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Short communication

Cadmium adsorption by rhizobacteria: implications for New Zealand pastureland

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Abstract

Cadmium (Cd) accumulation by plants is a concern for human health and for decreased productivity of plants and animals. Uptake of Cd by plants is also fundamental in the process of phytoremediation, whereby plants are used to remove Cd from polluted soils. Plants have a substantial microflora associated with their rhizospheres that influence nutrient mobilisation and availability to the plant. This study investigated the effects of Cd on the growth and Cd uptake of *Pseudomonas fluorescens* (CR3), a *lux*-marked rhizoplane bacterial isolate from clover-root tips, as well as four other bacterial isolates from the clover (*Trifolium* spp.) rhizosphere. The bacteria studied are from a ryegrass (*Lolium perenne*)/clover pastureland near Palmerston North. In the first study, CR3 was grown in a media containing 0, 0.1, 0.5, 1 and 5 mg l^{-1} Cd. Optical density, a luminescence measurement of metabolic activity and Cd adsorption were measured over a 48-hour period. There was no significant retardation of growth in media containing less than 1 mg l^{-1} Cd. The lag phase was significantly longer in the 1 and 5 mg l^{-1} treatments. The metabolic activity in these treatments, as indicated by luminescence, was also significantly lower during the exponential growth phase. Cadmium accumulated in the bacteria to circa is 100 times that of the Cd concentration of the media in which they were grown. Accumulation of Cd was also assessed under different growth-media pH conditions and with different bacterial isolates. Cadmium absorption by cells decreased with reduced pH and absorption varied between different species. Sequestration of Cd by rhizosphere microorganisms may have an important influence on plant Cd uptake. Further research is still required, however, to establish whether the accumulation of Cd by rhizobacteria inhibits, or accelerates, Cd uptake by the host plant.

Keywords: Cadmium; Pastureland; Phytoremediation; Rhizobacteria; New Zealand

1. Introduction

Cadmium is a non-essential, toxic, heavy metal that is a common contaminant in soil. Elevated levels of Cd reduce productivity, endanger human health and could even be used as non-tariff barriers to exports. Soil Cd pollution is cumulative with levels increasing over time until eventually the soil may become unusable for production. Common sources of soil Cd contamination are:

1. application of Cd-rich phosphatic fertilisers (Loganathan and Hedley, 1997),

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2. application of municipal sewage sludge (Gardiner et al., 1995),
3. runoff or leaching from roadways (Morrison and Florence, 1990), and base-metal mines (Robinson et al., 1998),
4. deposition from base-metal smelter emissions (Robinson et al., 1998).

Central to the problem of soil Cd contamination and its remediation is plant Cd uptake. Kuboi et al. (1986) investigated Cd accumulation in different families of higher plants. Plant Cd uptake is also important in the process of Cd phytoextraction, where crops of plants are grown to accumulate Cd in the above ground portions. The plant material is removed from the site and placed in an area where it does not pose a risk to the environment (Chaney, 1983).

To date, attempts to decrease plant Cd uptake in agricultural/horticultural systems have focused on using or creating varieties that take up less Cd (Yeargan et al., 1992) and decrease the bioavailability of Cd in the soil (Hooda and Alloway, 1996). The bioavailability of Cd in soils is a function of its solubility (Ernst, 1996) with pH and organic matter content being the main controlling factors (Grey et al., 1998). The production of metal-binding compounds by roots such as phytochelatins has been considered as a mechanism of Cd sequestration by the plant (Salt et al., 1995).

The activities of the large population of bacteria inhabiting the rhizosphere can also be expected to influence Cd uptake by plants. Rhizobacteria produce metal-chelating agents called siderophores (Leong, 1986). These have the effect of scavenging Fe^{3+} and their production is stimulated by the presence of heavy metals (van der Lelie et al., 1999). Heavy metal tolerance by bacteria has been reviewed by Silver and Phung (1996). Tolerance is facilitated by plasmid systems that encode active transport and exclusion systems called chemiosmotic pumps, whereby Cd is moved from the cytoplasm to the periplasmic space outside the cell. Scott et al. (1986) showed that Cd is absorbed into bacterial capsular coatings, whilst Cotoras et al. (1992) demonstrated that different sorption capacities of metals by different bacteria types is correlated more to the size of the organism than to the age of the culture.

Rhizobacteria encounter soil solution before it enters the root and the sequestration of Cd by rhizobacteria from soil solution may play an important part in plant Cd uptake. However, the role played by bacteria in plant Cd uptake is still poorly understood.

This paper reports experiments measuring the effect of increasing Cd levels on rhizobacteria survival, growth and activity. A *lux*-marked rhizobacterium evaluated as a biosensitive indicator to the effect of Cd on growth and metabolic activity of rhizobacteria (Amin-Hanjani et al., 1993). The ability of different rhizobacteria to sequester Cd was measured and the effect of pH on Cd-adsorption by rhizobacteria. Additional studies were conducted with *Rhizobium leguminosarum* bv. *trifolii* to determine the Cd characteristics of this organism.

2. Materials and methods

2.1. Study area

The clover and ryegrass pasture studied was at Massey University's No. 1 dairy farm, Palmerston North (latitude 40.2°S, longitude 175.4°E). The soil type was Manawatu silt loam (FAO soil classification: eutric fluvisol; New Zealand classification: recent alluvial) which has a pH of 5.7, total organic carbon content of $63\,g\,kg^{-1}$ and an exchange capacity of $13.4\,cmol(+)\,kg^{-1}$.

2.2. Bacterial strains

The bacterial strains used in this study were rhizosphere and rhizoplane isolates from the study area described above. *P. fluorescens* CR3 is a clover root-tip isolate that was chromosomally *lux*-tagged with the minitransposon Tn*5luxAB*::*Tc*^{r} (Russell, pers. commun.). Details of strains are given in Table 1.

2.3. Culture media and growth conditions

Bacterial strains were inoculated into a mineral-salts' (MS) broth medium (Williams and Wollum, 1981). Luria Bertani broth (LB) and agar (LBA) (Amin-Hanjani et al., 1993) were used to grow *Pseudomonas* strains and CR3 (Amin-Hanjani et al., 1993) at 28°C overnight. *R. leguminosarum* bv. *trifolii* was cultured on tryptone yeast (TY) and agar (TYA) medium (Beringer, 1974) at 28°C for 48 h. MS medium without glucose amendment was used for characterising

Table 1
Bacterial strains used in the study

Bacterial strain	Relevant properties	Reference or source
P. fluorescens CR3 (C)	Transconjugant clover root-tip isolate carrying Tn*5luxAB*	C.N. Russell
R. leguminosarum bv. *trifolii* NZP561 (R)	Wild type nodulates *Trifolium repens*	D.B. Scott
Brevundimonas sp. KR013 (A)	Rhizoplane isolate from clover/ryegrass pasture; oxidase negative	C.N. Russell
Pseudomonas sp. KR017 (B)	Rhizosphere soil isolate from clover/ryegrass pasture; oxidase negative	C.N. Russell

the effect of Cd on the different rhizobacterial strains. The effect of pH on Cd uptake by CR3 was performed in phosphate buffer at pH 5, 5.5, 6 and 6.5. The pH was measured in the solution after the cells were added.

2.4. Growth and luminescence measurements during batch growth

CR3 was grown in LB overnight to late log-phase growth in batch culture. Two 1 ml aliquots were spun in a microcentrifuge for 2 min, re-suspended in phosphate buffer (pH = 7.0) and the process was repeated twice. A 0.1 ml aliquot was added to triplicate flasks of MS containing 0, 0.1, 0.5, 1 and 5 mg l^{-1} Cd (as $CdCl_2 \cdot 2\frac{1}{2}H_2O$). The flasks were incubated at 28°C on an orbital shaker rotating at 150 rpm. Samples were removed at regular intervals and the optical density (A_{550}) measured. Light output was measured using integrated output over a 10 s period of continuous mixing. The results were expressed in relative light units (RLU) one minute after the addition of 2 μl of aldehyde solution (33% (v/v) of *n*-decyl aldehyde (Sigma):ethanol) in 1 ml samples. A Wallac Bio-orbit 1251 luminometer was used.

2.5. Variable pH and Cd^{2+} concentrations with CR3

Overnight cultures (4 × 100 ml) of CR3 in LB were washed twice in phosphate buffer (pH = 7.0) by centrifugation (12 000 × *g*, 18°C, 5 min), and then re-suspended in 200 ml of phosphate buffer at pH 4.0, 5.0, 6.0 and 7.0. Optical density measurements were also made. Aliquots of 10 ml were placed into PVC universal bottles and 0, 0.5, 1, 5 and 100 mg l^{-1} Cd^{2+} was added in 100 μl aliquots. The cell suspensions were agitated on a rotary mixer (100 rpm for 15 min). For bioassay standardisation, the cells were then immediately pelleted by centrifugation (12 000 × *g*, 18°C, 5 min).

2.6. Rhizobacterial strains and Cd^{2+} concentrations

This experiment was set up exactly as described immediately above, except log-phase cultures (100 ml) of *R. leguminosarum* bv. *trifolii* (R), RK013 (A), RK017 (B) and CR3 (C) were re-suspended in 200 ml of MS without glucose.

2.7. Cadmium uptake

The quantity of Cd^{2+} taken up by cells grown in the MS broth with varying Cd concentrations was measured by flame atomic absorption spectrophotometry using a GBC 904 atomic absorption spectrophotometer. Residual Cd^{2+} concentration in the culture supernatant was determined.

2.8. Cell dry weight

CR3 was inoculated into triplicate flasks of MS as described above and the optical density and biomass readings were made at regular intervals. Biomass was measured by filtering 5 ml of culture media through a 0.45 μm millipore membrane, drying the filter overnight at 105°C and weighing. Control membranes were included which were filtered with sterile MS only.

2.9. Statistical analysis

Significant differences between treatment means were determined by analysis of variance using Microsoft® Excel 97.

3. Results and discussion

3.1. Growth and luminescence in broth culture with varying cadmium concentrations

Fig. 1a shows cell growth of the aerated broth cultures of CR3 in MS at the five different Cd concentrations. Exponential growth curves were fitted and growth rates were significantly reduced ($P < 0.05$) for the 1 and 5 mg l^{-1} Cd treatments. As Cd concentration increased cultures exhibited longer lag phases, the control and the 0.5 mg l^{-1} Cd treatments had the shortest lag phase of 8 h. The term 'lag phase' is defined as the adjustment time of the bacterial inoculum to the growth media where there is a significant physiological activity but little growth in numbers. Exponential growth rates were different and regression analysis showed that optical density was correlated to time ($r = 0.978$, $P < 0.001$). The maximum specific growth rate, μ_{max} (where $dx/dt = \mu_{max} x[1 - x/x_f]$, x = population and x_f = final population in maximum phase) for the control, 0.1 and 0.5 mg l^{-1} Cd treatments was reached after 22 h. In the 1 and 5 mg l^{-1} Cd treatments, μ_{max} was reached after 25 and 30 h, respectively.

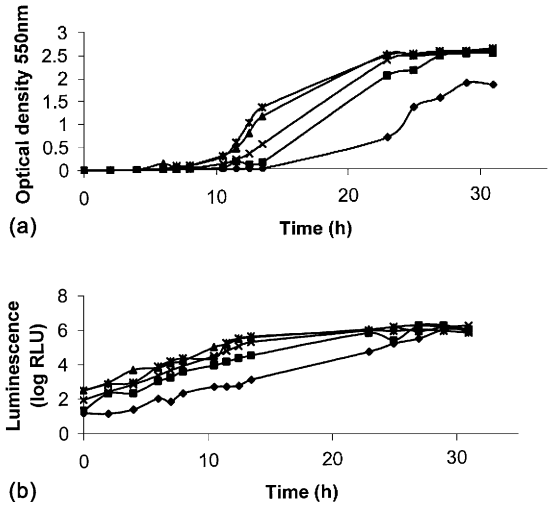

Fig. 1. Growth of *lux*-strain CR3 in batch culture as measured by (a) optical density ($OD_{550\,nm}$) and (b) luminescence (log (RLU)) with Cd^{2+} concentrations at 5 mg l^{-1} (◆) and 1 mg l^{-1} (■), 0.5 mg l^{-1} (▲), 0.1 mg l^{-1} (×) and control (0 mg l^{-1}) (∗) strains grown in MS.

Increased Cd concentration decreased the rate of luminescence increase (Fig. 1b). Luminescence was correlated with time and increase of cell numbers ($r = 0.904$, $P < 0.001$). The initial reduction in luminescence in the higher Cd treatments disappeared after 30 h, where upon luminescence became the same in all treatments. As luminescence is a measure of metabolic activity (Meikle et al., 1994), the equality of luminescence after 30 h indicates adaptation of the bacteria to elevated Cd levels. Scott and Palmer (1990) showed that pseudomonad species can have detoxification systems that precipitate Cd internally. McGrath et al. (1999) used a *lux*-marked biosensor to provide a common basis for the expression of toxic thresholds in soils. However, if adaptation to Cd occurs, detection of Cd toxicity in situ may not be possible. It should be noted that the solution Cd concentrations (0–5 mg l^{-1}) used in this study are higher than those expected (0–0.04 mg l^{-1}) for normal agricultural soils (Andersson, 1976; Tiller et al., 1993).

3.2. Absorption of Cd by CR3 and pH effects

Cadmium absorption by CR3 in solutions with varying Cd concentrations and varying pH, increased with increasing Cd in solution (Fig. 2). The bioaccumulation coefficient is classified as the bacterial biomass (dry weight): solution metal concentration quotient. This decreased with increasing solution Cd

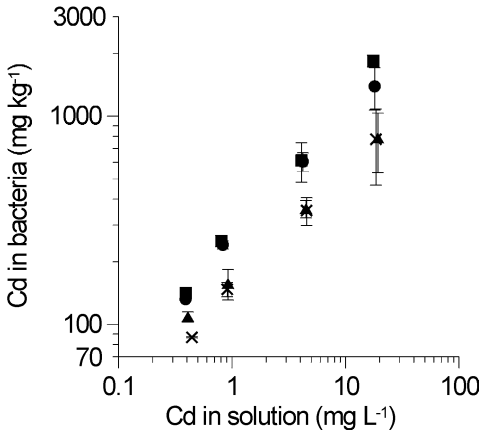

Fig. 2. Cadmium adsorption by *P. fluorescens* at pH 6.5 (■), 6.1 (●), 5.4 (▲) and 5.1 (×). Bacterial concentrations reported on a dry matter basis.

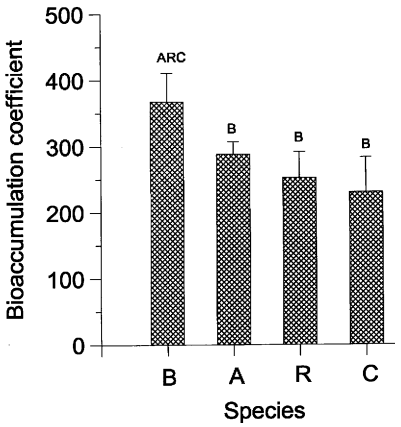

Fig. 3. Average bioaccumulation coefficients (bacteria / solution cadmium concentration quotients) for four rhizobacterial species. (A) *Brevundimonas* sp. KR013, (B) *Pseudomonas* sp. KR017, (C) *P. fluorescens* CR3, (R) *R. leguminosarum* bv. *trifolii* NZP561. Letters above the bars indicate species that are significantly ($P < 0.05$) different.

concentration. At pH 6.5, the average bioaccumulation coefficient was 231. A decrease in pH caused a significant ($P < 0.01$) reduction in the amount of Cd adsorbed by the bacteria. The average bioaccumulation coefficient at pH 5.1 was lower than 112. The reduced accumulation in acid conditions is consistent with the decreased stability of compounds where metals are complexed with organic ligands such as Cd bound to bacterial cell walls.

Bioaccumulation values of rhizobacteria in situ will obviously differ in absolute terms to those reported in Fig. 3 because our experiments neither replicated the chemical environment of the root zone nor measured the long-term effect of Cd exposure on adsorption. Nevertheless, our results do indicate that rhizobacteria have the potential to sequester Cd from soil solution.

4. Potential implications

4.1. Rhizobacterial cadmium in a New Zealand pastureland

Table 2 shows the distribution of biologically active Cd in a typical New Zealand pasture. The results have been calculated using the average Cd concentration in

Table 2
Balance of biologically active cadmium from a hectare of New Zealand soil with a total Cd concentration of 0.4 mg kg^{-1}, depth 0.1 m and moisture content of 33%[a]

Pastureland	Mass (t ha^{-1})	[Cd]	Mass of Cd (g ha^{-1})
Soil water	330	0.005	1.65
Shoots	3	0.1	0.3
Roots	3	0.15	0.45
Bacteria	0.2	1.5	0.3
Fungi	0.5	Unknown	Unknown

[a] Biomass data are from Clark and Paul (1970).

New Zealand pasturelands (0.4 mg kg^{-1}) which results in 0.005 mg l^{-1} Cd in the soil solution (Loganathan and Hedley, 1997). The Cd concentration of the rhizobacteria was calculated using bioaccumulation coefficients from Fig. 3 using *Pseudomonas* KR017. The Cd concentration in the pastureland soil solution is lower than the concentrations used in Fig. 3. Bioaccumulation coefficients decrease with increasing solution concentration (Fig. 2), so our calculation of Cd adsorption is likely to be conservative. Table 2 indicates that although the biomass of rhizobacteria is small relative to the other biota, the amount of Cd associated with these organisms may be comparable to that contained in the roots. Rhizobacteria therefore have potential to enhance plant Cd uptake. Upon cell death, the Cd will be released in the vicinity of the root, some of which may be taken up by the plant. Additionally, the removal of Cd from soil solution will result in an increased desorption of Cd by soil particles.

Gadd (1990) demonstrated that some fungal species accumulated Cd and other heavy metals. The interaction of Cd with soil fungi, which have a biomass twice that of rhizobacteria per unit area (Table 2), should represent a fertile area for future research.

4.2. Relative absorption of cadmium by different rhizobacteria

Bioaccumulation coefficients for the four rhizobacteria studied are shown in Fig. 3. Cadmium accumulation was significantly ($P < 0.05$) greater in *Pseudomonas* KR017 than *R. leguminosarum* bv. *trifolii* and CR3, although there was no significant difference between the latter. These results indi-

cate that the species of bacteria associated with the rhizosphere have different affinities for Cd accumulation. This may be correlated to organism surface area as reported by Cotoras et al. (1992). It may be possible to enhance or inhibit plant-Cd uptake by rhizosphere inoculation with different strains of rhizobacteria.

5. Conclusions

Cadmium temporarily reduces both the growth rate and luminescence of the rhizoplane bacterial biosensor. Luminescence is therefore an excellent measure of bacterial activity since detection is far more sensitive than optical density. Luminescence output from a *lux*-marked rhizobacterium should be an effective indicator of increasing Cd concentrations, activity and growth rate, however, long-term assays will be ineffective as bacteria adapt to the increased Cd concentrations.

Rhizobacteria sequester Cd from solution. The pH and the species of bacteria present affect the degree of sequestration. A significant amount of biologically active Cd may be associated with rhizobacteria in pasturelands.

Future work should involve plant uptake experiments where plant Cd uptake from sterile soils is compared with the uptake from soils inoculated with various rhizobacterial strains.

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