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Isolation and characterization of cobalt-sensitive mutant of *Neurospora crassa*Krishnapuram Rashmi^{1,Δ}, Jeevigunta Naveena Lavanya Latha^{2*,Δ}, Pamarthi Maruthi Mohan¹¹Department of Biochemistry, Osmania University, Hyderabad-500 007, India²Department of Biotechnology, Krishna University, Machilipatnam-52001, Andhra Pradesh, India

PEER REVIEW

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Comments

This is a valuable research work in which authors have demonstrated the development of a hyper-accumulator strain of *N. crassa* with defined mechanism for its sensitivity. Furthermore, it was also shown to have cross sensitivity to other metal ions which is advantageous for a strain in processing the ability to remove multiple metal types using a single strain.

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ABSTRACT

Objective: To isolate and demonstrate the mechanism of metal transport in cobalt-sensitive mutant (CSM) of *Neurospora crassa* (*N. crassa*).

Methods: Isolation of CSM of *N. crassa*, I_{50} determination, growth measurements, metal ion uptake studies and sexual crosses were performed to determine the mechanism of sensitivity and locus.

Results: CSMs of *N. crassa* were isolated by mutagenesis with diethyl sulfate. More than 500 isolates were screened and out of these isolates, CSM-I was 5-fold and CSM-II was 10-fold sensitive to Co on liquid medium as compared to the wild type. Compositional analysis of cell wall revealed the decrease in total phosphate content. *N. crassa* CSM bound much less cobalt to cell wall fraction than wild type. The data indicated closer linkage between resistance and mating type locus (mat), which is, located on LG I.

Conclusions: A CSM of *N. crassa* is 5-fold more sensitive than wild type and cross sensitive to nickel and copper and hyper-accumulates 2–4 fold more toxic metal ions over wild type. The mechanism for sensitivity is decreased in cobalt-binding to cell wall fraction and increased intracellular uptake. *N. crassa-acm-3* morphologically resembles the CSM, cobalt-sensitive and maps to similar locus.

KEYWORDS

Metal toxicity, Cobalt sensitivity, Metal transport, Cobalt-sensitive mutant, Hyper-accumulation, *Neurospora crassa*

1. Introduction

Microorganisms when exposed to toxic concentrations of metal ions are known to evolve resistant strains. A number of metal-resistant strains of fungi have been isolated, but only in a few cases the mechanism of resistance has been investigated in detail[1–4]. Resistant strains of cobalt (Co), nickel (Ni), zinc (Zn), mercury and cadmium (Cd) have been so far isolated and characterized for bioremediation purposes[4–11]. Continuous exposure of *Neurospora crassa* (*N. crassa*) to Co and Ni ions was shown to result in stable resistant mutants, which does not revert back to sensitivity when cultured on metal-free medium[6,9]. The first Co-

resistance *N. crassa* (*cor*) was shown to be 10-fold more resistant to Co and Ni when compared with the wild type[6]. Co transport, which is energy-dependent in the wild type, was by a passive mode in the resistant strain. A *cor* non-identical to the above described strain was characterized and resistance was mapped to linkage group IIIIR[12]. The primary mechanism for Co-resistance was found to be due to transport block for Co ions[13]. Apart from the decreased uptake, growth in excess Co was shown to result in synthesis of a novel Co binding protein (CBP), which is over produced in *cor* strain accounting for 12% of the total soluble protein. About 80% of the total Co of the soluble fraction was found to be located in the CBP fraction of the resistant strain.

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The same in the case of the wild type was only 25% under comparable conditions^[14]. Co-resistance was also studied in wall-less mutant (*Cor-sl*) of *N. crassa* wherein the effects of cell wall will not influence the metal ion effects^[15]. *Cor-sl* is 10-fold more resistant to Co when compared to wild slime, and cross-resistant to copper (Cu) (10-fold) and Ni (3-fold). Like in case of wild type filamentous *N. crassa*, CBP was shown to be overproduced and responsible for Co-resistance. Three Ni-resistant strains of *N. crassa* designated NiR1, NiR2 and NiR3 were isolated by gradual adaptation of the parent wild type *N. crassa* on agar media containing different concentrations of Ni^[8]. All three strains were 4-fold resistant to Ni while they differed in their cross-resistance to Co and Cu. Three non-identical Zn-resistant strains of *N. crassa* have also been isolated and characterized^[9]. Amongst microorganisms very little is known regarding metal-sensitivity. In comparison to the number of resistant fungi characterized only a few reports are there regarding metal-sensitivity. Calcium (Ca)-sensitive mutants (*cls7-cls11*) of *Saccharomyces cerevisiae* (*S. cerevisiae*) showing pet phenotype with lesions in a system for maintaining intracellular Ca homeostasis has been reported. Biochemical studies have demonstrated that these mutations are related to defects in vacuolar membrane H⁺ATPase^[16]. Ca-sensitive (*cls4*) mutant of *S. cerevisiae* ceased dividing in the presence of 100 mmol/L of CaCl₂, producing large, unbudded cells. While its DNA replication and nuclear division continued after interruption of normal budding, the *cls4* mutant exhibited defect in bud formation in Ca rich medium. The Ca content and uptake activity were same as that of the wild type strain suggesting the primary defect of the mutation was not in Ca transport system^[16]. Cd-sensitive mutants of *Arabidopsis thaliana* were deficient in forming Cd-peptide complexes^[17]. The work presented herein aimed to describe the isolation and characterization of CSM of *N. crassa*.

2. Materials and methods

2.1. Isolation of CSM of *N. crassa*

CSM of *N. crassa* was isolated by filtration enrichment method by chemical mutagenesis. Conidiospores (10⁸/mL) were inoculated in 10 mL basal medium and allowed for germination for 2 h at 100 r/min. Germinating conidiospores were treated with 0.3% diethyl sulfate (DES) and incubated for 16 h at (28±1) °C in a rotary incubator shaker (100 r/min). The mutagenised conidia were washed twice with sterile basal medium (centrifugation 5000 r/min for 5 min) to remove DES^[9]. The pellet was then suspended in 10 mL of 0.1 mmol/L Co containing basal medium. After 48 h of incubation, the culture was filtered through glass wool and

the filtrate was centrifuged (5000 r/min for 5 min). The spore suspension was placed on agar medium without Co. Single colonies were picked up and transferred to basal medium slants. Initially, the isolates were tested for their Co-sensitivity on the agar medium containing 0.1 mmol/L Co. The isolates, which failed to show growth after 24 h were further, tested for Co-sensitivity on liquid medium in comparison with wild type *N. crassa*.

2.2. Fifty percent growth inhibitory concentration (*I*₅₀) determination

Conidiospores of *N. crassa* were inoculated into 10 mL basal medium containing metal ion and incubated at (28±1) °C for 3 d. The mycelia were harvested and washed thrice with glass-distilled water, pressed free of excess moisture with filter paper and dried over night in hot-air oven at 60–80 °C. Growth was quantitated by measuring dry weight of the mycelia. *I*₅₀ value for metal ion was derived from graphical plots of growth verses metal ion concentration.

2.3. Growth measurement

N. crassa wild and CSM were inoculated on agar medium containing sorbose and different concentrations of Co *i.e.* nil, 0.1, 0.25, 0.5 and 1.0 mmol/L in Petri plates. Colony diameter was measured in cm at 24 h, intervals for 3 d. Hyphal growth of *N. crassa* wild and CSM in growth tubes was measured (cm) as described by Ryan *et al*^[18]. Conidiospores were inoculated on agar medium containing nil, 0.1, 0.25, 0.5 and 1.0 mmol/L Co in Ryan's growth tubes (25 cm×1 cm). Hyphal elongation was measured at 24 h, intervals for 3 d.

2.4. Metal uptake

Metal ion uptake was studied by culturing of *N. crassa* wild and CSM in the basal medium containing different concentrations of metal ion (during growth) or suspending pre grown mycelial mats (3 d) in 10 mL basal medium containing 5 mmol/L metal ion as in earlier studies^[8]. Metal taken up by mycelia was quantitated, following acid digestion by atomic absorption spectrophotometry (AAS) using a Perkin Elmer 2380 spectrophotometer^[8]. To determine the distribution of metal ion *i.e.* bound to the cell surface and that taken up by the mycelia (intracellular), mycelia were washed thoroughly and suspended in 10 mL of ethylene diamine tetraacetic acid (EDTA) (10 mmol/L, pH 7), for 3 min to strip surface bound metal ion. Mycelia were then removed, washed, dried and weighed. The metal ion content in the EDTA extract and that remaining with the mycelia was estimated, after acid digestion, by AAS. Metal content was determined after subjecting to wet acid digestion as described earlier^[8].

Mycelia (30–50 mg dry weight) were digested, in 50 mL conical flasks with 5 mL of concentrated nitric acid and 1 mL of 70% perchloric acid, slowly to dryness on a sand bath. The residue was further digested with a 1:1 mixture of nitric acid and hydrochloric acid (2 mL) and finally with 1 mL of HCl. The final residue was dissolved in a suitable volume of millipore water and metal ions estimated by AAS.

2.5. Diethyl-aminoethanol-cellulose (DEAE-cellulose) chromatography of cell-free extracts

The method reported earlier for fractionation of Co in cell-free extracts^[14]. *N. crassa* wild and CSM were cultured 10 mL basal medium containing 0.1 mmol/L of Co in 50 mL conical flasks for 72 h at (28±1) °C. The mycelia were then harvested, washed and homogenized with an equal weight of acid-washed sand in 10 volumes (w/v) of Tris buffer (50 mmol/L, pH 6.5). In each case, the homogenate was centrifuged for 5 min at 600 *g* to remove sand and debris and the supernatant was centrifuged at 15000 *r/min* for 30 min. To determine the concentration of free ionic and protein-bound Co in cell-free extracts, an aliquot (equivalent to 100 µg Co) was loaded on a DEAE-cellulose column (5 mL bed volume) pre-equilibrated with 50 mmol/L Tris buffer, pH 6.5. The DEAE-cellulose column was washed with three bed volumes of equilibration buffer and bound proteins were eluted with a linear gradient of NaCl (0–1 mol/L) in the same buffer at a flow rate of 1 mL/min. Fractions (1 mL) were collected and monitored for protein at 280 nm and Co was estimated using AAS.

2.6. Cell wall isolation

The method of Schmit *et al.* was used for cell wall preparation^[19]. Mycelia of *N. crassa* wild and CSM (10 g) was homogenized using mortar/pestle by gradual addition of sodium dodecyl sulfonate (20 mL) and stirred for 4 h and then incubated for 16 h at room temperature. The homogenate was washed several times with double distilled water to remove sodium dodecyl sulfonate and treated sequentially with chloroform: methanol (2:1) followed by drying at room temperature. Dried cell wall preparations was ground to powder, weighed and used as suspension.

2.7. Phosphate estimation

Phosphate in the cell walls was estimated by modified Fiske-Subba Row method^[20]. *N. crassa* wild and CSM cell walls (20 mg) were taken and heated with 2 mL of 5 mol/L H₂SO₄. To the phosphorus containing solution, 4.1 mL of millipore water, 0.5 mL of 5 mol/L H₂SO₄, 0.2 mL of 5% ammonium molybdate and 0.2 mL of Fiske-Subba

Row (0.25% of 1-amino-2-naphthol-4-sulfonic acid, 15% of sodium bisulfite and 0.5% of sodium sulfite) reagent were added in succession with mixing. The solution was heated for 7 min at 100 °C and the color was read at 830 nm.

2.8. Sexual crosses in *N. crassa*

N. crassa FGSC 2489 mating type A was grown in Petri plates containing Westergaard Mitchell crossing medium adjusted to pH 6.0. The conidial suspension of CSM (mating type a) was spread on top and further incubated at 25 °C. After 3–4 weeks, the plates were exposed to light to release ascospores and collected the ascospores in distilled water and heat shocked at 70 °C for 30 min to kill the vegetative population and to initiate germination of ascospores. The ascospores were then plated in basal medium containing 1.5% agar and 1% sorbose to obtain individual colonies. Single colony isolates were tested for Co-sensitivity on agar plates containing 0.1 mmol/L Co, which permits wild type growth, but not CSM upto 1 d. To determine the loci for Co-sensitivity, *N. crassa* CSM was crossed with a tester *alcoy* strain which contains three unlinked reciprocal translocations with conveniently visible markers linked to groups I through VI. The genotype of *alcoy* is *al* T (IR; IIR); *cot* (IIIR; VI); *ylo* (IIIR; VI)^[21]. The ascospores were collected and heat shocked at 70 °C for 45 min and plated on agar medium containing 0.2% sucrose and 1% sorbose to obtain colonial growth. Single colonies were transferred onto agar slants and incubated initially at 34 °C for 24 h. Under these conditions the *cot* (colonial temperature sensitive) spores showed colonial growth. These were marked and numbered. The culture tubes were then incubated at 25 °C for 2–3 d and scored for albino, (white) and conidial separation (on inverting and tapping the culture tube, the conidiospores get detached) and among the non-albino for yellow. All the progeny were for Co-sensitivity both on solid and liquid medium.

3. Results

3.1. Isolation of Co-sensitive strains

Germinating conidiospores of *N. crassa* subjected to chemical mutagenesis with diethyl sulfate were screened for CSMs by filtration enrichment procedure. More than 500 isolates were screened on the agar medium, and 13, which displayed relative Co-sensitivity as compared to wild type, were further screened on the liquid medium. The obtained I₅₀ values indicate variable values for Co-sensitivity. Among these isolates, two of them (C3 and C9), which showed Co-sensitivity on both solid as well as liquid medium, were chosen for further study. CSM-I was 5-fold sensitive and CSM-

II was 10-fold sensitive to Co on liquid medium as compared to the wild type. Control weights of mycelia (without Co) were much lower (50%–80%) than wild type in CSM-I (Figure 1). CSM-II showed very poor growth characteristics and hence CSM-I was chosen for further study.

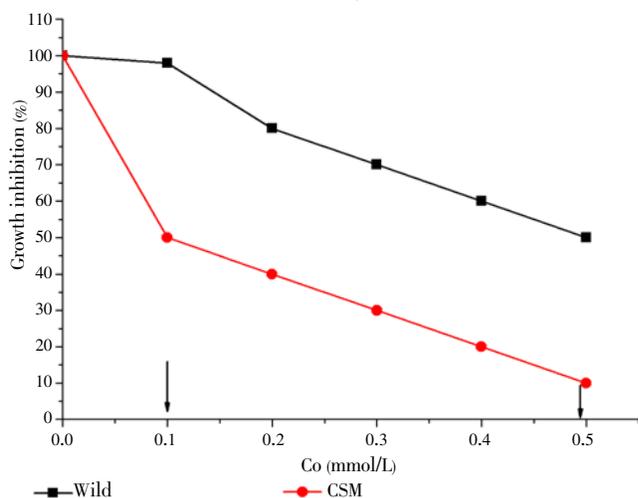


Figure 1. Record of dry weights of *N. crassa* strains grown in different concentrations of cobalt for 3 d.

A pure culture of CSM-I was obtained by repeated plating

on sorbose medium and single colony isolation. One of the pure Co-sensitive cultures, exhibiting good growth, was selected for further characterization and referred to hereafter as CSM. *N. crassa* CSM was crossed with wild type (mating type A) and progeny showing good growth and Co-sensitivity (mating type a) was selected for further detailed study.

3.2. Measurement of growth by colony diameter and hyphal elongation

Growth of wild type *N. crassa* and CSM were evaluated by measurement of colony diameter on sorbose agar medium plates and hyphal elongation in Ryan’s growth tubes. The results obtained clearly distinguish *N. crassa* wild and CSM. While CSM failed to grow on 0.1 mmol/L, 0.25 mmol/L Co containing sorbose-agar medium, the wild type *N. crassa* showed excellent growth on Day 1. However, CSM managed to grow by Day 2 and Day 3 and a 2–3 fold growth inhibition due to Co was observed in case of CSM when compared to wild type (Figures 2 and 3). On Ryan’s race tubes hyphal elongation of both wild and CSM showed similar growth at 1 d in Co medium upto 0.25 mmol/L. At higher concentration

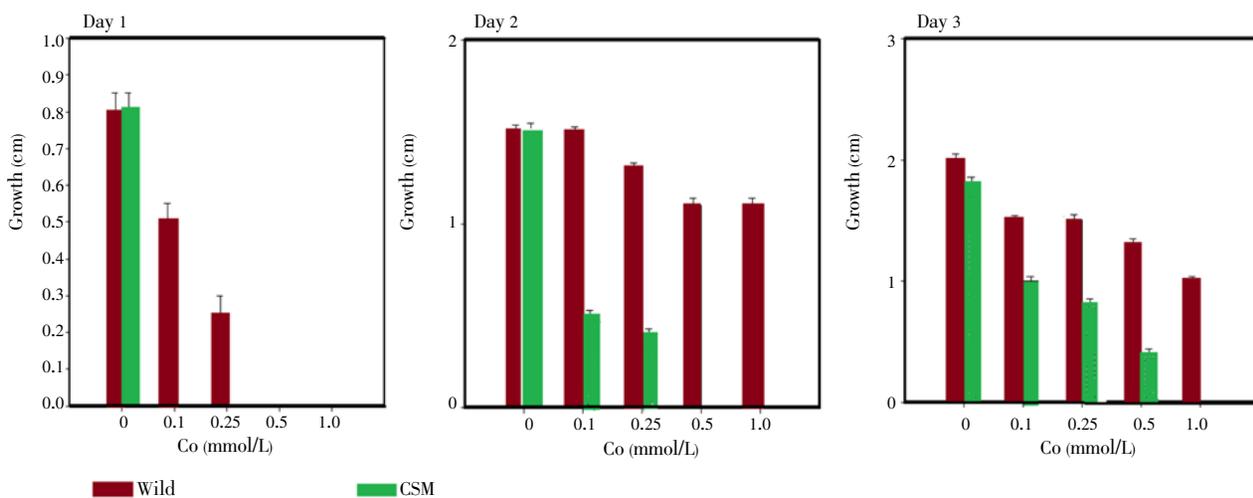


Figure 2. Colony diameter (cm) of *N. crassa* wild and CSM inoculation in petri plates for 3 d in different concentrations at 24 h intervals.

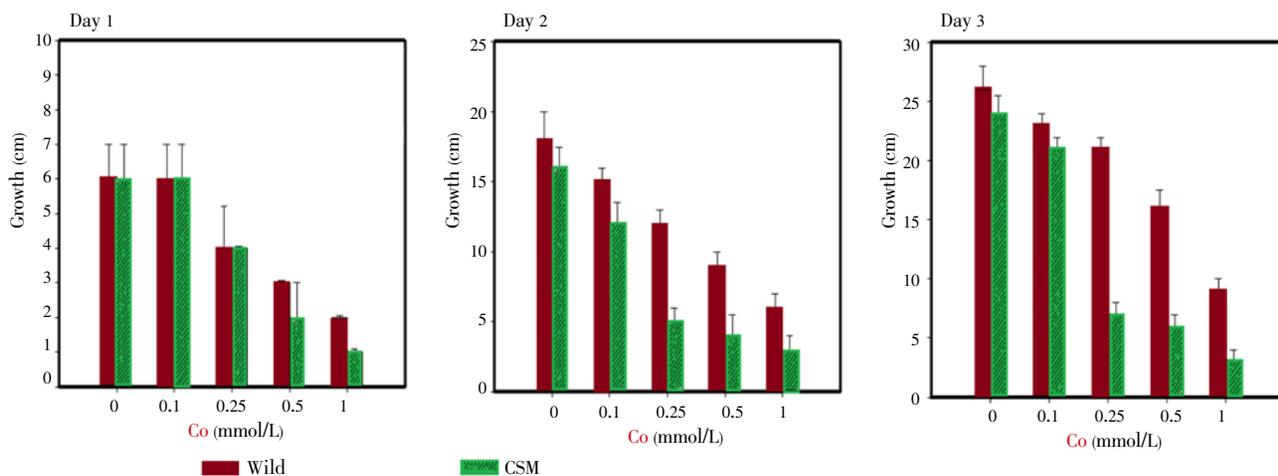


Figure 3. Hyphal elongation of *N. crassa* wild and CSM inoculation in petri plates for 3 d in different concentrations in Ryan’s growth tubes at 24 h intervals.

(0.5 mmol/L and 1.0 mmol/L) 2–fold growth inhibition of hyphal elongation was observed in CSM when compared to wild type. CSM exhibited a 2–3 fold growth inhibition on 0.25, 0.5 and 1.0 mmol/L Co containing basal medium than wild type at Day 2 and Day 3 periods. It may be noted that the growth in controls (without Co) as 10%–12% less in case of CSM as compared to wild type *N. crassa*.

3.3. Co uptake

Co taken up by mycelia and distribution between cell wall and intracellular fractions was determined in wild type and CSM *N. crassa* grown in presence of Co (50 µmol/L). Most of the Co (68%) was intracellular and relatively smaller fraction was observed in cell wall fraction (32%). In case of wild type *N. crassa* most of the Co was located in cell wall bound fraction (72%) and the remaining (28%) was intracellular at 0.05 mmol/L Co (Table 1). The same pattern of uptake was also observed at 0.1, 0.25 mmol/L Co concentration tested. At higher concentrations like 0.5 and 1.0 mmol/L only wild type showed growth but CSM does not grow at these concentrations.

Table 1

Cobalt uptake and distribution. *n* (%).

<i>N. crassa</i> strains	Total	Cell wall	Intracellular
Wild	22 (100)	16 (72)	6 (28)
CSM	86 (100)	28 (32)	58 (68)

Short-term Co uptake and distribution between cell wall and intracellular fractions were studied using mycelial mats of wild type and CSM *N. crassa*. In wild type *N. crassa* Co uptake in cell wall fraction increases with concentration and a 2–fold increase is observed at 5 mmol/L as compared to CSM at 5 min while at 2 h there is 4–fold increase. Change in Co binding to cell wall fraction between 5 to 120 min in presence of 0.25 mmol/L Co, there is a significant increase at higher concentration with increase in the period of incubation. Under similar experimental conditions for CSM strain there were no major differences in Co uptake in cell wall fraction either with time or Co concentration (Figures 4a and 4b). Co accumulation into intracellular fractions showed significant uptake was observed only at 30 min above 0.25 mmol/L Co concentration, which increased rapidly

thereafter. A similar pattern of results was observed in both *N. crassa* wild and CSM strain. However, overall uptake of Co in case of CSM is distinctively higher (2–3 fold) than wild type *N. crassa* (Figures 4c and 4d).

3.4. Cross-sensitivity

Since metal-resistant strains of fungi generally exhibit cross-resistance to other related toxic ions. This aspect towards Ni, Cu, Zn and Cd was examined in CSM strain. Obtained results showed that the CSM strain was about 2–fold more sensitive to Ni and 2–fold more sensitive to Cu but not sensitive to Zn and Cd as compared with the wild type *N. crassa* (Table 2). Once again, CSM accumulates 2–4 fold less Ni in the cell wall fraction, in comparison with the wild type, but accumulated 4–fold higher concentration in the intracellular fraction, as in case of Co. While CSM accumulated 2–fold more Cu into the intracellular fraction and 2–fold less Cu in the cell wall fraction (Table 3). The overall observation in case of CSM was that there was a decrease in metal ion binding to cell wall fraction and a concomitant increase in intracellular concentration in comparison to wild type *N. crassa*.

Table 2

I₅₀ (mmol/L) of different metal ions.

Metal ions	Wild	CSM
Ni	0.40±0.10	0.20±0.10
Cu	1.20±0.10	0.45±0.05
Zn	1.26±0.10	1.26±0.10
Cd	0.05±0.02	0.05±0.03

Table 3

Metal uptake and distribution.

<i>N. crassa</i> strains	Ni (µg/100 mg)		Cu (µg/100 mg)	
	Cell wall	Intracellular	Cell wall	Intracellular
Wild	170	60	186	65
CSM	35	210	52	106

3.5. Fractionation of Co in soluble mycelial extracts

A CBP, which was overproduced, and bound most of intracellular Co in *cor* strain was earlier shown to be involved in resistance^[14]. The same in case of wild type was produced in relatively small quantities and bound around 25% of intracellular Co. In order to see whether the CBP levels

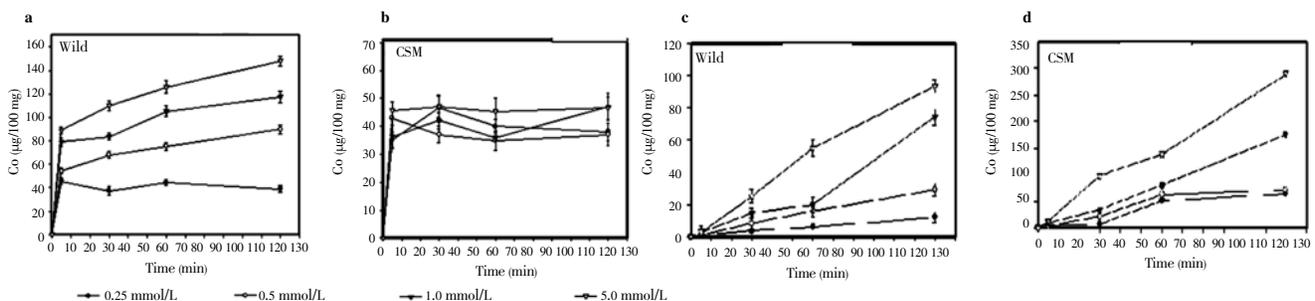


Figure 4. Cobalt uptake in cell–wall fraction (a & b) and accumulation into intracellular fraction (c & d).

responsible for Co-sensitivity are the same was studied in the CSM. DEAE-cellulose chromatography of cell-free extracts of the wild type and CSM grown at their respective I_{50} Co concentration (0.1 and 0.5 mmol/L) indicated that in CSM most of the Co (80%) was found in the flow-through fraction and the rest (20%) in the DEAE-cellulose bound CBP fraction. While in case of wild type 28% of Co was observed in CBP fraction (Table 4).

Table 4

DEAE-cellulose chromatography of cobalt extracts.

Fractions	Co (%)	
	0.1 mmol/L	0.5 mmol/L
Total loaded	100	100
CBP bound	28	20
Flow-through	72	80

Datas are shown in average value of three experiments with duplicates.

3.6. Co binding to isolated cell wall preparations

In live mycelia CSM bound 2–4 fold less Co to the cell wall fraction, in comparison with the wild type *N. crassa*, but accumulated 4-fold higher concentration into the intracellular fraction. Isolated cell wall preparations were used to study Co binding in *N. crassa* strains. Interestingly, cell wall preparation of CSM [2.3 ± 0.2 $\mu\text{mol}/100$ mg] showed 2-fold lesser Co binding when compared to wild type *N. crassa* [0.9 ± 0.1 $\mu\text{mol}/100$ mg]. Compositional analysis showed similar protein content, however, 2-fold lower levels of phosphate [6.0 ± 0.25 $\mu\text{mol}/100$ mg] were observed both to CSM and wild type. Comparison of the infrared radiation spectra of wild and CSM cell wall preparation from mycelia was made. There were notable changes in the spectrum of CSM in the region when compared to wild type between 1000 – 400 cm^{-1} , which can be assigned for C–N and C–O stretching frequencies.

3.7. Mapping of Co-sensitive locus

N. crassa CSM (mating type a) characterized was from a genetically pure culture, which was obtained following a back cross with parental mating type A. Progeny obtained from a cross between wild type (mating type A) and CSM strain (mating type a) were analyzed on agar plates containing 0.1 mmol/L of Co. Of the 68 progeny tested 23.5% (16 isolates) were Co-sensitive and the rest were like the wild type (Figure 5). All the 16 isolates, which were sensitive on solid medium, were tested for Co-sensitivity on liquid medium. Among these 7 progeny were sensitive in liquid medium with an of Co (0.1 mmol/L) similar to CSM, while the rest of them exhibited I_{50} in between 0.2–0.5 mmol/L. In order to determine to which mating type they belong, the Co-sensitive progeny were crossed with two opposite mating types (A or a) of wild type. The results obtained in Figure 5 shows that 63% of mating A and the remaining were of mating type a.

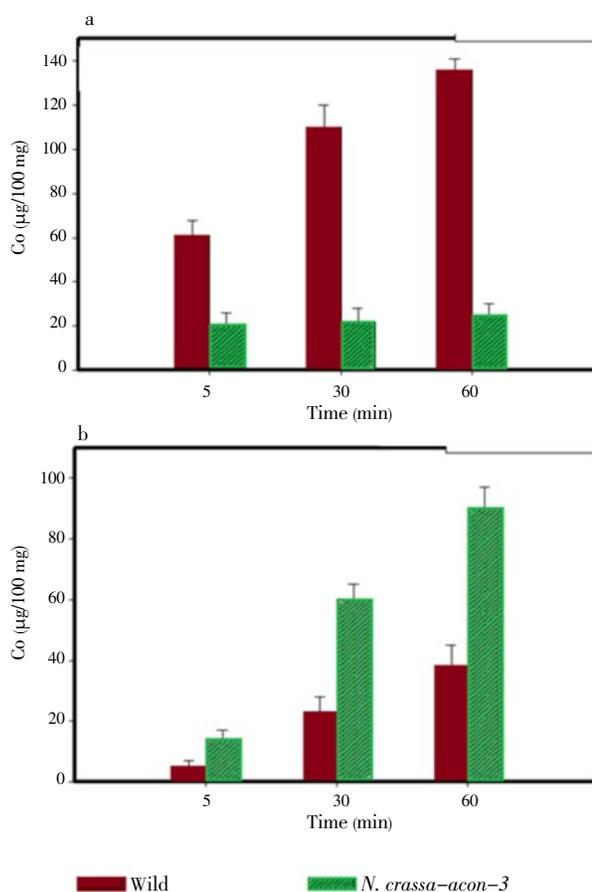


Figure 5. Cobalt uptake in aconidiation mutant of *N. crassa*. a: Cell wall fraction; b: Intracellular fraction.

To determine Co-sensitive locus, *N. crassa* CSM was crossed with a tester *alcoy* strain, which contained three unlinked reciprocal translocations with conveniently visible markers linked to groups I through VI. The genotype of *alcoy* is *al T* (IR; IIR); *cot* (IIIR; VI); *ylo* (IIIR; VI). The data obtained shows that 50 progeny tested (18%) were found to be Co-sensitive and the rest behaved like wild type. Among these, 9 isolates were sensitive on solid medium. Out of these 9 isolates, 3 were sensitive on liquid medium (*cot* type). The least recombination was noticed with *cot* indicating linkage to IV or V.

3.8. Co-toxicity in aconidiation mutant of *N. crassa-acon-3*

N. crassa CSM resembles the aconidiation mutant (*acon*) morphologically and also does not produce visible conidiospores, the same was used to check for Co-toxicity. The results showed that I_{50} of *N. crassa-acon-3* mutant [0.1 ± 0.03 mmol/L] was 5-fold sensitive to Co as in CSM [0.1 ± 0.02 mmol/L] when compared to wild type [0.5 ± 0.1 mmol/L]. Short-term Co uptake and distribution of Co in *N. crassa-acon-3* was studied. Mycelial mats of *N. crassa* wild and *N. crassa-acon-3* (3 d) were floated in medium containing Co (1 and 5 mmol/L) for 5, 30, 60 and 120 min respectively. *N. crassa-acon-3* accumulated 3–6 fold less Co at the cell surface, in comparison with the wild type *N. crassa*, but accumulated 3-fold higher concentration

into the intracellular fraction. Two aconidiation mutants *N. crassa-acon-2* and *N. crassa-acon-3* were used and only *N. crassa-acon-3* showed distinctively Co-sensitivity with an I_{50} of around 0.1 mmol/L, similar to CSM.

4. Discussion

Major metabolic pathways of carbohydrate, nitrogen and amino acids are the primary sites for the action of toxic metal ions. Continuous exposure to toxic metal ions was shown to result in resistant strains[6,8,14]. Resistant strains for Co, Ni and Zn have been characterized and resistant loci were also mapped[12,14]. Transport block at the level of metal ion uptake and intracellular detoxification mechanisms were shown to be involved in metal-resistance. In comparison to the number of resistant fungi characterized only a few reports are there regarding metal-sensitivity. Ca-sensitive mutants (*cls7-cls11*) of *S. cerevisiae* showing pet phenotype with lesions in a system for maintaining intracellular Ca homeostasis has been reported. Biochemical studies have demonstrated that these mutations are related to defects in vacuolar membrane H^+ ATPase[16]. Ca-sensitive mutant (*cls4*) of *S. cerevisiae* ceased dividing in the presence of 100 mmol/L of Ca chloride, producing large, unbounded cells. While its DNA replication and nuclear division continued after interruption of normal budding, the *cls4* mutant exhibited defect in bud formation in Ca rich medium. The Ca content and uptake activity were the same as that of the wild type strain suggesting the primary defect of the mutation was not in Ca transport system[16]. Cd-sensitive mutants of *Arabidopsis thaliana* were deficient in forming Cd-peptide complexes[17]. In another report by Gardarin *et al.*[22], it was shown that the increased Cd was targeted towards endoplasmic reticulum. Amongst microorganisms, very little is known regarding metal-sensitivity. Such a property of organisms to remove toxic metal ions from very low and heterogeneous solutions can have a specific application in bioremediation. The present study involves the development of a Co hyper-accumulator strain, mutants of *N. crassa*.

CSMs of *N. crassa* were isolated by filtration enrichment technique following mutagenesis with DES. More than 500 isolates were screened on the solid medium of which only 13 isolates showed Co-sensitivity on solid medium. Further screening on the liquid medium resulted in only 2 mutants and a large background of wild type were observed. Out of these isolates, only two were found to be Co-sensitive on liquid medium. CSM-I was 5-fold sensitive and CSM-II was 10-fold sensitive to Co on liquid medium compared to the wild type. Growth response on Co media by colony diameter, hyphal elongation and liquid medium clearly showed that the isolate was distinct from wild type and showed clear Co-sensitivity. The apparent large background of wild type shows that it is due to the limitation in the technique employed. Another interesting feature to be noted is that many isolates, which showed Co-sensitivity on agar medium, were not sensitive in liquid medium. This observation suggests that

these could be non-identical mutants and the method for selection of mutants is an important criterion. However, a mutant which is sensitive both on agar and liquid media is selected for detailed study.

Divalent cations interact with the cell walls in two distinct ways as characterized by Fuhrmann and Rothstein in yeast cells[23]. Co uptake in *N. crassa* was shown to have an initial rapid binding to mycelial cell wall which is completed in 10 min followed by a slower transport into the intracellular space[24]. Most of the studies in general have not given importance to the distribution of the toxic metal ions on cell wall and intracellular fractions. The present study highlights the importance of this aspect. It is the intracellularly accumulated toxic metal ion, which determines toxicity, while cell wall fraction serves mostly to restrict metal ion entry. In this context, greater binding of Co ions to cell wall was shown to be one of the mechanisms for resistance in *cor* strain[24]. While in the present study, decrease in Co binding is observed in CSM, which is likely to contribute to overall sensitivity. In addition, hyper-accumulation of 2–4 fold excess of toxic metal ions (Co and Ni) is also observed in intracellular fraction, which is the major reason of the observed sensitivity of the isolate. Both in growth and short-term Co uptake experiments, hyper-accumulation of toxic metal ions were observed in CSM as compared to wild type *N. crassa*. Even the isolated cell wall preparation bound distinctively less Co in case of CSM *N. crassa* as compared to wild type. Compositional analysis of cell wall revealed decrease in total phosphate content, which could contribute to the observed features.

The importance of cell wall binding in determining sensitivity and resistance appear logical from the present study using both the CSM *N. crassa* and *cor* mutants. Another interesting aspect observed in the present study is that inspite of higher levels of Co binding observed in *cor* strain, 70% of cell wall bound Co was released into the medium in 10 min, which in wild type, it was 50% and in CSM it was only 33%. This observation suggests that the cell wall could act as a biosensor for distinguishing toxic and essential elements. In a recent study, *N. crassa* wall-bound toxic metal ions are not to be taken up into intracellular fraction. In fact, those which escape being trapped on cell wall, were shown to be accumulated[24,25]. In this present study, *N. crassa* CSM binds much less Co to cell wall fraction than wild type. The same was also observed in isolated cell wall preparations. Also compositional analysis showed more than 2-fold decrease in total phosphate content in cell wall preparation of CSM, which could be a potential Co-binding group. Taken together a decreased binding of cell wall fraction could lead to increased access to metal transporters of membrane fraction leading to enhanced intracellular accumulation. Changes in cell wall composition of *N. crassa* were earlier shown in Cu toxicity in which chitosan, phosphate and amino groups were increased. Further cell wall preparation from Cu toxic *N. crassa* accumulated 12% Cu by weight, which accounts for 90% of the total mycelial Cu. On the other hand hyper-accumulation also could be due to increased activity of membrane transporters, which needs more in depth study. At present about 26 putative

divalent cation transporters are identified from *N. crassa* genome sequence[26]. Putative Ni–Co transporters, apart from magnesium transporters are present. Earlier studies have implicated Co transport via magnesium transport pathway based on its antagonistic behavior, while in the present study, magnesium ions are unable to reverse toxicity of Co like in wild type *N. crassa*. This suggests that magnesium transporter might not be involved in enhanced accumulation of Co in CSM. Further studies will be needed to understand the role of other putative metal ion transporters. It is a well-known fact that toxicity of metal ions is due to the free ionic forms, which can interact with the toxic site(s), while the protein bound, or complex form of the metal is unable to interact with the same. The CBP is overproduced in *cor* strain in which about 80% of the total Co of intracellular fraction was found to be fraction of the resistant strain[14]. A marginal decrease in levels of CBP (8%) was observed in cobaltoprotein levels were observed in CSM, as compared to wild type. The difference is more likely within the experimental error cannot be the major reason for sensitivity.

A number of metal-resistant strains of fungi have been isolated and several of their biochemical characteristics have been determined. However, only in few cases metal-resistant loci have been mapped. In *N. crassa* genetic loci for metal-resistance to Co, Cd, Zn, chromium and vanadate have been mapped. While in a cross between ZNR-3 (a) strain with wild type A to obtain opposite mating type resistant strain, only 8% of Zn-resistance progeny were found to be A mating type. Normally, the progeny obtained are expected to show equal number of mating types (A and a). The data indicated closer linkage between resistance and mating type locus (mat), which is, located on LG I. To determine the loci for Co-sensitivity, *N. crassa* CSM-I was crossed with a tester *alcoy* strain[21] which contains three unlinked reciprocal translocations with conveniently visible markers linked to groups I through VI. Out of the 50 progeny tested, 18% were found to be Co-sensitive and the rest behaved like wild type. The least recombination was noticed with cot indicating linkage to IV or V. Hence the Co-sensitivity appears to be closely linked to cot locus. Morphologically, CSM resembles the aconidiation mutant of *N. crassa* including the characteristic Co-sensitivity and uptake and the locus also appears to similar LG IV. In the absence of more extensive mapping data, it is difficult to conclude whether CSM and *N. crassa*-*acon-3* are similar. The present data does not permit fine structural analysis of very closely linked multiple loci. Nevertheless, it is clear that these studies have broadly localized Co-sensitivity to chromosome IV of *N. crassa*.

Microbial bioremediation of radioactive waste is increasingly considered as a potential alternative to the conventional organic ion-exchanger based treatment. The application of Co and Ni-resistant strains in removal of toxic metal ions from high concentrations has also been demonstrated. In the above studies, wild type *N. crassa* was found to have superior ability to remove metal ions from low non-toxic levels as compared to resistant mutants[27]. It was presumed that if resistant strains from a wild type organism

can be developed, it should also be possible to isolate a sensitive strain. Such sensitive strains in comparison to the parental wild type should be able to hyper-accumulate toxic metal ions from very low and heterogeneous solutions. One such requirement is in the removal of ^{60}Co from nuclear power reactor effluents. Nuclear power reactors are made up of alloy containing Co and due to high energy and intensive reactions corrosion products develop and need periodical decontamination. The decontamination solution contains high concentration of nonradioactive iron, chromium, and Ni in complex form with organic chelators. The major problem in this effluent is the ^{60}Co gamma emitting radionuclide with half-life of 5.26 Y. It is the current practice to process the spent decontamination solution by employing synthetic organic ion-exchangers, which due to their limited exchange capacity result in generating large waste volume. Chemical approaches are available for metal remediation, but are often inefficient and expensive to apply and lack the specificity required to treat target metals against a background of competing ions. However, due to the limited ion exchange capacity for Co ion, saturation with large excess of iron present a large quantity of resin is generated posing problems of solid waste management. Such a disposal either requires a number of ion-exchange columns or subsequently tile holes for the safe burial of the resin bearing columns. This requirement increases the cost of such waste treatment. Co removal from a mixture of metal ions by CSM was 2–3 fold more efficient than wild type *N. crassa*. Understanding the removal of metal-complexes is difficult because several coupled geochemical and microbiological processes interact to influence the transport. Although the application of bioremediation technology to radioactive-waste treatment has attracted much interest, studies have generally been confined mostly to laboratory. Technical challenges associated with large-scale cleanup of highly complex wastes must be overcome prior to the full commercial realization of the technologies currently under consideration.

Conflict of interest statement

We declare that we have no conflict of interest.

Comments

Background

Metal toxicity and metal ion interactions are evergreen fields of research interest. Everywhere we will come across many forms of metal pollutions in air, water or land. The microbes are getting adapted or becoming tolerant to the particular environment with diverse mechanisms. They may serve as transport blocks to the particular pollutants. As the need is still not satisfied and the urge becomes increasing day by day because of the urbanization, finding a strain that can accumulate more metal content than a resistant one is of great importance that focuses in the present study.

Research frontiers

The present study explores a new dimension in the field of metal bioremediation using fungi in developing a novel Co-sensitive mutant of *N. crassa*, which is a hyper-accumulator strain if Co as well as other metal ions are with well defined mechanism of action.

Related reports

There are reports about the development of Ca-sensitive mutant of *Aspergillus*. Gardarin *et al.* found that the increased Cd was targeted towards endoplasmic reticulum.

Innovations and breakthroughs

CSM isolation by filter enrichment method, cell wall isolation and estimation of its phosphorus, *etc.*, are presented in this article.

Applications

From the study it seems that CSM can be used in the field of bioremediation and remove ⁶⁰Co from bioreactors at atomic research institutions.

Peer review

This is a valuable research work in which authors have demonstrated the development of a hyper-accumulator strain of *N. crassa* with defined mechanism for its sensitivity. Furthermore, it was also shown to have cross sensitivity to other metal ions which is advantageous for a strain in processing the ability to remove multiple metal types using a single strain.

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