vicibactin would enhance or alleviate aluminum toxicity under such conditions. An enhancement of toxicity would suggest siderophore-mediated uptake of Al, while alleviation would indicate binding as a means of detoxification. The initial plan was to compare aluminum toxicity to cells of *R. leguminosarum* WSM710 grown under high- and low-iron regimes, and therefore producing different concentrations of vicibactin. An initial complication was the need to grow cells at sufficiently low pH to keep Al(III) in solution. Another was the reproducible achievement of sufficiently iron-limiting conditions at pH 5.5 to derepress vicibactin synthesis, possibly because there is a sharp repression of vicibactin synthesis as the pH falls from 5.5 to 5.0 (Dilworth *et al.* 1998).

Little is yet known about the regulation of the genes for siderophore biosynthesis in the root nodule bacteria, beyond the evidence that they must be iron-repressed. However, these bacteria express several outer membrane proteins in response to iron-limitation (Reigh & O'Connell 1993; Carson *et al.* 1994; Jadhav & Desai 1994; Patel *et al.* 1994; Roy *et al.* 1994; Fabiano *et al.* 1995; Levier & Guerinot 1996), suggesting that bacteria tightly control siderophore-mediated iron acquisition. Very small changes in environmental iron conditions could significantly affect siderophore biosynthesis and uptake. Although the initial experiments on the effect of added iron concentration on aluminum toxicity were nominally carried out under the same conditions, even small differences in external iron concentration could have affected siderophore production and/or uptake and subsequent aluminum toxicity.

The trihydroxamate siderophore vicibactin binds to aluminum, although at a much slower rate than for Fe(III). Aluminum is clearly toxic to *R. leguminosarum* WSM710, but its complexation by vicibactin alleviates rather than increases that toxicity. Indeed, the preformed aluminum-vicibactin complex appears to be non-toxic, although the Al:vicibactin stoichiometry in preparations of the complex has not been

determined and may be less than the 1:1 ratio expected. Simultaneous addition of equimolar amounts of Al(III) and vicibactin also indicate that complexation results in detoxification. The data suggest, however, that a solution of 25 μM Al and 25 μM vicibactin still has sufficient uncomplexed Al(III) to exert toxic effects on cells of WSM710; the residual uncomplexed Al(III) from a mixture of 50 μM aluminum and 50 μM vicibactin appears sufficient completely to prevent growth. However, excess of vicibactin (100 μM) (added to a culture containing 50 μM Al(III) complexed sufficient AI(III) to permit growth.

Other examples of siderophores affecting aluminum toxicity can also be interpreted as detoxification by complexation. Thus, the production of the hydroxamate siderophore schizokinen by *Bacillus megaterium* ATCC 19213 protected against the toxicity of both aluminum and chromium (Davis *et al.* 1971). Aluminum stress also increased schizokinen synthesis; one explanation is that interference with iron uptake or processing leads to increased siderophore production, but it could also be argued that complexation of schizokinen by aluminum lowers a perceived external concentration and elicits further synthesis. With a hydroxamate-negative strain (SK11) of *B. megaterium* 19213 that showed greater sensitivity to aluminum than did the wild-type (Byers *et al.* 1967), addition of either Desferal (1 mg ml^{-1}) or iron (1 mg ml^{-1}) to the growth medium reversed the toxicity of 10 to 200 μM aluminum. Addition of hydroxamate to the SK11 strain under iron-limiting conditions did not return the growth rate to that of the wild type. It therefore seems more likely that addition of hydroxamate siderophore to the mutant strain SK11 in the presence of aluminum alleviates its toxicity by binding the aluminum and making it unavailable to the cell rather than by increasing growth through enhanced iron uptake.

Complexes between siderophores and aluminum (Emery 1971) can be transported into bacterial cells, indicating that it is the conformation of the metal ligand complex and not the identify of the coordinated metal that determines if it is transported. Thus, cells of *B. megaterium* have been shown to use the hydroxamate siderophore schizokinen to transport aluminum via the siderophore transport receptor at low concentrations of the metal (0–37 μM) (Hu & Boyer 1996). Neither study showed toxicity to aluminum taken up by siderophore-mediated transport, presumably because aluminum cannot be released from the complex by reduction and the complex therefore simply accumulates as a non-toxic species. Even if the Al(III) were released from the complex, it is unlikely to exert toxic effects (Flis *et al.* 1993). This is because the intracellular pH of neutrophilic bacteria like *B. megaterium* and *R. leguminosarum* is slightly alkaline (Booth 1985; O'Hare *et al.* 1989), under which conditions the dominant Al species is likely to be the insoluble $Al(OH)_3$. For *R. leguminosarum* WSM710, however, it is not known if the aluminum-vicibactin complex simply remains in the culture medium, is bound to the outer membrane or in the periplasm, or is actually taken up into the cytoplasm. As with *B. megaterium*, aluminum is unlikely to become toxic intracellularly for the same reasons – lack of a release mechanism, and precipitation of any released Al(III) as $Al(OH)_3$ at the slightly alkaline cytoplasmic pH. Resolution of these questions will require use of techniques sensitive enough to establish the localisation of the aluminum-vicibactin complex, inside or on the cell surface.

Acknowledgements

This work was supported by an AFRC Studentship and by a Wain Fellowship to NJR.

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