Bull. Environ. Contam. Toxicol. (2004) 72:1122–1127

DOI: 10.1007/s00128-004-0360-x

Bulletin of Environmental Contamination and Toxicology

Sequestration of Nickel and Copper by *Azotobacter chroococcum* SB1

G. Vasundhara, G. Jayashree, G. Muraleedhara Kurup

Applied Biochemistry Laboratory, School of Biosciences, Mahatma Gandhi University, Kottayam, PIN-686560, Kerala, India

Received: 4 May 2003/Accepted: 10 March 2004

Though nitrogen fixers are recommended as good bio fertilizers, their efficiency in a polluted environment is a problem of great concern. In the present paper we have focused on one of the major pollutants ,heavy metals viz nickel and copper and their toxicological effects on the nitrogen fixer, *Azotobacter chroococcum* ; Nickel and copper are reported to be toxic inspite of their biological importance(Dalton et al, 1985).Some organisms are reported to accumulate heavy metals inspite of their toxicity(Fuchs, Thauer 1988;Maier et al,1990) But no reports are available on the effects of nickel and copper on the nitrogen fixing capacity of *Azotobacter sp* .We have investigated the efficiency of nitrogen fixation and induction of thiols as a function of heavy metal binding peptides in *A. chroococcum.*

MATERIALS AND METHODS

Azotobacter sp. was isolated from soil by standard micro-biological methods and identified by standard procedure.(Henzyl,1994). The organism was isolated in Jensens' medium, consisting of Sucrose 20.0g, potassium monohydrogen phosphate 1.0g , sodium chloride 0.5g , magnesium sulphate 0.1g , calcium carbonate 2.0g, sodium molybdate 0.005g, agar 15.0g, distilled water 1000ml (pH7.0).(Subba Rao,1977)

To 20ml broth culture at log phase (48 hrs) in 100ml rubber stoppered bottles, sterile nickel chloride and copper chloride solutions were added to get a final concentration of 0.5,1.0,5,10 and 25 ppm in the cultures. Broth culture without metal was taken as the control. Enzyme activity was measured after 24h, 48h and 72h of metal treatment .

Nitrogenase activity was determined by acetylene reduction assay (ARA).(Hardy et al,1968). The broth cultures were incubated with 10% acetylene and ethylene formed at 1hr, 3hr,and 5hr of time intervals was estimated by gas chromatograph(Shimadzu3C-Poropak) using flame ionisation detector.

250 ml Jenson's broth culture (Subba Rao,1977) of the bacteria at 48 hrs. was treated with metal chloride solution so that the final concentration in the medium was 0.5 ppm and 1ppm. Broth culture without metal was taken as the control. After 24hr of treatment, the bacteria were separated by centrifugation at 1800 rpm for 20 min at 4^0 C. The pellet was sonicated by applying 5-10 cycles of 20 sec burst of energy at an intensity of

Correspondence to: G. Muraleedhara Kurup

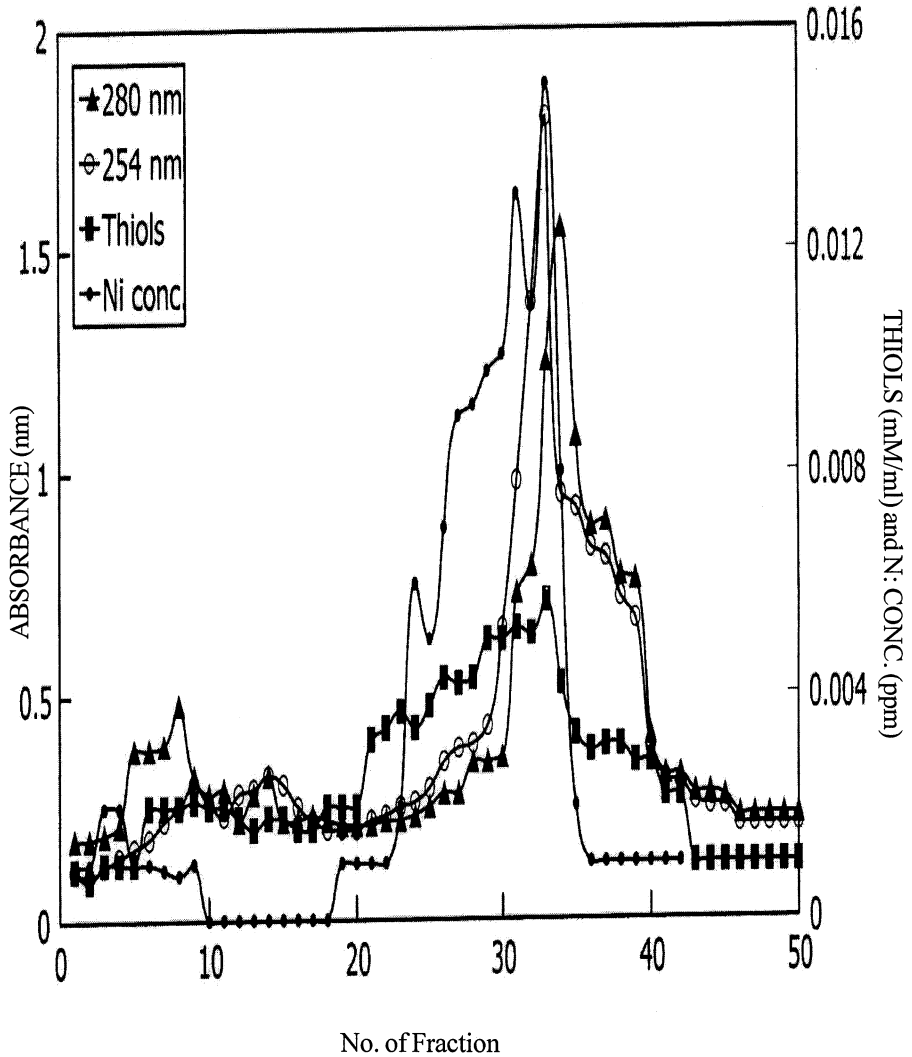

Figure 1 Nickel sequestration by *A. chroococcum.*

100-110mV. Cell debris were removed by centrifugation at 6000rpm. Supernatant was analysed for nickel ion concentration by atomic absorption spectrophotometry (Dannielsson etal,1978;1982)and the total thiols were estimated as described earlier (Boyne and Ellman, 1972).

Supernatant was applied to a column packed with Sephadex G-50 gel and eluted with Tris buffer (0.1 M) pH 8.0. In each case, 50 fractions were collected at a rate of 4 drops per minute. The absorbance of each fraction was read at 254 nm and 280 nm. The samples and the fractions were analysed for metal ion concentrations and for total thiols.(Dannielsson etal,1978;1982, Boyne and Ellman ,1972). Protein content in enzyme extracts were determined by Lowry's method.(Lowry et al,1951)

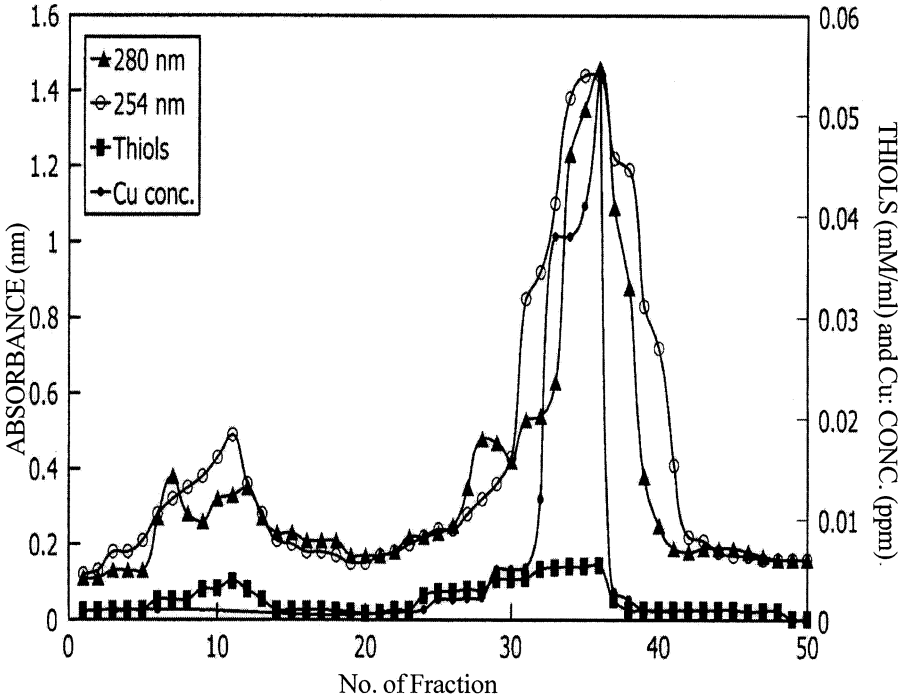

Figure 2 Copper sequestration by *A. chroococcum*

Table 1. Nitrogenase activity in *Azotobacter sp.* under nickel stress (n moles of ethylene produced /mg protein /min)

Conc (ppm)	Mean ± S.D		
	24h	48h	72h
0	68.94± 41.31	145.50± 46.12	167.20 ± 7.67
0.5	56.25± 12.32	58.95 ± 9.66	185.13 ± 4.16
1	39.89± 4.07	50.02 ± 6.38	80.69 ± 8.68
5	ND	ND	ND
10	ND	ND	ND
25	ND	ND	ND

$P<0.05$. Average of six values
ND - Not detected

Table 2. Nitrogenase activity in *Azotobacter sp.* under copper stress. (nM/mg/min)

Conc (ppm)	Mean ± SD		
	24 h	48h	72h
0	68.93± 41.3	145.50±46.12	167.19±7.67
0.5	38.15± 1.96	80.34± 15.68	141.59± 6.41
1	32.56± 3.81	47.25± 6.63	61.97± 3.04
5	ND	ND	ND
10	ND	ND	ND
25	ND	ND	ND

$P<0.05$, Average of six values

ND-Not detected

Table 3. Metal sequestration and thiol production in *Azotobacter sp.*

Metal	Metal conc. Applied. (ppm)	Thiol Conc. (mM/ml)	Metal conc. Absorbed (ppm)
Ni	0.5 1	0.0422 0.1339	0.008 0.13
Cu	0.5 1	0.061 0.099	0.0071 0.19
Control		0.026	ND

ND - Not detected

RESULTS AND DISCUSSION

Nitrogen fixation in *Azotobacter sp* . is affected by different levels of nickel and copper. There was a complete abolishing of nitrogenase at 5, 10 and 25ppm of nickel in all cases (Table 1). A partial inhibition was observed at 0.5 and 1ppm in all the cases except for a significant recovery at 0.5ppm after 72h of nickel treatment. Nitrogenase activity was decreased at higher concentrations of copper viz.5ppm, 10 ppm and 25ppm (Table 2). But only a partial inhibition at 0.5 and 1 ppm. *Azotobacter* was found to accumulate both nickel and copper (Table 3). When the concentration of the metal in the medium was increased, a corresponding increase in metal uptake was observed. An increased production of thiols was observed with increase in metal concentration (Fig 1 & 2)

No reports are available on the effects of nickel or copper on nitrogen fixation with respect to thiol production as s stress defying mechanism in *Azotobacter sp.* and perhaps this is the first report on the same. Our studies indicated a recovery in nitrogen fixation at 0.5 ppm after 72hrs. Hence 0.5 ppm nickel may be stimulatory to *Azotobacter* sp. Whether nickel is being chelated by the thiols produced by the bacterium needs further study. The organism might have developed defense mechanisms to circumvent the toxicity caused by heavy metals viz. synthesis of heavy metal binding peptides(Steffans,1990;Boyle,1984). At very high concentrations no recovery of enzyme was observed because of the failure of defense mechanisms to alleviate the stress.

Thiol production was greater at 1 ppm of nickel compared to copper, but metal absorption was found to be greater at 1 ppm of copper. Production of thiol is primarily due to the synthesis of phytochelatins , potential biomarkers of heavy metal exposure in plants(Depledge et al,1994).Metal accumulation by an organism provides a better indication of metal in the environment which is likely to affect the aquatic system than most types of direct chemical analysis(Boyle,1984)

It is a matter of fact that the efficiency of nitrogen fixation is adversely affected by nickel and copper except nickel at 0.5 ppm where an increase in nitrogen fixation was observed.Since nitrogenase is highly sensitive to heavy metal stress, it may be suggested as an index to monitor heavy metal pollution in farmlands where *Azotobacter chroococcum* is utilized as fertilizer.Production of thiols provide an accessible method to assess heavy metal pollution and may be preferred to heavy metal binding peptides.Our studies establish the efficiency of *Azotobacter chroococcum* as a biofertiliser as well as heavy metal remover.

Acknowledgements. Vasundhara is thankful to UGC for providing SRF for the work and Jayashree is grateful to M.G. University for JRF.Authors are grateful to Prof. Jacob Chacko, Department of Oceanography, CUSAT, Kochi and Dr. Raveendran, Scientist, Pollution Control Board, Kochi for their valuable help.

REFERENCES

Boyle TP (1984) The effect of environmental contaminants on aquatic algae In : Elliot Shubert (Ed) Algae as Ecological Indicator ,Academic Press , p 237- 255.

Boyne AF, Ellman GC (1972) A methodology for analysis of tissue sulfhydryl components. Anal Biochem 46: 639-653

Dalton DA, Evans H J , Hanns F J(1985) Stimulation by nickel of soil microbial urease activity and urease and hydrogenase activities in soybeans grown in a low nickel soil. Plant Soil 88 : 245-258

Danielsson LG , Magnersson B, Westerland S (1978) An improved metal extraction procedure for the determination of trace metals in water by AAS with electrothermal atomisation . Anal Chem Acta 98 : 47-57

Danielsson LG , Magnersson B ,Westerland S (1982)Trace metal determination in estuarine water by electrothermal AAS ,after extraction of dithiocarbamate complexes into freon. Anal Chem Acta 144: 183-188

Depledge MH,Weeks JM, Bjerregaard P (1994) Heavy metals, In: Petercalow(Ed) Hand book of Ecotoxicology.Oxford Blackwell scientific publications 79-105.

Fuchs DA , Thauer RK(1988) Nickel in biology, In: Jack RL Jr (Ed)The Bioinorganic Chemistry of Nickel, VCH Publishers

Hardy RWF, Holsten RD, Jackson EK , Burns PC (1968)The acetylene –ethylene reduction assay for nitrogen fixation: Laboratory and field evaluation, Plant Physiol 43: 1185-1207

Henzyl WR (1994) Bergy's manual of determinative bacteriology 9 th Ed,Williams and Wilkins,USA ,p134-136.

Lowry OH, Rosebrough N J, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent . J Biol Chem 193: 265-268

Maier RJ, Pihl TD, Stults L, Sray W (1990) Nickel accumulation and storage in Bradyrhizobium japonicum. Appl Environ Microbiol. 56: 1905-1911

Steffans JC (1990) The heavy metal binding peptides of plants. Ann Rev Plant physiol . Plant Mol Biol 41:553-575

Subba Rao NS (1977) Soil Micro-organisms and plant growth .Oxford and IBH Publishing Co, N. Delhi