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A STUDY ON INVOLVEMENT OF METAL-BINDING PROTEIN(S) FOR THE BIOSORPTION OF SOME SELECTED HEAVY METALS

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ABSTRACT

The present research finding have shown that extracellular protein(s) of growing cells of *Pseudomonas aeruginosa* DSGPM4 strain are responsible for giving withstand power in presence of toxic heavy metals.

To study the role of cadmium, nickel and copper resistance, a preliminary test was done by treating the cells with different concentrations of proteinase K. To perform the test highly resistant isolates DSGPM4 was inoculated in nutrient broth and incubated overnight at 37^0C. Reaction tubes were prepared by adding overnight cultures in two different concentrations of proteinase K i.e. 1 mg/ml and 2 mg/ml in the ratio of 1:1 and incubated for 2 hours at 37^0C. After incubation, 20 ml of each sample was spread over selective plates containing two different concentrations of $PbCl_2$ and $NiCl_2$ and $CuCl_2$ i.e., 1mM and 2 mM in growth medium (1x A) as well as on control plates. Control plates were prepared by adding 1 mg/ml (C1), and 2 mg/ml (C2) of proteinase K whereas, other control plates containing 2mM of $PbCl_2$ (C3) and 2mM of $NiCl_2$ (C4) and $CuCl_2$ (C5) salts but no proteinase K.

To find out whether the extracellular protein has any role in metal resistance, preliminary test was conducted by treating the plasmid bearing isolate (DSGPM4) and a control DSGPM4 with protease.

This study reports the accumulation of some heavy metals (Cu, Ni and Pb) by growing cells of *Pseudomonas aeruginosa* DSGPM4 strain and the purification and molecular characterization of related heavy metal binding proteins.

Key words: Protease K; Extracellular proteins; DSGPM4.

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Introduction

Microorganisms have evolved several mechanisms to withstand the toxic effects of heavy metals. One of the common mechanisms is the induction of metals binding proteins following the uptake of metals into the cells. A well studied class of metal binding protein is called metallothioneins (MT) or low molecular weight cysteine-rich proteins. A unique property of this class of proteins is their inducibility in response to heavy metals (1). The isolation of bacterial cadmium proteins from *P.putida* and cysteine-rich proteins from *P. Cepacia* grown in Au (I) may have a common role in detoxification of heavy metals (2). Metallothioneins have been isolated from diverse organisms including mammals, yeast, algae and fungi. Their presence has been found in metal homeostasis and detoxification (3).

The monitoring of sensitivity or tolerance of the microorganisms have a primary importance in the preparation of inocula for bioremediation of heavy metal contaminated soils. Although some metals (Cu, Zn) are considered as essential and play an important role in maintaining the biochemical activity, a huge number of them are toxic even in very small concentrations. Microorganisms and the bacteria in particular, have developed different tools to protect themselves by the oxidative stress produced by the metals. Usually this is expressed in detoxification mechanisms, including compartmentalization, binding, efflux etc (4) in tolerant strains. On the other hand, heavy metals helps to formation of the hydroxide radicals in redox reactions, which are highly reactive molecules and they play a role in a number of deleterious reactions such as peroxidation of lipids which conducts to membrane disruption and oxidation of proteins causing their inactivation. This can lead to alteration in structure and function of these molecules (5).

To understand the function of genes in an organism, it is not only required to know which genes are expressed, when and where but also what protein end products are and under which conditions they accumulate in certain tissues (3,6). There are large number of specific proteins reported in various genera of bacteria that showed increase in their level of expression, upon adverse conditions such as heat, toxic elements and nutrient limitations. In post economic investigations, proteomics is one of the best strategies used to reveal the dynamic expression of whole proteins in cells and their interactions. The term 'proteome' is used here to describe the complex state of an organism under defined conditions (7).

This study reports the accumulation of some heavy metals (Cu, Ni and Pb) by growing cells of *Pseudomonas aeruginosa* DSGPM4 strain and the purification and molecular characterization of related heavy metal binding proteins.

Methodology

Strains and growth conditions:

The strain used in this study was DSGPM4, isolated from waste water sample. Culture was maintained as slants in nutrient agar media. Experiment was carried out in liquid nutrient broth at 28^0C with the presence or absence of 200 ppm of respective metal salts till reaching O.D. 0.05 at 550 nm. The cells were then harvested by centrifugation at 5000 rpm for 15 min.

Study of the Involvement of Protein in Metal Resistance:

To study the role of cadmium, nickel and copper resistance, a preliminary test was done by treating the cells with different concentrations of proteinase K (7,8).To perform the test, highly resistant isolates of DSGPM4 was inoculated in nutrient broth and incubated overnight at 37^0C. Reaction tubes were prepared by adding overnight cultures in two different concentrations of proteinase K i.e. 1 mg/ml and 2 mg/ml in the ratio of 1:1 and incubated for 2 hours at 37^0C. After incubation, 20 ml of each sample was spread over selective plates containing two different concentrations of $CdCl_2$ and $NiCl_2$ and $CuCl_2$ i.e., 1mM and 2 mM in growth medium (1x A) as well as on control plates. Control plates were prepared by adding 1 mg/ml (C1), and 2 mg/ml (C2) of proteinase K whereas, other control plates containing 2mM of $PbCl_2$ (C3) and 2mM of $NiCl_2$ (C4) and $CuCl_2$ (C5) salts but no proteinase K.

Preparation of cell extract:

The pellets were resuspended and washed, first in 0.02 m $MgCl_2$ and after that in deionized water. Each time the cells were obtained by centrifugation at 6000 rpm, 4^0C for 15 min. The pellets were resuspended in 500µl lysis buffer (thiourea 2M, urea 7M, CHAPS 4%, DTT 1% and carrier ampholytes 2%), followed by repeated ultrasonication with intervals in ice. Finally, the extracts were centrifuged at 12000 rpm, 4^0C for 15 min and the supernatants were stored at -20^0C.

Protein purification:

The cells were separated by centrifugation at 12,000 × g for 10 min and used as crude protein sample. The purification of the protein was done at 4ºC. The crude protein was precipitated with ammonium sulfate at a concentration corresponding to 80% saturation. The resultant precipitate was collected by centrifigation at 15,000 × g for 20 min, dissolved in 50 mM sodium phosphate buffer (pH 7.0), and dialyzed against the same buffer and applied to the DEAE-sepharose column. The elution was done from 0-0.5 M NaCl. The active fractions from DEAE-sepharose column were combined, mixed with the same volume of 3 M ammonium sulfate and put onto Phenyl 5 PW column, which was previously equilibrated with 20 mM sodium phosphate buffer (pH 6.8) containing 1.5 M ammonium sulfate. Adsorbed proteins were eluted with a descending linear gradient of ammonium sulfate. The active fraction from phenyl 5 PW was dialyzed against 5 mM sodium phosphate buffer of pH 7.0. The dialyzed protein solution was put onto a hydroxyapatite column previously equilibrated with the same buffer. The absorbed protein was eluted with a linear gradient of 5-100 mM sodium phosphate buffer (pH 7.0). The active fraction was eluted at 50 mM sodium phosphate buffer. Purity of protein in the fractions from column chromatography was analyzed by SDS-PAGE (9).

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Estimation of Protein Content in Samples:

For estimation of protein concentration in prepared samples dilutions were prepared in duplicates in such a way that total volume in each tube must be 0.2 ml. Blank was also prepared at the same time without sample (10).

Quantitative Analysis of Protein (SDS-PAGE Gel Electrophoresis):

Each protein sample was analysed quantitatively by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) using 12% (w/v) acrylamide gel. A mini gel apparatus of BIO-RAD was employed for electrophoresis (Atto-AE-6400, Dual mini slab) (11).

Results

To study the role of cadmium, nickel and copper resistance, a preliminary test was done by treating the cells with different concentrations of proteinase K (2,4,12).To perform the test highly resistant isolates DSGPM4 was inoculated in nutrient broth and incubated overnight at 37^0C. Reaction tubes were prepared by adding overnight cultures in two different concentrations of proteinase K i.e. 1 mg/ml and 2 mg/ml in the ratio of 1:1 and incubated for 2 hours at 37^0C. After incubation, 20 ml of each sample was spread over selective plates containing two different concentrations of $CdCl_2$ and $NiCl_2$ and $CuCl_2$ i.e., 1mM and 2 mM in growth medium (1x A) as well as on control plates. Control plates were prepared by adding 1 mg/ml (C1), and 2 mg/ml (C2) of proteinase K whereas, other control plates containing 2mM of $PbCl_2$ (C3) and 2mM of $NiCl_2$ (C4) and $CuCl_2$ (C5) salts but no proteinase K.

To find out whether the extracellular protein has any role in metal resistance, preliminary test was conducted by treating the plasmid bearing isolate (DSGPM4) and a control DSGPM4 with protease. After treating with protease, isolate was not able to grown in metal containing plates with respective metals. Inability to grow in presence of metal salts indicated the possibility of presence of some extracellular protein presumably some other outer membrane proteins. Further quantitative analysis of total protein content of DSGPM4 test and control revealed that higher protein content in presence of respective metal salts as compared to control. This result suggests that some plasmid mediated protein is produced in presence of respective metal salts.

Table:1 Effect of Protease K on Pb, Ni and Cu resistance

DSGPM4 (Control)	**Growth**
Protease K (1mg/ml)	+++
Protease K (2mg/ml)	+
$CdCl_2$ 2mM	+++
$NiCl_2$ 2mM	+++
$CuCl_2$ 2mM	+++
DSGPM4 (Test)	**Growth**
$PbCl_2$ 2mM + Protease K (2mg/ml	No growth
$NiCl_2$ 2mM+ Protease K (2mg/ml)	No growth
$CuCl_2$ 2mM+ Protease K (2mg/ml)	No growth

Note: +++ : Proper growth, + : Moderate growth,

Table: 2 Comparison of total protein quantity in presence and absence of metals in dry cell of DSGPM4

Metal used (200 ppm)	**Average Protein concentration in µg/mg of bacterial cell for Test DSGPM4**	**Average Protein concentration in µg/mg of bacterial cell for control DSGPM4**
Lead	2800	1780
Nickel	2570	
Copper	2480	

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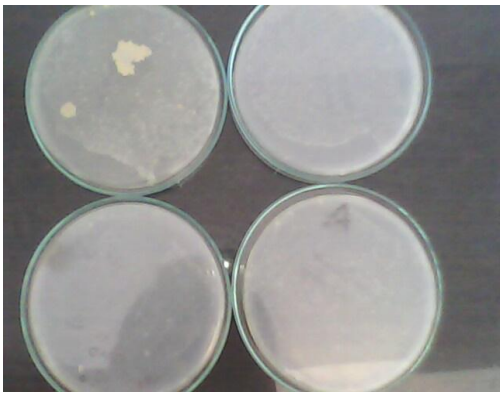
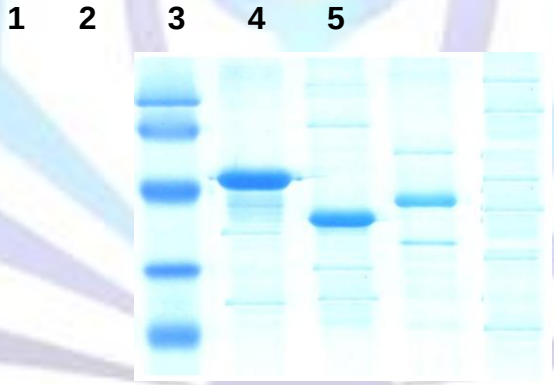
Fig:1 Effect of Protease K on Pb, Ni and Cu resistance- Top left: Control DSGPM4,Top right :Protease K + Lead Salt, Down Left: Protease K+ Nickel salt, down right: Protease K+ Copper salt.



To determine the enhanced accumulation of the metal inside the cell, may be related to the synthesis of bacterial proteins, attempts were made to extract proteins from the cultures of bacteria grown in presence of respective metal salts. Bacterial proteins were extracted from the dry cells. The pellets were resuspended in 500µl lysis buffer (thiourea 2M, urea 7M, CHAPS 4%, DTT 1% and carrier ampholytes 2%), followed by repeated ultrasonication with intervals in ice. Finally, the extracts were centrifuged at 12000 rpm, 4^0C for 15 min and the supernatants were stored at -20^0C. Further, protein was precipitated by ammonium sulphate precipitation and further purified based on hydroxypatite column chromatography. Selected fractions which absorbed strongly at 260 nm and 280 nm were analysed using the SDS-PAGE. SDS-PAGE electrophoresis analysis of protein samples of DSGPM4 isolate revealed the presence of additional protein bands in parental isolates that was missing in control derivatives. Result of the SDS-PAGE showed that all proteins are of low molecular weight and ranging from 85 kD to 15 kD in presence of lead, copper and nickel. In picture, it has been shown that in presence of lead, there are four bands and their molecular weight are 65kd, 48kD, 40kD and 18kD respectively. In presence of nickel, there were also four bands and their molecular weight were 85kD, 45kD, 32kD and 15kD respectively. In case of copper, 75kD, 48kD, 35kD and 16kD bands were found. In case of protein sample in absence of metals, there were 7 bands in gel of different molecular weights (118kD, 90kD, 85kD, 65kD, 40kD, 30kD and 10kD respectively).

Fig:2 SDS PAGE Electrophoresis: Lane1: Molecular weight marker, Lane2: Purified protein in presence of lead, Lane3: Purified protein in presence of nickel, Lane4: Purified protein in presence of copper and Lane 5: Purified protein extract in absence of lead, nickel and copper.



DISCUSSION

From the experiments that have been carried out, there were at least four types of proteins, which might have a common role of binding to lead, copper and nickel respectively. These proteins may possibly function to detoxify Pb^{+2}, Cu^{+2} and Ni^{+2} respectively, and thus allowing the organisms to survive. It should also be noted that the proteins extracted from the cell pellet in presence and absence of metal showed a lot of difference in their quantity. Maximum difference was found in presence of lead, then nickel and at last copper. It may be that lead creates much more stress in bacterial cell to survive as well as to adjust in the lead contaminated environment. The most heavy metal tolerant isolates recovered were Gram-negative in types. Many studies have shown that Gram-negative bacteria are more tolerant to heavy metals than Gram-positive types, this metal tolerance can be attributed to the interactions between the bacterial cell wall and the metal ions resulting in their detoxification (13,14,15). Regarding the toxicity, it was shown in this study that for all isolated strains the order of toxicity of the metals tested was found to be Pb >Ni > Cu.

Protein analysis of the most abundant strains strongly indicates that further efforts have to be made to fully understand this metal contaminated environment. This includes further isolation surveys and detailed characterization of the isolated populations in order to define their ecophysiological roles.

Further work in this area would include amino acid analysis, circular dichoism and optical rotary dispersion. This will further aid in protein characterization and if there is any resemblance to metallothionein, whose function is well understood.

There was a preliminary evidence for the presence of the metallothionein like cadmium binding protein in *E.c*oli (13,14), whereas a metallothionein like protein was purified from *P.putida* (9,13). This indicated the possibility that cadmium resistance in gram –ve bacteria results from the production of metallothionein like protein.

Metallothioneins mediated mechanisms have been reported in bacteria, cyanobacteria, algae, fungi, and yeast (15). Metallothioneins are generally reported as small cysteine rich polypeptide that can bind essential metals such as Cu and Zn as well as non-essential metal like Cd and function is metal detoxification (16). Metallothioneins synthesis is generally regulated by heavy metals in bacteria (9,11,17).

Among bacterial isolates like *Pseudomonas sp., Alcaligenes sp.,* and a cyanobacterium, *Synechoccus* were studied for production of metal binding proteins in response to cadmium and zinc (4,6,9). Bacterial metallothionein probably represents the independent evolutionary development of animal and plant metallothionein, since the sequence is shorter and contains fewer cysteine residues (15,17). Copper resistance in *Saccharomyces cerevisiae* is mediated by the induction of 6573 dalton cysteine-rich protein (5,9,11). This yeast metallothionein may play a potential role in metal recovery (8, 13,17). These metal binding proteins are responsible for complexing metal ions (7,11,15).

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