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Cobalt-resistance in wall-less mutant (*fz; sg; os*-1) of *Neurospora crassa*

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Abstract

A cobalt-resistant wall-less mutant (slime) of *Neurospora crassa* was obtained by repeated sub-culturing of the sensitive wall-less mutant (W-sl) on agar medium containing toxic concentrations of cobalt. Resistance was stable on culturing Cor-sl on cobalt-free medium up to 15 weekly subcultures. Cor-sl is 10-fold more resistant to cobalt when compared to W-sl. It is also cross-resistant to Cu (10-fold) and Ni (3-fold). Cobalt accumulated by Cor-sl during growth and in short-term uptake experiments was lower when compared to W-sl. Cells previously loaded with cobalt was released into medium in both mutants, while in case of Cor-sl most of cobalt taken up (> 80%), was released back into the medium when compared to W-sl. Metabolic inhibitor (Sodium azide) and magnesium ions inhibited cobalt uptake in both the mutants. Fractionation of cell-free extracts showed that most of the cobalt (70%) taken up by Cor-sl was bound to an inducible protein fraction which bound to DEAE-Cellulose, while in W-sl only 20% of cobalt was associated with this fraction. Subcellular localization of cobalt in W-sl indicated most of it to be cytoplasmic (70%) while nuclei and mitochondria had 10% and 5% respectively. In case of Cor-sl, mitochondrial cobalt accounted for only 2% while no significant differences were noted for other fractions. Our data implicate both transport block and intracellular sequestration of cobalt to play a major role in resistance.

Introduction

Metal ions like Co^{2+}, Ni^{2+}, Cu^{2+} etc., when present in excess interfere with the major metabolic pathways leading to toxicity in *Neurospora crassa* (Healy *et al.* 1955; Subramanyam *et al.* 1983; Maruthi Mohan & Sastry 1983; Gadd 1993). Exposure of *N. crassa* to cobalt and nickel was shown to result in stable resistant mutants which are several fold more resistant than the sensitive wild type (Venkateswerlu & Sastry 1973; Maruthi Mohan & Sastry 1983). The nickel-resistant isolates Ni-R1, Ni-R2 and Ni-R3, were non-identical to each other with respect to cross-resistance, metal transport and resistance mechanisms. Nickel resistance was shown to be due to a transport block in *N. crassa* Ni-R3, while intracellular mechanism appears to function in the hyperaccumulating strains Ni-R1 and Ni-R2 (Maruthi Mohan & Sastry 1983; Maruthi Mohan *et al.* 1984). In the first cobalt-resistant isolate of *N. crassa*, alteration in transport and iron metabolism was observed (Venkateswerlu & Sastry 1973, 1979). In a recently characterized cobalt-resistant strain of *N. crassa* (*cor*) partial transport block due to alteration in cell wall binding of cobalt was reported. Further an intracellular mechanism due to induction of a specific cobaltoprotein, overproduced by this strain was also shown to play a major role in cobalt resistance (Sajani & Maruthi Mohan 1997, 1998). Also three non-identical zinc-resistant strains of *N. crassa Zn-R1, Zn-R2* and *Zn-R3* obtained both by adaptation to Zn^{2+} and by chemical mutagenesis were found to have transport block (Rama Rao *et al.* 1997).

Exposure of fungi to toxic metal ions is known to result in resistant strains by acquiring any of the following two general mechanisms: (a) transport block leading to decreased accumulation of toxic metal ions; (b) intracellular compartmentalization into organelles/sequestration by specific metal binding proteins (Gadd 1993). The metal-resistant mutants of

fungi in general and *N. crassa* in particular are those which were derived from filamentous forms containing thick cell walls which bind significant quantities of metal ions to the anionic sites therein. This binding, which constitutes between 10% to 90% of the total mycelial metal ion taken up by the organism presents a problem in characterizing the actual uptake kinetics of the membrane transporter system. In order to discount cell-wall effects on metal transport and to better understand the mechanisms of resistance, a wall-less mutant of *N. crassa* was used to develop a cobalt-resistant strain. The characteristics of the resistant strain in comparison with the sensitive wall-less mutant are presented in relation to the mechanism of cobalt-resistance.

Materials and methods

Organism

The wall-less mutant of *Neurospora crassa* (FGSC# 1118) also referred to as W-sl was obtained from the Fungal Genetics Stock Center, Kansas City, USA.

Chemicals

Metal salts used were $CoSO_4.7H_2O$, $NiSO_4.7H_2O$, $CuSO_4.5H_2O$ and $MgSO_4.7H_2O$. All chemicals are analytical grade products of Sarabhai M.Chemicals Ltd. Baroda, India. ^{57}Co and ^{58}Co were purchased from BRIT, Mumbai, India. DEAE-Cellulose is from Sigma Chemical Company, St.Louis, USA. Nutrient broth and yeast extract are 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 (Vogel 1956) supplemented with 0.75% Yeast extract and 0.75% Nutrient broth. Metal toxicities and uptake studies were conducted in basal medium described earlier (Venkateswerlu & Sastry 1973). 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. For the preparation of slants 3% agar was included in basal medium. Liquid cultures were grown and incubated at 28 ± 1 °C in a rotary shaker incubator at 100 rpm for 24 h (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. For isolation of cobalt-resistant strain, *N. crassa* wall-less mutant (W-sl) was grown on basal medium slants containing cobalt.

Isolation of cobalt-resistant wall-less mutant of N. crassa

The general procedure followed was that described by Maruthi Mohan and Sastry (1983). Slime cells (W-sl) from active cultures were transferred to slants of basal medium containing cobalt (2–10 mM). After satisfactory growth was obtained repeated transfers were done in the same concentrations of cobalt, which resulted in progressively better growth. After 10 to 20 weekly subcultures resistance to cobalt was tested by measuring growth (counting cell number by Hemocytometer) as a function of cobalt concentration in basal medium. When no further increase in cobalt-resistance was observed as seen by changes in I_{50} values (50% growth inhibitory concentration), single colonies were isolated by plating out on agar medium containing 10 mM cobalt. One of the isolates was chosen for further detailed studies and referred to as *N. crassa* Cor-sl. To check the stability of resistance, Cor-sl was maintained on cobalt-free media for 15 weekly subcultures.

Cobalt uptake

Cobalt uptake (^{58}Co) during growth was performed in 10 ml medium in 50 ml conical flasks incubated at 28 °C in rotary shaker (100 rpm) incubator for 24 h. In short term uptake studies 10^8 cells in 10 ml medium were allowed to take up ^{57}Co (1μ Ci in 0.3 mM cobalt) for required times and at various indicated concentrations. The cells were washed by pelleting at 3000 *g* for 5 min in cold cobalt medium (5 mM) and cobalt taken up was measured in the pellet by mini-gamma counter. To check for the release of cobalt into medium, cells were allowed to take up cobalt for 30 min, washed in isotonic medium and resuspended in cobalt-free medium for 30 min centrifuged (1000 *g* for 10 min) and radioactivity of the supernatant was measured.

Metal analysis

Radioactive cobalt (^{57}Co sp.activity = 10 Ci/mole or ^{58}Co sp.activity = 500 mCi/mole) was included as

tracer to measure cobalt uptake by cells (or as indicated separately in experiments) and was measured in cell pellets or cell-free extracts as required using a γ-counter (LKB-Wallac 1275 Mini γ-counter) or liquid scintillation counter (Beckmann).

Organellar distribution of cobalt

Slime cells were grown in 50 ml basal medium containing cobalt (0.3 mM with 10 μCi of ^{57}Co) in 250-ml flasks. The cells were harvested, washed and the cell pellet was suspended in 5 ml buffer (0.25 M Sucrose, $MgCl_2$ 5 mM, $CaCl_2$ 10 mM, glycerol 20%, Tris 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 centrifugations contain cell debri. 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. Radioactivity of suitable aliquots from each step was determined to measure cobalt.

DEAE-cellulose chromatography

Cobalt distribution in cell-free extracts was studied by the method described by Sajani and Maruthi Mohan (1998). Slime cells were grown in 10 ml medium containing cobalt (0.3 mM containing 5 μCi of ^{57}Co for 24 h). The cells were harvested, washed and re-suspended in 5 ml of 50 mM Tris-Cl pH 6.5, homogenized and centrifuged for 5 min at 1000 *g* to remove cell debris and the supernatant was centrifuged at 12,000 *g* for 30 min. The supernatant fraction was collected and the protein content was determined by the procedure of Lowry *et al.* (1951) and cobalt content (cpm) was determined. An aliquot of the supernatant containing 2 mg protein was used to load on DEAE-Cellulose column (10 ml bed volume) pre-equilibrated with Tris-Cl buffer (50 mM, pH 6.5). The column was washed with three bed volumes of equilibration buffer and bound protein fraction was eluted with a 0.3 M of NaCl in the same buffer at a flow rate of 1 ml min^{-1}. Fractions (3 ml) were collected and monitored for protein at 280 nm and for cobalt by γ-counter.

Note: Unless indicated all the experiments were repeated 3–5 times and representative data are shown. A variation up to 15% was observed between separate experiments.

Table 1. Isolation of cobalt-resistant wall-less mutant of *N. crassa.*

Cobalt (mM)	Number of subcultures(weekly)	I_{50} for cobalt (mM)
nil	–	0.35 ± 0.07
1	10	0.35 ± 0.08
2	15	0.35 ± 0.07
4	15	1.0 ± 0.2
10	20	3.5 ± 0.7

N. crassa wall-less mutant was subcultured on agar medium containing increasing concentrations of cobalt as indicated. I_{50} = 50% growth inhibitory concentration of Co^{2+}. Values shown are averages derived from graphical plots of growth versus concentration from 3 separate experiments ($\pm$ S.D).

Results

Isolation of cobalt-resistant wall-less mutant

In an effort to isolate a cobalt-resistant wall less mutant of *N. crassa*, the sensitive mutant (W-sl) was repeatedly sub cultured (10 to 20 weekly subcultures) in agar medium containing various concentrations of cobalt (2–10 mM). Initial growth was slow but improved with increasing number of subcultures. The results of Table 1 show I_{50} values (50% growth inhibitory concentration for cobalt derived from graphical plots of growth versus cobalt concentration in liquid medium) which indicate that *N. crassa* W-sl adapted to 1 mM or 2 mM cobalt do not show any resistance to cobalt, while with 4 mM a 2–3-fold resistance was observed when compared to the unadapted cultures. However a 10-fold was obtained in cultures adapted to 10 mM cobalt. The I_{50} value is 0.35 mM for W-sl strain, while it is 3.5 mM for 10 mM cobalt adapted cultures (Figure 1). To obtain a genetically homogenous culture 10 single colony isolates of the 10-fold cobalt-resistant cultures were tested for resistance and all of them showed similar I_{50} values for cobalt (about 3.5 mM) and hence one of them was chosen for detailed studies and referred to hereafter as cobalt-resistant *N. crassa* or as Cor-sl. Cobalt resistance was found to be stable and unaltered when Cor-sl was subcultured on cobalt-free medium for 15 weekly subcultures. Most of the metal-resistant strains of *N. crassa* isolated so far exhibited cross-resistance to related toxic metal ions and the same

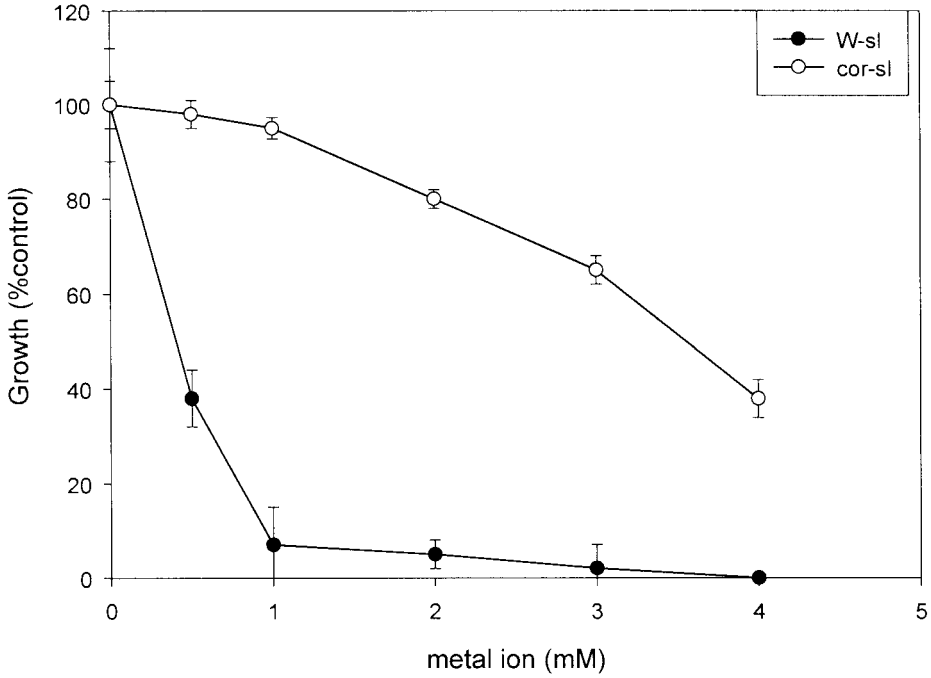

Fig. 1. Cobalt toxicity in W-sl and Cor-sl. Slime cells (10^8) were grown in the indicated concentrations of cobalt for 24 h at 28 ± 1 °C. Cell number was measured by hemocytometer to determine the growth.

was studied in Cor-sl. Cor-sl is three-fold resistant to Ni (I_{50}-2 mM) and 10-fold resistant to copper (I_{50}-3 mM) when compared to W-sl (I_{50} for Ni-0.3 mM; Cu-0.3 mM). However it was not resistant to cadmium or zinc.

Cobalt transport

Cobalt toxicity and uptake was examined for both *N. crassa* W-sl and Cor-sl. The I_{50} value of cobalt (50% growth inhibitory concentration) was found to be about 0.35 mM Co^{2+} for W-sl and 3.5 mM Co^{2+} for Cor-sl. When cobalt uptake values are compared Cor-sl accumulated relatively less cobalt than W-sl, especially between 0.3 mM to 0.5 mM cobalt concentration in growth medium (Figure 2). However, at relatively higher concentrations at which the sensitive W-sl does not grow, Cor-sl accumulates higher levels with resultant growth inhibition (Figure 3).

Since *N. crassa* Cor-sl accumulated relatively lower levels of cobalt when compared to W-sl cobalt uptake was studied in short-term uptake experiments to understand the alterations in the cobalt transport system. Cobalt uptake from medium containing 0.1 mM cobalt was found to be linear with Cor-sl taking up less cobalt at all the time points studied up to 60 min. The rates of uptake (nmoles/10^8 cells /10 min) were calculated to be 0.35 for W-sl and 0.12 for Cor-sl, respectively (Figure 3). Cobalt uptake as a function of concentration also showed lower accumulation by Cor-sl when compared to W-sl at all the concentration (1–4 mM). At 4 mM cobalt concentration W-sl accumulated 60 μmoles/10^8 cells, while it is around 30 μmoles in case of Cor-sl. (Figure 4).

Table 2. Effect of magnesium on cobalt uptake.

	Cobalt (nmoles)	
	− Mg	+ Mg
W-sl	2.0 ± 0.3 (100%)	1.0 (50%)
Cor-sl	1.5 ± 0.2 (100%)	1.1 (42%)

Slime cells (10^8) were allowed to take up cobalt (0.1 mM containing 1μCi of ^{57}Co) from 10 ml medium in presence and absence of magnesium (0.1 mM). Values shown are average of 2 separate experiments (± S.D)

Cobalt transport in the filamentous form of *N. crassa* was shown to be by an energy-dependent mechanism sensitive to respiratory inhibitors (Venkateswerlu & Sastry 1979). However in the first cobalt-resistant isolate of *N. crassa* was insensitive to metabolic inhibitors (Venkateswerlu & Sastry 1979).

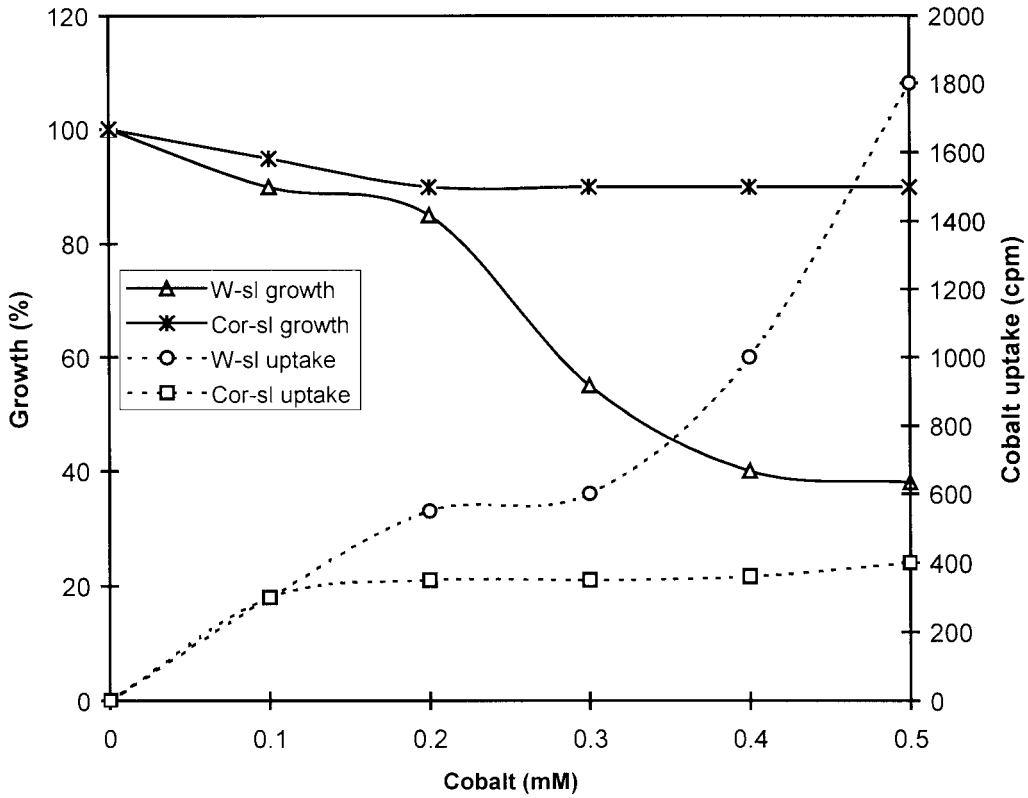

Fig. 2. Cobalt toxicity and uptake in *N. crassa* W-sl and Cor-sl. Slime cells (10^8) were grown in the indicated concentrations of cobalt (^{58}Co = 500 mCi/mole) for 24 h at 28 ± 1 °C. Number of cells was recorded as measurement of growth and cobalt uptake was determined using liquid scintillation counter.

Hence the same was examined in the wall-less mutants. In W-sl and Cor-sl 80–90% inhibition of cobalt uptake was observed in the presence of metabolic inhibitor sodium azide, indicating that cobalt transport is energy- dependent in both the mutants (Figure 5). Magnesium is known to suppress cobalt uptake severely in the filamentous *N. crassa* and hence it was presumed to be by magnesium transport pathway. The same was investigated in slime mutants. Cobalt uptake in presence of equimolar concentration of magnesium (0.1mM each), was found to be decreased by 50% in W-sl and 26.8% in case of Cor-sl strain (Table 2). To see if the overall lower ability for cobalt by *N. crassa* Cor-sl is due to the release of cobalt already taken up by the cells, efflux of cobalt from slime cells was examined. Around 25 to 45% of the total cobalt taken up by cells was found to be released into the medium (Table 3) in both W-sl and Cor-sl mutants with the later showing relatively higher efflux, in spite of lower accumulation. Though magnesium ions inhibited the overall cobalt uptake, it did not influence the efflux of cobalt in both the strains.

Table 3. Efflux of cobalt in the wall-less mutants of *N. crassa*.

	Cobalt (nmoles)			
	W-sl		Cor-sl	
Mg^{2+}	Intracellular	Effluxed	Intracellular	Effluxed
Nil	2.8 ± 0.3	1.1 ± 0.2	0.7 ± 0.1	0.6 ± 0.1
0.1 mM	1.6 ± 0.2	0.7 ± 0.1	0.5 ± 0.1	0.4 ± 0.1

Slime cells (10^8) were allowed to take up cobalt for 30 min as described in Methods. Cobalt remaining with cells (intracellular) and that released into medium (effluxed) was estimated. Values shown are average of 2 separate experiments (± SD).

Fractionation of cellular cobalt

One of the mechanisms by which cell could evade toxicity due to metal ions is compartmentalization into vacuoles or cellular organelles. Hence the distribution of cobalt in the same was studied comparatively between W-sl and Cor-sl strains. The data presented in Table 4 show that 12% of the total intracellular

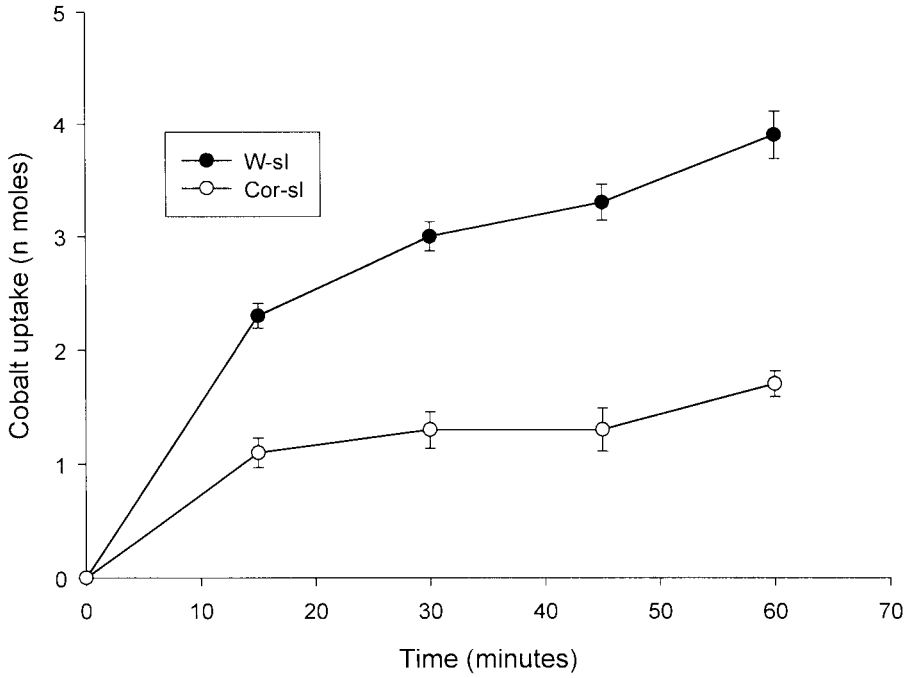

Fig. 3. Time course of cobalt uptake in slime mutant of *N. crassa*. Cells (10^8) were suspended in 10 ml medium containing with cobalt (0.3 mM containing 1 μCi of ^{57}Co) for different time points, washed and the cobalt uptake was measured by γ-counter. Error bars are $\pm$ SD values from 2 separate experiments.

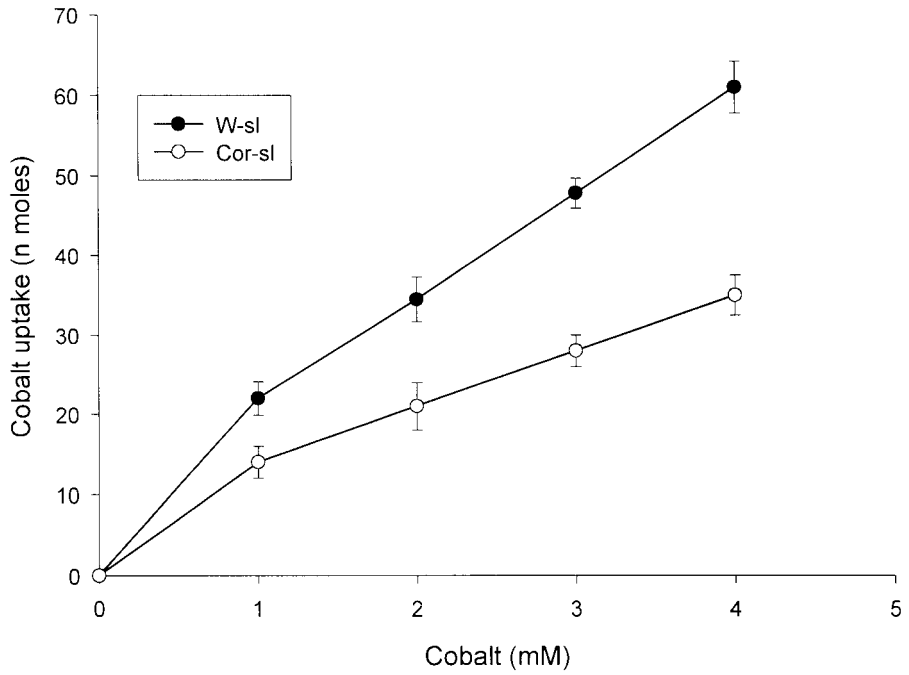

Fig. 4. Cobalt uptake in wall less mutant strains of *N. crassa*. Slime Cells (10^8) were incubated for 60 min at different concentrations of cobalt (^{57}Co = 10 Ci/mole). Mean values ($\pm$ SD) are from 3 separate experiments.

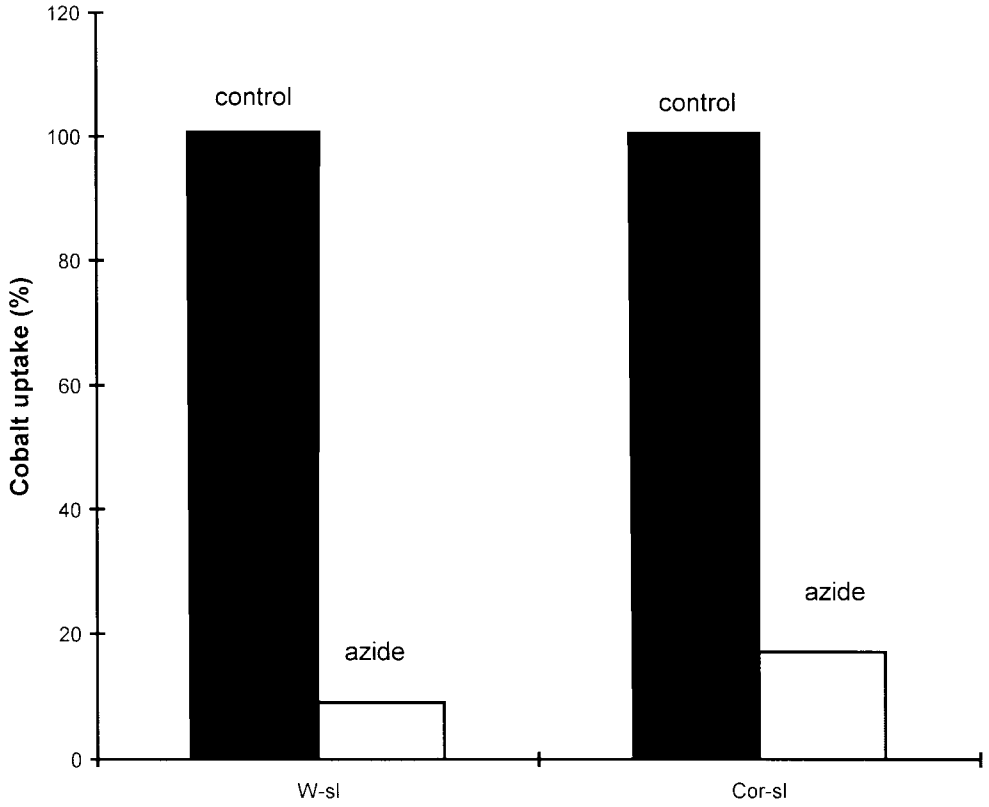

Fig. 5. Effect of metabolic inhibitors (azide) on cobalt uptake. Slime cells (10^8) were allowed to take up cobalt ($^{57}Co = 10$ Ci/mole) as in Figure 3 (with and without azide) for 60 min, washed and the cobalt taken up was measured by γ-counter. Average values form two seperate experiments are shown.

Table 4. Organellar distribution of cobalt in wall-less mutants of *N. crassa*.

	Cobalt (cpm)	
	W-sl	Cor-sl
Cell free extracts	4.5×10^5 (100%)	4.0×10^5 (100%)
Nuclear fraction	5.8×10^4 (12%)	4.3×10^4 (11%)
Mitochondrial fraction	2.3×10^4 (5%)	6.6×10^3 (2%)
Post mitochondrial fraction	3.8×10^5 (69%)	2.5×10^5 (65%)

Slime cells were grown in 50 ml basal medium containing cobalt (0.3 mM containing 10 μCi of ^{57}Co) for 24 h. The cells were washed and fractionated as described in the methods.

Table 5. DEAE-Cellulose column chromatography of cell-free extracts.

	Cobalt (cpm)	
	W-sl	Cor-sl
Protein loaded	27,457 (100%)	30,017 (100%)
Flowthrough	22,243 (81%)	6,480 (21.5%)
Protein eluted with NaCl	3,350 (19%)	26,759 (78.5%)

Slime cells were grown in the presence of cobalt (0.3 mM containing 5 μCi of ^{57}Co) in 10 ml basal medium for 24 h, washed and homogenised with Tris-Cl buffer pH 6.5. Protein (2 mg) was loaded on to DEAE-Cellulose column equilibrated with Tris-Cl buffer pH 6.5. Protein bound to the column was eluted with 0.3 M NaCl in the same buffer. Cobalt (^{57}Co) of cell-free extract, flowthrough and eluted fraction was estimated in γ-counter.

cobalt was found to be in the nuclear pellet, whereas 5% was found to be in mitochondrial pellet in W-sl. In Cor-sl 11% was found in the nuclear pellet and 2% in the mitochondrial fraction. Majority of cobalt (70%) was found in the cytosolic fraction in both the strains (Table 4). Since no major difference was noted in the above data, further studies were undertaken to look for any cobalt binding proteins in cell-free extracts. Fractionation of cobalt of cell-free extracts of Cor-sl by DEAE-Cellulose column chromatography showed majority of cobalt (70%) was found to be in the protein bound fraction eluted with 0.3M NaCl in Tris buffer (pH 6.5). In W-sl only 30% of the

total cobalt was found to be in protein bound fraction and 70% in the flow through fraction (Table 5). The protein bound cobalt fraction was further purified by Sephadex G-50 gel filtration Chromatography. A single cobaltoprotein was purified, which constitutes about 18% of the total protein of cell-free extracts. It was found to be similar to the cobaltoprotein (CBP) purified from the filamentous cobalt-resistant strain of *N. crassa* (Sajani and Maruthi Mohan 1998) as indicated by the cobalt/protein ratio and characteristic absorption spectrum.

Discussion

Fungi in general are known to develop resistance for a variety of toxic metal ions (Ashida 1965). The methodology employed to obtain resistant strains of fungi, organisms involved and the metal ion appears to have a role in determining the type of resistant mechanisms elaborated. Cobalt-resistant strains of *N. crassa*, which have been obtained by adaptation to 4–16 mM cobalt, resulted in a 10-fold resistant strain than the sensitive wild type, while adaptation to 32–50 mM cobalt resulted in 20-fold resistant strain (Sajani & Maruthi Mohan 1997). Further the above cobalt-resistant strains were found to be non-identical to the first cobalt-resistant isolate, which was characterized by similar adaptive procedures (Venkateswerlu & Sastry 1973). In the present study using the wall-less mutant a 10-fold resistant *N. crassa* Cor-sl was obtained by the adaptation to 10 mM cobalt while adaptation to lower concentrations (2–4 mM) either showed no resistance or a mere 2-fold resistance was observed. Hence metal resistance appears to be a complex phenomenon, which needs thorough investigation in order to define the fundamental nature of events responsible for particular characteristic metal-resistant strains.

Metal resistant strains of fungi and *N. crassa* in particular are known to be cross-resistant to related toxic metal ions. Cobalt and nickel resistant strains of *N. crassa* characterised so far are not cross-resistant to copper. The present isolate (Cor-sl) is not only cross-resistant to nickel (3-fold) but surprisingly cross-resistant to copper (10-fold). This is for the first time such an observation is made amongst metal-resistant *N. crassa*.

Studies so far reported on metal resistance in yeast and fungi have come out with two broad mechanisms by which resistant strains are able to avoid toxicity i) transport block ii) intracellular detoxification by binding proteins (Mehra & Winge 1991). In most cases generally any one mechanism is supposed to be responsible for resistance. Recent work from this laboratory on isolation and characterization of a cobalt-resistant strain of *N. crassa* cor was the first report to show that both the mechanisms are elaborated in the same mutant, which makes this highly resistant to cobalt. The transport block interestingly was demonstrated to be due to a decrease in cobalt binding to cell wall fraction (Sajani & Maruthi Mohan 1997), while the intracellular mechanism involves overproduction of a cobaltoprotein (CBP), which sequesters majority of the intracellular cobalt (Sajani & Maruthi Mohan 1998). Hence, in the present study using the wall-less mutants of *N. crassa* in which the cell-wall effects can be discounted was employed. As expected the rapid phase of cobalt binding to surface was not observed in the present study with these mutants. The data have been compared to the filamentous wall containing mutants. A transport block was observed in the *N. crassa* Cor-sl, which accumulates relatively less cobalt both during growth and in short, term uptake studies. This transport block resulted in 2–3-fold lower levels of accumulation of the toxic ion. In addition release of cobalt already accumulated by the cells also appear to have a role in maintaining lower levels of intracellular cobalt. This is corroborated by the fact that apart from accumulating lower levels of cobalt a relatively higher percentage of the same are released into growth medium by Cor-sl strain when compared to W-sl. This release of intracellular cobalt was insensitive to both magnesium ions and metabolic poisons. Though similar release of cobalt was observed in the wall containing cobalt-resistant mutant of *N. crassa* the results were not definitive due to the presence of cell-wall which could also release the passively bound cobalt (Sajani & Maruthi Mohan 1997). The above data appears to be logical in the sense that in order to maintain a homeostatic control for the normally required metal ions like Mg such systems are required. Since, cobalt uptake is sensitive to magnesium ions in both mutants though to a level extent in Cor-sl cobalt ion appear to use the Mg transporter. However, in case of efflux of cobalt, presence of magnesium ions did not influence the release of cobalt suggesting that there might be other independent pathways, which warrants further investigations.

In the first cobalt-resistant strain uptake of cobalt ions was shown to be by passive mechanism insensitive to metabolic poisons. This is in contrast to wild

type wherein it is by an energy-dependent mechanism (Venkateswerlu & Sastry 1970, 1979). Cobalt uptake was sensitive to sodium azide in both the sensitive W-sl and Cor-sl strains in the present study.

Major intracellular mechanisms for metal-resistance, which are more common in the simple eukaryotic yeast and fungal system are elaboration of metal binding proteins especially metallothioneins. In the *N. crassa* cor the filamentous strain, most of the cobalt was shown to be sequestered by a cobaltoprotein (CBP), which has been characterized and found to constitute upto 13% of total protein of cell-free extracts. In the present study lower quantities of cobalt (25–30%) in W-sl and higher levels (70%) in Cor-sl was observed in the cobaltoprotein fraction bound to DEAE-Cellulose like in the filamentous *N. crassa* (Sajani & Maruthi Mohan 1998). However significantly higher quantities of CBP was expressed by the wall-less Cor-sl mutant which accounts up to 18% of the total protein which suggests that this is the major mechanism responsible for intracellular detoxification. Though the Cor-sl is equally cross-resistant to copper, no over expression of CBP was observed suggesting a separate mechanism which is under study. It is noteworthy that very few cobaltoproteins have been described in nature, most of which are enzymes (Kobayashi & Shimizer 1999). The cobaltoprotein (CBP) of *N. crassa* is indeed a novel protein whose role in resistance is described for the first time in all the cobalt-resistant mutants including the present study. One another intracellular detoxification mechanism suggested is concentration of toxic metal ions into vacuoles/organelles. Especially in yeast lithium ions was shown to be concentrated in vacuoles (REF). Quantitation of cobalt in various organellar fractions showed that nuclei and mitochondria accumulate significant quantities of cobalt. Although the above constitute less than 20% of the total cobalt accumulated these could disturb critical cellular functions. Till date such quantitation as described in the present study above was not undertaken in *N. crassa* and fungi in general. This is due to difficulties presented by thick cell-wall which requires harsh conditions of disruption which results in organellar damage. It should be noted that in the sensitive W-sl strain of *N. crassa* 5% of the total cobalt was observed in mitochondrial fraction while in the Cor-sl only 2% was observed. This lower accumulation most likely is due to non-availability of free cobalt ions, which are mostly bound to the cobaltoprotein. This indirectly also would contribute to decrease in possible inhibition of critical mitochondrial functions. A more thorough examination of the above facts is required before any conclusion can be drawn.

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