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Comparison of Ni-sensitive and Ni-resistant strains of *Nostoc muscorum*

R.K. Asthana, A.L. Singh and S.P. Singh*

A wild-type Ni-sensitive (Ni^s) strain of *Nostoc muscorum* ISU spontaneously yielded mutants resistant to inhibition by 40 μM Ni with a frequency of about 10^{-7}. A Ni-resistant (Ni^r) mutant was deficient in the activities of urease and uptake hydrogenase. Cellular Ni uptake in the Ni^s strain was dependent on concentration (40 to 120 μM) and time (0 to 30 min) (V_{max} = 0.51 nmol/μg protein.min; K_m = 92 μM). The Ni bioconcentration factor for such cells ranged between 0.95×10^3 and 1.89×10^3. Ni uptake in spheroplast preparations from Ni^s cells followed almost the same trend as intact cells except that the bioconcentration factor was slightly less [$(0.82 \text{ to } 1.39) \times 10^3$]. In contrast, Ni uptake in the Ni^r intact cells was not concentration dependent and also the uptake was saturated, even at 40 μM, within 10 min. Spheroplasts from the Ni^r strain showed a Ni bioconcentration factor of 1.19×10^3 compared with 4.41×10^3 for intact cells. The invariably lower Ni uptake by spheroplasts was attributed to altered membrane transport properties.

Key words: Bioconcentration, Cyanobacterium, Ni^r, Ni^s, nickel, spheroplasts.

The role of Ni as a nutrient for microbes has been emphasized (Hausinger 1987) and Van Baalen & O'Donnell (1978) observed Ni-dependent growth of *Oscillatoria* sp. 3NT. Interest in the Ni nutrition of cyanobacteria has established the element's essential role in the control of urease activity in *Anabaena cylindrica* (Mackerras & Smith 1986) and of the hydrogenase associated with Ni uptake in this species (Daday & Smith 1983) and other species (Papen *et al.* 1986) including *Mastigocladus laminosus* (Pederson *et al.* 1986). Uptake of Ni has been studied in *An. cylindrica* (Campbell & Smith 1986), *Alcaligenes eutrophus* (Lohmeyer & Freiderich 1987), *Scenedesmus* sp. ATCC 11460 and *Chlamydomonas* UTEX 89 (Gleason & Wood 1988). At high concentrations, Ni is toxic to cyanobacterial growth, photosynthesis, N_2 fixation, nutrient uptake and ATPases (Stratton & Corke 1979; Babich & Stotzky 1983; Asthana *et al.* 1990, 1992). The development of Ni-resistance in *Synechococcus* ATCC 17146 was accompanied by the biosynthesis of cellular polymers, and the inability of cells to accumulate Ni (Gleason & Wood 1988).

The present communication compares Ni-sensitive (Ni^s) and Ni-resistant (Ni^r) strains of *Nostoc muscorum* ISU in terms of their activities of uptake hydrogenase and urease, and of Ni uptake by intact cells and spheroplast preparations.

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Materials and Methods

Experimental Organism and Growth Conditions

The parent strain, *Nostoc muscorum* ISU (*Anabaena* ATCC 27893, from R. Haselkorn, University of Chicago, USA), was grown axenically in growth medium as described by Gerloff *et al.* (1950) at 24 ± 1°C with illumination at 50 $\mu Em^{-2} s^{-1}$, with a 18/6 h light/dark cycle. The basal medium was supplemented with 5 mM KNO_3, 1 mM NH_4Cl or 1 mM urea to give media named nitrate-, ammonia-, or urea-medium, respectively. Medium lacking a fixed nitrogen source was termed N-less medium.

Isolation and Enrichment of Mutants

The general methodology for Ni-sensitivity tests was that of Asthana *et al.* (1990). As the cells could not grow in 40 μM Ni in any fixed-N media, nickel resistant (Ni^r) mutants could be selected by the method described elsewhere (Singh & Singh 1978). The Ni^r mutant clones were subsequently maintained on N-less medium containing 40 μM Ni.

Determination of Heterocyst Frequency

Heterocyst frequency was determined microscopically, and expressed as percentage of total cells.

Nitrogenase Activity

In vivo activity was measured by monitoring the amount of ethylene produced in the acetylene reduction technique (Stewart *et al.* 1968): 2.0 ml cyanobacterial suspension (350 μg protein/ml) was held in a 8.5 ml glass vial, sealed with a rubber stopper, containing an atmosphere of 10% (v/v) acetylene. The general methodology adopted was as described by Asthana *et al.* (1990); activity was expressed as nmol ethylene formed/μg chlorophyll *a*. h.

Uptake Hydrogenase Activity

Activity was measured according to the method of Tel-Or *et al.* (1977). The cyanobacterial cells were harvested, washed, and resuspended in 2 ml N-less medium held in a 15 ml glass tube which was then sealed with a rubber stopper. H_2 (99.99% purity) was injected with a syringe (final mixture 2%, v/v), and the tubes were incubated under a photoflux of 50 $\mu Em^{-2} s^{-1}$ at 28 $\pm$ 2°C. Gas samples (0.5 ml) were withdrawn from the tubes, and injected into a gas chromatograph fitted with sieve column. Argon was used as carrier (flow rate 20 ml/min). H_2 served as control. Uptake hydrogenase activity was expressed as nmol H_2 consumed/μg chlorophyll *a*. h.

Urease Activity

Urease activity was monitored in terms of ammonia released, using an ammonium electrode. Urease specific-activity was expressed as nmol NH_3 released/mg protein.min.

Protein and Chlorophyll a Measurements

Protein was estimated by the method of Lowry modified by Herbert *et al.* (1971) using lysozyme as standard. Chlorophyll *a* was estimated by the method of Mackinney (1941).

Ni Uptake

Nostoc muscorum, grown for 6 days, was centrifuged, washed repeatedly with triple glass-distilled water and suspended in phosphate buffer (0.2 M, pH 8.6). Ni uptake from $NiCl_2$ was measured from 40 to 120 μM. Spheroplasts of Ni^s and Ni^r cells were prepared by the method of Biggins (1967) and Ni uptake was determined in mannitol/phosphate buffer (0.4 M, pH 8.6). The uptake experiments were conducted in light (50 $\mu Em^{-2} s^{-1}$) at 24 $\pm$ 1°C. Samples were removed at intervals, centrifuged (3000 × g; 75 s) and monitored for cellular Ni using atomic absorption spectrophotometry at 232 nm. The general methodology adopted was essentially that which Singh and Yadava (1985) used for Cd.

Results and Discussion

Genetically-stable clones of *N. muscorum* growing with 40 μM Ni in diazotrophic growth medium arose with a frequency of about 10^{-7}. Such nickel resistant (Ni^r) clones were maintained on diazotrophic solid medium containing 40 μM Ni. One such Ni^r mutant clone and its parent clone (Ni^s) were grown in bulk in ammonium-medium containing a standard concentration of Ni (1 μM). Such cultures were subsequently transferred to various nitrogen media for comparison of growth, heterocyst frequency and nitrogenase activity (Table 1). Direct comparison, only possible with NO_3^- or NH_4^+ as nitrogen source, showed that the diazotrophic growth and nitrogenase activity of the Ni^r strain were much lower than that of the Ni^s strain. The Ni^r strain failed to grow with urea as nitrogen source, possibly the result of inactivation/non-availability of the enzyme system metabolizing urea. Data (Table 2) show the negligible activity of uptake hydrogenase and urease in the Ni^r strain of *N. muscorum* which supports the conclusions from the growth experiments (Table 1). Such observations led us to look into the intracellular build-up of Ni in the Ni^r strain as this could have resulted in the non-availability of the metal for the synthesis of the enzymes under investigation. Heavy metals also bind with the phosphate reserves in cyanobacterial cells (Jensen *et al.* 1982), with other cell components of microscopic algae (Vallee & Ulmer 1972) and even with organic polymers in nature (Karapanagiotis *et al.* 1990). In a recent report, the intracellular binding of Ni with polymers, in a class of Ni-tolerant mutants of *Synechococcus* ATCC 27146, was taken as one means of development of metal tolerance (Gleason & Wood 1988).

Figure 1 shows that Ni uptake by *N. muscorum* cells increased linearly up to 120 μM Ni. Ni uptake in the Ni^s strain was similar to that of Zn uptake in *Cladophora glomerata* (McHardy & George 1990), in which there was rapid cation uptake in the first 10 min, but no increase in metal uptake beyond 20 min. The bioconcentration factors for Ni^s cells ranged from (0.95 to 1.89) × 10^3 compared

Table 1. Growth, heterocyst frequency, and acetylene reducing activity in 6-day-old cultures of the parent (Ni^s) and Ni^r mutant strains of *N. muscorum* in different nigrogen media.*

	Parent			Ni^r mutant		
	Growth (mg dry wt/ml)	Heterocyst frequency (%)	ARA†	Growth (mg dry wt/ml)	Heterocyst frequency (%)	ARA†
Control (N-less medium)	0.35	5 to 6	12.6	0.18	5 to 6	7.4
NO_3^--medium	0.53	0	0.0	0.47	0	0.0
NH_4^+-medium	0.36	0	0.0	0.36	0	0.0
Urea medium	0.50	0	0.0	0.0	0	0.0

* The source of inocula for growth in the various nitrogen media was a NH_4^+-grown culture.
† Acetylene reducing activity, expressed in nmol ethylene formed/μg chlorophyll *a*.h.

Table 2. Uptake hydrogenase and urease activities of the parent and Ni^r strains of *N. muscorum* in 6-day-old cultures in N-less medium with 1 μM Ni.

Enzyme	Parent (Ni^s)	Mutant (Ni^r)
Uptake hydrogenase (nmol H_2 consumed/μg chlorophyll *a*.h	8.6	0.5
Urease (nmol NH_3 formed/mg protein.min)	6.3	0.1

with 2.2 × 10^3 for the green alga *Scenedesmus* ATCC 11460, 3.3 × 10^3 for *Synechococcus* ATCC 17146 and 1.1 × 10^1 for *Oscillatoria* UTEX 1270 (Gleason & Wood 1988). Spheroplasts from Ni^s cells followed a similar trend but had a lower Ni uptake (60%) and bioconcentration factor [(0.82 to 1.39) × 10^3] than the intact cells (compare Figures 1 and 2). The lower Ni uptake of the spheroplasts can be attributed to significant inhibition of PS II activity, as reported in *Anacystis nidulans* (Ward & Myers 1972). Lineweaver-Burk plots for Ni uptake in Ni^s intact cells and spheroplasts gave a common K_m of 92 μM while V_{max} values were 0.51 and 0.39 nmol/μg protein.min, respectively. Similar comparisons between whole cells and protoplasts of *Saccharomyces cerevisiae* revealed that the former accumulated Cu with an efficiency 18 times greater than the latter (Gadd *et al.* 1984); this was attributed to the resistance offered by protoplasts to Cu-influx. The observed lowering of Ni uptake in spheroplasts can also be ascribed to altered permeability, as reported in similar preparations of *Spirulina platensis* by Robinson *et al.* (1982). These investigators also noted that

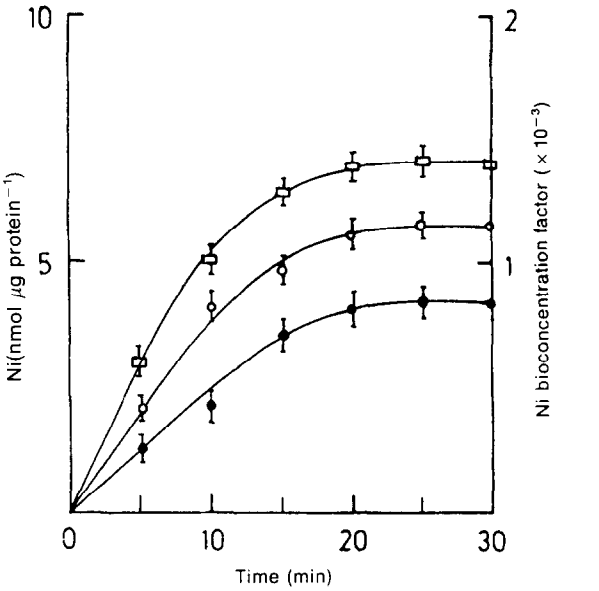

Figure 2. Ni uptake in spheroplasts from *N. muscorum* (Ni^s) with Ni at 40 (●), 80 (○) and 120 (□) μM.

spheroplasts do not retain CO_2-dependent O_2-evolution activity.

Figure 3 shows that Ni uptake by Ni^r cells remained unchanged if Ni concentration was increased from 40 to 120 μM [in contrast to that of the Ni^s strain (Figure 1)], suggesting that the Ni^r strain failed to accumulate Ni exceeding 40 μM (the concentration to which it was resistant). However, the bioconcentration factors suggested that the Ni^r strain concentrated Ni 2.33 times more (4.41 × 10^3) than the Ni^s strain (1.89 × 10^3) from 40 μM Ni

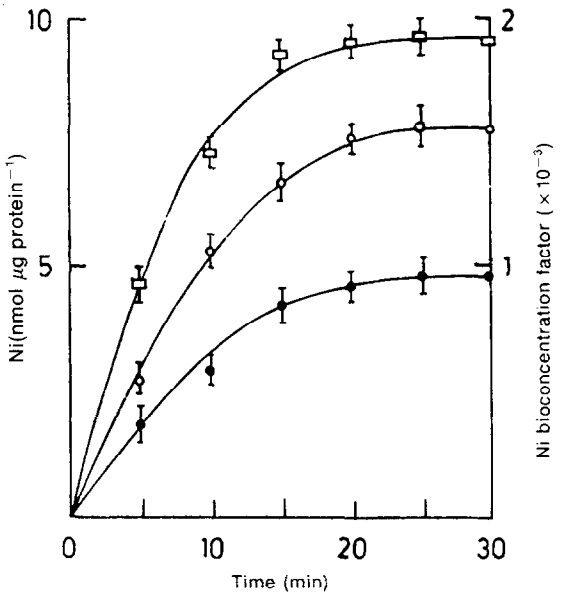

Figure 1. Ni uptake into *N. muscorum* (Ni^s) with Ni at 40 (●), 80 (○) and 120 (□) μM.

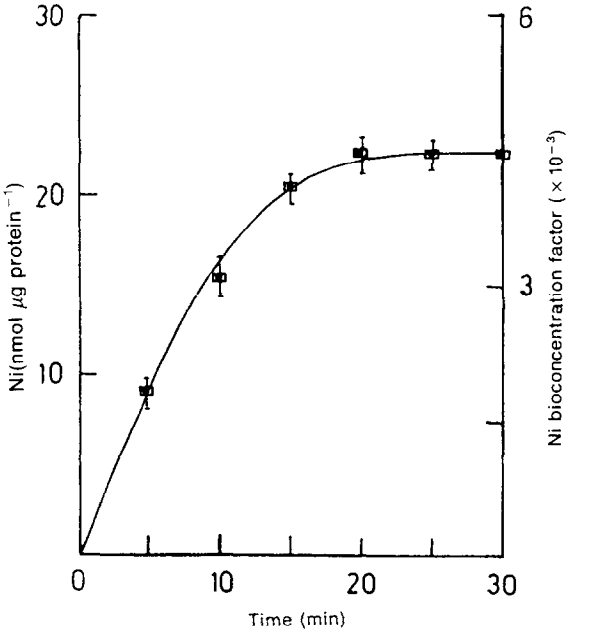

Figure 3. Ni uptake in *N. muscorum* (Ni^r) cells with Ni at 40 (●) and 120 (□) μM.

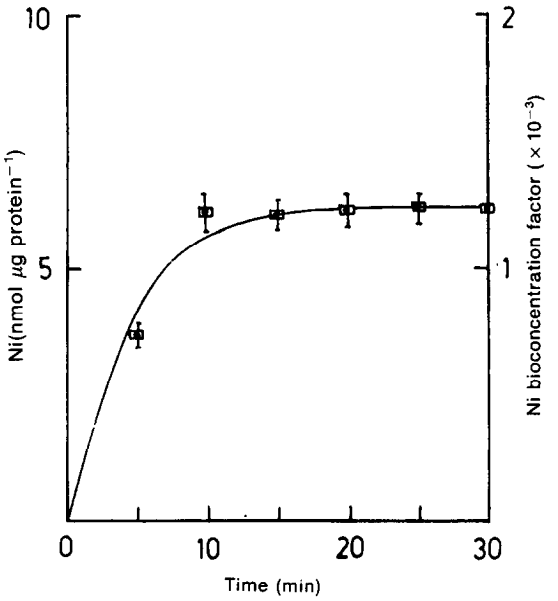

Figure 4. Ni uptake in spheroplasts from the Ni^r strain of *N. muscorum* with Ni at 40 (●) and 120 (□) μM.

uptake medium. Such observations concur with the normal growth in and/or resistance of Ni^r strain to 40 μM but not 120 μM Ni. The lower saturating concentration (40 μM) for Ni uptake in the Ni^r strain, compared with that for the Ni^s, could also arise through altered membrane permeability and/or any other factor leading to the loss of the concentration-dependence of Ni-uptake found in Ni^s cells. Spheroplasts from the Ni^r strain followed a similar trend in Ni uptake to the Ni^r intact cells, although the intact cells concentrated 3.6 times more Ni (4.41×10^3) than the spheroplasts (1.22×10^3; Figure 4). Similar comparisons in a Cu-resistant strain of *Saccharomyces cerevisiae* revealed that intact cells maintained relatively high Ni accumulation, 1.5 times more than the spheroplasts (Gadd *et al.* 1984).

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