CHARACTERIZATION OF METAL BINDING PROTEIN FROM MICROORGANISAMS

Synopsis of the Ph.D. Thesis
Submitted to Osmania University
For the Degree of
Doctor of Philosophy
in
Biochemistry

By

Shanti Kumari Lunavat

Department of Biochemistry
Osmania University
Hyderabad - 500 007, T.S.
INDIA.

2015

Metal ions are gaining increased attention due to their importance in mineral nutrition and toxicity studies. Microorganisms have been used for leaching of minerals from ores, concentration of precious metals and also in bioremediation of toxic metal ions from polluted effluents. Due to their proximity to metal ions, microorganisms have developed homeostatic mechanism for maintaining the concentration of metal ions. Metal transport in microorganism occurs by an initial rapid phase of biosorption followed by energy-dependent bioaccumulation into the cells. Highly metal-specific affinity mechanisms have been described for physiological important metal ions such as Ca and Mg, which are required in bulk quantities, and also for trace elements (Cu, Co, Ni, Zn etc) in some microorganism. With the complete genome sequencing of a large number of microorganisms hitherto unknown genes with putative functions for transport/binding are beginning to be identified. On the other hand a large number of genes with unknown function have been observed in organism such a *Pseudomonas putida KT2440* and other *pseudomonas* species. Especially the genes for metal binding proteins of *Pseudomonas putida KT2440* have not been characterized and till date two duf family metal binding proteins were identified in Cu (I) bound CsoR from *Mycobacterium tuberculosis,* Cu (I) and Cu (II) CsoR from *Thermus thermophilus* HB8.

Heavy metal tolerance and homeostasis has been well studied in *P. putida* KT2440 and *P. putida* CD2 (Canovas *et al*, 2003; Hu and Zhao, 2007). Expression studies using quantitative reverse transcription-PCR showed the induction of *mrdH and mreA* by cadmium, chromium, zinc, cobalt and nickel respectively. Since, both *mrdH* and *mreA* are part of the genomic island in *Ppu* KT2440, transcriptional response of the flanking mobile genetic elements Tn*4652* (*tnpA* and *tnpR* components) IS*1246*

transposase was also studied. Recent studies from our laboratory showed that that *mrdH* is novel heavy metal efflux transporter exhibiting broad substrate specificity form *P. putida* KT2440. In association with *mrdH*, *mreA* encoding a putative cytoplasmic protein has been identified (Haritha *et al.*, 2009).

There is also a lack of systematic study of metal binding proteins molecular characterization from *Pseudomonas putida* KT2440. Such analyses could bring out unique features of metal binding specificity related to metal homeostasis and its physiological significance. Based up on the availability of genome sequence of *Pseudomonas putida* KT2440, we have identified a conserved hypothetical putative metal binding gene (PP_2969) in the vicinity of *mrdH* in *P. putida* KT2440.

In the present study, the *mreA* gene from *Pseudomonas putida* KT2440 was characterized by constructing a recombinant *E. coli*. Thus the present thesis work was undertaken with the following specific objectives:

1. *Insilico* validation of a metal binding protein from *Pseudomonas putida KT2440.*
2. Characterization of putative metal binding protein properties in bioengineered *Escherichia coli.*

The work carried out is presented as five chapters. These include introduction in Chapter I, materials and methods in Chapter II, The experimental results and discussed in chapters III & IV, followed by a summary in Chapter V.

CHAPTER I: Introduction

A consolidated review of fundamental aspects related to metal ion interactions in microorganisms has been presented in this chapter. It encompasses the background literature pertaining to heavy metal resistance mechanisms in microbes with a special emphasis on bacterial efflux systems. The status of the genus Pseudomonas in the post genomic era and various facets of *Pseudomonas* metal biology were covered in detail to understand the lacunae and project the importance of the present study.

CHAPTER II: Materials and Methods

The second chapter describes the growth and culture conditions and the various experimental techniques used in the present study. The procedures followed for chromosomal/plasmid DNA isolation, polymerase chain reaction (PCR), restriction digestion, ligation, DNA transformation and screening of positive transformants, semi-quantitative reverse transcriptase PCR are given in detail. The techniques employed for extraction and estimation of total cellular proteins, their electrophoretic separation by SDS-PAGE and western blotting are elaborated. The protocols followed for transcription and translational expression of above gene during metal stress and its accumulation assays of cells are also described.

CHAPTER III: *Insilico validation of a Metal binding protein from Pseudomonas putida KT2440.*

On the basis of a bioinformatics analysis, we have identified putative metal binding sites for metal binding and amino acids were 40, 65, and 69, has been postulated

to ribbon like structure. In blast analysis strongly suggests that MreA protein was Nickel binding 55 out of 100 proteins were hypothetical *Pseudomonas* sequences and 1 protein showed Copper binding RcnR/FrmR. Pair wise Alignment of MreA and copper binding protein RcnR (61% identical). Homology modelling of MreA was generated from 5-88 by using M4T server and templates were 2HH7, 4ADZ, 1OUZ (RMSD - 1.20Å, Energy is -3392.598 KJ/mol). Molecular docking of MreA and Zink ion was accomplished on Autodock. Metal binding sites C40, H65 and C69 were replaced with Alanine (Alanine scanning).

CHAPTER-IV: Characterization of putative metal binding protein properties in bioengineered *Escherichia coli*

This chapter deals with the isolation, characterization and functional validation of MreA in heterologous systems. The Atomic absorption spectroscopy analysis reported here, the protein was showing the binding of metals as follows Zn>Cr>Cd. This was further evident and confirmed by QRT-PCR and western analysis. Metal binding efficiencies of wild type proteins compared with site directed mutants of MreA, which were generated by primer, based substitution of Alanine amino acid. As a part of *in-vivo* analysis wild type and mutant strains were subjected for transcriptional, translation and metal accumulation analysis by using quantitative Real-Time PCR, western and AAS in the presence and absence of ligands (i.e. metals ions).

Chapter V: Summary

As a result of *insilico* analysis, MreA protein existing as dimer and all metal ions are interacting with homo dimer complex only. Metal binding positions are 65, 69 amino acid residues of monomer1 and 40^{th} residue of monomer2. Alanine scanning confirms the importance of suspected residues. As a part of cloning and overexpression studies, above identified gene was amplified by PCR and further over expressed into *E.coli* expression systems. Ni-NTA metal chelate affinity chromatography was employed for purification of over expressed protein and confirmed by anti-his antibodies. Purified protein was injected into rabbits for getting polyclonal antibodies. Metal induction studies were analyzed by Q-PCR and western analysis in wild type, recombinant bacterial systems as well. LC-MS/MS was used for identification of apparent molecular weight of the recombinant protein using peptide maps and sequencing of some of the peptides. As a part of functional study, metal binding studies were undertaken by Atomic absorption spectroscopy (AAS) and the metal binding or accumulation efficiencies were as follows Zn>Cr>Cd. This was further evident and confirmed by QRT-PCR and western analysis. Metal binding efficiencies of wild type proteins compared with site directed mutants of MreA, which were generated by primer, based substitution of Alanine amino acid.

As such, MreA may be deployed as a potential candidate for enhancement of metal accumulation and tolerance in diverse organisms, which will be explored in the fields of medical and environmental biotechnological applications.