CONSTRUCTING METAL BINDING PROTEINS FOR USE IN BIOTECHNOLOGY

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Abstract

To combat environmental pollution caused by heavy metals and fulfillment of energy demand of humankind, this research was focused on constructing metal binding proteins for use in biotechnology. To achieve this objective, complementary degenerate putative metal (copper ion) binding motifs were designed to create a library of potential copper ion binding short peptides which have applications in bioremediation of heavy metal (metal sequestering) from the polluted environment.

These putative copper ion binding motifs were expressed downstream of the *malE* gene of pMALp2x plasmid. The *malE* gene codes for a fusion protein called maltose binding protein (MBP) so the designed motifs were expressed along with MBP as small peptides in *E. coli* DH5α cells. Upon sequencing of the recombinant pMALp2x plasmids, seven putative copper ion binding peptides (9-10 amino acids) were generated from these putative copper binding motifs. Each peptide contains a putative copper ion binding site, made up of Cys, His, Tyr and Arg amino acids. The fusion proteins were purified by maltose affinity chromatography. Resulting proteins were concentrated and measured. The recombinant *E. coli* DH5α cells expressing the putative copper binding peptides were grown on copper sulfate gradient plates containing the selective LB medium to check the resistance or Cu^{++} metal binding properties of these recombinant cells. Recombinant cells harboring short peptides as a fusion to MBP protein were quite resistant to growth on copper sulfate in comparison to the control cells. It indicated that these peptides can bind copper metal and from variation between growth of cells on gradient plates, it was proposed that all these peptides interact with copper with more or less binding efficiency ultimately these recombinant cells can be used for bioremediation or heavy metal sequestering to clean up the environment. The other possible use of these putative copper binding peptides in fusion with OprP, a porin protein of *P. aeruginosa* PA01, to make it a redox active electron transporter for future research in electricity generation using a microbial fuel cell (MFC).

To fulfill the energy demand a novel mutagenesis approach was applied to OprP, to make it a redox active electron transporter by changing amino acids Tyr^{62}, Asp^{80} and Asp^{94} to His, His and Cys, respectively which create a potential copper binding site into channel region of this protein. OprP from *P. aeruginosa* PA01 was selected due to its inherent high specificity for anions transport across the cell and a well studied crystal structure which offers the chance of mutagenic studies to make it possible electrons transporter. The *oprP* gene was amplified, cloned into *E. coli* DH5α & TOP10 cells and native OprP porin was expressed in *E. coli* DH5α cells. The designed putative copper binding peptides might be fused to it and express the resulting fusion protein in a suitable bacterium. To analyze the redox active properties of this recombinant bacterium a biofilm will be created on the anode of an MFC to check its electric potential or electricity generation capacity.

Acknowledgements

I am always owe to my parents and family who have been my major motivation during this research period and words are eloquent to thanks for their untiring help, co-operation and affection which always stood by my side at all time of ups and down.

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Last but not the least; I bow my head before ALMIGHTY GOD for showering his blessing upon me always.

Declaration by author

I declare that all of the work presented in this thesis is my own.

Dharmender Kumar

CHAPTER-I

1.1 Introduction

The single most critical challenge facing humanity today is energy. Due to globalization, the conventional energy resources around the world are going to decline but the demand for the energy continues to increase day by day. This makes renewable and non-polluting sources of energy one of the greatest demands of peoples worldwide. In search of clean sources of energy, attention has turned to the extraction of energy from environmental bio-wastes which could be an important and rich source of energy. To extract energy from bio-wastes it is necessary to degrade the bio-waste by suitable means without any further environmental pollution. Degradation of organic wastes by microorganisms is accompanied by the production of electrical energy without any environmental pollution (Potter, 1911; Lovely, 2006 & 2008; Lovely and Kelly, 2008). So production of energy from bio-wastes with the help of either a single or a diverse range of microbial population in a special device called microbial fuel cell (MFC) might be a better option (Lovely and Kelly, 2008). It is well known that microorganisms can produce a wide variety of fuels such as ethanol, hydrogen and methane, from the degradation of the organic wastes or bio-wastes but less known about the conversion of bio-wastes into electricity by microorganisms using a device called microbial fuel cell (MFC)

1.1a Microbial fuel cell

A simple device that is capable to convert chemical energy from a diverse range of carbonic/organic waste into electrical energy using microorganism (bacteria) as biocatalyst, is known as microbial fuel cell (Shukla *et al.*, 2004; Lovely, 2006). It is usually made up of two electrodes, a cathode and an anode, which are put separately in two different compartments separated by a thin membrane known as proton exchange membrane; these

two electrodes are joined by an electrical circuit (Fig. 1). In the anodic chamber, organic compounds/ bio-wastes are oxidized or degraded by microorganisms into CO_2, protons and electrons as described in chemical reaction 1. In an MFC a wide range of substrates are utilized to produce the electrical energy for example cellulose, sucrose, glucose, starch, methanol, ethanol, butyrate, formate, acetate, amino acids, proteins and some inorganic substances like acid mine drainages and sulfides (Heilmann and Logan, 2006; Liu *et al*., 2007; Cheng *et al*., 2008; Clauwaert *et al*., 2008; Ishii *et al*., 2008 and Rabaey *et al*., 2008). The substrate which is most commonly used in MFC is acetate due to its inertness towards alternative microbial metabolic conversions of fermentative to methanogenesis pathways at normal room temperature. It can also produces ~ 98% columbic efficiencies of MFC (Rabaey *et al.*, 2005) and up to 115 $W.m^3$ power outputs (Cheng and Logan, 2007) for a diversity of mixed anodophilic microbial biofilm.

Bacterial culture in aerobic conditions use O_2 as final electron acceptor in the production of H_2O but no oxygen is present in the anodic chamber where bacterial culture is added in an MFC so bacterial cells need to change their natural soluble electron acceptor to an insoluble electron acceptor of an MFC know as anode. This switching ability of the bacterial cell makes it easy to transport electrons to the anode of an MFC, as shown in Fig. 2 converting a complex organic fuel to electricity using diversity of microorganisms. Synthesized electrons by microbial metabolism, transfer to the anode via three primary mechanisms (1) direct transfer which involves proteins located on the surface of the bacteria (2) by use of small, redox reactive molecules which can 'shuttle' electrons from bacteria to electrode surfaces by diffusion-limited processes, and (3) by small electrically conductive appendages, pili also known as microbial or bacterial nanowires as depicted in the Fig. 3 (Reguera *et al*., 2005 and Lovely, 2008). Finally the electrical current (electrons) is

transmitted from anode to the cathode via an electrical circuit with a resistor. The resulting potential difference in volt between these two electrodes (anode and cathode) with electrons flow (ampere) results in the electrical power (watt) generation. The synthesized protons are transferred to the cathode via proton exchange membrane, these protons react with the environmental oxygen and electrons to produce H_2O and complete the circuit of an MFC as shown in the reaction 2. This project is relevant to the first mechanism involving direct electron transfer via bacterial cell surface proteins. A rational synthetic biology approach was used to modify the protein structure by genetic engineering (mutagenesis) to make an efficient electron transporter. The intention was to create a chain of redox active metal by using copper, a redox active transition metal. It is required in trace amounts from µM to mM for survival and growth of various types of microorganisms. It occupies two different redox state +1 and +2 under the same physiological redox conditions (Cascella *et al.*, 2006). Copper ions have important roles in biochemical processes for examples redox reactions, transport of dioxygen species and transfer of electrons (Zhaoxiang *et al.*, 2010) so putting copper on either side of the OprP porin might allow electrons transfer from the periplasm to the outside of cell and thus be useful in electricity generation using MFCs.

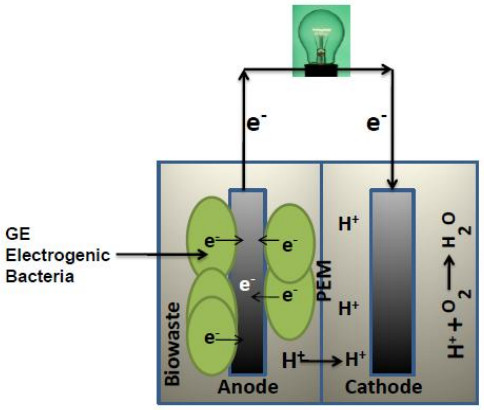


Figure 1: Design of a microbial fuel cell (MFC). Electricity generation from organic waste in the anodic compartment of an MFC. The bio-waste is oxidized completely by genetically engineered bacterial cell bio

film on the anode and transfer of electrons by various mechanisms to the fuel cell electrode. In the anodic compartment the conditions are anaerobic to microaerophilic while in the cathodic compartment the conditions are aerobic.

Note: GE-genetically engineered, PEM- proton exchange membrane, H^+ and e^- are protons and electrons, respectively. Picture adapted from Bruce. E. Logan, 2008.

Reaction 1. On Anode : $C_XH_YO_Z + H_2O \longrightarrow CO_2 + e^- + H^+$

Reaction 2. On Cathode: $O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$

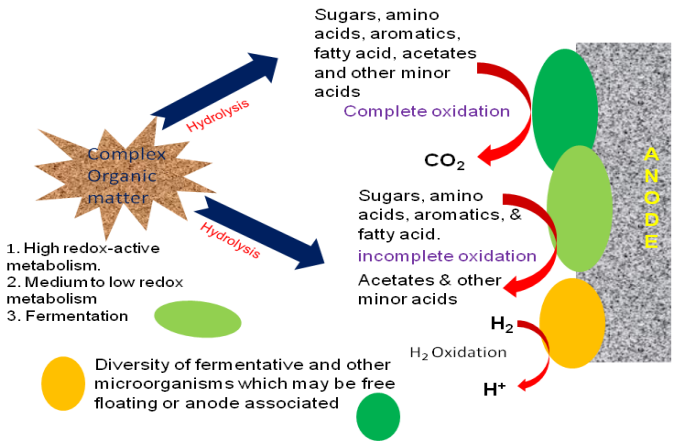


Figure 2: Microbial degradation of complex organic waste into electricity in the anodic chamber of an MFC. Complex organic waste is hydrolyzed first into fermented constituents called incomplete degradation by diversity of microorganisms while some microbes can completely oxidize the carbonic/organic wastes (complete degradation) using an electrode as sole electrons acceptor. The products of fermentation (acetate and some minor acids) then fully oxidize into CO_2 that will be the electrons source for current generation. The produced H_2 from fermentation could be electrons source. Picture adapted from Lovely, 2008.

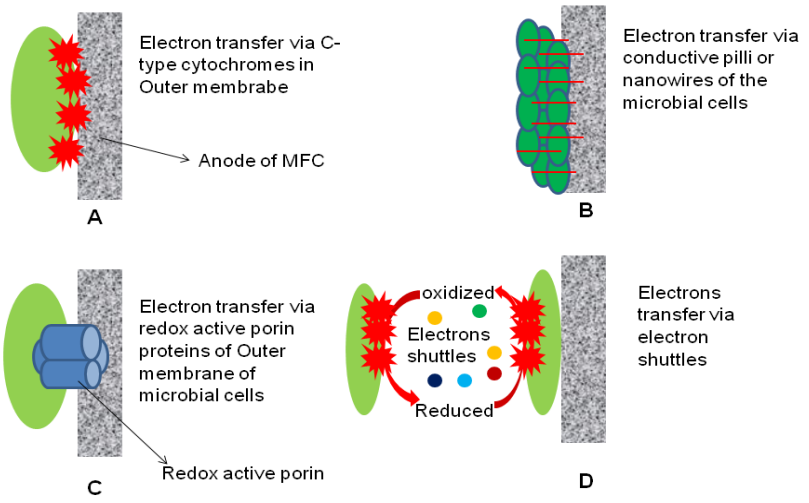


Figure 3: Cartoon represents the possible electrons flow mechanisms to anode in an MFC. Red spiked structures in A & D denote surface cytochromes of bacterial cell, red lines in B show pili called bacterial nanowires and dots of different colours in D are the electron shuttles present in the media conferring conductivity. Picture adapted from Lovely, 2008.

This project was focused on constructing a genetically engineered nonpathogenic bacterial cell that will be able to transmit electrical current (electrons) to the anode by using a genetically engineered version of the naturally occurring outer membrane porin protein, OprP from the Gram negative bacteria, *P. aeruginosa* PA01. It can be achieved by modifying residues in the OprP channel to bind a redox active metal like copper (Fig. 4) to transfer the electrons from the periplasmic space to outside of cell; the proposed model for this transfer of electrons is shown in Fig. 5. A similar model of electrons flow to the anode in an MFC via the membrane cytochromes from *G. sulfurreducences* is shown in Fig. 6.

The second approach of making OprP a redox active transmembrane electron transporter is to design small copper binding peptides and fuse them on either side of the channel. In order to alter the electrical conductive properties of the protein product, the existing gene of the OprP was manipulated using site directed mutagenesis.

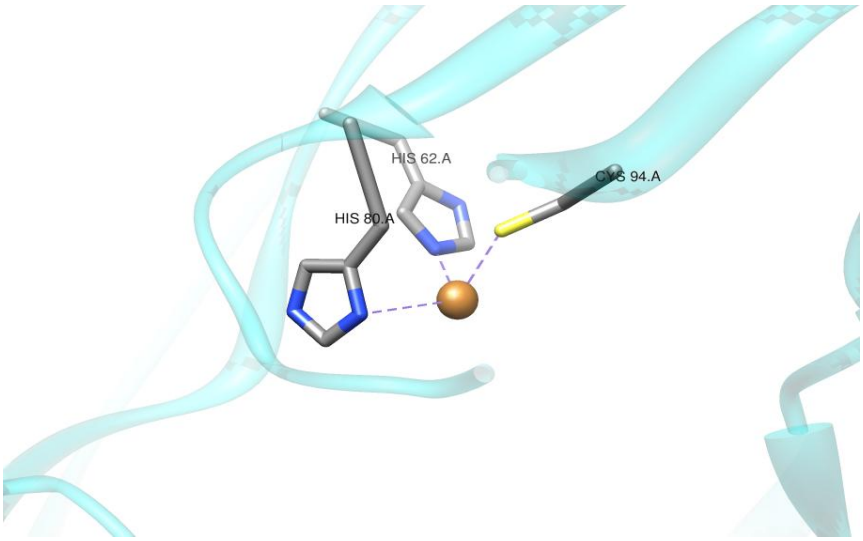


Figure 4: Generating a copper ion-binding site in the OprP monomer by mutation of residues Tyr^{62}, Asp^{80} and Asp^{94} to His, His and Cys, respectively. The structure was made using UCSF chimera.

The proposed interactions are:

His^{62} and His^{80} make bonds with copper ion via N from imidazole ring

Cys^{94} make bond with copper ion via S from the thiol group.

Fourth coordination may be completed by carboximide, O atom of Asn^{78} side chain or Peptidal N of Tyr^{79}

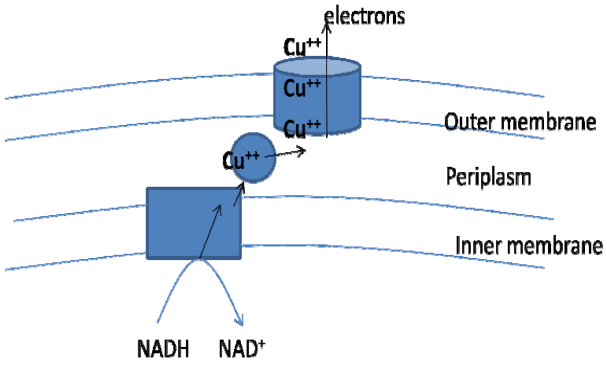


Figure 5: Proposed electron transfer model via genetically engineered redox active OprP protein from *P. aeruginosa* PA01. To make it redox active the Cu^{++} binding site was created which help in redox reactions and transfer of electrons from periplasm to outside of the cell. NADH derived from oxidation of organic matter into the cytoplasm of the cells and energy conservation results from pumping of protons through inner membrane associated with electron transport. Subsequent electrons flow to the anode will occur via redox

active outer membrane channel protein OprP, which has been proposed for cells in direct contact with anode in an MFC.

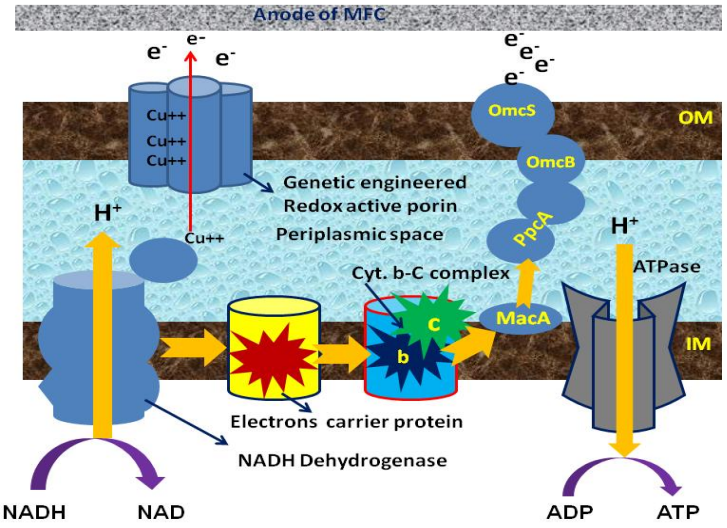


Figure 6: *G. sulfurreducens* electron transfer model along with the proposed genetically engineered OprP porin in the outer membrane. Transfer of electrons to the anode was depicted using cell surface cytochromes OmcS and OmcB which are main components of the respiratory chain of *G.sulfurreducens* for extracellular Fe (III) reduction. The OmcS and OmcB get electrons from inner membrane cytochromes bc complex and periplasmic, Ppca binding protein, respectively to make an electron conduit. The electrons and protons are synthesized by oxido-reduction reactions of organic matter; converting NADH to NAD by NADH dehydrogenase and ADP to ATP by ATPase enzymes, respectively. Generated electrons are transferred to the electrons carrier proteins in the inner membrane and protons in the periplasm of the cell. The energy conservation takes place by proton pumping across the inner membrane. Picture adapted from Lovely, 2008.

1.2 Cell envelope of Gram negative bacteria

Gram negative bacterial cell envelope is composed of three distinct parts; the inner membrane, periplasmic space and outer membrane (Fig. 6). They all have an important role in maintaining the equilibrium between the intracellular and extracellular environments of the cell. Each of them also plays a role in uptake of nutrients from the extracellular environment and transport of toxic components and other metabolites out of the cell. In addition many bacteria also produce surface structures such as capsules, pili and flagella. These surface structures help in providing resistance to the phagocytic action of neutrophils

and to antibiotics, locomotion and sensing of environmental conditions, respectively (Costerton *et al*., 1981).

1.2a Inner membrane

Gram negative bacterial inner membrane is asymmetrical bilayer of phospholipids with polar head group facing toward either the cytoplasm or periplasmic space (Nikaido and Vaara, 1987). The phospholipids have hydrophilic head and hydrophobic tails and these tails facing toward each other and associated by strong, non-polar interactions. The lipids arrangement of membrane provides a formidable barrier to the non-specific transfer of hydrophilic molecules and a little impediment in the transport of moderately hydrophobic molecules across the membrane. A variety of different proteins are also interspersed with the lipid molecules which help in serving many functions for example proteins ATPase help in active transport of the molecules by maintaining an electrochemical gradient and conservation of energy across the membrane (Dassa and Hofnung, 1985). Others proteins help in translocation for example proteins of Sec and Tat pathway which bind specific substrates and finding their way into periplasmic space in conjunction with other inner membrane associated proteins. Some proteins work as pumps across the membrane helping in expulsion of noxious compounds from inside of the cell (Li *et al*., 1995). Other class of proteins acts in synthesis of various components of the cell envelope (Tuomanen, 1986).

1.2b The periplasmic space

A gelatinous aqueous layer that separates the two membranes (outer and inner) of a Gram negative bacterial cell is called the periplasmic space. This gelatinous substance is called peptidoglycan, made up of linear strands of two different amino sugars, N-acetyl glucosamine and N-acetylmuramic acids. This peptidoglycan layer provides a stabilizing

force in the cell envelope and shows covalent or strong non-covalent attachment to the various proteins in outer membrane of bacteria (Nikaido and Varra, 1987).

In addition to stabilizing role, the periplasmic space acts in the determination of the molecules which enter the cell cytoplasm as well as the rate at which these molecules cross the membrane. Various substrate binding proteins have been observed to reside into the periplasmic space having specificity towards molecules which are necessary for the growth and survival of the bacterial cell. These substrate binding proteins ensure the continuous flow of the molecules through the outer membrane by maintaining a low concentration of free substrate into the periplasmic space for examples the phosphate binding protein (OprP) of *P. aeruginosa* and maltose binding protein of *E. coli* have shown to be responsible for keeping the free periplasmic concentration of their respective substrates far below that of the exterior of the cell (Kellerman and Ferenci, 1982; Poole and Hancock, 1984). Donnan/electrical potential is maintained across the outer membrane which helps in transfer of charged molecules into the periplasmic space. This Donnan/electrical potential is enhanced by periplasmically localized fixed charges (Stock *et al*., 1977).

1.2c The outer membrane

Gram negative bacterial outer membrane is also composed of a number of phospholipids molecules like the inner membrane. In addition, this layer contains a lipopolysaccharide (LPS) layer which is only found in the bacterial cell wall (Nikaido and Varra, 1987). The molecules of LPS layer are made up of lipid A core and a hydrophilic polysaccharide tail. Lipid A anchors in the membrane while the hydrophilic tail protrudes away from the surface. The outer membrane is arranged in such a way that LPS layer forms the external face while the phospholipids line the inner face of the membrane. The LPS molecules attach covalently and non-covalently to the proteins of outer membrane (Hancock and

Karunaratne, 1990). The stability of outer membrane is maintained by different kinds of divalent cations such as Mg^{++} (Hancock *et al*., 1994).

This membrane also composed of a number of proteins like other biological membranes; these proteins perform various functions for example transport of molecule, structural stability of membrane and sensing the environmental signal etc.

OmpA, one of the major proteins of *E. coli* cell that maintains structural stability of the outer membrane in addition to its channel forming activity (Sugawara and Nikaido, 1992). Braun lipoproteins of *E. coli* also play a role in stabilizing the outer membrane structure it is localized on the inner face of the outer membrane and is covalently attach to the peptidoglycan layer of the periplasmic space (Braun and Sieglin, 1970). Some other outer membrane proteins of *P. aeruginosa* which act as channels include OprP, OprC and OprD.

The outer membrane of Gram negative bacteria acts as an excellent barrier to non-specific transport across the cell. This is due to the presence of hydrophilic LPS side chains on the external surface which limit the uptake of hydrophobic molecules and internal nonpolar nature of lipid core which does not permit transport of hydrophilic compounds. This impermeability of the outer membrane is facilitated by inclusion of different proteins which help in the uptake of different kinds of nutrients from the environment. The difference in permeability of the Gram negative bacterial outer membrane to the inner membrane is because of the difference in numbers and types of the proteins as well as their expression.

1.3 Functional and structural aspects of porin proteins of Gram negative bacteria

1.3a Functional aspects

Porin proteins are the outer membrane-spanning water filled channels of Gram negative bacteria whose normal function is to allow small molecules (nucleotides or sugars etc.) across the outer membrane. The presence of these porins in Gram negative bacterial outer

membrane provides these organisms with a means to obtain various growth factor and nutrients from the extracellular environment which are essential for the survival and growth of the cell (Jeanteur *et al.*, 1991). Functional analyses of these porins can be done by different techniques for examples uptake experiments using whole cell expressing the porin of interest, model membrane studies of purified porin via different biophysical techniques etc. Biophysical techniques provide a finer level of understanding of the porin proteins as they allow better control of the environment to which the porins are exposed. Based on these studies porin proteins are classified into two functional categories, general diffusion porins and specific porins. The majority of the porins studied to date have been shown to form non-specific channels allowing passive diffusion of different molecules across the outer membrane below their size exclusion limit hence they are chemically non-specific. Porins which have been shown to perform these functions in *E. coli* are OmpF and OmpC. Functional analyses of these porins demonstrates that the channels share many features i.e. their diameter is of slightly more than 10 Å and the exclusion limit is approximately 600 Daltons (Nikaido and Rosenberg, 1983). The channels from the OprF and OmpC have also been shown to exhibit a slight to moderate selectivity towards cations over anions. However, both of these proteins differ in their single channel conductance measured using an artificial membrane system. Purified OmpF has an average conductance of its channel 2.1 nS in 1 M KCl when reconstituted into a bilayer. By using the same method, OmpC shows conductance of 1.5 nS (Nikaido and Rosenberg, 1983). These functional differences have been shown to be due to the difference in the electrochemical nature of the amino acid residues which are present in the channel of these porins. Proteins analogous to these two porins have been recognized in other Gram negative bacterial outer membrane (Lee and Schnaitman, 1980).

Another nonspecific porin is PhoE of *E. coli* which is expressed in the outer membrane under limiting phosphate conditions (Overbeeke and Lugtenberg, 1980; Poole and Hancock, 1986). The production of this porin under phosphate deprivation is related to selectivity for anions; the negatively charged phosphate ions more easily enter the cell. PhoE can transport most anions as efficiently as phosphate so it is also a nonspecific diffusion porin (Benz *et al*., 1984). The channel conductance and exclusion limit of PhoE is similar to OmpC and OmpF and the amino acid sequence of PhoE is 30% similar to OmpC and OmpF (Jeanteur *et al*., 1991).

Specific class of porins contains a substrate binding site for specific solutes. This site makes these porins specific to molecule than other of comparable size (Siehnel *et al.*, 1990 and Sukhan and Hancock, 1995). LamB of *E. coli* expressed in response to particular growth conditions of maltose so it is a maltose specific porin. It expressed in the outer membrane under low concentrations of carbon sources other than maltose (Schwartz, 1987). Examination of this protein by various techniques has explored that this exhibits a distinct preference for maltose and maltodextrin sugars transport into *E. coli* cells (Wandersman *et al*., 1979; Benz *et al*., 1986). Several other proteins which behave like porins have been identified in *P. aeruginosa*. These include nonspecific OprF as well as specific porin like OprC, D and OprP of *P. aeruginosa* (Hancock *et al*., 1982). Most constitutively expressed porin of *P. aeruginosa* is OprF. The size exclusion limit of this is much higher than that of *E. coli* porins. It was measured 3000 Daltons *in vitro* conditions. Data from *in vivo* conditions suggest the channel diameter is 15.6 Å. Despite higher channel diameter of OprF, the diffusion rate of a solute through it is about two orders of magnitude lower than that of OmpC and OmpF (Benz and Hancock, 1981; Nikaido *et al.*, 1991). This is because of only a small fraction of OprF porin forms the large channels while the majority of other

porins make channels with considerably smaller exclusion limits. The Conductances of this porin are 0.34 nS (major) and 5 nS (minor) as suggested by lipid bilayer experiments (Benz and Hancock, 1981; Nikaido *et al*., 1991). The *E. coli* porin OmpA has been shown to form channels which share some features with OprF of *P. aeruginosa*. (Sugawara and Nikaido, 1992). The OprD and P of the *P. aeruginosa*, expressed in the outer membrane during specific environmental conditions. Wild type expression of OprD is low and the conditions essential for increased expression have not been fully understood. The proposed binding site of OprD is thought to be specific for basic amino acids (Trias and Nikaido, 1990). *P. aeruginosa* growth under phosphate limiting conditions (lower or equal to 0.2 mM) induces the expression of outer membrane proteins, P & O (Hancock *et al.*, 1982). The properties of channel formed by OprO are quite similar to other porins but different from OprP. The OprO channels show low affinity with a high capacity to both PO_4^- and Cl^- ions than OprP. It shows 10 fold higher affinity to pyrophosphate and monophosphate than OprP.

The functional differences in the porins appear due to specific regions of amino acid sequence and only small numbers of individual amino acids (Sukhan and Hancock, 1995). Insertion and deletion mutagenesis in porin's genes showed that only certain parts of the expressed porins were permissive for these changes (Boulain *et al.*, 1986; Bosch and Tommassen, 1987; Rocque and McGroarty, 1990) and it seemed likely that the sites which were permissive for alterations (insertion or deletion) were located in the loop regions which connect the individual β strands. Foreign epitopes insertion into these permissive sites allowed the identification of surface exposed loops. (Charbit *et al*., 1986; Agterberg *et al*., 1987).

1.3b Structural aspects

Structural studies of a number of general diffusion porins have been determined by biophysical techniques i.e. crystallographic and spectroscopic techniques. Based upon these studies porins are made of three similar monomers, each of which is a 16-stranded β-barrel. These β-barrels are connected by loops exposed on either side of the membrane. The surface exposed loops are longer and more variable than periplasmic loops which are short and regular.

The information regarding structure of specific porins are less comparable to nonspecific porins so the list of specific porins is rather shorter. LamB and Tsx in *E. coli* and OprB, OprD, OprO, and OprP in *P. aeruginosa* are some examples of specific porins (Sukhan *et al*., 1995).

A number of topological models available based on the data generated by previously described techniques and methods; the most accurate available to date is X-ray crystallography for the assessment of three dimensional structures of the proteins. The structure of porin from *R. capsulatus* based on X-ray diffraction data showed that this porin is arranged in a complex of three identical monomers (Weiss *et al*., 1990) and these monomers were arranged around a central axis, tilted at slight angle of 16^0. Each monomer adopted the confirmation of β-barrel, composed of 16 stranded. This barrel is enclosed by a central water filled channel. The amino acid sequences of the β-barrel tended to alternate between hydrophilic and hydrophobic residues so one side of the β-barrel was faced to the interior of the channel and the other side faced the lipid core of the membrane. The larger surface exposed loops of OmpF β-strands seemed to play a much more essential role in determination of the channel characteristics than the smaller periplasmic loops. Six of the eight surface exposed loops (loops 1, 4, 5, 6, 7 and 8) were shown to form an umbrella like

structure (Fig. 7) over the opening of channels (Sukhan, 1996). This umbrella structure limits the size of molecules which can pass through the channels. Third surface exposed loop positioned in such a way that allows channels to exert a substantial influence over the passage of molecules through the channel. This loop is folded back into the channel and creates an eyelet structure approximately half way down the channel. This eyelet is believed to constrict the internal diameter of the pore and further reduce the channel exclusion limit (Sukhan, A., 1996). The crystal structure of the highly homologous *E. coli* general diffusion porins, OmpF and PhoE were found similar to *R. capsulatus* porins (Cowan *et al*., 1992) but as the amino acid sequence of *R. capsulatus* porins does not show a great deal of homology with these porins so this is somewhat surprising. Likewise *R. capsulatus* porin, *E. coli* porins also have third surface exposed loops which fold back into the channel and the second loop that acts to interlock the monomers (Van der ley *et al*., 1987; Benson *et al*., 1988). The loop regions were shown to be the same section that were permissive for the mutations. To verify the location of the loops, specific mutations were carried out in the amino acid sequences of these porins and this showed that certain amino acids which located in and around these loops define individual channel's characteristics (Misra and Benson, 1988). The specific channel characteristics were also confirmed by combinatorial mutagenesis in the OmpF, OmpC and PhoE (Benz *et al*., 1989). The experiments showed that the third surface exposed loop contains a number of residues that play an essential role in the function of the channels. Mutations between amino acids 108 and 133 in OmpF is responsible for cations selectivity (Benson *et al*., 1988). Other studies have shown that Lys^{125} in PhoE serves to change the ion selectivity of the channel (Bauer *et al.*, 1989). All of these amino acids are present in the third surface exposed loop. Thus, these results strengthen the proposal of an interior location for this particular third surface exposed loop.

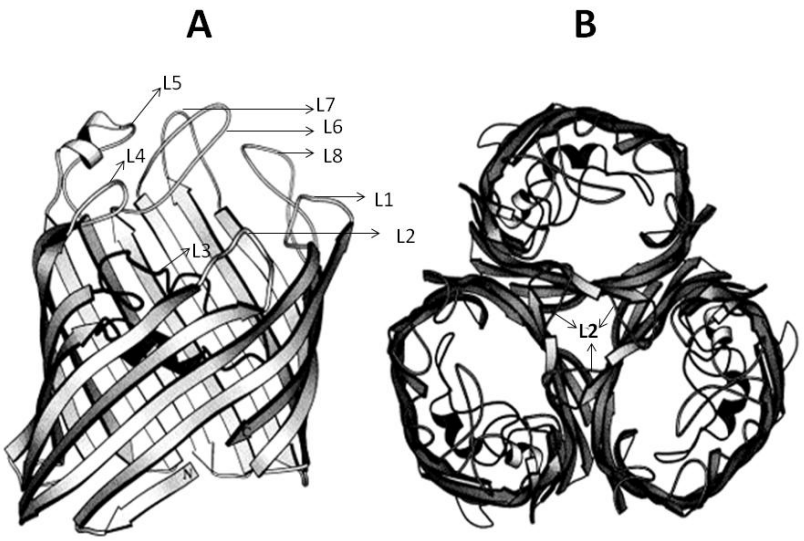


Figure 7: **β-barrel structure of OmpF porin from *E. coli*.** Picture adapted from Sukhan, 1996.
A. Umbrella type structure formed by the surface exposed loops 1, 4, 5, 6, 7 and 8 over opening of the channel in a monomer
B. The second loop acting to interlock the monomers of the porin.

As the structure of LamB (maltose specific porin) from *E. coli* was found to contain large amount of β sheet structure, it was supposed the topology would be identical to that of other better studied porins of this family. However, there exists little similarity between the amino acid sequence of LamB and other porins (Jeunteur *et al*., 1991). As this consists of approximately 25% more residues than other porins. Crystal structure of this porin showed the structure is quite similar to other porins with only one exception; the β barrel was composed of 18 strands rather than 16 (Schirmer *et al*., 1995).

Mutagenesis experiments explored that amino acids situated in both second and third surface exposed loops were essential for substrate specificity for maltose (Heine *et al*., 1987). In addition to these, certain residues arranged throughout the sequence direct the functions of the channel. Another example of porin is OmpX, a long β-barrel porin of *E. coli* cell having 8 antiparallel β-strands with high copies number in the outer membrane. It

is a monomeric porin of 148 amino acids which made up of 3 periplasmic and 4 external surface loops (Lai *et al*., 2004).

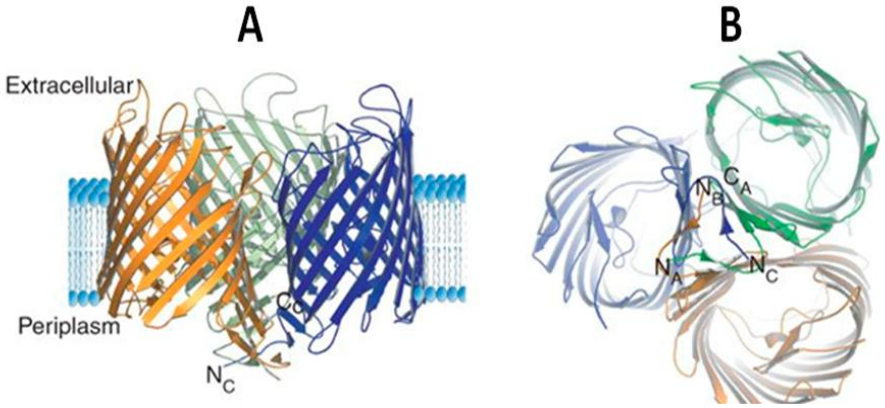
1.4 OprP protein of *P. aeruginosa* PA01

1.4a Characteristic of OprP protein:

Earlier biochemical and biophysical studies of OprP documented its many features which are closely related to other bacterial porins for example a trimeric structure with high β-sheet (68%) content and resistance to sodium dodecyl sulfate (SDS), several proteolytic enzymes and antibodies (Trevor *et al.*, 2006). This porin is expressed under low phosphate condition (<0.15 mM) so is important for inducible phosphate transport system under phosphate starvation for survival and growth of the *P. aeruginosa* (Siehnel *et al*., 1990; Trevor *et al*., 2006).

The molecular weight of this porin is 48 kDa with 440 amino acids. The crystallographic structure at 1.9 Å resolution of OprP (Trevor *et al*., 2006) revealed that it is composed of 16 antiparallel β-strands. The barrel is slightly elliptical in nature as shown in (Fig. 8) having dimensions laterally of ~ 3-3.5 nm and ~ 5 nm in height. The OprP trimer is stabilized by an extended periplasmic *N*-terminus strand exchange through a tricorn hat like structure while in all other porins (PhoE and OmpF etc.) the integrity of oligomeric structure is stabilized by the exoplasmic L2 loop. This tricorn hat like structure completes the 15 and 16 strands of β-barrel of each monomer. The periplasmic loop L7 and extracellular loops L3 & L5 are prominent in the OprP structure. L3 loop has α-helices which extend deep into the lumen of barrel and constrict the pore size. The extracellular L5 loop creates an electropositive sink (made up of Lys residues) inside the pore surface by running along toward the centre of the channel to attract anions (Trevor *et al*., 2006). On the extracellular surface each monomer forms a large funnel. These funnel have three

distinct Arg residues ladders which spiral down into the constriction zone near periplasmic face at ~3.5 Å where phosphate is coordinated. These ladders mediate PO_4^- specific movement through outer membrane of the *P. aeruginosa*. OprP is highly anion selective due to phosphate binding sites. It transports phosphate with an affinity 100 fold higher than other anions (Hancock and Benz, 1986; Trevor *et al*., 2006). The phosphate binding site in the pore region is made up of side chains of five residues: two from L3 loop (Arg^{133} & Ser^{125}) and a single residue from strand B1 (Arg^{34}), B2 (Tyr^{62}) and L2 loop (Asp^{94}) (Zeth *et al*., 2000; Hancock and Brinkman, 2002; Trevor *et al.*, 2006; Pongprayoon *et al*., 2009).



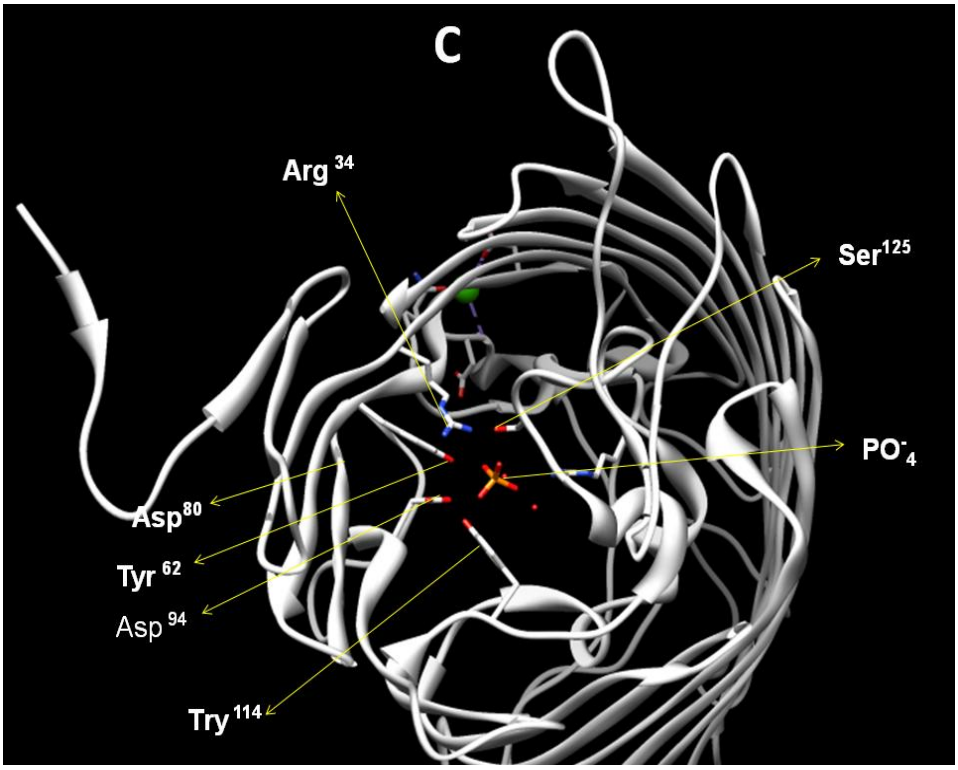


Figure 8: OprP of *P. aeruginosa* PAO1. (A) Side view of OprP subunits in green, blue and orange flanked by phospholipid bilayer (B) OprP tricorn *N*-terminal strands through the pore region from the periplasmic space, *N* & *C* termini of each subunit are also visible. (C) 3D structure of OprP single subunit (developed by UCSF chimera) showing close proximity of Tyr^{62}, Asp^{80} and Asp^{94} which could potentially be changed to His, His and Cys to make a possible copper binding site (Fig. 4). Pictures adapted from Trevor *et al.*, 2006.

1.4b Why OprP porin for this project

P. aeruginosa OprP protein seems like a good choice for creating a copper metal binding site in the pore region via site directed mutagenesis. A copper binding site in a protein can form by two His residues and rest of the coordination completed by a nearby Cys or other residues of the native protein or some inserted passenger peptides/protein (Regan, 1993). For example copper in the electron transfer (ET) site of cuperdoxin domains is anchored to a Cys and two His residues while a Met, Leu or Phe may present at 4^{th} axial position (Jeuken, 2001).

OprP is highly specific for anion transport and the X-ray crystal structure and simulations of anion transport through OprP channel are well documented (Trevor *et al.*, 2006; Pongprayoon *et al.*, 2009). Recently, much research effort has been focused on designing novel metal binding sites because metal can perform structural and catalytic functions within the protein. Some examples of naturally occurring metal binding proteins or metalloproteins are thermolysin, alcohol dehydrogenase and zinc fingers of eukaryotic transcription factors with Zn (II) binding site. Calmodulin and Troponin with Ca^{++} binding site. Catalytic function including redox activity of the protein, associated with metal binding site of the proteins for example Cu site of Azurin of *P. aeruginosa* , Pseudoazurin of *A. faecalis*, Plastocynin of *P. nigra* and Rusticyanin of *T. ferrooxidans* for electron transport in the bacterial cells (Jeuken, 2001). Most of designing strategies concentrate on acquiring accurate orientations of 2^{nd}, 3^{rd} or 4^{th} amino acids which form the binding site for metal ions (Regan, 1993). Tyr^{62}, Asp^{80} and Asp^{94} appear to be in close proximity in the structure of OprP (Fig. 8C) and could potentially be changed to His, His and Cys to make a potential copper binding site (Fig. 4). Cuperdoxin's type-1 copper binding site is present at a distance of 5-7 Å eccentrically from the outer surface. The copper ion in this protein is anchored by strong bonds with three amino acid residues a Cys and two His which coordinate by their S^{γ} and N^{δ} atoms, respectively in a pyramidal or a trigonal planar geometry. The 4^{th} coordination is completed with a weaker ligand, containing either Met or some time Glu with their S^{δ} or O^{e} atoms, respectively by occupying the axial position (Fig. 9). In certain instances, for example in azurin, a 2^{nd} axial group interacts weakly to backbone carbonyl oxygen which found at the opposite axial position (Jeuken, 2001). Azurin is an important electron carrier protein which exchanges electrons with other

proteins having redox properties for example cytochrome c551 or nitrite reductase, by using a Cu ion which is protein-bound.

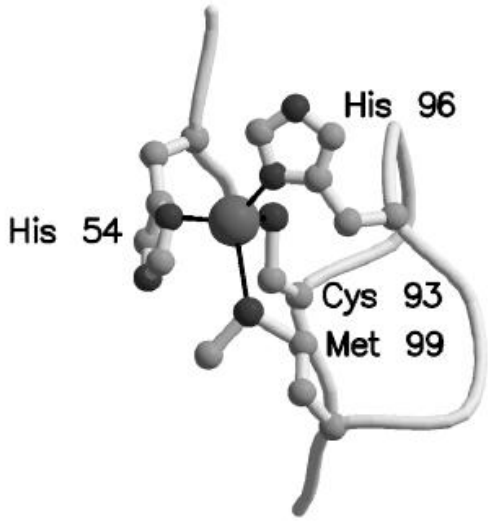


Figure 9: Active-site structure of wild type amicyanin (*Paracoccus versutus*) picture adapted from Salgado *et al.*, 1997.

1.5. Insertion of metal binding peptides/proteins in bacterial outer membrane proteins for use in biotechnology

Bacterial cells expressing metal binding proteins have applications from bioremediation of toxic heavy metals, bio-adsorption and recovery of rare metals to energy generation (Kouichi and Mitsuyoshi, 2010). Recombinant DNA technology offers methods for anchoring metal binding peptides/proteins to bacterial outer membrane proteins (Kotrba *et al*., 2011). Gram-negative bacterial outer membrane proteins offer the good choice for fusion of foreign proteins/peptides.

OmpA of *E. coli* allows insertion of foreign oligopeptide in the 2^{nd}, 3^{rd} and 4^{th} loops which are surface exposed (Freudl *et al.*, 1986 and Freudl, 1989). LamB of *E. coli* tolerates insertion in a number of sites but the most commonly used one is the outer loop.

The upper size limit for display of the heterologous peptides is 60-70 amino acids. A successful display example of 232 amino acids long polypeptide in the position of 153rd residue of staphylococcal protein A has been achieved by Steidler *et al.*, 1993. OmpC can allow insertions of up to the 449 amino acids long polypeptides at position 162 and 289 (Xu and Lee, 1999, Cruz *et al*., 2000 and Baek *et al*., 2010). Another example is OmpX which tolerates fusion of heterologus passenger peptides on the 2nd external loop (Kotrba *et al*., 2011).

Overall to anchor or insert foreign peptides/proteins into the outer membrane, the targeting protein or peptides should be onto the surface of cells and avoiding the periplasmic proteolytic cleavage. In this type of fusion experiment the size of the foreign proteins/peptides as well as the presence of specific amino acids in the foreign proteins/peptides plays a great role which can significantly affect the final topology of made fusion product. Functional groups of the outer membrane also have essential role i.e. the polar head groups of phospholipids assist metal binding primarily on the inner side of the outer membrane. LPS of the outer membrane can provide carboxyl as well as phosphoryl groups for divalent metal ions binding; the *E. coli* peptidoglycan layer makes bond with metal ions via the COOH group of D-glu of the peptide stem and the OH group of the glycan backbone (Hoyle and Beveridge, 1983).

1.6 Metal-protein interactions (with the emphasis on copper interactions with proteins and peptides)

An understanding of metal-protein interactions is provided by Cu-peptides/proteins interaction in solution and the gas phase. In the solution, side chains from the basic amino acids (Arg, Lys and His) are protonated, that reduces the binding energies of copper ions to *N*-donor ligands; in the gas phase, copper ions show highest preferences for binding to Arg,

Lys, and His amino acids (Shields, 2000). Most of the previous research focused on the gas phase copper-peptide interaction to specific amino acids and how the relative binding energies of copper ions to peptides affect the complex fragmentation reactions (Prudent and Girault, 2008). Lim and Vachet, 2003 developed a method for determination of the Cu^{++} binding sites in copper metalloproteins which is based on the oxidation reactions catalyzed by copper metal ion and mass spectrometry. Metal catalyzed oxidation reactions were used for oxidation of the amino acids in the copper binding sites and mass spectrometry helped in identification of the amino acids which have been oxidized.

Aims of the project

1. Express OprP of *P. aeruginosa* PA01 with a view to recombinant construction of a metal binding site.
2. Create a library of Cu-binding peptides.

CHAPTER-II

Materials and Methods

2.1 Reagents & chemicals

All reagents & chemicals used during this research were of analytical and molecular biology (MB) grade, purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Loughborough, UK). The media for culturing of microorganisms were prepared according to manufacturer's instructions using sterile H_2O. The Buffer and reagent solutions were prepared according to the established protocols. The antibiotics, ampicillin and kanamycin were purchased from the Melford, Ipswich, UK.

2.2 Bacterial strains & plasmids

In this research *P. aeruginosa* PA01 and *E. coli* DH5α strains were used throughout. The *P. aeruginosa* PA01 was used for *oprP* gene amplification by colony PCR. *E. coli* DH5α cells were used for all cloning and transformation of the *oprP* gene as well as the host for all expression, production and purification studies of OprP protein as well as MBP fusion proteins (maltose binding protein fused with synthetic putative metal binding peptides) experiments. The gene encoding OprP was originally cloned into the general cloning vector pCR®2.1-TOPO (Fig. 3 in appendix) obtained from the TOPO TA cloning kit (Invitrogen™ life technologies). This plasmid was used for transformation into competent TOP10 and DH5α *E. coli* strains for OprP expression & purification studies. The pMALp2x plasmid used for fusion and expression of small putative metal binding peptides was obtained from New England BioLabs.

2.3 Primers for amplification of *oprP* gene of *P. aeruginosa* PA01

The oligonucleotides strands of nucleic acid serving as a start point for synthesis of DNA called primers. These are essential for replication of DNA due to the enzyme, DNA polymerase which catalyzes the DNA synthesis process by adding new nucleotides to only an existing DNA

strand. This enzyme starts replication at 3' end of the primer, and copy the opposite strand. Primers are synthesized chemically with a length ~ 20 bases. All primers were purchased from Eurofins MWG Operon, Ebersberg, Germany.

2.3a General rules for designing of PCR primers

For amplification of the specific DNA fragment by PCR. Primer designing is a most critical parameter for successful PCR amplification. Poorly designed primer results into primer dimer formation and cause reaction failure. Some time poorly designed primers can result in little or no product by nonspecific amplification, even when all the other parameters are properly optimized (Arun and Saurabha, 2009).

2.3b Length of primer

Primer's specificity is controlled by the length and annealing temperature of the PCR reaction. Primers with a length of 18-24 bases are very specific if the annealing temperature is within a few degrees of the primer melting temperature (T_m).

2.3c Primer GC content and T_m

For a specific amplification, primers must have a reasonable GC content. A twenty bases long primer with 50% GC content normally has T_m value in the range of 56-62°C which can provide a sufficient thermal window for efficient annealing. T_m defines the dissociation temperature of primer/template duplex which is calculated by the equation, **T_m=81.5 + (0.41% G+C content) - (675/length of primer) - % mismatches**. A primer with lower T_m than the reaction annealing temperature may fails to hybridize and extend the target DNA and if the T_m is higher than the annealing temperature then mishybridization occur which leads to extension along the DNA sequence at an incorrect location (Dieffenbach *et al*., 1993). So primers pair should have well matched GC content and T_m.

2.4 Primer design for *oprP* gene

For cloning into the general cloning vector pCR®2.1-TOPO and expression vector pET (see Fig. 4 in appendix) the *oprP* gene location was searched in the *P. aeruginosa* PA01 chromosome from online nucleotide database and found it started from the 3672549 and stopped at 3671226 base pairs (see Fig. 6 in appendix). Then 21-22 nucleotides were selected from both 5'and 3' ends of the *oprP* gene and restriction sites *Nde*I, *Xho*I or *Sac*I were engineered to incorporate at the 5' end of the gene (Fig. 10). After that three bases were incorporated before the engineered restriction sites. Finally the melting temperature (T_m) of the designed primer sequences was calculated for cloning into pET and TOPO vectors, respectively.

The pET expressions system has a strong T_7 promoter and tight regulation for the expression of the protein. Two sets of primers were designed (Table 1) one without a promoter region (A) and another with an upstream promoter region (B) to analyze which combination of the primers give the bests result for amplification of *oprP* gene.

Primer's names	Sequence 5'—3'	Vol. for 100pmol/µl	T_m (°C)	GC content	Restriction sites
OprUP_S	gatGAgctcctgttttcggcctcg (24 ntds)	226	66.1	58.3%	*Sal*I
OprUP_N	ttaccATatgattcgcagacactcg (25ntds)	276	61.3	44%	*Nde*I
OprDwn_X	atccTcGaggcaatttcatgacgc (24 ntds)	321	62.7	50%	*Xho*I
OprDwn_X_2	gaacTcgAGctgcaggcgcatcac (24 ntds)	225	67.8	62.5%	*Xho*I

Table 1: Designed primers for *oprP* gene amplification along with their special features.

Up primers: OprUP_N and OprUP_S

Down primer**:** OprDwn_X and OprDwn_X_2

Note: N- *NdeI*, X- *XhoI* and S- *SacI* restriction sites and capital letters in colored region show engineered site to make proper restriction sites.

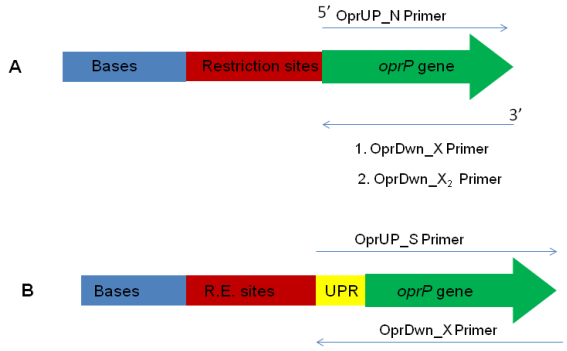


Figure 10: Primer design strategy for *oprP* gene of *P. aeruginosa* PA01 for cloning in to *Nde*I site on pET vector. Note: UPR- upstream promoter region of the *oprP* gene.

2.5 Media and growth conditions for *OprP*

E. coli and *P. aeruginosa* cells were cultivated at 37°C either on Luria-Bertani (LB) agar or broth. The *P. aeruginosa* strain was initially streaked onto LB plates and allowed to grow overnight at 37°C. A single colony was aseptically scraped from the plate by a sterile tip of a pipette and put into the PCR reaction mixture for amplification of *oprP* gene.

The recombinant *E. coli* strains were grown overnight in LB broth (10 g tryptone, 5 g yeast extract, 5 g NaCl/liter) or on LB agar (2%) plates. When cells were harboring the recombinant plasmid, media was supplemented with 50 µg/ml kanamycin. In addition, 1 µl/ml from 1M stock of isopropyl-β-D-thiogalactoside (IPTG) was included in the culture to induce the expression of plasmid encoded protein.

2.6 Media and Growth conditions for MBP fusion protein

For expression of the MBP fusion protein the recombinant pMALp2x plasmids harboring synthetic putative metal binding motif (Fig. 16B) were transformed into competent *E. coli* DH5α cells and allowed to grow at 37°C in rich LB media containing 10 g tryptone, 5 g yeast

extract, 5 g NaCl and 2 g glucose per liter along with sterile 100 µg/ml ampicillin and 100 mM IPTG.

2.7 Molecular markers

Standard molecular markers (Q-Step 4) for analysis of DNA and prestained protein ladder (Page Ruler) for protein analysis were purchased from the YOR BIO, Yorkshire Bioscience Ltd, UK and Fermentas, Life science, USA, respectively.

2.8 *oprP* gene of *P. aeruginosa* PA01

2.8a PCR amplification of *oprP* gene

The *oprP* gene was amplified by taking a single colony of overnight culture of *P. aeruginosa* PA01 from the LB agar plate and set up a programme for 35 cycles in thermo cycler machine (see Table 1 in appendix). The reaction mixture was prepared in a 0.5 ml ependorff tube by mixing this sterile single colony of *P. aeruginosa* with Go Taq flexi buffer, $MgCl_2$, dNTPs mixture, forward and reverses primers, Taq DNA polymerase and finally made desired volume with sterile water. The reaction mixture with exact concentrations of each component is shown in Table 2 of appendix. This reaction mixture was subject to thermocycles for the specific time. Following the PCR, DNA was separated by agarose gel electrophoresis.

2.8b Agarose gel electrophoresis

For analysis of the PCR product of *oprP* gene, gel electrophoresis was used. It is commonly used to separate the components of the PCR reaction product using Midi-Sub Gel system (Biorad, Hercules, CA, USA) at a constant voltage of 100V. 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA) was used in the electrophoretic tank. For staining the SYBERSafe DNA stain (Invitrogen, Camarillo, CA, USA) was added in the molten TBE agarose at a concentration of 0.01% (v/v). 6x DNA sample buffer (0.25% (v/w) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% (v/v) glycerol) was added to the PCR product to

concentration of 1x. For analysis of DNA bands 5-12 µl of the samples were loaded into the gel after mixing with 1x DNA sample loading buffer. For estimation of the product size Q-Step 4 DNA ladder was run along with the samples. A constant current of 85-100 volts were applied to run the gels for one hour. After that DNA bands were observed using UV trans-illuminator and photographed using a high speed Polaroid camera.

2.8c Desired DNA band extraction from agarose gel

oprP DNA band from the agarose gel was extracted using gel extraction kit (Qiagen) with the help of a clean, sharp scalpel over a UV trans-illuminator and purified from the gel using QIAquick Spin kit.

2.8d Transformation of *E. coli* cells

The pCR®2.1-TOPO vector was used for *oprP* gene and pMALp2x plasmid for putative metal binding motifs which conferring resistance either to ampicillin (Amp) or kanamycin (Kan) antibiotics. These antibiotics were used at standard concentrations from 50 µg/ml (Kan) to 100 µg/ml (Amp) during this research. The plasmid conferring resistance to Amp was transformed into chemically competent one shot® TOP10 and DH5α *E. coli* cells, using the protocol described in the TOPO TA cloning kit. For transformation into chemically competent one shot® TOP10 *E. coli* cells a cloning reaction was set up containing PCR product (4µl), salt solution (1.0 µl) and pCR®2.1-TOPO vector (0.5 µl); mixed this reaction gently and incubated at 22-23°C for 5 min. Then whole of reaction mixture (5.5 µl) was poured into 30 µl vial of one shot® chemically competent *E. coli* cells and mixed gently. Incubated this vial on ice for 30 min and heat-shocked the cells at 42°C for 30 seconds without shaking and transferred to ice immediately. Finally 250 µl of LB broth at room temperature was added, capped the vial tightly and shaken horizontally with speed of 200 rpm at 37°C for 1 hour. For optimal growth of One Shot® TOP10 *E. coli* cells selective plates of LB medium containing 50 µg/ml kan+ 20

µg/ml X-gal were prepared and placed at 37°C. Finally, 10-50 µl of transformation mixture from each vial was spread on these pre-warmed LB medium plate and incubated at 37°C overnight.

2.8e Analyzing transformants

Two to six white & light blue colonies were taken and cultured overnight in LB broth containing 50 µg/ml kanamycin and plasmid DNA of the transformed cells was isolated using QI Aprep Spin Miniprep Kit. After plasmid isolation, it was cut by *Eco*RI restriction enzyme to confirm the presence and correct orientation of the *oprP* gene. The construct [recombinant plasmid (pCR®2.1-TOPO+oprP gene)] was sequenced (Technology facility, Department of Biology) to confirm that the *oprP* gene was cloned in the correct orientation and the sequence was correct (See sequenced gene result in Fig. 6 of appendix).

2.8f Sub-cloning of amplified PCR product

Sub-cloning of the *oprP* gene was performed using competent *E. coli* DH5α cells in accordance with the TOPO TA cloning kit. The transformed *E. coli* DH5α cells were grown on selective LB media plates containing 50 µg/ml kanamycin+20 µg/ml X-gal and incubated overnight at 37^0C. Plasmid DNA was isolated using QI Aprep Spin Miniprep Kit and the DNA concentrations measured by a Nanodrop ND8000 spectrophotometer.

2.8g Restriction digestion of the cloned fragment

To confirm the presence of the *oprP* gene in to the pCR®2.1-TOPO vector a restriction digest mixture was prepared containing the sterile water (7.0 µl), cloned TOPO plasmid (12.0 µl), *E.co*RI enzyme (1.0 µl) with its respective EcoRI buffer (2.0 µl). Incubated this mixture at 37°C for 2 hours and run on 1% agarose gel at 100 volts for 2 hours. The *E.co*RI restriction enzyme cut the recombinant TOPO plasmid at two sites, at ~ 1.25 kb and ~ 0.7 kbp. Hence 3 bands were produced at nearly 4.0, 1.25 and 0.7 kb.

2.8h Sequencing of recombinant plasmid harboring *OprP* gene of *P. aeruginosa* PA01

The recombinant TOPO plasmid was sequenced with M13-20 forward and M13-26 reverse primers by Technology facility at the University of York, UK.

2.9 Expression and analysis of OprP protein

2.9a Whole cell lysate preparation

The recombinant and wild type strain of *E. coli* DH5α cells were grown in 10 ml of selective LB broth at 37°C with shaking until the OD_{600} was 0.8-1. (OprP $^{\pm}$ +50µg/ml kanamycin ± 1 µl/ml from 1M IPTG). The sample was prepared for SDS-PAGE analysis, pelleting of samples were done by centrifugation at 4500 rpm for 5-10 minutes and then dissolution of the pellets in 1x SDS-PAGE buffer (1M Tris-Cl pH 8, 20% SDS, 100% glycerol, β-mercaptoethanol and bromophenol blue). The samples were gently mixed and boiled at 95°C for ~ 5 minutes in water bath. Cooled samples were run on 12.5% SDS-PAGE gels at 200 volts for 1.30 hours. Gels were stained for 1 hour and destained overnight under shaking condition (15 rpm) in destaining solution.

2.9b Periplasmic, cytoplasmic and outer membrane fraction preparation

Recombinant and wild type *E. coli* DH5α cells were cultivated in selective LB broth at 37 °C under shaking conditions in a 2 liter flask till at OD_{600} reached 1, centrifuged at 4500 rpm for 5 minutes, the pellets were transferred along with 1 ml LB broth into an ependroff tubes and centrifuged again at 12000 rpm for 5 minutes. The Resulting pellets were resuspended in 1 ml of (10 mM Tris, pH 8+500 mM sucrose+3mM EDTA+1 mg of lysozyme) solution. The samples were incubated for 30 min at 30°C and centrifuged for 5 min at 12000 rpm. Resuspended the pellets in 1ml of (10 mM Tris pH 8+10 µl of DNase1 mixture) and incubated for 30 min at room temperature and centrifuged for 5 min at 1200 rpm. This time supernatant was cytoplasmic fraction and pellet was outer membrane fraction. Resuspended this outer

membrane fraction in equal amount of Tris, pH 8 and Mel's SDS loading buffer and boiled at 95°C-10 min in water bath. Analyzed this outer membrane fraction by 15% SDS-PAGE.

2.9c SDS-Polyacrylamide gel electrophoresis

Protein electrophoresis or SDS-Polyacrylamide gel electrophoresis was performed using 12.5-15% resolving gel concentration. The compositions of the gels are given in the Table 5A & 5B of appendix and all other descriptions of sample preparation, purification and analysis are described in the results and discussion section. Electrophoresis was carried at 200 volts for 1.30 hours till the dye front reach the bottom of the gels. For estimation of the product size Pre stained Page Ruler^{TM} was run along with the samples (purchased from the Fermentas, Life science). OprP was visualized by staining the gels in a solution of 45% methanol/45% H_2O/10% acetic acid containing 0.28% coomassie brilliant blue dye for 1 hour and then destained overnight before taking the picture of the gels.

2.10 Designing putative copper metal binding motifs

The putative copper metal binding motifs I and II were designed according to Kotrba, 1999. Both were complementary to each other and had degeneracy (shown in red and green) in the sequences.

Metal binding motif I $^{5'}$AATTCGGTYRTGGCYRCCCGYRTGGCYRCGGTTGAG$^{3'}$

Metalbindingmotif II $^{3'}$GCCARYACCGRYGGGCRYACCGRYGCCAACTCCTAG$^{5'}$

These motifs form a ds DNA and encode 9-10 amino acids long putative metal binding peptides starting from the Gly and ending with a stop codon. Keeping in mind that the motifs should code for small peptides containing Cys, His, Arg, Tyr and Pro amino acids with consecutive Gly residues, in alternate positions (Kotrba, 1999). The intention here was to synthesize a library of partially degenerate peptides containing potential copper binding sites as Cys and His are potent Cu^{++} binding ligands and Gly acts as spacer in binding to the Cu^{++} ion.

All peptides were proposed to bind Cu^{++} ion coordinated by His, Cys, Arg and Tyr. Peptides that interact strongly with a copper ion would be detected through biochemical and biophysical studies. These peptides would have potential uses in Biotechnology for example in heavy metal sequestering or bioremediation. Further attaching these peptides to OprP of *P. aeruginosa* PA01 to make it a redox active electron transporter might have application in electricity generation using an MFC. Synthetic genes for designed putative copper metal binding motifs were purchased from eurofins MWG Operon, Ebersberg, Germany.

2.11 Protein fusion and purification by pMAL™ system

The pMALp2x plasmid (see Fig. 5 of the appendix) is a novel vector which provides a simple procedure for fusion, expression and purification of small peptides or proteins. It has a strong "tac" promoter and the malE translation initiation signals to give optimum expression level of the proteins/peptides (putative metal binding motifs) (Amann *et al*., 1985 and Duplay *et al.*, 1984), and the expressed fusion is purified by one-step maltose affinity chromatography (Kellerman *et al*., 1982).

The *malE* gene fused to the *lacZα* gene and expressed with or without its signal sequence. For insertion of the desired coding sequence restriction sites are available between *malE* and *lacZα*. The desired insert inactivates the β-galactosidase α-fragment activity of the *malE-lacZα* fusion, resulting into blue to white color change of α-complementing *E. coli* DH5α cells on X-gal plates upon transformation.

The signal peptide which is present on pre-MBP successfully export the fusion proteins into the periplasm and allows proper folding and disulfide bond formation in the periplasm, as well as it allows purification of the fusion protein from the periplasmic space of the cells (Lauritzen *et al.*, 1991).

This pMALp2x system also contains the *lacI* gene, which codes for the Lac repressor which keeps expression level low from P_{tac} promoter in absence of IPTG induction. The pMALp2X vector also encodes for a cleavage site (Factor X_a) that cleaves after a four amino acid recognition sequence, so that few or no vector-derived residues are attached to the made fusion (Nagai *et al.*, 1984 and 1987).

Expressed fusion proteins, from the pMALp2x system typically constitute 1–5% of the total cellular protein. From maltose affinity purification the yield of fusion protein ranges up to 10–400 mg/liter culture. The yield of the proteins depends on the sequence which is fused to *malE*. Sometime either the fusion is degraded by *E. coli* proteases or during purification the fusion may be binds poorly to the column or might be insoluble.

The origin of DNA replication, also present that allows single-stranded DNA production by infection of pMALp2x plasmid harbouring cells with a helper phage (bacteriophage M13) (Sambrook *et al*., 1989). This single-stranded plasmid DNA helps in sequencing using either NEB primers or primers which are available in sequencing facility at Biology department of University of York, UK.

For putative metal binding motifs expression, the designed motifs (DNA sequences) were inserted downstream of the *malE* gene into the pMALp2x vector, the *malE* gene encodes the maltose binding protein (MBP). The putative motifs were expressed as MBP-peptide fusion proteins.

2.11a Construction of the Fusion Plasmid

For production of the fusion peptides in the pMALp2x plasmid, the putative copper metal binding motifs must be inserted into the polylinker region which contains restriction sites for cloning of the desired fragment. The designed putative metal binding motif ds DNA was inserted between the *E.co*RI and *Bam*HI restriction sites after double restriction digestion of

pMALp2x with these enzymes. These restriction enzymes are used to generate an end. It is desirable to use an enzyme that generates 3´ overhang at the end of gene to direct the orientation of the insert and increase ligation efficiency in the final step. The enzymes must not cut within the gene of interest. The putative metal binding motif should be in the same translational reading frame of *malE* gene.

2.11b Transformation of the fusion plasmid in to *E. coli* DH5α cells

The pMALp2x plasmid with inserted ds DNA of putative copper metal binding motifs was transformed in to competent *E. coli* DH5α cells and these recombinant cells were grown on selective LB media plates, containing various concentrations of ampicillin at 37^0C overnight. The recombinant plasmids were isolated using mini prep kit (Invitrogens, Life technologies).

2.11c Sequencing of the putative copper metal binding motifs

Putative copper metal binding motifs in the pMAL vectors were sequenced using M13-47 and *malE* primers available from the sequencing facility lab of Biology department, University of York and New England Biolabs, respectively. The malE Primer initiates sequence 78-81 bases upstream of primary polylinker site near the 3´ end of *malE*. So the malE primer is used for sequencing the single stranded DNA produced by plasmid's M13 origin. The 3´ end of the putative metal binding motifs was sequenced from the lacZα side using M13/pUC sequencing primers (NEB # S1224S this is same as M13-47). The metal binding motifs sequences were found in the exact position in between *E.co*RI and *Bam*HI restriction sites. Sequences of the pMAL vectors can be found from New England Biolabs web page at www.neb.com. All sequenced metal binding motifs were translated by ExPASy: Translation tool.

2.11d Testing metal binding efficiency of recombinant *E. coli* DH5α cells

Recombinant *E. coli* DH5α cells harboring putative metal binding motifs in the pMAL plasmid were grown at 37^0C overnight on Cu^{++} gradient plates (Fig. 19A) from lower to higher concentrations (0.0 to 10.0 mM) of copper sulfate and then analyzed which clones showed better result toward higher concentration of the copper sulfate.

2.11e Production, purification and concentration of MBP-Peptide fusion protein (Maltose binding protein attached to putative metal binding peptides)

The fusion proteins were expressed in the periplasm of the *E. coli* DH5α cells using the standard protocol for pMAL fusion protein expression (New England BioLabs.) Detailed procedures for the production, purification and concentration of the MBP-peptides fusion protein is given in the Results section. Fused MBP with different metal binding peptides were purified by affinity chromatography. For this 2.5x10 cm column was packed with amylose resins (New England BioLabs). These resins have binding capacity of 6-8 mg MBP-fusion protein per ml. Purified fusion proteins concentrations were measured by Bradford assay, calculated the proteins concentrations and yields as described in Results and plotted graphs for proteins concentrations and protein yields from each samples along with control MBP and γ-globulin proteins (See the γ-globulin standard protein concentrations in Table 6 of appendix)

Results and discussion

OprP protein expression from *P. aeruginosa* PA01

Creating a library of copper metal binding peptides

CHAPTER-III

3. OprP protein expression from *P. aeruginosa* PA01

3.1 Primers designing for amplification of *oprP* gene

Two sets of forward and reverse primers were designed to amplify the *oprP* gene:

Forward primers: OprUP_S, 24 nucleotides long containing the *Sal*I engineered restriction site and the native promoter of the gene, *oprP*. This design was used to establish whether the gene will amplify optimally or not using this primer. When colony PCR was performed a faint DNA band was appeared in agarose gel electrophoresis so it was assumed that the *oprP* gene was weakly hybridized with the designed primer and not amplified properly by PCR, so the need of other forward primer which can hybridize properly to the gene product and leads to optimal amplification for the correct size of the gene product. Thus OprUP_N was designed, containing 24 nucleotides and an *Nde*I engineered restriction site to clone the *oprP* gene into pET expression vector. The pET system is widely used for high level protein expression due to its strong T_7 promoter and tight regulation. T_7 is a strong phage promoter which relies on a T_7 phage encoded RNA polymerase of the host bacterial cell. This offers high level expression and tight regulation of the desired protein. Expression of *oprP* gene downstream of the T7 promoter can be regulated by *lacI* repressor of the pET vector. The His6 tag helps in protein purification.

Reverse primer: Two reverse primers were designed having the same *Xho*I restriction site (see Table 1 of Material and Methods section) to clone in the polylinker region of pET vector. The positions of the primers were different with respect to the gene to look which set of the primers give better hybridization for optimum amplification of the *oprP* gene by PCR. OprDwn_X started very far (76 bases) from the stop codon and OprDwn_X_2 just started after the stop codon of the *oprP* gene. Both contain 24 nucleotides.

The *oprP* gene was amplified using all of these primers in three sets: in one set forward primer OprUP_N, used with reverse primer, OprDwn_X (Fig. 11A), in second set forward primer, OprUP_S used with reverse primer OprDwn_X2 (Fig. 11A) and in third set forward primer OprUP_N, used along with reverse primer OprDwn_X2 in a PCR reaction (Fig. 11B). A good amplification of the *oprP* gene product was obtained by the combination of OprUP_N with OprDwn_X2 primers. Thus, the gene was optimally amplified by using OprUP_N primer in combination with OprDwn_X2 primer.

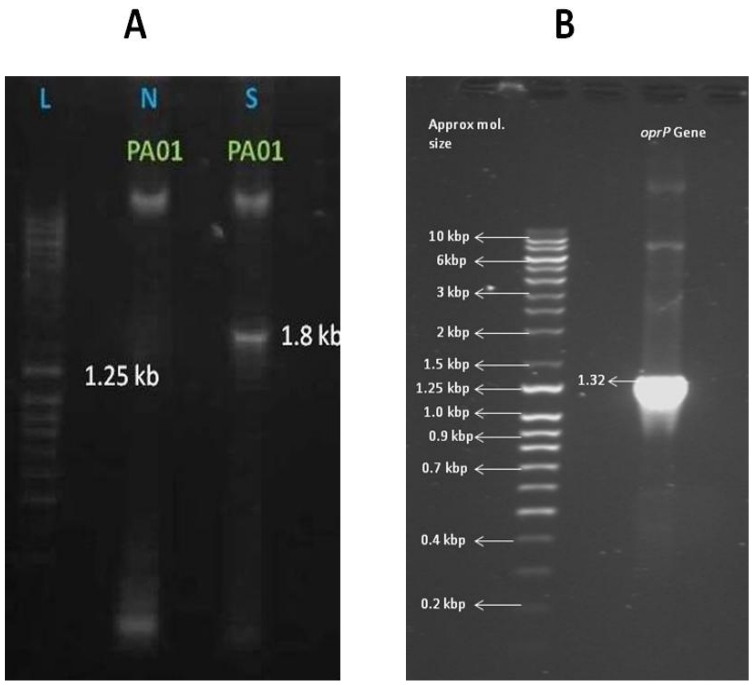


Figure 11: Amplified PCR product of *oprP* gene of *P. aeruginosa* PA01 with different set of primers.
(A) L is Q-Step 4 DNA ladder (5 µL), N is primer set: OprUP_N + OprDwn_X, S is primer set: OprUP_S + OprDwn_X2. (B) *oprP* gene amplification with primer set Opr UP_N +OprDwn_X2. *oprP* was amplified optimally as represented by a thick DNA band at 1.32 kbp position. All PCR products loaded in wells were 10 µL. The *oprP* gene product size is 1.32 kbp and *oprP*+ Up stream promoter region (UPR) = 1.8 kbp.

3.1a Extraction of desired DNA band from agrose gel

The PCR product of the *oprP* gene extracted from the agarose gel using a gel extraction kit (Qiagen) with the help of a clean, sharp scalpel over a UVP-dual intensity transilluminator and purified from the gel using QIAquick Spin kit. After extraction the DNA concentration was measured with a Nanodrop spectrophotometer ND8000 from the Genomic lab of the Biology department at University of York, UK and found to be 124.80 ng/µl.

3.1b Cloning and sub cloning of *oprP* gene

Cloning and sub cloning of the *oprP* gene product were performed in competent *E. coli* cells (one shot® TOP10 and DH5α) using the TOPO® TA cloning kit. Plasmids were isolated using a Mini Prep kit (Invitrogen Life Technologies) and the amount of DNA measured using a Nanodrop spectrophotometer ND8000 and found to be 315 and 440.3 ng/µl from the TOP10 and DH5α *E. coli* cells, respectively.

3.1c Analyzing transformants (Gel electrophoresis and sequencing)

After isolation of plasmids and measuring the concentration of plasmid DNA restriction digests by *Eco*RI restriction enzymes were performed to confirm the presence of the *oprP* gene into the TOPO vector. The digestion mixture was prepared as described in Materials and Methods. The digestion mixture along with native (uncut) TOPO plasmid was run on an agarose gel. Three bands were observed from the digested mixture of the recombinant plasmid. First at ~ 4.0 kbp, second at ~ 1.25 kbp and the third at ~ 0.7 kbp. From the undigested mixture only one band was observed near 5.0 kbp. So the *Eco*RI enzyme cut in two positions (i.e. 1.25 and 0.7 kbp) of the TOPO plasmid. From this it was confirmed that *oprP* gene was present in the TOPO plasmid. To confirm its orientation recombinant TOPO plasmid was submitted for sequencing to the Genomics Laboratory of Biology department, The University of York. Sequencing result of the cloned gene is shown in Fig. 6 of appendix section.

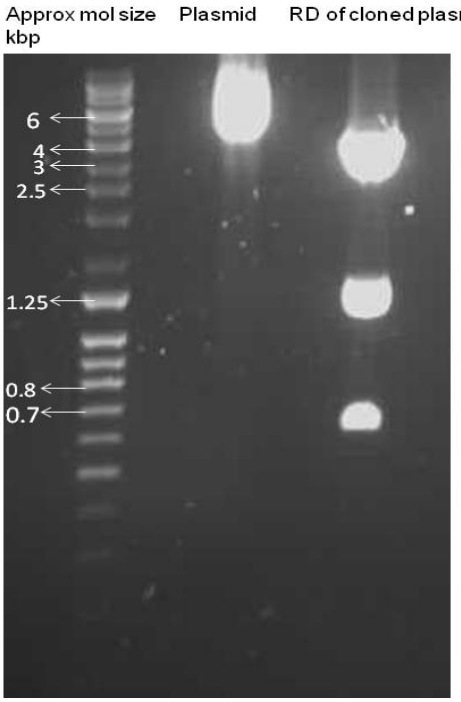


Figure12: Gel electrophoresis of the restriction digested recombinant plasmid (pCR®2.1-TOPO+ *oprP* gene) along with control (pCR®2.1-TOPO). Lane 1: 5 µL Q-Step4 DNA ladder; Lane 2: 10 µL of the undigested pCR®2.1-TOPO plasmid and Lane 3: 10 µL of the *E.co*RI digested recombinant pCR®2.1-TOPO plasmid. Gel was prepared by 1% agarose in TBE buffer and run at 100 volts for 2 hours.

3.1d Sequencing result of cloned *oprP* gene of *P. aeruginosa* PA01

Recombinant TOPO plasmid was sequenced by using M13-20 forward and M13-26 reverse primers, the *oprP* gene was found downstream of the *lacZ* α gene. The plasmid sequence was edited and only the gene sequence is represented (see Fig. 6 in Appendix).

3.2 OprP Protein expression in recombinant *E. coli* DH5α cells

3.2a Whole cell lysate preparation

Recombinant and empty plasmid harboring strains of *E. coli* DH5α cells were grown in selective LB broth at 37°C under shaking conditions up to the OD_{600} reached 0.8-1. (OprP $^{\pm}$ +50µg/ml kanamycin ± 1 µl/ml from 1 M IPTG). Cells lysates were run on 12.5% SDS-PAGE gels after making pellet suspensions in 1x SDS-PAGE buffer and boiling at 95°C for 5 minutes

in a water bath. The gel was run at 200 volts for 1 hour 30 minutes. After staining and destaining, different proteins bands appeared but most prominent band was appeared at 34 kDa in all samples and there was no significant difference in this bands size and intensity between all the different samples (Fig. 13) thus the target protein OprP (48kDa) did not express with prominent band and not distinguishable from other proteins based on the intensity of the bands at different location in the gel.

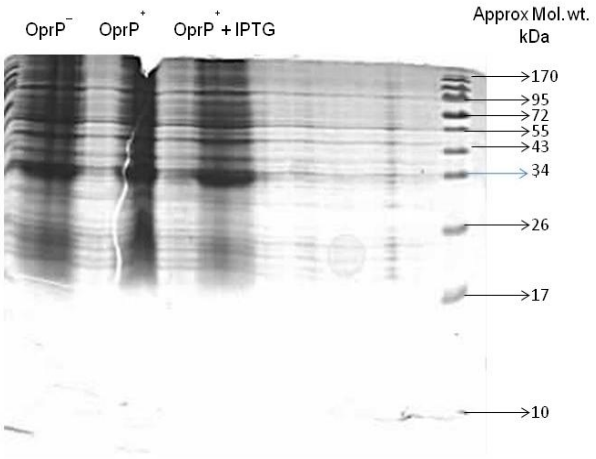


Figure 13: SDS-PAGE analysis of recombinant and wild type *E. coli* DH5α cells.

Protein induction was performed by IPTG at 37°C under shaking condition. Samples were analyzed before and after induction.

OprP$^-$: *E. coli* DH5α cells lysate harboring empty TOPO plasmid.

OprP$^+$: *E. coli* DH5α cells lysate harboring cloned TOPO plasmid before induction with IPTG.

OprP$^+$+IPTG: *E. coli* DH5α cells lysate harboring cloned TOPO plasmid after induction with IPTG.

3.2b Periplasmic, cytoplasmic and outer membrane fraction preparation:

Recombinant and empty plasmid harboring strains of *E. coli* DH5α cells were cultivated in same manner as for lysate preparation in selective LB broth at 37 °C. Centrifugation was performed at 4500 rpm for 5 minutes and the resulting pellets were transferred to a sterile ependorff tubes along with 1 ml LB broth for further centrifugation at 12000 rpm for 5 minutes.

Resuspended this pellet in 1 ml of a solution containing 10 mM Tris (pH 8) +500 mM sucrose+3mM EDTA+1 mg of lysozyme and incubated at 30°C for 30 minutes then centrifuged again at 12000 rpm for 5 minutes. This separated the spheroplast (pellet) from the periplasm (supernatant). The periplasmic fraction was run on SDS-PAGE and the pellet (spheroplast) in 1ml of 10 mM Tris (pH 8) +10 µl of DNase1 solution, incubated for 30 minutes at room temperature and centrifuged for 5 min at 1200 rpm. This time supernatant was the cytoplasmic content and pellet was the outer membrane fraction. The cytoplasmic content was run on SDS-PAGE and the outer membrane content was resuspended in Tris buffer (pH 8) and Mel's SDS loading buffer in an equal amount (See composition in Table 6 of Appendix) and boiled at 95°C for 10 minutes in water bath. The outer membrane fraction/content was analyzed along with the periplasmic and cytoplasmic fractions by 15% SDS-PAGE. In the cytoplasmic content very faint bands were observed at ~ 48 kDa. It suggested minimal OprP was present in the cytoplasm. In the periplasmic fraction/content bands at ~ 48 kDa were slightly denser than the cytoplasmic fraction so some OprP present in periplasm. In outer membrane fraction the band at ~ 48 kDa was much denser than those in cytoplasmic and periplasmic fraction, so OprP was more expressed in the outer membrane of the cells under these differential centrifugation conditions and PO_4^- limiting conditions of the LB medium. The OprP will not express in outer membrane unless the cells are grown on low phosphate medium (0.15 mM or less PO_4^-) because expression of cloned *oprP* gene is regulated by PO_4^- concentration of the growth medium (Siehnel *et al.*, 1990) which is required for the cultivation of the recombinant *E. coli* DH5α cells. In all fractions (cytoplasmic, periplasmic and outer membrane) several other proteins also expressed in different location as analyzed by SDS-PAGE. Like whole cell lysate the most prominent band on 34 kDa was appeared in all samples.

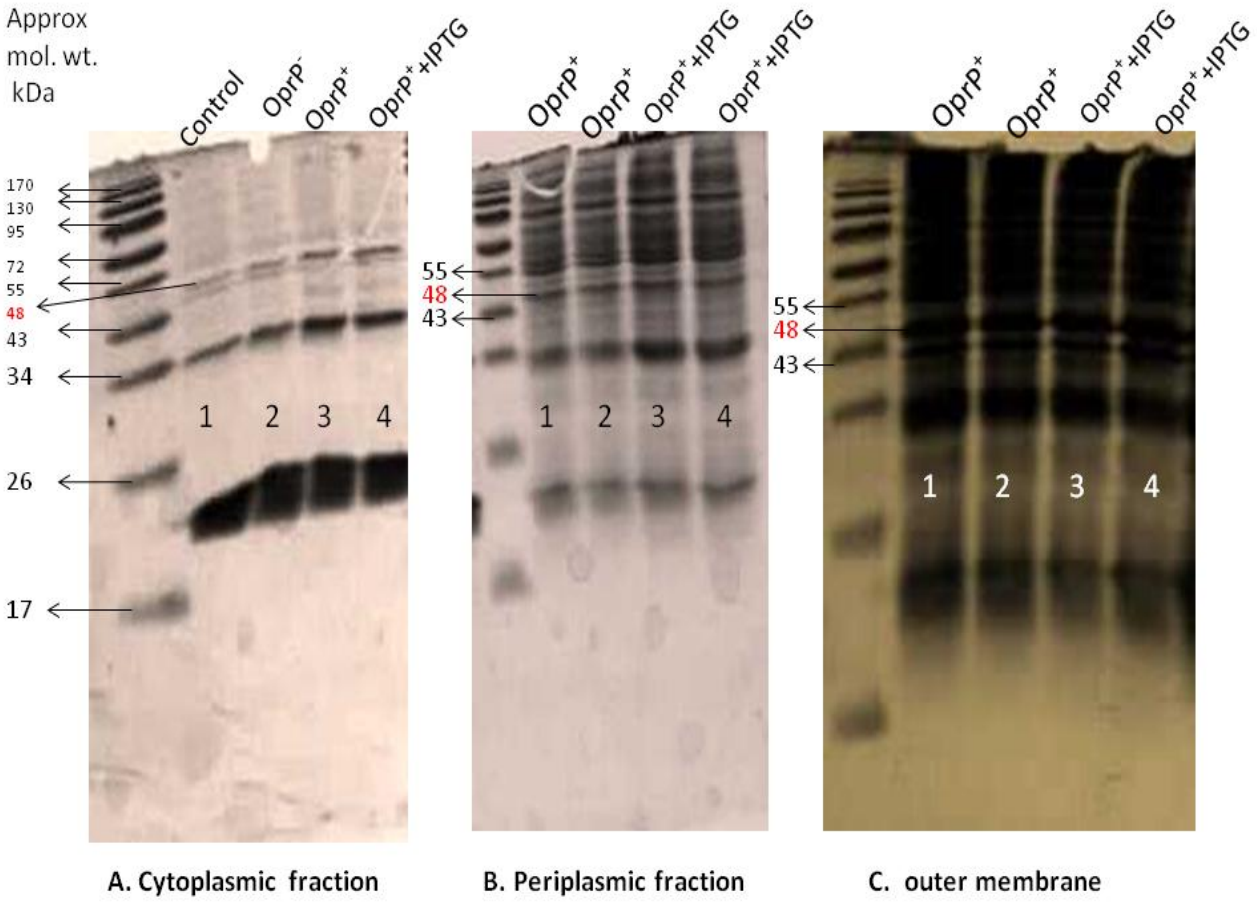


Figure 14: SDS-PAGE analysis of recombinant and wild type *E. coli* DH5α cells

Control: Cells without TOPO plasmid

$OprP^-$: Cells harboring empty TOPO plasmid

$OprP^+$: Cells harboring recombinant TOPO plasmid

$OprP^+$+IPTG: Cells harboring recombinant TOPO plasmid and protein expression was induced by IPTG.

CHAPTER-IV

4.1 Creating a library of copper metal binding peptides

4.1a Designing Cu-metal binding peptides

For rational design of peptides with specificity for a particular metal, cooperation of the cell structures (cell wall or its individual constituent i.e. outer membrane proteins) and relative affinities of metal binding peptide should be taken in to consideration. The biomolecules selectivity for metal ions is described by HSAB principle (hard & soft acids and base) and the Irving-Williams stability constants series of divalent ions which referred to relative stability of the complex formed by metal ions. For high-spin divalent ions complexes of first row transition metals, the stability constant of a complex formation follows, Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II) order. This order holds a wide variety of ligands (Irving & Williams, 1953) thus stability constant for copper ion is quite high. Two complementary degenerate oligonucleotide motifs were designed encoding a putative copper metal binding peptide library.

Metal binding motif I $^{5'}$AATTCGGTYRTGGCYRCCCGYRTGGCYRCGGTTGAG$^{3'}$

Metal binding motif II $^{3'}$GCCARYACCGRYGGGCRYACCGRYGCCAACTCCTAG$^{5'}$

Metal binding motif I was flanked at the 5' and 3'ends by *E.co*RI and *Bam*HI sites and motif II was flanked by *Bam*HI and *E.co*RI cohesive termini, respectively. Here Y is for pyrimidine base (T or C) and R is for purine base (A or G). Such design allows specific insertion into unique *Bam*HI and *E.co*RI sites of the pMALp2x vector to express fusion proteins/peptides downstream of *malE* gene. Both motifs encode small degenerate peptides of 9-10 amino acids long (Fig. 18) having Cys, His, Arg, Tyr and Pro with consecutive Gly residues, in alternate positions (Kotrba, 1999). The intention here was to generate a library of degenerate peptides, containing potential copper binding sites (Fig. 15) for use in biotechnology.

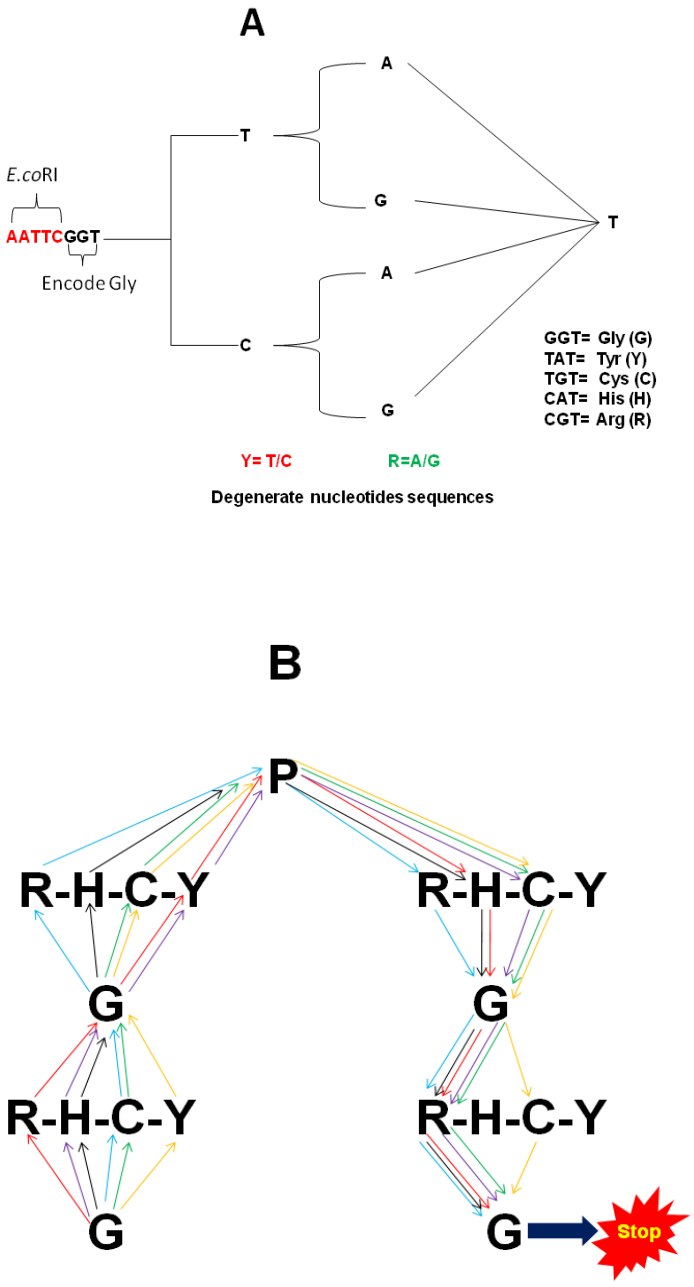


Figure 15: Design strategy for putative Cu- metal binding peptides.

(A)Shows how the metal binding motif I sequence will code for different amino acid residues of putative degenerate copper binding peptides. Likewise the metal binding motif II sequence codes for different residues of putative degenerate copper binding peptide. Y: T/C Pyrimidine base, R: A/G Purine base.

(B)The generation of a putative copper metal binding peptide library. Different colored lines show different degenerate peptides with sequences starting with Gly and ending with a stop codon.

G- Gly, R- Arg, H- His, C- Cys, Y- Tyr and P- Pro amino acid residues.

4.1b The pMALp2x plasmid construction

The pMAL vector was designed in such a way that it allowed insertion of ds DNA sequence of putative metal binding motif downstream of the *malE* gene in the polylinker region of the pMALp2x plasmid (Fig. 16). For this, the pMALp2x plasmid DNA was digested with *E.co*RI and *Bam*HI simultaneously (double digestion) to avoid star activity associated with either of these enzymes. The putative metal binding motif was inserted between the *E.co*RI and *Bam*HI sites. Insertion of this putative metal binding motif interrupted the *lacZ*α gene and inactivated the β-galactosidase α-fragment activity of the *malE-lacZα* fusion, resulting into blue to white color change of *E. coli* DH5α cells on X-gal plates upon transformation.

The signal sequence of *mal*E directed the made fusion protein through the cytoplasmic membrane into the periplasmic space which ultimately purified from the periplasm by one-step Maltose affinity chromatography (New England BioLabs, 2003)

The hybrid P_{tac} promoter offered protein yields, 282.5 mg/liter (MBP1), 347.5 mg/liter (MBP2), 217.5 mg/liter (MBP3), 190 mg/liter (MBP4), 120 mg/liter (MBP5), 90 mg/liter (MBP6) 125 mg/liter (MBP 7) and 107.5 mg/liter (MBP control), respectively (Fig. 24). Thus the highest protein yield (347.5 mg/liter) from the sample 2 which harbor the ^{N}GHGYPCGRC^{C} peptides sequence in fusion with MBP. The lowest protein yield (90 mg/liter) from sample 6 which contains the ^{N}GHGHPRGCG^{C} putative metal binding peptide in fusion with MBP protein. The control MBP sample also produced a good protein yield (107 mg/liter). The plasmid DNA was sequenced using M13-47 and *malE* primers available from the sequencing facility lab of Biology department, University of York and New England Biolabs, respectively (Fig. 17). The protein yield may be affected by the type of peptide coding sequence fused to *malE* gene, or might be the fusion is degraded somehow by *E. coli* proteases. Another possible

reason for low protein yield during purification might be poorly binding of the fusion protein to the respective column as in the case of sample 6.

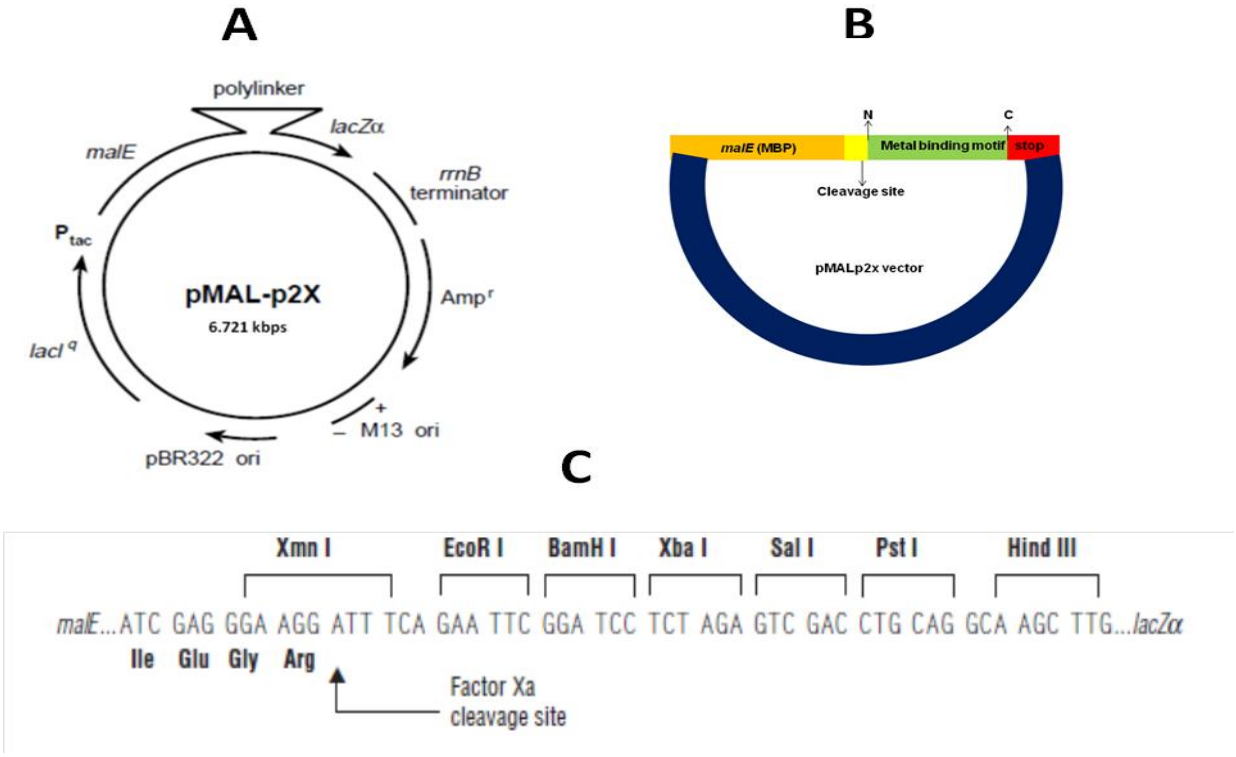


Figure 16: pMALp2x plasmid construction for insertion of putative metal binding motif in-frame with Maltose Binding Protein (MBP) (A) Genetic map of pMALp2x plasmid depicting different component.
(B) Putative metal binding motif inserted downstream of *malE* gene in between the *E.co*RI and *Bam*HI sites.
(C) Polylinker region of the pMALp2x plasmid showing *malE* signal sequences along with Xa cleavage and restriction endonuclease sites

4.1c Experimental design for expression of metal binding motifs in-frame with Maltose Binding Protein

In order to express the fusion protein (MBP attached to putative copper metal binding peptide), the DNA coding for the metal binding motifs (I+II) was re-suspended, mixed and denatured at 95°C for 5 minutes in a water bath and allowed to cool at room temperature to form duplex

DNA. Ligation reactions (7 µl double digested pMALp2x plasmid with *E.co*RI and *Bam*HI restriction enzymes + 1 µl combined metal binding motifs + 1 µl of (10x) ligase buffer + 1 µl of T_4 DNA ligase) were set up with a control reaction, (7 µl double digested pMALp2x plasmid with *E.co*RI and *Bam*HI restriction enzymes + 1 µl sterile dH_2O + 1 µl of (10x) ligase buffer + 1 µl of T_4 DNA ligase). These ligation reactions were incubated at room temperature overnight; in each reaction mixture 200 µl of competent *E. coli* DH5α cells were added and incubated overnight at 37^0C. Blue white screening for transformed *E. coli* DH5α cells harboring the fusion pMALp2x plasmid were carried out on a selective LB plate (having 50 µg/ml ampicillin, 40 µg/ml X-gal and 100 µM IPTG). Plasmid DNA was isolated using a Mini Prep kit (Quigen) and sequenced with M13-47 and *malE* primers. The putative metal binding motifs were found between the *E.co*RI and *Bam*HI restriction sites of pMALp2x plasmid as shown in Fig. 17.

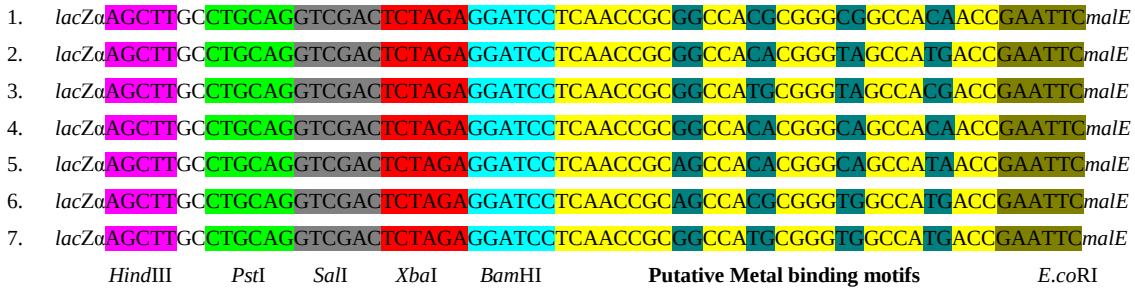


Figure 17: Nucleotide sequences of all seven degenerate putative copper metal binding motifs, helpful in generating peptides library for copper metal binding. The variable positions in the putative metal binding motif sequences are indicated by dark cyan color.

The nucleotide sequences of each isolated motif were translated using the ExPASy: translation tool http://web.expasy.org/translate/ (Fig. 18).

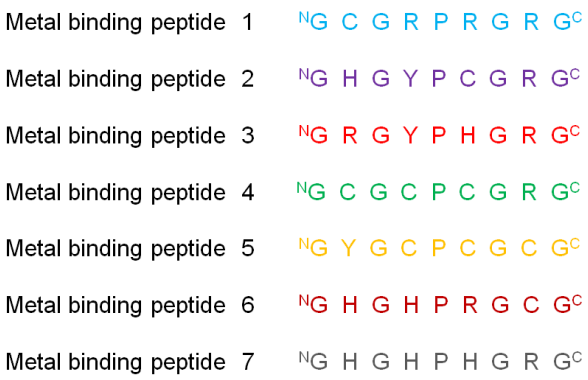


Figure 18: Library of putative copper metal binding peptides. Different colors denote the design strategy for each peptide as shown in Fig. 15.

4.2 Examination of Cu^{++} metal binding properties of recombinant *E. coli* DH5α cells harboring short peptides as a fusion to MBP protein

Control & recombinant *E. coli* cells harboring the recombinant pMALp2x plasmid were streaked out on the copper sulfate gradient plates (Fig. 19A) from lower (0 mM) to the higher concentration (10 mM) of copper sulfate (Fig. 19B). The gradient plate was incubated at 37°C overnight and the growth of the control and recombinant *E. coli* cells was observed. The cells expressing the peptides appeared to be resistant to copper sulfate when compared to control cells. This indicates that these peptides can bind copper ions. From the variation in growth between samples on gradient plates it can be assumed that all of these peptides will interact with copper with more or less binding efficiency. After these further biochemical and biophysical analysis of these peptides might be useful for creating copper metal binding sites in bacterial proteins for use in biotechnology. So the potential uses of these recombinant cells are in bioremediation or heavy metal sequestering and these putative copper metal binding peptides might also be attached to OprP of *P. aeruginosa* PA01, to make it a redox active porin for future research in energy generation.

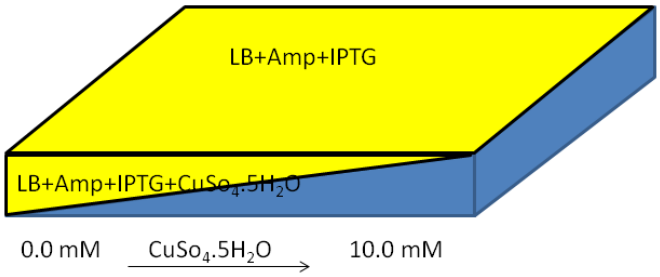


Figure 19A: **Copper sulfate gradient plate**

Made by first pouring the LB growth medium (50 ml) with ampicillin (50 µg/ml), IPTG (100 mM) and copper sulfate (10 mM) diagonally in sterile conditions under laminar air flow chamber and allowed to cool after that this copper gradient was covered by the LB medium with ampicillin and IPTG excluding copper sulfate. After solidification different recombinant *E. coli* cells were streaked along with the control and incubated over night at 37^0C.

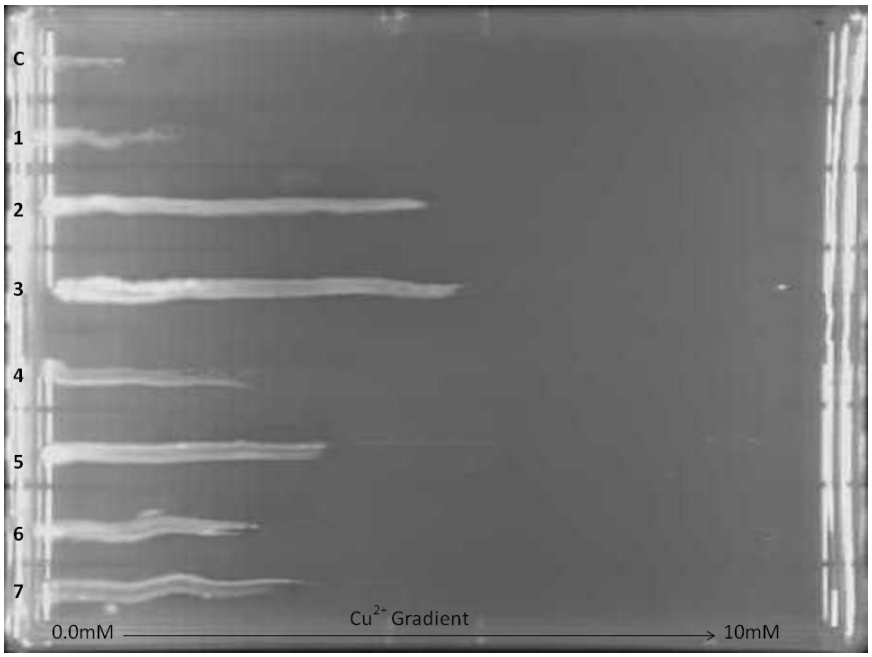


Figure 19B: Growth of recombinant *E. coli* cells harboring different Cu-metal binding peptides with control. Control: *E. coli* DH5α cell having empty pMALp2x plasmid, 1-7: *E. coli* DH5α cells harboring fusion pMALp2x plasmid. The numbering 1 to 7 is as in Fig. 18.

4.3 Production of MBP-Peptide fusion protein (Maltose binding protein attached to putative metal binding peptides) (New England BioLabs^{R} $_{inc, 2003}$)

Five hundred milliliters LB+glucose broth (containing 5 g trypton+ 2.5 g yeast extract +2.5 g NaCl+1 g glucose) was prepared in clean 2 liter flask, autoclaved & sterile ampicillin was added to 50 µg/ml. This broth was inoculated with an overnight culture (10 ml) recombinant plasmid harboring *E. coli* DH5α cells and grown the cells up to OD_{600} ~0.5. Added the IPTG to a final concentration of 0.3 mM and allowed to further incubation for 2 hours at 37°C. The protein expression time depends on different factors i.e. temperature of the incubation of the cells, stability of the fusion protein and host strain. After expression harvested the cells at 4000 x g for 20 min by centrifugation and discarded the supernatant. The pellet was resuspended into 200 ml Tris-sucrose solution having composition (30 mM Tris-HCl, 20% sucrose, pH 8.0) (used 80 ml/gram of cells's wet weight). EDTA was added to 1.0 mM with further incubation at room temperature for 5–10 min under shaking conditions. All the supernatant was removed by centrifugation at 8000 x g for 20 min at 4°C and the resulting pellet was resuspended in 200 ml of 5 mM $MgSO_4$ (ice-cold). Shaken this suspension at 35 rpm for 10 min in an ice bath and subjected to centrifugation at 8000 x g for 20 min at 4°C. Finally 1 M Tris-HCl, pH 7.4 (8 ml) was added to this (cold osmotic shock) supernatant which contains the periplasmic fraction. The periplasmic fraction was run on 10% SDS-PAGE (Fig. 20) by mixing of an equal volume of 4x protein gel SDS-PAGE sample buffer and boiling at 95°C for 5min in a water bath. Different protein band appeared at different location in the gel but the most prominent was appeared at ~40 kDa as expected the weight of the maltose binding protein (MBP). Small size of the putative metal binding peptides (9-10 amino acids long) did not show the major change in the location of the MBP band in the gel. Samples 1 and 2 expressed the proteins at ~ 40 kDa. Bands size and intensity were quite similar in both samples. In sample 3 and 6 protein bands

appeared slightly below the 40 kDa. While in samples 4 and 5 bands size were slightly above 40 kDa, thus in these samples the putative metal binding peptides increased the size of fusion protein. In sample 7 two prominent bands appeared both were slightly above the position of 40 kDa.

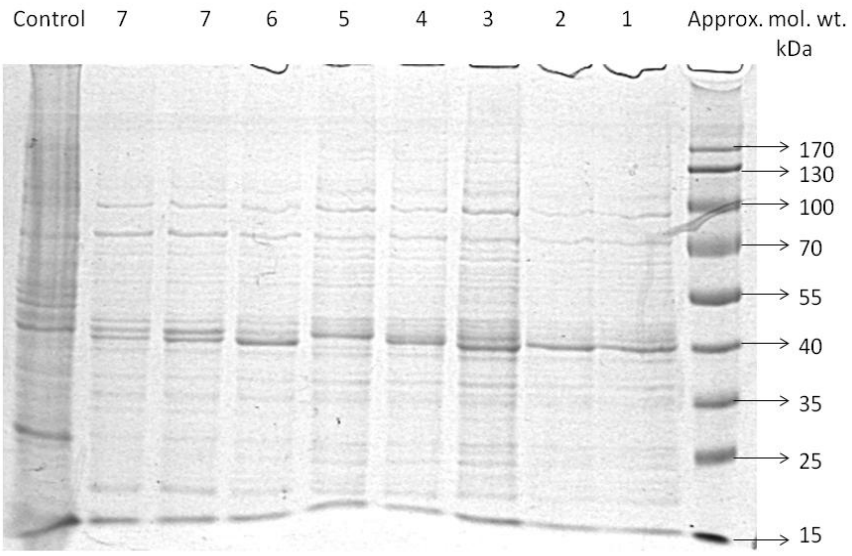


Figure 20: SDS-PAGE of MBP fusion proteins along with the control. Lane 1-7: *E. coli* DH5α cell harboring recombinant pMALp2x plasmid expressing MBP protein attached with different putative metal binding peptides. Lane 8: control, *E. coli* DH5α cell harboring empty pMALp2x plasmid so express only MBP protein at 40 kDa. The numbering 1 to 7 is as in Fig. 18.

4.3a Purification of MBP-Peptide fusion protein by affinity chromatography

Amylose resins suspension (10 ml) was poured into a 1.6 x 10 cm column and washing of the packed column was performed by 8 column volumes of Column Buffer (80 ml) (see composition in Table 7 of appendix). The resins amount determines the amount of fusion protein production. Purified periplasmic extract/content was loaded with a flow rate of [10 x (diameter of column in cm)^2] ml/hour]. It was 426 µl/min for 1.6 cm column. Once again column was washed with 12 column volumes of Column Buffer (120 ml). Finally, the fused

protein was eluted with Column Buffer + 10 mM maltose. Fifteen fractions, 3 ml each were collected through the column. The fusion protein usually started to elute within the first 5 fractions, and was easily detected by Bradford protein assay (Fig. 23). All the collected fractions were pooled in Viva Spin concentrator, MWCO 5000 (Sigma Aldrich,UK) and the protein was concentrated. These affinity purified concentrated fractions were run on 10% SDS-PAGE (Fig. 22). From samples 1 to 7 prominent bands were appeared ~ 40 kDa except in sample 5, might be the sample was overheated (protein denaturation) during sample preparation on heating block or the fusion protein from sample 5 eluted out with other impurity proteins of *E. coli* which could disturb in binding of the maltose binding protein fusion to the amylase resigns in the column. When concentration and buffer exchange procedures were carried out by Viva spin^{TM} concentrator and PD-10 column, respectively the impurity proteins may be eluted down in the concentrator and pure fusion protein was retained in the upper chamber of the concentrator which further gave a prominent band ~ 40 kDa after SDS-PAGE analysis (Fig. 22).

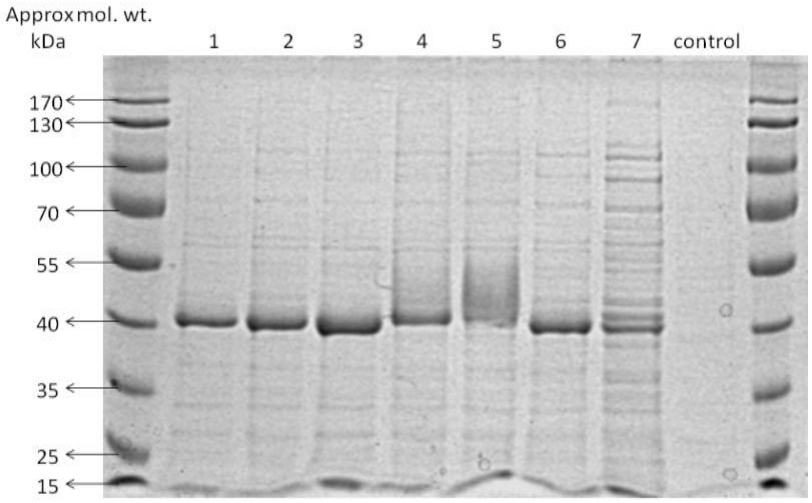


Figure 21: SDS-PAGE of the purified MBP fusion proteins along with the control.

Lane 1-7: MBP fusion proteins taken from the mixture of first five fractions eluted with column buffer and maltose. Control: eluted sample from the mixture of last five fractions. The numbering 1 to 7 is as in Fig. 18.

4.3b Concentration of periplasmic fusion protein fraction by 20 ml Viva spin™ concentrator

A Viva spin™ MWCO-5000 concentrator (Sigma Aldrich,UK) was filled with the maximum volume (20 ml) of the fusion protein sample and centrifuged at 4500 rpm for 23 min at 4°C. When the desired degree of concentration was achieved (1.5 ml) the concentrator was removed from the centrifuge and the concentrated protein sample was recovered from the bottom of the concentrator pocket with pipette.

4.3c Buffer exchange of the fusion protein by PD-10 Desalting column

The PD-10 columns were equilibrated with 25 ml elution buffer (2 mM Tris-HCl, pH 8) and the flow-through discarded. Then 2.5 ml of fusion protein samples were added and the flow-through discarded. The protein samples were eluted with 3.5 ml buffer (2 mM Tris-HCl, pH 8) in 4 differnt fractions. The protein concentration was measured by Bradford assay and run on a 10% SDS-PAGE (Fig. 22). In all samples a clear band was appered ~ 40 kDa position. Thus only the fusion proteins were purified by this maltose affinity chromatography after buffer exchange of the samples.

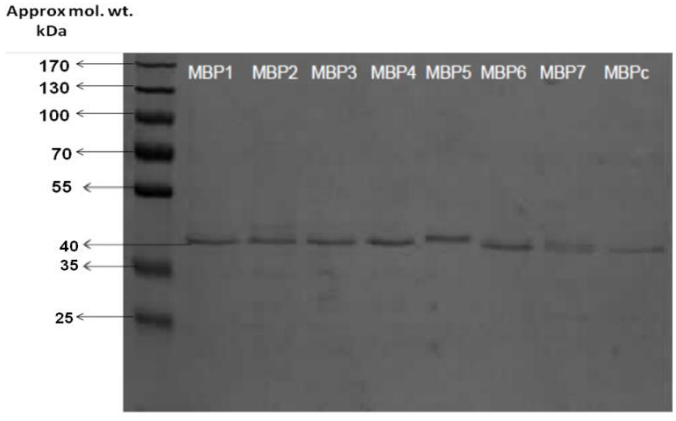


Figure 22. SDS-PAGE of viva spin™ concentrated MBP fusion proteins along with the control MBP.
Lane 1 : molecular weight marker, Lane 2-8: concentrated maltose binding protein attached with seven different putative metal binding peptides (MBP1-7). Lane 9: MBP.

4.3d Measuring the fusion protein concentration by Bradford assay

The dye reagent (1x) was removed from 4°C storage and allowed to warm to room temperature. Inverted the 1x dye reagent bottle for a few times before use. Twenty µl of each standards and unknown protein samples solution were pipetted into separate clean disposable cuvettes and 1 ml of 1x dye reagent added to each cuvette and vortexed. All cuvettes were incubated for 7 min at room temperature and measured the absorbance at 595 nm. Calculated the total protein concentration for each sample in µg/ml and yield in mg/litre as shown below in the (Fig. 23 and 24)

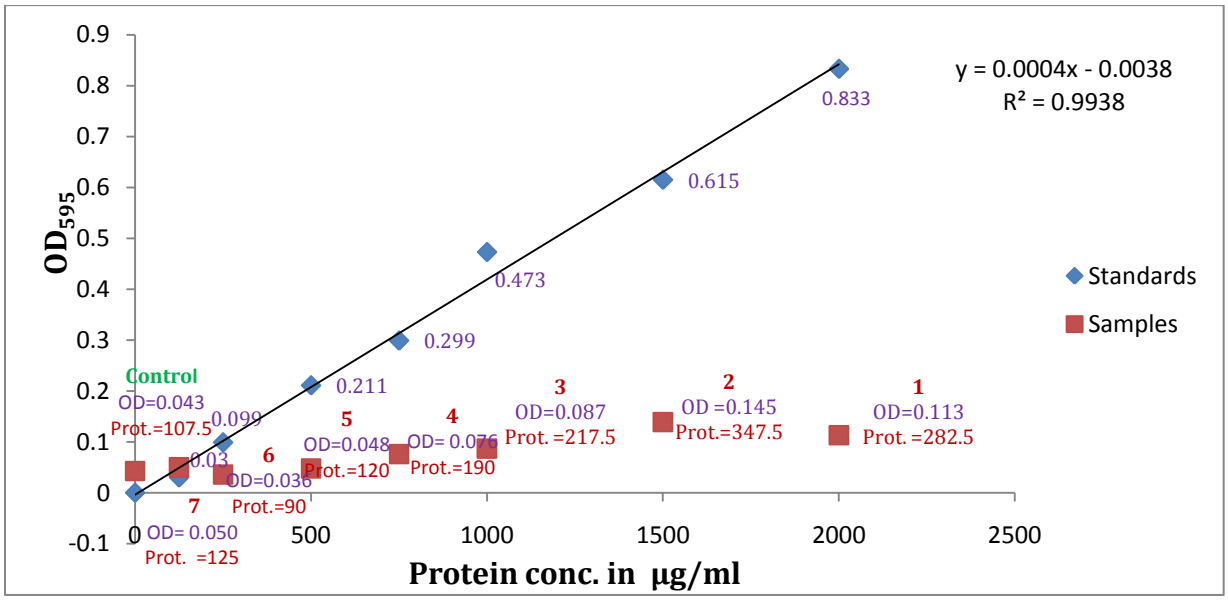


Figure 23: Fusion protein concentration along with γ- globulin standards by Bradford assay.

Note: 1-7 Samples: MBP+ putative copper metal binding peptide fusion proteins, Control: MBP protein only OD- Optical density at 595 nm, Standards- γ- globulin protein.

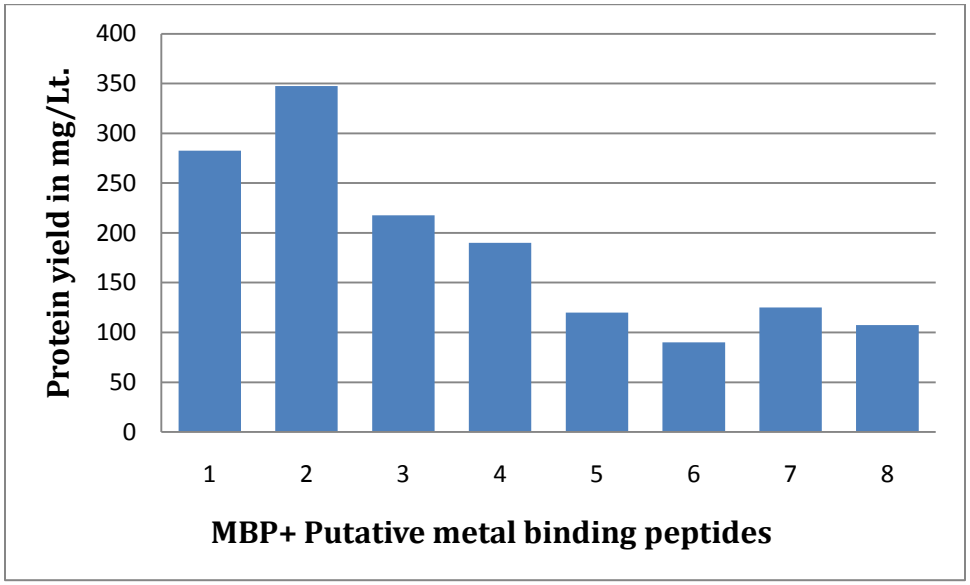


Figure 24: Fusion proteins yield along with control MBP.

1-7: MBP+ putative copper metal binding peptide fusion proteins, 8: Control MBP without any fusion peptide. Sample 2 shows the highest (347.5 mg/liter) and sample 6 lowest (90 mg/liter) protein yield, respectively. The protein yield of the samples depends on the expression and purification strategies of the individual fusion protein along with the control MBP.

CHAPTER-V

5.1 Summary and future directions

Energy is the most critical challenge facing humanity in the modern era due to industrialization and globalization consumption of energy is going to increase and the resources to get the energy are declining day by day. To fulfill the energy need energy is extracted from various sources such as wind, sea, plants and also from environmental pollutants. Extracting energy from environmental pollutants is a good approach to solve the problem of pollution as well as producing clean source of energy. For examples organic pollutants (waste) can be rich sources of energy if these wastes are degraded by diversity of microorganisms then energy can be extracted. The idea of generating electricity from organic waste with the help of microorganisms was proposed by Potter in 1911. Since then researchers have been trying to develop an efficient device to convert chemical energy from organic waste into electricity using different microbes as biocatalysts; this device is called a microbial fuel cell (MFC) (Shukla *et al*., 2004; Lovley, 2006) (Fig. 1). So MFCs can provide the possible way of extracting electrical current from different types of complex organic wastes (Lovley and Kelly, 2008).

In an MFC microbes degrade various organic wastes completely or incompletely via oxidation or fermentation processes, respectively. The microorganisms which completely degrade organic waste use an MFC electrode as an electron acceptor. Fig. 3 illustrates the possible electrons transfer mechanisms to the anode in an MFC.

This project was focussed on constructing a genetically engineered version of a nonpathogenic bacterial cell that will be able to transmit electrical current (i.e. electrons) to the anode *via* a channel protein, a naturally occurring outer membrane protein, called a porin, of Gram-negative bacteria, *P. aeruginosa* PA01. The proposed model for this transfer of electrons is shown in Fig. 5. The initial target for this project was the OprP porin from *P. aeruginosa* PA01

whose gene was amplified by PCR, cloned into *E. coli* DH5α cells and expressed. Purifications trials were in progress by using affinity chromatography. After purification of native porin, the electrochemical analysis *in vivo* and *in vitro* would be done to demonstrate the electrons transfer efficiency of this channel protein; also the other electrical conductive properties will be analyzed. Then the site directed mutagenesis of OprP porin would be carried out to alter its electrical conductive properties. The functional consequences of these changes could be assessed in the mutated protein product and finally the electricity generation potential of the recombinant cells expressing mutated OprP would be measured using an MFC. *P. aeruginosa* OprP seems like a good choice for electron across the cell as the X-ray crystal structure of OprP and simulations of anion transport through the OprP channel are well documented (Trevor *et al.*, 2006; Pongprayoon *et al*., 2009). So a rational approach was applied to generate a redox active metal ion (Cu^{++}) binding site in OprP channel by site directed mutagenesis to make it electrons transporter (Fig. 4). Tyr^{62}, Asp^{80} and Asp^{94} appear to be in close proximity in its 3D structure of OprP (Fig. 8C) and could potentially be changed to His, His and Cys (Fig. 4) to make a possible copper binding site.

Another possible approach to make OprP a potential redox active porin is, to synthesize small peptides with potential copper binding properties, which could be attached to OprP on either side (extracellular or periplasmic side) to make it a potential redox active transmembrane electron transporter. In this regard recombinant DNA technology offers methods for anchoring metal binding peptides to the outer membrane proteins of both Gram-positive and Gram-negative bacteria. (Kotrba *et al*, 2011). Previous studies documented that outer membrane proteins of Gram-negative bacteria are good anchoring proteins for intramolecular fusion of passenger proteins and peptides (Freudl *et al.*, 1986; Freudl, 1989 and Kotrba *et al*, 2011). To achieve this a peptide library of small peptides was created in which every peptide code for a

potential copper-binding site. To design the desired peptides, two complementary degenerate oligonucleotide motifs were designed according to Kotrba, 1999 which encode putative copper binding peptide library having a single potential copper binding site per peptide.

Metal binding motif I $^{5'}$AATTCGGTYRTGGCYRCCCGYRTGGCYRCGGTTGAG$^{3'}$
Metal binding motif II $^{3'}$GCCARYACCGRYGGGCRYACCGRYGCCAACTCCTAG$^{5'}$

These motifs were expressed downstream of *malE* gene in pMALp2x plasmid in host *E. coli* DH5α cells. The recombinant pMALp2x plasmids were sequenced and the putative Cu^{++} binding motifs with degeneracy at four places were found in between the *Bam*HI and *E.co*RI sites of pMALp2x plasmid (Fig. 17). The sequences were translated by bioinformatics ExPASy translation tool and a library of putative copper metal binding peptides was synthesized (Fig. 18). The Cu^{++} binding properties of recombinant *E. coli* DH5α cells harboring these peptides as a fusion to MBP protein were examined on a copper sulfate gradient plate (Fig. 19A & B). Cells expressing these peptides were resistant to copper sulfate in comparison to the control cells (Fig. 19B). It indicated that these peptides can bind copper and from the variation in growth between different samples on gradient plates, it can be assumed that all of these peptides will interact with copper with more or less binding efficiency. Further biochemical and biophysical analysis of these peptides might be useful for creating copper binding sites in bacterial proteins for use in biotechnology. The other potential use of these recombinant cells expressing the small peptides will be in bioremediation or heavy metal sequestering to clean up environment (Kotrba, 1999 & Kotrba *et al*., 2011). These peptides might also be attached to OprP of *P. aeruginosa* PA01, to make it a potential redox active electron transporter for future research in energy generation. Fusion proteins (MBP+ putative metal binding peptides) were expressed and purified from the recombinant *E. coli* DH5α cells (New England BioLabs^{R} $_{inc,\ 2003}$) and measured the protein concentrations by Bradford assay. The highest protein

concentration was found in sample 2 (347.5 µg/ml) as shown in the Fig. 23. So the possible outcome of this research is, creating a library of putative copper metal binding peptides, which will be used in the area of bioelectricity generation and heavy metal sequestering to clean up the environment from the heavy metals and fulfill the necessity of electricity for humankind, respectively.

APPENDIX

5'atgattcgcagacactcgtgcaaaggggtggggagcagtgttgcctggagtttgctgggcctggcgatttccgcg
cagagcctggccgggaccgtgaccaccgacggtgccgacatcgtgatcaagaccaagggcggcctcgaagtcgcca
ccaccgacaaggaattcagcttcaagctcggcggccgcctgcaggccgactacggccgcttcgacggctactacac
gaacaacggcaataccgccgatgccgcctacttccgccgcgcc**cac**ctggagttcggcggcaccgcctaccgggac
tggaagtaccagatcaactac**cac**ctgtcgcgcaacgtcggcaacgacagcgccggctacttt**tgc**gaagcttcgg
tcacctataccggcttcaacccggtcaacctgaagttcggccgcttctacaccgacttcggcctggaaaaggccac
cagctccaaatgggtcaccgcgctggagcgcaacctcacctacgacatcgccgactgggtcaacgacaacgtcggt
accggtatccaagccagctcggtggtgggcggcatggctttcctctctggcagcgtgttcagcgagaacaacaacg
ataccgacggcgacagcgtcaagcgctacaacctgcgcggcgtgttcgcgccgctgcacgagccgggcaacgtggt
gcacctgggcctgcaatacgcctatcgcgacctggaggacagcgcggtggatacccggatccgcccgcgcatgggc
atgcgcggcgtctccaccaatggcggcaacgatgccggcagcaatggcaaccgcggtctgttcggcggcagttcgg
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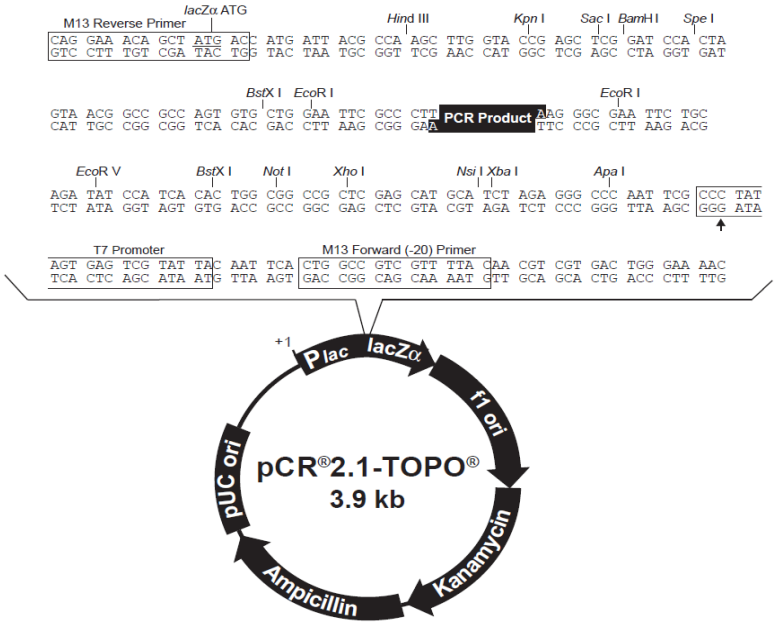
gtgaaggtttccgccaactacgtcaaggcgaagaccgacaagatcagcaatgccaacggcgacgatagcggcgatg
gcctggtgatgcgcctgcagtacgtgttctga3'

Figure 1: Nucleotide sequence of *Oprp* gene from *P. aeruginosa* PA01. The sites identified correspond to amino acids close to one another of the native OprP in 3D structure. Conversion of T-C, G-C and GA-TG to code for His, His and Cys could create a blue copper binding site (called 'Type 1' copper).

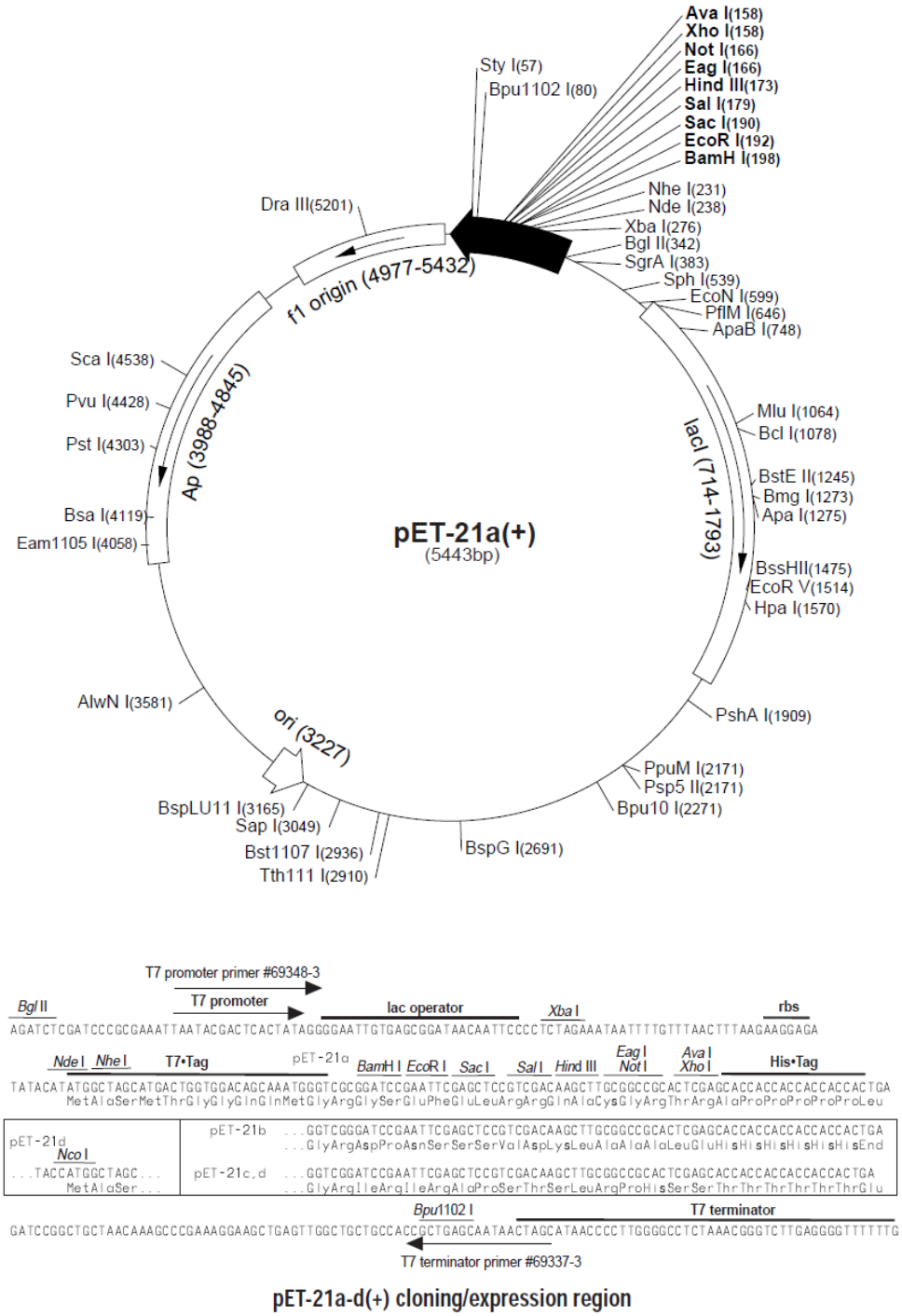
Figure 2: Secondary structure of the OprP of *P. aeruginosa* PA01. Red stars showing the site for mutagenesis for creation of potential copper binding site by changing Tyr^{62}, Asp^{80} and Asp^{94} in His, His and Cys, respectively.



Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Figure 3: **Genetic map of TOPO vector, a general cloning vector for *E. coli* cells.**



pET-21a-d(+) cloning/expression region

Figure 4: Genetic map of pET21-a (+) vector for expression of *oprP* gene *P. aeruginosa* PAO1 in *E. coli*. This vector has T7 strong promoter for tight regulation of expression of gene and His tag used for purification of expressed protein. It is ideal vector for most of protein expression study in *E. coli* cells.

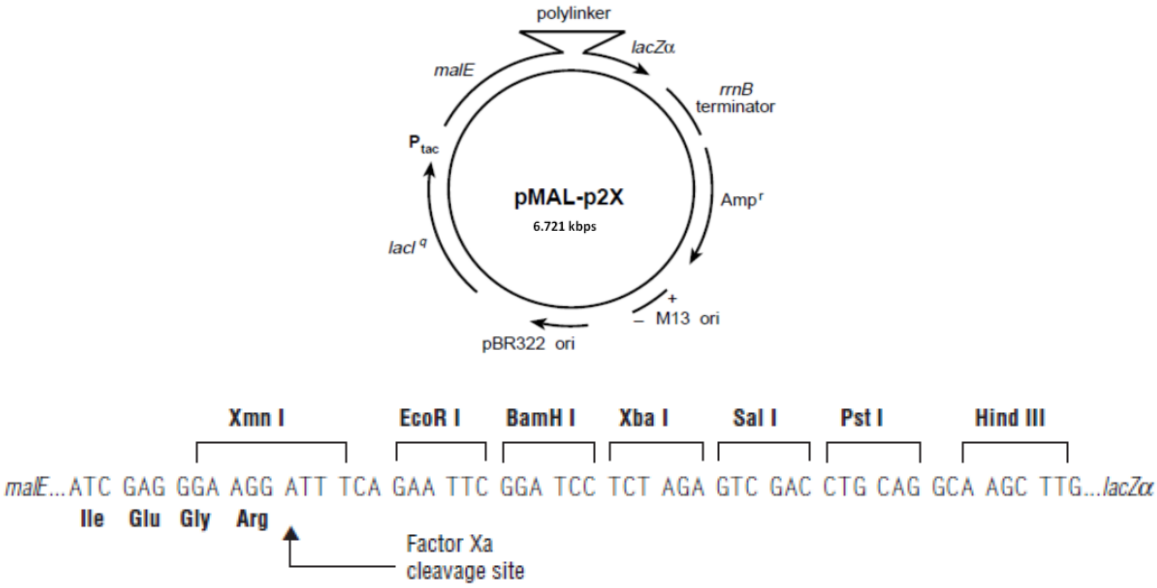


Figure 5: Genetic map of pMALp2x plasmid along with its polylinker regions

```
3671221 agcgcc3′tcag aacacgtact gcaggcgcat caccaggcca tcgccgctat cgtcgccgtt
3671281 ggcattgctg atcttgtcgg tcttcgcctt gacgtagttg gcggaaacct tcaccgcctc
3671341 gttggcgtac cagttgacgc ccagggtatg ggtcttgccc ttggcgtcgc cgacctcgcg
3671401 ggtggcgctg tcgacgacga tgttgtcatc ctcgaccttg atcgagtcgt agcggtagaa
3671461 cagctcccag gcgccgattt ccttgttctc cggcttgatg gtgtcgaact tggcgccgtc
3671521 cagcttgtag aggcgcggct cgccggtgag ggtgtaggcc agttgcgcgt aatagccgga
3671581 ggccttgaga tcctcgcggt cgcgctcggc cttgaccgtg cgccgcaggt actcggcctg
3671641 ggccgagaag gcacccagcg cccaggcgcc ttccaggccc cagaccgagt cgtccttcca
3671701 cagcccctcg actgccgaac tgccgccgaa cagaccgcgg ttgccattgc tgccggcatc
3671761 gttgccgcca ttggtggaga cgccgcgcat gcccatgcgc gggcggatcc gggtatccac
3671821 cgcgctgtcc tccaggtcgc gataggcgta ttgcaggccc aggtgcacca cgttgcccgg
3671881 ctcgtgcagc ggcgcgaaca cgccgcgcag gttgtagcgc ttgacgctgt cgccgtcggt
3671941 atcgttgttg ttctcgctga acacgctgcc agagaggaaa gccatgccgc ccaccaccga
3672001 gctggcttgg ataccggtac cgacgttgtc gttgacccag tcggcgatgt cgtaggtgag
3672061 gttgcgctcc agcgcggtga cccatttgga gctggtggcc ttttccaggc cgaagtcggt
3672121 gtagaagcgg ccgaacttca ggttgaccgg gttgaagccg gtataggtga ccgaagcttc
3672181 gtcaaagtag ccggcgctgt cgttgccgac gttgcgcgac aggtcgtagt tgatctggta
3672241 cttccagtcc cggtaggcgg tgccgccgaa ctccaggtag gcgcggcgga agtaggcggc
3672301 atcggcggta ttgccgttgt tcgtgtagta gccgtcgaag cggccgtagt cggcctgcag
3672361 gcggccgccg agcttgaagc tgaattcctt gtcggtggtg gcgacttcga ggccgccctt
3672421 ggtcttgatc acgatgtcgg caccgtcggt ggtcacggtc ccggccaggc tctgcgcgga
3672481 aatcgccagg cccagcaaac tccaggcaac actgctcccc acccctttgc acgagtgtct
3672541 gcgaatcat5′c aggtaa
```

Figure 6: Sequencing result of the *oprP* gene of the *P. aeruginosa* PA01.

Note: gene is encoded by complementary strand.

gcgaatcatc aggtaa Reverse complement of PCR Primer OprUP_N (ttaccATatgattcgcagacactcg)

gaacacgtact gcaggcgcat cac Complement of PCR Primer OprRDwnX2 (gaacTcgAGctgcaggcgcatcac)

Pre-denaturation	94°C	5.0 min.
Denaturation	94°C	30 sec.
Annealing	55°C at least 1 min per kb to amplify at 72°C	30 sec.
Extension	72°C	8.0 min.
Final extension	16°C	∞

Table 1: **PCR reaction condition for amplification of *oprP* gene**

Components	**Volume**
Go Taq flexi buffer	10 µl
25 mmol $MgCl_2$	3.0 µl
10 m mol dNTPs Mixture	1.0 µl
Forward primers	1.0 µl up primer [OprUP_N] or [OprUP_S]
Reverse primers	1.0 µl down primer [OprDwn_X] or OprDwn_X_2
Sterile water	33.5 µl
Colony *P. aeruginosa* PA01& PA14	Single
TaqDNA polymerase	0.5 µl
Total volume	**50.0 µl**

Table 2: PCR reaction mixture for the amplification of the *oprP* gene.

Components	**Volume**
Fresh PCR product	4.0 µl
Salt solution	1.0 µl
pCR® Blunt II -TOPO® vector	0.5 µl
Final volume	**5.5** µl

Table 3: Reaction set up for TOPO® cloning reaction

pMALp2x Plasmid DNA	5.0 µl
Sterile dH2O	11.0 µl
Promiga multicore buffer	2.0 µl
*BamH*1	1.0 µl
*E.co*RI	1.0 µl
Total	**20.0 µl**

Table 4: Restriction digestion mixture for pMALp2x Plasmid.

dH_2O	3.2 ml
Stacking gel buffer	1.3 ml
Acrylamide	0.5 ml
APS	25.0 µl
TEMED	8.0 µl

Table 5A: SDS-PAGE Stacking gel concentration

Components	**10%**	**15%**
dH_2O	4.0 ml	2.4 ml
Resolving gel buffer	2.5 ml	2.5 ml
Acrylamide	3.3 ml	5.0 ml
APS	50 µl	50 µl
TEMED	8.0 µl	8.0 µl

Table 5B: SDS-PAGE resolving gels concentration

Bromophenol blue	0.25%
Dithiothreitol (DTT)	0.5 M
Glycerol	50%
sodium dodecyl sulfate (SDS)	10%
Tris-Cl	0.25 M, pH 6.8

Table 6: Mel's SDS loading buffer (5X)

Per liter	**Final conc.**
20 ml 1.0 M Tris-HCl (pH 7 .4)	20 mM Tris-HCl
11 .7 g NaCl	200 mM NaCl
2 .0 ml 0 .5 M EDTA	1 mM EDTA
154 mg DTT	1 mM DTT

Table 7: Column Buffer composition for MBP-Peptide fusion protein purification by affinity chromatography

1 ml Standard Assay

Tube #	Standard Volume (µl)	Source of Standard	Diluent Volume (µl)	Final [Protein] (µg/ml)
1	70	2 mg/ml stock	0	2,000
2	75	2 mg/ml stock	25	1,500
3	70	2 mg/ml stock	70	1,000
4	35	Tube 2	35	750
5	70	Tube 3	70	500
6	70	Tube 5	70	250
7	70	Tube 6	70	125
8 (blank)	–	–	70	0

Table 6: Standard Bradford assay for protein determination in solution.

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