Iron metabolism in the opportunistic pathogen *Pseudomonas aeruginosa* requires two ferritin-like molecules, *Pa* FtnA and *Pa* BfrB

By

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ABSTRACT

FtnA is an iron storage protein in *Pseudomonas aeruginosa* which is capable of storing about 4500 Fe^{3+} ions in its inner core. According to earlier classifications of this protein *Pseudomonas aeruginosa* was proposed to have two bacterioferritins coded by two different genes *bfrA* and *bfrB* which coexists. Our research was focused on an idea that *Pseudomonas aeruginosa* have one bacterioferritin and one bacterial ferritin which acts as iron storage proteins instead of a heterogeneous bacterioferritin. To strengthen our hypothesis we first cloned *bfrA* gene in *Pseudomonas aeruginosa* and purified *Pa* FtnA to homogeneity. Crystallization studies were conducted to solve the structure of *Pa* FtnA protein which reveals that *Pa* FtnA is composed of 24 identical subunits coordinates together to form a spherical hollow sphere which helps in storage of iron in its inner cavity. Experiments performed in crystallo suggested that iron ions are oxidized at ferroxidase center and taken in to the inner cavity. The most interesting detail found by crystal structure was presence of a third iron site near ferroxidase center at different pH and buffer conditions which is similar to "Site C" in many known bacterial ferritins. Even experiment performed in vitro also showed Fe^{2+} is oxidized before storing in the inner cavity. Previous observation made by sequence alignment of *Pa* FtnA with *Pa* BfrB and other known bacterioferritins showed absence of M52 residue in *Pa* FtnA which also shows that *Pa* FtnA is a bacterial ferritin not bacterioferritin as this binds to Heme in *Pa* BfrB which is a characteristic feature.

Earlier studies suggested protein-protein interaction as a key step in releasing iron from iron storage protein BfrB in *Pseudomonas aeruginosa*. This indicates inhibition of this PPI with inhibitors should prevent the bacteria from releasing iron. In order to apply the developed inhibitor for *P. aeruginosa* to other pathogenic Gram-negative bacteria, first we need to understand the

structure and functional similarity of these pathogenic bacteria in comparison to *P. aeruginosa*. This can be achieved by doing multiple sequence alignment. This helps us understand the possible structure and function that can form by selected bacteria in comparison to *P.aeruginosa*. If these bacteria contain similar type of residues at important iron regulation sites than the possible mechanism even in these selected Gram-negative bacteria will be similar to *Pseudomonas aeruginosa*. Hence we can apply same inhibitors to inhibit PPI even in these bacteria.

Presence of these storage proteins is one mode of releasing iron during iron limiting conditions for bacteria to survive, other mode of action is by sequestering iron from host cells by releasing siderophores and hemophores. To study phenotype changes in biofilm formation in *Pseudomonas aeruginosa* under iron starvation conditions in presence of iron chelators, we choose transposon mutants which were purchased from University of Washington, Manoil lab. Experiments were performed using succinate minimal media in presence and absence of iron chelators with different mutants. Iron chelators such as BP (2,2' Bipyridine) and DTPA (diethylene triamine pentaacetic acid) were used for this study which showed, the mutant in presence of iron chelator have retarded growth whereas mutants and wild type in absence of iron chelators did not show any effects on its biofilm formation. These studies showed release of pyoverdine a type of siderophore by *Pseudomonas aeruginosa* and also showed release of pyocyanin a phenazine with DTPA. This concludes that initially bacteria tends to release pyoverdine for iron acquisition but when it is not getting enough iron for its growth and under high stress conditions it tends to release phenazine for iron acquisition and start showing a slow growth. We did observe planktonic difference but not phenotype changes with the medium used.

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DEDICATION

TO

My Great Parents

(Mr. Chandra Shekar Nama and Mrs. Indrani Nama)

AND

My Loving Husband

(Dr. Prakash Manikwar)

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ABBREVIATIONS

ABC	ATP-binding cassette
APS	Advanced photon source
Bfr	Bacterioferritin
Bfd	Bacterioferritin associated ferredoxin
BLAST	Basic Local Alignment Search Tool
BLOSUM	Block Substitution Matrix
BP	2,2’ Bipyridine
Bp	Base pair
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
Ec	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EMBOSS	European Molecular Biology Open Software Suite

ESI-MS	Electrospray ionization mass spectrometry
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella* species, *Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* specie
EtBr	*Ethidium bromide*
Ftn	Ferritin
FPLC	*Fast protein liquid chromatography*
F-Primer	Forward primer
Has	Heme acquisition system
HasAP	Heme acquisition system Pseudomonas aeruginosa
HasR	Heme acquisition system receptor
Hah	Hemagglutinin (HA) epitope and a hexahistidine (h) motif
H-chain	Heavy chain
HemO	Heme oxygenase
IE	Inside end
Inh	Inhibitor protein
IPTG	Isopropyl-β-D-thiogalactosidase
IS	Insertion site
JCVI-CMR	J. Craig venter institute - Comprehensive Microbial Resource
KEGG	*Kyoto Encyclopedia of Genes and Genomes*
lacZ	β-galactosidase
LB	Luria *Broth*
L-chain	Light chain
MSA	Multiple sequence alignment

Mwt	Molecular weight
NADPH	Nicotinamide adenine dinucleotide (phosphate)
NCBI	National Center for Biotechnology Information
OD	Optical density
OE	Outside end
Pa	Pseudomonas aeruginosa
PAM	Point accepted mutation
PCR	Polymerase chain reaction
PDB	Protein data bank
PhoA	Alkaline phosphatase
Pmf	Proton motive force
PMSF	Phenylmethylsulfonyl fluoride
PPI	Protein-Protein interactions
RNA	Ribonucleic acid
R-Primer	Reverse primer
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SM	Succinate minimal media
TBE	Tris base,boric acid and EDTA
TCEP	Tris (2-carboxyethyl)-phosphine hydrochloride
Tn	Transposon
Tnp	Transposase
Tris	Tris(hydroxymethyl)aminomethane
TSG	Tris-Hcl, sodium chloride and Glycerol

UV-Vis	Ultraviolet-visible
UW	University of Washington
Wt	Wildtype

CHAPTER I

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic gram negative bacterium, which is found in soil, hospitals, water and any place that has moisture (*1, 2, 3*). It mostly affects people who are immune compromised such as AIDS patients, burn victims, cancer patients, and also hospitalized patients whose immune system is not so strong. *P. aeruginosa* can infect tissues of the body, such as the lungs , cornea, gastric system, injured bones and joints, urinary tract, etc., causing severe infections(*4*) sometimes leading to surgery to remove that particular tissue. This bacterial infection can be treated by using multiple drugs or by surgery, but recently it was discovered that most of *P. aeruginosa* have developed resistance against these antibiotics (*5*). This leads to problems treating patients suffering with AIDS or cancer because to treat the actual disease, *P. aeruginosa* needs to be eradicated completely

About 60% of cystic fibrosis (CF) patients are infected with *P. aeruginosa* which causes a respiratory infection and leads to death. When it starts growing it forms biofilms in the air ways and becomes very difficult to treat. About 30,000 children and adults are infected by cystic fibrosis in the USA and about 70,000 are infected worldwide (*6*). Iron is an important factor to biofilm formation in CF patients. Recent findings by Kaneko.Y. et al., suggests that inhibition of iron dependent processes in the bacteria's system can prevent formation of biofilms. For example, they substituted Ga with iron which is almost similar to Iron, and noticed inhibition in biofilm formation, and killed the bacteria in vitro (*7*). This indicates that the decrease in iron concentration prevents or declines the growth and pathogenicity of *P. aeruginosa*; therefore understanding the mechanism of iron uptake, storage and usage by microorganisms is very important.

Importance of Iron in pathogenic bacteria:

Iron is the most important element for survival of many microorganisms in nature as it is an essential cofactor in many enzymatic functions. *Pseudomonas aeruginosa* also depends on iron for its survival and pathogenicity. Most of the iron in the human body is available from ferritins, hemosiderin, and heme. Extracellular iron is bound to iron binding proteins like transferrin and lactoferrin. The majority of iron (II) is not freely available due to incomplete O_2 reduction, resulting in the toxic formation of super oxide radicals and hydrogen peroxide (Reaction 1). Available ferrous ions can also react with hydrogen peroxide and produce free hydroxyl radicals (Fenton reaction) (Reaction 2) (*8*) which can damage DNA strands, the process of lipid peroxidation, and the degradation of biomolecules (*9*). These two reactions together are called the iron catalysed Haber-weiss reactions (Reaction 3) (*10*). Mammalian cells may contain approximately 10^{-18} M concentration of free available Fe^{3+} which is practically zero (*11*). These microorganisms have developed different mechanisms to steal lower concentrations of iron (Fe^{3+}) available in host cells and store the iron in iron storage proteins such as Ferritins, Bacterioferritins and DNA binding proteins of starved cells (Dps) to use for its survival (*12*). One of the most important mechanisms is the release of siderophores which act as Fe^{3+} chelating molecules that sequester iron from the host cells and help in bacterial growth and survival (*13, 14, 15, 16,*).

Reaction 1: Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$$

Reaction 2:

$$O_2^{\cdot -} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

Reaction 3: Haber-weiss reactions

$$H_2O_2 + O_2^{\cdot -} \rightarrow OH^- + OH^{\cdot} + O_2$$

Role of Siderophores and Hemophores in iron acquisition

Iron homeostasis is maintained in the human body by iron storage proteins, extracellular proteins, or by binding to the protoporphyrin ring in hemoproteins. This causes virtually no free iron available for bacterial growth. To overcome this shortage, these organisms depend on proteins that bind to iron or heme comprising molecules. There are two different mechanisms involved to sequester iron from the host. First is to sequester iron directly from the iron or heme source; whereas the second mechanism involves release of low molecular weight compounds or iron chelating molecules called siderophores to sequester iron(*17*)Another compound, a hemophore, is secreted to sequester iron from heme and is transferred by a transporter protein (*18, 19*). Siderophores and hemophores are considered as indirect sources for iron and heme and help maintain the iron level in the bacteria.

Siderophore:

The main reason for low availability of iron is its poor solubility, so the most challenging aspect for siderophores is to dissolve the iron and prevent it from binding to oxygen and precipitating as an iron oxide. There are two types of sources available for iron called direct and indirect. Compounds involved in direct sources include iron in the body (*20, 21, 22,*), iron from transferrin, lactoferrin (*23*), and ferritins (few bacterial species) (*24*, 25, 26,). Indirect sources come from siderophores which are secreted only under iron limitation conditions.

Siderophores are low molecular weight, high affinity iron chelating molecules that are secreted by prokaryotes and eukaryotes to sequester iron. The success of siderophores sequestering iron is dependent on a variety of factors. These include the stability of iron after it is bound, the environmental conditions in which it is released, the denticity of the ligand, the binding site on the siderophore, and the connecting chains on the binding site. The denticity of the ligand is important to the stability of the complex because the more binding sites available, the more stable the complex. This is seen in the comparison of a hexadentate complex which is more stable than a bidentate complex (*27*). The binding sites can be made up of hydroxamate, catecholate, and hydroxy-carboxic acid groups which form stable complexes by occupying two sites on the central iron atom. The structure of the siderophores also include their stability, for example, a cyclic siderophore forms a more stable complex with iron compared to acyclic compounds (*27*, *28*).

The general mechanism of iron uptake by siderophores in gram negative bacteria involves the most important outer membrane receptors which is TonB complex dependent. The TonB complex is present in the periplasm and is comprised of 3 different proteins called ExbB, ExbD and TonB. The outer membrane receptor is linked to TonB via an N-terminal end of the protein which contains 5 conserved residues called the TonB box (*29*, *30*). These receptors have a gated mechanism with TonB. The energy from the proton motive force (pmf) is transported by TonB in order to open this gate and transfer the ferrisiderophore to the periplasm where this ferrisiderophore bound to a periplasmic binding protein is transported to the cytoplasm via a transporter. Reduction of Fe^{3+} to Fe^{2+} is carried out in the cytoplasm where it separates from this periplasmic binding protein which is later sent to be recycled or undergo enzymatic destruction by esterases (*31, 32*) (Scheme 1). However, the mechanism of iron uptake in *Pseudomonas*

aeruginosa is a little different than the above mechanism, which is explained in detail in chapter 4.

Hemophores:

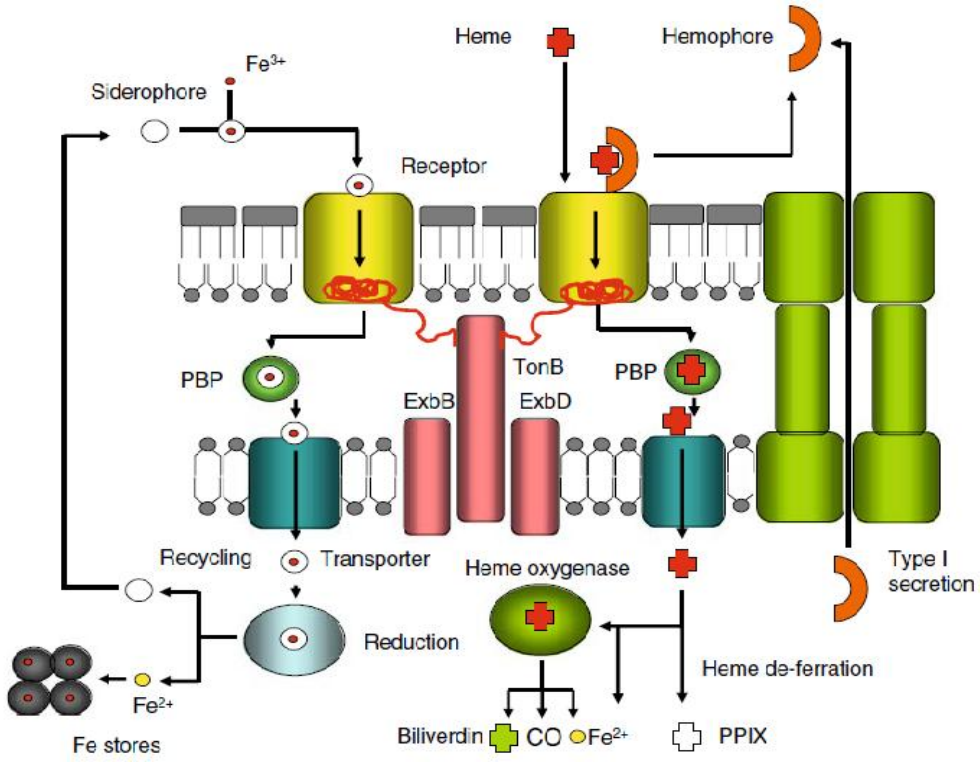
Hemophores are the protein molecules which have very high affinity for heme and help to transport it to appropriate receptors of the outer membrane. Hemophores also have direct and indirect types of sources to acquire heme. Direct sources include heme (*33*), hemoglobin (*34*), heptoglobin-hemoglobin (*35, 36*), hemopexin (*37*), albumin, myoglobin, and cytochrome b. Indirect sources are HxuA (which is found in *H. influenzae*) (*38*) and HasA (which is found in *Serratia marcescens, Pseudomonas aeruginosa, P. fluorescens, Yersinia pestis, and Y. enterocolitica*).

Heme is a major source of iron and is degraded to release the iron in the cytosol. The general mechanism of heme uptake involves the capture of heme by hemophores which transfer it to receptors on the outer membrane and release the heme in the periplasm. With help of ABC permeases, heme transfers to the inner membrane receptors which direct it to the cytoplasm where with the help of the enzyme, heme oxygenase, degrades to release Fe^{2+}, biliverdine, and CO. The hemophores may also undergo heme de-ferration and releases only Fe^{2+} and keep its tetrapyrole ring (*39, 40*). The binding of heme and the transfer of heme from the hemophore to the outer membrane is energy independent whereas, the basal amount of TonB and a high level of Pmf are is required for heme transport and release of the hemophore (Scheme 1).

Heme uptake in *Pseudomonas aeruginosa* contains 2 systems, the Phu and Has systems (*41*). In the Phu system, heme is extracted by hemoproteins directly, and in the Has system,the HasAp hemophore is involved in sequestering iron and transferring it to the HasR receptor. The

heme is then transported to the cytoplasm and is taken by the PhuS protein and is transferred to the heme oxygenase, which is encoded by hemO, and helps in the release of Fe^{2+} and biliverdine. The heme is always in coordination with another protein because free heme is toxic causing a Fenton like reaction. (*42, 43, 44*)In the Has system, transport of heme requires either TonB or HasB receptors which have the same function in nature (*45*).

Under iron starvation condition *Pseudomonas* and other microorganisms not only secrete siderophores and hemophores to sequester iron from host cells, but they also depend on their iron reservoirs known as iron storage proteins for their survival.



Scheme1: Showing mechanism of iron uptake by siderophores and hemophores in gram negative bacteria (*40*).

Iron storage proteins:

These are proteins which store iron intracellularly and release iron whenever it is necessary. These iron storage proteins are very important for the survival of the organism in iron depleting conditions. Three different types of iron storage proteins have been identified. They are ferritins, bacterial ferritins, and DNA binding proteins (Dps); although Dps have only been identified in prokaryotes, but there are few organisms which possess all three iron storage proteins. The major function of ferritins and bacterioferritins is to oxidize highly reactive ferrous iron (Fe^{2+}) to ferric form (Fe^{3+}), store the ferric mineral in the form of ferrihydrite or ferric phosphate (depending on presence of phosphate) in a storage cavity, and then release the stored iron when there is less available iron extracellularly. Dps proteins are useful to protect chromosome from iron induced free radical damage.

DNA binding proteins from starved cells (Dps):

Dps was discovered by Roberto Kolter et al. in 1992 from *E.coli* cells subjected to starvation conditions, hence the name DNA binding proteins from starved cells. Dps belongs to the ferritin family which contains a dodecameric sphere with 4,3,2 symmetry and is formed from 12 identical subunits. These subunits unite to form a hollow spherical sphere with an inner diameter of ~ 4.5 nm and an external diameter of ~ 9.0 nm, which has a capacity of storing about500 iron atoms (*46*). Iron in the form of Fe^{2+} is oxidized to Fe^{3+} and stored in the storage cavity. Oxidation of iron takes place in the ferroxidase center which is present in between two adjacent subunits. Two iron atoms are coordinated by amino acid residues present in the ferroxidase center (*47, 48*) (Figure 1).

The major function of Dps family proteins is not iron storage but to protect DNA from oxidative stress (*49*). The hydroxyl radical which is produced as a result of the Fenton reaction in

the presence of Fe^{2+} and hydrogen peroxide acts as an oxidative agent for proteins, lipids and DNA(*50, 51*) which deactivates the function of these molecules. To protect their function, Dps uses hydrogen peroxide as a catalyst to oxidize Fe^{2+} instead of molecular oxygen and decreases the production of hydroxyl radical(*52, 53*). Dps production is believed to increase in the stationary phase because during this phase of growth, the amount of nutrients available cause less oxidative stress, and the bacterial cells do not have sufficient energy to synthesize new enzymes or proteins. During this phase, bacteria directly interact with DNA and form a stable nucleoprotein called a biocrystal which helps to protect DNA from reactive oxygen species (*54, 55, 56*). All Dps proteins are capable of sequestering iron, whereas in *E.coli* and *Mycobacterim smegmatis,* Dps can bind both iron and DNA; although, all Dps proteins can function as DNA protecting proteins in oxidative stress conditions (54, 57).

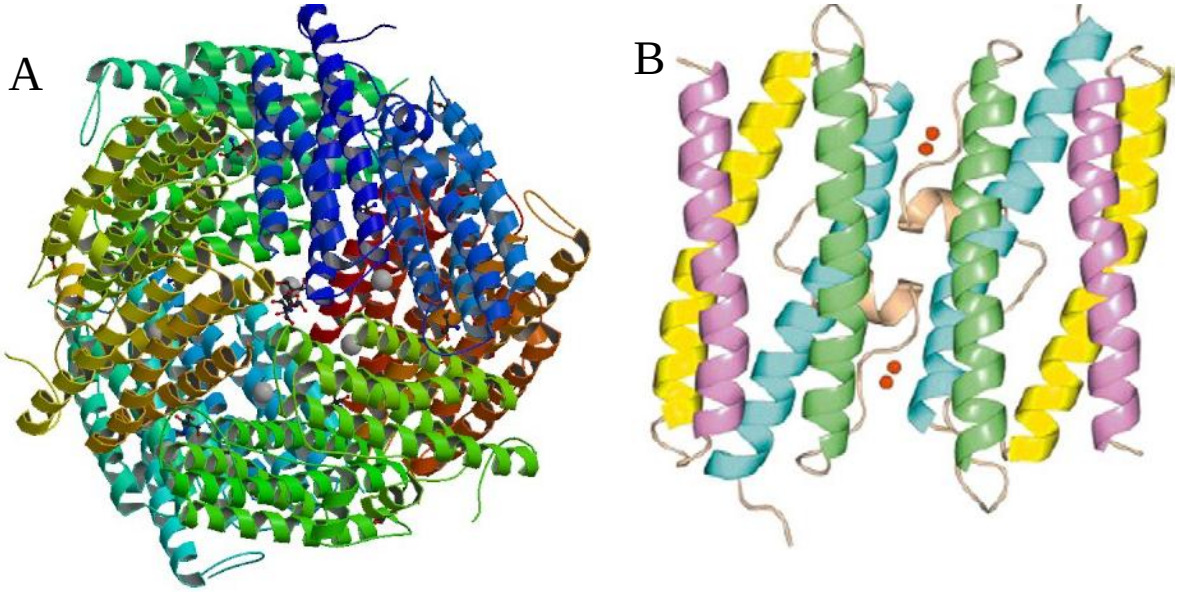


Figure 1: (A) Crystal structure of *E.coli* Dps (12 mer) (PDB 1L8H), (B) Dimer subunit from *Bacillus brevis* (PDB 1N1Q).

Bacterioferritin (Bfr)

Bacterioferritins are iron storage proteins from the ferritin family, which were earlier known as cytochrome b (*58*). These are heme containing proteins which help in iron storage and detoxification in the bacterial cell. The quaternary structure of bacterioferritins is made up of 24 identical subunits united to form a polymeric structure. These 24 subunits arrange in a 4, 3, 2 octahedral symmetry forming a spherical hollow cavity in the center which is capable of storing approximately 4,500 iron atoms. Each subunit is made up of four helices arranged parallel to each other and a fifth short helix which is perpendicular to the central axis of the helices bundle (*59*). Two subunits are paired to form a dimer with the heme, since 12 heme moieties are present in a single protein molecule. The heme is coordinated by Met 52 (methionine) which is located in between two subunits (*60, 61*).

The most important feature of bacterioferritins which helps in iron storage is the presence of a ferroxidase center. The ferroxidase center is a dinuclear ion center which is present in the middle of each subunit. Ferroxidase centers of Bfr are symmetric and made up of histidines and glutamic acids which bind to two iron ions and oxidizes Fe^{2+} to Fe^{3+} and store it in the central cavity. There are 3 important steps which take place during iron storage at the ferroxidase center: (*62*) (1) binding of Fe^{2+} ions at the ferroxidase center, (2) oxidation of Fe^{2+} to Fe^{3+} and (3) mineralization of Fe^{3+} into inner cavity. The other important feature of Bfrs is the presence of eight 3-fold pores, six 4–fold pores, and B pores which have been assumed to help in iron release. Studies in our research group on *Pseudomonas aeruginosa* BfrB have showed that the 4-fold pores play a vital role in iron release under iron limiting conditions (*63*) (Figure 3).

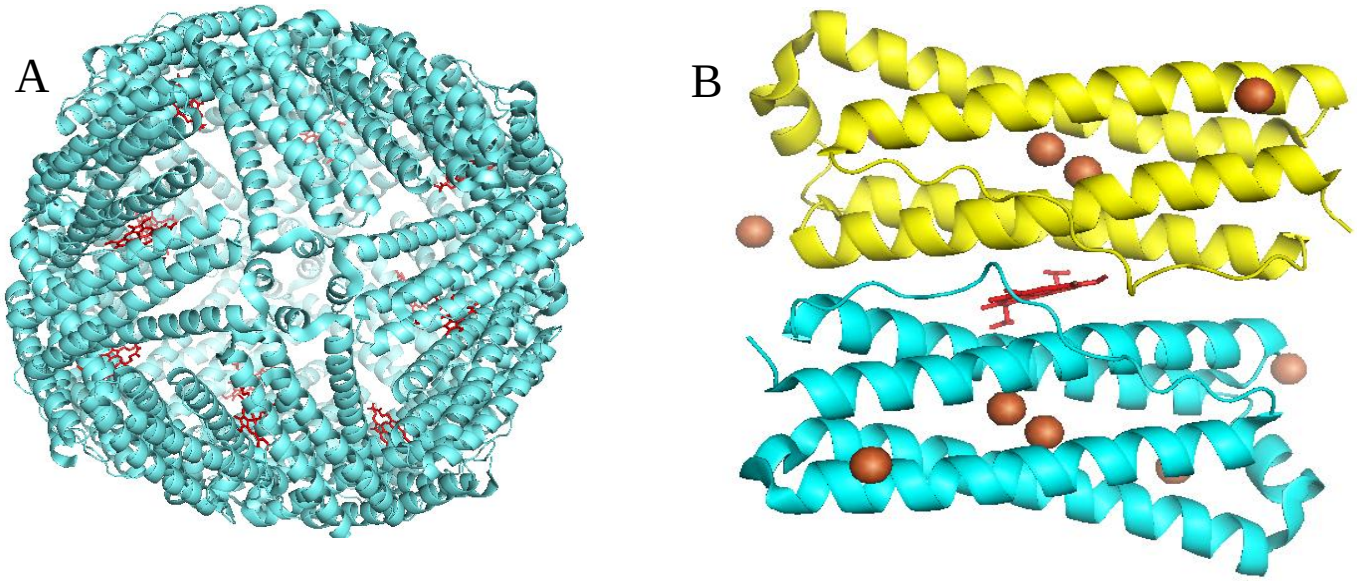
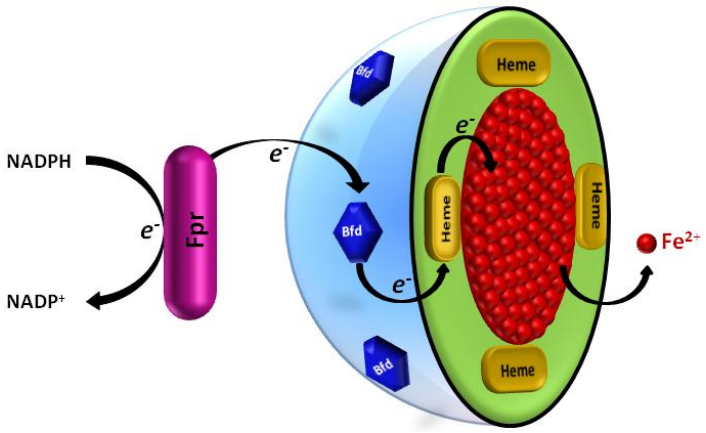


Figure 2: (A) 24-mer structure of *Pa*. BfrB with heme (Red) (PDB 3IS7), (B) *Pa*.BfrB subunit showing a diiron ferroxidase center.

Proposed mechanism of iron release from inner core in *Pseudomonas aeruginosa* from our research group indicates presence of protein interactions. In a solution containing *Pa* BfrB, a regulatory protein (*Pa* Bfd) and a reductase (*Pa* Fpr) are added along with NADPH, which suggests on addition of NADPH to a solution this initiates electron release form reductase protein *Pa* Fpr which in turn transfer electrons to [2Fe-2S] cluster of *Pa* Bfd on reduction this protein transfer electrons reduce heme present in *Pa* BfrB which helps in reducing ferric mineral (Fe^{3+}) which is present in inner core and releases it as soluble free iron in the form of Fe^{2+} which is utilized by bacterial cell *(63)*.



Scheme 2: Proposed mechanism of iron release in Pa BfrB

Ferritins (Ftn):

Ferritins are another type of storage proteins which are present in both prokaryotes and eukaryotes. These are also polymeric proteins made up of 24 subunits (Figure 3)which are arranged in a 4, 3, 2 octahedral symmetry forming a hollow central cavity with a molecular mass of about 480 kDa, an outer diameter of ~120 Å, and an inner diameter of about 80 Å. These are non-haem binding proteins, but are capable of storing about 4500 iron atoms in its central cavity. Vertebrate ferritins are composed of two different subunits called the H (Heavy)-chain and L (Light)-chain that are 55% identical in sequence (*64, 65*). These two are present in different ratios which is dependent on the species and place of origin. For example, H subunits are found more in ferritins extracted from the heart and brain and have lower average iron contents that are less than 1000 Fe atoms per molecule. The L chain is abundant in ferritins from the liver and spleen which have a relatively high average iron content of 1500 Fe atoms per molecule (*66*). The major difference between these two subunits is the presence of the ferroxidase center. For example, H chain subunits have a ferroxidase center which helps in oxidizing free iron (Fe^{2+}) to insoluble iron (Fe^{3+}) and thus helps in storing the mineralized iron in the storage cavity, whereas

the ferroxidase center is not located in L-chain subunit causing it to oxidize iron slowly (*67*). Studies on amphibians (frogs) show presence of a third type of subunit called an M subunit which differs only by 19 amino acids, but resembles the H-chain subunit in sequence (*68*).

Each of these 24 subunits has four long helices and one short fifth helix as in the bacterioferritin (Figure 3B). These subunits arranged in a sphere forming 4-fold, 3-fold and 2-fold symmetry axes. In higher organisms residues at the 3-fold axes are hydrophilic in nature. In vertebrates and plants the residues like aspartates and glutamates, which are conserved at side chains, mainly helps in binding metals like Cd^{2+}, Zn^{2+}, Tb^{3+} or Ca^{2+} (*69,70, 71*); hence, 3-fold channels are considered as the main route for iron transport and also consider as the oxidation site(*71, 72, 73, 74, 75, 76, 77*. These residues are not conserved in invertebrates and in *EcFTN* and H.*pylori* instead have several hydrophobic residues at this axis and no metals were found at 3-fold interface (*78, 79, 80*). The 4-fold pores are lined by hydrophobic residues whose function is not clear, but it is believed that these pores may be helpful in diffusion of O_2 and H_2O_2 in the ferritin (*81, 82, 83, 84*).

Bacterial ferritins are slightly different from mammalian ferritins which show about 22-24% identity to H chains and 18-21% identity to L chains of mammals and show only 14% similarity with each other (*78, 85*). Most studies have been completed on the bacterial ferritin from *E. coli* (EcFtnA) which is also a globular protein composed of 24 identical subunits that unite to form a central hollow cavity. The ferroxidase center is present in the center of a subunit as in bacterioferritins, but they do not have a haem moiety. It also has six 4-fold pores, eight 3-fold pores which have both hydrophobic and hydrophilic residues. The difference between eukaryotic ferritin and bacterial ferritin is the presence of another type of pores called the B

pores whose function is not known, but it is assumed to have the same function as the 3- fold channels of eukaryotic proteins (*59, 84*).

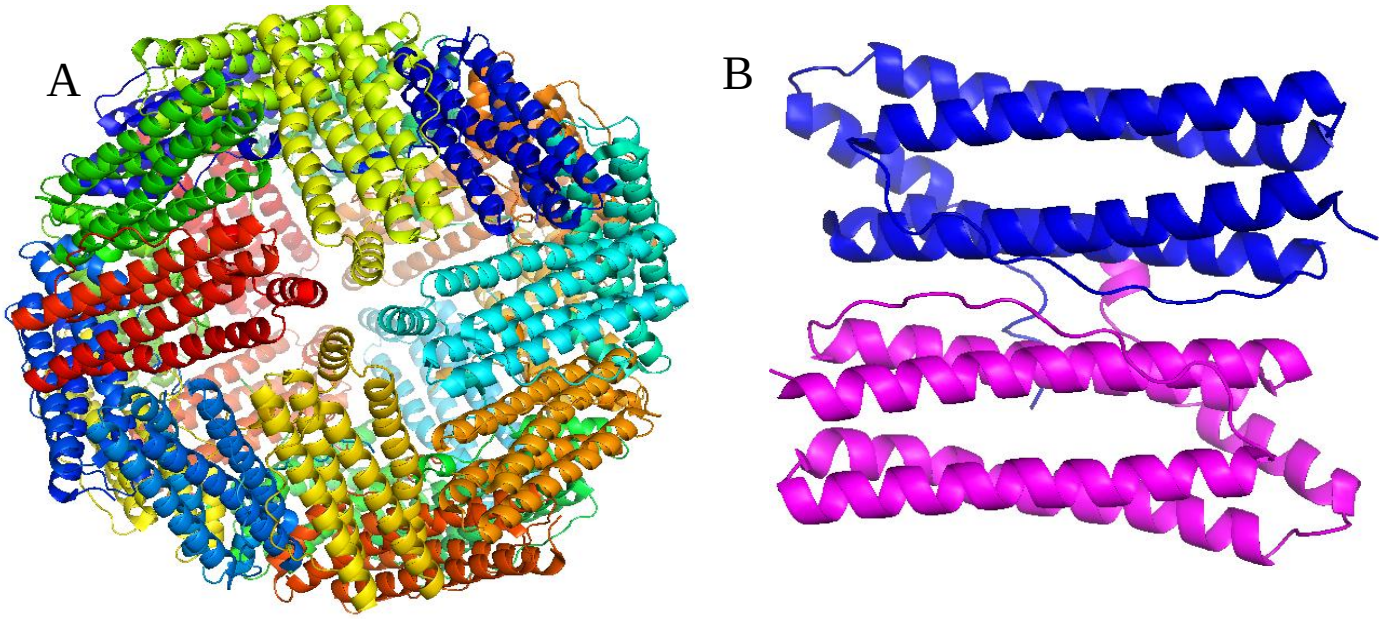


Figure 3: (A) 24 sub units and (B) dimer subunits of ferritin molecule from *Pyrococcus furiosus* (PDB 2X17).

Research problem/ rationale:

Iron is an essential nutrient for many biological processes to occur in prokaryotes and eukaryotes. It is the second most abundant metal in the earth's crust. However, because of the unstable nature of soluble Fe^{2+},which oxidizes easily to produce insoluble Fe^{3+} in the presence of oxygen and resulting in the Fenton reaction, iron is harmful to the organism. Despite of its large presence in nature it is available in the insoluble form, therefore, many organisms developed unique mechanism to sequester this iron from external sources by siderophores and internal sources from storage proteins where it stores excess iron and uses it whenever it is required.

P. aeruginosa is a ubiquitous opportunistic pathogen which invades immune compromised patients and is responsible for infections in patients suffering from cystic fibrosis, burn victims etc.,. *P. aeruginosa* also depends on iron for its pathogenicity and virulence. Due to the presence of low amounts of usable iron in the host cell at physiological pH, these organisms developed many strategies to acquire insoluble iron atoms by releasing siderophores to the external environment to sequester iron and transport it to storage proteins where it is stored as an iron mineral.

Pseudomonas aeruginosa has different storage proteins which help store excess available iron and release it in necessary conditions. It is known that in *P. aeruginosa* bacterioferritin, a type of storage protein is encoded by two genes which coexist as BfrA and BfrB. Studies conducted on the BfrA gene revealed interesting and important findings on functional and structural characteristic of this protein. The absence of heme, the lower number of important amino acid residues present during sequence alignment and X-ray crystallographic studies suggest the presence of a bacterial ferritin (*Pa*.FtnA) which resembles the *E.coli* ferritin (FtnA) in its structure. The presence of site c in the ferroxidase center, which is a characteristic feature of bacterial ferritin (*Ec*.FtnA), and the mechanism of iron release in this bacterial ferritin is different when compare to bacterioferritin (*Pa*.BfrB). The *Pa*.BfrB is involved in different protein-protein interactions, such as the necessity of the proteins Bfd and Fpr for the release of iron; whereas in *Pa*.FtnA, only Fpr is involved. Protein–protein interactions, structural details, and some important findings are explained in chapter 2.

To predict protein-protein interactions and the protein complex structures, sequence alignments were performed which revealed interesting information on amino acid sequence, which helped us understand the structure and connections between proteins to perform their

function. To search BfrB and Bfd like molecules in Gram-negative pathogenic bacteria, and To figure out whether similar type of iron release mechanism exist in these selected bacteria we performed sequence alignment of Bfr's and Bfd like molecules in selected Gram-negative bacteria with *Pa*.BfrB and *Pa*.Bfd.

The alignment between BfrB's and Bfd's give information on residues which are conserved and gives an idea on residues involved in this two protein interaction which helps in iron release in *Pa*.BfrB. The details of the software's used and an explanation of the findings are given in chapter 3.

It is believed that *Pseudomonas* under iron starvation conditions release siderophores. These small organic molecules, which have a high affinity for Fe^{3+}, sequester iron from surroundings and release it into the cell. This action involves many proteins and helps in the bacteria's growth by converting Fe^{3+} to Fe^{2+}. This process can occur by changing the physiological pH or by releasing reducing agents which can convert non- soluble iron to the soluble state. The type of siderophores released depends on the species and environmental conditions in which it is growing. We conducted a few studies on *Pseudomonas aeruginosa* PAO1 strain along with transposon mutants to study the growth effect in comparison with the amount and type of siderophore released by this strain in aerobic conditions under an iron depleting environment. The iron limiting conditions were created by iron chelating agents to see the release of siderophores in a forced environment with wildtype and transposon mutants. In the presence of iron chelator at different concentrations, which have high affinity for Fe^{3+},in comparison to Fe^{2+} showed some interesting results regarding the growth of wildtype and mutants and also the type and amount of siderophore released along with pyochelin. These results were explained in chapter 4.

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CHAPTER II

Overproduction, purification and characterization of bacterial ferritin A in *Pseudomonas aeruginosa*

Introduction

Ferritins are one of the iron storage proteins which are involved in storage of iron and release of it in iron limiting conditions. Ferritin-like molecules are found in both eukaryotes and prokaryotes (*1*). Eukaryotic ferritins are composed of two similar subunit types, H and L, which assembles into a 24mer structure (*2*). In bacteria two types of ferritin-like molecules are present called as bacterioferritins (Bfr) and ferritins (Ftn) (*3, 1*). Ferritins are composed of 24 identical subunits that arrange into a globular protein forming an inner cavity for storage of iron. Detail structure of ferritin in general is explained in chapter 1.

Former studies suggest that, bacterioferritin isolated from *Pseudomonas aeruginosa* is a heterogenous protein consist of two types of subunits called α and β, whose ratio of occurrence was based on the type of sample (*4, 5*). Later it was stated that, *aeruginosa* contain two genes encoding ferritin-like molecules termed as *bfrA* and *bfrB* (*6*) which encodes two bacterioferritins called as BfrA and BfrB. An amino acid sequence alignment of the proteins comparing *bfrA* gene with other known bacterioferritin amino acid sequences showed absence of Methionine 52 (M^{52}) *(7)* in *bfrA* sequence. As stated earlier in chapter 1, M^{52} is a unique feature of bacterioferritin which is capable of binding heme which helps in electron transport. This observation suggests *bfrA* in *Pseudomonas aeruginosa* is incapable of binding heme which leads to a new hypothesis that *bfrA* is not a bacterioferritin but may be a bacterial ferritin (*7*). This implies that iron storage and management in the cytosol of *Pseudomonas aeruginosa* is performed by two different

proteins (bactertial ferritin and bacterioferritin) but not just a single bacterioferritin composed of two distinct subunits.

Ferritin-like molecule helps in storage of iron as ferric mineral which involves oxidation of Fe^{2+} to $Fe^{3+.}$ For oxidation process it require ferroxidase center which is present in bacterial ferritins and bacterioferritins. Ferroxidase center generally present in center of each subunit (*8*) in all eukaryotic ferritins, bacterial ferritins and bacterioferritins, but still residues of ferroxidase center in all these proteins are not conserved (Figure 1).

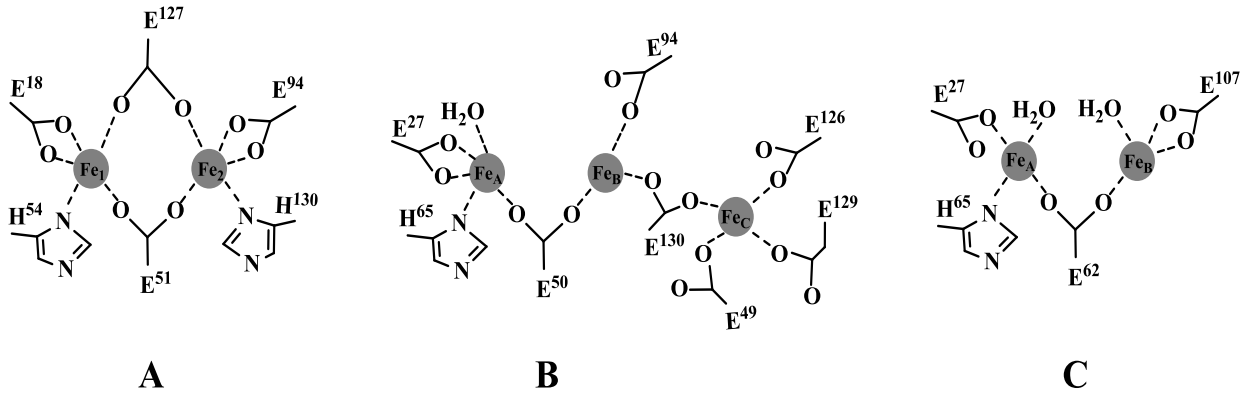

Figure 1: Schematic representation of the (A) symmetrical ferroxidase center typical of bacterioferritins (Bfr) where Fe_1 and Fe_2 are bridged by two Glu residues (numbering as in Pa BfrB), (B) ferroxidase center seen in *E.coli* and archaeal Ftn showing site C (Fe_C) iron in addition to ferroxidase iron Fe_A and Fe_B (numbering as in *Ec* FtnA), and (C) Ferroxidase center of human H-ferritin adapted from crystal structure of its Tb^{3+} derivative (*13*).

According to available genetic information of *P.aeruginosa*, there are two independent ferritin-like molecules function as storage proteins. For bacterioferritins, it was noticed in many organisms including *P.aeruginosa* that Bfr genes are present next to bfd gene (bacterioferritin-

associated ferredoxin). In *Pseudomonas aeruginosa*, under iron starvation conditions *bfrB* gene is down regulated (*9*) and *bfd* gene is up regulated (*10*). Invitro studies on *Pa* BfrB suggest *Pa* bfd acts as a regulator in the release of iron from inner core (*7*). But in case of *Pa* bfrA in *P.aeruginosa*, it was observed that *Pa* bfrA gene is present next to *katA* gene, which encodes a catalase active in all growth phases. Some mutant studies on *Pseudomonas aeruginosa* bfrA gene suggests a decrease in catalase activity of wildtype, while it did not showed any effect in bfrB mutants (*6*), which implies that bfrA and bfrB genes in *Pseudomonas* assembles into independent ferritin-like molecules that play different but independent roles in iron management.

To emphasize our hypothesis we have to successfully develop a method to express (*Pa* "BfrA") bacterial ferritin protein of *Pseudomonas aeruginosa* and purify it to homogeneity for studying structural details.

Materials and method:

Cloning of *P. aeruginosa bfrA*:

The gene encoding *Pa* FtnA (PA4235) was synthesized, subcloned into a pET11a vector, and sequenced (GeneScript Corp., Pisctaway, NJ). The gene was engineered with silent mutations introducing codons favored by *E. coli* (*11*) and with *NdeI and BamHI* restriction sites at the 5' and 3' ends, respectively, for subcloning. The pET11a/*bfrA* construct was transformed into *E. coli* BL21DE3 Gold cells (Stratagene) for protein expression

Expression and purification of *Pa FtnA*:

A single colony of *E. coli* BL21(DE3) competent cells harboring the recombinant pET11-a/*bfrA* construct was cultured overnight at 37 °C in 50 mL of LB medium containing 100 μg/mL

ampicillin. The 50 mL culture was used to inoculate 1 L of fresh LB medium (100 μg/mL ampicillin), which was shake-incubated (200 rpm) until the optical density at 600 nm (OD_{600}) was 0.6. The temperature was then lowered to 30 °C, and the culture was allowed to reach an OD_{600} of 0.8 before protein expression was induced by addition of IPTG (isopropyl 1-thio-D-galactopyranoside) to a final concentration of 1 mM. Cells were cultured for an additional 4 h at 30 °C before they were harvested by centrifugation and stored at -20 °C. Different conditions and steps have been optimized to obtain homogenous and 24 mer form of *Pa* FtnA. In first approach buffer conditions used for cell lysis was 20 mM Tris (pH 7.6), DNase and 1 mM TCEP (1 mM tris (2-carboxyethyl)-phosphine hydrochloride) (Fisher). The resultant supernatant was dialysed against 20 mM sodium phosphate (pH 7.6) and loaded on to hydroxyapatite column equilibrated with same buffer used for dialysis. The resultant fractions with protein are dialysed and loaded on to butyl sepharose column which was equilibrated with 50 mM sodium phosphate (pH 7.0) (Sigma Aldrich) with 0.7 M ammonium sulfate ($(NH_4)_2SO_4$) (Fisher). In second approach included one more chromatography column called phenyl sepharose for purification of protein which is equilibrated with 50 mM sodium phosphate, 0.6 M $(NH_4)_2SO_4$ and 1 mM TCEP (pH 7.0) followed by crystallization by using crystallization conditions in 1:1 ration of protein and crystallization solution (0.1 M sodium cacodylate trihydrate pH 6.5 (Hampton Research) , 5 M NaCl (Fisher), 5.55 M (NH_4)2SO_4 (Fisher)) and loaded dissolved crystals on to FPLC which was equilibrated with 20 mM sodium phosphate, 1 mM TCEP (pH 7.4), obtained impure protein was used to set up crystals for second time as purification step and loaded dissolved crystals on to sephacryl (S-300) size exclusion column [90 cm X 2.5 cm (inside diameter)] which was equilibrated with 20 mM sodium phosphate, 1 mM TCEP (pH 7.4). Next approach was to introduce ammonium sulfate cut method for purification. Different conditions used in this method are, lysis was performed with PBS (phosphate buffered saline) 15 mM KH_2PO_4 (monobasic

potassium phosphate), 8 mM Na_2PO_4 (disodium phosphate), 0.15 mM NaCl, 3.0 mM KCl (potassium chloride), 1 mM TCEP (pH 7.4) along with DNase, protease inhibitor. Resultant supernatant is loaded on to ion exchange column which is equilibrated with 20 mM Sodium phosphate (pH 7.4). Resultant protein was subjected to 20% to 50% ammonium sulfate cut and loaded these fractions on to second ion exchange column for further purification. Fractions with *Pa* FtnA are pooled and concentrated to 18 mg/mL and left at 4 °C overnight which showed crystals but of very less yield. Final approach which was successful in purifying *Pa* FtnA is explained below, Cell paste was resuspended (3 mL/g of cell paste) in 50 mM Tris-Base buffer (pH 7.6) containing 10 mM EDTA, 0.5 mM PMSF, protease inhibitor cocktail (Sigma Aldrich), and DNase (Sigma Aldrich) and lysed using a constant cell disruptor at 20 psi. Cell debris were pelleted by centrifugation at 4 °C and 19500 rpm for 45 min, and the supernatant was loaded onto a Q-Sepharose fast flow column [12 cm X 2.5 cm(inside diameter)] equilibrated with 20 mM Tris-Base and 1 mM EDTA (pH 7.6) at 4°C. The column was washed with 3 bed volumes of the same buffer, and the protein was eluted with a linear gradient (from 0 to 600 mM) of NaCl. Fractions containing *Pa* FtnA were pooled and dialyzed against 4 L of 20 mM Tris-HCl and 1 mM EDTA (pH 7.6) at 4 °C, and the resultant solution was loaded onto a second Q-Sepharose fast flow column [12 cm X 2.5 cm (inside diameter)] and eluted as described above. Fractions containing *Pa* FtnA were pooled, and NaCl was added to a final concentration of ~500 mM, to prevent protein precipitation during concentration by ultrafiltration (Ultracel 50K; Millipore). The concentrated solution (~4 mL) was loaded onto a Sephacryl S-300 (GE Healthcare) size exclusion column [90 cm X 2.5 cm (inside diameter)] equilibrated with 50 mM Tris-HCl, 200 mM NaCl, and 1 mM EDTA (pH 7.6) at 4 °C. Fractions containing *Pa* FtnA were pooled, concentrated, and then loaded a second time onto the Sephacryl S-300 column, which typically resulted in homogeneous protein, as judged by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) (15%). In some preparations, it was necessary to pass the sample a third time through the Sephacryl S-300 column to obtain pure *Pa* FtnA.

Determination of molecular mass of *Pa* FtnA:

The molecular mass of a *Pa* FtnA subunit was obtained by mass spectrometry using an ESI-MS Q-TOF mass spectrometer (Micromass Ltd., Manchester, U.K.). To this end, a 5 μM sample of *Pa* FtnA in 50 mM phosphate bufer (pH 7.4) was loaded onto a 0.5 mm inside diameter C4 reverse phase column (MC-5-C4, 300 Å pore size, Micro Tech). Elution was conducted by running an acetonitrile/isopropyl alcohol/water linear gradient of 4% acetonitrile/min at a rate of 10 μL/min from 20 to 60% acetonitrile (bufer A consisted of 99% water, 1% acetonitrile, and 0.08% formic acid; bufer B consisted of 80% acetonitrile, 10% isopropyl alcohol, 10% water, and 0.06% formic acid).

The molecular mass of 24-mer *Pa* FtnA was estimated by FPLC (AKTA, Amersham Pharmacia Biotech) using a size exclusion column [Superdex 200 Prep, 60 cm X 16 cm (inside diameter) (GE Healthcare)], equilibrated with 50 mM sodium phosphate (pH 7.4), 150 mM NaCl, and 1 mM TCEP. The column was calibrated with a set of molecular mass standards (GE Healthcare) that included ferritin (440000 Da), aldolase (158000 Da), conalbumin (75000 Da), and carbonic anhydrase (29000 Da).

Mineralization of *Pa* FtnA:

Recombinant *Pa* FtnA, as isolated, contains a small amount of iron in its core, ~10-15 iron ions per *Pa* FtnA 24-mer. To load *Pa* FtnA with iron, we prepared a solution of ferrous ammonium sulfate in a glovebox (Anaerobic environment) (Coy Laboratories), placed the solution in a container capped with a rubber septum, and removed the container from the anaerobic chamber. Concentrated

HCl was added to the ferrous ammonium sulfate solution (50 μL/100 mL) through a septum using a Hamilton microsyringe needle; the resultant solution was added to a stirred solution of 0.2 μM *Pa* FtnA in 50 mM Tris (pH 7.4) at ambient temperature. Aliquots, which delivered approximately 10% of the total iron necessary to load each *Pa* FtnA molecule with ~500 iron ions, were added approximately 15 min apart. The content of iron in the core was determined using a previously reported method (*7*, *12*). A similar procedure (*7*) was used to load *Pa* BfrB with ~550 iron ions. The number of iron atoms was calculated by using iron ferrozine assay in which 50 μL of the protein was added to 50 μL HCl and wait for 15 min so that it denatures the protein and releases iron Then added 50 μL of ascorbic acid (0.025 M) , 250 μL of saturated sodium acetate and 100 μL of Ferrozine (0.005 M) solution and left for 15 min for iron to react, formation of purple color indicates presence of iron-ferrozine complex which gives Uv-vis spectra at 562 nm (Figure 3A)) with extinction coefficient of 27.9 mM-1 cm-1 (13).

Iron release from central core in *Pa* FtnA and *Pa* BfrB:

To study the release of iron stored in *Pa* FtnA and *Pa* BfrB, the proteins were mineralized with ~500 iron ions and placed in an anaerobic glovebox (*7*). Experiments to investigate the release of iron stored in *Pa* FtnA and *Pa* BfrB were conducted in the same anaerobic chamber, in a manner analogous (*7*) that reported to previously for the release of iron from *Pa* BfrB. Reactions were conducted in a 1.0 cm path length cuvette equipped with a magnetic stirring bar and containing a 3 mM solution of 2,2'- bipyridyl (bipy) in 20 mM potassium phosphate (pH 7.6). For the study of *Pa* FtnA, a few microliters from a stock solution of *Pa* FtnA and *Pa* Fpr were added to the cuvette to make the solution 0.25 μM in *Pa* FtnA and 10 μM in *Pa* Fpr. The reaction was initiated by addition of excess NADPH to a final concentration of 1.5 mM and the progress monitored by following the time-dependent changes in the intensity of the band at 523 nm upon formation of the $[Fe(bipy)_3)]^{2+}$

complex. A similar procedure was used to study the release of iron from *Pa* BfrB, except that the cuvette also contained apo *Pa* Bfd, at concentrations described in the caption of Figure 7. Apo *Pa* Bfd was prepared in situ from holo *Pa* Bfd, using a procedure described previously (*7*). In short, holo *Pa* Bfd was added to a stirred cuvette containing 3 mM bipy in 20 mM potassium phosphate (pH 7.6). Sodium dithionite (5 mM) was added to stoichiometrically reduce the Fe^{3+} in holo *Pa* Bfd to Fe^{2+}. Capturing of the ferrous ion by bipy was monitored by the time-dependent increase in the 523 nm absorbance, which was followed until it reached a plateau with intensity corresponding to the theoretical value calculated from the amount of holo *Pa* Bfd placed in the cuvette. At this point, the solution containing apo *Pa* Bfd was reconstituted with *Pa* Fpr and *Pa* BfrB and the iron mobilization reaction initiated by the addition of NADPH, as described above. Experiments involving apo *Pa* Bfd and *Pa* FtnA were conducted in an analogous manner. Please note that throughout these studies, Bfd stands for the C43S mutant of Bfd, which has previously been shown to behave like wild-type Bfd but can be expressed (*7*) higher yield and is in more stable to storage and manipulation.

Crystallization and Data Collection:

Pa FtnA was concentrated to 8.0 mg/mL in 100 mM Tris buffer (pH 7.6) containing 200 mM NaCl and 1 mM EDTA for crystallization. Crystals were grown under aerobic conditions in Compact Jr. sitting-drop vapor diffusion plates (Emerald Biosystems) using 0.5 μL of protein and 0.5 μL of crystallization solution equilibrated against 100 μL of the latter. Crystals were obtained from three different conditions at 20 °C. (a) pH 6.0 crystals were obtained from Wizard2 #2 (Emerald Biosystems) 35% (v/v) 2-methyl-2,4 pentanediol, 100 mM MES (pH 6.0), and 200 mM Li_2SO_4 (Appendix 1),. Crystals were transferred to a fresh drop of crystallization solution, which served as a cryoprotectant prior to the crystals being frozen in liquid nitrogen for data acquisition. (b) pH 7.5

crystals were obtained from Wizard 2 #5 (Emerald Biosystems), 20% (v/v) 1,4-butanediol, 100 mM HEPES (pH 7.5), and 200 mM NaCl (Appendix 1). Crystals were transferred to a solution containing 80% crystallization solution and 20% glycerol for approximately 30 s before being frozen in liquid nitrogen for data collection. (c) pH 10.5 crystals were obtained from Wizard 2 #39 (Emerald Biosystems), 20% (w/v) PEG-8000, 100 mM CAPS (pH 10.5), and 200 mM NaCl (Appendix 1). Crystals were transferred to a solution containing 80% crystallization solution and 20% PEG400 for approximately 30 s before being frozen in liquid nitrogen for data collection. To prepare samples with iron bound in the ferroxidase center, crystals obtained at each of the three pH values were soaked aerobically for 15 min in their respective crystallization solution containing 50 mM $FeCl_2$. Doubly soaked crystals were prepared by aerobically soaking crystals in 50 mM $FeCl_2$ for 15 min, followed by aerobically soaking them for 30 min in the same crystallization solution without $FeCl_2$. Single crystals at each of the pH values were transferred to the corresponding cryoprotection solution described above before being frozen in liquid nitrogen for data collection. X-ray diffraction data were collected at 100 K at the Advanced Photon Source (APS) IMCA- CAT, sector 17-BM, using an ADSC Quantum 210 CCD detector for apo pH 10.5 crystals at a λ of 1.0000 Å. Diffraction data for apo (pH 7.5) and Fe-soaked (pH 7.5) crystals were collected using a Mar 165 CCD detector at wavelengths (λ) of 1.0000 and 1.6531 Å. Data at 1.6531 Å were collected to obtain an anomalous signal from potentially bound Fe ions. Although the X-ray fluorescence K-edge emission peak of Fe has a maximum at approximately 1.74 Å, at 1.6314 Å prominent anomalous signals can be observed for Fe ions. Data for pH 6.0 and doubly soaked pH 7.5 *Pa* FtnA crystals were collected at APS sector 17-ID using a Pilatus 6M pixel array detector at a λ of 1.7401 Å, which was at the Fe absorption edge.

Results

Optimizing conditions to purify *Pa* FtnA

In the first approach, used similar procedure as described earlier to purify *Pa* BfrB *(7)* except included one more hydrophobic column (butyl sepharose) for purification, which never produced any pure protein (Figure 2A). In second approach we decided to use butyl and phenyl sepharose column based on results from previous attempt which showed better removal of higher molecular weight impurities with butyl sepharose column and no purification was observed with hydroxyapatite. As obtained protein with these columns was still impure, crystals were grown under three different concentrations of protein such as 8 mg/mL, 4 mg/mL and 6.6 mg/mL, which failed to produce crystals but instead, showed formation of white cloudy precipitate which on centrifuged separated precipitate and supernatant, supernatant showed homogenous protein band with 8 mg/mL concentration but the yield was very low. The results with other two concentrations are not satisfactory as impurities were present in both supernatant and pellet. Confirmation of purity was performed using SDS-PAGE (Figure 2B).

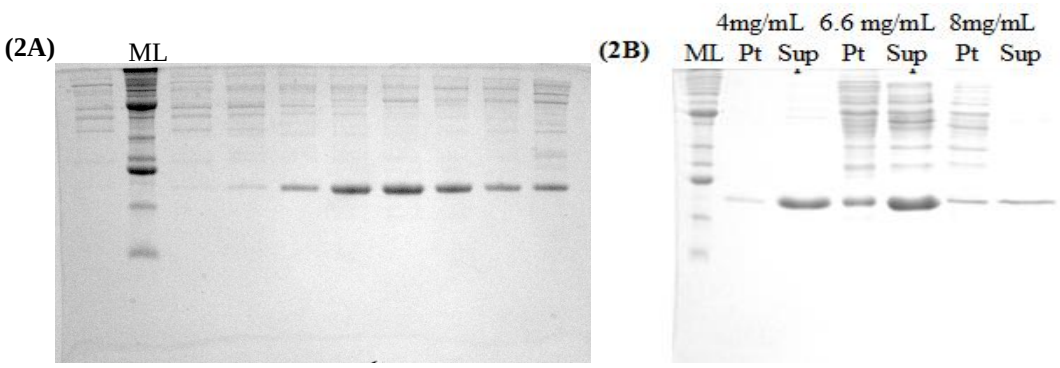

Figure 2: (A) 15% SDS-PAGE of 1st approach showing fractions with impurities after from butyl sepharose column. (B) 15% SDS-PAGE of second approach showing results of crystals at different concentrations. 4 mg/mL showed very less pellet formation and showed impurities in supernatant and 6.6 mg/mL supernatant (Sup) and pellet (Pt) both show high molecular weight impurities, whereas at 8 mg/mL impurities are present only in supernatant and precipitate is pure. 'ML' in the figure indicate molecular ladder.

As these ferritin proteins are known to be stable in phosphate buffer, PBS buffer was used for lysis along with TCEP as a reducing agent (to prevent formation of disulfide bonds during lysis as *Pa* FtnA has cysteine in each subunit). Protein after ion exchange column was subjected to 20% to 50% ammonium sulfate cut whose results showed presence of impurities in bot supernatant and pellet (Figure 3A). Protein sample after ion exchange was heated to 50 °C as *Pa* FtnA is stable at this temperature but most of the impurities are not, so they tend to precipitate leaving *Pa* FtnA in supernatant and pure, the resultant supernatant was loaded on to Q-sepharose column and the fractions were concentrated to 18 mg/mL and kept at 4 °C for crystallization which was successful but 15% SDS-PAGE results showed presence of 18 kDa band along with another small band at ~ 15 KDa (Figure 3B), which indicates denaturing of protein during process of purification. Repeated same procedure without heating protein and used two Q-sepharose column along with size exclusion column (Sephacryl 300) for purification, still saw presence of two bands, which indicates PBS buffer is not suitable for lysis of *Pa* FtnA, because presence of a small band at 15 KDa is observed even in 1st Q-sepharose column.

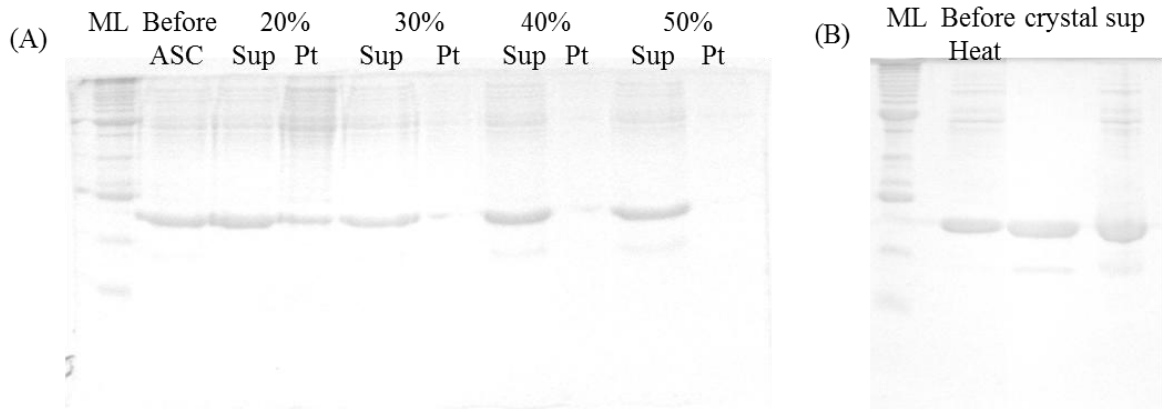

Figure 3: (A) Results of ammonium sulfate cut (15% SDS-PAGE) showing presence of impuritiesin both pellet and supernatant (B) Results of heating protein at 50 °C (15% SDS-PAGE) shows crystal obtained are from pure protein but contain both 18 KDa and 15 KDa bands and supernatant (Sup) with impurities and protein band.

FtnA expressed in *E.coli* BL21DE3 cells were able to purify to homogeneity with the procedure mentioned in materials and methods and the results were confirmed by obtaining ~ 18 KDa single band in SDS-PAGE gel (Figure 4A). This sample has to be loaded on to FPLC for separating dimers and 24mers (Figure 4B). The molecular weight of 24mer was further confirmed by ESI-MS (electronspray ionization mass spectrometry) which gave a molecular weight of 17939 Da which is very close to calculated value from the amino acid sequence. Further the 24 mer formation and molecular weight of the protein is confirmed by size exclusion column calibrated with known standards along with eukaryotic ferritin standard from GE healthcare gave an elution volume similar to elution volume of standard ferritin giving the molecular mass of 436.3 KDa which is in good agreement with calculated value of 430.6 KDa obtained from amino acid sequence (Figure 4B). These results conclude the purification of *Pa* FtnA as 24mer.

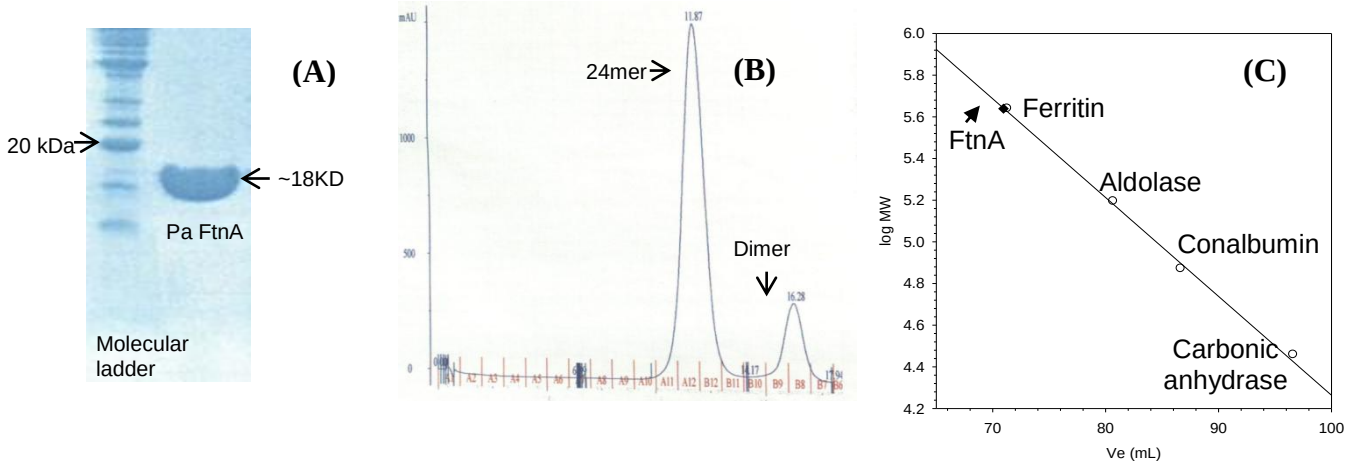

Figure 4: (A) 15% SDS-PAGE gel showing purified *Pa* FtnA by single band. (B) Calibration curve to determine molecular weight of purified *Pa* FtnA by comparing to elution volumes of standards such as ferritn (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), and carbonic anhydrase (29 kDa).

Mineralization of central core in *Pa* FtnA

Mineralization was performed as stated earlier, and where successfully able to mineralize FtnA

with ~520 (20 iron atoms per molecule). The analysis of iron content was performed using iron-ferrozine assay (Figure 5A). The spectra obtained during process of mineralization showed absorption spectra only at ~280 nm without any peak at 415 nm as in *Pa* BfrB. The increase in spectra at absorption 280 nm for every addition of ~10% of the total iron prepared anaerobically indicates Fe^{2+} is oxidized to Fe^{3+} and transferred to central core efficiently in *Pa* FtnA (Figure 5B).

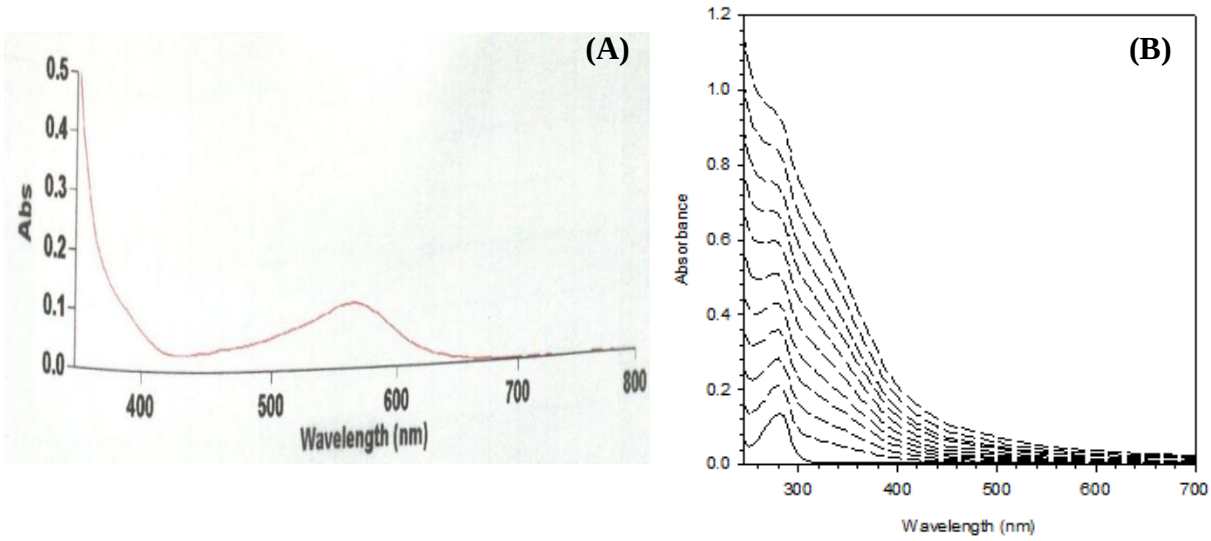

Figure 5: (A) UV –Vis absorption spectra showing Fe-ferrozine complex (Abs 562 nm). (B) Spectra showing mineralization of iron core in *Pa* FtnA. Spectrum of solid like is obtained before adding Fe^{2+}, and spectra of dashed lines obtained after every addition of 50 iron atoms at an interval of 15 min.

Release of Iron from central core in anaerobic conditions

Experiments conducted to release iron from inner core of *Pa* FtnA were performed in anaerobic chamber. Mineralized FtnA with ~500 iron ions was used for this study. Release of iron was initiated by adding NADPH to a cuvette which contained mineralized *Pa* FtnA and bipy. The time dependent formation of $[Fe(bipy)_3]^{2+}$ was monitored by increasing growth of absorption spectra at 523 nm. The

normalized results for this experiment are showed in (Figure 6A). The solid triangle in the graph are result of *Pa* FtnA (0.25 µM) and NADPH without presence of Fpr suggest no release of iron, whereas empty dots in the graph are the result of addition of NADPH to a solution containing *Pa* FtnA and Fpr, the increase at absorbance 523 nm with time suggests release of iron gradually and reached plateau. These results are completely different from results of *Pa* BfrB iron release experiments which showed Bfd was also required for electron transport to release iron (*7*), the solid dots are result of reconstitution of *Pa* FtnA with *Pa* Fpr and *Pa* Bfd which did not provided any different results than *Pa* FtnA without *Pa* Bfd. This observation of iron release in *Pa* FtnA is different than iron release mechanism observed previously in *Pa* BfrB, which shows dependence on *Pa* Bfd for release of iron from the inner core. To compare the results of *Pa* FtnA with *Pa* BfrB, experiments performed on *Pa* BfrB in similar conditions as *Pa* FtnA with different ratios of *Pa* bfd shows in the absence of *Pa* Bfd *Pseudomonas aeruginosa* bacterioferritin showed slow release of iron whereas in presence of *Pa* Bfd the rate of iron release however increase. Trend of accelerated release of iron is noticed with different ratios of *Pa* BfrB and *Pa* Bfd. The results are shown in (Figure 6B).

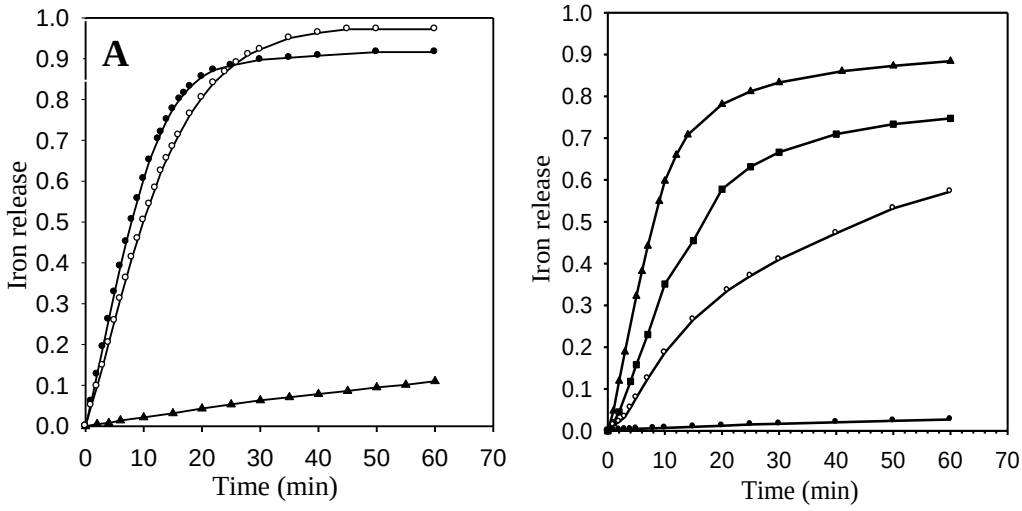

Figure 6: (A) Time dependent release of iron from *Pa* FtnA upon addition of NADPH (final concentration of 1.5 mM) to 20 mM phosphate buffer (pH 7.6) in different conditions. Graph

with solid triangles (▲) contain only *Pa* FtnA (0.25 μM), open circle (○) contain *Pa* FtnA (0.25 μM) and *Pa* Fpr (10 μM), and solid sphere (●) contain *Pa* FtnA (0.25 μM), *Pa* Fpr (10 μM) and apo *Pa* Bfd (10 μM); the apo Bfd/FtnA ratio was 40) **(B)** Iron release in Pa BfrB upon addition of NADPH (final concentration of 1.5mM) to a solution containing Pa BfrB (0.37 μM), Pa Fpr (15 μM), and apo Pa bfd with different molar ratios of Bfd:BfrB of 0 (●), 5 (○), 15 (■), and 40 (▲)

Crystallization

Crystals are obtained at different pH conditions such as pH 6.0, 7.5, and 10.5. Crystals obtained from this conditions and pH were diffracted for its structures. These obtained crystals were devoid of iron hence termed as as-isolated *Pa* FtnA. These crystals when once got structures were soaked in crystallization solution with dissolved $FeCl_2$ (Fe-soaked *Pa* FtnA) to freeze the action of ferroxidase center and were again soaked in crystallization solution to remove iron form the protein (doubly soaked *Pa* FtnA).

Discussion

Bacteria in general contain two types of storage proteins known as bacterial ferritin and bacterioferritin. As mentioned earlier Pseudomonas aeruginosa was thought to have two different bacterioferritin subunits. Earlier studies in our group showed bacterioferrtin (Pa BfrB) can be expressed separately and experiments showed proper function of that protein. In the process of optimizing purification conditions for Pa FtnA noticed few interesting differences in protocol for bacterioferritin and bacterial ferritin. The hydrophobic columns like butyl sepharose, phenyl sepharose and hydroxyapatite failed to remove any type of impurities. PBS (Phosphate buffered saline) was denaturing protein during lysis even in presence of TCEP and EDTA, which was overcome by changing the lysis buffer to Tris-HCl.

Addition of EDTA in Tris-HCl prevented appearance of band at 15 KDa which shows a chelating agent is necessary when cells are breaking in case of Pa FtnA than a reducing agent like TCEP. One more interesting observation was phosphate buffers are not actually suitable for purifying *Pa* FtnA, as protein was not stable in this buffer and shows precipitation during concentration and denatures it. Precipitation was even seen with Tris-HCl buffer but was controlled by adding NaCl at higher concentrations. With these observations a new protocol was designed successfully for purifying *Pa* FtnA but always observed formation of both dimer and 24 mer which were separated by sizing as final step for purification and separation.

Studies conducted on *bfrα* subunits suggests the product obtained from it can form 24mer a globular protein which is colorless indicating absence of heme. The crystals obtained from different conditions were also colorless and pyramidal in shape. The ferroxidase center was active and showed presence of diiron site along with active third iron site, which is in resemblance with site C in ferritin.

Experimental observation showed that proteins involved in release of iron in *Pa* ftnA are different than *Pa* BfrB. It was found that *Pa* BfrB releases iron efficiently in presence of *Pa* Bfd and *Pa* Fpr whereas in absence of *Pa* bfd results showed very slow release of iron. In contrast, *Pa* FtnA was able to release iron efficiently in presence of only *Pa* Fpr without any involvement of *Pa* Bfd in electron transfer. These observations suggests that *Pa* Fpr mediates the flow of electrons from NADPH to heme which in turn, releases electron to the mineral in *Pa* BfrB which reduces Fe^{3+} to Fe^{2+} and releases form inner core. In *Pa* FtnA only NADPH and *Pa* Fpr was enough to successfully release iron form inner core without presence of *Pa* Bfd. Experiments conducted in presence of *Pa* Bfd did not showed any appreciable difference. The above observations are in contrast with previous results obtained by *Pa* BfrB *(7)* suggests, *Pa* FtnA and *Pa* BfrB are two different proteins in *Pseudomonas aeruginosa* which helps in storage of iron.

Conclusion:

Successfully optimized conditions and developed a protocol to purify *Pa* FtnA to homogeneity. The crystals obtained from this homogenous protein were colorless indicating absence of heme. Important observation made by these crystals was presence of third iron site in the ferroxidase center which resembles site C in ferritins. Experiments showed *Pa* FtnA require different regulatory proteins for iron release from inner core when compared to *Pa* BfrB. These observations confirmed that *Pseudomonas aeruginosa* contain both bacterioferritin (*Pa* BfrB) and bacterial ferritin (*Pa* FtnA) as iron storage proteins instead of two distinct bacterioferritin subunits.

Appendix I: Protein Crystallization Conditions-Wizard 11 random sparse matrix Crystallization Conditions.

1 10% (w/v) PEG-3000 acetate pH 4.5 Zn(OAc)2

2 35% (v/v) 2-methyl-2,4-pentanediol MES pH 6.0 Li2SO4

3 20% (w/v) PEG-8000 Tris pH 8.5 MgCl2

4 2.0 M (NH4)2SO4 cacodylate pH 6.5 NaCl

5 20% (v/v) 1,4-butanediol HEPES pH 7.5 NaCl

6 10% (v/v) 2-propanol phosphate-citrate pH 4.2 Li2SO4

7 30% (w/v) PEG-3000 Tris pH 7.0 NaCl

8 10% (w/v) PEG-8000 Na/K phosphate pH 6.2 NaCl

9 2.0 M (NH4)2SO4 phosphate-citrate pH 4.2 none

10 1.0 M (NH4)2HPO4 Tris pH 8.5 none

11 10% (v/v) 2-propanol cacodylate pH 6.5 Zn(OAc)2

12 30% (v/v) PEG-400 cacodylate pH 6.5 Li2SO4

13 15% (v/v) ethanol citrate pH 5.5 Li2SO4

14 20% (w/v) PEG-1000 Na/K phosphate pH 6.2 NaCl

15 1.26 M (NH4)2SO4 HEPES pH 7.5 none

16 1.0 M sodium citrate CHES pH 9.5 none

17 2.5 M NaCl Tris pH 7.0 MgCl2

18 20% (w/v) PEG-3000 Tris pH 7.0 Ca(OAc)2

19 1.6 M NaH2PO4/0.4 M K2HPO4 phosphate-citrate pH 4.2 none

20 15% (v/v) ethanol MES pH 6.0 Zn(OAc)2

21 35% (v/v) 2-methyl-2,4-pentanediol acetate pH 4.5 none

22 10% (v/v) 2-propanol imidazole pH 8.0 none

23 15% (v/v) ethanol HEPES pH 7.5 MgCl2

24 30% (w/v) PEG-8000 imidazole pH 8.0 NaCl

25 35% (v/v) 2-methyl-2,4-pentanediol HEPES pH 7.5 NaCl

26 30% (v/v) PEG-400 CHES pH 9.5 none

27 10% (w/v) PEG-3000 cacodylate pH 6.5 MgCl2

28 20% (w/v) PEG-8000 MES pH 6.0 Ca(OAc)2

29 1.26 M (NH4)2SO4 CHES pH 9.5 NaCl

30 20% (v/v) 1,4-butanediol imidazole pH 8.0 Zn(OAc)2

31 1.0 M sodium citrate Tris pH 7.0 NaCl

32 20% (w/v) PEG-1000 Tris pH 8.5 none

33 1.0 M (NH4)2HPO4 citrate pH 5.5 NaCl

34 10% (w/v) PEG-8000 imidazole pH 8.0 none

35 0.8 M NaH2PO4/1.2 M K2HPO4 acetate pH 4.5 none

36 10% (w/v) PEG-3000 phosphate-citrate pH 4.2 NaCl

37 1.0 M K/Na tartrate Tris pH 7.0 Li2SO4

38 2.5 M NaCl acetate pH 4.5 Li2SO4

39 20% (w/v) PEG-8000 CAPS pH 10.5 NaCl

40 20% (w/v) PEG-3000 imidazole pH 8.0 Zn(OAc)2

41 2.0 M (NH4)2SO4 Tris pH 7.0 Li2SO4

42 30% (v/v) PEG-400 HEPES pH 7.5 NaCl

43 10% (w/v) PEG-8000 Tris pH 7.0 MgCl2

44 20% (w/v) PEG-1000 cacodylate pH 6.5 MgCl2

45 1.26 M (NH4)2SO4 MES pH 6.0 none

46 1.0 M (NH4)2HPO4 imidazole pH 8.0 NaCl

47 2.5 M NaCl imidazole pH 8.0 Zn(OAc)2

48 1.0 M K/Na tartrate MES pH 6.0 none

All formulations are made with ultrapure ASTM Type I water and sterile-filtered stock solutions. Store at 4-25 ºC.

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CHAPTER III

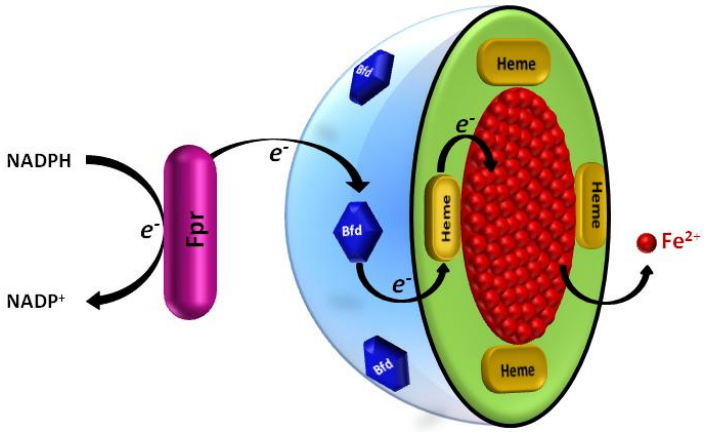
In search of BfrB and Bfd-like molecules in Gram-negative pathogens

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium which is responsible for infections in immune compromised people, cystic fibrosis patients and burn victims *(1)*. Structural and functional studies of proteins involved in the survival of this organism give an insight on its mechanism of pathogenicity and virulence. Hence identification of these functionally significant proteins is important. Prior studies in our group suggest protein-protein interactions (PPI) as one of the modes of action for its survival *(2)*. Hence, knowing the necessary amino acid residues responsible for its interactions helps in developing methods to prevent these interactions as a possible means to control the growth of organisms. It also helps in designing inhibitors to prevent PPI and also helps in drug designing once we understand its molecular structure and mechanism of action.

Bacterioferritin (BfrB) in *Pseudomonas* is known to play a major role in iron storage and iron release to maintain homeostasis *(2)*. Proposed mechanism of iron release in *Pseudomonas* involve *Pa* Bfd and *Pa* Fpr. Addition of NADPH, activates *Pa* Fpr protein as it is NADPH dependent. The electrons from *Pa* Fpr are mediated by *Pa* Bfd and transferred to the heme moiety; electrons from heme help in reducing Fe^{3+} in the inner core to Fe^{2+} and release Fe^{2+} *(2)* (scheme 1). In iron starvation conditions *bfrB* gene is down regulated, whereas *bfd* gene is upregulated (250 fold) and *fpr* gene is upregulated (3 fold) *(3)*. This observation suggests that under iron deficient conditions in *Pseudomonas aeruginosa* it triggers a response to activate *Pa* Bfd and *Pa* Fpr. Therefore production of these proteins which bind to storage proteins such as

Pa BfrB and transfer electrons to release iron in the form of Fe^{2+} for its survival. Former studies suggested role of Bfd and Fpr in iron release from BfrB *(2)* but did not shed light on the residues involved in the protein-protein interactions. Hence understanding these interactions is important to study means for inhibiting them and prevent the growth of *Pseudomonas aeruginosa*. In order to achieve our goal, first, we should know important residues which are involved in structure and function of these proteins in *Pseudomonas aeruginosa*.



Scheme 1: Showing pathways of electron transfer to inner core and release of Fe^{2+}.

Important known structural details of *Pa* BfrB and *Pa* Bfd:

***Pa* BfrB:**

Presence of Met 52 binds heme which helps in electron transfer for release of iron *(2).* This is known to be a characteristic feature of bacterioferritins. Ferroxidase center helps in iron uptake whereas four fold pores, three fold pores and B pores are known to be one of the conduits for iron release, thus knowing these residues is also important. As known iron is stored in the form of Fe^{3+} in iron storage proteins, which indicates that an oxidation step to convert Fe^{2+} to Fe^{3+} is crucial for these proteins. Consequently, knowing residues which form this ferroxidase

center and their location is important. Residues which are present at ferroxidase center are E18, E51, E94, E127, H54 and H130 *(3).* Structural studies on *Pa* BfrB shows three fold pores are made up of positive and negatively charged residues arranged alternatively in layers. Residues involved in forming three fold pores are three V106, and three H112 in first layer, in second layer three E109 and three R117, and the third narrow path is made up of three N118 and three N122. Depending on the observation made it is believed that iron can move in and out of these four fold pores in bacterioferritin. The residues are arranged in the form of layers even in four fold pores, they are, top layer consist of four E147, central layer in this pore is narrow and consist of four N148 and four Q151 and the innermost layer consist of S152, H155 and R39. B pores are other form of opening which is formed at the intersection of three subunits. The residues that line B-pores are D132 and T136 and D34 from different subunit *(4).*

Bfd:

Pa Bfd is ferredoxin protein containing 73 amino acid residues which contain four conserved cysteine residues arranged as **C**-X-**C**-X31-34-**C**-X2-**C** *(5)* in a unique manner which is known to acts as ligand in 2Fe – 2S cluster formation. It is believed that these cysteines may somehow help in electron transfer to release iron from inner core. The cysteines which form 2Fe – 2S cluster are C4, C6, C38 and C41.

The information obtained by structural details of *Pa* BrB and *Pa* Bfd is used to compare for similar type of possible interactions to be expected from similar proteins in other pathogenic organisms. If most of the known pathogenic organisms possess similar type of PPI as in *Pseudomonas aeruginosa,* than inhibitors developed for the interaction of *Pseudomonas* proteins should also be able to act on other pathogenic bacteria. Organisms can have similar type of

interactions only if they have comparatively similar type of amino acid residues which dictates its structure and functions. Different methods were used to study and understand amino acid similarity of *Pseudomonas aeruginosa* with other known pathogenic organisms.

The goal of this study is to probe whether other pathogenic organisms also have similar type of BfrB and Bfd interactions. If we observe similarities then we can extend our understanding of *Pa* BfrB and *Pa* Bfd PPI interactions to these pathogenic organisms.

One way to identify conserved residues is to perform sequence alignments. Some of the programs and substitution matrices used for sequence alignments in this study are BLAST (Basic Local Alignment Search Tool) (*6*), CLUSTAL W (*7, 8*), EMBOSS supermatcher (the European Molecular Biology Open Software Suite) and BLOSUM (Block Substitution Matrix)) *(9, 10).*

Methods:

Performing blast for similar protein sequence

Required sequences of BfrB (bacterioferritin) and Bfd (bacterioferritin associated ferredoxin) of *Pseudomonas aeruginosa* were found from *Pseudomonas aeruginosa* genome database. The appropriate sequence was submitted in FASTA format in the box provided for entering query sequence in the NCBI BLAST (*National Center for Biotechnology Information*-Basic Local Alignment Search Tool) tool, default settings were chosen and the algorithm selected to perform the search was blastp (protein-protein BLAST). Clicking BLAST button performed protein search in the whole database. Sequences were selected based on percentage similarity and pathogenesis of the corresponding organism, especially organisms which fall under ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella* species,

Acinetobacter baumannii, *Pseudomonas aeruginosa, and Enterobacter* species) category and other pathogenic organisms were selected.

JCVI-CMR and KEGG: To determine functional relationship between proteins

To study relationships between two proteins of *Pseudomonas aeruginosa,* BfrB and Bfd, we wanted to confirm the position of these two genes in different organisms, which was carried out using JCVI-CMR (J Craig Venter Institute - Comprehensive Microbial Resource) and KEGG (Kyoto Encyclopedia of Genes and Genomes) sites which are available for free.

To search for sequence of a particular protein in J Craig Venter Institute - Comprehensive Microbial Resource *(11),* a locus ID (Example: locus ID for *Pa* BfrB is PA3531) for that particular protein is entered into the space provided to obtain information of that protein. From the given options select "region view" to see the position of the genes and their transcription directions which is denoted by arrows. For the amino acid sequence select another option on the home page called "JCVI sequence".

Information for few other organisms is found in KEGG (Kyoto Encyclopedia of Genes and Genomes) database *(12).* Opening the KEGG link and entering the organism name or directly search for organism name in Google with KEGG extension will open KEGG database with details of that particular organism. The text box with the name of the desired protein opens a new window with all the information like locus ID (entry), gene function, length, molecular weight etc., clicked on option called genome map to obtain the information on location, orientation (direction) and position (adjacent or not adjacent to each other).

Improving sensitivity of protein alignment (ClustalW)

ClustalW is one of the oldest software used to perform protein alignment of large sequences successfully. Here, for our study we used ExPASy website which is a SIB Bioinformatics Resource Portal which gives access to many scientific databases and software tools. Under heading called proteomic tools and search for subheading called sequence alignment and selected option called multiple, where we found ClustalW link at PBIL. Enter query sequence along with appropriate sequence of all other organisms in FASTA format and used default settings except for output order select option called input so that all the sequences are aligned against query sequence. It's always important to enter query sequence as first sequence followed by other known sequence. When Clustal W performs all its steps, it provides results with aligned sequence.

Calculating identity and similarity percentage

To determine identity and similarity of the query sequence in comparison to multiple set of sequences, we used a program called EMBOSS (European Molecular Biology Open Software Suite) 6.3.1 supermatcher. This program was suitable to compare multiple sequences at a time and good for analyzing large sequences. It can accept any type of input files but we choose FASTA format *(13)*. Under the input section for ' asequence' option we entered query sequence or sequence of interest and under ' bsequence' entered all other sequences whose functions are known. We adjusted a few more parameters before performing the run, such as the Matrix file to Eblossum62, as we don't know how far apart these sequences are in the evolutionary chain so it's always better to choose blosum62 instead of blosum40 or blosum80 *(11)*; we chose Gap opening penalty of 10 and gap extension penalty of 2 for all sets to get proper value for identity and similarity, because if gap open penalty or gap extension penalty is zero than we will get a value which is higher than expected. Alignment width and Word length

for initial matching is left as default *(9)*. The alignment output format is chosen as "simple" and gave a proper name for alignment and clicked on run to get output file.

Results

Genome analysis:

Genome analysis of *Pseudomonas aeruginosa* revealed presence of *bfrB* gene adjacent to *bfd* gene indicated by forward arrows showing similar transcription direction. Interesting observation was the presence of these two genes adjacent to each other in the entire selected pathogenic Gram negative bacteria, suggesting the evolutionary relatedness of these two proteins (Figure 1). The *bfrB* gene and bfd gene are given an ID called locus ID which indicates its position in the genome. A table is generated with locus ID's to show presence of *bfrB* and *bfd* gene adjacent to each other in almost all pathogenic organisms (Table 1).

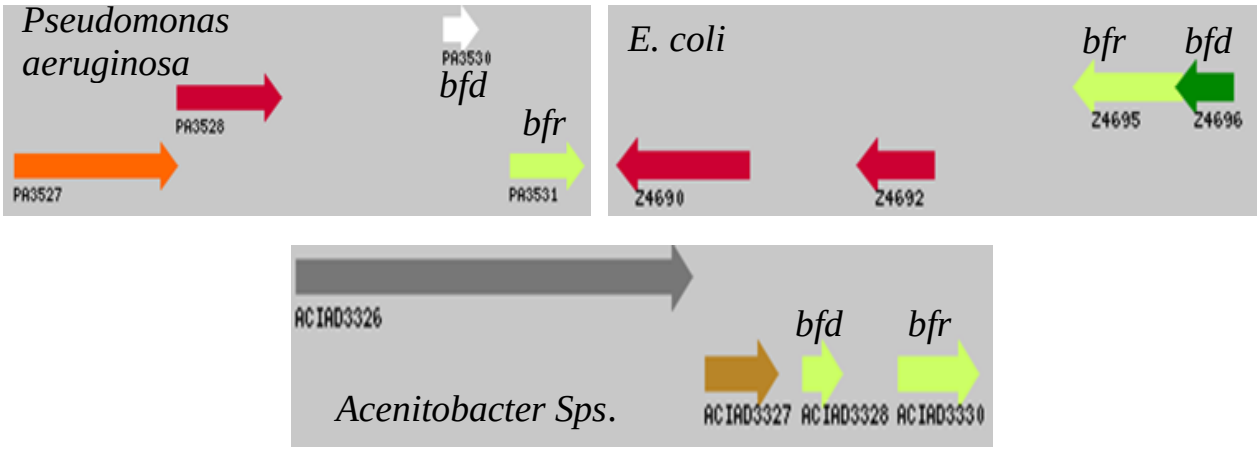


Figure 1: *bfr* and *bfd* genes are adjacent to each other in *Pseudomonas aeruginosa, E.coli* and *Acenitobacter sps*.ADP1 and arrow showing transcription direction

Table 1: Showing Locus ID of *bfr* and *bfd* genes in different organisms

S. No.	Microorganism	BfrB	Bfd
1	*Pseudomonas aeruginosa PAO1*	PA3531	PA3530
2	*Escherichia coli O157:H7 EDL933*	Z4695	Z4696
3	*Escherichia coli K12-*	b3336	b3337
4	*Salmonella enterica serovar Typhi Ty2*	t4062	t4061
5	*Yersinia pseudotuberculosis IP32953*	YPTB3700	YPTB3701
6	*Vibrio cholerae El Tor N16961*	VC_0365	VC_0364
7	*Shigella flexneri 2a 2457T*	S4407	S4406
8	*Yersinia enterocolitica subsp. enterocolitica 8081*	YE3925	YE3926
9	*Erwinia carotovora atroseptica SCRI1043*	ECA4033	ECA4034
10	*Citrobacter koseri ATCC BAA-895*	CKO_04743	CKO_04744
11	*Acinetobacter baumannii ATCC 17978*	A1S_3175	A1S_3174
12	*Klebsiella pneumoniae 342*	KPK_0396	KPK_0394
13	*Enterobacter cloacae subsp. cloacae ATCC 13047*	ECL_04700	ECL_04701

14	*Serratia sp. AS12: SerAS12_4645*	SerAS12_4645	SerAS12_4646

Importance of consensus motifs (*Pa* Bfr, *Pa* Bfd, *Pa* Fpr and *Pa* FtnA)

The sequence of *Pseudomonas aeruginosa* proteins are aligned against several known Gram negative pathogenic bacteria using ClustalW. Sequences of *Pa* BfrB, *Pa* FtnA, *Pa* Fpr and *Pa* Bfd from *P. aeruginosa* were used as query sequences and compared similar sequences in pathogens which fall under ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella* species, *Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species) category and some other pathogenic species.

The output of the alignment gave a properly aligned multiple sequence with conserved residues and residues with less similarity. At the end of the alignment, all these conserved and less conserved residues are represented by symbols in the last row. Residues in red color with symbol ' *' are conserved residues which means all the residues are same in this region (Example: The entire row contain only "H" histidine) and are believed to play an important role in the function of that particular protein, whereas residues with green color and symbol ' :' are strongly similar which means not all the residues are same but contain at least two different amino acid residues in the entire row (Example: "H" Histidine and "Y" tyrosine) and blue color with ' .' symbol indicates these residues are weakly similar and may have repeats of three (Example: "G" glycine, "D" Aspartic acid and "S" serine).

The aligned sequences of *Pa* BfrB and *Pa* Bfd (Figure 2) with other pathogenic sequences gave an average percentage similarity and identity of 83.37% and 68.4% respectively which shows resemblance of bacterioferritin protein of *Pseudomonas aeruginosa* with other

pathogenic bacteria. The results of bacterioferritin alignment shows conserved residues for ferroxidase center, four fold pores and B-pores with almost all selected organisms (Figure 2). This indicates the mechanism of iron uptake, iron release or oxidation of Fe^{2+} to Fe^{3+} involve almost similar pathways as studied in *Pa* BfrB.

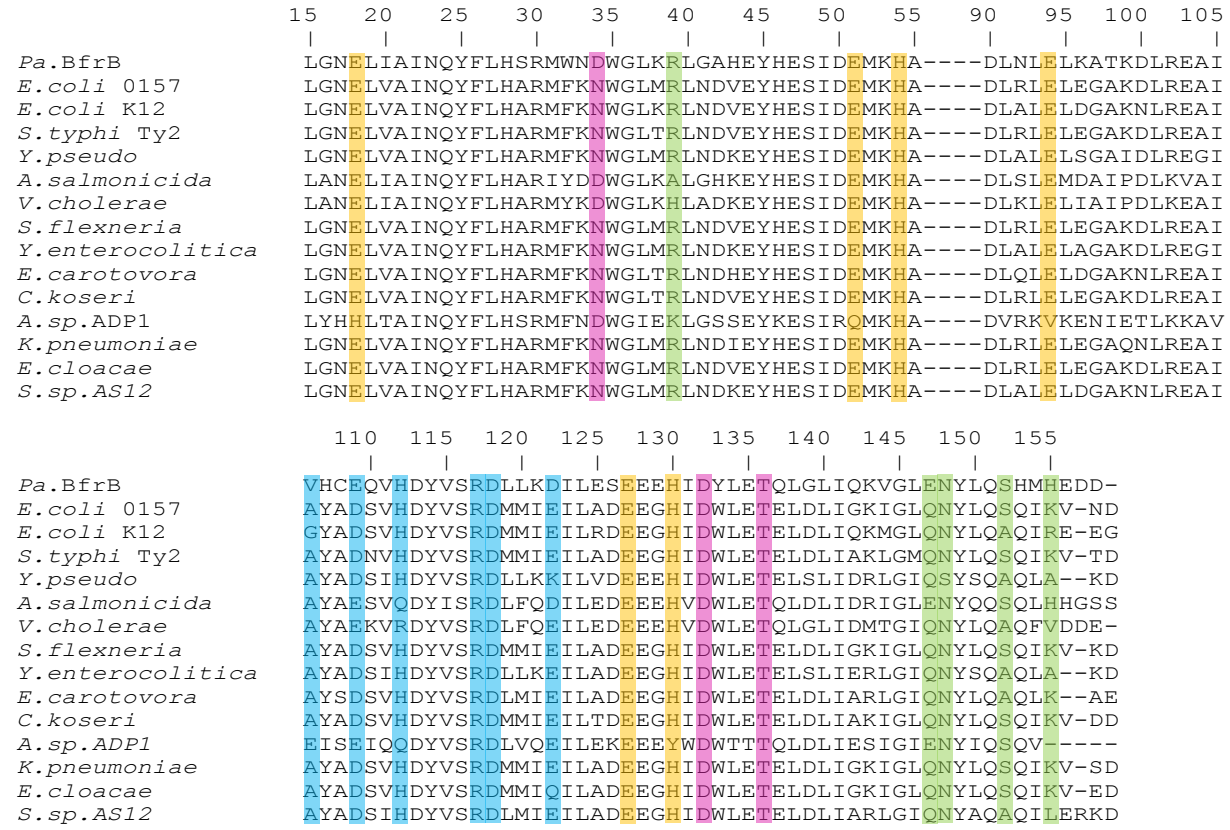


Figure 2: Sequence alignment of bacterioferritins in comparison to *Pseudomonas aeruginosa*. Residues involved in ferroxidase center (Orange), four fold pore (Green), three fold pore (Blue), and B-pores (Pink) are highlighted

Sequence alignment of *Pa* Bfd with other pathogenic organisms shows presence of conserved cysteine with similar type of arrangement **C**-X-**C**-X31-34-**C**-X2-**C** (Figure 3). Hence, these organisms may also possess similar electron transfer from this cluster to enable iron release

from bacterioferritin. The average similarity and identity percentage of *Pa* Bfd obtained in comparison with other pathogenic organisms are 66.75% and 40.97% respectively.

```
                          5   10   15   20   25   30   35   40
                          |    |    |    |    |    |    |    |
Pa.Bfd                    MYVCLCQGVTDNQIRDAIYEGCCS-YREVREATGVGTQCGKCACL
E.coli 0157               MYVCLCNGVSDKKIRQAVRQFHPQSFQQLRKFIPVGNQCGKCIRA
E.coli K12                MYVCLCNGISDKKIRQAVRQFSPHSFQQLKKFIPVGNQCGKCVRA
S.Typhi Ty2               MYVCLCNGISDKKIRQAVRQFHPQSFQQLRKFIPVGNQCGKCIRA
Y.pseudo                  MYVCLCNAVSDKVIRKAVRQHQPHTVKQLRQLVPIGSDCGKCIRQ
A.salmonicida             MYVCLCRGITDTQIRKAVQAGKSE-FRQLKQSLEVGAQCGKCVRM
V.cholerae                MYVCLCHGVSDKKIRRLVAEQGITDIKGIKRCTALGSQCGKCVRQ
S.flexneri                MYVCLCNGVSDKKIRQAVRQFHPQSFQQLRKFIPVGNQCGKCIRA
Y.enterocolitica          MYVCLCNAVSDKVIRNAVRQHHPHTIQQLRQLVPIGTDCGKCIRQ
E.carotovora              MYVCLCNAISDKAIRNAVRQHQPQSMQQLRKLVPIGTDCGKCIRQ
C.koseri                  MYVCLCNGVSDKKIRQAVRQFHPQSFQQLRKFIPVGNQCGKCVRA
```

Figure 3: All the pathogenic organisms studied contain four conserved cysteine like *Pseudomonas aeruginosa* which are highlighted in yellow

The conserved residues other than cysteines in Bfd may play an important role in binding to Bfr which aids protein-protein interactions that helps in iron release.

Crystal structure of BfrB-Bfd complex of *P.aeruginosa* proposed from our lab *(5)* shows residues which involve in this complex formation. Comparison of theses residues with other selected Gram-negative pathogenic bacteria shows this type of interface is present in all these selected bacteria (Figure 4).

(A)

```
                          5     35    40
                          |     |     |
Pa.Bfd                    MYVCL-----GTQCGK
E.coli 0157               MYVCL-----GNQCGK
E.coli K12                MYVCL-----GNQCGK
S.typhi Ty2               MYVCL-----GNQCGK
Y.pseudo                  MYVCL-----GSDCGK
A.salmonicida             MYVCL-----GAQCGK
V.cholerae                MYVCL-----GSQCGK
S.flexneri                MYVCL-----GNQCGK
Y.enterocolitica          MYVCL-----GTDCGK
E.carotovora              MYVCL-----GTDCGK
C.koseri                  MYVCL-----GNQCGK
```

(B)

```
                                  70          80
                                  |           |
Pa.BfrB                   LPNLQDLGKLLIGENTQE
E.coli 0157               IPNLQDLGKLGIGEDVEE
E.coli K12                LPNLQDLGKLNIGEDVEE
S.typhi Ty2               IPNLQDLGKLGIGEDVEE
Y.pseudo                  IPNLQELGKLNIGEDVEE
A.salmonicida             LPNLQDLGKLRIGETVEE
V.cholerae                IPNLQDLGKLMIGEDVQE
S.flexneria               IPNLQDLGKLGIGEDVEE
Y.enterocolitica          IPNLQDLGKLNIGEDVEE
E.carotovora              IPNLQDLGKLNIGEDVEE
C.koseri                  IPNLQDLGKLGIGEDVEE
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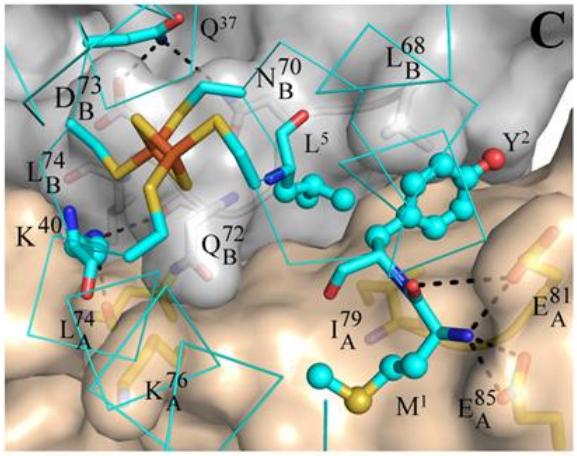

Figure 4: (A) (B) Alignments of *Pa*.Bfd like molecule and *Pa*.BfrB respectively with other selected Gram-negative pathogenic bacteria. Highlighted residues in yellow are involved in BfrB-Bfd complex formation. (C) Two subunits od BfrB A and B are shown in grey and wheat respectively and Bfd in cyan, Oxygen in red, nitrogen in blue, sulfur in yellow and iron in orange.

DISSCUSION

The sequence alignment studies suggest that Gram negative pathogenic bacteria which are selected for this study resemble proteins of *Pseudomonas aeruginosa* in their structure and functions as *Pa* BfrB and *Pa* Bfd. This is confirmed by presence of conserved regions for ferroxidase center, four fold pores, three fold pores and B-pores in case of Bfr and presence of four conserved residues in Bfd alignment. The close similarity of these pathogenic bacterial proteins with *Pseudomonas aeruginosa* proteins implies the mechanism of iron uptake and iron release even in these proteins of other pathogenic organisms can be similar.

Presence of highly conserved residues at interface in wide variety of Gram-negative bacteria in relation to *Pa*-BfrB-Bfd complex, suggest that these arrangement may be common in pathogenic bacteria to maintain iron homeostasis.

CONCLUSION

Genome analysis of *Pseudomonas aeruginosa* and other pathogenic organisms using JCVI-CMR and KEGG showed the presence of *bfr* and *bfd* genes next to each other which have similar transcription direction. This suggests a possible evolutionary importance of these two genes in the iron management. Bfd like sequence are present in these selected pathogenic bacteria with a unique arrangement of four conserved cysteines which indicates even in these bacteria [2Fe-2S] cluster of bfd may help in release of electrons during iron release. Presence of Similar residues in BfrB sequence in selected pathogenic bacteria in comparison to BfrB sequence in *P. aeruginosa* suggests formation similar type of structure even in these bacteria as *P.aeruginosa* and may form similar type of channels which may involve in maintaining iron homeostasis. Presence of conserved residues at BfrB-Bfd interface indicates clustering of *bfrB* and *bfd* genes is common in wide number of bacterial species which suggests this type of interaction of Bfd and Bfr is common for regulating iron from inner cavity. Hence inhibitors which can inhibit interactions of BfrB-bfd complex in *P.aeruginosa* can also inhibit similar type of interactions in other pathogenic Gram-negative bacteria.

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CHAPTER IV

Effect of transposon mutants BfrB and Bfd on biofilm growth in *Pseudomonas aeruginosa*

Introduction:

Pseudomonas aeruginosa contain storage proteins such as *Pa* BfrB and *Pa* FtnA to store iron. The iron homeostasis in this bacterium is maintained by these storage proteins by taking in excess Fe^{2+} and stores it as Fe^{3+} in its inner cavity and releases this stored Fe^{3+} in the form of Fe^{2+} during iron deficiency conditions with the help protein-protein interactions. In order to study the phenotype changes in biofilm formation of *Pseudomonas aeruginosa* under iron starvation conditions mutants in absence of these storage proteins were used. Instead of mutating, we purchased transposon mutants from University of Washington transposon library for better efficiency. As these mutants are prepared by high throughput method we need to confirm presence of this transposon before performing further experiments on biofilm formation.

Transposition was discovered in Maize by Barbara McClintocks in 1983, and named it as dissociation locus (Ds locus) (*1*). Later it was termed as transposition or jumping gene or junk DNA because the transposon did not have any specific function of its own. Transposition is defined as the moving of genetic information from one place to another place in a genome. This movement can occur within the genome or from outside the genome with the help of a plasmid (*2*). The three elements required for this process are: [1] transposon [2] transposase and [3] target DNA. Transposon is part of the DNA sequence which does not have any primary function of its own, but is able to alter the functions of other genes. Transposase is an enzyme that helps in

cutting, moving, and pasting of the transposon in the target DNA (*3*). Transposon insertion can occur two ways. The first is cut and paste where the whole transposon DNA sequence is selected and gets cut and pasted at different site in genome. The second is copy and paste where the original transposon sequences stays in its original site, but forms a duplicate copy that is inserted in a different location. Any of this progression causes sudden mutations, gene fusion, and rearrangement.

Transposons are believed to play an important role in evolution of any organism including prokaryotes and eukaryotes. The unique feature of a transposon is its ability to associate with any gene in the genome irrespective of any specific binding sites. This indicates the possibility of a transposon element insertion at several places in a genome during the course of evolution (*4*). It has been shown that about 40% of the human genome has been changed or rearranged during the course of evolution with transposons (*3*).

Transposable elements which have antibiotic resistant genes are mostly studied to understand their mechanism and to be used as mutagens. It is believed that because of the prolonged use of antibiotics in agriculture and medicine it has caused environmental changes for the bacteria and forced their growth under difficult conditions. This may have caused transposable elements to move from place to place to adapt to the changes and be able to survive in unfavorable conditions and make the bacteria resistant to specific antibiotic (*4*). Tn5 transposon, which is kanamycin resistance, is mostly studied to understand the function and mechanism of the proteins it replaces.

Tn5 is a 5,700 bp large composite element which has two inverted insertion elements called IS50R and IS50L. The IS50 have a unique arrangement which contain an outside end

(OE) and an inside end (IE) at the periphery and a central region that encodes for antibiotic resistance. The most useful feature is the presence of a flexible central region which can be modified to require antibiotic resistance when preparing transposons in vitro (Figure 1). IS50 with both outside ends has a higher rate of transposition (100-1000 fold) than IS50 with both inside ends, but IS50 with one IE and one OE acts the same as IS50 with both OE. Both these, OE and IE, have 19 bp which help in transposase recognition (*4*). IS50R encodes two proteins called transposase (tnp) and inhibitor proteins (Inh); whereas IS50L encodes nonfunctional counterparts of tnp and Inh proteins. The presence of the ochre codon makes the proteins nonfunctional (*2*, *5*).

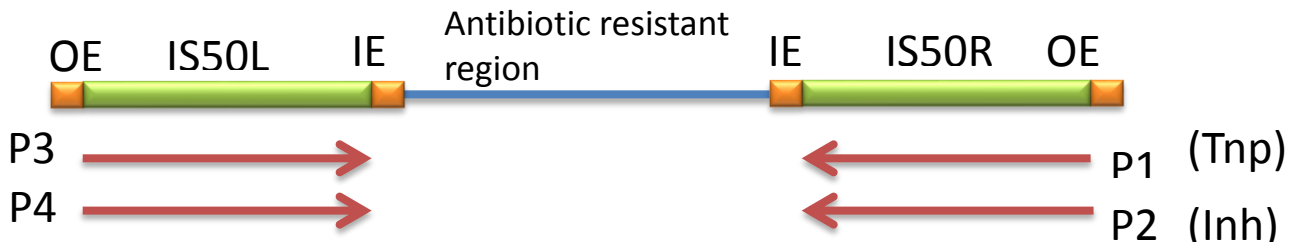


Figure 1: Transposon Tn5 bearing IS50L and IS50R insertion sites at the end, both IS50 elements are delineated by 19-bp sequence, outside and inside ends which are linked with antibiotic resistance region. Tnp or P1 (transposases) and Inh or P2 (inhibits transposition) which are translated into same reading frame in IS50R whereas IS50L contain an ochre codon which helps in release of P3 and P4

Pseudomonas aeruginosa (Pa) mutants for these studies are derived from IS50L element of the transposon Tn5. Insertion sites of the transposon are termed as IS*phoA/hah* (4.83Kbp) and IS*lacZ/hah* (6.16kbp) (*6*) (Figure2) which are a modified form of IS*phoA/in* and IS*lacZ/in* (*7*).

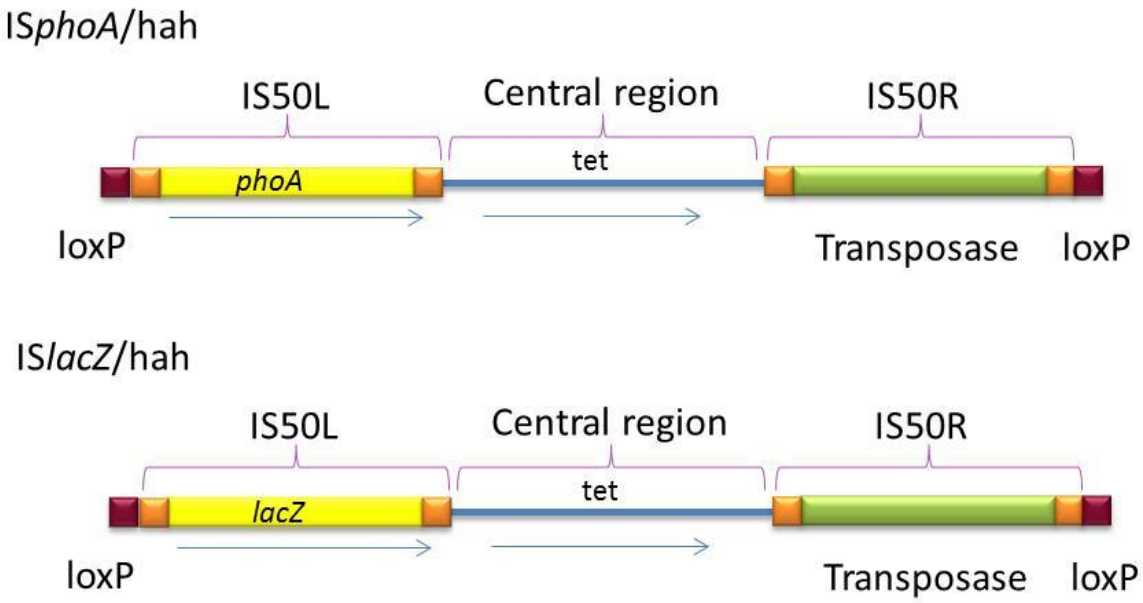


Figure 2: Transposon insertion sites IS*phoA/hah* (4.83kbp) and Is*lacZ/hah* (6.16kbp) showing tetracycline (tet) resistant central region with phoA (alkaline phosphatase) gene and lacZ (β-galactosidase) gene insertion at two IS50L elements respectively. Also shows loxP, cre recognition site and P, neomycin phosphotransferase promoter.

These two transposons are relatively similar, but *phoA* encodes for alkaline phosphatase and *lacZ* encodes for β galactosidase. To design these transposon elements for *pa* mutants, Manoil and coworkers inserted influenza hemagglutinin (HA) epitope and a hexahistidine (h) motif in TnphoA and TnlacZ hence the term "IS*phoA/hah* and IS*lacZ/hah* " which is a 63 codon insertion and is made to be tetracycline resistant. Presence of the loxP site at the end of the transposase region has two flanking 13 bp inverted repeats (Figure 3) on both the ends of the transposon, which helps in site specific recombination. The loxP site is made of a specific 34-base pair (bp) sequence consisting of an 8 bp core sequence where recombination takes place and, two flanking 13 bp inverted repeats (*8*) (Figure 3) on either ends of transposon, which helps

in site specific recombination. These transposons can be used in a wide variety of organisms, since these are generated based on a broad host range technique.

Figure 3: LoxP site contains specific 34-base pair (bp) sequence of which 8bp are core sequence (spacer) where recombination takes place and two flanking 13 bp inverted repeats on either ends of transposon helps in site specific recombination.

PhoA and lacZ is inserted at the IS50L side of theTn5 transposon. As stated earlier, the IS50L has nonfunctional counterparts, so deletion of this part will not affect the Tn5 transposon. Gene fusion of phoA or lacZ at this site will be favorable instead of the IS50R because of its function (transposase). To generate phoA gene fusion, a truncated phoA gene is inserted in the left IS50 element of Tn5 transposon. This transposon is transpositioned on to the target plasmid or chromosome at the permissive site without disturbing the function and folding of the protein .The created transposons are inserted into different genes of *Pseudomonas aeruginosa* and generated the transposon mutants by coworker of Manoil lab from University of Washington (*9, 10*). We choose Tn Pa BfrB, Tn Pa FtnA as these are known storage proteins and Tn bfd as this forms protein-protein interaction with Pa BfrB to release iron and Tn HasAP and wildtype as controls. Mutants used for these studies are given in Table 1. The presence of the transposon had to be confirmed before studies were conducted because they were developed by high throughput methods which could lead to the error of mixed colonies and the proposed inserted transposon not being present in the mutant.

TABLE 1: *Pseudomonas aeruginosa* Transposon mutants used for this study

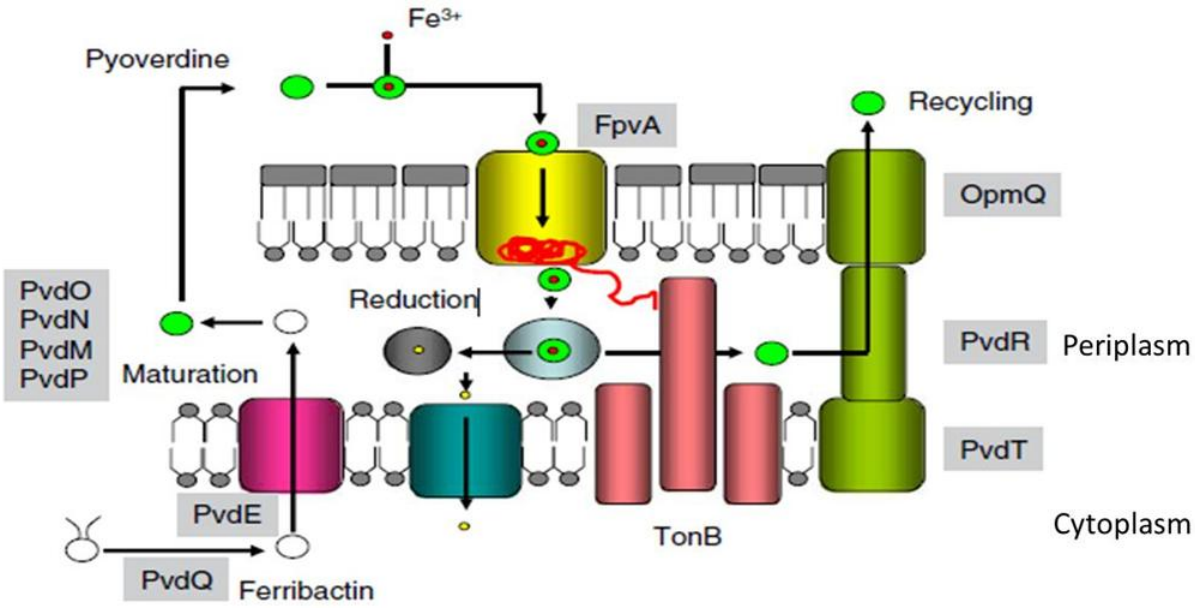
Strain Name	**Location**	**PA ORF**	**Transposon**	**Gene product**	
PAO1	Wild type MPAO1				
PW6979	lacZbp01q1C04	PA3531	ISlacZ/hah	Bacterioferritin	bfrB
PW6980	lacZwp05q4A04	PA3531	ISlacZ/hah	Bacterioferritin	bfrB
PW6978	phoAwp10q1A09	PA3530	ISphoA/hah	conserved hypothetical protein	Bfd
PW6977	phoAwp02q3E10	PA3530	ISphoA/hah	conserved hypothetical protein	Bfd
PW8188	lacZwp01q4F06	PA4235	ISlacZ/hah	Bacterioferritin	FtnA
PW8189	phoAbp02q2C12	PA4235	ISphoA/hah	Bacterioferritin	FtnA
PW6747	phoAwp04q2H07	PA3407	ISphoA/hah	heme acquisition protein HasAp	hasAp
PW6748	phoAwp06q3G11	PA3407	ISphoA/hah	heme acquisition protein HasAp	hasAp

Studies on the transposon mutants provide information on essential genes in a genome (*11*). As part of our interest in understanding how iron metabolism and homeostasis affect the growth of *P. aeruginosa*, growth studies were conducted with transposon mutants of iron storage proteins and heme transporter proteins under aerobic conditions as an attempt to observe phenotypes differences.

Mechanism of iron uptake by pyoverdine in Pseudomonads

The general mechanism of iron uptake in *Pseudomonads* is as follows, a pyoverdine precursor called ferribactin which undergoes de-acylation via the PvdQ acylase enzyme in the

cytoplasm. The enzyme is non-fluorescent and is transported from the cytoplasm to the periplasm by the ATP-binding cassette (ABC) transporter PvdE (*12, 13, 14, 15, 16*). Insertion of a chromophore into ferribactin is mediated by the proteins PvdO, PvdN, PvdM and PvdP by a process called maturation (*15, 16*). Pyoverdine binds to Fe^{3+} once it is transported from the periplasm to extracellular space. This ferripyoverdine is recognized by the FpvA receptor and carries it to the periplasm where Fe^{3+} is released from pyoverdine and is reduced to Fe^{2+}. It is then passed to the cytoplasm where it is mineralized and stored by iron storage proteins. Pyoverdine without Fe^{3+} is recycled again to extracellular space to capture more Fe^{3+} by a tripartite efflux pump which is formed by 3 different proteins, PvdT, PvdR, and OpmQ (*16*) (Scheme 1).



Scheme 1: Mechanism of iron uptake by pyoverdine in pseudomonads *(45)*.

Experimental section:

PCR protocol for transposon mutant:

Materials:

Transposon mutant strains listed in Table 1 and related primers in Table 2 were ordered from UW genome sciences (Manoil lab), 10X TSG buffer (Tris-HCl, sodium chloride and Glycerol), 10 mM dNTP mix, 25 mM $MgCl_2$ and TSG plus DNA polymerase where purchased from Lambda Biotech, dimethyl sulfoxide (Fisher), PCR experiments are performed using a Mini cyclerMJ research, PTC-150)

TABLE 2: Specific and flanking primers used for different strains to perform PCR

Strain Name	**PA ORF Gene product**	**Gene**	**Specific primer**	**Flanking primer**
PW6979	PA3531	bfrB	GGGTAACGCCAGGGTTTTCC	CCAAGGTGTTACCGACAACC
PW6980	PA3531	bfrB	GGGTAACGCCAGGGTTTTCC	TCCCTCTTTCAGAAGAACCG
PW6978	PA3530	Bfd	CGGGTGCAGTAATATCGCCCT	GAAGTGGGAGTCGATGGAAA
PW6977	PA3530	Bfd	CGGGTGCAGTAATATCGCCCT	AAGGTGAAGTCCAGCGGATA
PW8188	PA4235	FtnA	GGGTAACGCCAGGGTTTTCC	GATACCGGCAAGACCCTGTA
PW8189	PA4235	FtnA	CGGGTGCAGTAATATCGCCCT	GTGATCGAGCACAACCTGG
PW6747	PA3407	hasAp	CGGGTGCAGTAATATCGCCCT	ATCAGCAGGGTCATGTGGTT
PW6748	PA3407	hasAp	CGGGTGCAGTAATATCGCCCT	GAGGACATTGCGGCAGTC

Methods:

LB agar plates for wild type and Tn mutants were prepared separately as plates required for streaking wildtype does not require tetracycline whereas plates used to streak mutants were prepared by adding tetracycline to a final concentration of 5 µg/mL. These plates were incubated for 12 to 15 h at 37 °C to grow single colony cultures. Selected three to four colonies obtained from these plates and prepared stab cultures. These newly prepared stab cultures from single colonies are streaked again and resultant colonies are used to conduct PCR for confirmation of transposon (Figure 4).

In a PCR tube add all the reagents with its proper volume as shown in Table 3. To this mixture add a single colony from petri plate obtained after streaking plates with newly prepared stab cultures and mix properly before leaving it on Mini cycler for PCR reaction.

TABLE 3: PCR Volumes

Reagents	**Volume**
Autoclaved nanopure water	4.7 µL
DMSO	0.5 µL
10X TSG buffer	1.0 µL
25 mM $MgCl_2$	1.5 µL
10 mM Mix dNTPs	0.2 µL
TSG enzyme	0.1 µL
Primer 5 pmol/ µL	1.0 µL
Primer 5 pmol/ µL	1.0 µL

Single colony	Pick entire colony

Conditions used for PCR (19) are given in Table 4. Tests were conducted on at least 16 colonies obtained from one strain. Results were confirmed by a 0.8% agarose gel.

TABLE 4: PCR conditions

Steps	**Condition**	**Temperature**	**Time**
1	Initial denaturation	94 ° C	10 min
2	Denaturing	94 ° C	30 sec
3	Annealing	64 ° C	30 sec
4	Extension	72 ° C	3 min
5	Go to step 2	30 cycles then to step 6	
6	Final extension	72 ° C	7 min
7	Hold at	04 ° C	

DNA agarose gel preparation:

Materials:

Agarose (Sigma aldrich), 1X TBE buffer (Tris base (10.9 g/L)(Chem Cruz), boric acid (5.5 g/L) (sigma aldrich) and EDTA (0.46 g/L) (Fisher)) and ethidium bromide (8 µg/mL EtBr) (Fisher bio reagents), and DNA ladder (Lambda biotech)

Methods:

Agar (0.8 g) was dissolved in 100 mL of 1X TBE buffer by microwaving for 45 sec to 60 sec until agarose dissolved. Remove this mixture from microwave and leave it at room temperature then add 8 µL (8 µg/mL) final concnetrations (stock solution purchased is 10 mg/mL) of ethidium bromide (EtBr) when solution reaches to a temperature of 50°C-55°C. EtBr was added at low temperatures to avoid the release of carcinogenic fumes. Prior to pouring, the case was properly wiped with ethanol.12 mm thick combs were added to the holders for one hour until the gel was solidified.

Experimental procedure for aerobic growth studies of *Pa* mutants:

Materials:

Potassium phosphate dibasic (K_2HPO_4), magnesium sulfate heptahydrate, sodium chloride, calcium chloride dehydrate and copper sulfate pentahydrate (Fisher chemicals), potassium phosphate monobasic (KH_2PO_4), manganese sulfate monohydrate and sodium molybdate dehydrate (MP biomedicals), ammonium sulfate, nitrilotriacetic acid, cobalt chloride hexahydrate, zinc sulfate heptahydrate, iron III chloride hexahydrate and boric acid (Sigma aldrich) and succinic acid disodium salt (Acros organic).

Method:

Succinate minimal media (SM media) was used to study the growth patterns in *Pseudomonas aeruginosa* transposon (Tn) mutants. Iron rich succinate minimal media (*18*) (SM media A) consists of: 35 mM succinic acid disodium salt, 3 mM $(NH_4)_2SO_4$, 35 mM K_2HPO_4, 22 mM KH_2PO_4 and .0017 mM $FeCl_3.6H_20$) and another media called succinate minimal media (SM media B) (35 mM Succinic acid disodium salt, 3 mM $(NH_4)_2SO_4$, 35 mM K_2HPO_4, 22 mM KH_2PO_4) are used to maintain iron rich and neutral media. To these two media,

10 mL/L of Wolin salt (composition is given in Table 5) was added. Succinic acid and ammonium sulfate were filtered through 0.2 µm filter paper and added to both SM media and SM media B after autoclaving. A single colony of wild type and Tn mutants were incubated for 13 to16 h in 10 mL of SM media A at 230 rpm and 37 °C. Overnight culture was centrifuged at 3500 rpm for 15 min at 20 °C to separate pellet from supernatant. The pellet was resuspended with 3 mL of SM media B gently with a pipette. 600 µL of the this suspension was added to 100 mL SM media B present in the conical flask which contained the control (without chelating agent) and the chelating agent. These flasks were incubated at 37 °C at 235 rpm for 12 h. Growth of Tn mutants were monitored for every 1 h by checking cell density and the release of pyoverdine related to growth.

TABLE 5: composition of Wolin salt

S.No	Chemicals	Concentration
1	Nitrilotriacetic acid	15.7 mM
2	Magnesium sulfate heptahydrate	24.3 mM
3	Sodium chloride	17.1 mM
4	Manganesesulfate monohydrate	6 mM
5	Calcium chloride dihydrate	0.68 mM
6	Cobalt (II) chloride hexahydrate	0.42 mM
7	Zinc sufate heptahydrate	0.35 mM
8	Boric acid	0.32 mM
9	Sodium molybdate dihydrate	0.041 mM
10	Copper sulfate pentahydrate	0.04 mM

Results:

Confirmation of transposon mutations:

In order to study growth pattern in *Pseudomonas aeruginosa* under iron limiting conditions we purchased transposon mutants from University of Washington Manoil. Confirmation of transposon mutation was important to complete before proceeding with further experimentation. A protocol was determined to store the mutants as stab cultures: Once received, samples were streaked for single colonies on to LB agar plates with 5µg/ml tetracycline and incubated for 13 to 14 h h at 37 °C. A few colonies were randomly picked and stabbed into the prepared stab culture tubes and were labeled as stab culture 1, stab culture 2 etc. These single colonies were used to streak LB agar plates with tetracycline (5 µg/mL) to obtain daughter colonies. Obtaining daughter colonies was important because transposon insertion may not be present in all colonies which are obtained from the original stock (Figure 4). PCR was performed under conditions mentioned in Table 4, to increase the intensity of DNA bands in the agarose gel, a few steps where modified for the PCR studies. A whole single daughter colony was used to do PCR studies instead of a few microliters of one boiled colony (*19*) because, adding only few micro liters of this boiled colony to perform PCR was giving very faint band and sometimes these bands were not visible on agarose gel to confirm mutation which was overcome by adding whole colony to each PCR tube as it increases the concentration.

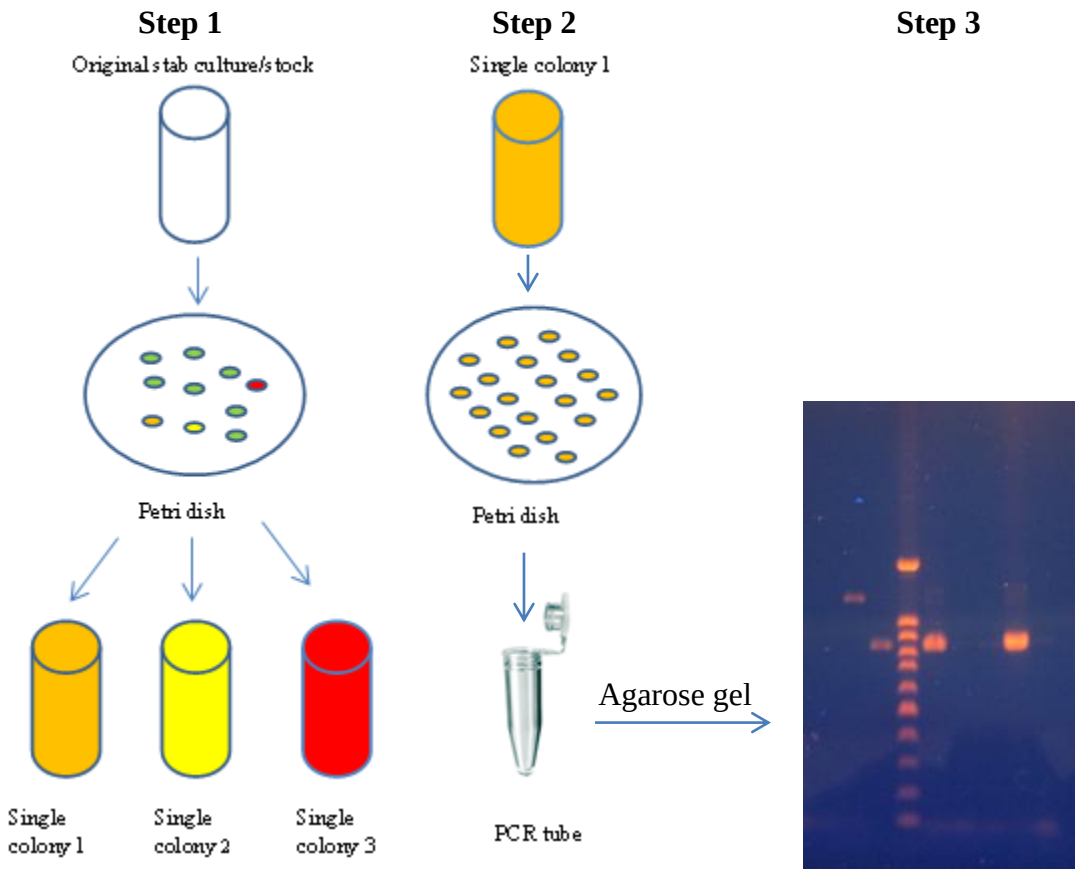


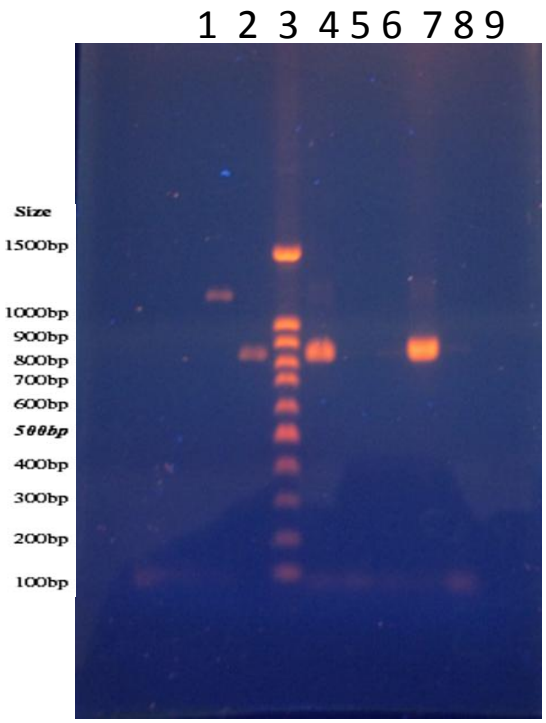
Figure 4: Step 1: showing preparation of single colony stab culture. Step 2: obtaining daughter colonies from single colony stab culture and performing PCR. Step 3: DNA agarose gel showing result of PCR product.

To do PCR, two primers, the forward and reverse are needed. Selection of these primers depends on the type of insertion they are harboring. There are two different insertion types present in these mutants called the LacZ and PhoA site obtained by gene fusion. Hence, we have to use the LacZ-148 primer for mutants holding LacZ insertion site and Hah-138 primer for mutants having PhoA insertion site. These two primers LacZ and Hah are called specific primers. In order to proceed with PCR, we need another primer which depends on the type of orientation of the transposon. If orientation is in forward direction, then it is expected to see a PCR product with the forward primer, but not with the reverse primer and vice versa for mutants with reverse

orientation. These forward and reverse primers are called flanking primers. Selection of the primers was decided from information provided by the Manoil Lab Table 2. In the case of Tn BfrB (PW6979 and PW6980) and one strain of Tn FtnA (PW8188) LacZ is used as specific primer because they contain LacZ insertion in the transposon which will recognize by this primer and reads the frame if its orientation is correct and gives a positive result, if the orientation of the transposon is not correct even addition of LacZ primer will give a negative results (no band in agarose gel) indicating absence of transposon in that particular colony, and for the other strains like Tn FtnA (PW8189), TnBfd (PW6977, PW6978), and Tn HasAp (PW6747, PW6748) Hah was used as the specific primer Because of presence of hah insertion site on transposon.

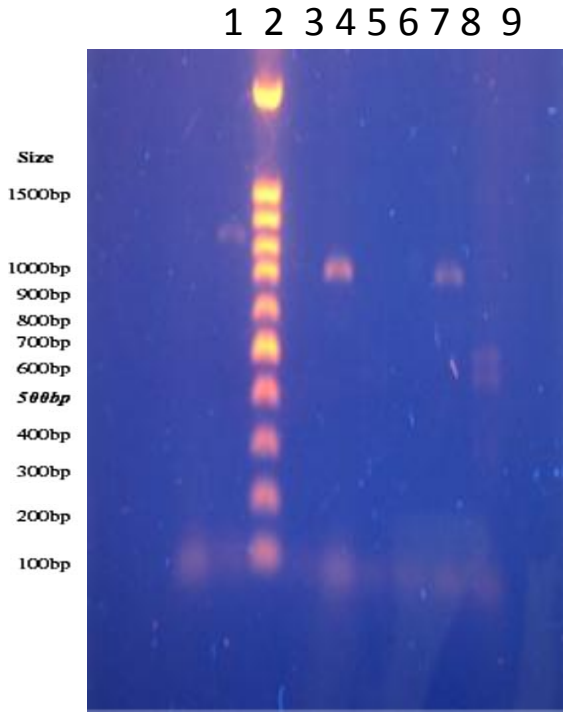
For confirming the presence of the transposon, DNA agarose gel with EtBr was used. The PCR product was loaded on to the 8% agarose gel with a final concentration of 0.8µg/ml EtBr. It was important to adjust the right concentration of EtBr for gels because higher concentration can mask the bands with background effect and lower concentration will not give proper resolution. The size of the comb and volume of the loading sample was also important to get high resolved bands. Here we used 12 mm thick combs and loaded 10µl of sample. The sample was prepared by adding 2µL of sample buffer to each PCR product and mixed by a pipette. Results were verified with a 1Kb or 100 bp DNA ladder for molecular weight confirmation. We were successfully able to confirm the presence of the transposon mutants in all the colonies picked from all strains except BfrB (PW6980) (stab culture 1) which gave us negative results indicating the absence of the transposon in that colony which shows that the original stab culture contained a mixed population (Figure 5 and 6). The expected molecular weight for the wildtype and Tn mutants for all strains are given in Table 1.

(A) Bfd A09 (PW6978)



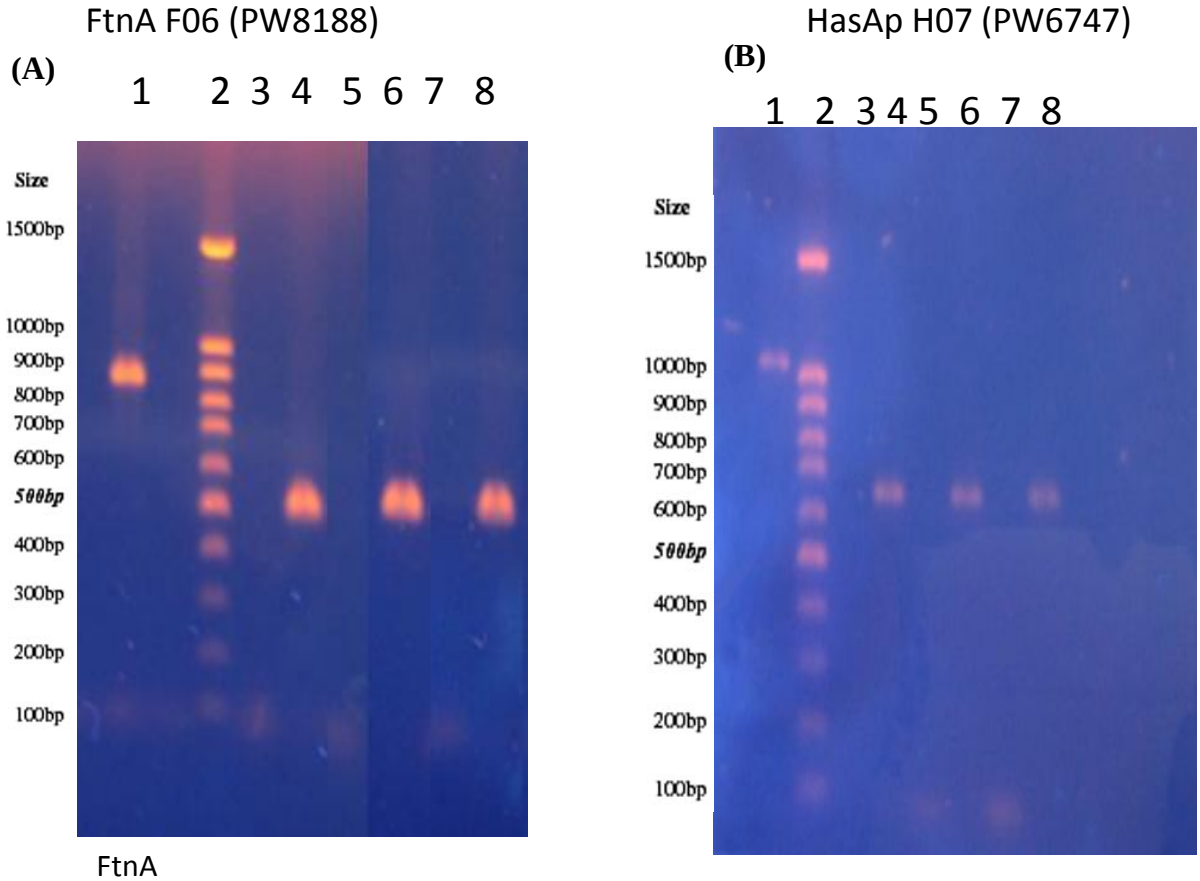
Bfd
1: wt F+R primer
2: Tn Bfd Hah+F (Stab culture 1)
3: 100bp ladder
4: Tn Bfd Hah+F (Stab culture 2)
5: Tn Bfd Hah+R (Stab culture 2)
6: Tn Bfd F+R (Stab culture 2)
7: Tn Bfd Hah+F (Stab culture 3)
8: Tn Bfd Hah+R (Stab culture 3)
9: Tn Bfd F+R (Stab culture 3)

(B) BfrB A04 (PW6980)



BfrB
1: wt F+R primer
2: 1Kb ladder
3: Tn BfrB LacZ+F (Stab culture 1)
4: Tn BfrB LacZ+F (Stab culture 2)
5: Tn BfrB LacZ+R (Stab culture 2)
6: Tn BfrB F+R (Stab culture 2)
7: Tn BfrB LacZ+F (Stab culture 3)
8: Tn BfrB LacZ+R (Stab culture 3)
9: Tn BfrB F+R (Stab culture 3)

Figure 5: (A) 0.8 % DNA agarose gel analysis of *Pa* wt showing presence of bfd in lane 1 and presence of transposon insertion can be seen as a band in *Pa* Tn mutants indicating absence of Bfd gene (lane 2, 4, 7). (B) *Pa* wt showing presence of BfrB (lane 2) and presence of insertion site in Tn *Pa* BfrB mutants (4 and 8) negative results for presence of transposon was found in stab culture 1 (lane 3).



FtnA
1: wt F+R primer
2: 100bp DNA ladder
3: Tn FtnA LacZ+F(Stab culture 1)
4: Tn FtnA LacZ+R (Stab culture 1)
5: Tn FtnA lacZ+F(Stab culture 2)
6: Tn FtnA LacZ+R (Stab culture 2)
7: Tn FtnA LacZ+F (Stab culture 3)
8: Tn FtnA LacZ+R (Stab culture 3)

HasAP
1: wt F+R primere
2: 100bp Ladder
3: Tn HasAp LacZ+F (Stab culture 1)
4: Tn HasAp LacZ+R(Stab culture 1)
5: Tn HasAp LacZ+F (Stab culture 2)
6: Tn HasAp lacZ+R (Stab culture 2)
7: Tn HasAp LacZ+F (Stab culture 3)
8: Tn HasAp LacZ+R (Stab culture 3)

Figure 6: (A) 0.8% DNA agarose gel showing presence of insertion in isolated *Pa* Tn FtnA culture. (B) Shows presence of insertion site in *Pa* Tn HasAp culture after PCR.

Influence of iron chelators on growth of Pa Tn mutants:

Like many microorganisms, *Pseudomonas aeruginosa* also requires iron for its survival. It is known that they release different type of siderophores at different conditions to sequester iron externally for its survival *(22)*. To understand which proteins are important for its survival, and to study effects of iron chelator on growth of *Pseudomonas*, studies were conducted on transposon mutants along with wildtype by using succinate medium with and without Iron chelators. Addition of iron chelators helps in binding iron if any present in the media forcing *Pseudomonas* to secrete siderophores for its growth. Iron chelators were chosen based on their binding constant to Fe^{3+} ions. Studies were conducted using two different chelators (1) 2,2' Bipyridine (BP) and (2) *diethylene triamine pentaacetic acid* (DTPA) whose binding constant for Fe^{3+} are $\log_{10}= 16.3 \pm 0.01$ and $\log_{10} = 28.6 \pm 1$ respectively. Binding constants for different iron chelators for Fe^{2+} and Fe^{3+} are shown in Table 6.

TABLE 6: Binding constants of different iron chelators to Fe^{2+} and Fe^{3+}.

S.No	**Compound**	**Fe^{2+}**	**Fe^{3+}**	**Reference**
1	Ferrozine	$\log_{10} = 17$		24
2	Quercetin	$\log_{10} = 6$		25
3	BP	$\log_{10} = 17.4$	$\log_{10} = 16.3$	26, 27, 28, 43
4	EDTA	$\log_{10} = 14.33$	$\log_{10} = 24.23$	29, 30, 31, 32, 43
5	DTPA	$\log_{10} = 16.5$	$\log_{10} = 28.6$	29, 33, 34, 43,44
6	8HQS		$\log_{10} = 11.6$	35
7	8HQ		$\log_{10} = 14 \pm .04$	36, 37
8	HBED		$\log_{10} = 39.68$	38, 39
9	EDDHA		$\log_{10} =35.09$	38

10	Aniline		$\log_{10} = 70 \pm 3$	40
11	Thiourea		$\log_{10} = 7 \pm 2$	40
12	ascorbic acid		$\log_{10} = 28.0 \pm 2$	40
13	Pyoverdine		$\log_{10} = 32$	41
14	Transferrin		$\log_{10} = 22.7$	42

Studies conducted to see phenotypical changes in biofilm formation using Transposons:

These studies were conducted in presence of iron chelators such as 2,2' Bipyridine (BP) and *diethylene triamine pentaacetic acid* (DTPA). Experiments were performed using different concentrations of BP and DTPA to see the effect of chelator on growth of the strains. Common observations made for growth of the Tn mutants along with wildtype in presence of BP as a chelator include a lag phase for the control (All Tn strains including wildtype grown in media without any chelator) around 2 h to 3 h. All controls along with wild type in presence of BP at the 4^{th} h showed colloids, by the 8^{th} h all strains of the transposon mutants in presence of BP (250 µM and 500 µM) showed a green color indicating the release of a siderophore (pyoverdine) in to the media for sequestering iron Whereas, in the presence of higher concentration of BP (750 µM) none of the Tn mutant strains showed release of pyoverdine. By the 12^{th} h all the Tn mutant strains and wildtype in presence and absence of BP showed release of pyoverdine. The siderophore secreted by *Pseudomonas aeruginosa* in this study is concluded as pyoverdine based on the absorption spectra obtained at 400nm (*20*).

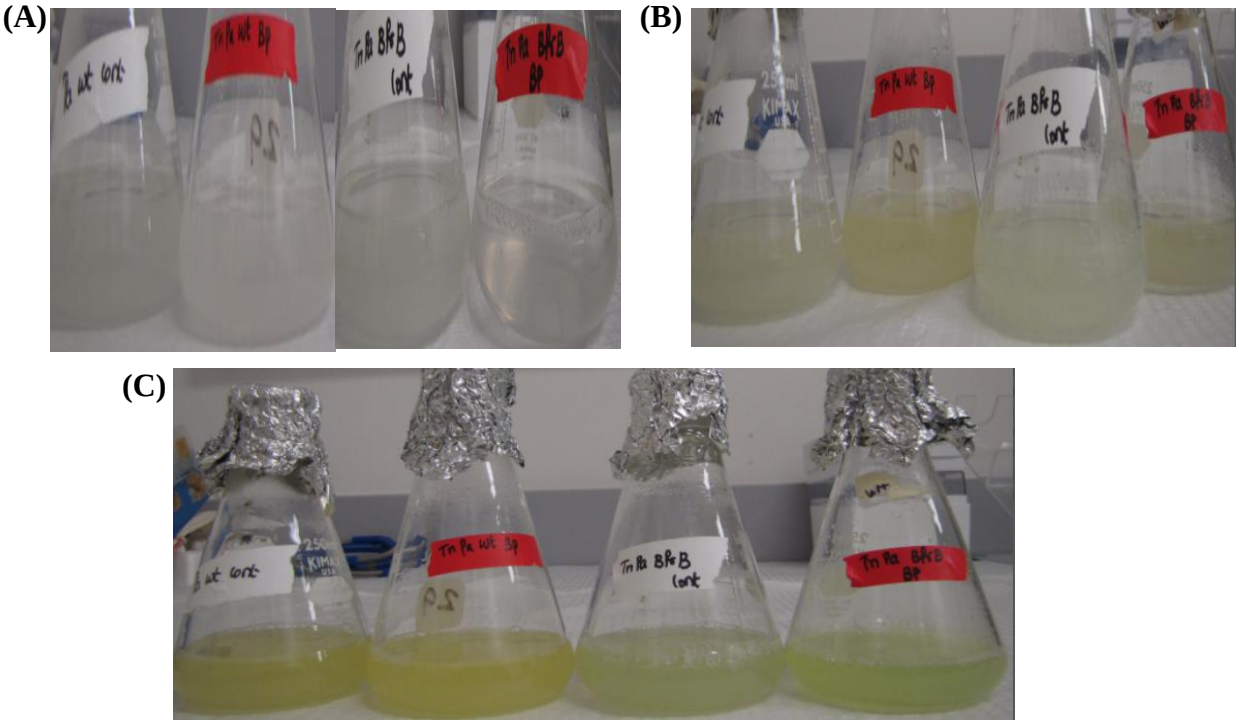


Figure 7: (A) Cultures of wildtype (control and in presence of chelator (500 µM BP)) and Tn BfrB mutant control (without chelator) samples at 4th h showing colloid (B) Cultures of wildtype and Tn BfrB mutant controls at 8th h showing no release of pyoverdine (no green pigment) and Wildtype and Tn BfrB mutants in presence of chelator (500 µM BP) shows release of pyoverdine (green pigment). (C) Cultures of wildtype and Tn BfrB mutant starins both controls and in presence of BP showed release of pyoverdine by 12th h.

At concentration of 250 µM BP Tn mutants did not show much of inhibition in their growth, but just observed increased lag phase to 1hr compare to controls (without chelators) (results not shown). This suggests mutants where trying to adapt to new conditions and started growing normally. The concentration was increased to 500 µM to see its effect on the growth of Tn mutants. At this concentration strains did show slow growth of Tn mutants compared to the controls. As we are forcing Tn mutants to grow in stressed conditions, they required some time to adjust to the conditions resulting in an increased lag phase until the 4th or 5th hour and then

started growing normally. They reached the plateau by the 12th h (Tn HasAP and Tn FtnA), but Tn BfrB and Tn Bfd where growing much slower when compared to the other two strains and never reached plateau by the 12th hour. This specifies that BfrB and Bfd are important for *Pseudomonas* survival. With 500 µM even wildtype in presence of BP was affected, but eventually managed to grow normally by releasing more pyoverdine (results not shown). In the case with 750 µM, all the Tn mutants and wildtype control grew normally as expected, but Tn mutant strains did not show much growth until the 12th hour (Figure 8). This represents that the specific genes absent from the genome have important roles in iron regulation and bacterial growth.

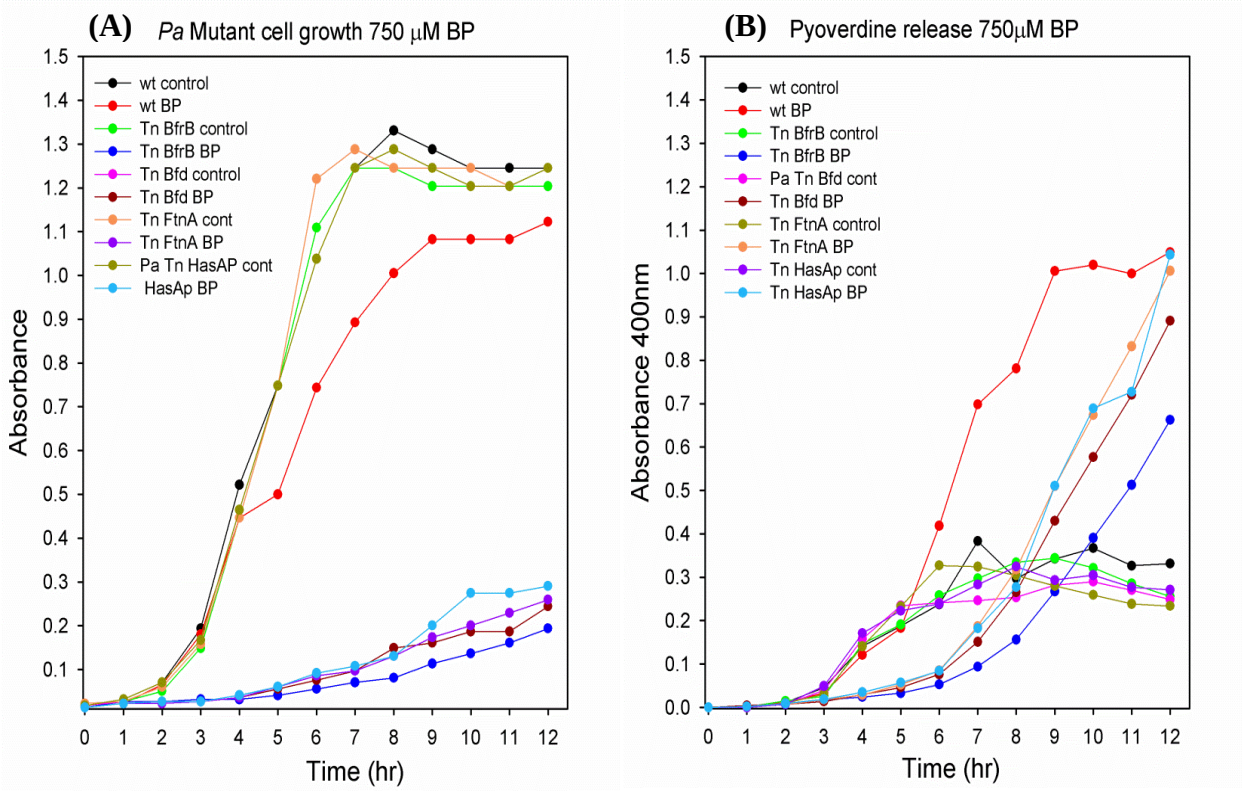


Figure 8: (A) Graph showing growth (OD 0.6 nm) of wildtype and Tn mutants in presence and absence of 750 µM BP (B) Graph showing release of pyoverdine (Absorbance 400 nm) in wildtype and Tn mutants in presence and absence of BP

Observation on biofilm formation in presence of *diethylene triamine pentaacetic acid* (DTPA):

DTPA binds Fe^{3+} more strongly than BP according to its binding constant as shown in Table 6. Different concentration (250 µM, 500 µM and 750 µM) of DTPA are used to study growth pattern in *Pseudomonas aeruginosa* wildtype and transposon mutants (Tn BfrB, Tn Bfd, Tn FtnA and Tn HasAp). Even with less concentration of 250 µM DTPA showed retarded growth in all Tn mutants and wildtype which are forced to grow with iron chelator. At this concentration all controls showed a lag phase of about 3 h and reached exponential state by 6^{th} hr to 7^{th} h. All Tn mutants with wildtype showed a lag phase at 6^{th} h and reached stationary phase by 11^{th} h (Figure 9). We were able to study growth of cultures by measuring density for every 1 h at OD 600 nm. Appearance of change in color and density was clearly observed at different intervals. Initially all the cultures were color less and clear. At 4^{th} h all the controls appeared as white dense precipitate representing growth of cells but at this point all strains with DTPA were still transparent specifying very less growth or no growth at all. By 8^{th} h all the cultures are colorless but strains with DTPA started showing white dense precipitate representing growth of the cells whereas controls became denser than initial but still no color. By 10^{th} h even though there was less growth by strains with DTPA except wildtype started turning dark green in color which represents pyoverdine. Wildtype color was different than other Tn mutants (Figure 10). May be it is releasing a different chromophore than others. By 12^{th} h all Tn mutants with DTPA showed dark green color pigment whereas wildtype with DTPA and all controls showed yellow-green color pigment.

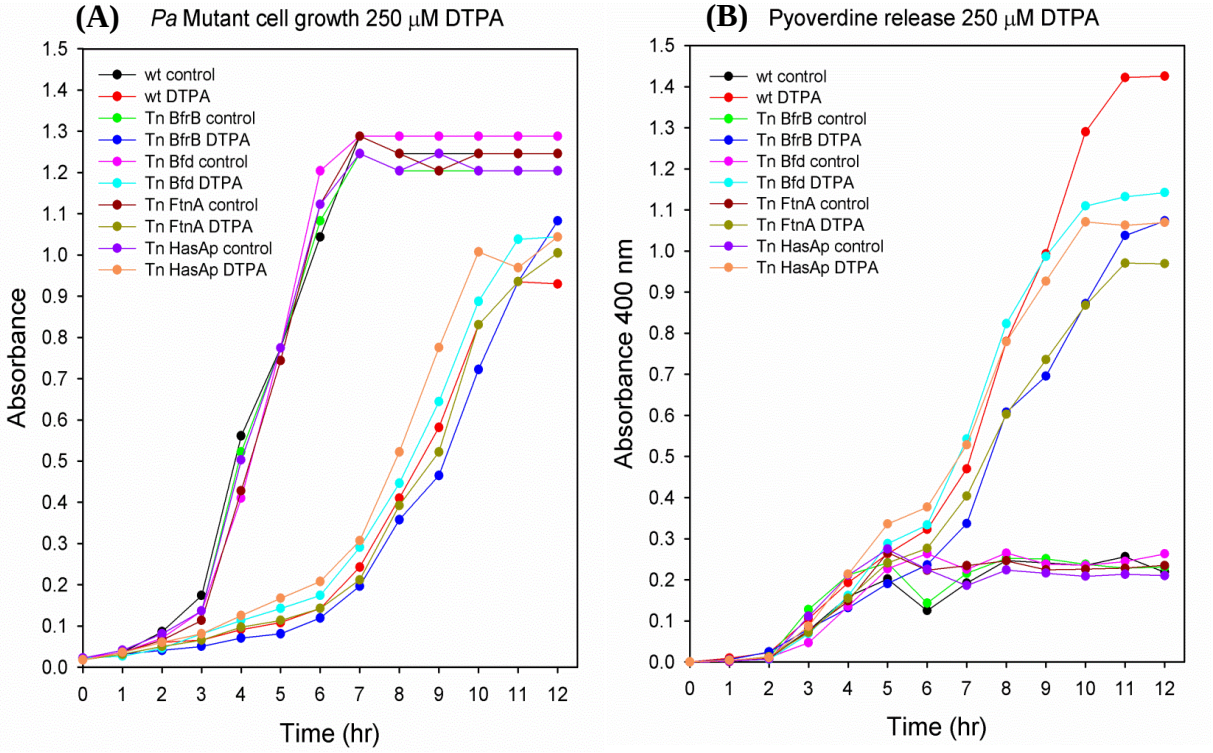


Figure 9: (A) Growth of Pseudomonas aeruginosa wildtype and Tn mutants in presence and absence of DTPA (250 µM) and (B) Release of pyoverdine by wildtype and Tn mutants

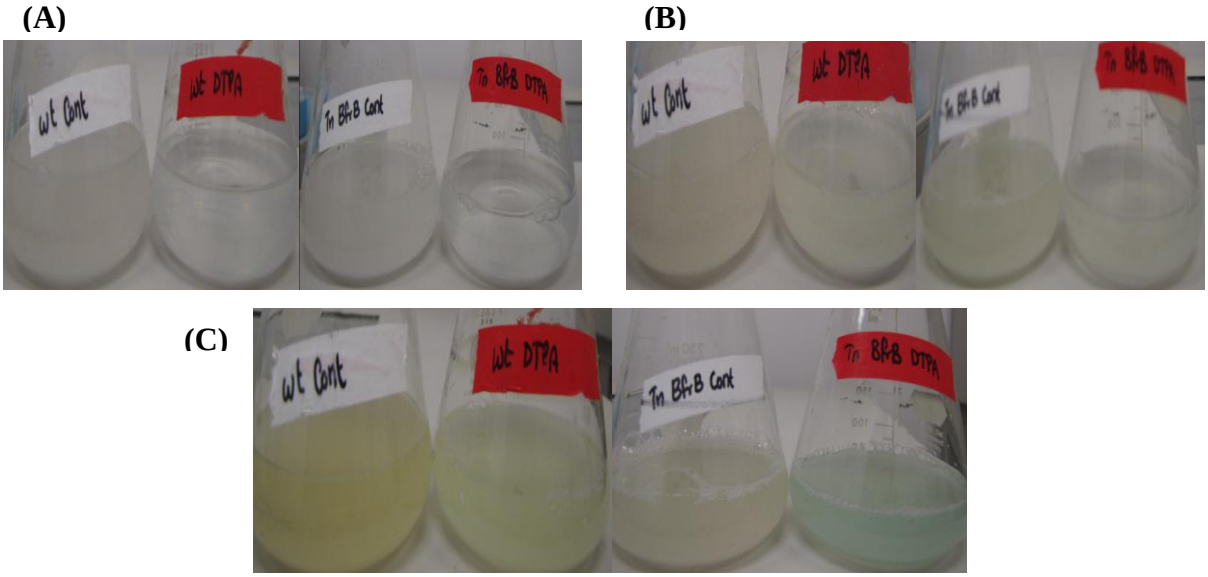


Figure 10: Cultures of Pseudomoas aeruginosa wildtype and Tn BfrB showing release of pyoverdine at different time periods in presence and absence of DTPA (250 µM) (A) at 4th h (B) at 8th h (C) 12th h

500 µM DTPA showed more retardation in growth than 250 µM. When compare to controls all the strains (wildtype and Tn mutants) showed very less growth and never reached plateau by 12th h. They had a very long lag phase of about 7 h to 8 h whereas lag phase for controls lasted for 2 h to 3 h with an exponential state started at 8th h. For controls lag phase of 3 h, exponential state from 4 h to 7 h or 8 h and remained in stationary phase till 12th h (Results not shown).

750 µM DTPA did not show much different results than 500 µM DTPA. Even with this concentration all the strains with DTPA showed more retarded growth and had a lag phase till 8th hour to 9th h and started exponential state from 10th h controls have same results as 500 µM DTPA (Figure 11).

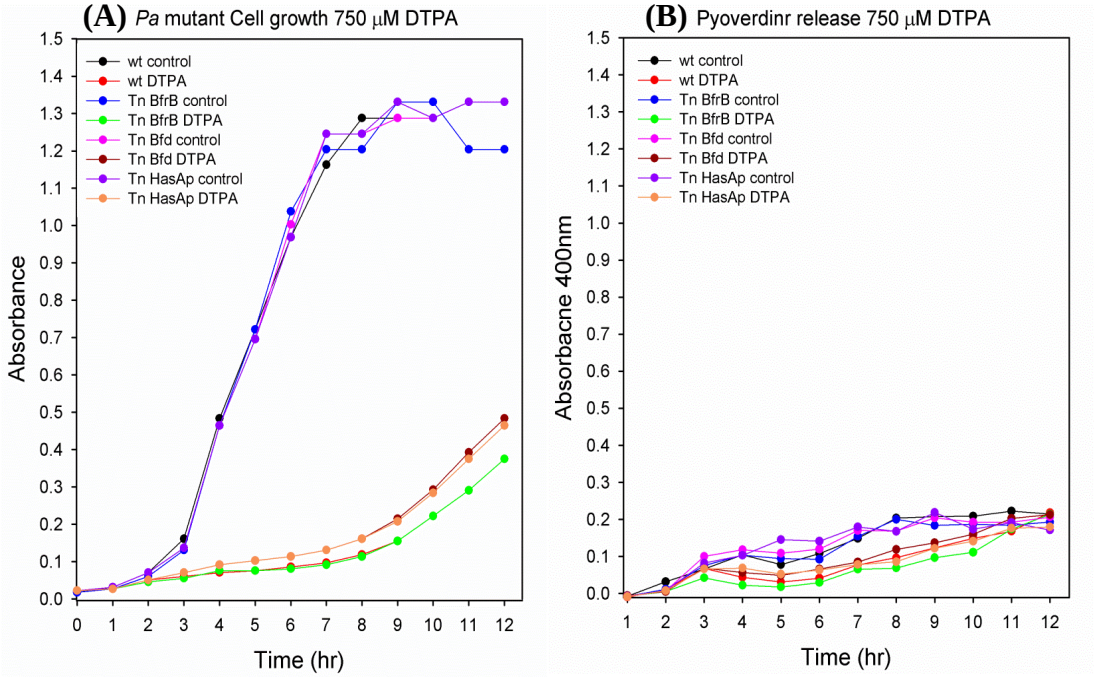


Figure 11: Graph showing growth pattern and pyoverdine release in presence of 750 µM DTPA (A) Growth graph of wildtype and Tn mutants. (B) Pyoverdine release by wildtype and Tn mutants

Discussion:

Transposon confirmation:

A PCR study followed by agarose gel electrophoresis was used to confirm presence of transposon. Results (Figure 5 and 6) confirms presence of transposon insertion in all the strains except in one stab culture of Pa Tn BfrB. Band with forward primer for Pa Tn BfrB and Pa Tn Bfd confirms presence of transposon in forward direction and a band with reverse primers for Pa Tn FtnA and Pa Tn HasAP confirms presence of transposon in reverse orientation. Absence of any molecular weight bands by wildtype in presence of specific and reverse or forward primer indicates positive result as transposon insertion is absent in wildtype.

Study on biofilm formation in presence of chelators

Growth studies are executed in aerobic conditions in presence and absence of Iron chelators to monitor siderophore secretion under iron limiting condition. Fe^{3+} chelators are used to monitor growth of wildtype and transposon mutants at 37°C. *Pseudomonas aeruginosa* under iron limiting conditions releases siderophore to sequester iron form surroundings. Pseudomonads in general releases different type of siderophores to survive but type of siderophores produced depend on species. In general one species of *Pseudomonas* produce only one type of pyoverdine but this is not true with *Pseudomonas putida* and *Pseudomonas aeruginosa* it is capable of producing 3 different types of pyoverdines (*22*) *Pseudomonas aeruginosa* is capable of producing pyocyanin a type of phenazine in iron limiting conditions (*23*).

In this study we demonstrated that under iron deficient condition *Pseudomonas aeruginosa* wildtype releases pyoverdine for capturing iron and Tn mutants which we studied

showed some difficulties in growing as concentration of iron chelators was increased. They struggled to grow by releasing more amount of pyoverdine at some concentrations and showed very minimal growth and unable to secrete pyoverdine at strong concentrations of iron chelators. Studies were performed using 250 µM, 500 µM and 750 µM concentrations of BP and DTPA in succinate minimal media at 37°C.

Spectroscopic analysis of pyoverdine release:

Pyoverdine has a characteristic peak at 400nm in succinate media and also produces a peak at 320nm which was believe as a peak of a purple compound. Even though purple color is not visible to eyes in any samples, absorption spectra showed an increasing peak of 320nm along with 400 nm in all Tn mutants without chelator with increase in time. As BP has a characteristic broad peak from 200 nm to ~330 nm we are unable to see any peak or shoulder at 320 nm indicating purple compound. In case of DTPA as it don't have any characteristic peak as with absorption spectra obtained with spectrophotometer Tn mutants with DTPA showed an increased peak at 310 nm and 600 to 750 nm along with 400 nm. This explains that Tn strains with DTPA releases something else other than pyoverdine. But the peaks at 310 nm and a hump at 600 nm to 750 nm suggest release of pyocyanin a phenazine which is believed to produce by *Pseudomonas aeruginosa* under poor conditions which enhances its the ability survive (*23*).

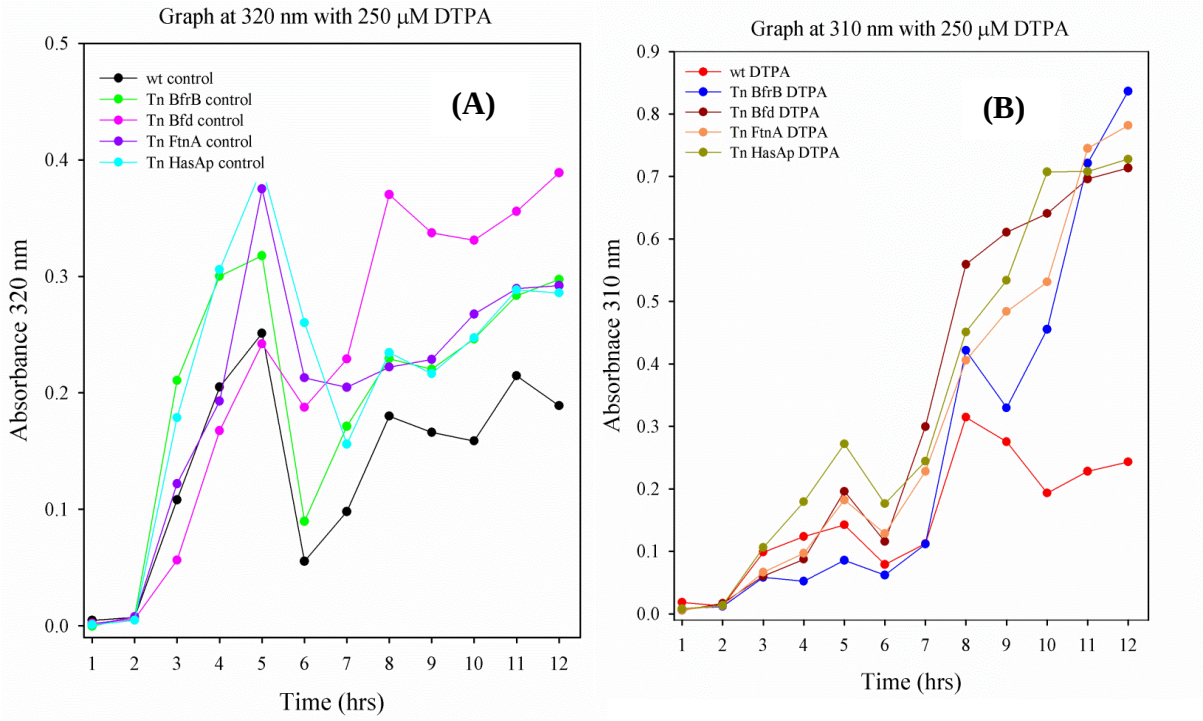


Figure 12: A) Graph showing increase in peak at 320 nm absorbance by increase in time, both in *Pa* wild type and *Pa* Tn mutant's controls without iron chelating agent (DTPA). (B) shows increase in absorbance spectra at 310 nm in *Pa* Tn mutants with time whereas retarded increase is observed in wt, these results were obtained from *Pa* wildtype and *Pa* Tn mutant's in presence of 250 µM DTPA.

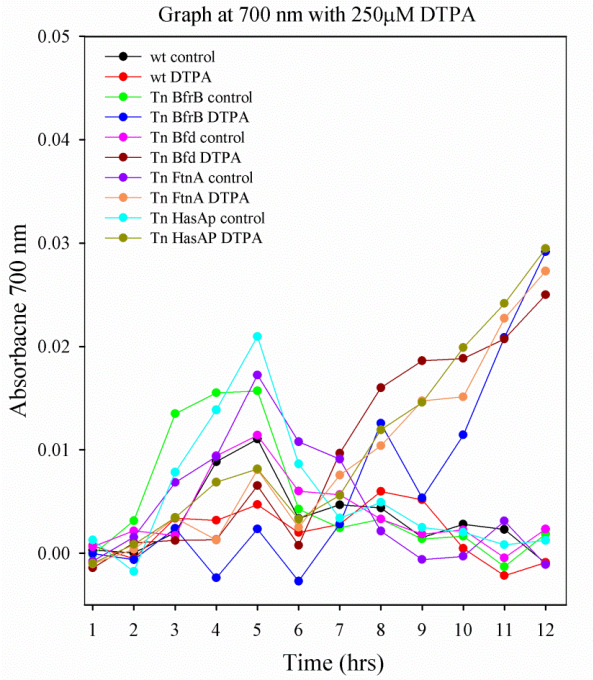


Figure 13: Increase in absorbance at 700 nm in *Pa* Tn mutant's with time and wildtype with and without chelators at 250 µM DTPA and *Pa* Tn mutant's without DTPA did not show any increase in spectrum at absorbance 700nm.

Release of this compound suggests during lag phase these mutants are releasing pyoverdine to sequester available iron from the media as Fe^{3+} and mean while reducing this Fe^{3+} to Fe^{2+} with help of this pyocyanin which helps in survival of Tn mutants. As this peak is visible only in Tn mutants and not in wildtype indicates that lack of particular gene in mutants is unable to process conversion of Fe^{3+} to Fe^{2+}forcing them to release pyocyanin. Presence of these peaks only in Tn mutants with DTPA and not with controls also indicates its struggle to sequester iron in succinate minimal media with a strong competitor. We think release of pyocyanin along with pyoverdine in strains with DTPA helps them to show growth by 8 h in experiments conducted with 500 µM and 750 µM DTPA when compare to BP where these peaks are absent which

causes no growth in strains at 750 µM BP. As these results are preliminary more experiments has to be performed to conclude these assumptions.

Conclusion:

We successfully identified and separated colonies of *Pseudomonas aeruginosa* PAO1 with transposon mutants as shown in Table 7 by conducting PCR studies. Growth studies of these strains along with wild type as control are performed in succinate medium at 37°C in shaker incubator for 12 h under aerobic condition in presence and absence of Iron chelator. Growth of *Pseudomonas aeruginosa* PAO1 wild type and Tn mutants was monitored using cell density by checking OD 600 nm for every 1 h and simultaneously monitoring release of pyoverdine for every 1h by absorption spectrophotometer.

The experimental results suggests in presence of chelators Tn mutants show impairment in growth at higher concentration (500 µM and 750 µM). In forced environment *Pseudomonas aeruginosa* secretes pyoverdine to sequester iron but it also capable of releasing pyocyanin a phenazine under iron limiting condition as in Tn mutants with DTPA. DTPA and BP more readily bind to Fe^{3+} than pyoverdine and acts as strong chelators. Results did not show distinct phenotype changes in biofilm formation with transposon mutants in succinate media indicating this media may not be preferable for this type of studies.

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CHAPTER V

SUMMARY

Pseudomonas aeruginosa is a ubiquitous, opportunistic gram negative bacterium which depends on iron for its survival and pathogenicity. *Pseudomonas* developed a mechanism to sequester iron from host cells and store in iron storage proteins like bacterioferritin, bacterial ferritin and DNA binding proteins in the form of Fe^{3+} for use when required. To sequester iron during iron limiting conditions, *Pseudomonas* acquired different strategies like releasing siderophores (pyoverdine) or hemeophores (Has) (*1*) to bind iron and transfer it into cells for its survival.

It was hypothesized that *Pseudomonas* contains two bacterioferritin proteins which coexist and termed as BfrB and BfrA. Recent studies suggest the product of bacterioferritin is separate, and BfrB can be purified and isolated as a separate protein (*2*). To understand the other type of so called bacterioferritins existence, structure and function was important as it was not classified properly. In the process of purifying and isolating BfrA, the researchers found a few interesting details of this BfrA protein in *Pseudomonas* with the help of different experiments and techniques like sequence alignment which helped us in naming and classifying BfrA protein as bacterial ferritin (*Pa* FtnA) but not bacterioferritin (*Pa* BfrB).

The other modes of survival during iron limiting conditions include the secretions of siderophores in search of available iron in host cells and stealing it. To understand the type of

siderophore released and to strengthen the idea of the importance of storage proteins in the presence of iron chelator studies were performed on transposon mutants. All results were drawn to conclusion and summarized as below.

1. **Isolation, purification and crystallization of *Pseudomonas* bacterial ferritin (*Pa* FtnA)**

Pa FtnA was expressed successfully and purified to homogeneity by using column chromatography. Crystal structures were obtained from purified protein under different conditions and pH as shown in chapter 2 (Figure 5, 6 and 7), which revealed the presence of a distinct ferroxidase center that resembles the ferroxidase center of ferritins from *E.coli*, *Pyrococcus furiosus* etc., (chapter2 Figure 1). The presence of site c is one of the unique features of ferritin molecule, and this in *Pseudomonas* indicates the product of the BfrA gene as bacterial ferritin but not bacterioferritin. Few more interesting details which strongly suggests this product as bacterial ferritin lacks methionine residue at the 52^{nd} place as in bacterioferritin which helps in binding heme which, in turn, helps in the release of electrons during iron release. In addition, the presence of heme moiety is a characteristic feature of bacterioferritin, and its absence in this protein raises many questions about its classification.

This research also suggests that *Pa* FtnA has different regulatory mechanisms to release iron from the inner core than *Pa* BfrB (*2*) does. It was demonstrated that *Pa* BfrB required NADPH, Bfd and Fpr for transferring electrons to the inner core and reducing Fe^{3+} to Fe^{2+} and releasing Fe^{2+}, but the current experiments show only NADPH and Fpr are sufficient in releasing iron from the *Pa* FtnA inner core. The presence of the Bfd gene adjacent to bacterioferritin was observed in many organisms as an evolutionary adaptation which has a functional role of

releasing iron in BfrB, but any such protein was not present adjacent to *Pa* FtnA; instead, it was located next to a catalase protein (*katA*)which is characteristic of ferritin proteins.

The experimental observations and results revealed that the product of the BfrA gene in *Pseudomonas aeruginosa* is not a bacterioferritin; instead, it is a bacterial ferritin and, hence, termed as ‘ *Pa* FtnA’ not as *Pa* BfrA.

2. Use of computational tools to identify protein-protein interactions in pathogenic bacteria

Genome search for bacterioferritin protein and Bfd in many pathogenic organisms revealed that almost all these bacteria have *bfd* gene adjacent to *bfr* gene similarly like *Pseudomonas aeruginosa*, whereas transcription orientation of these two genes varies from to genome. For our study we choose organisms which have transcription orientation in same direction for both these proteins similar to *Pseudomonas aeruginosa.* Alignment of BfrB sequence of Pseudomonas aeruginosa with all these pathogenic bacteria showed a similarity and identity percentage of 83.37% and 68.40% respectively. All these sequence showed conserved regions at residues which have important functions in *Pseudomonas aeruginosa*, for example all the residues involve in ferroxidase center (oxidation of Iron), four fold pore, three fold pore and B-pores (These pores involve in iron uptake and release) (Chapter 3, Figure 2). These conserved regions indicate, the structure and function of BfrB in all these selected pathogenic bacteria will be similar to that of Pseudomonas. Few residues which are conserved but do not know the function are believed to have some unknown function and think few may involve in protein-protein interactions. Even sequence alignment of Bfd protein with these pathogenic bacteria gave satisfactory results. All these Bfd like proteins contain 4 conserved cysteines as in Pseudomonas

which is arranged as **C**-X-**C**-X32-**C**-X2-**C** (Chapter 3, Figure 3). These residues are involved in iron-sulfur cluster formation in Pseudomonas which helps in electron transport during iron release. This resemblance indicates all these bfd like proteins in these pathogenic bacteria may contain similar structural formation and may have same function. The similarity in these two proteins in all selected bacteria implies these may also have similar type of residues involve in protein-protein interaction as in Pseudomonas which helps in iron release during iron deficiency conditions. Inhibitors which are studied in our lab to block BfrB-Bfd interaction in Pseudomonas can also show similar type of blocking even in these pathogenic bacteria.

3. **To study phenotypical changes in biofilm formation in pseudomonas aeruginosa by transposon mutants**

The experimental studies on transposon mutants suggest that under iron limiting conditions *Pseudomonas aeruginosa* is forced to release siderophores to acquire iron from host cells and transport it into the bacterial cell for its survival. Studies with chelating agents like BP and DTPA gave interesting results with wildtype and transposon mutants.

Experiments performed at different concentrations showed at higher amounts the growth of Tn mutants was retarded in presence of BP, and the release of pyoverdine was more abundant in Tn mutants with chelators than Tn mutants without chelators, This indicates Tn mutants need some time to adjust to new environments and then secrete siderophores called pyoverdines and steal iron from external environments and improves its growth; this was observed with lower concentrations of chelators in media with Tn mutants, but in presence of higher concentration (750 µM) of chelators, the growth of Tn mutants was totally stopped but could still release pyoverdine and is observable by absorbance spectra with a peak at 400nm (*3*) which shows their

struggle to survive. This is because in presence of higher concentrations of chelators all the available iron is chelated by BP, and even after releasing pyoverdine in this condition they could not acquire iron from these strongly bound chelators and transport it to bacterium for its growth.

The biofilm formation in presence of DTPA was almost similar to BP, but it is noteworthy to mention a few observations which suggest that under iron impaired conditions *Pseudomonas* not only releases pyoverdine but is also capable of releasing pyocyanin, a type of phenazine. Release of pyocyanin was confirmed by results obtained in presence of DTPA in Tn mutants and wildtype at higher concentrations (500 µM and 750 µM) (Figure 12 and 13, chapter 4), which showed a peak at a range of 600nm to 750nm (*4*) and gradually increases with time which also caused Tn mutants in presence of DTPA to show minor growth. The graph for DTPA showed extended lag phase with an increase in concentration; at the beginning of the exponential stage the researchers observed the release of pyocyanin, which demonstrates that at initial stages it releases pyoverdine, but when it senses the release of pyoverdine is not helping in sequestering iron, the bacterium is forced to release some other molecule to sequester iron for its survival.

These results did not show much of phenotypical changes in biofilm formation by *Pseudomonas aeruginosa* in succinate minimal medium.

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