Bull. Environ. Contam. Toxicol. (1991) 46:450–455

Environmental Contamination and Toxicology

Impact of Heavy Metals on an Arctic Rhizobium

Vasu D. Appanna

Department of Chemistry, Laurentian University, Ramsey Lake Road, Sudbury, Ontario, P3E 2C6, Canada

Metals play a dual role in biological systems. In trace amounts, they are essential for all life forms due to their involvement in numerous physiological processes. In elevated concentrations, metals tend to act as potent inhibitors of cell growth and developments. The latter fact has become a major cause of concern due to the increased bioavailability of metals as a consequence of industrially induced acid rain and wastes (Huag 1984: Wright et al. 1980).

Bacteria belonging to the genus **Rhizobium**, when residing in the root nodules of leguminous plants, fix nitrogen and thus contribute very significantly to the global nitrogen budget (Burris 1980). Although there is a paucity of data concerning the effects of metal pollutants on these agronomically important organisms, their negative impact on the nitrogen fixing ability of these microbes is evident (Holding and Lowe 1971): Huang et al. 1974). As rhizobia from root nodules of arctic legumes have been demonstrated to contribute significantly to the ecological balance in this region (Karagatzides et al. 1985), the impact of some metals, found in elevated amounts in acidic surroundings (Havas and Hutchinson 1983), on this unique **Rhizobium** has been assessed. In this paper the ability of the microbe to tolerate abnormal levels of manganese and aluminum is reported and the effectiveness of iron in reversing cadmium toxicity is also discussed.

MATERIALS AND METHODS

All chemicals were reagent grade. Folin Ciocalteu's phenol reagent was obtained from Anachemia, serum albumin from Sigma, yeast extract and agar from Difco Laboratories. Root nodules of **Oxytropis maydelliana** from Melville Peninsula near Sarcpa Lake (68°, 33'N, 83°, 19'W) Northwest Territories, Canada, were the source of the **Rhizobium** used in this study. It was maintained on yeast extract mannitol (Vincent 1970) containing 2% w.v agar. Slants were kept at 4°C and were subcultured monthly. The liquid medium was as described by Appanna and Preston (1987) and was supplemented as required with various concentrations of filter-sterilized (0.22 μm, millipore filters) test metals. For growth experiments 100 ml of medium was inoculated with 0.5 ml of

a stationary phase control culture (unamended with test metals). Cells were grown at 23°C in 250 ml conical flasks rotated at 250 rev min^{-1}.
At appropriate time intervals, the cells from 10 ml samples of culture fluid were harvested by centrifugation at 16,000 x g for 20 min at 4°C, washed twice with 0.85% saline and treated with 4 ml of 0.5M NaOH
at 90°C for 10 min. The protein content of 100 µl aliquots was estimated with Folin Ciocalteu's phenol reagent (Lowry et al. 1952) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

In the control cultures, a yield of 49.6 µg protein per ml of culture was obtained after 1.0 day. Stationary phase was reached in 4 days at which time the cell yield corresponded to 500 µg of protein per ml of culture. when the medium was supplemented with 700 µM Mn^{2+}, a faster growth rate was observed and stationary phase was reached after 3 days with a cell yield similar to that in the control medium. There was no major disruption in the growth pattern of the **Rhizobium** cultured with 440 µM Al^{3+}. Although a slightly longer time was required for the culture to attain stationary phase (5.5 days), the cell yield was not affected. The presence of 300 µM Cr^{3+} in the culture medium considerably perturbed growth. Bacterial multiplication was evident only after 2.0 days of incubation (36.4 µg of protein per ml of culture) and there was a marked diminution in cell yield; at stationary phase a 25% decrease in protein concentration was recorded. In the presence of heavy metals like Cd^{2+}, Zn^{2+} and Co^{2+}, the **Rhizobium** showed a markedly different response. When the medium contained either Zn^{2+} (20 µM) or Co^{2+} (20 µM), a prolonged lag phase of about 5 to 6 days resulted and in both instances the cultures reached stationary phase after a further 4 days. However, the cell yield was significantly diminished to approximately 45% compared to that in the control medium (Figure 1).

When exposed to heavy metals like Zn^{2+}, Cd^{2+}, Ni^{2+} and Co^{2+}, the arctic **Rhizobium** N_{11} showed marked different patterns of tolerance. In the presence of Zn^{2+} (20 µM) and Co^{2+} (20 µM) this microbe grew with diminished cell yield after prolonged lag periods (Figure 1). Such an adaptation may be due to the alteration of permeability leading to partial or total exclusion of these metals. After a similar lag phase, no zinc uptake was discerned (Falla and Weinberg 1977). Cd^{2+}, a cation closely related to Zn^{2+}, arrested cell growth completely when present in the medium at a concentration of only 20 µM. Cd^{2+} uptake in some bacteria appears to occur by means of the Mn^{2+} active transport system (Laddaga and Silver 1985). since Mn^{2+} stimulated the growth of the arctic **Rhizobium** N_{11}, it was hoped that if these two metals (Cd^{2+} and Mn^{2+}) were entering the cells via the same route, Mn^{2+} in a relatively high concentration would block the uptake of Cd^{2+}, thereby neutralizing its toxic effect. But when the microbe was

grown in the presence of Cd^{2+} (20 µM) and Mn^{2+} (700 µM) no improvement in cellular multiplication could be discerned. Thus Cd^{2+} would appear to be exerting its toxic effect through mechanism(s) other than its entry into cells via the Mn^{2+} transport system. However, when both Cd^{2+} and Fe^{3+} were included in the growth medium, a reversal of the inhibitory effect of Cd^{2+} was observed. This clearly points to an antagonism between Cd^{2+} and Fe^{3+} and implies that the mechanism of Cd^{2+} toxicity may involve a disruption of iron nutrition in this bacterium. Ni^{2+} appears to be toxic via other mechanism(s), as Fe^{3+} was unable to reverse the inhibitory effect of Ni^{2+} on this **Rhizobium**. The two trivalent metals chromium and aluminum are known to arrest bacterial growth in low concentrations (Davis et al. 1971):

When Cd^{2+} (20 µM) was included in the medium, cellular multiplication was completely arrested even after 10 days of incubation. However, as shown in Figure 2, when Fe^{3+} (100 µM) was present in the Cd^{2+} supplemented medium, growth was detected. After 2 days, a protein concentration of 92 µg per ml of culture was recorded and cell yield at stationary phase was almost the same as in control cultures. The toxicity due to Cd^{2+} was not ameliorated by inclusion of Mn^{2+} (700 µM) in the medium. Ni^{2+} (20 µM) also exhibited toxic properties on the microbe and completely abolished cellular division, but in this case no improvement in growth was apparent when Fe^{3+} (100 µM) was included in the medium (Table 1)

Figure 1

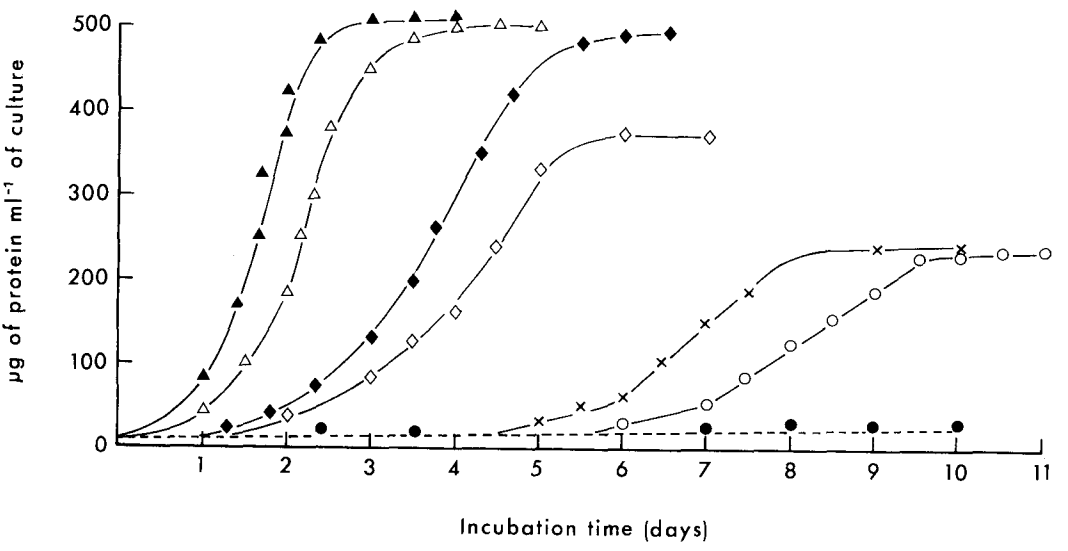

Figure 1. Growth of arctic **Rhizobium** N_{11} in media containing various metals. Δ - Δ, Control (medium unamended with test metal); ▲ - ▲, Mn^{2+} (700 µM); ◆ - ◆, Al^{3+} (440 µM); ◇ - ◇, Cr^{3+} (300 µM); x - x, Co^{2+} (20 µM); O - O, Zn^{2+} (20 µM); ● ----- ●, Cd^{2+} (20 µM).

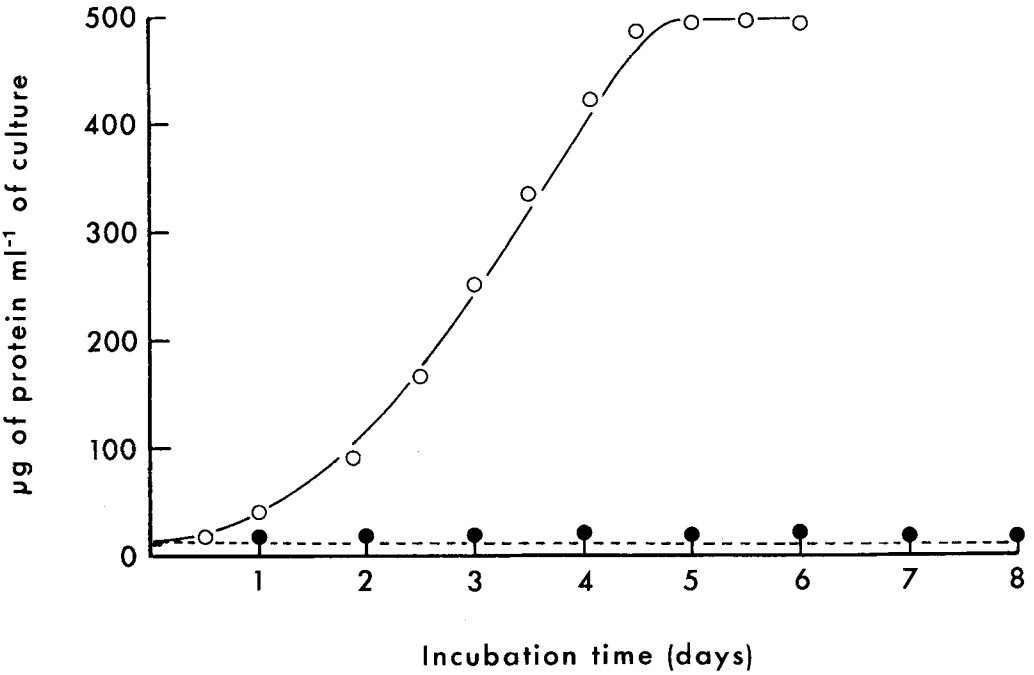

Figure 2. Effect of Fe^{3+} on Cd^{2+} toxicity. ● - ●, growth of arctic **Rhizobium** (N_{11}) in Cd^{2+} (20 µM); O ---- O, growth of arctic **Rhizobium** (N_{11}) in Cd^{2+} (20 µM) and Fe^{3+} (100 µM).

Table 1. Effect of various metals on growth rate and cell yield

Additions	Mean generation time (hrs)	Cell yield (µg of protein ml^{-1} of culture
None	11.7	500
Mn^{2+} (700 µM)	8.6	504
Al^{3+} (440 µM)	15.9	498
Cr^{3+} (300µM)	23.6	376
Zn^{2+} (20 µM)	26.9	225
Co^{2+} (20 µM)	25.2	228
Cd^{2+} (20 µM), Fe^{3+} (100 µM)	18.3	482

The data presented in the paper illustrate the diverse responses elicited by the arctic **Rhizobium** in the presence of different metallic stresses. Mn^{2+} (910 µM) has been demonstrated to cause a decline in cell yield and cell growth in **Leptothrix discophora** and it was suggested that Fe^{3+} deprivation as a consequence of excess of manganese might be responsible for these phenomena (Adams and Ghiorse 1985).

The arctic **Rhizobium** N_{11} was able to grow in 700 μM Mn^{2+} without any apparent decrease in cell yield and, in fact, Mn^{2+} seems to have actually stimulated growth. Similar observations have been made in other rhizobia cultivated in much lower concentrations of Mn^{2+} (Wilson and Reisenauer 1970). This metal is involved in a wide variety of enzymes, especially catalase and superoxide dismutase (Williams 1982), that may have a beneficial effect on growth. Munns and Keyser 1981). The apparent resistance of this **Rhizobium** to these metals requires further studies.

In conclusion, this report illustrates the ability of the arctic **Rhizobium** N_{11} to tolerate various metals and, possibly, disparate detoxification mechanisms may be operative. Of particular interest, however, is the finding that this microorganism, unlike other temperate and tropical rhizobia (Wilson and Reisenauer 1970); Munns and Keyser 1981) seems to be refractory to fairly high concentrations of manganese and aluminum, both present in elevated amounts in acidic environments. It may be of significance to evaluate the impact of these two metals on the efficiency of nitrogen fixation by this arctic **Rhizobium.** Recently, we have demonstrated the elaboration of altered exopolysaccharides biomolecules essential to this vital process in rhizobia in the presence of manganese (Appanna and Preston 1987); Appanna 1988).

Acknowledgments. The author is grateful to Professor A.S. Perlin, Chemistry Department, McGill University, Montreal, Quebec, for laboratory facilities and helpful discussions and to Ms. Claire Raymond, for typing the manuscript. Part of this work was inititated at the Lady Davis Institute for Medical Research, Montreal, Quebec.

REFERENCES

Adams L.F., Ghiorse G. (1985) Influence of manganese on growth of a sheathless stain of **Leptothrix discophora.** Appl Environ Microbiol 49:556-561

Appanna VD (1988) Alteration of exopolysaccharide composition in **Rhizobium meliloti** J5-1 exposed to manganese. FEBS Lett 50:141-144

Appanna VD, Preston CM (1987) Manganese elicits the synthesis of a novel exopolysaccharide in an arctic Rhizobium. FEBS Lett 215:79-82

Burris RH (1980) The global nitrogen budget. In: Nitrogen Fixation (Newton WE, Orme Johnson WH eds). University Park Press, Baltimore, pp. 7-16

Davis WB, McCauley MJ, Byers BR (1971) Iron requirements and aluminium sensitivity of a hydroxamic acid requiring strain of **Baccilus magaterium.** J Bacteriol 105:589-594

Falla ML, Weinberg ED (1977) cyclic accumulation of zinc by **Candida utilis** during growth in batch culture. J Gen Microbiol 99:85-97

Haug H (1984) Molecular aspects of aluminum toxicity. CRC Rev Plant Sci 1:345-367

Havas M. Hutchinson TC (1983) The smoking hills: natural acidification of an aquatic ecosystem. Nature 301:23-27

Holding, AJ, Lowe JF (1971) Some effets of acidity and heavy metals in the rhizobium leguminous plant association. In: Biological Nitrogen Fixation in Natural and Agricultural Habitats (Lie TA, Mulder EG eds). Plant and Soil special volume, pp. 153-166

Huang CY, Bazzaz FA, Vanderoef LN (1974) The inhibition of soybean metabolism by Cd and Pb. Plant Physiol 54:122-124

Karagatzides JD, Lewis MC, Schulman HM (1985) Nitrogen fixation in the high arctic tundra at Sarcpa Lake, Northwest Territories. Can J Bot 63:974-979

Laddaga, RA, Silver S (1985) Cadmium uptake in **E. coli** K-12. J Bacteriol 162:1100-1105

Lowry OH, Rosebrough N, Farr AL, Randall RJ (1952) Protein measurements with Folin phenol reagent. J Biol Chem 193:265-275 Munns DN, Keyser HH (1981) Response of **Rhizobium** strains to acid and aluminium stress. Soil Biol Biochem 13:115-118

Vincent JM (1970) A manual for the practical study of the root nodule bacteria. IBP Handbook No. 15, Blackwell Science Publ, Oxford

Williams RJP (1982) Free manganese (II) and iron (II) cations can act as intracellular cell controls. FEBS Lett 40:3-10

Wilson DO, Reisenauer HM (1970) Effect of manganese and zinc ions on the growth of **Rhizobium**. J Bacteriol 102:729-732

Wright RF, Conroy N, Dickson WT, Harriman R, Henriksen A, Schofield CL (1980) In: Proceedings of the International Conference on the Ecological Impact of Acid Precipitation (Drabos D, Tollan A eds). SNSF Project, Sandefjord, Norway, pp. 371-379.

Received November 25, 1989; accepted October 1, 1990.