Mechanism of nickel resistance in a cobalt-resistant wall-less mutant of *Neurospora crassa* (fz; sg; os-1)

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A cobalt-resistant wall-less mutant of *N. crassa* (Cor-sl) characterized previously was also found to be 3-fold more resistant to nickel when compared to the parent wall-less mutant (W-sl). The Cor-sl strain accumulates relatively lower amounts of nickel when compared to W-sl. Sub-cellular fractionation showed significant quantities of nickel to be associated with nuclear and mitochondrial fractions in both the wall-less mutants. However significant differences were observed in vacuolar fractions of W-sl and Cor-sl strains. Fractionation of cell-free extracts on Sephadex G-10 column resolved nickel into two peaks, of which the peak II in Cor-sl constituted 70% of nickel, while the same in W-sl was about 30%. A 3-fold increase in histidine content was observed in case of Cor-sl as compared to W-sl strain, suggesting its role in Ni-resistance.

**Keywords**: *Neurospora crassa*, Nickel, Resistance, Transport, Wall-less mutant

Nickel toxicity in terms of toxic responses in a number of prokaryotes and eukaryotes is a well-studied phenomenon. Major toxic effects of nickel include chromosomal damage, alteration of enzyme activity, inhibition of protein and RNA synthesis, and decrease in ATP pool. Nickel toxicity in *Neurospora crassa* (fz; sg; os-1) was shown to result in accumulation of pyruvate and α-ketoglutaric acid. The induction of metallothionein by animal cells does not contribute to increased tolerance.

A number of metal-resistant strains of *N. crassa* have been characterized and their mechanisms studied. The first cobalt-resistant strain of *N. crassa* was 10-fold resistant to cobalt and nickel. The mechanisms of resistance were suggested to be due to alteration in iron metabolism, which is altered, in resistant strain. In a later cobalt-resistant isolate a transport block and intracellular sequestration of cobalt by a novel cobaltoprotein were shown to be the mechanisms for resistance. Cobalt and nickel resistance was earlier mapped to the same locus on LG III of *N. crassa*. Three non-identical nickel resistant mutants of *N. crassa* which were 4-fold resistant to nickel but differed in cross-resistance to cobalt were also characterized. Transport block and Ni-hyperaccumulation were observed in the above strains. However in all three zinc-resistant strains of *N. crassa* a transport block was observed. Metal resistant strains in general exhibit cross-resistance to related metal ions. Heavy metal resistant mutants of microorganisms in general are not cross-resistant to nickel. However in *N. crassa* cobalt and nickel-resistant strains are cross-resistant to one another. Cobalt-resistance in *N. crassa* (Cor) was shown to involve a transport block and induction of cobaltoprotein. Recently cobalt-resistant wall-less mutant of *N. crassa* (Cor-sl) was isolated and characterized. The mechanism of resistance was shown to involve both transport block as well as overproduction of a cobaltoprotein like in the normal filamentous cor mutant of *N. crassa*. The Cor-sl was also cross-resistant to nickel and copper. In this paper the mechanism for cross-resistance to nickel (which is different from cobalt resistance) is examined and discussed in relation to known mechanisms.

**Materials and Methods**

**Organisms**—The wall-less mutant of *Neurospora crassa* (FGSC#1118) referred to herein as W-sl was obtained from Fungal Genetics Stock Center, Kansas City, USA. The cobalt-resistant strain (Cor-sl) was obtained by adaptation to toxic levels of cobalt. Both, *N. crassa* W-sl and Cor-sl mutants are also commonly referred to as ‘slime’.
Chemicals—Metal salts used were CoSO₄·7H₂O, NiSO₄·7H₂O, CuSO₄·5H₂O and MgSO₄·7H₂O. All chemicals were of analytical grade products from Qualigen and Sarabhai M.Chemicals Ltd, Baroda, India. ⁶⁵Ni (Sp. activity 8 mCi/g) was purchased from BRIT, Mumbai, India and Sephadex G-50 from Sigma Chemical Company, ST, Louis, USA. Nutrient broth and yeast extract were products of Hi-Media (Mumbai, India).

Media and growth conditions—Cultures of the wall-less mutants (W-sl and Cor-sl) were grown and maintained in Vogel’s medium supplemented with 0.75% yeast extract and 0.75% nutrient broth. Sorbitol (0.25 M) was added to the medium as an osmotic stabilizer and pH of the medium was adjusted to 5.6 with dilute NaOH. Metal toxicities and uptake studies were conducted in basal medium with added 0.25 M sorbitol. Liquid cultures were grown and incubated at 28 ± 1°C in a rotary shaker incubator at 100 rpm for 24 hr (Labline, USA). Growth was measured by counting cell number using a Hemocytometer (Neubauer, Germany). Metal ions were added to the basal medium separately after autoclaving to the desired concentrations. Cells were harvested by centrifugation at 1000 g for 10 min in a clinical centrifuge.

Nickel estimation—Nickel content of the cells and those required in the medium were determined by Atomic Absorption Spectrophotometry (AAS) (Perkin-Elmer Model 2830). In most experiments radioactive ⁶⁵Ni (0.8 mCi/g) used was estimated as follows. After incubation, cultures were pelleted by centrifugation at 3000 rpm in a clinical centrifuge for 10 min, the cell pellet was washed with nickel-free basal medium (twice) and solubilized in 0.5 N NaOH. Suitable aliquots were added to scintillation cocktail [PPO (2, 5 diphenyl oxazole) and [POPOP (1, 4-bis (5-phenyl-2-oxazollyl) benzene] in toluene and radioactivity was measured using Liquid Scintillation Counter (Beckman LS 1801).

Sub-cellular distribution of nickel—Slime cells were grown in 50 ml basal medium containing nickel (8 mCi/³⁵Ni) in 250 ml flasks and sub-cellular fractionation procedure was performed. Briefly, the cells were harvested, washed and the cell pellet (10⁶ cells) was suspended in 5 ml buffer (sucrose 0.25 M, MgCl₂ 5 mM, CaCl₂ 10 mM, glycerol 20%, Tris-Cl pH 7.5), homogenized with a teflon pestle for 25 strokes. To the homogenate 5 ml of the same buffer was added and centrifuged at 1000 g for 10 min. Supernatant (S1) was collected and the pellet was resuspended and centrifuged at 1000 g for 10 min. Supernatant (S2) was collected. The pellets from above centrifugation contain cell debris. The supernatants (S1 and S2) were pooled and centrifuged at 5000 g for 20 min to obtain the nuclear pellet. The resulting supernatant was centrifuged at 12,000 g for 20 min to obtain mitochondrial pellet. Supernatant obtained was collected and added to a gradient made by layering 4 ml of 1.8 M sucrose and a continuous 6 ml of 1.8 M sucrose-1 M sorbitol gradient. The gradient was centrifuged at 43,000 g and the vacuolar pellet was suspended in 1.8 M sucrose. Radioactivity of suitable aliquots from each step was determined to measure nickel.

Histidine estimation—Cultures were filtered through 0.2 µm membrane and washed with 50 ml ice-cold basal medium. The filters were immersed in 4 ml of double distilled water and heated to 100°C for 15 min in a water bath. After cooling to room temperature the samples were diluted to 10 ml, centrifuged at 10,000 g for 15 min and aliquots of the supernatant were used for histidine estimation by an automated amino acid analyser (Pharmacia LKB plus) as in earlier studies.

Gel filtration column chromatography—Cell-free extracts were prepared and separated on Sephadex G-50 column (2.5 x 40 cm). The column was pre-equilibrated in Tris-Cl buffer (50 mM, pH 6.5). Fractions (5 ml) were collected and analysed for nickel by AAS. For separation of small molecular weight peptides or amino acids associated with nickel, cells were disrupted by sonication in ammonium bicarbonate (30 mM) and high molecular weight proteins were removed by extraction with chloroform-butanol (10:1 v/v) mixture and the aqueous phase was used for separation on Sephadex G-10 column (2.5 x 45 cm) equilibrated with ammonium bicarbonate (30 mM). Fractions were analysed for nickel by AAS and histidine by automated amino acid analyser. Sub-cellular fractionation procedures used are similar to those described earlier.

Results
The cobalt-resistant wall-less mutant of N. crassa was found to grow on agar medium containing 2-10
mM nickel, while the sensitive W-sl strain did not show growth even up to 7 days. Resistance to cobalt was further quantitated in liquid basal medium. Fig.1 shows that *N. crassa* Cor-sl is 2-3 fold more resistant to nickel when compared to W-sl strain. The % growth (without Ni²⁺) of Cor-sl mutant is 36.7% less than that of parental W-sl strain. The I₅₀ value of nickel in Cor-sl and W-sl were 2 mM and 0.8 mM respectively. Nickel uptake (*⁶³Ni*) measured under the conditions showed that W-sl accumulates twice as much nickel when compared to the Cor-sl strain at their respective I₅₀ concentrations of nickel.

Nickel uptake—Nickel uptake in short-term experiments was next studied and the results are shown in Fig.2. *N. crassa* Cor-sl accumulated much less nickel up to 120 min when compared to W-sl mutant. Ni²⁺ uptake by 30 min was about 15 nmoles/mg protein, which increased marginally to over 20 nmoles by 120 min for Cor-sl strain. In W-sl strain it was around 24 nmoles at 30 min, which increased to 35 nmoles by 120 min. In both the strains Ni²⁺ uptake was drastically inhibited (80-90%) by sodium azide (Fig. 3). The effect of magnesium ions (at equimolar concentration of nickel) was also examined and the results indicate that both the strains Ni²⁺ uptake was inhibited by 20-30% (Fig. 4).

In order to see if there is any efflux of nickel ions already taken up by wall-less mutants, cells were allowed to take up nickel (*⁶³Ni*), washed and resuspended in Ni²⁺-free medium. The Ni²⁺ remaining with cells and that released was estimated. The total nickel accumulated by W-sl and Cor-sl were 41.9 and 32.6 nmoles respectively and about 25% was released by the former and 39% by the latter strain.

The distribution of nickel (*⁶³Ni*) in various subcellular fractions was undertaken and the results are presented in Table 1. A significant quantity of nickel was observed in nuclear (30-31%) and mitochondrial (5-6%) fractions of both W-sl and Cor-sl. However significant differences were noted in the vacuolar fraction wherein 16.3% of total nickel was observed.
in W-sl strain in comparison to 3.6% in case of Cor-sl strain.

In the earlier work, cobalt-resistant wall-less mutant of *N. crassa* (Cor-sl) was shown to overproduce a cobaltoprotein, which binds most of the cellular cobalt ions and constitutes up to 12% of the total protein\(^1\). In order to see whether nickel accumulated by the Cor-sl strain is also bound by the cobaltoprotein, similar fractionation procedures were employed (DEAE-Cellulose chromatography). The results indicated that unlike cobalt, most of the nickel (>90%) was found in the flow through fractions of the column. Hence cell-free extracts were separated on Sephadex G-50 gel filtration column and most of the nickel was eluted at the bed volume of the column and none was observed in the void volume. However separation of extracts on Sephadex G-10 columns resolved nickel into two peaks. Peak II contained the free nickel as it was calibrated to be the V\(_b\) of the column. In Cor-sl strain more than 70% of the nickel was observed to be in Peak II fraction in comparison to the W-sl strain, which harboured about 30% (Fig. 5). This prompted us to look for small molecular weight compounds to which nickel is bound. The amino acid histidine is known to be a chelator of nickel ions, which also eluted in peak II fraction and hence the same was estimated in extracts of both the wall-less mutants under control (no nickel) and in presence of nickel. In *N. crassa* Cor-sl there is 3-fold increase in histidine content when grown in presence of nickel, while such an increase in W-sl was not observed (Table 2). About 4.2 μmoles of histidine (per g protein) was present in W-sl, while it was estimated to be 21 μmoles in Cor-sl strain. When histidine was estimated from pooled fractions of peak II, Cor-sl contained 5-6 times more histidine in comparison to W-sl.

**Discussion**

Microorganisms when adapted to toxic concentrations of metal ions are known to develop resistance and the mechanism of resistance depends upon both the organism and the specific metal ion under study. One general phenomenon observed with resistant strains of most organisms is that they exhibit cross-resistance to closely related metal ions. In *N. crassa* most of the resistant strains characterized

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Nickel (μg/10(^8) cells)</th>
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<tbody>
<tr>
<td></td>
<td>W-sl</td>
</tr>
<tr>
<td>Cell lysate</td>
<td>330</td>
</tr>
<tr>
<td>Cell debris</td>
<td>42</td>
</tr>
<tr>
<td>Nuclei</td>
<td>90</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>19</td>
</tr>
<tr>
<td>Vacuoles</td>
<td>54</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>116.5</td>
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**Table 1**—Sub-cellular distribution of nickel in slime cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nickel Histidine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mm) (μmoles/g protein)</td>
</tr>
<tr>
<td>W-sl</td>
<td>ND  4.4</td>
</tr>
<tr>
<td>Cor-sl</td>
<td>1.0  4.2</td>
</tr>
<tr>
<td></td>
<td>ND  6.4</td>
</tr>
<tr>
<td></td>
<td>1.0  21.0</td>
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</tbody>
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**Table 2**—Histidine content of extracts in wall-less mutants of *N. crassa*

ND = Not detectable.

![Fig. 4—Effect of magnesium on Ni\(^{2+}\) uptake](image1)

![Fig. 5—Sephadex G-10 column chromatography](image2)
till date exhibit cross-resistance to other related metal ions. For example the first cobalt-resistant strain of *N. crassa* was equally resistant to nickel\(^9\). The later isolate of the same exhibited 20-fold resistance to cobalt and 4-fold cross-resistance to nickel\(^{19}\). In three non-identical nickel-resistant strains of *N. crassa* a variable cross-resistance pattern to cobalt was observed\(^{13}\). Two strains (Ni-R1 and Ni-R2) were in fact more resistant to cobalt than to nickel, one of them (Ni-R3) was comparatively less resistant to cobalt. Nickel-resistant mutants of *Saccharomyces cerevisiae* were shown to be more sensitive to cadmium and copper than wild type. But no significant differences were observed with respect to uptake as compared to wild type. However inhibitory effects of nickel on RNA and protein synthesis were less pronounced in mutants.\(^{19}\)

The more recently characterized zinc-resistant mutants were cross-resistant to cobalt but not to nickel\(^{14}\). The present cobalt-resistant wall-less mutant which is 10-fold resistant to cobalt but only 2-3 fold resistant to nickel. One prominent feature commonly seen in metal-resistant mutants of *N. crassa* was that the growth was markedly lower than the parent type. In the present mutant (Cor-sl) also a significant decrease in growth (36%) was observed when compared to W-sl strain. Hence it appears that cross-resistance is a complex phenomenon useful in characterization of organisms resistant to nickel when compared to W-sl strain. In general the Cor-sl mutant accumulated relatively less nickel when compared to W-sl strain. In the present study it is clear that nickel is not bound to any protein similar to that of cobaltoprotein\(^{11}\). Separation on Sephadex G-50 gel filtration column indicated that most of the nickel eluted as a single peak near the Vi of column and was observed at the void volume (Vo) of the column where high molecular weight proteins elute. Separation on Sephadex G-10 column however resolved nickel in two peaks wherein significant differences between Cor-sl and W-sl were noted. Peak II in Cor-sl strain contained major fraction of nickel (70%), while the same was relatively much less in W-sl strain (30%) (Fig.5). The remaining nickel (70%) was seen in peak I fractions. Peak II contained free nickel ions along with small molecular weight component, which could not be resolved under the experimental conditions. On analysis, histidine was found in significant quantities in peak II fraction which was found to be 5-6 times more in Cor-sl strain as compared to W-sl. Hence it appears that nickel is chelated most likely to histidine. Histidine is a well-known high-affinity chelator of nickel ion, which was estimated in extracts of both the wall-less mutants. Interestingly there was a 3-fold increase in the free histidine levels in cell-free extracts of Cor-sl strain grown in presence of nickel. This was not observed in W-sl strain of *N. crassa* (Table 2). Most likely this could contribute to peak II fraction obtained on Sephadex G-10 column. Significant increase in free histidine levels was observed in nickel hyperaccumulating plants\(^{20}\). In *N. crassa* copper toxicity on nitrate N-medium was shown to result in accumulation of intermediate compounds of the histidine biosynthetic pathway\(^{21}\). Marked differences in amino acid levels were observed under copper toxic conditions\(^{22, 23}\). The overall distribution of nickel was similar in sub cellular fractions of both wall-less mutants of *N. crassa* except in vacuolar fraction. In Cor-sl (3.6%) a 4-fold decrease in nickel content was observed when compared to W-sl (16.3%). Vacuoles were shown to accumulate most (85%) of lithium ions in *S. cerevisiae*. In this study enlarge vacuoles were observed\(^{24}\). The novel feature which emerges from the present study, for the first time is that cross-resistance mechanisms are different from that of the mechanisms existing for the primary metal ion to which an organism is resistant.

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