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Zinc resistance in *Neurospora crassa*

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Three non-identical Zn^{2+}-resistant strains of *Neurospora crassa* have been isolated. ZNR-1 and ZNR-2 strains were obtained after repeated subculturing of wild type *N. crassa* on Zn^{2+}-containing agar media (8 mM and 16 mM), while ZNR-3 was isolated after mutagenesis with diethyl sulfate, followed by selection on Zn^{2+} agar plates (16 mM). All three ZNR strains showed two- to threefold resistance to Zn^{2+} in liquid media when compared with the wild type. However, growth measured by hyphal elongation clearly distinguished between the resistant strains (ZNR-3 > ZNR-2 > ZNR-1 >>> wild). The ZNR-2 and ZNR-3 strains were also cross-resistant to Co^{2+}, while ZNR-2 alone was cross-resistant to Cu^{2+}. Both Mg^{2+} and Fe^{3+} reversed the growth inhibition caused by Zn^{2+}; Mg^{2+} by suppression of Zn^{2+} uptake and Fe^{3+} without affecting the same. Assay of catalase, iron-binding siderophores and glutathione in Zn^{2+} toxicity revealed significant increases in catalase and glutathione levels in the ZNR-2 strain when compared with the wild type. Kinetics of Zn^{2+} uptake by preformed mycelia showed a rapid initial phase of uptake followed by a slower phase. The rates of Zn^{2+} uptake measured after leaching surface-bound metal with EDTA revealed that ZNR strains have significantly reduced Zn^{2+} uptake rates when compared with the wild type. The overall data suggest a partial transport block for Zn^{2+} uptake as the major mechanism for resistance in ZNR strains. Genetic analysis of ZNR strains showed that in the ZNR-3 strain the *znr* locus maps close to the mating type locus (*mt*) of *N. crassa* LG I, while that of ZNR-1 and ZNR-2 is linked to LG IV associated with chromosomal aberration.

Keywords: linkage, *Neurospora crassa*, resistance, uptake, zinc

Introduction

Microorganisms, when exposed to toxic concentrations of metal ions, are known to evolve resistant strains (Ashida 1965). Metal-resistant strains can also be obtained spontaneously or by mutagenic procedures followed by selection on metal-containing media. Metal resistance in general could be due to two broad mechanisms: (i) a primary transport block at the level of metal transport; and (ii) intracellular mechanisms such as compartmentalization into vacuoles or sequestration by specific proteins (metallothioneins). While the former is more common in bacterial systems, the latter finds more favour with the eukaryotic yeast and fungal systems (Mehra & Winge 1991).

Several metal-resistant strains of fungi have been isolated but in only a few cases has the mechanism of resistance been investigated in detail (Ashida 1965, Gadd 1993). The yeast system has been extensively investigated with respect to the role of metallothioneins in resistance (Mehra & Winge 1991, Gadd 1993). However, amongst filamentous fungi very little is known regarding metal resistance mechanisms except in the case of *Neurospora crassa*. Strains of *N. crassa* resistant Co^{2+}, Ni^{2+}, Hg^{2+} and Cd^{2+} have been isolated and characterized (Landner 1971, Venkateswerlu & Sastry 1973, Maruthi Mohan & Sastry 1983, Levine & Marzluf 1989). In the cobalt-resistant *N. crassa* (Co-R), an

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alteration in iron metabolism or compartmentalization of the toxic ions has been suggested to be the mechanism of resistance. In one of the nickel-resistant mutants (Ni-R3) a severe transport block was shown to be the mechanism for resistance (Maruthi Mohan *et al.* 1984). Further, a hyperaccumulating nickel-resistant strain of *N. crassa* (Ni-R2) has been exploited for removing toxic metal ions from aqueous media (Shravan Kumar *et al.* 1992). Metal resistance loci for cobalt, nickel and cadmium have been mapped in *N. crassa* (Levine & Marzluf 1989, Wilson *et al.* 1992).

Zinc resistance determined by plasmids has been reported for *Alcaligenes eutrophus* (Nies & Silver 1989) and *Staphylococcus aureus* (Tynecka *et al.* 1981, Nucifora *et al.* 1989). In the above cases resistance was shown to be due to efflux of Zn^{2+}. Further, Zn^{2+} resistance due to specific zinc-binding proteins has been shown in *Alcaligenes eutrophus* (Remacle & Vercheval 1991) and *Thiobacillus thiooxidans* (Sakamoto *et al.* 1989). Very little is known regarding zinc resistance in the more complex eukaryotic fungal systems, except in yeast where a gene conferring Zn^{2+} resistance has been characterized (Kamezano *et al.* 1989). Zinc transport in yeast cells was shown to be mediated by a system that also transports Mg^{2+} and Mn^{2+} (Fuhrmann & Rothstein 1968). In *Sporobolomyces roseus*, *Saccharomyces cerevisiae* and *Neocosmospora vasinfencta*, Zn^{2+} uptake was shown to be biphasic, with a rapid initial phase (independent of metabolic energy) followed by a second phase (dependent on metabolic energy) (Paton & Budd 1972, Mowll & Gadd 1983, White & Gadd 1987). More recent studies indicated that two systems were involved in Zn^{2+} transport, with low and high affinities for Zn^{2+} (Budd 1988, 1989). Although zinc requirements and toxicity have been studied in *N. crassa* (Anderson-Kötto & Hevesy 1949, Sastry *et al.* 1962a), very little is known regarding Zn^{2+} resistance in fungi. In the present study characterization of Zn^{2+}-resistant strains and mapping of resistance loci in *Neurospora crassa* has been undertaken with a view to study the mechanism of resistance.

Materials and methods

Chemicals

The metal salts $ZnSO_4.7H_2O$ and $CuSO_4.7H_2O$, and glutathione reductase were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were analytical grade products of Qualigens (Bombay, India).

Strains

The wild type *Neurospora crassa* (FGSC # 4200), and the standard tester strains *alcoy* (FGSC # 3661), *ser-3 un-16 acr-3* (a) (LG I marker, FGSC # 5079) and *pdx-1 cot-1 cys-4* (A) (LG IV marker, FGSC # 4151) of *N. crassa* were obtained from the Fungal Genetics Stock Center, Kansas City, KS, USA.

Media and growth conditions

N. crassa strains were grown in 10 ml basal medium in 50 ml conical flasks for 72 h at 28 ± 1ºC as described by Sastry *et al.* (1962a). The pH of the medium was adjusted to between 4.8 and 5.0 with dilute HCl or NaOH as required. For preparation of slants, 3% agar was included in the basal medium. For the preparation of agar slants with higher concentrations of metal ions, 0.4 g sodium β glycerophosphate and 0.015 g of KCl were used instead of KH_2PO_4 for 100 ml basal medium. Metal ions were supplemented in the medium separately after autoclaving to provide the required concentrations. After incubation the mycelia were harvested and washed thoroughly with glass distilled water, dried overnight in an oven at 80ºC and weighed to measure growth. 50% growth inhibitory concentrations (I_{50}) of metal ions were derived from the graphical plots of growth versus metal ion concentration.

Hyphal elongation

To determine growth and resistance of different strains of *N. crassa*, hyphal elongation of *N. crassa* strains was studied in race tubes (Ryan *et al.* 1943) with a diameter of 1 cm, a length of 25 cm, and both ends of the tube bent at a 45º angle in the same plane. The tube was filled to half its diameter with either basal medium for normal growth, or medium with zinc (8 or 16 mM) for resistance studies. Conidiospores were inoculated at one end of the tube and the rate of hyphal elongation was observed and recorded at regular intervals of time.

Metal analysis

Mycelial metal ion content was determined after subjecting the mycelia to a wet digestion procedure (Sastry *et al.* 1962b). To 30–50 mg dry weight of mycelia, were added 5 ml of concentrated HNO_3 and 0.5 ml of 70% $HClO_4$; the mixture, in 50 ml conical flasks, was heated slowly to dryness on a sand bath. The residue was further digested with a mixture of 2 ml HNO_3 and 2 ml HCl and the resulting residue was finally digested with 1 ml HCl. The residue was dissolved in a suitable minimal volume of distilled water and aliquots were taken for estimation of metal ions by atomic absorption spectrophotometer (Perkin-Elmer, Model 2380).

Isolation of resistant strains

Zinc-resistant strains of *N. crassa* were obtained by repeated subculturing of wild type on agar slants contain-

ing toxic concentrations of Zn^{2+} in basal medium and also by chemical mutagenesis using diethyl sulfate. Spores from a seven day old culture of *N. crassa* wild type were inoculated on agar slants containing 8 mM Zn^{2+}. After four to five biweekly subcultures spores were transferred to agar slants containing 16 mM Zn^{2+}. After 20 subcultures on 8 mM and 16 mM Zn^{2+} media resistance to zinc was determined in liquid media.

For chemical mutagenesis, germinating conidiospores in 10 ml medium (10^8 per ml) were treated with 0.1 ml diethyl sulfate for 2 h and plated on agar medium containing 16 mM Zn^{2+} and 1% sorbose with 0.2% sucrose (to obtain conidial growth). A few colonies were picked up and analysed for Zn^{2+} resistance in liquid cultures and one of the isolates was selected for further study.

To obtain genetically homogeneous Zn^{2+}-resistant cultures the conidiospores from a resistant isolate were plated on agar medium containing 1% sorbose, and five single colonies were isolated. These were tested for their resistance to Zn^{2+} in liquid culture. The Zn^{2+}-resistant strains isolated after subculturing on 8 mM and 16 mM Zn^{2+} are designated as ZNR-1 and ZNR-2, respectively, and the one obtained by chemical mutagenesis is referred to as ZNR-3. All the Zn^{2+}-resistant strains were repeatedly subcultured on Zn^{2+}-free medium and tested for stability of resistance to Zn^{2+}.

Assay of catalase, glutathione and iron-binding siderophores

N. crassa mycelia (approximately 1 g fresh weight) of both wild and resistant strains, grown at different concentrations of Zn^{2+}, were washed extensively with ice cold water, and homogenized at 0–4ºC with acid washed sand and 5 ml of cold phosphate buffer (pH 7.0). The homogenates were centrifuged in the cold at $10\,000 \times g$ for 30 min and the supernatants were collected. The residues were further extracted with 3 ml of the same buffer and centrifuged as above. The supernatants from the first and second extractions were pooled and suitable aliquots were used to measure the catalase activity by a permanganate titration procedure (Venkateswerlu & Sastry 1973). Catalase activity was expressed as micromoles of H_2O_2 decomposed per mg of protein in five minutes. Protein content of the extract was determined by the method of Lowry *et al.* (1951). In extracts prepared from mycelia total glutathione (reduced (GSH) + oxidized (GSSG)) was estimated by the method of Akerboom & Sies (1981), and expressed as nmoles per mg of protein.

Iron-binding siderophores excreted into the culture medium were estimated by the method of Neilands (1974). To 3 ml of culture medium, 1 ml of $FeCl_3.6H_2O$ (1 mg ml^{-1}) solution was added and centrifuged. Absorption of the supernatants was measured at 440 nm.

Zinc uptake

To study the kinetics of Zn^{2+} transport in *N. crassa* strains, mycelia grown for three days were washed with distilled water and pressed free of excess moisture with filter paper. The mycelia were suspended in 10 ml basal medium with 1 mM Zn^{2+} for different time intervals in a rotary shaker at 100 rpm and 28ºC. At the end of the incubation period the mycelia were washed with distilled water and dried. Metal ion uptake was estimated by atomic absorption spectrophotometry after acid digestion. In similar experiments the mycelia were treated with 10 ml of EDTA (10 mM) for 5 min to remove surface-bound Zn^{2+}. The Zn^{2+} remaining with the mycelia after EDTA treatment and the Zn^{2+} leached into the EDTA solution were esti-mated after acid digestion by atomic absorption spectrophotometer.

Mapping of zinc-resistant loci

ZNR(a) strains were crossed with wild type (A), and the zinc-resistant progeny thus obtained were used in crosses with the alcoy FGSC # 3661 (A) strain to determine the linkage group associated with resistance. The crosses were done on petri plates containing Westergaard and Mitchell's (1947) crossing medium containing 1.5% agar, and the progeny were then analysed. ZNR-1/ZNR-2(a) and ZNR-3(A) strains were further crossed with marker strains *pdx-1 cot-1 cys-4*(A) and *ser-3 un-16 acr-3*(a), respectively and the progeny obtained were analysed. Zinc resistance in the progeny of the above crosses was tested by their ability to grow on 8 mM Zn^{2+}-containing agar media within two to three days.

Results

Isolation of zinc-resistant strains

Zinc-resistant strains of *N. crassa* were obtained by repeated subculturing of wild type *N. crassa* on zinc-containing agar media. Two non-identical zinc-resistant strains, referred to as ZNR-1 and ZNR-2, were obtained after 20 biweekly subcultures on 8 mM and 16 mM Zn^{2+}-containing agar media, respectively. *N. crassa* ZNR-3 was obtained by mutagenesis with diethyl sulfate followed by selection on 16 mM zinc-containing agar medium. All three zinc-resistant strains were two to three-fold resistant to zinc in liquid cultures, with I_{50} (50% growth inhibitory concentration of zinc) values around 7–8 mM Zn^{2+} as compared with wild type *N. crassa* (I_{50} 3 mM) (Table 1). Further, when subcultured repeatedly on zinc-free medium (20 subcultures), zinc-resistant strains exhibited no reduction in resistance, indicating that they have stable resistance.

To obtain genetically homogeneous zinc-resistant cultures, five single colonies from each of the above strains were isolated by plating conidiospores on 1% sorbose-containing agar plates (to obtain conidial growth), and their resistance to zinc was determined. The results in Table 2 show that all the individual

single colonies have similar resistance patterns to zinc and hence are genetically homogeneous.

Zinc toxicity

Although all three Zn^{2+}-resistant strains have similar I_{50} values in liquid culture, growth by hyphal elongation on 8 mM and 16 mM zinc-containing agar media clearly distinguished between them (Figure 1). The order of hyphal elongation is ZNR-3 > ZNR-2 > ZNR-1 >>> wild type, at all three time points studied.

Zinc toxicity and uptake in liquid cultures were studied in all three strains and the data are shown in Figure 2. The results clearly show that all three strains are two to threefold more resistant to Zn^{2+} as compared with wild type *N. crassa*. However, uptake of Zn^{2+} by the three strains is lower in magnitude than that found with the parent *N. crassa*. Zinc uptake values, derived from the data of Figure 2 at half maximal growth (I_{50}), were (μg per 100 mg dry weight): wild, 550; ZNR-1, 530; ZNR-2, 550; and ZNR-3, 540, respectively. At 3 mM Zn^{2+}, the mycelial zinc content was (μg per 100 mg dry weight): wild, 550; ZNR-1, 260; ZNR-2, 250; and ZNR-3, 380. It should be noted that ZNR strains take up less Zn^{2+} than the parent *N. crassa*. Together with the patterns of Zn^{2+} uptake at the growth inhibitory concentration, these data show that there exists a partial transport for Zn^{2+} in the resistant strains; by implication, this is the underlying resistance mechanism.

Generally metal-resistant strains of microorganisms display cross-resistance to closely related metal ions, hence this aspect was also examined with Co^{2+} and Cu^{2+}. While ZNR-1 was not found to be cross-resistant to Co^{2+}, Cu^{2+} or Ni^{2+} (data not shown), ZNR-2 and ZNR-3 were fourfold (I_{50} 2 mM) and twofold (I_{50} 1 mM) resistant to Co^{2+} as compared with the wild type (I_{50} for Co^{2+} is 0.4 mM) (Figure 3). In the case of Cu^{2+}, only ZNR-2 showed twofold resistance (I_{50} 1.5 mM of Cu^{2+}) when compared with the wild type (I_{50} 0.7 mM of Cu^{2+}) (Figure 4). Cross-resistance to Ni^{2+} was not observed for any of the three strains (data not shown). Once again, a partial transport block for cobalt and copper ions is displayed in ZNR strains.

Metal toxicities in *N. crassa* are known to be reversed by physiologically important metal ions (Sastry *et al.* 1962a) and hence the effects of Mg^{2+} and Fe^{3+} in zinc toxicity were studied in all three strains. In each case an almost complete reversal of zinc toxicity was obtained by Mg^{2+} and Fe^{3+}. In the case of Mg^{2+} the ratio of Mg^{2+}: Zn^{2+} required for 90% reversal of inhibition for the wild type is 2:1, while in the ZNR strains it is about 1:1 (Table 3). In all cases suppression of Zn^{2+} uptake was observed. However, more efficient reversal of toxicity by Mg^{2+} was observed in ZNR strains. On the other hand, iron reverses the Zn^{2+} toxicity without suppression of Zn^{2+} uptake in all strains of *N. crassa* studied (Table 3) indicating that the mechanism involved is intracellular rather than at the level of the transport system.

As zinc toxicity is reversed by iron, the effect of Zn^{2+} toxicity on catalase and excretion of iron-binding siderophores (which are involved in iron metabolism) into growth medium was studied in wild type and ZNR strains. The results in Table 4 indicate that at I_{50} concentrations of Zn^{2+} the levels of catalase increased marginally in the wild type, while in the ZNR-2 strain they increased by 61%. However there was no change with respect to iron-binding siderophore levels excreted into the medium. This is in contrast with Co^{2+} toxicity in *N. crassa*, which severely impairs iron metabolism (Venkateswerlu & Sastry 1973). Since glutathione is known to be involved in cellular protection in metal toxicities, it was studied in Zn^{2+} toxicity. The results indicated that in the wild type there is a 30%

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

Strains	Concentration of Zn^{2+}	Number of subcultures (biweekly)		(I_{50})
		Zn^{2+} medium	Zn^{2+}-free medium	
Wild	Nil	–	20	3.0 ± 0.5
ZNR-1	8.0	20	20	8.0 ± 0.5
ZNR-2	16.0	20	20	8.0 ± 0.6
ZNR-3	*16.0	–	20	6.5 ± 0.5

N. crassa wild type was subcultured on agar medium containing 8 mM or 16 mM zinc. I_{50} = 50% growth inhibitory concentration of Zn^{2+}. Values shown are derived from graphs of four separate experiments (± standard deviation) (for further details, see text).

**N. crassa* conidia were treated with diethyl sulfate followed by selection on agar medium containing 16 mM Zn^{2+}.

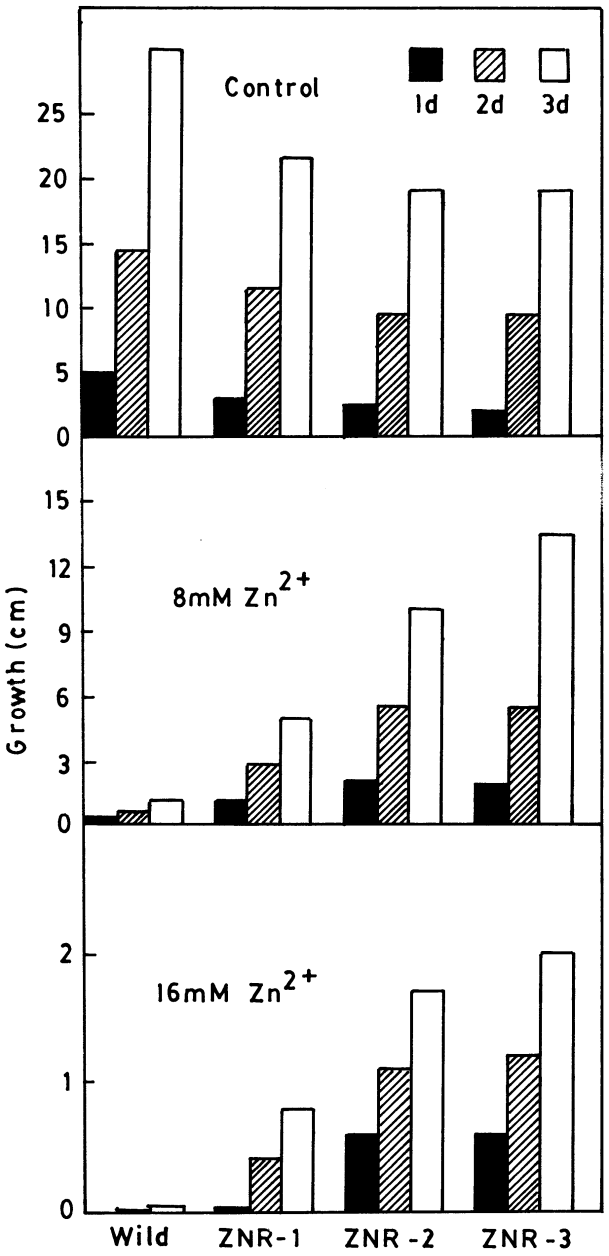

Figure 1. Hyphal elongation rates of *N. crassa* strains. Typical results of at least three experiments are shown (standard deviation ± 5%).

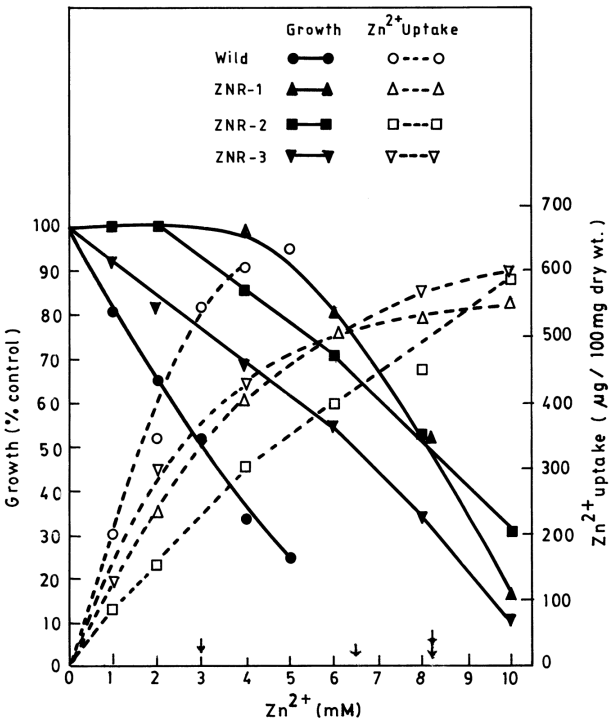

Figure 2. Zn^{2+} toxicity in *N. crassa* strains. *N. crassa* strains were grown in the indicated concentrations of Zn^{2+} for three days at 28 ± 1°C. Mycelial dry weights were recorded to measure growth and Zn^{2+} uptake was determined by atomic absorption spectrophotometry after acid digestion (for details see Materials and methods). Control (100% growth) mycelial weights (mg): wild, 42; ZNR-1, 39; ZNR-2, 40; ZNR-3, 40. Values shown are means derived from four separate experiments, each with duplicate samples (standard deviation ± 10%).

Table 2. Zn^{2+} resistance in single colony isolates of ZNR strains

Strains	I_{50} for Zn^{2+} (mM)
Wild	3.0 ± 0.4
ZNR-1	8.0 ± 0.4
ZNR-2	8.0 ± 0.6
ZNR-3	6.5 ± 0.6

N. crassa strains were plated on sorbose media to obtain colonial growth. Five single colonies from each strain were isolated and tested for Zn^{2+} resistance. The I_{50} (50% growth inhibitory concentration) values for Zn^{2+} represent the means of five isolates ± standard deviation (for further details, see text).

increase of glutathione levels at 6 mM zinc concentration, which is highly toxic for this organism. At 2 and 4 mM zinc concentration glutathione levels were relatively unaffected. However, in the case of the ZNR-2 strain at 4 mM zinc, which is relatively less toxic (10–15% inhibition) there was a fourfold increase in glutathione levels which thereafter decreased to two- to threefold at 8 mM zinc (the concentration which causes 50% growth inhibition (I_{50}) for the ZNR-2 strain). Glutathione, catalase and siderophore levels of ZNR-1 were similar to ZNR-2 values, while those of ZNR-3 were

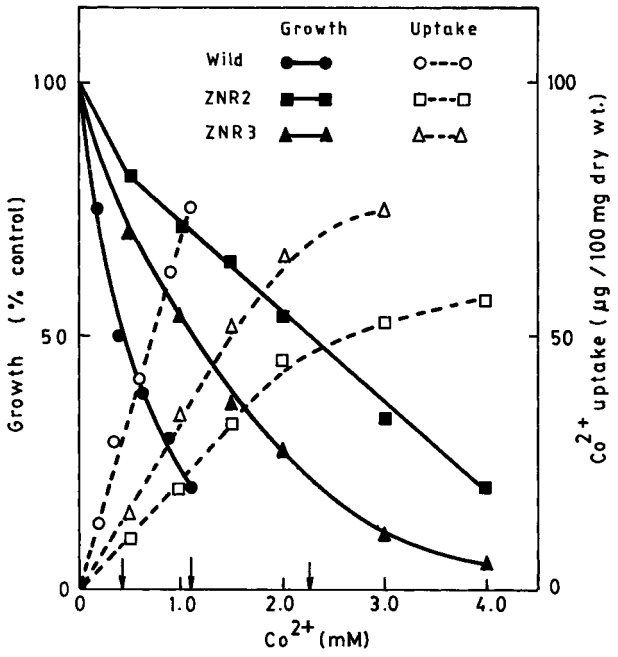

Figure 3. Co^{2+} toxicity in *N. crassa* strains. Experimental details were as described for Figure 2. Control (100% growth) mycelial weights (mg): wild, 42; ZNR-2, 41; ZNR-3, 40. Values shown are means derived from three separate experiments, each with duplicate samples (standard deviation ± 15%).

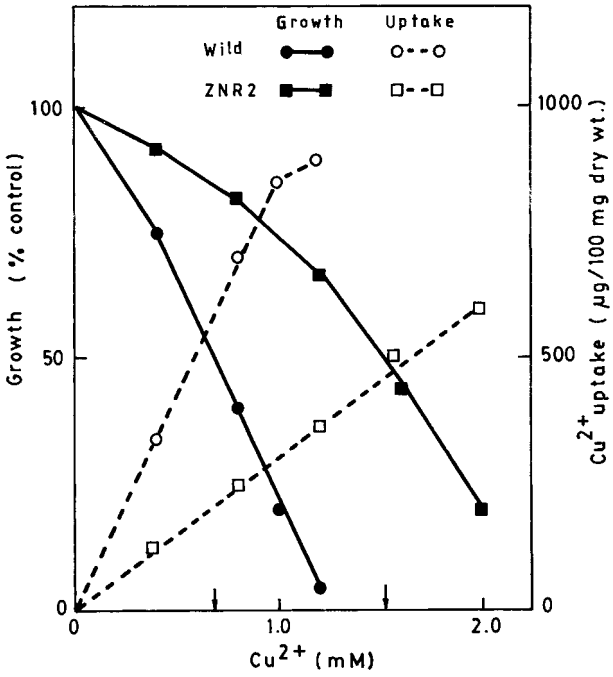

Figure 4. Cu^{2+} toxicity in *N. crassa* strains. Experimental details were as described for Figure 2. Control (100% growth) mycelial weights (mg): wild, 42; ZNR-2, 39. Values shown are means derived from three separate experiments, each with duplicate samples (standard deviation ± 5%).

Table 3. Reversal of zinc toxicity by Mg^{2+} and Fe^{3+} in *N. crassa*

Strain	Supplements to medium			Growth (mg dry wt)	Zn^{2+} uptake (µg per 100 mg dry wt)
	*Concentration of Zn^{2+} (mM)	Mg^{2+} (mM)	Fe^{3+} (mM)		
Wild type	nil	–	–	42	–
	3.0	–	–	20	550
	3.0	6.0	–	41	300
	3.0	–	10.0	46	520
ZNR-1	nil	–	–	40	–
	8.0	–	–	21	530
	8.0	6.0	–	40	308
	8.0	–	10.0	36	508
ZNR-2	nil	–	–	40	–
	8.0	–	–	20	502
	8.0	6.0	–	39	290
	8.0	–	10.0	37	525
ZNR-3	nil	–	–	43	–
	6.0	–	–	21	540
	6.0	6.0	–	42	250
	6.0	–	10.0	37	520

*Zn^{2+} concentration which results in 50% growth inhibition.
N. crassa strains were grown at their respective I_{50} concentrations of Zn^{2+} for 72 h along with Mg^{2+} or Fe^{3+}. (Minimal concentrations of Mg^{2+} and Fe^{3+} required for complete reversal of growth inhibition only are indicated.) Results shown are typical values from at least three experiments. Standard deviation ± 8%. (For further details, see text.)

comparable to wild type *N. crassa*; hence, separate data for these strains are not shown.

Zinc uptake by mycelia

A partial transport block for Zn^{2+} was noticed in ZNR strains in growth experiments (Figure 1), hence Zn^{2+} uptake was studied using pregrown mycelia. For these studies wild type, ZNR-2 and ZNR-3 strains were used. The kinetics of Zn^{2+} uptake (Figure 5A) clearly indicate a rapid phase of uptake (5 min), presumably due to cell surface binding, followed by a slower rate of uptake into the cells. To determine the actual Zn^{2+} uptake by cells, surface-bound metal was leached out with EDTA (10 mM) and the zinc remaining with mycelia was determined. The results are shown in Figure 5B. The rates of uptake calculated from this data are (μg per 100 mg per h): wild, 48; ZNR-2, 24; ZNR-3, 14. These data indicate that the amount of cell surface bound Zn^{2+} in wild type and ZNR strains is not very different (estimated to be 40 ± 7 μg per 100 mg dry weight), although the uptake rates are distinguishingly different. It should be noted from Figure 5 that zinc concentration at zero time (20 and 10 μg per 100 mg) is that which is associated with the control mycelia before and after EDTA treatment. Taken together, the above data suggest that a partial transport block due to reduced affinity of the zinc transporter system (presumably similar to the Mg^{2+} transporter) confers resistance to Zn^{2+} in *N. crassa* ZNR strains.

Genetic loci

Genetic crosses of resistant strains with linkage group tester strains indicated at least two separate loci for zinc resistance. In the case of ZNR-1 and ZNR-2 there appears to be a chromosomal aberration. Crosses with the tester *alcoy* strain indicated that resistance is associated with the *cot* marker (LG IV and V). Further crosses with *pdx-1 cot-1 cys-4* showed ambiguous recombination percentages between *pdx-1/cot-1* (37.5%) and *pdx-1/cys-4* (38%), indicating possible chromosomal aberration on LG IV. However, in the ZNR-3 strain the resistance mapped to LG I close to the *mt* locus (7%). The results of the cross between the tester strain *ser-3 un-16 acr-3* and ZNR-3 (Table 5) indicate that the zinc resistance is located to the left of *ser*.

Discussion

The work presented herein describes the isolation and characterization of three non-identical Zn^{2+} resistant mutants of *N. crassa*. Two different methods have been used to select Zn^{2+}-resistant mutants: (i) gradual adaptation of wild type *N. crassa* on toxic concentrations of Zn^{2+}; and (ii) mutagenesis with diethyl sulfate followed by selection of Zn^{2+}-containing media. Both methods yielded mutants with distinct non-overlapping zinc resistance loci. In ZNR-1 and ZNR-2 the resistance was linked to LG IV, while in ZNR-3 it was located to LG I. The interesting feature which emerges herein is that constant exposure to toxic levels of Zn^{2+} yields mutants with aberrations in chromosomes. Using a similar strategy three cadmium-resistant mutants of *N. crassa* had been isolated earlier; these have distinct cadmium resistance loci mapping at LG II and LG VII (Levine & Marzluf 1989). However, in this case the mechanism of resistance is not clear. Earlier studies from our laboratory have characterized three Ni^{2+}-resistant strains of *N. crassa* which displayed either a partial transport block (as in Ni^{R3}) or a hyperaccumulating character (as in Ni^{R1} and Ni^{R2}) (Maruthi Mohan & Sastry 1983). Based on these data the authors suggested more than one

Table 4. Effect of Zn^{2+} toxicity on glutathione, catalase and iron-binding siderophores

Zn^{2+} (mM)	Glutathione[1]		Catalase[2]		XFe[3] (O.D. at 440 nm)	
	Wild	ZNR-2	Wild	ZNR-2	Wild	ZNR-2
Control	6.5	7.4	180	172	0.047	0.077
2.0	5.7	6.5	192	186	0.043	0.079
4.0	7.8	30.7	198	210	0.037	0.078
6.0	8.7	25.0	205	254	0.043	0.068
8.0	–	17.9	–	278	–	0.071

[1]Glutathione (oxidized plus reduced) is expressed in nmoles per mg of protein.

[2]Catalase activity units are micromoles of H_2O_2 decomposed per mg of protein in five minutes. Values shown are averages of triplicates from three experiments (standard deviation up to ± 10%).

XFe = iron-binding siderophores.

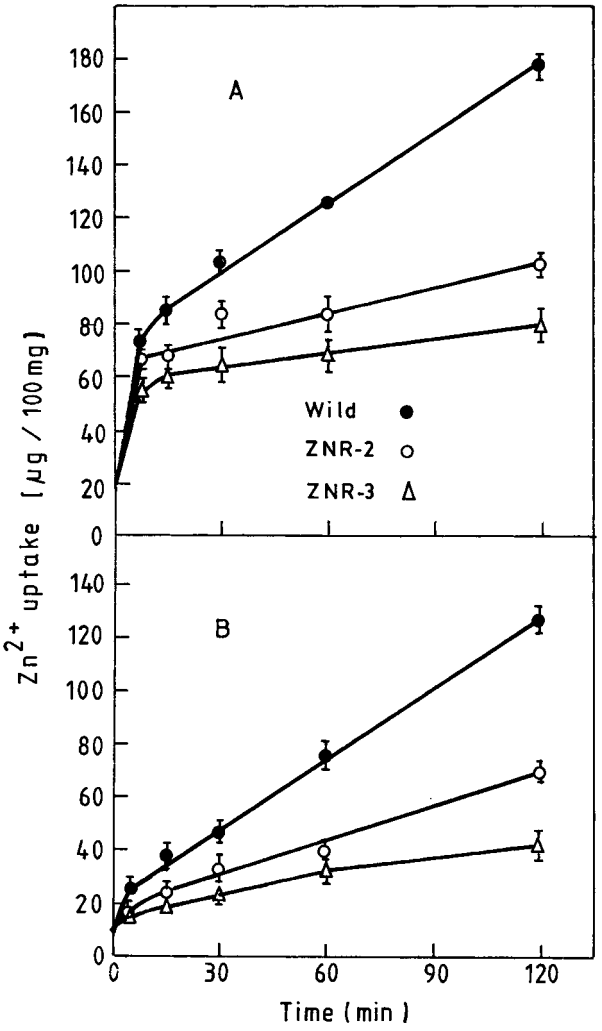

Figure 5. Kinetics of Zn^{2+} uptake by *N. crassa* strains. Mycelia (3 day old) were floated in 10 ml basal medium (without Mg^{2+}) containing Zn^{2+} (1 mM) and incubated on a rotary shaker (100 rpm) at 28°C. (A) The mycelia were washed with distilled water, dried and the Zn^{2+} concentration was determined by atomic absorption spectrophotometry after acid digestion. (B) After washing with distilled water the mycelial surface-bound Zn^{2+} was leached with 10 ml EDTA (10 mM) for 5 min and the Zn^{2+} remaining was determined. Values shown are derived from three separate experiments, each with duplicate samples. Vertical bars represent standard deviations.

locus for these two types of strains. Later studies have located the resistance loci for cobalt and nickel to LG III in a cobalt-resistant strain of *N. crassa*, which is also cross-resistant to nickel (Wilson *et al.* 1992). The present findings are in agreement with the above studies in that more than one genetic locus could be involved in metal resistance in *N. crassa*.

Table 5. Mapping of *znr* locus of *N. crassa* ZNR-3 strain

Parental				
	znr	+	+	66
	+	*ser*	*mt*	*un*	28
Single crossovers				
	znr	*ser*	*mt*	*un*	2
	+	+	+	+	4
	+	*ser*	+	+	2
Double crossovers				
	znr	*ser*	+	*un*	1
	+	+	*mt*	*un*	1
Simple recombination percentages				
	znr-ser	7.7	*znr-mt*	7.7
	znr-un	8.7	*ser-mt*	3.8
	ser-un	2.9	*mt-un*	1.0

Analysis of progeny (104) from a cross of ZNR-3 (A) × *ser-3 un-16 acr-3*(a) (acriflavin resistance is not scored).

It should be noted that the mutations at both loci resulted only in reduced uptake of Zn^{2+} by mycelia, though at different rates. Further, both ZNR-2 and ZNR-3 strains show different levels of resistance to cobalt but ZNR-2 alone shows cross-resistance to copper, once again due to reduced uptake of these ions. Hence, it appears that metal resistance in general, and Zn^{2+} resistance in particular, is a complex phenomenon in *N. crassa* involving different non-overlapping genetic loci.

In the present study a partial transport block appears to play a major role in the Zn^{2+} resistance. Although all three resistance strains display non-identical characteristics such as hyphal elongation rates and cross-resistance patterns, the overall Zn^{2+} uptake is only half that of parental *N. crassa*; this could explain the two- to threefold resistance of the ZNR strains in liquid cultures. Whether this is a character expressed only during growth of *N. crassa* over a three day period or an inherent ability was studied using pregrown mycelia. The kinetics of Zn^{2+} uptake (Figure 5A, B) clearly indicated that with 1 mM Zn^{2+} in the medium the rapid phase of adsorption resulted in almost identical quantities of Zn^{2+} binding to mycelial surface of wild type and ZNR strains. However, the slower phase of Zn^{2+} accumulation (after the rapid initial phase) clearly showed that uptake rates of ZNR-2 and ZNR-3 were distinctly lower than that of the wild type (Figure 5B). These results suggest that there could be a change in the affinity of the Zn^{2+} transporting system of the ZNR strains, caused by mutation(s) affecting genes which code for one or other of the

different subunits of the multisubunit transporting system(s).

Toxicity of Co^{2+}, Ni^{2+} and Zn^{2+} has been studied earlier in *N. crassa* (Sastry *et al.* 1962a). These workers showed that Mg^{2+} causes the reversal of growth inhibition due to the above ions by suppression of their uptake. Cobalt transport in *N. crassa* has been shown to be via an energy-dependent transporter presumably similar to Mg^{2+} transporters (Venkateswerlu & Sastry 1970). However, cobalt uptake in a cobalt-resistant strain was shown to be by a passive process and was not suppressed by Mg^{2+} (Venkateswerlu & Sastry 1979). In the present study Mg^{2+} reversed Zn^{2+} toxicity by suppression of uptake in both wild type and resistant strains; however, the ability of Mg^{2+} to reverse the toxicity is doubled in resistant strains when compared with the wild type. Similarly, Fe^{3+} also reverses the zinc toxicity but without suppression of its uptake. The conditioned iron deficiency due to cobalt ions results in reduction of catalase activity and excretion of iron-binding siderophores (Healy *et al.* 1955, Padmanabhan & Sarma 1964, 1966). In the present study there was a marginal increase in catalase activity in the wild type, and about a 60% increase in the ZNR-2 strain, while levels of iron-binding siderophores remained unaltered. These results confirm the earlier studies that Zn^{2+} toxicity does not cause iron deficiency (Healy *et al.* 1955). However, the higher levels of catalase in the ZNR-2 strain compared with the wild type at their respective I_{50} concentrations of Zn^{2+} indicates a possible role in detoxification mechanisms, as this has been implicated in reducing the toxic oxygen radicals generated during metal toxicity in animal systems (Lee & Ho 1994).

Glutathione is suggested to be involved in cellular protection during early Cd^{2+} exposure to mammalian cells (Singhal *et al.* 1987). Glutathione and metallothionein have been implicated in detoxification of copper in *N. crassa* (Germann & Lerch 1987). However, in the plant system it was shown that the GSH concentration decreases in Cd^{2+} toxicity and is also present in low levels in a Cd-tolerant strain of *Datura innoxia* compared with a sensitive strain (Delhaize *et al.* 1989). This was suggested to be due to depletion of glutathione pools for the synthesis of phytochelatins, for which glutathione is a precursor. In the present study there was only marginal increase of glutathione in the wild type *N. crassa* but a threefold increase in ZNR-2 strain, suggesting a possible role for glutathione in Zn^{2+} resistance. A phytochelatin from *N. crassa* was isolated from cultures grown in the presence of cadmium (Kneer *et al.* 1992); however, its role in resistance was not studied. As in the case of ZNR-2 *N. crassa*, methyl mercury-resistant rat pheochromocytoma cell lines, which accumulate lower levels of methyl mercury, showed a fourfold increase in glutathione levels compared with the sensitive cells (Miura *et al.* 1994). Further studies are required to interpret the role of glutathione and catalase in zinc resistance in *N. crassa*.

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