Mechanism to combat cobalt toxicity in cobalt resistant mutants of *Aspergillus nidulans*

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Abstract Characteristics of fungal species tolerant to high levels of metals in natural environment can be amplified by isolation and selection of resistant mutants. Step-by-step culturing led to identification of highly stable Co-resistant (Co^R) mutants of *A. nidulans*. Based on two distinct morphological features, Co-resistant mutants were categorized as Co^RI and Co^RII. The two mutants varied in their growth behavior and colony morphology that were reflected in supplemented as well as unsupplemented growth media over the generations. As compared to the Co^RI, Co^RII mutant exhibited sparse mycelia and conidiation but secreted higher amount of melanin. Co^R mutants could tolerate up to 2.5mM Co in the medium, however, required a threshold concentration of 0.25mM Co for optimal growth and germination. Absence of Co in the medium caused a stressful situation for the Co^R mutants and led to the secretion of a white extracellular precipitate found to be a glycoprotein. In response to interactions with Co-ions, Co^R mutants produced oxalic acid and bioprecipitated Co as Co-oxalate providing scope for metal reclamation as well as oxalic acid extraction. The mutants could help to recover the insoluble Co-oxalate salt from aqueous solutions by entrapping it in their growing mycelial meshwork.

Keywords *Aspergillus nidulans* · Cobalt · Cobalt oxalate · Mutants · Precipitate · Resistance

Introduction

Metals are the integral part of all ecosystems and occur in both elemental and ore forms in nature. In fact life has evolved in the environment rich in various metals and therefore, all cells have incorporated certain metal ions into their essential cellular functions. Due to industrial and anthropogenic activities, the ecosystems are increasingly subjected to heavy metal pollution¹. Consequently, the life forms are continuously being exposed to potentially toxic conditions, and therefore most organisms have evolved mechanisms of metal homeostasis and metal resistance to cope-up with varying amounts of metals in their environments. This requires the cellular ability to recognize the metal species as well as the relative concentrations^{2,3}.

The heavy metal cobalt (Co), finds extensive use for industrial purposes like production of steel, and for electroplating, and for the production of pigments⁴. Like many other heavy metals, Co is an essential metal needed in trace amount by the organisms for a range of metabolic activities and structural organization. It is an essential component of vitamin B₁₂ and is an integral requirement for enzymes like transcarboxylase and amidino aspartase⁵. However, at higher concentrations, Co is toxic to the cells as it inhibits cellular respiration and enzymes of citric acid cycle⁶. Environments polluted with metals lead to the selection of resistant organism via molecular mechanisms. Such resistant microorganisms constitute a subject of increasing importance as they can be exploited to minimize metal pollution. Bacteria and fungi belonging to various genera

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are known to resist metals^{6,7}. Filamentous fungus, because of its characteristic expanding growth is a good eukaryotic model system to study metal-microbe interactions. We therefore chose *Aspergillus nidulans* as the experimental organisms for this study, as this fungus has been found in industrially polluted soils. Cobalt resistant strains were developed and characterized for their physiological characters including intracellular Co-localization. The significant findings are reported in this communication.

Materials and Methods

Organism and the growth conditions: A metal sensitive strain *riboA₁*, *biA₁* of *Aspergillus nidulans*, obtained from Culture Collection Centre, Glasgow, UK, was used as the original wild type strain (Co^s). The wild type as well as the mutant strains, selected in this study were normally grown at 37° C for 120 h on either supplemented Czapeck-Dox medium (CDM) or complete medium (CM) and assessed for their growth behaviour⁸. Molar stock solution of cobalt sulphate (CoSO₄ · 7H₂O) was used for developing Co-resistant mutants.

Development of Co-resistant mutants: Cobalt resistant (Co^R) mutants of *A. nidulans* were obtained by stepwise selection against increasing concentration of Co on Czapeck-Dox agar medium^{3,8}. The process led to the selection of Co^R mutants that exhibited good growth and conidiation at 2.5mM of Co. Beyond this concentration, growth of the mutant strains was inhibited. Therefore, 2.5mM Co was considered as maximum tolerable concentration (MTC) of the metal.

Biochemical analysis of culture secretions: The fungus secreted a pink coloured extracellular precipitate in Co-containing medium and a white precipitate in Co-free medium. Both types of precipitates were analyzed.

The extracellular pink precipitate was insoluble in water and was collected by filtering the culture medium through a muslin cloth. The culture filtrate containing the precipitate was processed differently for the analysis of Co and the associated anionic radical. For Co assay, the culture filtrate was filtered on a dried and pre-weighed Whatman No. 1 filter paper, dried at 80°C till the constant weight was obtained, and incinerated at 600°C for 6 h. The ash was dissolved in 2 ml of 1N HCl and subjected to Co assay by Atomic Absorption Spectrophotometer (AAS-Shimadzu: AA-640-13) at 240.7 nm after appropriate dilutions in deionised distilled water.

For the detection of anionic radical, the culture filtrate was centrifuged at 10,000 x g for 20 min. The pink

precipitate was washed with distilled water and subjected to chemical analysis. Since the oxalate is known to be insoluble pink-coloured salt of Co, the tests for the detection of oxalate radical were conducted. Tests were also carried out to examine the possibility of citrate radical.

For the assay of white precipitate, the Co-free culture broth containing the precipitate was centrifuged. The pellet was washed extensively and subjected to solubility tests in weak acids, weak alkalis, strong acids, hot alkalis as well as in several organic solvents. Tests for the presence of polysaccharides and proteins were carried out. Maackia amurensis agglutinin test was performed to identify the complex sialylated carbohydrate chains and the type of sialic acid linkage.

Maackia amurensis agglutinin (MAA) assay: The test is based on the principle of lectin binding to the carbohydrate moiety of the glycoprotein. The lectin used was conjugated with the steroid hapten digoxigenin, which enabled the immunological detection of bound lectins. In order to identify specific carbohydrate structure, the lectins, which selectively recognize the terminal sugars, were used. This enabled the identification of the nature of the carbohydrate chain in the glycoproteins. The galactose recognizing Maackia Amurensis Agglutinin (MAA) was the lectin used in the present test. MAA specifically identifies sialic acid linked -(2-3) to galactose

Transmission electron microscopy: TEM was carried out to identify the cellular sites of Co sequestration in the mutants. Mycelia, grown in Co-containing (MTC-level) liquid Czapeck-Dox medium for 72 hours, were used for this study. The sections were viewed and photographed through transmission electron microscope (CM-10 Phillips).

Results and Discussion

Development of Co-resistant mutants: Fungi can interact with the metals and accumulate them by employing different physico-chemical mechanisms and transport system of varying specificity⁹. The selection by step-by-step culturing of wild type Co sensitive strain *riboA₁*, *biA₁*, on medium containing increasing concentrations of Co, provided mutants having tolerance to 2.5 mM Co. All the mutants retained this level of resistance, even after several subculturing on Co-free medium. The heritable acquisition of metal resistance by continuous training has been reported for other fungi including *A. nidulans*^{3,8}.

Two categories of Co-resistant (Co^R) mutants, having two distinct colony morphologies were identified, and designated as Co^RI and Co^RII. Co^RI mutant had relatively

Fig. 1 Co^R mutants grown for 120 h on supplemented Czapeck-Dox agar containing 2.5 mM CoSO_4 at 37°C. A: Co^RI , B: Co^RII . Growth of the wild type Co^S strain is inhibited at this concentration of Co (marked by).

Fig. 2 Growth of Co^S and Co^R strains on supplemented Czapeck-Dox agar at 37°C for 120h. A: Co-free medium, $\circ$ = Co^S , $\triangle$ = Co^RI , $\square$ = Co^RII . B: Co (2.5mM) containing medium, $\bullet$ = Co^S , $\blacktriangle$ = Co^RI , $\blacksquare$ = Co^RII .

small colonies with dense mycelia, bright green conidia and little melanin secretion (Fig. 1A). Co^RII mutant had sparse mycelia and conidiation but little larger colonies and secreted melanin (Fig. 1B). In terms of colony diameter, Co^RI and Co^RII after 120 h of growth on CDM containing Co registered about 70 and 93 percent growth, respectively, as compared to Co^S strain on Co-free medium (Fig. 2). At least 50% inhibition of conidial germination by 0.7mM Co and complete inhibition by 2mM Co of wild type strains

of *A. nidulans* has been reported. The two Co-resistant mutants cob A^r and cob B^r required 4- and 2- folds more Co to bring about similar inhibition¹⁰.

On Co-containing medium both Co^R mutants exhibited two phases of conidiation. The first phase of conidiation occurred between 48–72 h, followed by a period of vegetative growth and then the second phase of conidiation occurred between 96–120 h. This phenomenon was more pronounced in Co^RII mutant than in Co^RI mutant. On Co-free agar

medium, the conidiation was completed by 72 h. Co-resistant mutants of *Neurospora crassa* exhibited change in conidial colour suggesting the possible interference in pigment production¹¹.

Growth and morphological characteristics: The growth of Co^R mutants was affected by the absence of cobalt in the medium. In the absence of Co, Co^R mutants appeared to be under stress as their hyphae were very sparse and tended to spread in the medium as compared to Co^S colonies. The growth of mutants in media with different concentrations of

Co revealed that Co^RI and Co^RII mutants formed larger colonies in the presence of 0.25mM Co than in absence of Co on Czapeck-Dox agar (Fig. 3). The colonies were compact with densely packed mycelia and good conidiation. This demonstrated that the low concentration of 0.25 mM of Co was optimal and synergistic to the growth of Co^R mutants.

Almost similar trend of growth stimulation by low doses of Co was observed when the mutant strains were cultured on CM agar. More than 0.25mM Co in CM led to gradual reduction in the colony diameter and interference in the pigmentation of conidia (Fig. 4). Both the mutants exhibited a

Fig. 3 Inhibition of hyphal extension of Co^S and Co^R strains at the end of 120 h by increasing concentration of Co on Czapeck-Dox agar at 37°C. The relative hyphal growth is expressed as log % area of the colonies. ● = Co^S, ▲ = Co^RI, ■ = Co^RII

Fig. 4 Colonies of Co^R mutants on CM agar containing Co at 37°C after 96 h. A: CM+0.25mM Co, B: CM+2.5mM Co.

Fig. 5 Growth of Co^{R} mutants in CM agar devoid of Co. A: Co^{RI} strain. The stunted growth of the mutant covered by a white precipitate secreted by the deformed mycelia. B: Co^{RI} strain. A part of the colony showing the mycelia entrapped in the white precipitate. C: Co^{RII} strain. The mutant secreted much less amount of precipitate visible as a halo surrounding the colony (marked by).

transient stage of reduced pigmentation in conidia especially in the central region of the colonies. At the end of 120 h, however, all the conidia acquired the green colour that was brighter than the colour observed in sensitive strain.

In Co-free CM, a concentric zone of white halo was found around the colonies of Co^{R} mutants accompanied by deformed mycelial growth after 72 h. Halo zone consisted of a white precipitate secreted by the mycelium. In Co^{RI} strain, whose growth was highly inhibited, there was a very dense white precipitate (Fig. 5 A, B), while in Co^{RII} strain, the halo as well as precipitation was less pronounced and the former was visible only under the microscope (Fig. 5 C).

In liquid CDM, the growth of the mutants was comparable to that obtained on solid medium. Stimulating effect of low concentration of Co (up to 0.25 mM) on the growth of both categories of Co^{R} mutants was further evident. The mutants did not exhibit normal germination of conidia and growth in Co-free medium. Normal filamentous structure and conidiation was found lacking. The colonies appeared unusually dense and compact and the abnormal mycelia bore hyaline, thick-walled structures of uncertain morphol-

ogy. However, at very few regions of the colonies, normal filamentous branching could be observed after 24 to 48 h (Fig. 6 A,B). Addition of Co to the medium restored the normal growth and conidiation. The data of the germination of conidia also clearly showed higher conidial germination (colony forming units) of Co^{R} mutants than Co^{S} on medium containing Co and vice-versa (Fig. 7).

In liquid CM, Co^{RI} mutant showed deformed mycelial growth and produced higher amount of white precipitate as compared to Co^{RII} strain and the medium appeared turbid. Addition of divalent cations Co, Ni, Zn, Fe, and Cu in the culture medium restored the normal growth of the mutants. The extracellular precipitate disappeared from the medium over the period of 16 hours, leaving the medium clear. It can be suggested that many of these cations through their interaction with each other or other components of the growth media improved the availability of certain essential nutrients. Such a role has been assigned to Cd in Cd^{R} mutants of *A. nidulans*¹².

In Co-containing liquid CDM a distinct phenomenon was observed. By 24 h, the mycelia acquired a distinct pink colour (Fig. 8 A) and a pink coloured substance started

Fig. 6 Mycelia of Co^R mutant grown in supplemented liquid Czapeck-Dox medium at 37°C after 48 h. (X1280). A: Co-free medium; mycelia appeared dense and compact with unusual morphology. B: Co (2.5mM) containing medium; presence of Co led to normal filamentous mycelial growth

Fig. 7 Percentage of CFUs (colony forming units) of Co^S and Co^R strains on supplemented Czapeck-Dox agar. A: Co^S , B: Co^{RI} , C: Co^{RII} , = Co-free medium, = Co (2.5mM) containing medium.

precipitating and accumulating at the bottom of the culture flask. By 48 h, the pink precipitate formed in Co^{RI} culture was about 4.2% and in Co^{RII} culture about 6% of the weight of the biomass. During the subsequent growth, the amount of precipitate was found to decrease. An examination of the mycelia under the microscope showed the entrapment of the pink precipitate in the mycelial network (Fig. 8B). Acquisition of a distinct pink colour by the Co^R mutants is therefore, suggestive of sequestration of Co. In *C. blakesleena*, most of the Cu taken up was located in the cell wall and gave blue colour to the fungus^{13–15}.

Characterization of precipitates: The analysis of insoluble pink precipitate formed in liquid CDM containing 2.5mM Co by Co^R mutants revealed it to consist of cobalt oxalate.

There was little decrease of pH in the growth medium containing 2.5mM Co after 24 h growth. However by 48 h of growth, when the pink precipitation was maximum the pH increased slightly and continued to increase up to 96 h till the biomass degradation commenced as also observed during normal growth of the fungus. A trend of lowering pH was not observed when Co^S strain was grown in Co-containing medium. This suggested that the secretion of a weak acid like oxalic acid was the property of Co^R mutants. Slight increase in pH beyond 48 h of growth of Co^R mutants indicated the cessation of oxalic acid secretion by the mycelia, and consequent decrease in the amount of pink precipitate. Utilization of ligand mechanism and release of a mild organic acid (oxalic acid) that precipitated the metal as Co-oxalate, a resistance mechanism, is also reported in other studies^{16,17}.

Fig. 8 Co-accumulation by Co^{R} mutants grown in Co (2.5mM) containing supplemented liquid Czapeck-Dox medium at 37°C for 48 h. A: Pink coloured mycelial balls of Co^{R} mutants. B: Mycelial meshwork of Co^{R} mutant with trapped insoluble Co -oxalate precipitate (marked by $\uparrow$) (X1280).

Fig. 9 Transmission electron micrographs of the sections of mycelia of Co^{S} and Co^{R} strains A: Co^{S} , B: Co^{RI} , C: Co^{RII} (X15000). Intracellular Co -deposition is shown as electron dense area in the cytoplasm (marked by $\uparrow$).

The analysis of white extracellular substance, produced by Co^{R} mutant under stress in Co -free medium, revealed it to be insoluble in water, weak acids as well as alkalis

and organic acids but soluble in hot alkali and concentrated acid. It gave weak but positive reaction to Biuret and Millon's test for proteins and strong positive reaction

to the phenol-sulphuric acid and anthrone reagents for the carbohydrates

The sugar-moiety of the stress precipitate appeared to be a sialic acid molecule terminally linked at -(2-3) to galactose. This was shown by performing the Maackia amurensis agglutinin test (MAA-test) that specifically recognizes the sialylated carbohydrate chains as well as type of sialic acid linkage. Similar tests conducted for the identification of mannose and other terminal sugars yielded negative results. The precipitate did not exhibit any mobility under the influence of the electric field on SDS-PAGE, but yielded positive result as a pink spot in the dot blot test for proteins using Ponceau S reagent.

Fungi are known to produce polymers of glycans under stress conditions. In the medium devoid of nitrogen or carbon source, species of *Aspergillus* and *Penicillium* secrete a hot water extractable -glucan, the nigeran, that gets deposited in the hyphal walls¹⁸. The presence of heavy metals can also elicit such a response from the fungus. Cu, Fe and Mg have been reported to elevate nigeran deposits in many species of *Aspergillus* and *Penicillium*¹⁹. Nigeran deposition on the cell walls is generally interpreted as a mechanism to maintain the integrity of the cells under hostile conditions. In certain fungi, under stress condition the cell walls are thickened and are accompanied by the formation of chlamydospore structures¹⁹. The results in our study, although did not confirm deposition of nigeran, there was indeed a secretion of a glycoprotein by Co^R mutants in the absence of Co in the medium. Though, the precise reason for such a secretion is still not clear, the glycoprotein could possibly be a byproduct of an incomplete pathway and its further metabolism might depend on certain factors or enzymes that require Co or other divalent cations. However, attempts to substitute Co by vitamin B-12 (cyanocobalamine) neither promoted the growth nor prevented the precipitation.

Intracellular localization of Co: The distribution of Co at various locales in the cell was examined by transmission electron microscopy. Thin sections of 72 h old mycelial of Co^R and Co^S strains cultured in Co-containing medium showed the normal hyphal structure with no evidence of any significant structural abnormalities. The pattern of accumulation and distribution of the metal in the two categories of mutants were different. In Co^RII mutants, the mycelia showed cell surface deposition and intracellular electron dense deposits dispersed in the entire cytoplasm (Fig. 9). Co^RI mutant showed electron dense regions in granulated form distributed in the entire cytoplasm. Deposition of U (Uranium) extracellularly in *S. cerevisiae*, and in the bacterium, *Pseudomonas aeruginosa* did not bring about

apparent structural changes²⁰. Cd, however, was compartmentalized in vacuoles in *S. cerevisiae* with no apparent alteration of the cell interior and organelles²¹. In *N. vasinfecta*, Zn was deposited either in large, irregular deposits located just inside the hyphal membrane or in small discrete spots concentrated mainly in the nucleus²². The hyphae of Cu-grown *C. blakesleeana* were larger in diameter with rough granular surface and thicker cell walls, and the cytoplasm and mitochondria appeared damaged¹³.

These results suggest that in some fungi including *A. nidulans*, there may not be any structural changes due to extracellular or intracellular deposits of certain metals, while in some other fungi, these changes may occur.

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References

- Gadd GM (1990) Fungi and yeasts for metal accumulation. In: Microbial Mineral Recovery (Ehrlich H & Brierley CL eds). McGraw-Hill, NY, pp 249–275
- Gadd GM (1994 F) Interactions of fungi with toxic metals. In: The Genus *Aspergillus*: From taxonomy and genetics to industrial application (Powell KA, Renwick A & Peberdy JF eds). (FEMS Symposium No. 69), Plenum Press, NY, pp 361–374
- Tomsett AB (1994) Genetics and Molecular Biology of Metal Tolerance in Fungi. In: Stress Tolerance of Fungi (Jennines DH ed). Marcel Dekker INC, NY, pp 69–95
- Nies DH (1992) Resistance to cadmium, cobalt, zinc and nickel in microbes. Plasmid 27:17–28
- Wood HG & Zwolinski GK (1976) Transcarboxylase: Role of biotin, metals, and subunits in the reaction and its quaternary structure. Crit Rev Biochem 4:47
- Gadd GM (1992) Metals and microorganisms: A problem of definition. FEMS Microbiol Lett 100:197–204
- Saxena P, Bhattacharyya AK & Mathur N (2006) Nickel tolerance and accumulation by filamentous fungi from sludge of metal finishing industry. Geomicrobiology Journal 235: 330–340
- Tripathi P & Srivastava S (2007) Development and characterization of nickel accumulating mutants of *Aspergillus nidulans*. Indian J Microbiol 47:241–250
- Gadd GM (1988) Accumulation of metals by microorganisms and algae. In: Biotechnology-A comprehensive Treatise (Rehm HJ ed). Special Microbial Process. Vol 6 (b). VCH Verlagsgesellschaft Weinheim, Germany pp 401–433
- Tomsett AB, Hodges KE, Cooley RN & Thurman DA (1989) Metal resistance in *Aspergillus nidulans*. In: Metal Ion Homeostasis-Molecular Biology and Chemistry (Hamer DH & Winge DR eds). Alan R. Liss, NY, pp 375–384

11. Venkateswerlu G & Sastry KS (1973) Interrelationships in trace-element metabolism in metal toxicities in a Cobalt-resistant strain of *Neurospora crassa*. *Biochem J* 132: 673–680
12. Cooley RN, Haslock HR & Tomsett AB (1986) Isolation and Characterization of Cadmium-resistant Mutants of *Aspergillus nidulans*. *Curr Microbiol* 13:265–268
13. Venkateswerlu G & Stotzky G (1986) Copper and cobalt alter the cell wall composition of *Cunninghamella blakesleeana*. *Can J Microbiol* 32:654–662
14. Venkateswerlu G & Stotzky G (1989) Binding of metals by cell walls of *Cunninghamella blakesleeana* grown in the presence of copper and cobalt. *Appl Microbiol Biotechnol* 31:619–625
15. Venkateswerlu G, Yoder MJ & Stotzky G (1989) Morphological, ultrastructural and chemical changes induced in *Cunninghamella blakesleeana* by copper and cobalt. *Appl Microbiol Biotechnol* 31:204–210
16. Fomina M, Hillier S, Charnock JM, Melville K, Alexander IJ & Gadd GM (2005) Role of oxalic acid over-excretion in toxic metal mineral transformations by *Beauveria caledonica*. *Applied and Environmental Microbiology* 71: 371–381
17. Gadd GM (2004) Mycotransformation of organic and inorganic substrates. *Mycologist* 18:60–70
18. Bobbitt TF, Nordin JH, Roux M, Revol JF & Marchessault RH (1977) Distribution and conformation of crystalline nigeran in hyphal walls of *Aspergillus niger* and *Aspergillus awamori*. *J Bacteriol* 132:691–703
19. Gupta R & Mukerji KG (1982) Nigeran production in some *Aspergillus* and *Penicillium* species. *Folia Microbiol* 27: 38–42
20. Strandberg GW, Shumate SE & Parrott JR (1981) Microbiol cell as biosorbents for heavy metals: accumulation of uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa*. *Appl Environ Microbiol* 41(1):237–245
21. Volesky B, May H & Holan ZR (1993) Cadmium biosorption by *Saccharomyces cerevisiae*. *Biotech Bioeng* 41:826–829
22. Paton WHN & Budd K (1972) Zinc uptake in *Neocosmospora vasinfecta*. *J Gen Microbiol* 72:173–184