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# Isolation and Characterization of Strontium Resistant Mutant of *Neurospora crassa*

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## ABSTRACT

Strontium is a trace element that has no known essential biological role. The divalent cation, $Sr^{2+}$, displays physicochemical properties similar to those of the abundant and biologically essential cations $Ca^{2+}$ and $Mg^{2+}$. Abundant release of strontium is known to be the effect of radionuclide explosions. As there is no special strain for the removal of strontium from the effected environments, a strontium-resistant strain of *Neurospora crassa* (SRR) was obtained by repeated subculturing of the wild type on strontium containing agar medium. *Neurospora crassa* SRR was obtained by repeated subculturing, then the stability of the mutant was compared with wild type in terms of uptake, cellular partitioning and cross-resistant to other metals. *Neurospora crassa* SRR is twelve fold more resistant to strontium ions compared with the wild type. Resistance was stable on repeated subculturing of SRR on strontium-free media. *Neurospora crassa* SRR is also cross-resistant to calcium (fourfold). Higher concentrations of calcium ions are required to reverse growth inhibition due to strontium toxicity in *Neurospora crassa* SRR, compared with the wild type. The mechanism of strontium uptake is shown to be primarily due to binding of strontium to mycelia and cell walls. Efflux of mycelial strontium was also observed in wild type and strontium-resistant *Neurospora crassa*. The characteristics of SRR in comparison with wild type *Neurospora crassa* are discussed in relation to the mechanisms of strontium resistance.

**Key words:** Strontium binding, strontium-resistant, strontium uptake, metal-resistant, *N. crassa*

## INTRODUCTION

Microorganisms are intimately involved in metal biogeochemistry with a variety of processes. Recent environmental pollution with anthropogenic sources of metals has increased the need for research concerning microbial resistance as well as remediation. Metal resistance has been extensively studied in the bacterial system (Silver and Phung, 2009). 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 (Azevedo *et al*., 2009; Gadd, 2010; Pocsi, 2011; Antonella *et al*., 2013). Metallothioneins mainly serve for protective function, limiting the intracellular concentration of reactive heavy metal ions and shielding cellular structures from the harmful influences of toxic metals such as cadmium, mercury, platinum, bismuth, silver and gold. Reduction of toxic metal ions is more widely reported in bacteria than in fungi. It was also shown in yeasts *Candida albicans* and *Saccharomyces cerevisiae*. Similarly, reduction of $Ag^{2+}$ to $Ag^{0}$, $Cu^{2+}$ to $Cu^{+}$, $Te^{2+}$ to $Te^{0}$ and $Se^{2+}$ to $Se^{0}$ was reported (Ehrlich, 1997; Beveridge and Doyle, 1989). A number of

cobalt, nickel and zinc resistant strains of *Neurospora crassa* are known at present. In several of these, metal ion uptake patterns are altered such that, either there is a partial transport block, possibly due to altered specificities of the carrier system, or a diminished binding of the toxic metal ions to the cell wall or even hyperaccumulation in some cases. Cobalt resistance was also studied in wall-less mutant of *Neurospora crassa* wherein the effects of cell wall will not influence the metal ion effects (Rajyalaxmi *et al*., 2003).

The alkaline earth metal $Sr^{2+}$ is a common ground water contaminant found at DOE field sites. Strontium (Sr) is found in the environment in a variety of different compounds and is chemically analogous to calcium; hence, there is a tendency for $Sr^{2+}$ to be incorporated into bone (Brown *et al*., 2006). However, little is known about the biological impact of $Sr^{2+}$ toxicity on microbial systems or the underlying mechanisms enabling resistance against $Sr^{2+}$ ions.

The potential use of metal-resistant microorganisms in the treatment of heavy metal contaminated wastewater plants has become more important (Shakibaie *et al*., 2008). Different biomass types, such as bacteria, fungi and algae, have been screened and studied extensively by many authors over the past decades with the aim of identifying highly efficient metal removal biological systems (Kapoor and Viraraghavan, 1995; Vieira and Volesky, 2000; Herrero *et al*., 2005). In the present study we describe the isolation and characterization of strontium resistant mutant of *Neurospora crassa*.

**Media and growth conditions:** *Neurospora crassa* 74-OR23-IVA (obtained from Fungal Genetics Stock Center (FGSC), Kansas city, USA) and the strontium-resistant strain were grown in 10 mL basal medium in 50 mL conical flasks for 72 h at 28±1°C as described earlier (Rashmi *et al*., 2014). Metal ions were supplemented in the basal medium to provide the required concentrations.

To obtain colonial growth, glucose was replaced by 1% sorbose and 0.2% sucrose. Fifty percent growth Inhibitory Concentration ($IC_{50}$) values for metal ions were derived from graphical plots of growth verses metal ion concentration.

**Isolation of strontium-resistant strain of *Neurospora crassa*:** The general procedures followed were as described by Sajani and Mohan (1997) and Rashmi *et al*. (2014). Spores from 7 day old cultures of *Neurospora crassa* 74-OR23-IVA (wild type) were transferred onto 3% agar slants made with basal medium containing the desired concentration of strontium (4, 8, 16, 32 and 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 strontium by measuring growth as a function of strontium concentration in the medium. When no further increase in strontium resistance was noted, the conidia were plated on 1% sorbose agar medium containing the same level of strontium employed for adaptation, sucrose (0.2%) and nicotinamide (0.001%). Several colonies were subcultured and strontium resistance concentration of strontium resulting in 50% growth Inhibition ($I_{50}$) was determined.

**Strontium uptake:** Metal ion uptake was studied by incubating preformed mycelial mats (72 h) in 20 mL basal medium containing strontium as in earlier studies (Mohan and Sastry, 1984). Metal taken up by mycelia was estimated, following acid digestion (Venkateswerlu and Sastry, 1973), by Atomic Absorption Spectrophotometry (AAS) using a Perkin-Elmer 2380 spectrophotometer.

To determine the strontium 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 strontium. Mycelia were then removed, washed, dried and weighed. The strontium content in the EDTA extract and that remaining with the mycelia was estimated, after acid digestion, by AAS.

**Metal analysis:** Metal content was determined after subjecting mycelia to wet digestion as described earlier (Anupama *et al*., 2014). 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 l: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 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 strontium-resistant strains:** Strontium-resistant strain of *Neurospora crassa* was obtained by repeated subculturing of wild type *Neurospora crassa* on agar medium containing 0-50 mM strontium. Table 1 shows that after 25 such biweekly subcultures, a 3.5 fold resistance to strontium ($IC_{50}$ 36 mM) was observed when compared with the parental *Neurospora crassa* ($IC_{50}$ 10 mM). It should be noted that adaptation on progressively increasing concentrations of strontium, up to 50 mM, resulted in strains which were 3.5 fold resistant, while selection on a higher strontium concentration (50 mM) resulted in strains which were resistant. The strontium-resistant strains so obtained were found to be stable mutants; subculturing on strontium-free media (20-30 biweekly subcultures) did not result in loss of resistance. The strontium-resistant isolates were found to be genetically homogeneous the values for strontium 50% growth inhibitory concentration of metal ion $IC_{50}$ of eight single colony isolates from each of the adapted cultures were almost identical. A single isolate from the 50 mM strontium adapted culture, referred to hereafter as the SRR strain, was selected for further detailed studies. Strontium toxicity and accumulation were examined in this strain and the data were compared with the parental wild type *Neurospora crassa*.

Table 1: Isolation of strontium-resistant of *Neurospora crassa*

| Strain No. | $Sr^{2+}$ concentration (mM) | No. of subcultures (biweekly) | *No. of subcultures on Sr-free (biweekly) | $IC_{50}$ for $Sr^{2+}$ (mM) |
|---|---|---|---|---|
| 1 | 0 | 20 | 24 | 10.0±0.4 |
| 2 | 2 | 20 | 22 | 10.5±0.5 |
| 3 | 4 | 18 | 22 | 12.2±0.4 |
| 4 | 8 | 18 | 24 | 15.0±0.7 |
| 5 | 16 | 20 | 20 | 18.0±0.9 |
| 6 | 24 | 22 | 24 | 19.2±1.0 |
| 7 | 32 | 18 | 24 | 36.0±2.0 |
| 8 | 50 | 20 | 20 | 36.0±2.2 |

*Neurospora crassa* wild type was subcultured on agar slants (biweekly) containing $Sr^{2+}$, $IC_{50}$-50% growth inhibitory concentration). Values shown are averages from three separate experiments (±SD), *No. of subcultures (biweekly) during which, to date, resistance to Strontium remained unaltered

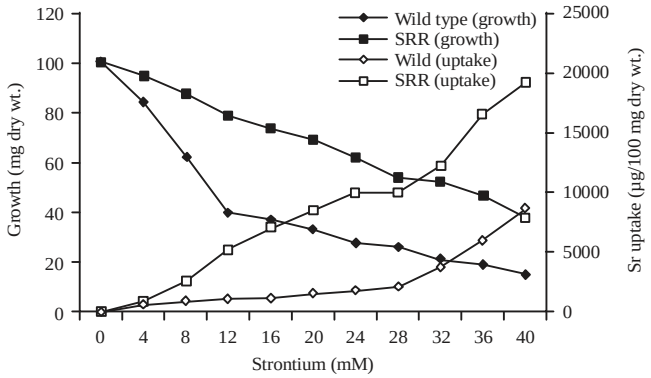


Fig. 1: Strontium toxicity in *Neurospora crassa*. *Neurospora crassa* strains were grown in 10 mL basal medium in 50 mL conical flasks for 72 h at 28±l°C. Strontium was included at the required concentrations as indicated. After incubation, the mycelia were washed, dried and Strontium was estimated, following acid digestion, by Atomic Absorption Spectrophotometry (AAS). Mycelial weights of controls (taken as l00% (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; $Ca^{2+}$ uptake, open symbols

The results presented in Fig. 1 show that growth of parental *Neurospora crassa* was inhibited (>80%) by 25 mM strontium, with an $IC_{50}$ around 10 mM, while the SRR strain was relatively unaffected under similar conditions of growth. *Neurospora crassa* (wild type) accumulated relatively higher concentrations of strontium (up to five fold) compared with the SRR strain. Growth inhibition for the SRR strain was observed at a 3.5 fold higher concentration of strontium than that of wild type *Neurospora crassa*, with an $IC_{50}$ value of about 36 mM (Fig. 1). Consequently, higher levels of strontium were accumulated by the mycelia.

**Effect of calcium and magnesium on strontium toxicity:** Since metal-resistant strains of fungi generally exhibit cross-resistance to other related metal ions, this aspect was also examined in the SRR strain. Figure 2 shows that the SRR strain is over four fold ($IC_{50}$ 12 mM) more resistant to calcium compared with the wild type ($IC_{50}$ 3.2 mM). Once again, the SRR strain accumulates more calcium than does wild type *Neurospora crassa*. No cross-resistance to magnesium was observed (data not shown).

Strontium toxicity in *Neurospora crassa* is known to be reversed by calcium when an excess of these ions is included in the growth medium (to be published elsewhere). Calcium ions were found to reverse completely the growth inhibition caused by strontium in *Neurospora crassa* wild type and the SRR strain (Table 2). Calcium reversed growth inhibition by suppression of strontium uptake in both *Neurospora crassa* strains, the SRR strain requiring a tenfold higher ratio of Ca (Sr:Ca = 1:4) compared with the wild type (Sr:Ca = 1:2).

**Effect of metabolic inhibitors:** In an earlier isolated metal-resistant strains of *Neurospora crassa* (cor, NiR, ZnR), metal uptake was found to be insensitive to respiratory poisons, while in the wild type uptake was sensitive (Mohan and Sastry, 1984; Sajani and Mohan, 1997;

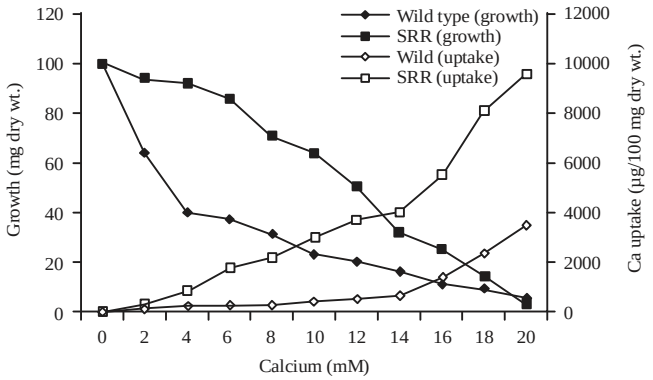


Fig. 2: Calcium toxicity in *Neurospora crassa*. *Neurospora crassa* were grown as in Fig. 1. Calcium was included at the required concentrations as indicated. Mycelial weights of controls (l00% mg dry wt were: wild type, 42±5 and SRR, 50±5. The arrows indicate $I_{50}$ values. Typical results from at least three experiments are shown. Growth, closed symbols; $Ca^{2+}$ uptake, open symbols

Table 2: Calcium ions were found to reverse completely the growth inhibition caused by strontium in *Neurospora crassa* wild type and the SRR strain

| | Metal concentration (mM) | | | |
|---|---|---|---|---|
| Strain | Sr | Ca | Growth (mg dry wt.) | Sr uptake (µg/100 mg dry wt.) |
| *Neurospora crassa* (wild type) | 0 | | 46.0±2.0 | ND |
| | 10 | | 22.5±1.7 | 840±52 |
| | 10 | 3.2 | 41.0±2.9 | 412±26 |
| *Neurospora crassa* (SRR) | 0 | | 58.0±3.6 | ND |
| | 36 | | 30.0±1.5 | 2962±132 |
| | 36 | 5.0 | 55.0±4.2 | 1254±76 |

*Neurospora crassa* strains were grown at their respective $I_{50}$ concentrations of Sr for 72 h along with Ca. (Minimal concentrations of Ca required for complete reversal of growth inhibition only are indicated). Growth and Strontium uptake values shown are means derived from duplicates of two separate experiments (±SD), ND: Not detectable

Table 3: Effect of respiratory inhibitors on Strontium uptake by *Neurospora crassa* strains

| | Inhibition (%) | | |
|---|---|---|---|
| Strain | Control | Sodium azide (1.0 mM) | 2,4-dinitrophenol (5 mM) |
| Wild type | 521 | 296 (43.2) | 184 (64.7) |
| SRR | 2550 | 1172 (54) | 1049 (58.8) |

Preformed mycelia (72 h) were floated for 1 h in 20 mL basal medium containing 5 mM Strontium. Metabolic inhibitors were included as indicated. At the end of the incubation the mycelia were harvested, washed, weighed and acid digested to estimate Strontium by AAS. Average values of two separate experiments are shown (SD up to ±15%)

Rao *et al*., 1997). To examine this, the effects of sodium azide and 2,4 dinitrophenol on strontium uptake were studied using preformed (72 h) mycelial mats. Table 3 shows a distinct suppression of strontium uptake in both the wild type and the SRR strain. It should also be noted from control data (without inhibitors) that strontium uptake by the SRR strain is about one-half to one-third of that of the wild type *Neurospora crassa*.

**Strontium partitioning:** In *Neurospora crassa* SRR partial transport block for strontium 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 difference in strontium accumulation by the SRR strain is due to increased surface binding and/or to intracellular

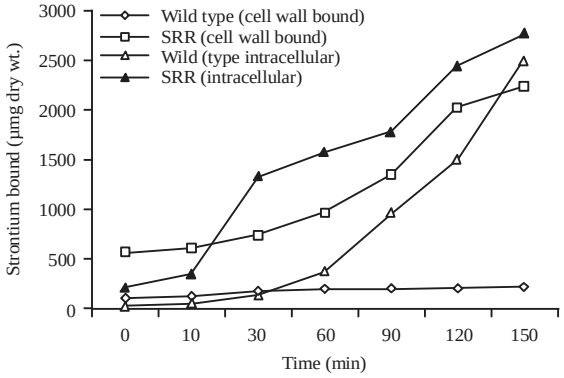


Fig. 3: Strontium partitioning between surface and intracellular fractions. Preformed mycelia (72 h) were floated in 20 mL basal medium containing Strontium (10 mM). The mycelia were harvested at the indicated time points, washed and incubated in 10 mL EDTA solution (10 mM) for 5 min to leach out surface bound Strontium. The mycelia were then dried, weighed and digested. The Strontium content of both the above fractions was determined by AAS. Data points are average values from two separate experiments, each in duplicate (±SD)

Table 4: Strontium binding by cell walls of *Neurospora crassa*

| Strain | Sr taken up (µg/100 mg dry wt.) |
|---|---|
| Wild type | 3320 |
| SRR | 5040 |

Cell walls (~100 mg dry wt) of *Neurospora crassa* strains were suspended in 10 mL basal medium containing 5 mM $Sr^{2+}$ 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 Strontium was estimated by AAS. Mean values from three separate experiments, each in duplicate, are shown (±SD)

accumulation. To study the partitioning of strontium between the above two fractions, preformed mycelial mats (72 h) were allowed to take up strontium for various time periods and surface bound strontium was leached with EDTA. Strontium remaining with the mycelia was assumed to be intracellular.

Figure 3 indicates that surface bound strontium (EDTA leachable fraction) in the SRR strain is significantly higher than in the wild type, at all time points. The strontium of the intracellular fraction (Fig. 3) is also less in the SRR strain than in the wild type, but is not as significant. Further cell wall preparations from the wild type and the SRR strain were used to determine strontium sorption. Table 4 supports the above results in that *Neurospora crassa* SRR cell walls bind only about 25% of strontium compared with the wild type.

**Strontium efflux:** To determine if there is any efflux of strontium that has already been accumulated, mycelia of *Neurospora crassa* wild type and the SRR strain were allowed to take up strontium (10 mM) for 3 h and were then washed and resuspended in a strontium-free medium. Table 5 indicates that the SRR strain releases relatively more strontium when compared with wild type *Neurospora crassa* under the experimental conditions. About 25% of the mycelial strontium was released; the SRR strain, which takes up less strontium, releases twofold more than the wild type. Strontium efflux was found to be a slow process influenced neither by metabolic inhibitors (data not shown).

Table 5: Strontium efflux by *Neurospora crassa* strains

| | Sr (μg/100 mg dry wt.) | |
|---|---|---|
| Strain | Bound to mycelia | Released by mycelia |
| Wild type | 411 | 123 |
| SRR | 1992 | 457 |

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

## DISCUSSION

It is well established that microorganisms acquire resistance to toxic metal ions in the environment. However, the mechanisms whereby they do so are less clear. 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 (Garcia-Toledo *et al*., 1985). However, resistance of *Neurospora crassa* to cobalt and nickel was shown to be stable (Mohan and Sastry, 1984; Sajani and Mohan, 1997) as observed with the cor strain in the present study.

In the present study, strontium-resistant strains have been derived by subculturing *Neurospora crassa* on different levels of cobalt. One of the striking findings is that SRR strain with 3.5 fold resistance is obtained when the strontium concentration is in the range 30-50 mM. Cross-resistance is exhibited to calcium (four fold) but not to magnesium ions.

One general reason for this mode of cross resistance between these two metal ions (calcium and strontium) is that, both elements belong to a common group II B in the periodic table sharing many features like similar atomic radius, valency and so on. Strontium uptake data indicate that a major portion of strontium taken up was bound to cell wall portion in SRR strain. The partitioning of strontium between the surface bound and intracellular fractions provides an understanding of the mechanism involved in resistance.

Cell surface binding is greatly increased in the SRR strain (both in preformed mycelia and in isolated cell walls) which in turn could lead to increased 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 *Neurospora crassa*, chitin/chitosan levels were shown to be altered in copper toxicity on a sole nitrate medium (Subramanyam *et al*., 1983). This could probably also contribute to the overall ability of this strain to accumulate more strontium. 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, 1993; Cervantes and Gutierrez-Corona, 1994), thereby possibly restricting entry into cells. However, in the present study, a increase in metal binding to cell walls is for the first time shown to be involved in strontium resistance in *Neurospora crassa*. Since binding to the cell wall is the primary site of interaction during metal uptake, a quantitative increase in binding could in turn result in increased accumulation into the intracellular fraction. However, this observation with SRR strain is in contrast with the earlier report made by Latha *et al*. (2005) that cell wall bound metal ions will not be taken up into the intracellular reason. However it can be noted that in their report the organism was not a resistance nor sensitive strain like SRR, secondly the organism tried to protect itself from the toxic environment. Whereas SRR strain was exposed to a relatively high concentrations of strontium for a prolonged time.

Since the *Neurospora crassa* SRR strain is only 3.5 fold more resistant to strontium than the wild type, this suggests that there is also a second mode, i.e., an intracellular transport mechanism for resistance. This is the subject of current investigation in our laboratory.

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