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Characterization of a cobalt-resistant mutant of *Neurospora crassa* with transport block

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A cobalt-resistant strain of *Neurospora crassa (cor)* was obtained by repeated subculturing of the wild type on cobalt-containing agar medium. *N. crassa cor* is twentyfold more resistant to cobalt ions compared with the wild type. Resistance was stable on repeated subculturing of *cor* on cobalt-free media. *N. crassa cor* is also cross-resistant to nickel (fourfold), but not to zinc or copper. Higher concentrations of iron and magnesium ions are required to reverse growth inhibition due to cobalt toxicity in *N. crassa cor*, compared with the wild type. Germinating conidia and mycelia of the *cor* strain accumulated lower levels of cobalt ions compared with the parent *N. crassa*. The partial transport block for cobalt uptake is shown to be primarily due to decreased surface binding of cobalt to mycelia and cell walls. Efflux of mycelial cobalt was also observed in wild type and cobalt-resistant *N. crassa*. The characteristics of *cor* in comparison with wild type *N. crassa* are discussed in relation to the mechanisms of cobalt resistance.

Keywords: cobalt binding, cobalt-resistant, cobalt uptake, metal-resistant, *N. crassa*

Introduction

Metal toxicities in *Neurospora crassa* were first studied by Healy *et al.* (1955) who suggested that cobalt toxicity specifically induces a 'conditioned iron deficiency' in this mold. Toxicities of cobalt, nickel and zinc were subsequently shown to be reversed by magnesium and iron, the latter without suppression of toxic metal uptake (Sastry *et al.* 1962). An iron-binding siderochrome was isolated and characterized both in cobalt toxicity and in iron deficiency (Padmanaban & Sarma 1964). Cobalt toxicity was further shown to inhibit the heme biosynthetic pathway by specifically inhibiting the rate limiting enzyme σ-aminolevulinic acid dehydratase (Padmanaban & Sarma 1966).

Strains of fungi resistant to metal ions have been obtained by repeated subculturing of wild type organisms on media containing toxic concentrations of metal ions (Ashida 1965, Ross 1975). However, the mechanisms underlying resistance in such strains have not been studied in detail. In the first isolated cobalt-resistant strain of *N. crassa* (Co^R), which is tenfold more resistant to both cobalt and nickel ions than the wild type, a Co–Fe antagonism does not obtain, and hence the mechanism of resistance was suggested to involve an alteration in iron metabolism (Venkateswerlu & Sastry 1973). Cobalt uptake, which is energy-dependent in the wild type (Venkateswerlu & Sastry 1970), is by passive diffusion in cobalt-resistant *N. crassa* (Venkateswerlu & Sastry 1979). The above studies did not however provide an understanding of the mechanism involved in resistance. Cobalt transport was also studied in three nickel-resistant strains of *N. crassa* (Ni-Rl, Ni-R2 and Ni-R3) which exhibit strain-specific cross-resistance and uptake rates for cobalt (Maruthi Mohan & Sastry 1983, 1984). Cobalt uptake was shown to be temperature- and energy-dependent, and was sensitive to related metal ions. With respect to nickel resistance, a transport block was shown to be the resistance mechanism in *N. crassa* Ni-R3, while an intracellular mechanism

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appears to function in the hyperaccumulating strains, Ni-R1 and Ni-R2 (Maruthi Mohan *et al.* 1984). Metal-resistant strains of fungi are highly desirable because of their utility in decontaminating toxic metal ions from polluted effluents. The efficiency of metal-resistant mutants was demonstrated using a nickel-resistant, hyperaccumulating mutant of *N. crassa* which has superior ability to the wild type in depleting Ni^{2+} from aqueous media (Kumar *et al.* 1992). Recently, optimal conditions for efficient removal of cobalt ions from highly toxic concentrations using wild type and cobalt-resistant *N. crassa* were also reported (Karna *et al.* 1996).

In the present paper, isolation and characterization of a cobalt-resistant mutant of *N. crassa*, nonidentical to that of a previous isolate (Venkateswerlu & Sastry 1973), is described. Transport block is shown to be the primary mechanism of cobalt resistance.

Materials and methods

Chemicals

Metal salts used were $CoSO_4 \cdot 7H_2O$, $NiSO_4 \cdot 7H_2O$, $CuSO_4 \cdot SH_2O$, $ZnSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$ and ferric ammonium citrate. All chemicals were of analytical grade, from Sarabhai M. Chemicals Ltd., Baroda, India. ^{58}Co (carrier free) was obtained from Bhabha Atomic Research Centre, Bombay, India.

Media and growth conditions

Neurospora crassa FGSC #4200 (obtained from Fungal Genetics Stock Center (FGSC), Kansas city, USA) and the cobalt-resistant strain (deposited at FGSC with #7290) were grown in 10 ml basal medium in 50 ml conical flasks for 72 h at 28 ± 1°C as described earlier (Maruthi Mohan & Sastry 1983). Metal ions were supplemented in the basal medium as sulfates to provide the required concentrations. To obtain colonial growth, glucose was replaced by 1% sorbose and 0.2% sucrose. I_{50} (50% growth inhibitory concentration) values for metal ions were derived from graphical plots of growth verses metal ion concentration (typically shown in Figures 1 and 2).

Isolation of cobalt-resistant strains

The general procedures followed were as described by Maruthi Mohan and Sastry (1983). Spores from 7 day old cultures of *N. crassa* FGSC #4200 (wild type) were transferred onto 3% agar slants made with basal medium containing the desired concentration of cobalt (4 mM, 8 mM, 16 mM, 32 mM, 50 mM). After satisfactory growth was obtained (2–4 weeks) repeated transfers resulted in progressively better growth, and further subculturing was possible within 7–10 days. After 18–20 such subcultures, the cultures were tested for resistance to cobalt by measuring growth as a function of cobalt concentration in the medium. When no further increase in cobalt resistance was noted, the conidia were plated on 1% sorbose agar medium containing the same level of cobalt employed for adaptation, sucrose (0.2%) and nicotinamide (0.001%). Several colonies were subcultured and cobalt resistance (I_{50} concentration of cobalt resulting in 50% growth inhibition) was determined.

Cobalt uptake

Metal ion uptake was studied by incubating preformed mycelial mats (72 h) in 20 ml basal medium containing cobalt as in earlier studies (Maruthi Mohan & Sastry 1984). Metal taken up by mycelia was estimated, following acid digestion (Venkateswerlu & Sastry 1973), by atomic absorption spectrophotometry (AAS) using a Perkin-Elmer 2380 spectrophotometer.

To determine the cobalt bound to the cell surface and that taken up by the mycelia (intracellular), mycelia were washed thoroughly and suspended in 10 ml of EDTA (10 mM, pH 7), for 5 min to strip surface bound cobalt. Mycelia were then removed, washed, dried and weighed. The cobalt content in the EDTA extract and that remaining with the mycelia was estimated, after acid digestion, by AAS.

For experiments involving cobalt uptake by germinating conidia, conidiospores from 7–10 day old slants were filtered through glass wool to remove hyphal fragments, and were then inoculated (10^8) in 10 ml basal medium in 50 ml conical flasks containing cobalt (1 mM containing 1 μCi ^{58}Co). The flasks were incubated in an environmental shaker (100 rpm) at 28°C. The germinating conidia were harvested by centrifugation in a clinical centrifuge at 5000 rpm for 5 min, at 1 h and 3 h time intervals, then washed thoroughly with cold cobalt solution (10 mM). The spores were solubilized by boiling in 1 N NaOH at 100°C. Suitable aliquots were spotted on discs of Whatman No.3 filter paper, dried and the radioactivity (β decay) was measured in a scintillation cocktail consisting of PPO (2,5 diphenyloxazole), POPOP (1,4 bis[5-phenyl-2-oxazolyl]- benzene) and toluene in a Beckmann LS 1801 liquid scintillation counter.

Cell walls were prepared by the method of Schmit *et al.* (1975), lyophilized and stored at 4°C. Catalase activity and iron-binding siderochrome were determined by methods followed in earlier studies (Venkateswerlu & Sastry 1973).

Metal analysis

Metal content was determined after subjecting mycelia to wet digestion as described earlier (Venkateswerlu & Sastry 1973). Mycelia (30–50 mg dry wt) 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

Table 1. Isolation of cobalt-resistant strains of *N. crassa*

Strain number	Concentration of Co^{2+} (mM)	Number of subcultures (biweekly)	*Number of subcultures on Co^{2+}-free medium	I_{50} for Co^{2+} (mM)
1	0	20	22	0.4 ± 0.1
2	4	18	22	4.0 ± 0.5
3	8	20	20	4.0 ± 0.4
4	16	20	21	4.0 ± 0.5
5	32	20	32	8.0 ± 1.0
6	50	18	20	8.0 ± 0.9

N. crassa wild type was subcultured on agar slants (biweekly) containing Co^{2+}. I_{50} (50% growth inhibitory concentration). Values shown are averages from three separate experiments (± SD).

*Number of subcultures (biweekly) during which, to date, resistance to cobalt remained unaltered.

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 distilled water and metal ions were estimated by AAS.

Note: Unless indicated, all experiments were repeated a minimum of three times in triplicate. Invariably, a variation of not more than 10% was observed within an experiment. However, between separate experiments a maximum variation of 20% was observed without any change in the overall pattern of results.

Results

Isolation of cobalt-resistant strains

Cobalt-resistant cultures of *Neurospora crassa* were obtained by repeated subculturing of wild type *N. crassa* on agar medium containing 4–50 mM cobalt. Table 1 shows that after 18–20 such biweekly subcultures, a ten- to twentyfold resistance to cobalt (I_{50} 4–8 mM) was observed when compared with the parental *N. crassa* (I_{50} 0.4 mM). It should be noted that adaptation on progressively increasing concentrations of cobalt, up to 16 mM, resulted in strains which were tenfold resistant, while selection on a higher cobalt concentration (32 or 50 mM) resulted in strains which were twentyfold resistant. The cobalt-resistant strains so obtained were found to be stable mutants; subculturing on cobalt-free media (18–32 biweekly subcultures) did not result in loss of resistance. The cobalt-resistant isolates were found to be genetically homogeneous the I_{50} values for cobalt (50% growth inhibitory concentration of metal ion) of six single colony isolates from each of the adapted cultures were almost identical. A single isolate from the 32 mM cobalt adapted culture, referred to hereafter as the *cor* strain, was selected for further detailed studies. Cobalt toxicity and accumulation were examined in this strain and the data were compared with the parental wild type *N. crassa*. The results presented in Figure 1 show that growth of parental *N. crassa* was inhibited (> 80%) by 2 mM cobalt, with an I_{50} around 0.4 mM, while the *cor* strain was relatively unaffected under similar conditions of growth. *N. crassa* (wild type) accumulated relatively higher concentrations of cobalt (up to fourfold) compared with the *cor* strain. Growth

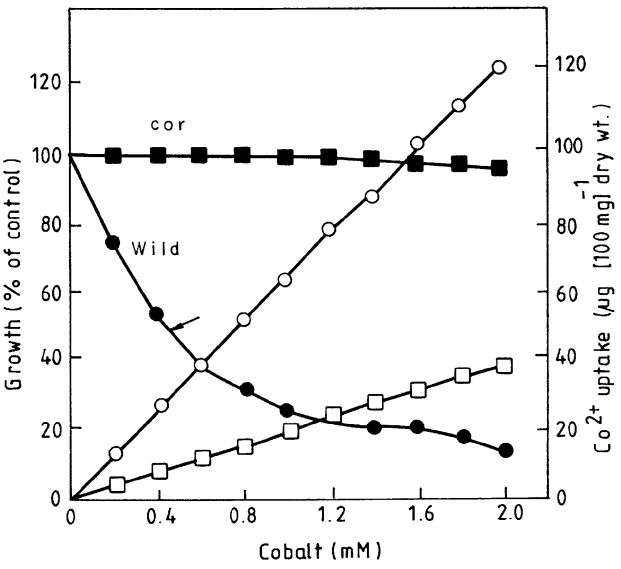

Figure 1. Cobalt toxicity in *N. crassa*. *N. crassa* strains were grown in 10 ml basal medium in 50 ml conical flasks for 72 h at 28 ± 1°C. Cobalt was included at the required concentrations as indicated. After incubation, the mycelia were washed, dried and cobalt was estimated, following acid digestion, by atomic absorption spectrophotometry (AAS). Mycelial weights of controls (taken as 100% (mg dry wt)) were: wild type (circles), 42 ± 5; and *cor* (squares), 38 ± 4. The arrow indicates 50% growth inhibition (I_{50}). The data shown are average values of four experiments (SD up to ±18%). Growth, closed symbols; Co^{2+} uptake, open symbols.

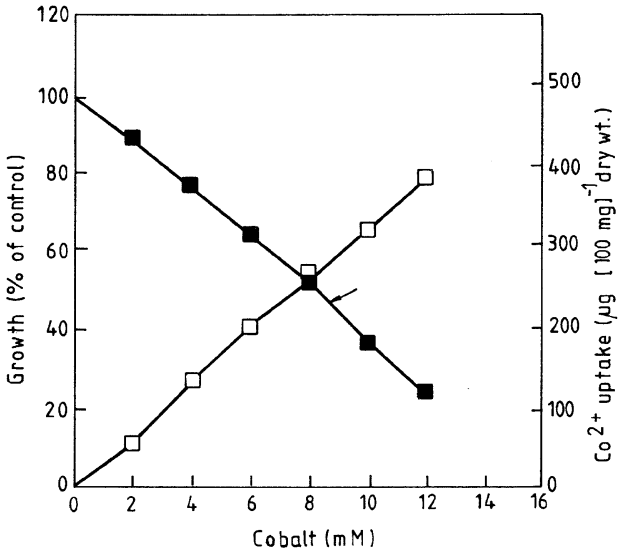

Figure 2. Cobalt toxicity in *N. crassa cor*. *N. crassa cor* was grown and processed as in Figure l. Mycelial weight of control (40 mg ± mg dry wt (100%)). The arrow indicates the I_{50} value. Typical results from at least six experiments, each in triplicate, are shown. Growth, closed symbols; Co^{2+} uptake, open symbols.

inhibition for the *cor* strain was observed at a twentyfold higher concentration of cobalt than that of wild type *N. crassa*, with an I_{50} value of about 8 mM (Figure 2). Consequently, higher levels of cobalt were accumulated by the mycelia.

Cross-resistance

Since metal-resistant strains of fungi generally exhibit cross-resistance to other related toxic ions, this aspect was also examined in the *cor* strain. Figure 3 shows that the *cor* strain is over fourfold (I_{50} 3.6 mM) more resistant to nickel compared with the wild type (I_{50} 0.8 mM). Once again, the *cor* strain accumulates much less nickel than does wild type *N. crassa*. No cross-resistance to copper and zinc was observed (data not shown).

Effect of magnesium and iron on cobalt toxicity

Cobalt toxicity in *N. crassa* is known to be reversed by magnesium and iron (ferric) when an excess of these ions is included in the growth medium (Sastry *et al.* 1962). However, in an earlier isolated cobalt-resistant *N. crassa* (Co^R), iron was unable to reverse cobalt toxicity (Venkateswerlu & Sastry 1973). Hence, the effect of magnesium and iron was studied in cobalt toxicity. Both these metal ions were found to reverse completely the growth inhibition caused by cobalt in *N. crassa* wild type and the *cor* strain (Table 2). Magnesium reversed growth inhibition by suppression of cobalt uptake in both *N. crassa* strains, the *cor* strain requiring a tenfold higher ratio of Mg (Co : Mg = 1 : 1) compared with the wild type (Co : Mg = 10 : 1). On the other hand, iron reversed growth inhibition due to cobalt toxicity without suppression of cobalt uptake. Reversal by iron in the *cor* strain also required higher concentrations than in the wild type. The minimal optimal concentrations of Mg and Fe required for complete growth reversal only are shown in Table 2.

^{58}Co uptake by germinating conidia

Since conidiospores of *N. crassa* were used as inoculum in all of our studies, it was of interest to study cobalt uptake in germinating conidia in the wild type and the *cor* strain. Table 3 indicates that conidia of the *cor* strain accumulate relatively less cobalt, both at 1 h and 3 h time periods of germi-

Table 2. Reversal of cobalt toxicity by Mg and Fe in *N. crassa*

Strain	Concentration of			Growth	Co uptake (µg per
	Co (mM)	Mg (mM)	Fe (mM)	(mg dry wt)	100 mg dry wt)
Wild type	0.0	–	–	42 ± 4	–
	0.4	–	–	21 ± 3	30 ± 3
	0.4	0.04	–	41 ± 3	12 ± 3
	0.4	–	0.04	39 ± 4	28 ± 4
cor	0.0	–	–	40 ± 5	–
	8.0	–	–	18 ± 4	250 ± 20
	8.0	8.0	–	39 ± 3	50 ± 6
	8.0	–	8.0	39 ± 4	258 ± 32

N. crassa strains were grown at their respective I_{50} concentrations of Co for 72 h along with Mg or Fe. (Minimal concentrations of Mg and Fe required for complete reversal of growth inhibition only are indicated.) Growth and cobalt uptake values shown are means derived from duplicates of two separate experiments (± SD).

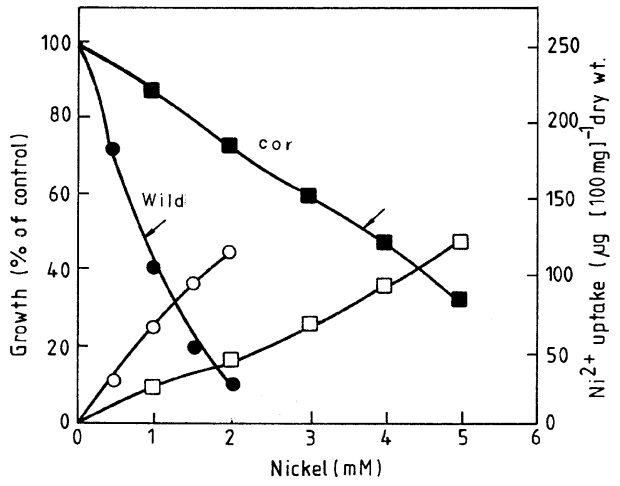

Figure 3. Nickel toxicity in *N. crassa*. *N. crassa* were grown as in Figure 1. Nickel was included at the required concentrations as indicated. Mycelial weights of controls (100% (mg dry wt)) were: wild type (circles), 42 ± 5; and *cor* (squares), 40 ± 5. The arrows indicate I_{50} values. Typical results from at least three experiments are shown. Growth, closed symbols; Ni^{2+} uptake, open symbols.

nation. Cobalt uptake was also studied using pregrown mycelia (72 h). The rates of uptake were found to be (μg of Co^{2+} per 100 mg dry wt per h): wild type, 35; and *cor*, 25, respectively. The cobalt uptake rate of the *cor* strain is approximately 30% less than that of the wild type *N. crassa*.

Effect of metabolic inhibitors

In an earlier isolated cobalt-resistant *N. crassa* (Co^R), cobalt uptake was found to be insensitive to respiratory poisons, while in the wild type uptake was sensitive (Venkateswerlu & Sastry 1970, 1979). To examine this, the effects of sodium azide and 2,4 dinitrophenol on cobalt uptake were studied using preformed (72 h) mycelial mats. Table 4 shows a distinct suppression of cobalt uptake in both the wild type and the *cor* strain. It should also be noted from control data (without inhibitors) that cobalt uptake by the *cor* strain is about one half of that of the wild type *N. crassa.*

Table 3. ^{58}Co uptake by conidiospores of *N. crassa*

	cpm mg^{-1} protein	
	1 h	3 h
Wild type	865 (100)	1820 (100)
cor	269 (31)	580 (31)

N. crassa conidiospores (10^8) were suspended in 10 ml basal medium containing 1 mM Co^{2+} (1 μCi of $^{58}Co^{2+}$ specific activity 500 mCi per millimole). Incubation was at 28°C in a rotary shaker (100 rpm) for 1 h and 3 h. The radioactivity of the spores was measured as described in Materials and methods. Numbers in parentheses indicate percentage values. Typical results from at least three experiments are shown.

Cobalt partitioning

In *N. crassa cor* partial transport block for cobalt uptake has been observed in growth experiments, in short-term uptake by preformed mycelia, and in germinating conidia. It was of interest, therefore, to determine whether the decreased cobalt accumulation by the *cor* strain is due to decreased surface binding or to intracellular accumulation. To study the partitioning of cobalt between the above two fractions, preformed mycelial mats (72 h) were allowed to take up cobalt for various time periods, and surface bound cobalt was leached with EDTA. Cobalt remaining with the mycelia was assumed to be intracellular. Figure 4A indicates that surface bound cobalt (EDTA leachable fraction) in the *cor* strain is significantly lower than in the wild type, at all time points. The cobalt of the intracellular fraction (Figure 4B) is also less in the *cor* strain than in the wild type, but is not as significant. Further cell wall preparations from the wild type and the *cor* strain were used to determine cobalt sorption. Table 5 supports the above results in that *N. crassa cor* cell walls bind only about 25% of cobalt compared with the wild type.

Table 4. Effect of respiratory inhibitors on cobalt uptake by *N. crassa* strains

Inhibitor	Strain	Inhibitor (mM)	Cobalt uptake (μg per 100 mg)	Inhibition (%)
Sodium azide	Wild	0	102	0
		0.1	18	82
		1.0	17	83
	cor	0	46	0
		0.1	23	51
		1.0	22	50
2,4 dinitro phenol	Wild	0	104	0
		0.1	74	28
		1.0	16	84
	cor	0	44	0
		0.1	22	52
		1.0	21	48

Preformed mycelia (72 h) were floated for 1 h in 20 ml basal medium containing 2 mM cobalt. Metabolic inhibitors were included as indicated. At the end of the incubation the mycelia were harvested, washed, weighed and acid digested to estimate cobalt by AAS. Average values of two separate experiments are shown (SD up to ± 15%).

Cobalt efflux

To determine if there is any efflux of cobalt that has already been accumulated, mycelia of *N. crassa* wild type and the *cor* strain were allowed to take up cobalt (2 mM) for 3 h, and were then washed and resuspended in a cobalt-free medium. Table 6 indicates that the *cor* strain releases relatively more cobalt when compared with wild type *N. crassa* under the experimental conditions. About 12-25% of the mycelial cobalt was released; the *cor* strain, which takes up less cobalt, releases twofold more than the wild type. Cobalt efflux was found to be a slow process influenced neither by metabolic inhibitors, nor by the presence of magnesium ions in the medium (data not shown).

Discussion

That microorganisms acquire resistance to toxic metal ions in the environment is well established. However, the mechanisms whereby they do so are less clear. One reason for this is that, except in certain specific instances, exactly how a toxic metal ion derails cellular metabolism and the system(s) that is its target is mostly unknown. Bacteria acquire resistance to toxic metals, but this is not stable (Webb 1970). Similarly, resistance of *Rhizopus stolonifer* and *Cunninghamella blakesleeana* to copper was shown to be unstable and was lost following subculture on copper-free medium (Garcia-Toledo *et al.* 1985). However, resistance of *N. crassa* to cobalt and

Table 5. Cobalt binding by cell walls of *N. crassa*

	Co^{2+} (per 100 mg dry wt)
Wild	32 (± 4)
cor	7 (± 2)

Cell walls (~ 100 mg dry wt) of *N. crassa* strains were suspended in 10 ml basal medium containing 5 mM Co^{2+} and incubated in a rotary shaker (100 rpm) at 28°C for 30 min. The cell walls were then washed, dried, weighed and acid digested, and cobalt was estimated by AAS. Mean values from three separate experiments, each in duplicate, are shown (± SD).

Table 6. Cobalt efflux by *N. crassa* strains

	Cobalt (μg per 100 mg dry wt)	
	Mycelia	Released (%)
Wild type	110	13 (12)
cor	56	15 (25)

Preformed mycelia (3 days) of *N. crassa* strains were floated for 3 h in medium containing cobalt (2 mM). The mycelia were washed and resuspended in cobalt-free medium (10 ml) to check for the release of cobalt. Cobalt released into the medium after 1 h and the remaining cobalt in the mycelia was estimated, after acid digestion, by AAS. Typical results from at least three experiments are shown.

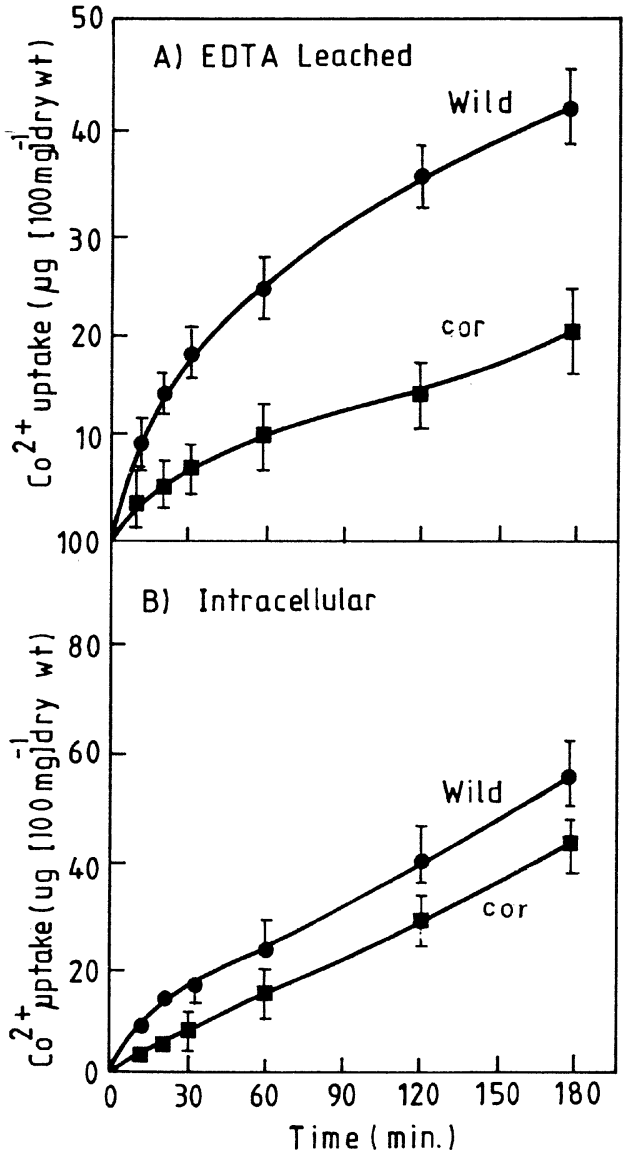

Figure 4. Cobalt partitioning between surface and intracellular fractions. Preformed mycelia (72 h) were floated in 20 ml basal medium containing cobalt (2 mM). The mycelia were harvested at the indicated time points, washed and incubated in 10 ml EDTA solution (10 mM) for 5 min to leach out surface bound cobalt. The mycelia were then dried, weighed and digested. The cobalt content of both the above fractions was determined by AAS. Data points are average values from two separate experiments, each in duplicate (± SD).

nickel was shown to be stable (Venkateswerlu & Sastry 1973, Maruthi Mohan & Sastry 1983) as observed with the *cor* strain in the present study. Studies on nickel resistance in *N. crassa* led to the suggestion that a plurality of nonallelic gene loci is associated with nickel resistance and that adaptation of *N. crassa*, depending on conditions employed, can lead to nonidentical resistant mutants (Maruthi Mohan & Sastry 1983). However, resistance to both cobalt and nickel was found to be closely linked to LG IIIR of *N. crassa* (Wilson *et al.* 1992).

In the present study, cobalt-resistant strains have been derived by subculturing *N. crassa* on different levels of cobalt. One of the striking findings is that a *cor* strain with twentyfold resistance is obtained when the cobalt concentration is in the range 32–50 mM, while strains with tenfold resistance result when the cobalt concentration is between 4 and 16 mM. Cross-resistance is exhibited to nickel (fourfold) but not to zinc or copper ions. Another important conclusion from the present study is that the *cor* strain differs in several respects from the earlier isolated Co^R strain (Venkateswerlu & Sastry 1973), although both were derived in the quest for cobalt-resistant mutants of *N. crassa*. For example, in contrast to Co^R, in the present isolate (*cor*), cobalt toxicity is counteracted by iron. A comparison of the characteristics of the wild type and the two cobalt-resistant mutants of *N. crassa* (including the *cor* strain from the present work and the earlier isolated co^R) highlights the salient differences. The complexity of the interaction between cobalt and iron is displayed in two ways: (i) the quantitative aspects of reversal differ between wild type *N. crassa* and the *cor* strain in that while normal growth is attained in the wild type when the Co : Fe ratio is 10 : 1, much higher levels of Fe (Co : Fe = 1 : 1) are demanded by the *cor* strain, although in neither case is growth reversal due to suppression of cobalt uptake; (ii) in the case of the *cor* strain, cobalt toxicity is not reflected either in changes in catalase activity *in vivo* or in the excretion of the iron-binding siderochrome (Table 7), which is an indicator of iron deficiency in wild type *N. crassa* (Padmanaban & Sarma 1964). The implication is that intracellularly accumulated cobalt is bound to, and adversely affects a different set(s) of intracellular sites which are obviously not those which lead to inhibition of heme synthesis. Counteractions of cobalt toxicity by magnesium in *N. crassa cor* is due to suppression of cobalt uptake. One general reason for this mode of antagonism between these two metal ions is that, apparently, cobalt ions are primarily taken up by the magnesium transporter (Venkateswerlu & Sastry 1979, Maruthi Mohan & Sastry 1984). A comparison of the pattern of reversal by magnesium in the wild type and the *cor* strain reveals that, quantitatively, magnesium is far more efficient in the wild type (Mg : Co = 1 : 10), than in the *cor* strain (Mg : Co = 1 : 1). This indicates that there is decreased affinity for cobalt in the *cor* strain.

Cobalt uptake data indicate that a transport block is the primary mechanism for resistance in the *cor* strain. The partitioning of cobalt between the surface bound and intracellular fractions provides an understanding of the mechanism involved in resistance. Cell surface binding is greatly decreased in the *cor* strain (both in preformed mycelia and in isolated cell walls) which in turn could lead to decreased accumulation into the intracellular fraction (not leachable by EDTA). Chitin and chitosan are known to be the major binding components for toxic metal ions (Muzzarelli *et al.* 1980). In *N. crassa*, chitin/chitosan levels were shown to be altered in copper toxicity on a sole nitrate medium (Subramanyam *et al.* 1983). It remains to be seen if a decrease in chitin/chitosan content is responsible for the decreased binding of cobalt to the cell walls of *N. crassa cor*. Wild type *N. crassa* shows an inherent ability to exclude cobalt ions, which is also observed in the *cor* strain. However, in the latter, which accumulates relatively lower levels of cobalt,

Table 7. Comparison of cobalt-resistant strains of *N. crassa*

Characteristic	*Wild	Co^R	*cor*
Co^{2+} concentration for adaptation (mM)	0	1.6–33	32
Resistance to Co	–	Tenfold	Twentyfold
Resistance to Ni	–	Tenfold	Fivefold
Reversal of Co toxicity by iron	+ve	–ve	+ve
Catalase levels in Co toxicity	Decreased	No change	No change
Iron-binding siderochrome in Co toxicity	Increases	No change	No change
Cobalt transport	Active	Passive	Active

Co^R derived from wild type *N. crassa* Em 5297 (Venkateswerlu & Sastry 1973) and *cor* from *N. crassa* OR #4200.

a greater percentage of the total cobalt is excluded in comparison with wild type *N. crassa*. This could probably also contribute to the overall ability of this strain to accumulate less cobalt. Further studies are necessary before such a conclusion can be drawn.

Cell walls of fungi have been implicated in metal resistance by acting as a barrier to toxic metals; the metals bind in excess to this site (Gadd l993, Cervantes & Gutierrez-Corona 1994), thereby possibly restricting entry into cells. However, in the present study, a decrease in metal binding to cell walls is for the first time shown to be involved in cobalt resistance in *N. crassa*. Since binding to the cell wall is the primary site of interaction during metal uptake, a quantitative decrease in binding could in turn result in decreased accumulation into the intracellular fraction. Reduced capacity for the energy-linked uptake of cobalt and nickel ions has been found to be the mechanism determining resistance in cobalt- and nickel-resistant strains of *E. coli*, *A. aerogenes* and *B. subtilis* (Webb 1970). In contrast to the above, a cobalt-resistant strain of *Proteus vulgaris* isolated by Neyland *et al.* (1952) was found to take up thirtyfold more cobalt than the wild type at the respective 50% growth inhibitory levels of cobalt. Further details regarding the mechanism of resistance are unknown. Metal resistance in bacteria is known to be mediated by plasmids. Plasmid mediated cobalt efflux was demonstrated to be the mechanism of cobalt resistance in *Alcaligenes eutrophus* (Nies & Silver 1989). In *E. coli* and *Salmonella*, R factors mediating resistance to cobalt, mercury and nickel have been reported by Smith (1967). A cobalt-tolerant *S.cerevisiae* selected by gradual adaptation was found to accumulate up to 9.9% of cobalt. However, the mechanism of resistance was not studied (Perlman & O'Brien 1954). More recently, the COT-1 gene, involved in cobalt accumulation and responsible for cobalt resistance in *S. cerevisiae*, was characterized (Conklin *et al.* l992).

Resistance to toxic metal ions could be due to two basic mechanisms: (i) a permeability block, which restricts entry of toxic metal ions into the cells; and (ii) intracellular compartmentalization or sequestration of metal ions. In the present study the *N. crassa cor* strain is shown to have transport block primarily due to decrease in cobalt binding to the cell wall. However, it should be noted that an overall two- to fourfold decrease in cobalt uptake by the *cor* strain compared with wild type *N. crassa* should ideally result in only a two- to fourfold resistance. Since the *N. crassa cor* strain is twentyfold more resistant to cobalt than the wild type, this suggests that there is also a second mode, i.e. an intracellular mechanism for resistance. This is the subject of current investigation in our laboratory.

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