Interrelationships in trace-element metabolism in metal toxicities in nickel-resistant strains of *Neurospora crassa*

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Three different Ni^{2+} -resistant strains of *Neurospora crassa* ($\text{Ni}^{\text{R}1}$, $\text{Ni}^{\text{R}2}$ and $\text{Ni}^{\text{R}3}$) have been isolated. All are stable mutants and are fourfold more resistant to Ni^{2+} than the parent wild-type strain. $\text{Ni}^{\text{R}1}$ and $\text{Ni}^{\text{R}2}$ are also sixfold more resistant to Co^{2+} , whereas $\text{Ni}^{\text{R}3}$ is only twice as resistant to Co^{2+} ; the former two are also twofold more resistant to Zn^{2+} , but $\text{Ni}^{\text{R}3}$ is not. These three strains also differ in sensitivity to Cu^{2+} . Toxicities and concomitant accumulation patterns of Ni^{2+} , Co^{2+} and Cu^{2+} have been examined in these strains. $\text{Ni}^{\text{R}1}$ and $\text{Ni}^{\text{R}2}$, despite quantitative individual differences, generally accumulate very high amounts of Ni^{2+} and Co^{2+} , and Mg^{2+} reverses the toxicities of these two ions by different mechanisms; Ni^{2+} uptake is suppressed, but not that of Co^{2+} . In $\text{Ni}^{\text{R}3}$, Mg^{2+} controls uptake of both Ni^{2+} and Co^{2+} . Studies indicate that two kinds of Ni^{2+} -resistant strains of *N. crassa* exist; one kind is resistant because it can tolerate high intracellular concentrations of heavy-metal ions, whereas the other is resistant because it can control metal-ion accumulation.

Metal-ion-resistant strains of fungi (Yanagishima *et al.*, 1949; Jurkowska, 1952; Perlman & O'Brien, 1954; Laskowski, 1956) and bacteria (Webb, 1970; Silver *et al.*, 1972; Baldry *et al.*, 1977; Mandelbaum & Kishuik, 1978; Pickett & Dean, 1979; Khazaeli & Mitra, 1981) have been obtained by repeatedly subculturing parent organisms on media containing toxic concentrations of heavy-metal ions. However, mechanisms underlying metal toxicities in such strains have not been studied in detail (Ashida, 1965; Ross, 1975).

Toxicities of Co^{2+} , Ni^{2+} and Zn^{2+} have been examined in *Neurospora crassa* Em 5297a (Healy *et al.*, 1955; Sivarama Sastry *et al.*, 1962b) and in the first cobalt-resistant strain (*N. crassa* Co^{R}) isolated previously in this laboratory (Venkateswerlu & Sivarama Sastry, 1973). In the parent wild-type strain (Em), toxicity of Co^{2+} induces an iron deficiency, and consequent effects on catalase (Healy *et al.*, 1955) and on growth (Sivarama Sastry *et al.*, 1962b) are reversed by supplemented Fe^{3+} , the latter without attendant suppression of Co^{2+} uptake. In contrast, such Co^{2+} – Fe^{3+} antagonism does not obtain in *N. crassa* Co^{R} , and the mechanism of resistance involves an alteration in its iron metabolism to insensitivity to Co^{2+} inhibition. Uptake of Co^{2+} in the Co^{R} strain also differs in several respects (Venkateswerlu & Sivarama Sastry, 1979) from that in the Em strain (Venkateswerlu & Sivarama Sastry, 1970). However, in both strains, growth inhibition

due to excess Co^{2+} , Ni^{2+} and Zn^{2+} is counteracted by Mg^{2+} , with parallel suppression of toxic-ion uptake, as in the case of *Aspergillus niger* (Adiga *et al.*, 1961) and in several bacteria (Abelson & Aldous, 1950).

N. crassa Co^{R} was found to be equally cross-resistant to Ni^{2+} , but not to Zn^{2+} or Cu^{2+} . Hence we decided to isolate strains specifically resistant to Ni^{2+} and examine such aspects of metal toxicities. Rather unexpectedly, three strains have been obtained, equally resistant to Ni^{2+} , but differing in cross-resistance to Co^{2+} , Zn^{2+} and Cu^{2+} . Also, two of these are notable in that Co^{2+} toxicity is reversed by Mg^{2+} without suppression of Co^{2+} uptake. In the present study these features of the Ni^{2+} -resistant strains have been examined in detail.

Materials and methods

Chemicals

Metal salts used were $\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. All chemicals were analytical-grade products from Sarabhai M. Chemicals Ltd., Baroda, India. Fe^{3+} –EDTA monosodium salt was obtained from BDH.

Media, growth and analytical procedures

Neurospora crassa Em 5297a wild-type (obtained from the Fungal Genetics Stock Centre,

Humbolt State University Foundation, Arcata, CA, U.S.A.), as well as the nickel-resistant strains were grown on 10 ml of basal medium in 50-ml conical flasks for 72 h at $30 \pm 1^\circ\text{C}$ (Venkateswerlu & Sivarama Sastry, 1973). Metal ions were supplemented in the basal medium as sulphates to provide the required concentration as indicated below. Fe^{3+} was supplemented as Fe^{3+} -EDTA complex. At the end of the growth period, the mycelia were harvested, washed thoroughly with glass-distilled water, dried overnight (60 – 80°C) in an oven and weighed to measure growth.

Isolation of nickel-resistant strains of *Neurospora crassa*

General procedures were as described previously (Venkateswerlu & Sivarama Sastry, 1973). Spores from a 7-day-old culture of *N. crassa* Em 5297a (wild-type) were transferred on to 3% agar slants made with basal medium with the desired concentration of Ni^{2+} (between 4 and 16 mM; see below). Growth was initially slow. After satisfactory growth was obtained (2–3 weeks), repeated transfers resulted in progressively better growth, and later subculturing was possible in 7–10 days. After seven to ten such subcultures, the organism was tested for resistance to Ni^{2+} by measuring growth as a function of medium Ni^{2+} concentration. When no further increase in Ni^{2+} resistance was evident, as shown by the I_{50} values (metal-ion concentration needed for half-maximal growth), each strain was purified to genetic homogeneity as described previously (Venkateswerlu & Sivarama Sastry, 1973), by plating out on agar plates containing basal medium with the concentration of Ni^{2+} employed in 'training', sucrose (0.2%), sorbose (1.5%) and nicotinamide (0.001%). From the colonies obtained (7–10 days), several were picked out and subcultured, and each single-spore isolate checked for Ni^{2+} resistance. After two platings, each isolate was found to be genetically homogeneous.

Three different Ni^{2+} -resistant strains of *N. crassa* were obtained; these will be designated as Ni^{R1} , Ni^{R2} and Ni^{R3} respectively. The experimental protocol employed is summarized in Table 1.

Stability of resistance

Subculturing was done on Ni^{2+} -free basal media, containing either a normal ($50 \mu\text{g/ml}$) or minimal ($5 \mu\text{g/ml}$) concentration of Mg^{2+} and the strains periodically checked for metal resistance. Resistance patterns were fully stable.

Metal analysis

Metal content was determined after subjecting mycelia (30–50 mg dry wt.) to the wet-digestion procedure described previously (Sivarama Sastry *et al.*, 1962a). The residue was dissolved in 0.5 M-

HCl and portions were taken for determination of Ni^{2+} with dimethylglyoxime (Sandell, 1959), Co^{2+} with nitroso-R salt (Venkateswerlu & Sivarama Sastry, 1973), Cu^{2+} with oxalaldehyde (Gunnar Gran, 1956) and Zn^{2+} by Atomic-absorption measurements on a Varian Techtron Model 1000 instrument. Recoveries were checked by adding a known amount of metal salt to a control mycelium (grown on minimal basal medium alone) and subjecting it to the wet digestion above-mentioned, and were invariably between 95 and 100%.

Results

Characteristics of the Ni^{2+} -resistant strains

All the Ni^{2+} -resistant strains were stable and grew like the Em strain except that mycelial weights reached in 72 h on minimal medium were generally a little lower (values are indicated in legends to Figs. 1–4). Resistance was maintained indefinitely on subculturing in media with or without Ni^{2+} (see Table 1) irrespective of the medium Mg^{2+} level, in contrast with metal-resistant strains of bacteria, which revert to sensitivity on low- Mg^{2+} media (Webb, 1970).

Metal-ion toxicity in the Ni^{2+} -resistant strains

The effects of increasing concentrations of Ni^{2+} , Co^{2+} , Zn^{2+} and Cu^{2+} on the growth of the Ni^{2+} -resistant and Em strains and concomitant metal-ion accumulation are shown in Figs. 1–4.

Table 1. Details of the isolation procedure for Ni^{2+} -resistant strains of *Neurospora crassa*
For experimental details, see the text.

Strain	[Ni^{2+}] for 'training' (mM)	No. of subcultures		
		On Ni^{2+} medium*	Stability of resistance†	Stability on Ni^{2+} -free medium‡
Ni^{R1}	4	20	84	65
Ni^{R2}	8	20	60	25
Ni^{R3}	8	8	60	23
		followed by 16	followed by 40	

* Intervals of 10 days for Ni^{R1} and Ni^{R2} ; 12–15-day intervals for Ni^{R3} .

† Intervals of 10 days for Ni^{R1} and Ni^{R2} ; 15-day intervals for Ni^{R3} ; the concentration of Ni^{2+} in the medium was the same as in training, and resistance to both Ni^{2+} and Co^{2+} was unchanged over the number of subcultures indicated.

‡ Number of weekly subcultures on Ni^{2+} -free medium during which, to date, resistance to Ni^{2+} as well as to Co^{2+} was maintained unaltered.

Fig. 1. Ni^{2+} toxicity in *N. crassa* Em 5297a and in Ni^{2+} -resistant strains

For experimental details, see the text. Growth is shown by closed symbols; uptake is shown by open symbols. Dry weights of control mycelia (no Ni^{2+}) were (in mg): Em, 45; Ni^{R1} , 30; Ni^{R2} , 29; Ni^{R3} , 28.

Fig. 3. Zn^{2+} toxicity in *N. crassa* Em 5297a and in Ni^{2+} -resistant strains

For experimental details, see the text. Growth is shown by closed symbols; uptake is shown by open symbols. Dry weights of control mycelia (no Zn^{2+}) were (in mg): Em, 47.5; Ni^{R1} , 30; Ni^{R2} , 30; Ni^{R3} , 27.

Fig. 2. Co^{2+} toxicity in *N. crassa* Em 5297a and in Ni^{2+} -resistant strains

For experimental details, see the text. Growth is shown by closed symbols; uptake is shown by open symbols. Dry weights of control mycelia (no Co^{2+}) were (in mg): Em, 45; Ni^{R1} , 29; Ni^{R2} , 29; Ni^{R3} , 27.

Fig. 4. Cu^{2+} toxicity in *N. crassa* Em 5297a and in Ni^{2+} -resistant strains

For experimental details, see the text. Growth is shown by closed symbols; uptake is shown by open symbols. Dry weights of control mycelia (no Cu^{2+}) were (in mg): Em, 46; Ni^{R1} , 30; Ni^{R2} , 31; Ni^{R3} , 28.

Whereas the three Ni^{2+} -resistant strains of *N. crassa* are all about 4-fold more resistant to Ni^{2+} compared with the parent Em strain, they differ in other respects; I_{50} concentrations of Co^{2+} are 6-fold higher for Ni^{R1} and Ni^{R2} , but only 2-fold for Ni^{R3} ,

and likewise the I_{50} of Zn^{2+} is 2-fold higher for Ni^{R1} and Ni^{R2} , but is unchanged for Ni^{R3} .

Accumulation patterns for Ni^{2+} and Co^{2+} show that Ni^{R1} and Ni^{R2} are non-identical. Figs. 1–4 show that, generally, Ni^{R1} and Ni^{R2} consistently accumu-

Fig. 5. Effect of Mg^{2+} on Ni^{2+} toxicity in *N. crassa* Ni^{R1} , Ni^{R2} and Ni^{R3} .

For experimental details, see the text. Growth is shown by closed symbols; uptake is shown by open symbols. The toxic concentration of Ni^{2+} used was 4 mM in all the strains. The concentration of Mg^{2+} is that supplemented in addition to the level in the medium (2 mM). Weights of control mycelia (mg): Ni^{R1} , 30; Ni^{R2} , 29; Ni^{R3} , 28.

ate very high intracellular concentrations of toxic metal ions, in contrast with Em and Ni^{R3} . Further, one major difference between Ni^{R1} and Ni^{R2} is that, of the two, Ni^{R2} takes up more Ni^{2+} and Co^{2+} throughout the concentration range of these cations than does Ni^{R1} ; this feature is not, however, exhibited in respect of Zn^{2+} and Cu^{2+} . Metal resistance of Ni^{R1} and Ni^{R2} is evidently due to the ability of their metabolic machinery to tolerate high intracellular levels of toxic cations; in contrast, Ni^{R3} owes its resistant characteristics to its capacity to partially restrain metal-ion uptake.

N. crassa Co^R had been found to be unexpectedly twice as sensitive to Cu^{2+} as the Em strain (Venkateswerlu & Sivarama Sastry, 1973). The present data show this to be true also of the Ni^{2+} -resistant strains generally; Ni^{R3} , which assimilates less Cu^{2+} throughout the toxicity range than even the Em strain, is, however, less sensitive to this cation than Ni^{R1} or Ni^{R2} ; characteristic differences are emphasized by their responses to a level of 0.4 mM- Cu^{2+} in the medium (Fig. 4).

Reversal of metal-ion toxicity by Mg^{2+}

Since Mg^{2+} counteracts metal toxicities in micro-organisms by controlling uptake of toxic ions, this aspect has been examined in the Ni^{2+} -resistant strains. This general mechanism prevails in toxicity

Fig. 6. Effect of Mg^{2+} on Co^{2+} toxicity in *N. crassa* Ni^{R1} and Ni^{R2} .

For experimental details, see the text. Growth is shown by closed symbols; uptake is shown by open symbols. The toxic concentration of Co^{2+} used was 6 mM in both the strains. The concentration of Mg^{2+} is that supplemented in addition to the level in the medium (2 mM). Weights of control mycelia (mg): Ni^{R1} , 31; Ni^{R2} , 30.

Fig. 7. Effect of Mg^{2+} on Co^{2+} toxicity in *N. crassa* Ni^{R3} .

For experimental details, see the text. Growth is shown by closed symbols; uptake is shown by open symbols. The toxic concentration of Co^{2+} used was 2 mM. The concentration of Mg^{2+} is that supplemented in addition to the level in the medium (2 mM). Weight of control mycelia (mg): 26.

due to Ni^{2+} in all strains (Fig. 5), in that due to Zn^{2+} in Ni^{R1} and Ni^{R2} (Fig. 8), and in that due to Co^{2+} in Ni^{R3} (Fig. 7). However, in Ni^{R1} and Ni^{R2} , Mg^{2+} reverses growth inhibition caused by Co^{2+} without the concomitant suppression of toxic-ion accumulation (Fig. 6). This is extremely unusual and is reminiscent of a similar kind of interaction between Fe^{3+} and Co^{2+} in the Em strain (Sivarama Sastry *et al.*, 1962b).

Fig. 8. Effect of Mg^{2+} on Zn^{2+} toxicity in *N. crassa* Ni^{R1} and Ni^{R2}

For experimental details, see the text. Growth is shown by closed symbols; uptake is shown by open symbols. The toxic concentration of Zn^{2+} used was 6 mM in both the strains. The concentration of Mg^{2+} is that supplemented in addition to the level in the medium (2 mM). Weights of control mycelia (mg): Ni^{R1} , 30; Ni^{R2} , 29.

Effects of Fe^{3+} on metal toxicities

In other experiments it was found that Fe^{3+} , up to the maximal concentration tested (10 mM), has no beneficial influence on Ni^{2+} and Co^{2+} toxicities in any of the Ni^{2+} -resistant strains.

Discussion

The Ni^{2+} -resistant strains studied herein are genetically homogeneous and are stable mutants that do not revert to a more-sensitive form, in contrast with metal-resistant bacterial strains (Webb, 1970).

Comparison with *N. crassa* Co^R , obtained by adaptation on high- Co^{2+} media (Venkateswerlu & Sivarama Sastry, 1973), which is equally resistant to Co^{2+} and Ni^{2+} , shows that the Ni^{2+} -resistant strains are quite different from it. Whereas they are all 4-fold more resistant to Ni^{2+} , tolerances to Co^{2+} are not identical (Fig. 2). Co^R is not resistant to Zn^{2+} , whereas Ni^{R1} and Ni^{R2} (but not Ni^{R3}) are also 2-fold more tolerant to Zn^{2+} . Also whereas Ni^{R1} and Ni^{R2} , like Co^R , are twice as sensitive to Cu^{2+} , Ni^{R3} is a little less so. Careful analysis of responses to these various toxic ions shows that Ni^{R1} and Ni^{R2} are

similar, though non-identical, and that Ni^{R3} differs from both. Mycelial accumulation of Ni^{2+} and Co^{2+} , but not of Zn^{2+} and Cu^{2+} , is always higher in Ni^{R2} than in Ni^{R1} .

A comparison of the influence of Mg^{2+} and Fe^{3+} on metal toxicities in the three Ni^{2+} -resistant strains with corresponding effects of these ions in *Em* (Sivarama Sastry *et al.*, 1962b) and in *N. crassa* Co^R (see above) reveals the differences in metal-ion interactions obtaining in these strains. As in Co^R (but not in *Em*), Fe^{3+} has no beneficial effect on Ni^{2+} and Co^{2+} toxicities in Ni^{2+} -resistant strains. Mg^{2+} controls accumulation of Ni^{2+} and Zn^{2+} in all strains and of Co^{2+} in Ni^{R3} , and this is indicative of competition between Mg^{2+} and these toxic ions at the level of uptake. However, the unusual counteraction of Co^{2+} toxicity by Mg^{2+} in Ni^{R1} and Ni^{R2} without such control of Co^{2+} uptake suggests that, in these strains, Co^{2+} is not transported by the system that handles Mg^{2+} ; presumably competitive interactions between Mg^{2+} and Co^{2+} are restricted to specific intracellular sites.

Again, in Ni^{R1} and Ni^{R2} , although growth inhibition progressively increases with increase in the level of Co^{2+} in the medium, uptake is already nearly maximal at about 3 mM- Co^{2+} . This also suggests that the bulk of the Co^{2+} accumulation here observed may not necessarily be at sites at which Co^{2+} exerts its toxic effects intracellularly, and that only a fraction of the Co^{2+} taken up is at locations at which it is inhibitory. Progressive increase in association of Co^{2+} with such loci could be masked by the overall massive uptake, and consequently displacement from such sites by Mg^{2+} would be necessarily be evident, since total mycelial Co^{2+} content remains unaffected. In contrast, in Ni^{R3} , there is a general Mg^{2+} - Co^{2+} antagonism at all levels.

The two general mechanisms of adaptation to toxic metals in micro-organisms are: (i) by modification of cellular machinery to tolerance to high intracellular toxic-ion concentrations, and (ii) by alteration of permeability, leading to partial or total exclusion of metal ions (Ashida, 1965; Ross, 1975). The most significant feature displayed by the Ni^{2+} -resistant strains is that whereas Ni^{R1} and Ni^{R2} employ the first mode, Ni^{R3} utilizes the second alternative. It is noteworthy that the strains have been obtained by varying the Ni^{2+} concentration in media used for adaptation, Ni^{R3} resulting only from media with the highest Ni^{2+} level.

There are other comparable instances of resistant strains of micro-organisms exhibiting some of the features displayed by the strains studied in the present paper. Exposure of *Escherichia coli* to one alkylating agent results in an adaptive response, culminating in strains able to resist several similar compounds (Jeggo *et al.*, 1977, 1978; Jeggo, 1979). 5-Fluorodeoxyuridine uptake is decreased in some

fluorodeoxyuridine-resistant mutants of *Pediococcus cerevisiae*, but increased in others (Mandelbaum & Kishuik, 1978). Graded resistance to Cd^{2+} and Zn^{2+} , dependent on the 'training' concentration of heavy-metal ions, is known in *Pseudomonas* species (Pickett & Dean, 1979). Stepwise acquisition of resistance to oxacillin, involving several genes, has been reported in *Streptococcus pneumoniae* (Zig-helboim & Tomasz, 1979). In *N. crassa*, the Co^R strain described by us previously was the first of its kind (see above). Present results with the Ni^{2+} -resistant strains show that the consequences of using Ni^{2+} in place of Co^{2+} in media used for obtaining metal-resistant mutants are quite different.

It is tempting to speculate that Ni^{2+} is also possibly mutagenic in *N. crassa* and leads to resistant mutants such as those obtained with alkylating agents in *E. coli* (see above) and fluoro-deoxyuridine in *P. cerevisiae* (see above), with the additional feature that the nature of mutants obtained is Ni^{2+} -dose-dependent.

At any rate, the present studies indicate that more than one genetic locus is associated with Ni^{2+} resistance in *Neurospora crassa*. At least two sites would be needed to explain the features of Ni^{R1} , Ni^{R2} and Ni^{R3} . The mutation that leads to Ni^{R1} and Ni^{R2} is possibly different from that which leads to Ni^{R3} , which could involve a different locus, since the latter mutant exhibits low permeability to metal ions. In this context, it is noteworthy that Laskowski (1955) had previously isolated, similarly, different Li^+ -resistant mutants of *Saccharomyces* and had found four different loci associated with Li^+ resistance. The present study is the first to suggest that multiple loci may be associated with heavy-metal resistance in *Neurospora crassa*.

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