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**Investigating the mechanism used by
Pyocin S3 to target and kill susceptible
Pseudomonas aeruginosa strains**

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Declaration

I hereby declare that unless otherwise stated, all work presented here is my own and has not been submitted for any other degree at the University of Nottingham or any other institute of learning.

Faisal Albaqami

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that causes infections with a high mortality and morbidity rate. *P. aeruginosa* produces bacteriocins (known as pyocins) that kill strains of the same species. Therefore, pyocins may have potential as alternatives to antibiotics as novel narrow-spectrum antimicrobials. Pyocin S3 (PyoS3), contains a DNase domain that kills targeted prey bacteria. The DNase domain of PyoS3 is interesting because it does not have an H-N-H fold and does not share sequence homology with type E colicins and other DNase S-type Pyocins. Little is known about the mechanisms that PyoS3 uses to enter prey cells or the structure of its domains. PyoS3 has been suggested to utilise the Type 2 pyoverdine (PvDII) receptor FpvAll to target prey cells. FpvAll uses the TonB1 system for translocation, whereas some colicins that share sequence homology with S-type pyocins (type A and E9 colicins) use the Tol system for translocation. Thus, we hypothesized that PyoS3 utilises either TonB or Tol for translocation.

Genetic engineering was performed to separately overexpress TonB1, TolAIII, and two versions of PyoS3: the receptor and translocation domains (R+T), and the full-length protein with its cognate immunity protein (immS3) to prevent the producing cells from undergoing suicide. These proteins were purified via metal chelation affinity chromatography and size-exclusion chromatography (SEC). Analytical SEC was performed with the aim of detecting potential interactions between these proteins. Evidence for formation of a complex between PyoS3 R+T and TonB1 was obtained, but no complex was evident between PyoS3 R+T and TolAIII. This result supports the hypothesis that PyoS3 utilises FpvAll for uptake, since FpvAll is a TonB-dependent transporter.

To identify genes involved in the uptake and translocation of PyoS3 into prey bacteria, Tn5 transposon mutagenesis was conducted with the PSA 892 PyoS3-sensitive *P. aeruginosa* strain. Three mutants were analysed, mutants #44, #107 and #362. In comparison to the PSA 892 parental strain, mutant 44 and mutant 362 showed smaller and larger PyoS3 growth inhibition zones, respectively. The transposon insertions in mutant 44 is located in a gene

encoding the LuxR family transcriptional regulator VqsR and in the gene encoding a short-chain dehydrogenase reductase (SDR) family oxidoreductase in mutant 362. Both of these genes regulate quorum-sensing (QS) in *P. aeruginosa*; thus, QS may play a role in the uptake of PyoS3. Mutant 107 was completely resistant to PyoS3-mediated killing and contains a transposon insertion within the *pvdM* gene. PvdM is a protein that delivers ferribactin into the periplasm to the PvdQ protein for Pvd maturation, then the PvdQ delivers the Pvd to PvdRT-OpmQ efflux pump to release it out from the cell. In addition, PvdM shares sequence homology with TonB-dependent transporters, which might explain why the PvdM mutant was fully resistant to PyoS3.

Finally, we attempted to determine the crystal structure of PyoS3-Imm3. However the resulted crystal analysis using the Cambridge Structural Database determined the crystals were an imidazole salt. Furthermore, Crystals were successfully grown in a new set of screens; however, X-ray diffraction analysis could not be conducted due to Covid-19 restrictions. Future work could analyse the newly grown crystals to determine if they are proteinaceous and generate X-ray diffraction patterns to solve the structure of PyoS3.

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I would like to take this chance to sincerely thank my supervisors Christopher Penfold and Kim Hardie for their constant guidance, advice, and endless support during my PhD. Also, I would like to thank them from the bottom of my heart because of their support aside from my academic work, especially when they know about my beloved father's illness and when he passed away a couple of months back they were very understanding and supportive in every way possible, without their care I do not think I would reach to this stage in my academic studies.

I would also want to take this opportunity to thank Philip Bardelang, Thomas Warwick, Siyu Wu and Jean Dubern for their continuous support and for teaching me the methodology of my research and for being there for advice whenever I need them. Furthermore, I would, like to thank every member of Kim Hardie's group, all members of the Department of Life sciences and all of the Biodiscovery institute building personnel who helped me through my PhD course.

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Finally, I would like to dedicate this work to my hero, my beloved father; may he rest in peace. He was the first one to support me in this journey to pursue my higher education. He is always there for me and always giving me advice in my career and life, he also supports me whenever I need it. His passing took a toll on me mentally. I could not perform well in my work or in my normal life. In the end what I want to say is I will we will always remember you as my rock my friend and my mentor.

Statement of COVID impact

As a husband and a PhD student, the Covid-19 pandemic hit me hard physically and mentally. My wife who is severely asthmatic and my mother who is ill put me under a lot of stress because I'm always either looking after my wife so that she would not get infected or afraid to receive calls from my home country delivering bad news. During the pandemic, I lost a lot of people who are close to me. I lost my cousin who is my age, and we were close and also I lost my aunt who is like a grandmother to me.

In my PhD work, it did affect me tremendously because since the Covid-19 restriction have been placed, I stopped for a couple of months until we get into the labs and then when we got back the time limited to work in the lab because of the restriction prevented me to do long term experiment due to the limited access to the instruments to do them and due to the limited time I'm allowed into the labs and building as well as ordering materials to conduct experiments needed takes a lot of time to do and the materials to arrive. This meant that the final experiments that I must do could not be conducted, which affected the final results of my thesis. In addition to all of what has been stated I caught COVID three times during my writing up which exhausted me tremendously and affected my writing up greatly since I need more focus and energy to work. Furthermore, when everything went back to normal last year, in July my father suddenly got sick, so I had to go back to my home country to see him. From that time until early this year I was frequently travelling between the UK and my home country to check on my father since my big brother is in the military and cannot leave his post and my mother is ill and the rest of my siblings were young who do not know how to handle my father. On the 31st of March this year my father passed away and it did affect me so much that I could not get back to work until May. My supervisors were very supportive and were with me every step of the way and I could not make it without them in my difficult times.

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List of abbreviation

AHLs	<i>N</i> -acyl homoserine lactones
ATP	Adenosine triphosphate
BAM	β -barrel assembly machinery
bp	Base pair
CF	Cystic fibrosis
CPA	Common polysaccharide antigen
DNA	Deoxyribose nucleic acid
eDNA	Extracellular DNA
Mbp	Million base pairs
MDR	Multidrug resistant
dH ₂ O	Deionised water
EDTA	Ethylenediaminetetraacetic acid
HAIs	Healthcare-associated infections
h	Hour(s)
His	Histidine
IM	Inner membrane
IPTG	Isopropyl β -D-1-thiogalactopyranoside
ITC	Isothermal titration calorimetry
kbp	Kilobases
kDa	Kilo Dalton
LB	Lysogen broth
LPS	Lipopolysaccharide
Mbp	Million base pairs
mg	Milligram
min	Minutes
mL	Milliliters
M	Molar
MWCo	Molecular Weight Cut-off
NADH	Nicotinamide adenine dinucleotide

OD600	Optical density at a wavelength of 600 nanometers
OM	Outer membrane
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDB	Protein Databank
PG	Peptidoglycan
PMF	Proton motive force
PMSF	Phenylmethylsulfonyl fluoride
PQS	<i>Pseudomonas</i> quinolone signal
PvD	Pyoverdine
PyoS3	Pyocin S3
QS	Quorum sensing
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
TBDTs	TonB-dependent transporters
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
μg	Microgram
μl	Microliter
μM	Micromolar
UV	Ultraviolet
X-Gal	(5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside)
% (v/v)	Percent volume per volume
% (w/v)	Percent weight per volume
°C	Degree Celsius
βOMPs	β-barrel outer membrane protein

1 Chapter 1. General introduction

1.1 *Pseudomonas aeruginosa*:

P. aeruginosa is an aerobic, Gram-negative, rod-shaped bacterium that is motile, non-spore-forming and non-lactose-fermenting. This bacterium is oxidase-positive, which differentiates it from other Gram-negative bacteria (Fujitani, 2017). *P. aeruginosa* is widespread in the hospital environment and other places such as freshwater and soil. It can also be found in sinks, respiratory therapy equipment, swimming pools, medicine, antiseptics, and contaminated water (Livermore, 2002). The spread of *P. aeruginosa* may be due to its ability to colonize different surfaces, as well as utilize numerous compounds in the environment as an energy source (Lyczak et al., 2000, Favero et al., 1971, Lister et al., 2009).

The genome size of *P. aeruginosa* is 6.3 million base pairs (Mbp), encoding 5567 genes, larger than that of many other bacteria such as *Escherichia coli* K12 (4.64 Mbp, 4279 genes), *Staphylococcus aureus* N315 (2.81 Mbp, 2594 genes), and *Haemophilus influenza* Rd (1.83 Mbp, 1714 genes). Furthermore, the predicted proportion of regulatory genes for *P. aeruginosa* is higher than for all other sequenced bacterial genomes, and this is presumably related to its ability to adapt to different environments (Alhazmi, 2015).

1.2 Multidrug resistance in *P. aeruginosa*:

P. aeruginosa is found in the environment, and it is unlikely to cause infection in a host with a healthy immune system; as an opportunistic pathogen, it takes advantage of a compromised immune system to cause infection. *P. aeruginosa* is responsible for 10–20% of nosocomial infections in immunosuppressed patients, such as individuals suffering from acquired immunodeficiency syndrome (AIDS), chemotherapy-treated patients, cancer patients and transplant patients (Lyczak et al., 2000, Bodey et al., 1983, Franzetti et al., 1992).

P. aeruginosa is one of the leading infections found in burns patients, and causes the highest mortality rate (Lipový et al., 2010). *P. aeruginosa* is also the most significant pathogen causing pulmonary infection, and the highest cause of morbidity and mortality in cystic fibrosis (CF) patients. (Bhagirath et al., 2016). Mutations in cystic fibrosis transmembrane conductance regulator (CFTR), which transports chloride and bicarbonate ions across multiple epithelial surfaces, cause cystic fibrosis. When CFTR is dysfunctional, chloride and bicarbonate ions cannot be transported across epithelia, leading to multiple organ dysfunction, that leads to diseases such as meconium ileus, distal intestinal obstruction, exocrine pancreatic insufficiency, growth disturbances, male infertility, and chronic and recurrent pulmonary infections. Due to Impaired mucociliary clearance (MCC) via dehydrated airway surface liquid (ASL) and mucus the *P. aeruginosa* with its arsenal of toxins and its great ability to adhere to epithelial cells, the bacterium thrive in the lung of CF patients (Malhotra et al., 2019). Around 80% of CF patients at the age of 20 were found to be diagnosed with a chronic lung infection caused by *P. aeruginosa* (Bhagirath et al., 2016).

The ability of *P. aeruginosa* to form biofilms makes it difficult for effective antibiotic treatment because biofilms provide a physical barrier that protect bacterial cells against antibiotic penetration. *P. aeruginosa* can form biofilms on catheters and ventilators, thereby causing infections in patients, including urinary tract infections or ventilator-associated pneumonia (Vital-Lopez et al., 2015, Mulcahy et al., 2014).

P. aeruginosa is one of the most common infectious agents causing nosocomial infections mediated through the presence of antimicrobial resistance genes. *P. aeruginosa* can acquire such genes by horizontal gene transfer from different sources (such as other bacteria or bacteriophages), or endogenously through chromosomal mutation. Mechanistically, the low permeability of its outer membrane helps *P. aeruginosa* to resist certain antibiotics. In addition, the natural efflux systems in *P. aeruginosa* mediate antibiotic resistance, and this helps the bacterium to removing unwanted substances. There are 12 efflux pumps in *P. aeruginosa*, and the most studied are MexAB-OprM, MexCD-OprJ,

MexEF-OprN, and MexXY. An example of an efflux system contributing to bacterial resistance is the MexAB-OprM pump system (Figure 1.1), which is reported to pump out drugs such as β -lactams, chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides, tetracycline, and trimethoprim, thereby making *P. aeruginosa* resistant to these antibiotics (Livermore, 2002, Poole, 2011, Mesaros et al., 2007, Ivanov et al., 2022, Turk, 2011).

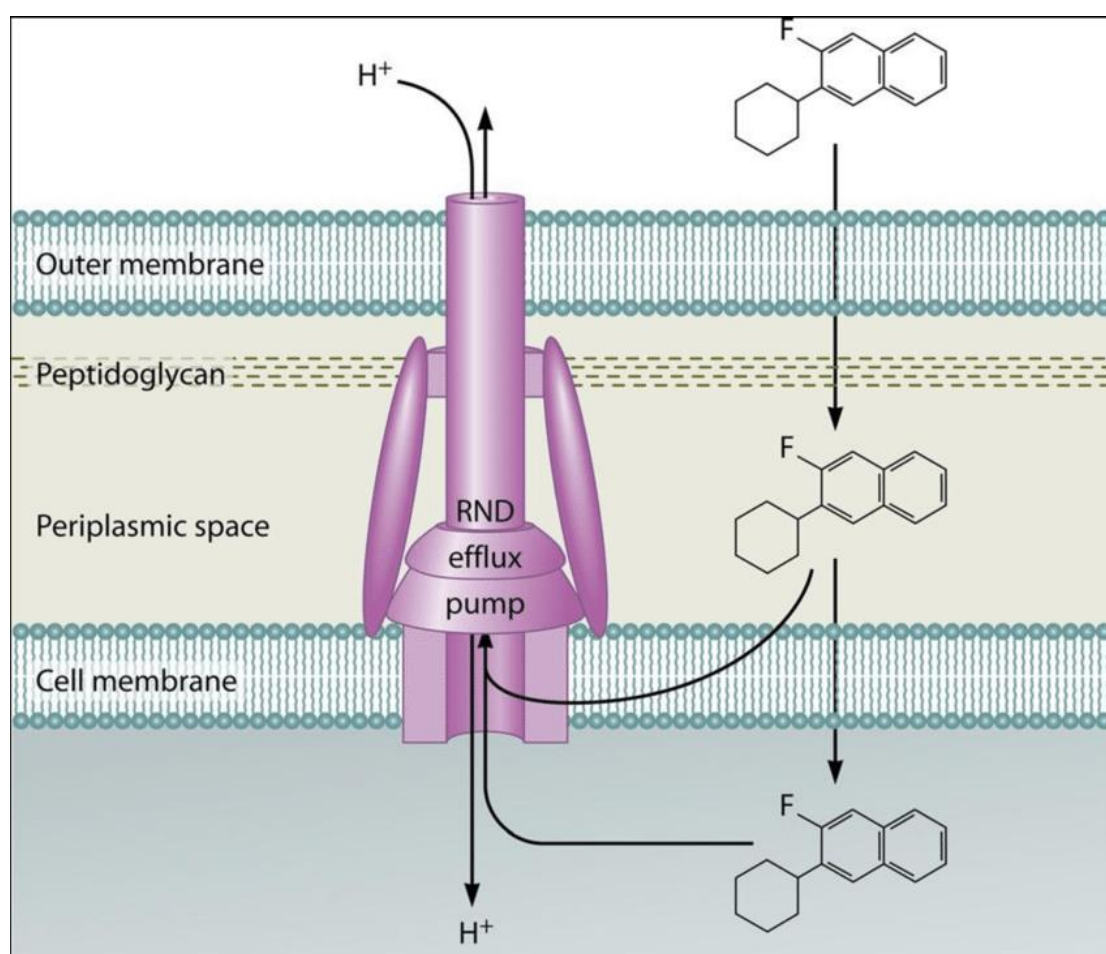


Figure 1.1: Schematic diagram of an archetypal efflux pump system. The pump consists of three components: (1) the pump (MexB) located in the cytoplasmic membrane attached to (2) the exit portal in the periplasmic membrane (OprM) via (3) the lipoprotein linker (MexA) (Livermore, 2002, Turk, 2011).

The rapid increase in resistant bacteria prevents the health benefits of antibiotics from being realized. Widespread resistance has led to a healthcare crisis due to the lack of development of new antibiotics by pharmaceutical companies and the overuse of drugs. One of the most common causative agents for healthcare-associated infections (HAIs) is *P. aeruginosa*. Yearly, multidrug resistant (MDR) *P. aeruginosa* is reportedly responsible for 13% of HAIs. Certain MDR *P. aeruginosa* strains have been discovered to be resistant to almost all kinds of antibiotics, including cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones (Ventola, 2015).

1.3 The Gram-negative bacterial cell envelope:

In the late nineteenth century, Hans Christian Gram first classified bacteria into two categories: Gram-negative bacteria and Gram-positive bacteria. He established this classification based on whether bacteria retained a purple-coloured stain or not. This stain became to be known as the Gram stain (Gram, 1884), with purple-dyed bacteria known as Gram positive and bacteria that do not retain the stain as Gram negative. Structural differences in the cell envelope make bacteria positive or negative to the Gram stain. In Gram-positive bacteria such as *Staphylococcus aureus*, the cell envelope consists of one cytoplasmic membrane and a cell wall of thick peptidoglycan (PG) exposed to the environment. By contrast, Gram-negative bacteria such as *P. aeruginosa* have a cell envelope consisting of an inner membrane (IM), a periplasmic space that contains a thin PG, then an outer membrane (OM), as shown in Figure 1.2 (Silhavy et al., 2010, Murray et al., 2020).

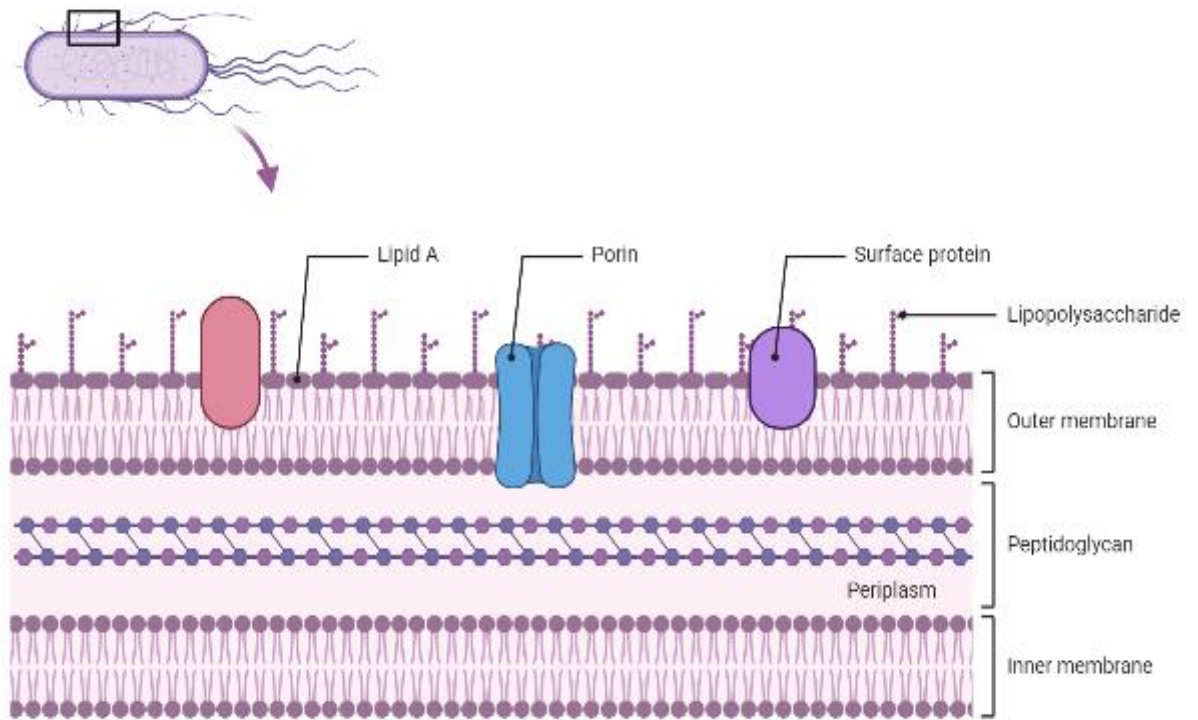


Figure 1.2: The Gram-negative bacterial cell envelope. *Pseudomonas aeruginosa* cell dimensions are 0.5 to 0.8 μm by 1.5 to 3.0 μm . Cells are similar to any other Gram-negative bacteria with an (IM), a periplasmic space containing a peptidoglycan layer, and an (OM) containing lipopolysaccharide (LPS), surface proteins, lipid A and porins that span to the periplasm (Nagoba and Pichare, 2020, Baron, 1996, Murray et al., 2020).

1.4 The outer membrane:

The OM of Gram-negative bacteria is a lipid bilayer with phospholipids on the inner side, such as phosphatidylglycerol, cardiolipin, and phosphatidylethanolamine. The outer side of the OM mainly consists of LPS. LPS consists of lipid A, which is a hexa- or hepta-acylated glucosamine disaccharide, and O-antigen, consisting of a polysaccharide core and varying polysaccharide chains (Raetz and Whitfield, 2002).

The OM works as a shield to protect bacteria from the outer environment, and as a permeability barrier that controls transportation of substances such as toxins and small diffusible metabolites important in nutrition. Regulation of the permeability of the OM is dependent on proteins, of which most are lipoproteins or β -barrel OM proteins. Lipoproteins are anchored into the inner side of the

OM via an acyl chain which is attached to an N-terminal cysteine residue (Sankaran and Wu, 1994, Grabowicz, 2019).

OM proteins (OMPs) have a β -barrel structure and they function as porins, specific ligand transporters, virulence factors, and enzymes. Porins allow the permeability of small molecules such as ions, amino acids, and small sugars. Specific ligand transporters such as BtuB take up and import large ligands like vitamin B12 (Delcour, 2009).

1.5 The inner membrane (IM):

The Gram-negative bacterial IM consists of a balanced bilayer of glycerophospholipids with the same structure as the inner side of the OM. The difference between eukaryotes and prokaryotes is that eukaryotes have intracellular organelles that play important roles in cellular processes. For example, energy is produced by mitochondria, lipids are synthesized by smooth endoplasmic reticulum (ER), the rough ER is where protein secretion occurs, and the cytoplasmic membrane contains receptors that sense the environment, and there are nutrient and waste product transport systems. By contrast, the lack of intracellular organelles in bacteria means that all membrane functions of eukaryotic organelles are carried out in the IM of the bacterial cell. Many of the proteins that function in lipid biosynthesis, transport, protein secretion, and energy production are located in the IM of the bacteria, and they are conserved (Raetz and Dowhan, 1990, Silhavy et al., 2010).

1.6 The periplasm:

The periplasmic space is stationed between the OM and the IM in Gram-negative bacteria. The periplasm is a gel-like matrix containing embedded peptidoglycan (Seltmann and Holst, 2002). The periplasm provides the cell with structural systems that add to its capability for enduring turgor pressure. Structural systems such as the PG and lipoproteins, multidrug efflux systems, and production platforms that participate in the secretion of uniquely structured beta-barrel proteins, lipoproteins, and glycerolphospholipids of the OM are

present in the periplasm (Miller and Salama, 2018). The periplasm can isolate harmful enzymes such as alkaline phosphatase and RNase A, which might indicate that the periplasm is an ancestor of the eukaryotic lysosome (Silhavy et al., 2010).

1.7 The peptidoglycan cell wall:

The PG is located within the periplasm. Structurally, the PG consists of repeating units of the disaccharide *N*-acetyl glucosamine-*N*-acetyl muramic acid, adjoined by a five amino acid stem peptide (Vollmer, 2008), and it provides bacterial cell shape due to its strength and rigidity. Through Braun's lipoprotein (Lpp), a covalent attachment links the PG and the OM (Braun, 1975). Additionally, they are linked via non-covalent interactions with OMPs and other lipoproteins (Park et al., 2012). On removing the PG, the bacterial cell can still be active as a spheroplast; however, the cell loses its shape and becomes highly vulnerable to lysis (Silhavy et al., 2010).

1.8 Virulence factors:

1.8.1 Siderophores:

Iron is an essential component for most organisms, but due to the low solubility of this element in nature, microorganisms have developed ways of scavenging iron in order to use it. Some bacteria such as *Borrelia burgdorferi* do not require iron for it to function or cause infection (Kim et al., 2021). Most Bacteria produce low-molecular-weight components called siderophores that bind to iron and transform it inside the cell as ferrisiderophores (Hider and Kong, 2010). Other iron transport system is found in bacteria called Feo and it is a type of iron transport system found in many bacteria such as *E. coli*, that differs from siderophore-dependent systems. Ferrous iron is transported instead of ferric iron (Andrews et al., 2003). *P. aeruginosa* produces two kinds of siderophore under iron-limited conditions: pyoverdine and pyochelin. In addition to its ability to bind and transport utilize iron, siderophores can perform other functions. For example, two secreted virulence factors produced by *P. aeruginosa*, an

endoprotease PrpL and an exotoxin A, have been found to be regulated by pyoverdine. Proteins FpvA, FpvR, and PvdS participate in the regulation process. When Pvd is present on the cell surface and interacts with the FpvA receptor protein, signaling is initiated. As a result of FpvA's interaction with the periplasmic domain of FpvR, a signal is transmitted through the cytoplasmic membrane to the cytoplasmic domain of FpvR. It is through this event that PvdS becomes active, interacting with RNA polymerase to initiate the transcription of genes that produce pyoverdine, exotoxin A, and PrpL protease (Lamont et al., 2002).

1.8.2 TOXINS:

P. aeruginosa secretes enzymes and toxins that affect the host (Figure 1.3). For example, alkaline elastase destroys some of the complement components and immunoglobulins of the host, which helps the bacteria evade the host immune response and invade host tissues (Howe and Iglewski, 1984, Oldak and Trafny, 2005). *P. aeruginosa* produces two phospholipase C enzymes (PLCs): hemolytic PLCs (PlcHR) and non-hemolytic PLCs (PlcN). Both can hydrolyze phospholipids in eukaryotic cells. In addition, high doses of purified PlcHR, when injected into mice, may cause vascular permeability, end-organ damage, and death (Orndorff et al., 1999) (Hassuna et al., 2020, Wiener-Kronish et al., 1993).

Furthermore, *P. aeruginosa* produces two ADP ribosyltransferase toxins, Exotoxin A (ExoA) and Exotoxin S (ExoS). ExoA, secreted by the Type II secretion system, is the exotoxin that causes most damage to host cells. It can cause tissue necrosis and can cross over into the bloodstream. In addition, ExoA inhibits protein synthesis by suppressing elongation factor-2 (EF-2), thereby leading to cell death (Pavlovskis et al., 1978, Javanmardi et al., 2019). ExoS can help *P. aeruginosa* to adhere to epithelial cells of the host. It plays a role in causing damage to immunoglobulin A and G, which contributes to macrophage resistance through depolymerization of actin filaments and can disrupt cytoskeletal organization. This could contribute to the resistance of *P.*

aeruginosa to macrophages (Ben Haj Khalifa et al., 2011, Javanmardi et al., 2019).

The type III secretion system (TTSS) is a channel through which *P. aeruginosa* injects toxins directly into the host cell. TTSS is a pilus-like structure which is used to translocate effector proteins through a needle-like appendage into the eukaryotic cell, forming a pore through the cell membrane. TTSS has other known toxins beside ExoS, including Exotoxin Y (ExoY), Exotoxin T (ExoT), and Exotoxin U (ExoU). ExoY is encoded in most isolated strains, but only recently was its effect on host cells explored. Additionally, it is believed to play a role in limiting the cytotoxicity induced by other effectors. Despite the presence of ExoY in a variety of *in vitro* phenotypes, it does not appear to be relevant in some *in vivo* models despite its contributions to corneal virulence. (Kipnis et al., 2006, Kroken et al., 2022) A minority of isolates express ExoU, one of the most potent A2 phospholipase enzymes, and these are classified as 'cytotoxic strains'. Furthermore, most *P. aeruginosa* invasive strains encode ExoT and with ExoS, and these strains can invade and thrive in multiple infection sites in hosts including bladder, lung, and cornea (Kipnis et al., 2006, Kroken et al., 2022).

1.8.3 Biofilms:

The ability of *P. aeruginosa* to form biofilms makes it difficult for antibiotics to work. These bacteria can form biofilms on catheters and ventilators, thereby causing infections in patients, and biofilms can cause urinary tract infection or ventilator-associated pneumonia (Vital-Lopez et al., 2015, Mulcahy et al., 2014). Biofilms are formed from communities of bacteria in a self-made extracellular polymeric substance (EPS) that holds the cells together and allows them to adhere to a surface, such that 85% of the biofilm's biomass is made up of EPS. The main components of EPS are biomolecules, exopolysaccharides, extracellular DNA (eDNA), and polypeptides, which make EPS highly hydrated (Rasamiravaka et al., 2015). There are three exopolysaccharides produced by *P. aeruginosa*: alginate, Pel, and Psl. These polysaccharides are vital for biofilm stability (Ghafoor et al., 2011, Le Mauff et al., 2022). Alginates, linear

polyanionic exopolysaccharides containing uronic acids, are produced by *P. aeruginosa* when CF patients are infected. Alginates in biofilms cause the susceptibility of bacteria to antibiotic treatments and the human immune system to be reduced. Pel is a polymer in which glucose is abundant and resembles cellulose. The biofilm-forming *P. aeruginosa* PA14 community relies on Pel for cell-to-cell interactions, acting as a structural scaffold for the community at the very beginning of the biofilm development process. The Psl polysaccharide contains mannose and galactose, supports the attachment of the first biofilm to the host, and facilitates the development of mature biofilms (Ghafoor et al., 2011, Le Mauff et al., 2022).

eDNA plays a vital role as a functional part of the biofilm matrix (Sarkar, 2020). Exposure to DNase can prevent the formation of biofilms (Swartjes et al., 2013, Sarkar, 2020). Biofilms deficient in eDNA are more sensitive to the detergent sodium dodecyl sulfate (Yang et al., 2007). One study observed a 90% reduction in biofilm formation within only 5 minutes of contact post-treatment with DNase-I+Mg²⁺, thus *in situ* biofilm prevention and reduction can be achieved by using DNase-I as an effective antibiofilm agent (Sharma and Pagedar Singh, 2018). During starvation periods, eDNA also plays a role as a source of nutrition for the bacteria (Mulcahy et al., 2010) (Finkel and Kolter, 2001).

1.8.4 Quorum sensing:

Quorum sensing (QS) is a widely known mechanism of bacterial cell-to-cell communication, and is involved in biofilm formation and expression of virulence genes (Figure 1.3) (Smith and Iglewski, 2003b, Smith and Iglewski, 2003a). QS involves the production of a small chemical signaling molecules in a population density-dependent manner. QS in *P. aeruginosa*, as in many Gram-negative bacteria, is controlled by signaling molecules called *N*-acyl homoserine lactones (AHLs). Two AHL QS systems regulate the expression of numerous *P. aeruginosa* virulence factors and genes. These two systems are known as LasI-LasR (LasIR) and RhII-RhlR (RhlIR). The production of the *N*-3-oxo-dodecanoyl homoserine lactone (3OC12-HSL) QS signal is a consequence of

the synthase LasI. Next, 3OC12-HSL binds to the transcription factor LasR, which activates the expression of genes including *lasI*, *rhII*, and *rhIR*. Since one of the genes upregulated is part of the LasI synthase, a positive feedback loop is triggered which rapidly ramps up the response. RhII initiates the production of the QS signal butyryl-HSL (C4-HSL), which then binds to the transcription factor RhIR. Expression of *rhII* and other genes is activated by RhIR (Waters and Bassler, 2005, Schuster et al., 2013). It was observed that for LasR induction of RhIR in *P. aeruginosa* strain PAO1, the QS system is required. For this reason, deletion of either *lasR* or *lasI* in PAO1 results in downregulation of the expression of genes activated by QS (Kostylev et al., 2019). Another *P. aeruginosa* QS signal, *Pseudomonas* quinolone signal (PQS), PQS acts primarily through the 2-heptyl-3-hydroxy-4-quinolone, which is connected to the LysR-type transcriptional regulator PqsR, to stimulate virulence genes. Furthermore, biofilm development and virulence factor generation are reduced in null mutants of the PQS system, including pyocyanin, elastase, lectin, and rhamnolipids. Moreover, the presence of PQS has been detected in sputum from CF patients infected with *P. aeruginosa*. Moreover, PQS is known to regulate 182 genes, including a large number that are independent of PQS (Lin et al., 2018, Warriar et al., 2021).

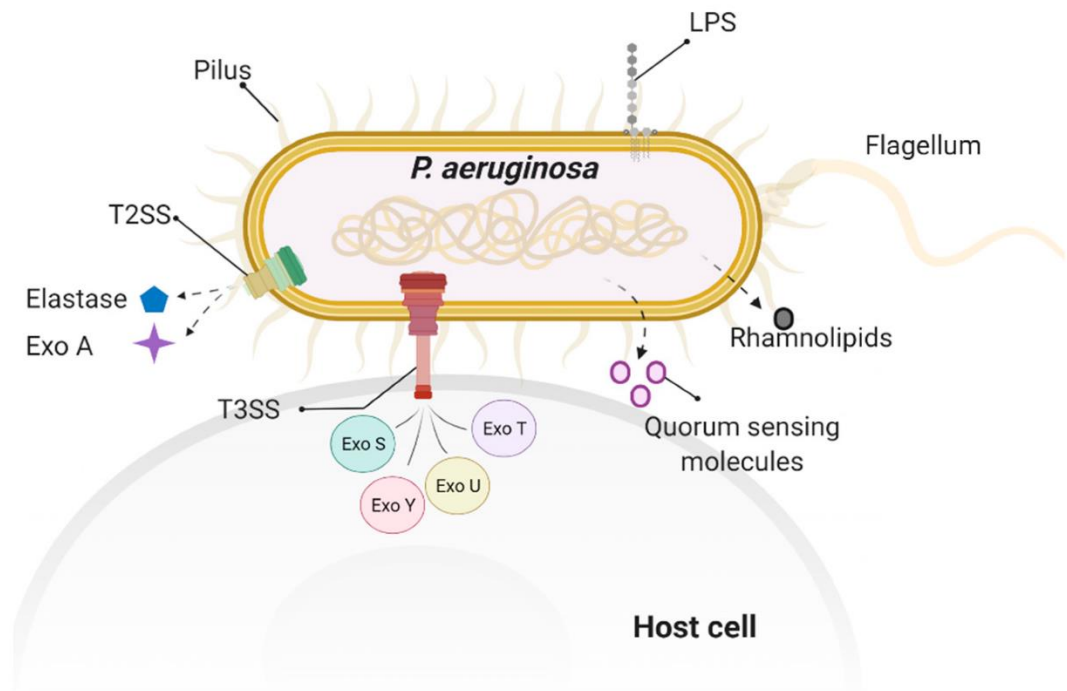


Figure 1.3: *P. aeruginosa* virulence factors. This bacterium has numerous virulence factors including surface factors such as the flagellum, pilus, and LPS. In addition, it secretes factors such as the type III secretion system (T3SS) and rhamnolipid, and it has quorum-sensing factors such as N-(3-oxododecanoyl)-L-homoserine lactone (Wagener et al., 2021).

1.9 Protein translocation systems in bacteria:

Due to the complicated structure of the cell envelopes of Gram-negative bacteria, specialized protein secretion systems have evolved. As a result, these bacteria can export their virulence factors to the cell surface or beyond, which allows them to have direct contact with target cells and the environment, from where they can scavenge nutrients or find protection against the host organism.

1.9.1 The Sec and Tat secretory systems:

The Sec system is essential for bacteria. As a front end to a number of subsequent protein-sorting and sub-cellular-targeting mechanisms in bacteria, the Sec pathway catalyzes the secretion of most proteins (Papanikou et al., 2007, Troman and Collinson, 2021, Korotkov et al., 2012a). Proteins are translocated via the Sec pathway in their unfolded state. The Sec system contains three parts: a protein targeting component, a motor protein, and a

SecYEG translocase (a membrane-integrated conducting channel). The Sec system secretes proteins that perform various roles, many of which are related to bacterial virulence (Papanikou et al., 2007, Troman and Collinson, 2021, Korotkov et al., 2012a).

Unlike the Sec pathway, folded proteins are secreted by the twin-arginine translocation (Tat) pathway. The Tat signal consists of a pair of arginines located in the S-R-R motif at the N-terminus of the folded protein. Proteins secreted via the Tat system in Gram-negative bacteria may remain periplasmic or be secreted out of cells through the Type II secretion system, unlike Gram-positive bacteria in which the Tat system releases proteins extracellularly. The Tat pathway is crucial for the physiology and survival of pathogenic and non-pathogenic bacteria. Moreover, for pathogenic bacteria such as *P. aeruginosa* to display full virulence in models of animal infection, they require a functional Tat pathway. One example of a Tat-secreted protein is the phospholipase C enzyme, a virulence factor for *P. aeruginosa*. In addition, the tat system is required for the *P. aeruginosa* PQS to function (Ochsner et al., 2002) (Robinson and Bolhuis, 2004) (Müller, 2005, Soh et al., 2021).

1.9.2 The β -barrel assembly machinery (BAM):

When β -barrel outer membrane proteins (β OMPs) are translocated via the Sec system into the periplasm, they must be inserted into the OM in an efficient folded form. This process is performed by the β -barrel assembly machinery (BAM) complex, which consists of five proteins: BamA, BamB, BamC, BamD, and BamE. BamA, the main component of BAM, is an Omp85 β -barrel with five polypeptide transport-associated domains (POTRAs). Lipoproteins BamB–E are anchored into the membrane through their N-terminal acyl chains (Wu et al., 2005, Leyton et al., 2015). For *E. coli*, only BamA and BamD are important, but for maximal OMP folding all components are essential (Mahoney et al., 2016). Periplasmic chaperones such as survival protein A (SurA) and seventeen kilodalton protein (Skp) help developing OMPs to maintain a translocation-competent conformation and protect against its proteolytic degradation (Schiffrin et al., 2016). It is thought that these β OMPs originate in

the cytoplasm, where polypeptide precursors of β OMPs are synthesized by ribosomes. There is an N-terminal signal peptide incorporated in each of these emerging β OMP precursors to route them to the SecYEG translocon. Proteolytic removal of the signal peptide then follows translocation of the emerging β OMP precursors across the intracellular membrane. There are several chaperones, including SurA, Skp, and FkpA, that interact with the emerging β OMPs once they have migrated to the periplasm. These chaperones escort emerging β OMPs to the OM where their folding and insertion is mediated by BAM (Sklar et al., 2007, Lundquist et al., 2021). In addition to BAM, β OMPs are also translocated by translocation and assembly module (TAM). TAM contains a 16-stranded β -barrel transmembrane domain at its C-terminus connected to three POTRA domains that reside within the periplasm. Although it is not as abundant or important in cell viability as BAM, mutation of TAM can lead to virulence and colonization deficiency (Stubenrauch and Lithgow, 2019).

1.9.3 Secretion systems:

There are a number of systems used by Gram-negative bacteria that assist host infection and evasion of the host immune system during infection. Examples include the secretion systems of virulence proteins. Sec/Tat-independent secretion systems cover the entire cell envelope and transport products outside the cell. Additionally, there are virulence proteins secreted into the cytoplasm of other cells. Gram-negative bacteria have six secretion systems (type I–VI), and each transports a different protein substrate (Green and Mecsas, 2016). Type I secretion systems (T1SSs) transport their substrates across the bacterial IM and OM in a one-step process, and they are found in numerous Gram-negative bacteria (Thomas et al., 2014). T1SSs bear a resemblance to a large family of ATP-binding cassette (ABC) transporters, making them able to export small molecules such as antibiotics and toxins out of the cell (Symmons et al., 2009). Several T1SSs may be resident in some bacteria, each transporting either one or more unfolded substrates (Delepelaire, 2004). These substrates differ in function and may be digestive enzymes (proteases and lipases), adhesins, heme-binding proteins, and proteins with repeats-in-toxins (RTX)

motifs. In general, T1SS substrates are Sec-independent. *P. aeruginosa* utilises T1SS for secretion of proteins such as the alkaline protease AprA and the heme acquisition protein HasA (Green and Mecsas, 2016, Bleves et al., 2010).

In most Gram-negative bacteria, Type II secretion system (T2SS) are conserved. Folded proteins are transported from the periplasm out of the cell. The reason for this is that the channel for T2SS can only be found in the OM. The Sec and Tat secretion pathways deliver the protein secreted via T2SS to the periplasm (Voulhoux et al., 2001, Korotkov et al., 2012b). A diverse range of substrates can be secreted out of bacterial cells by T2SSs. Some of these substrates are involved in pathogen virulence. T2SS is needed in some bacterial species to secrete more than one substrate, while other bacterial species utilize T2SS to transport only one protein. The secreted proteins perform a variety of functions, but they are mostly enzymes such as lipases, proteases, and phosphatases, as well as some carbohydrate-processing proteins (Cianciotto, 2005, Korotkov et al., 2012b).

Type III secretion system (T3SS) are channels through which *P. aeruginosa* injects toxins directly into the host cell. T3SSs consist of a pilus-like structure which is used to translocate the effector proteins through a needle-like appendage into the eukaryotic cell, forming a pore through the cell membrane. T3SS have four known toxins: ExoY, ExoS, ExoT, and ExoU and they are described in section 1.8.2 (Kipnis et al., 2006, Horna and Ruiz, 2021, Kroken et al., 2022).

A variety of substrates are secreted by Type IV secretion systems (T4SS), such as single proteins, protein-protein, and DNA-protein substrates. In addition, there is an ancestral relation between T4SS and bacterial DNA conjugation systems (Cascales and Christie, 2003). A wide range of cells can be targeted by substrates secreted by T4SS, including bacteria of the same or different species, and eukaryotic cells. Furthermore, transfer of substrates by T4SS can be made directly into the cytoplasm of the host cell. T4SS vary in function and can transfer DNA and proteins. For example, T4SS can transfer DNA via conjugation, effector protein translocation, DNA transfer, and uptake and

release of DNA and DNA/protein complexes transferred into the recipient cells directly. T4SS are not present in *P. aeruginosa* (Green and Meccas, 2016).

The type V secretion system (T5SS) is unique because it can secrete itself and cross the bacterial membrane via a membrane channel or secretion apparatus. The most notable T5SS proteins are virulence proteins that serve receptor-binding proteins and toxins. Examples of the virulence of T5SS include immunoglobulin A protease produced by *N. gonorrhoeae*, which causes host antibody cleaving, and the IcsA protein produced by *Shigella flexneri* that functions as an adhesin, as well as EstA that cleaves rhamnolipids in *P. aeruginosa*, which is important for biofilm formation (Green and Meccas, 2016, Brandon et al., 2003, Meuskens et al., 2019).

The type VI secretion system (T6SS) is used by numerous bacteria. It is a protein translocation apparatus that effector proteins are delivered through into eukaryotic and prokaryotic cells. This system was found to play a major role as a tool in inter-bacterial competition (Coulthurst, 2019). Bacteria deliver toxins into competitor bacterial cells via T6SS. Examples include phospholipase effectors PldA and PldB released by *P. aeruginosa* via T6SS. They act by delivering toxicity to the periplasm of target cells lacking the cognate immunity proteins. Furthermore, the T6SS component has 14 conserved genes, including TssA-M, hemolysin co-regulated protein (Hcp), VgrG-family protein, and the AAA+ ATPase ClpV that form key T6SS structures (Coulthurst, 2019, Förster et al., 2014). A cell-puncturing tip is formed by VgrG-family protein and an effector delivery tail-tube structure is formed by Hcp family. Moreover, ClpV and TssM are predicted to work as energizing components that are vital for T6SS secretion (Silverman et al., 2012). In addition, T6SS shows diversity in physiological function, including biofilm formation, stress responses, chronic infections, and inter-bacterial interactions (Hood et al., 2010, Schwarz et al., 2010, Zhang et al., 2013).

1.10 Energy transduction in Gram-negative bacteria

1.10.1 Proton motive force:

The proton motive force (PMF) is an electrochemical gradient across the IM involved in energy production. The cytoplasmic side of the IM is more alkaline than the periplasmic side, and this difference affects proton flow, which is needed for motility, secretion, Adenosine triphosphate (ATP) production, and secondary active transport. The electron transport chain maintains the PMF under aerobic conditions. It also includes IM protein complexes, iron-sulfur clusters, quinones, and cytochromes, which take part in oxidation-reduction chain reactions. Nicotinamide adenine dinucleotide (NADH) is usually the initial electron donor, through NADH dehydrogenase it transfers electrons to ubiquinone. An electron-rich ubiquinone (ubiquinol) then acts to transfer electrons to the terminal ubiquinol oxidase complex, which contains cytochromes of type b and d, or b alone. Furthermore, oxygen is eventually turned into water by a ubiquinol oxidase complex. The IM periplasmic side accumulates protons during electron transfer, and this creates the proton gradient (Anraku, 1988, Montville and Bruno, 1994).

1.10.2 The Ton and Tol systems:

The lack of ATP in the periplasm and the absence of an electrochemical gradient across the OM leads to challenges for bacteria in transporting substances across the OM (Allen et al., 2009). Thus, in Gram-negative bacteria, for the energy in the PMF to be transduced across the OM, trans-envelope systems such as Ton and Tol systems evolved (Figure 1.4) (Egan, 2018). The Ton system consists of three IM protein complexes: ExbB, ExbD, and TonB. TonB spans the width of the periplasm and comes into contact with OM TonB-dependent transporters (TBDTs). TonB transports energy to TBDTs from the PMF, which powers the uptake of specific nutrients such as iron-siderophores, divalent metals, carbohydrates, vitamins, and peptides (Ratliff et al., 2021). The Tol system includes IM complexes TolQ, TolR, and TolA, as well as the soluble periplasmic protein TolB, and the OM-anchored lipoprotein Pal (Szczepaniak et al., 2020). Deletion of *tol* genes causes hypersensitivity to detergents and antibiotics, blebbing in the OM, and O-antigen synthesis defects, indicating that

the Tol system plays a role in stabilizing the OM (Bernstein et al., 1972, Paterson et al., 2009). In response to the PMF, TolA and TonB undergo conformational changes (Germon et al., 2001).

All known structures of TBDTs are similar, with a C-terminal 22-strand β -barrel that has an N-terminal plug domain inserted into it (Noinaj et al., 2010). The TonB box is an N-terminal conserved sequence that is exposed to the periplasm by conformational changes induced via the TBDT extracellular face binding to the ligand. After this, the TBDT-TonB box interacts with the TonB subunit C-terminal periplasmic domain, which leads to a stable complex connecting TBDT physically to the IM (Josts et al., 2019, Sarver et al., 2018). What occurs after the formation of the TBDT/TonB complex has not been fully elucidated, but it is assumed that TonB alters the conformation of TBDT when it receives energy from the IM PMF (Ratliff et al., 2021). The ExbB and ExbD activation mechanisms are also currently unknown. However, these may rely on their connection with the TonB subunit, and may also rely on TBDT being fully ligand-loaded (Ratliff et al., 2021). Partial unfolding of the TBDT N-terminal plug would occur when the TonB C-terminal and TBDT-TonB box interact with each other because the pulling force of this interaction is strong enough to unfold this plug (Gumbart et al., 2007, Noinaj et al., 2012, Hickman et al., 2017). In comparison to TBDT, the TonB C-terminus can adopt more different orientations when it is bound to the TBDT-TonB box and becomes mobile (Sarver et al., 2018, Josts et al., 2019). *In vivo* studies have shown that the PMF is needed for the interaction between the TonB C-terminal domain and the ExbD C-terminal domain (Ollis and Postle, 2012, Ollis and Postle, 2011).

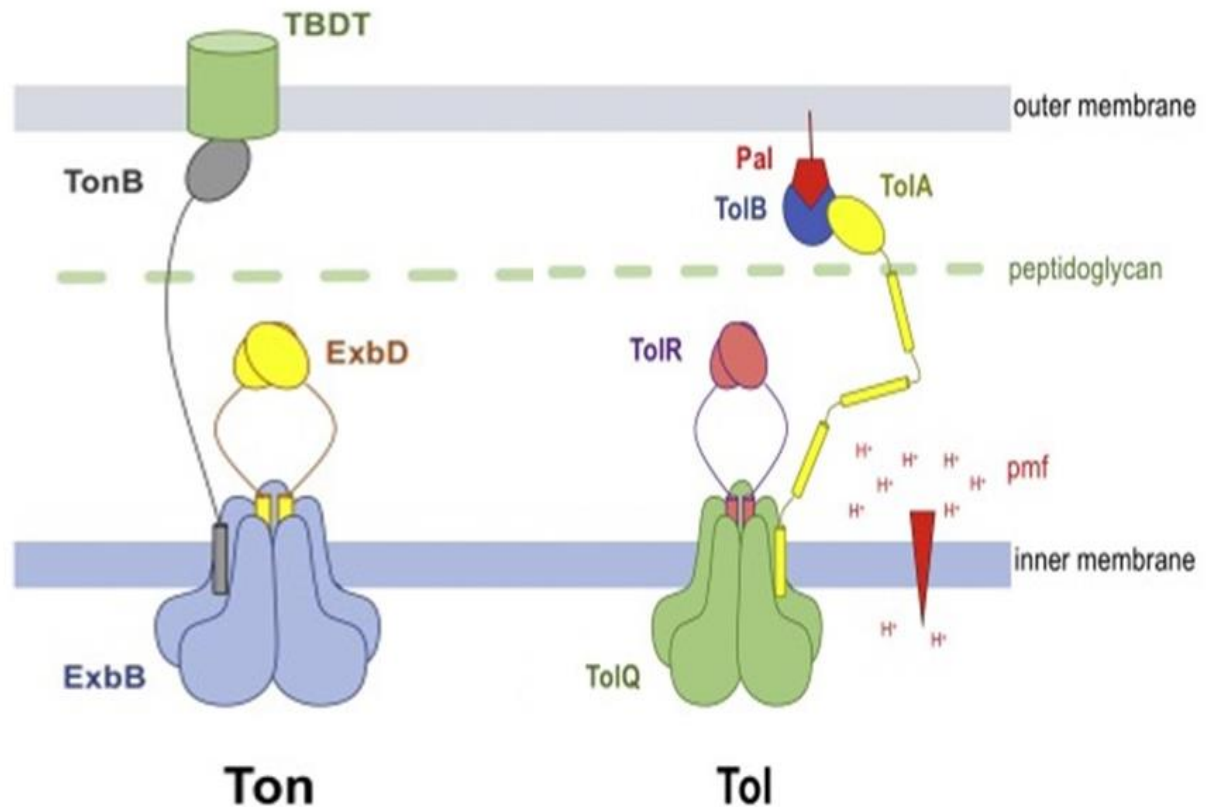


Figure 1.4: Tol and Ton systems. Ton is comprised of five proteins; ExbB (blue), ExbD (yellow), and at least one TonB protein (grey) is needed to form the Ton complex. TBDTs loaded with nutrients interact with the elongated TonB subunit along their OMs. Currently, the stoichiometry of the Tol complex is unknown, but the complex consists of five TolQs (green), two TolRs (purple), and at least one TolA (yellow), based on extensive homology with the Ton complexes. A periplasmic domain of TolA that is elongated interacts with the Tol-Pal complexes at the OM (blue-red) (Ratliff et al., 2021).

1.10.3 Iron acquisition and its metabolism:

An important factor in the outcome of bacterial infection relates to the acquisition iron and other metal ions. This is due to limited metal ion availability from the host through adoption of a nutritional immunity strategy (Palmer and Skaar, 2016, Cornelissen, 2018). Gram-negative bacteria compete with the host cell nutritional immunity strategy by capturing substrates containing iron via numerous iron uptake systems such as siderophores (Chu et al., 2010). Siderophore uptake involves an OM receptor, a periplasmic-binding protein, and an IM transporter (Chatterjee and O'Brian, 2018, Mosbahi et al., 2018). *P.*

aeruginosa acquires iron via the fluorescent siderophore Pyoverdine (PVD). There are three types of PVD, Pyoverdin type I (PvDI), Pyoverdine type II (PvDII) and Pyoverdine type III (PvDIII), of which PVDI is the most investigated in *P. aeruginosa* PAO1 (Bonneau et al., 2020). When PVDI is secreted into the environment, it chelates ferric iron (Fe^{3+}), producing a complex named ferripyoverdine (Konings et al., 2013, Albrecht-Gary et al., 1994). Ferripyoverdine is then recognized by two TBDTs, FpvAI and FbvB, and it is then bound and transported into the cell by one of them. The IM protein complex of TonB, ExbB, and ExbD provides the energy necessary for transport (Chatterjee and O'Brian, 2018, Brillet et al., 2007, Ghysels et al., 2004). TonB1, one of three TonB proteins, helps in the transport of ferripyoverdine (Clément et al., 2004, Schalk et al., 2001). FpvC and FpvF, two periplasmic proteins, bind to ferripyoverdine when it enters the periplasm (Brillet et al., 2012). PVDI releases iron in the periplasm via IM protein FpvG through an iron reduction mechanism. Three genes (*fpvH*, *fpvJ*, and *fpvK*) related to FpvG encode the proteins FpvH, FpvJ, and FpvK, whose functions are unknown. However, expression of these proteins is necessary for FpvG to show optimal activity (Ganne et al., 2017, Bonneau et al., 2020). FpvC, FpvDE, and FpvF are proteins that belong to a group of periplasmic-binding protein-dependent ABC transporters (Brillet et al., 2012). For the efficient dissociation of iron from PVDI, the three proteins FpvC, FpvDE, and FpvF are needed (Ganne et al., 2017, Bonneau et al., 2020). After iron is released from PVDI, a PvdRT-OpmQ efflux pump recycles apo-PVDI into the extracellular space, where PVDI can again chelate Fe^{3+} and carry it into the bacterial cell (Figure 1.5). In addition to FpvAI transporting iron through its N-terminal extension, known as its signalling domain, it can also regulate iron uptake and metabolism through this domain (Shen et al., 2002). The IM anti-sigma factor FpvR inhibits extra-cytoplasmic function (ECF) sigma factors FpvI and PvdS when ferripyoverdine is absent. When ferripyoverdine is transported under iron-limited conditions, the signalling domain of FpvAI contacts FpvR, which leads to FpvR TonB-dependent breakdown. This breakdown activates *fpvAI* expression and biosynthesis of PVD, due to the corresponding release of FpvI and PvdS (Rédly and Poole, 2005, Shirley and Lamont, 2009). Furthermore, PVD signaling regulates other virulence factors of *P. aeruginosa* such as ExoA (Kang et al., 2019).

There are two TBBDT transporters related to FpvAI, namely FpvAII and FpvAIII, which are receptors for PVDII and PVDIII (De Chial et al., 2003). In addition, there are other siderophore transporters, such as FptA that transports ferripyochelin, FoxA that transports ferrioxamine B, FiuA that transports ferrichrome, and HasR that transports heme (Cobessi et al., 2005, Llamas et al., 2006, Dean and Poole, 1993, Ochsner et al., 2000).

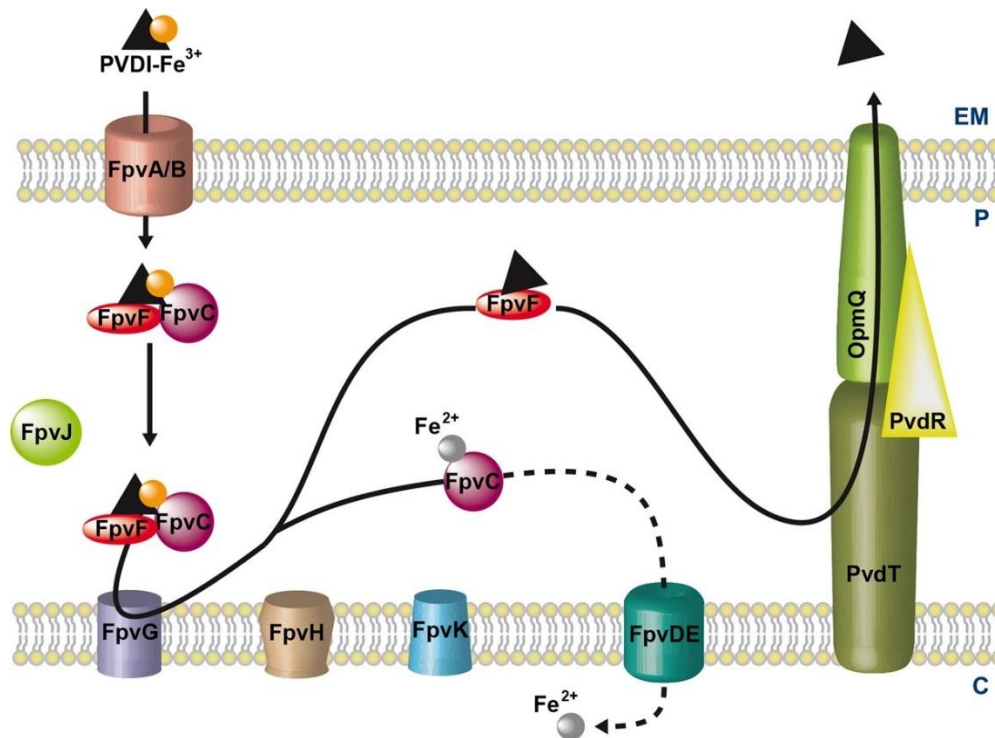


Figure 1.5: Model for enzymes that regulate the uptake of PVDI in *P. aeruginosa* (details are described above) (Bonneau et al., 2020)

Biosynthesis of pyoverdine starts in the cytoplasm and ends in the periplasm. PvdL, PvdI, PvdJ, and PvdD, which are non-ribosomal peptide synthases (NRPSs), synthesize an acylated ferribactin precursor in the cytoplasm through the involvement of PvdH, PvdF, and PvdA enzymes. These enzymes produce the non-proteinogenic amino acids L-Dab and L-fOHOrn. A siderosome is created by the organization of L-Dab and L-fOHOrn as a membrane-bound complex. The soluble thioesterase PvdG and the adenylation domain-activating protein MbtH may be involved in this biosynthesis process. Furthermore, PvdE exports the acylated ferribactin precursor into the periplasm, and PvdQ then deacylates it. After this, chromophore formation is catalyzed by PvdP.

Subsequently, PvdM, PvdN, and PvdO enzymes are responsible for the unknown maturation steps of the produced pyoverdine. The mature pyoverdine can be exported into the extracellular space via PvdRT-OpmQ (Figure 1.6) (Ringel et al., 2018, Ringel and Brüser, 2018, Schalk et al., 2020)..

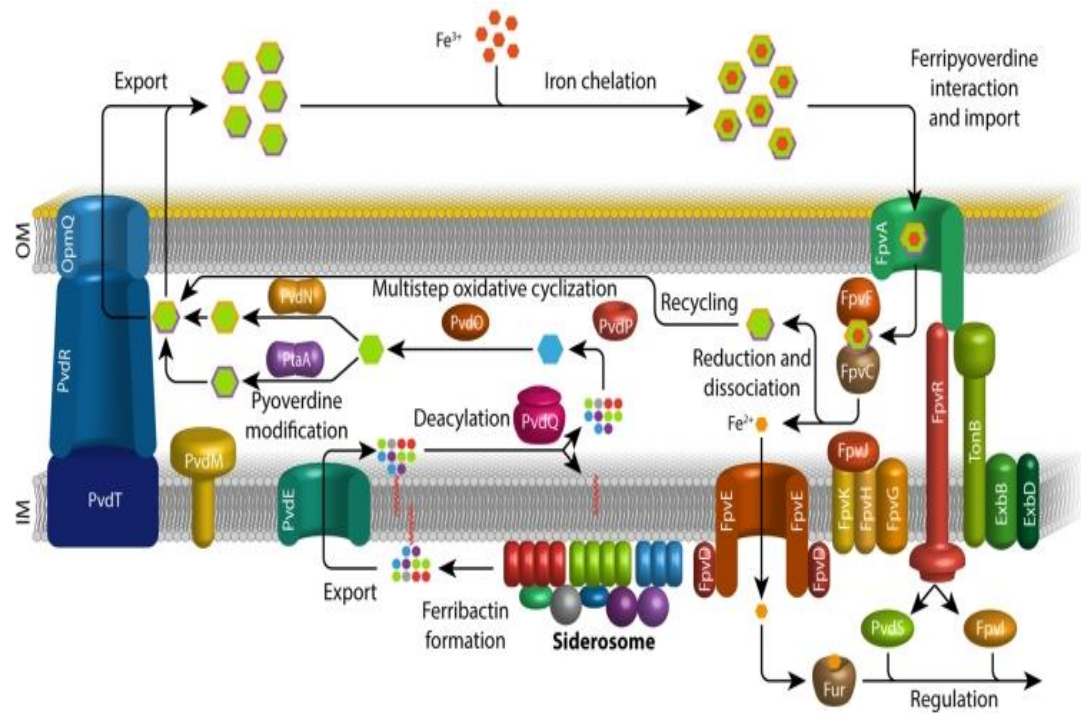


Figure 1.6: Illustration of pyoverdine biosynthesis (details are described above) (Ringel and Brüser, 2018).

1.11 Inter-bacterial competition:

Multicellular communities of bacteria compete and interact with each other rather than existing in isolation. Cytotoxic agents such as microcins, bacteriocins, contact-dependent growth inhibition (CDI), and T6SS effectors are responsible for growth inhibition or killing by direct contact between neighboring bacterial cells.

1.11.1 Contact-dependent growth inhibition (CDI):

Like other Gram-negative bacteria, in *P. aeruginosa* the CDI system consists of two secretion partners CdiA and CdiB. The CdiA exoprotein is displayed at the surface of the bacterial cell via CdiB. An entire filamentous stalk of CdiA is

formed, which facilitates the binding of the C-terminal toxin (CT) domain to a target cell, and hence its delivery. CdiI is an immunity protein encoded by CDI, and CDI-producing cells are protected from auto-inhibition by the binding of CdiI to CdiA-CT, neutralizing its toxin activity (Allen and Hauser, 2019).

1.11.2 Bacteriocins:

Bacteriocins are proteins produced by bacteria that kill or inhibit other bacteria without harming the producing bacterium, and they have a narrow killing spectrum. At least one bacteriocin is produced by 99% of all bacteria (Riley and Wertz, 2002). Examples in Gram-negative bacteria are colicins of *E. coli* and pyocins of *P. aeruginosa* (50). In response to stress, colicins can be expressed from pCol plasmids via the SOS pathway in *E. coli* (Papadakos et al., 2012). Expressed colicins are released into the extracellular space, which leads to the killing of strains closely related to the producing *E. coli* strain (Cascales et al., 2007a). Colicins contact the target cell via high-affinity binding to a specific OM receptor, often a TBDT or porin. Colicins are then translocated across the OM by utilizing the cell envelope components of the target cell and Ton and Tol systems, as well as the PMF (Cramer et al., 2018, Postle and Kadner, 2003). Colicins have multiple domains, including translocation, receptor binding, and cytotoxicity domains. When colicin nucleases cross the IM, the cytotoxic domain is cleaved by the ATP-dependent protease FtsH, which also mediates translocation into the cytoplasm (Kim et al., 2014, Walker et al., 2007). Colicins are produced alongside a cognate immunity protein that neutralizes the cytotoxic domain's activity by binding to it to prevent the producing cell from committing suicide. During translocation into the target cell, the immunity protein is released to liberate the enzymatic colicin, which is cytotoxic to the sensitive cell (Kleanthous and Walker, 2001).

1.11.3 *P. aeruginosa* pyocins:

The first evidence of *P. aeruginosa* bacteriocin production was provided by François Jacob, who found that a compound was released through bacterial lysis following treatment with a mutagenic agent such as ultraviolet irradiation

(wavelength 253.7 nm), which could kill susceptible bacteria from the same species after being absorbed onto its cell surface. Due to their similarity to colicins, and because the name originally used for *P. aeruginosa* was *Pseudomonas pyocyanea*, Jacob named the compounds pyocins (Michel-Briand and Baysse, 2002).

There are three types of pyocin: R type pyocin, F type pyocin, and S type pyocin. Pyocin R (five subgroup, R1–R5) is a rod-like structure resembling a bacteriophage, and it is nuclease- and protease-resistant. LPS is the main receptor of R type pyocins, and some other bacterial species have also been found to be sensitive to R types pyocin, such as *Neisseria gonorrhoeae* and *Haemophilus influenzae*. This suggests that some other bacterial species share similar receptor sites for R type pyocins with *P. aeruginosa* (Salazar et al., 2019, Govan, 1974). After being absorbed onto the receptor of the sensitive cell, the R type pyocin initiates intracellular secretion into the cell and arrests the synthesis of macromolecules, thereby resulting in pore formation and depolarization of the cytoplasmic membrane, which leads to cell death (Mei et al., 2021, Ge et al., 2020).

F type pyocins are nuclease- and protease-resistant bacteriocins, which are non-contractile *E. coli* phage lambda and have a flexible rod-like structure. There are three subtypes of F type pyocin (F1, F2, and F3) (Saha et al., 2021, Nakayama et al., 2000).

S type pyocins are soluble proteins, and unlike pyocin R and F, they are protease-sensitive. Pyocin S is organized in an operon consisting of two open-reading frames (ORFs), one of which encodes the bactericidal protein, and the other encodes the immunity protein. S type pyocins consist of a large protein that has killing activity and a small protein that harbors the immunity function (Ghequire and De Mot, 2014, Michel-Briand and Baysse, 2002). The pyocin-producing strain is protected by inactivating the cytotoxic domain of the protein via the immunity protein. The immunity protein binds the pyocin with high affinity (equilibrium dissociation constant [K_d] ~10–14 M) and high specificity (Joshi et al., 2015). In general, two separate genes that encode both the killing and immunity proteins of pyocins are organized in an operon. PrtN, PrtR, and RecA

proteins are responsible for regulating the transcription of pyocin genes (Matsui et al., 1993). Under stressful conditions, expression of pyocin genes is induced by PrtN, and the repressor PrtR inhibits PrtN transcription under normal conditions. Under stressful conditions, RecA forms a nucleoprotein filament that can bind to single-stranded DNA, which causes PrtR autocleavage, and thus pyocin production, due to PrtN derepression (Sun et al., 2014, Matsui et al., 1993).

After their biosynthesis, S type pyocins are released as a killing-immunity protein complex into the extracellular medium. These pyocins utilize the nutrient uptake pathways of target cells, which are important for target cells under nutrient-limited conditions. S type pyocins gain access to target cells via the N-terminal receptor-binding domain of the pyocin, which binds to a specific receptor on the target cell surface (Behrens et al., 2020a, Sano et al., 1993a). In the IM, the N-terminus then interacts with Ton or Tol-Pal systems and the PMF system that span the periplasm, mediating the transport of pyocin across the OM (Behrens et al., 2020a, McCaughey et al., 2016, White et al., 2017). Moreover, the immunity protein is released into the extracellular medium and the cytotoxic domain of the C-terminus enters the cytoplasm. Finally, the cytotoxic domain exerts its killing effect, leading to the demise of the target cell via a variety of mechanisms (Turano et al., 2020, Mora and de Zamaroczy, 2014, Chauleau et al., 2011).

Most S type pyocins show nuclease activities against DNA, rRNA, and tRNA (Ghequire and De Mot, 2014). Furthermore, the DNase domain catalytic cores of pyocins S1, S2, and AP41 carry a conserved H-N-H endonuclease motif (Parret and De Mot, 2002). Some colicins of *E. coli* (E2, E7, E8, and E9) have an H-N-H motif. The H-N-H motif is found in other enzymes that perform a variety of functions and is broadly spread across all kingdoms. The functions include apoptosis, homologous recombination, DNA repair, and mobile intron homing. Moreover, random cleavage of the bacterial genome is catalyzed by a single divalent metal ion group that is coordinated via the H-N-H motif of colicins and pyocins (Kühlmann et al., 1999, Walker et al., 2002, Joshi et al., 2015). The H-N-H domain comprises 32 amino acids located in the cytotoxic domain. The structure of the H-N-H motif resembles a zinc finger topology, with two

antiparallel β -strands and one α -helix ($\beta\beta\alpha$ -Me), and a metal ion is inserted between each (Kleanthous et al., 1999, Ko et al., 1999, Maté and Kleanthous, 2004, Joshi et al., 2015). The H-N-H motif has some residues that play an important role in DNA hydrolysis and ion binding. The H-N-H motif of colicins has been thoroughly investigated, but structural information remains lacking for pyocins (Turano et al., 2020). It is interesting to note that Pyocin S3 (PyoS3) has a completely different primary sequence without an H-N-H motif in its killing domain (Parret and De Mot, 2002, Ghequire and De Mot, 2014, Joshi et al., 2015). Thus, PyoS3 may have an unusual coordination with its cognate immunity protein and could be classified as a different kind of DNase pyocin.

Pyocin S1 was the first S-type pyocin isolated from a clear lysate of the *P. aeruginosa* P28 strain after treatment with R4 pyocin antiserum (ITO et al., 1970). Pyocin S2 was the second pyocin discovered, isolated from *P. aeruginosa* PAO1. Furthermore, pyocin AP41 is a pyocin produced by *P. aeruginosa* that are protease-sensitive, sharing similarity with pyocin S1 and S2. In addition, pyocin AP41 has a role in Pyocins R1 and S2 induction as well as phages F116L or PS17 (Sano and Kageyama, 1981, Michel-Briand and Baysse, 2002). PyoS3 was identified in the *P. aeruginosa* strain P12 isolated from CF patients (Duport et al., 1995). Pyocin S4 and S5 were identified during genomic analysis of the *P. aeruginosa* PAO1 sequence (Parret and De Mot, 2000). Pyocins SD1, SD2, and SD3 contain N-terminal domains that are homologous to those of pyocin S1, S2, and S3, respectively (McCaughey et al., 2016).

Pyocins S1, S2, S3, S8, and AP41 carry DNase activity, which causes cell death by DNA breakdown (Turano et al., 2020, Martínez-Alemán et al., 2020, Ferrara et al., 2017). Pyocins S1 and S2 also inhibit lipid synthesis in the cell, along with DNase activity (Sano et al., 1993b). Pyocin S4 has a structure resembling that of a tRNase in the C-terminal domain, while pyocin S5 has pore-forming activity in the C-terminal domain (Elfarash et al., 2012, Behrens et al., 2020d). There is close homology between the tRNase domain of colicin D and the C-terminal cytotoxic domain of SD1, SD2, and SD3 (McCaughey et al., 2016).

The pyocin S structure consists of an N-terminal region (240 amino acids) containing three domains: a receptor-binding domain, a domain with unknown activity that is absent in pyocin S2, and a translocation domain. The C-terminal region of pyocin S spans 130 amino acid residues and is reported to be responsible for the killing activity of the pyocin (Figure 1.7) (Mehina, 2021, Sano et al., 1993a).

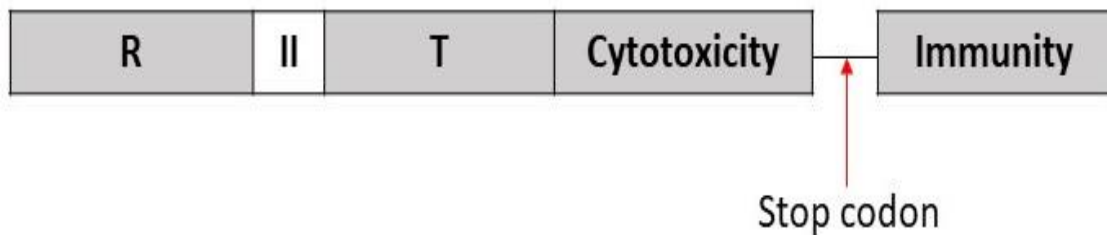


Figure 1.7: The domain structure of S type pyocins. The N-terminal domain is a receptor-binding domain (R); the second domain (II) has unknown activity and is absent in Pyocin S2; The third domain is a translocation domain (T); the cytotoxic domain contains the killing activity, followed by its cognate immunity protein (Im). There is a TGA stop codon in first ORF that contains the cytotoxicity, separating the Im protein in the second ORF, which has its own start codon (ATG), by a single T base (Michel-Briand and Baysse, 2002).

Some Pyocin S proteins can bind to common polysaccharide antigen (CPA), an LPS of *P. aeruginosa* strains. This binding helps pyocins to concentrate at the cell surface, which leads to more efficient localisation of its OM receptors. Pyocins S2, SD2, SD3. and S5 utilize CPA (McCaughey et al., 2016, Behrens et al., 2020a).

The OM pyoverdine receptor FpvAI acts as a receptor for pyocin type S1, S2, S4, and SD2. FpvAII is proposed to form the receptor for the S3 pyocin, but very little is known about PyoS3 import (Denayer et al., 2007, Elfarash et al., 2012, Baysse et al., 1999). Pyocin S5 utilises the ferric pyochelin transporter FptA as a receptor (Behrens et al., 2020b). Pyocin G, newly discovered through bioinformatics analysis, uses the TonB-dependent transporter hemin (Hur) as a receptor (Atanaskovic et al., 2020). The pyocin AP41 receptor has not yet been identified, but it is thought that the putative hemagglutinin transporter

FhaC might be responsible as a receptor and possible translocator, or only as a translocator (Atanaskovic, 2021).

A recent study confirmed the previous suggestion that S2 translocation is mediated through the TonB system. FpvAI is a TBDT responsible for importing the small siderophore ferripyoverdine (Fe-Pvd) through pairing it to the PMF via the IM protein TonB1. The N-terminal domain of pyocin S2 binds to FpvAI, and this binding causes conformational changes in the receptor. Additionally, the coupling of S2 with Fe-Pvd leads to translocation of pyocin S2 through FpvAI, which is similar to a translocation process used by the TBDT ligand. In addition, this pyocin initiates unfolding of TonB1 and shifting of the TonB1-binding epitope to the periplasm through S2 interaction. Following this, pyocin S2 is pulled through the tight channel of FpvAI (White et al., 2017).

To date, little is known about PyoS3. It has a unique DNase domain that does not have an H-N-H motif, and PyoS3 might utilize FpvAI as a receptor, hence it is probable that it utilises the TonB system for translocation (Duport et al., 1995, Baysse et al., 1999) .

1.12 Aims and objectives:

The emergence of antibiotic resistant bacteria has struck a huge blow to public, exacerbated by the slow pace of antibiotic discovery (O'Neill, 2016). The misuse and prolonged use of antibiotics has led to increased tolerance of bacteria to antibiotics. A major factor driving the increase in antibiotic resistance is the spread of resistant bacteria from person to person, or from a non-human sources such as livestock to humans. This has led to increasing pressure to discover new antimicrobial agents (Soltani et al., 2020). PyoS3 is poorly characterized in terms of the mechanism of cell killing and its full structural arrangement.

The aims of this study are as follows:

- It is hypothesized that PyoS3 interacts with TonB system TonB1 protein, or possibly the TolAIII protein of Tol-Pal system. A range of biophysical approaches will be employed to explore this.
- To identify any unknown interaction partners, a library of random mutants will be generated and screened for susceptibility to PyoS3.
- The structure of the entire PyoS3 protein has not been determined, and it is not clear how the receptor-binding, translocation, and catalytic domains are arranged relative to each other, and in relation to the cognate immunity protein. To shed light on this, the protein will be purified and subjected to crystallographic analysis.

By determining the structure and killing mechanism of PyoS3, it is hoped that there will be potential to exploit it as a novel antimicrobial.

2 Chapter 2. Materials and methods

2.1 Growth media:

Lysogen broth (LB) media (Oxoid Ltd, UK.) was used for cell growth unless otherwise stated. LB was prepared by dissolving 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL deionized water. The pH of the growth media was adjusted to 7.0 by adding 1 M NaOH, and water was added until the volume reached 1 L. LB agar was prepared by adding 0.8% (w/v) technical agar No. 3 (Oxoid), followed by autoclaving at 121°C on liquid cycle for 20 min at 15 psi for sterilization.

2.2 Antibiotics:

Ampicillin, gentamicin, and nalidixic acid were used in this study, and the concentrations of the stock and working solutions are shown in Table 2.1. Stocks were stored at -20°C for ampicillin, while gentamicin and nalidixic acid were stored at 6°C, and all were dissolved in water.

Table 2.1: Concentrations of antibiotics used in the study.

Antibiotics	Stock concentration	Working concentration
Ampicillin (salt), Sigma-Aldrich, UK	100 mg/mL	100 µg/mL
Gentamicin (liquid) Sigma-Aldrich, UK)	50 mg/mL	15 µg/mL
Nalidixic Acid (salt), Sigma-Aldrich, UK	30 mg/mL	30 µg/mL

2.3 Bacterial strains and plasmids:

Bacterial strains and plasmids used in this study are shown in Table 2.2 and Table 2.3.

Table 2.2: Strains used in this study.

Strains	Features/role in the study	Source or reference
<i>E. coli</i> DH5 α	cloning strain: <i>huA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	(Studier et al., 1990)
<i>E. coli</i> BL21 (DE3)	Expression host: <i>F- ompT hsdS_B (r_B-, m_B-) gal dcm</i> (DE3)	(Kwon et al., 2015)
ER2566 cells	Used for direct mutagenesis to remove the stop codon in TonB1. Genotype: B- <i>F- λ^- fhuA2 [lon] ompT lacZ::T7.1 gal sulA11 Δ(mcrC-mrr)114::IS10 R(mcr-73::miniTn10)(Tet^S)2 R(zgb-210::Tn10)(Tet^S) endA1 [dcm]</i>	New England Biolabs (NEB)
PSA 971	Cystic Fibrosis (CF) clinical <i>P. aeruginosa</i> strain: used as a sensitivity strain for activity testing of PyoS3 protein. Genotype:	Unknown epidemic strain isolated from CF a patient. Donated by Dr Matthew Hurley.
PSA 892	Cystic Fibrosis (CF) clinical <i>P. aeruginosa</i> strain: used as a sensitivity strain for activity testing of PyoS3 protein and in the transposon library experiment	Unknown epidemic strain isolated from a CF patient. Donated by Dr Matthew Hurley
S17-1 λ pir <i>E. coli</i> /PME9978	Donor strain containing the PME9978 plasmid to generate a transposon library. Genotype: thi	Obtained from Dr N. Wenner

	pro hsdR hsdM1 recA RP4-2-Tc::Mu-Km::Tn7	
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Table 2.3: plasmids used in the study.

Plasmids	Role in the study	Source or reference
pPYSS-3	Pyocin S3 genes in pBluescript KS+ vector. Used as template for PyoS3 R+T and PyoS3 DNA amplification	Kindly donated by Pierre Cornelis (Pierre.Cornelis@vub.ac.be) (Duport et al., 1995)
pGEM-T easy vector	Cloning vector: It contains T7 polymerase promoters flanking multiple cloning regions within the β -galactosidase enzyme's α -peptide coding region	Promega
pET21a vector (Novagene)	Expression vector: Contains an N-terminal T7 tag sequence as well as a His-tag sequence at the C-terminus	Novagen
pBAG1	Recombinant pET21a plasmid containing TolAIII DNA	Bashair AL-Gethami, MSc thesis

2.4 Growth conditions of bacterial strains:

Bacterial colonies were obtained after incubation of LB agar at 37°C overnight. Bacterial strains in LB broth were cultivated after being incubated overnight at 37°C in a shaking incubator at 200 rpm unless otherwise stated.

2.5 PCR amplification:

2.5.1 Primer design and synthesis:

Primers were designed using the Chromas program (Table 2.4). Restriction sites and tags were included in primer sequences. Primers were synthesized by Sigma-Aldrich (UK).

2.5.2 PCR conditions:

Mastercycler personal (Eppendorf) conventional PCR was used to amplify target DNA using a 50 µl reaction volume and 40 amplification cycles (Table 2.5).

Table 2.4: Primers used in restriction digestion in this study.

Primer	Sequence (5'–3')	Usage
S3 R+T forward (F)	<u>CATATGGCTGCACCA</u> CCGCCACTG	To clone the receptor (R) and Translocation (T) domains of the PyoS3 gene. The <i>NdeI</i> site (CAT ATG) was added to the N-terminus of the PyoS3 R +T domain gene.
S3 R+T reverse (R)	TTCTCGAGCGGCTTG GAAAAGACC	To clone the receptor (R) and Translocation (T) domains of the PyoS3 gene. The <i>XhoI</i> site (CTC GAG) was added to the C-terminus of the PyoS3 R +T domain gene.
Pyocin S3 F	<u>CATATGGCTGCACCA</u> CCGCCACTG	To clone the whole PyoS3 gene. The <i>NdeI</i> site (CAT ATG) was added to the N-terminus of the PyoS3 gene.
Pyocin S3 R	ATCTCGAGTTTAGAAC CAAGAAGAGC	To clone the whole PyoS3 gene. The <i>XhoI</i> site (CTC GAG) was

		added to the C-terminus of the PyoS3 gene.
TonB1stopt oGin_F	CAGCGCTCGCAAGGC CCGGC	Used to remove the stop codon of TonB1
TonB1stopt oGin_R	GGCGCAGTCTGCGCG CCG	Used to remove the stop codon of TonB1
TPNRL17- 1 F	AACAAGCCAGGGATG TAACG	Used to sequence the transposon mutant insertion
TPNRL13- 2 R	CAGCAACACCTTCTTC ACGA	Used to sequence the transposon mutant insertion

Table 2.5: Conventional PCR conditions and amplification procedures used in this study.

PCR		
PCR component	Volume	Final concentration
10× PCR buffer	5 µL	1×
2 mM dNTP	5 µL	200 µM
DNA template	5 µL	10 ng
Forward primer	5 µL	0.5 µM
Reverse primer	5 µL	0.5 µM
Jumpstart Taq DNA Polymerase (Sigma-Aldrich)	1 µL	2.5 units/µL
Nuclease-free water	24 µL	
Total volume	50 µL	
PCR conditions		
Cycle stage	Temperature	Time
Initial denaturation	95°C	2 min
Denaturation	95°C	45 s

Annealing	58°C	45 s	} 40 cycles
Extension	72°C	45 s	
Final extension	72°C	10 min	
Hold	4°C	Pause	

2.6 Gel extraction:

A DNA Gel Extraction Kit (Monarch) was used to extract DNA from agarose gels according to the manufacturer's instructions (18).

2.7 DNA ligation:

A Mastercycler personal (Eppendorf) was used to ligate DNA into the vector of interest as shown in Table 2.6. Reactions were performed overnight at 16°C and the resultant ligation mix was transformed into *E. coli* DH5α cells for screening or *E. coli* BL21 (DE3) cells for DNA expression. Transformed cells were incubated overnight at 37°C on a selective plate.

Table 2.6: Ligation reactions used in this study.

Ligation mix	Volume
Ligase enzyme	1 µL
pGEM-T easy vector/pET21a vector	1 µL
DNA (gel extraction DNA product)	Variable
10× ligase buffer	1 µL
dH ₂ O	To 10 µL
Total volume	10 µL

2.8 Site-directed mutagenesis (SDM):

A Q5 Site-directed Mutagenesis Kit (New England BioLabs) was used to generate single mutations in the TonB1 gene. The resulting PCR product was transferred into BL21 (DE3) and ER2566 cells. Primers used for SDM are shown in Table 2.4.

2.9 Chemically competent cells:

A 1:100 dilution of the bacterial strain from an overnight broth was inoculated into 50 mL LB and incubated for 2 h in a 250 mL flask until the OD_{600nm} reached 0.4–0.7, measured using a Thermo Biomate 3 Spectrophotometer, and 40 mL of bacterial culture was centrifuged using an Allegra X-22R instrument (Beckman Coulter) at 1008 ×g for 10 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 15 mL of 100 mM CaCl₂ (Sigma-Aldrich). Following this, the mixture was incubated on ice for 15 min, followed by centrifugation at 1008 ×g to harvest bacterial cells. The supernatant was discarded, and the cell pellet was resuspended in 2 mL of 100 mM CaCl₂.

2.10 Heat-shock bacterial transformation:

A 10 µL volume of plasmid DNA was transferred to 200 µL of the competent cells and incubated on ice for 30 min. Bacterial cells were then heat-shocked in a water bath at 42°C for 90 s, incubated on ice for 2 min, and 800 µL of SOC Outgrowth Media (New England BioLabs) or LB broth was added. Cells were incubated in a shaker incubator at 37°C for 45 min, streaked on a selective LB agar plate, and plates were incubated overnight at 37°C for colony observation.

2.11 Blue-white screening:

After transformation of the DNA pGEM-T easy vector into *E. coli* DH5α competent cells, cells were spread on an LB plate containing 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) with 20 mg/mL X-gal and 100 mg/mL ampicillin for blue-white screening. Plates were incubated overnight at 37°C for colony observation.

2.12 Plasmid extraction:

Plasmids were purified using a GenElute Plasmid Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions. Purified plasmids were transferred into bacterial cells of interest.

2.13 DNA and protein concentration measurement:

NanoDrop ND-1000 instrument (Nanodrop Technologies) was used to measure the concentration of the DNA in plasmid preps. In addition, the NanoDrop was

also used to measure the protein concentration at 280 nm and extinction coefficient, which estimated by ExPASy ProtParam tool (Gasteiger et al., 2005), was employed for the proteins (Table 2.7). In addition, the Bradford assay was used to measure the concentration of TonB1 protein (Bradford, 1976).

Table 2.7: extinction coefficient of each protein used in this study.

Protein	Extinction coefficient
PyoS3 R+T	11460 M ⁻¹ cm ⁻¹
TonB1	28125 M ⁻¹ cm ⁻¹
TolAIII	1490 M ⁻¹ cm ⁻¹
PyoS3	12950 M ⁻¹ cm ⁻¹

2.14 Restriction enzyme digestion of DNA:

For restriction enzyme reactions, reaction mixtures containing 500–1000 ng plasmid DNA, 1 µL restriction enzyme, 1 µL 10× restriction buffer and molecular biology grade H₂O (Fisher Scientific, UK) was added to reach the final volume. Reaction mixtures were incubated in a water bath at 37°C for 1 h, followed by incubation in a heating block for 15 min at 65°C. The resulting samples were analysed by agarose gel electrophoresis.

2.15 DNA analysis using gel electrophoresis:

Firstly, a 0.8% agarose gel was prepared by using agarose BioReagent for molecular biology (Sigma-Aldrich). Agarose powder was mixed with 1x tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer (<https://www.sigmaaldrich.com/GB/en/technical-documents/protocol/protein-biology/gel-electrophoresis/tae-and-tbe-running-buffers-recipe>). Next, 5 µL of SYBR Safe DNA Gel Stain was added to the mixture, gels were allowed to solidify, and they were placed in the electrophoresis apparatus in 1× TAE as running buffer. Electrophoresis was performed at 100 V for 40 min. For measurement of DNA sizes during electrophoresis, 5 µL of 100 bp or 1 kbp DNA ladder (New England Biolabs) was used. After electrophoresis, DNA

fragments were imaged using a Gel DOC XR+ instrument (BioRad) with Image Lab software.

2.16 Sequencing:

2.16.1 2DNA sequencing:

Recombinant plasmids were sequenced externally by Source Bioscience, UK and GENEWIZ, UK using 5 µL of sample at a concentration of 100 ng/µL.

2.16.2 Whole-genome sequencing:

The whole-genome sequences of *PSA 971* and *PSA 892* clinical *P. aeruginosa* strains used in this study were determined by MicrobesNG, UK. Sample preparation was performed according to the MicrobesNG protocol.

2.17 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE):

For protein analysis, SDS-PAGE was prepared using a 3% (v/v) of stacking gel and a 10% (v/v) separating gel (Table 2.8).

Table 2.8: SDS-PAGE gel components.

Stacking gel (3%)	Volume	Separating gel (10%)	Volume
1 M Tris-Cl, 6.8 pH	2 mL	1.5 M Tris-Cl, pH 8.8	2.5 mL
30% Acrylamide	400 µL	30% Acrylamide	5 mL
Water	1.45 mL	Water	2.39 mL
N,N,N',N'-tetramethylethylenediamine (TEMED)	5 µL	TEMED	10 µL
10% Ammonium persulfate (APS)	50 µL	10% APS	100 µL

2.18 Protein expression:

E. coli BL21 (DE3) cells containing recombinant expression plasmids were inoculated into 1 L LB + 1 mL ampicillin and cultured in an incubating shaker at 37°C until an OD_{600nm} of 0.6–0.9 was reached. Cells were then induced for

expression by adding 1 mM IPTG and culture for a further 4 h at 37°C. Uninduced and induced samples were collected and mixed with 2× SDS-PAGE loading buffer (125 mM Tris-HCl pH 6.8, 4 % SDS, 20% glycerol, 10% β -mercaptoethanol, 0.004 % bromophenol blue) in a 1:1 ratio, followed by boiling in a heating block at 100°C for 3 min. After this, induced and uninduced samples were loaded onto an SDS-PAGE gel, and 1× SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3) was used as transfer buffer. Protein size was estimated by comparison with a coloured protein standards ladder in the range of 11–245 kDa (New England Biolabs) Furthermore, induced cells were harvested via centrifugation, the pellet was resuspended in resuspension buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH 7), and stored at -80°C until needed for further analysis.

2.19 Protein purification by metal chelate affinity chromatography:

Stored induced protein expression cell cultures were thawed and immediately mixed with 100 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF) and isopropanol then subjected to pulse sonication using model 505 sonic dismembrator instrument (Fisher Scientific, Pittsburgh, USA) for 6 min at 15 microns. Next, the culture was centrifuged at 10,733 ×g for 1 h at 10°C, the supernatant was collected and loaded onto a 5 mL His Trap HP column (GE Healthcare, Chicago, Illinois, USA), and buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH 7) was used to wash the column to remove unwanted contaminants. The His Trap HP column was connected to an AKTApurime Automated Liquid Chromatography System (GE Healthcare), and buffer A (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH 7) and buffer B (50 mM NaH_2PO_4 , 300 mM NaCl, 500 mM imidazole, pH 7) were used to elute His-tagged proteins. Samples from fractions of interest were analysed by SDS-PAGE, then the remaining fractions were frozen in liquid nitrogen and stored at -80°C.

Due to the unavailability of the AKTApurime plus chromatography system and the AKTA pure chromatography system (Cytiva, UK), affinity chromatography purification of PyoS3 was performed on the bench. Five different buffers containing similar ingredients, but different imidazole concentrations were

prepared. All buffers contained 50 mM NaH_2PO_4 and 300 mM NaCl, but the imidazole concentrations in different buffers were 10 mM, 50 mM, 125 mM, 250 mM, and 500 mM. These different concentrations could mimic the gradient elution for affinity chromatography performed using the Akta system. A 10 mL volume of each buffer was passed through the column to elute proteins, and each fraction was analysed by SDS-PAGE.

2.20 Protein dialysis:

Purified TonB1 was dialysed using Fisherbrand 3000 MCW Spectra Por dialysis membrane (Fisher Scientific, UK) for low molecular weight cut-off.

2.21 Protein purification via size-exclusion chromatography (SEC):

Purified and dialyzed proteins were passed through a HiLoad 26/600 Superdex 75 pg column (GE Healthcare, UK) using an AKTAprime instrument. Protein was eluted by elution buffer (50 mM $\text{Na}_2\text{H}_2\text{PO}_4$, 150 mM NaCl, pH 7). The column used for SEC separates proteins based on their size. Fractions of interest were collected analysed by SDS-PAGE.

2.22 Protein concentration using a spin column:

Protein concentrators (Thermo Fisher Scientific, UK) were used to concentrate proteins. Two sizes of membrane were used: 3K molecular weight cut-off (MWCO) and 10K MWCO. The process was performed according to the manufacturer's instructions.

2.23 Analytical size-exclusion chromatography (Analytical SEC):

This technique was used to assess the initial interactions between purified proteins. S3 R+T and TonB1, and S3 R+T and TolAIII proteins were mixed in a tube to form two separate protein complexes. Three tubes of each complex were made. Each tube was incubated under different conditions to explore which conditions might support a binding complex between the pairs of proteins. The first tube was incubated for 1 h on ice, the second was incubated for 30 min at room temperature, and the third was incubated at 37°C for 10 min. After incubation, samples were passed through a Superdex 200 10/300 GL column (GE Healthcare) using an AKTA pure chromatography system (Cytiva, UK). Elution was carried out using elution buffer (50 mM $\text{Na}_2\text{H}_2\text{PO}_4$, 150 mM

NaCl, pH 7). TonB1 was separated using the same approach used for S3 R+T. Furthermore, S3 R+T+TonB1 complexes that had been incubated under different conditions were run one after the other through the same column using the AKTA pure chromatography system (Cytiva, UK) using the same buffer. Moreover, the steps were repeated for S3 R+T and TolAIII proteins to test their interactions. Columns separated protein complexes based on size in all cases. All eluates were assessed based on UV peaks, with each peak representing a protein or protein complex.

2.24 Isothermal titration calorimetry (ITC):

Purified protein after SEC was applied to a MicroCal PEAQ-ITC system to explore protein-protein interactions. A Hamilton syringe was used to fill the reference cell with 300 μ L ultrapure water (Sigma-Aldrich, UK), and any excess water was removed using the syringe. The same process was applied to the sample cell, but instead of ultrapure water, the 1st protein of interest was loaded into the sample cell. Furthermore, 80 μ L of the 2nd protein of interest was loaded into the injection syringe. The parameters for the experiment are shown in Table 2.9. The injection syringe was placed into the sample cell and the experiment started. Results were analysed using Malvern MicroCal ITC Systems software (Microcal Llc, Northampton, USA).

Table 2.9: ITC parameters for experiments.

Protein concentration in the injection syringe	Variable (μ M)
Protein concentration in the sample cell	Variable (μ M)
Number of injections	19
Temperature	37°C

2.25 Pyocin S3 activity against *P. aeruginosa* clinical strains using the stab test:

E. coli BL21 (DE3) colonies containing the plasmid encoding PyoS3 protein pFAII were stabbed into an LB plate, alongside a strain of bacteria not producing PyoS3 that served as a negative control, and plates were incubated overnight at 37°C. In addition, the PSA 892 clinical strain was incubated in 5

mL LB broth overnight at 37°C. Furthermore, the plate containing the *E. coli* BL21(DE3) pFAII strain and the negative control strain was placed face down on top of cloth soaked in chloroform for 30 min to lyse bacteria and release the cell contents. Next, 8 µL of the PSA 892 clinical isolate strain was mixed with 18 mL of soft-top agar and poured on top of the plate containing the *E. coli* BL21 (DE3) pFAII and negative control strains. The plate was incubated overnight at 37°C and observed for any zones of inhibition around the stabbed colonies.

2.26 Testing the purified pyocin S3 protein against the sensitive PA clinical strain:

A 6.7 µM sample of PyoS3 protein was diluted to 1:10, 1:100, and 1:1000 in 50 mM Na₂H₂PO₄, 150 mM NaCl, pH 7 buffer. The PyoS3-sensitive PSA 892 and PSA 971 clinical strains were incubated overnight at 37°C in 5 mL LB broth. A 50 µL sample of the sensitive strain was mixed with 5 mL soft-top agar and poured into a Petri dish. A 2 µL sample of PyoS3 protein at different dilutions was spotted on top of the soft-top agar in the plate and incubated overnight at 37°C. After incubation, inhibition zones were observed.

2.27 Transposon library:

2.27.1 Conjugation:

P. aeruginosa clinical strain PSA 892 was incubated in 5 mL LB broth overnight and the S17-1 *E. coli* (pME9978) strain was incubated in 5 mL LB broth + gentamicin overnight. A 1 mL volume from each strain was loaded into two different Eppendorf tubes and centrifuged for 2 min at 5000 ×g. The supernatant was discarded, and the previous step was repeated until a sufficient pellet was present in the tube. The pellet was resuspended in 1 mL of phosphate-buffered saline (PBS) and centrifuged for 2 min at 5000 ×g. The PSA 892 strain required more than 2 min, hence samples were centrifuged until the pellet was present in the tube. Next, 50 µL/mL of the supernatant used to resuspend the resulting pellet. Furthermore, the PSA892 and S17-1 *λpir E. coli* (pME9978) strains were mixed in one tube and 20 µL from the mixture was spotted onto an LB plate and incubated for 6 h at 37°C. After incubation, a loop was used to scrape the spotted colonies and they were resuspended in 500 µL PBS. A 1:1 series of

dilutions were made, and each dilution was spread on an LB + gentamicin+ nalidixic acid plate and incubated overnight at 37°C.

2.27.2 Mutant testing:

A replica plate was made for each colony. A sample of each colony was incubated in 5 mL LB + gentamicin + nalidixic acid in a tube and cultured overnight at 37°C. A 5 mL volume of soft-top agar was added to each well of the 12 well plates, and 50 µL of each mutant was added to each well. When the soft-top agar had solidified, 2 µL of 6.7 µM PyoS3 purified protein was spotted on top of each well and incubated overnight at 37°C. Any inhibition zones were observed after 24 h, and possible mutants with an interesting inhibition zone different to the wild-type control were characterized further.

2.27.3 Plasmid recovery and sequencing:

Each interesting mutant was incubated overnight in LB broth + gentamicin+ nalidixic acid to extract genomic DNA. Genomic DNA was purified using a GenElute Genomic DNA Miniprep Kit (Sigma-Aldrich). The rescue cloning method was then conducted (Larsen et al., 2002). Genomic DNA was digested using *EcoRV* restriction enzyme overnight in a water bath at 37°C. *EcoRV* enzyme activity was stopped by incubating the tube at 65°C for 15 min. Furthermore, digested genomic DNA containing the transposon insertion was end-repaired and circled using a ligation enzyme and a Mastercycler personal instrument (Eppendorf) overnight. Ligation mixtures are shown in Table 2.10. After ligation, mixtures were transformed into *E. coli* DH5α competent cells by heat shock transformation. Cells were streaked onto an LB + gentamicin + nalidixic acid plate and incubated at 37°C overnight. The resulting colonies were incubated in 5 mL LB broth + gentamicin + nalidixic acid tubes overnight at 37°C. Plasmids were extracted using a GenElute Plasmid Miniprep Kit (SIGMA-ALDRICH, UK) and sequenced using *pME9978* primers by GENEWIZ, UK.

Table 2.10: Ligations reactions used in the transposon library experiment plasmid rescue step.

Ligation mix	Volume
Ligase enzyme	5 µL
Genomic DNA (gel extraction DNA product) containing transposon insert	20 µL
10× ligase buffer	5 µL
dH ₂ O	20 µL
Total volume	50 µL

2.28 Genomic analysis of PSA 892:

The genomic sequences of PSA 971 and PSA 892 *P. aeruginosa* clinical strains were analysed using The Pathosystems Resource Integration Center (PATRIC), a bacterial bioinformatics tool (Brettin et al., 2015). PATRIC BLAST was used to determine the transposon insertion of each PSA 892 mutant. In addition, PATRIC BLAST was used as identify pyoverdine biosynthesis enzymes.

2.29 Pyoverdine (PvD) production and quantification:

Iron-limited medium was prepared (10 mL of x5 SOH, 100 µL of 1 M MgSO₄, 2 mL of 0.5 M Succinate, 5 mL of 1 M CaCl₂ and 5 mL of 10 % CoD). Then each strain that used to test PvD production was incubated for overnight at 37°C in 5 ml LB broth. After that each strain would be mixed with iron limited medium and the OD_{600nm} would be measured until the mixture reaches 0.05 OD_{600nm}. Then, 200 µL from each mixture was loaded into 96-well plate as triplicate. Furthermore, the 96-well plate containing the samples would be transferred into TECAN to quantify the cells growth in the iron-limited environment. The UV wavelength measurement would be at 600 nm, the temperature would be at 37°C and kinetic cycle would be 48 times (30 minutes each cycle). Then the result would be analysed via Microsoft Excel.

2.30 X-ray crystallography:

A sparse matrix screening experiment was conducted. A 2 μ L sample of purified PyoS3 was placed above the reservoir of each well of a 96 well plate, alongside a control drop containing sample buffer + crystallization mixture without protein. Each crystallization mixture was loaded into its designated place in the plate using a Mosquito robot (SPT Labtech, UK). Plates were sealed with sealing sheet (Molecular Dimensions, UK) and incubated at 27°C for 24 h to allow crystals to grow. After incubation, each screen was observed under a microscope to see if crystals had formed, incubation was continued, and observations were repeated daily. The screens used in this study were the Ligand-friendly Screen (LFS) and the PACT Premier (PACT) Screen (Molecular dimensions, UK), as well as a screen made in the lab that only contained different pH and concentration of ZnCl₂ and all of them used PEG 6000 20 % w/v and Ethylene glycol 10 % v/v as precipitant (Table 2.11 and Table 2.12).

UV-microscopy was used to measure UV fluorescence from tryptophan residues to confirm whether crystals were made from protein or salt. After confirmation, crystals of interest were picked, stored in liquid nitrogen, and shipped to the Macromolecular X-ray Diffraction Facility at The University of Birmingham for crystal diffraction experiments, which were controlled remotely from the Biodiscovery Institute at the University of Nottingham.

Table 2.11: premade commercial screens that used to generate PyoS3 crystals.

Screen name	Well number	Buffer	Salt	Precipitant 1	Precipitant 2
LFS	A12	-	0.01 M ZnCl ₂	PEG 6000 20 % w/v	Ethylene glycol 10 % v/v
LFS	B12	0.1 M MES pH 6.0	0.01 M ZnCl ₂	PEG 6000 20 % w/v	Ethylene glycol 10 % v/v
LFS	C12	0.1 M HEPES pH 7.0	0.01 M ZnCl ₂	PEG 6000 20 %w/v	Ethylene glycol 10 % v/v
LFS	D12	0.1 M Tris pH 7.5	0.01 M ZnCl ₂	PEG 6000 20 % w/v	Ethylene glycol 10 % v/v
PACT	C12	0.1 M Sodium Acetate pH 5.0	0.01 M ZnCl ₂	PEG 6000 20 % w/v	-
PACT	D12	0.1 M Tris pH 8.0	0.002 M ZnCl ₂	PEG 6000 20 % w/v	-

Table 2.12: screens made in the laboratory. It contains only ZnCl_2 as a salt source with different concentration and pH. PEG 6000 20 % w/v and Ethylene glycol 10 % v/v as precipitant.

ZnCl_2						
pH		6mM	7mM	8mM	9mM	10mM
	5.0	+	+	+	+	+
	5.5	+	+	+	+	+
	6.0	+	+	+	+	+
	6.5	+	+	+	+	+
	7.0	+	+	+	+	+
	7.5	+	+	+	+	+
	8.0	+	+	+	+	+
	8.5	+	+	+	+	+
	9.0	+	+	+	+	+
	9.5	+	+	+	+	+

3 Chapter 3.

Investigation of the interactions between Pyocin S3 and periplasmic proteins TonB1 and TolAIII.

3.1 Introduction

The translocation of pyocin S3 (PyoS3) has not been fully studied. Other S-type pyocins, including Pyocins S2 and S4, bind pyoverdine receptors. Ferripyoverdine receptors utilise the TonB system to import small metabolites. It is likely that the translocation of PyoS3 also occurs via the pyoverdine ferrisiderophore and TonB system (Behrens, Lowe et al. 2020). Although PyoS1, S2 and S4 use the ferripyoverdine (Fe-Pvd) receptor FpvAI, PyoS3 uses FpvAII to recognize and bind to susceptible bacteria (Baysse et al., 1999, Michel-Briand and Baysse, 2002).

The TonB-dependent transporter (TBDT) FpvAI imports the small siderophore Fe-Pvd powered by the proton motive force (PMF) via the inner membrane protein TonB1. The N-terminal domain of PyoS2 binds to FpvAI, which causes conformational changes in the receptor. Additionally, the coupling of PyoS2 with Fe-Pvd leads to translocation of PyoS2 through FpvAI, in a manner similar to a translocation process used by the native TBDT ligand (White et al., 2017).

There are three different TonB proteins in *Pseudomonas*: TonB1, TonB2 and TonB3. TonB1 is the main iron transport protein in *P. aeruginosa*. TonB1 is an energy-transducing protein that harnesses the PMF of the cytoplasmic membrane (CM) to energise the outer membrane (OM) receptors that import molecules such as ferrisiderophores. TonB1 requires the cytoplasmic membrane-associated proteins ExbB and ExbD to fully function (Shirley and Lamont, 2009).

TonB1 mutants have limited ability to transport iron through siderophores and utilize haem, consequently they grow slowly in iron-limited medium. The *TonB2* gene is not required for *P. aeruginosa* to grow in iron-limited medium: however, TonB1 and TonB2 double mutants grow much more slowly in iron-limited medium than single TonB1 mutants. This indicates that TonB2 may partially

complement the iron acquisition role of TonB1. There is no evidence that TonB3 plays a role in iron acquisition; however, TonB3 is required for twitching motility and assembly of extracellular pili (Shirley and Lamont, 2009).

Much less is known about the translocation pathway of PyoS3, though one study showed that PyoS3 is translocated into the cell by the ferripyoverdine receptor FpvAII and therefore uses the Ton (Baysse et al., 1999, Cornelis and Bodilis, 2009). The S-type pyocins share structural and functional similarities with colicins E2, E7, E8 and E9. Therefore, as PyoS3 appears to utilize the FpvAII cell surface receptor, it is possible that PyoS3 also interacts with TonB system, PyoS3 may also interact with the Tol-Pal system, as group A colicins as well as the E9 type colicins utilise the Tol system as a mode of translocation (Parret and De Mot, 2002, Cascales et al., 2007b). There are five proteins that constitute the Tol-Pal system: TolA, TolB, TolQ, TolR, as well as the peptidoglycan-associated lipoprotein (Pal). There are three domains in TolA, separated by clusters of glycine residues in the periplasm of the cell. TolA single transmembrane region (TolAI) is anchored to the cytoplasmic membrane, all of which are vital for interactions with the TolQ and TolR proteins within the membrane. TolA extended central domain (TolAII) spans the periplasm, and its C-terminal domain (TolAIII) binds to TolB and Pal proteins in the periplasm. A transmembrane protein called TolQ and TolR are responsible for the proton motive force-dependent activation of TolA. By interacting with Pal anchored on the outer membrane, the proton motive-dependent activation of TolA shuttles energy from the inner membrane through its interaction with the outer membrane. The periplasmic protein TolB is linked to the outer membrane by means of an interaction between its C-terminal domain with Pal and its N-terminal domain with TolA (Szczepaniak et al., 2020). In addition, it has been found that colicin A binds to TolA binding site TolAIII (Li et al., 2012). The purpose of the *in vitro* experiments in this Chapter were to determine whether PyoS3 utilises the TonB or TolA system of translocation. As PyoS3 utilises the PvdII receptor FpvAII, which uses the TonB system (Baysse et al., 1999), we hypothesized that PyoS3 binds to TonB1 or TolAIII. To demonstrate an interaction, *tonB1*, *tolAIII* plus DNA encoding the translocation and receptor domains of PyoS3 were PCR amplified and cloned into expression vectors to

produce high-quality purified proteins. The receptor (R) domain was cloned and co-expressed with the translocation (T) domain of PyoS3 as the translocation domain of colicins and S type pyocins are unstructured and previous work in our laboratory demonstrated a stable, structured protein could not be purified for *in vitro* assays.

Analytical size exclusion chromatography (Analytical SEC) was employed to assess whether the PyoS3 R+T domains interact with either TonB1 or TolAIII (Figure 3.1). The advantage of Analytical SEC is that it can be used to detect stable protein interaction complexes without the need to tag the purified proteins with non-native moieties. The protein interactions take place in solution, and the size and stoichiometry of the protein complexes can be estimated to identify protein partners and the optimal interaction conditions. Analytical SEC is simple to set up and the instrument is relatively inexpensive. The disadvantages are that complexes with a relatively low binding affinity can dissociate and may not be detected (Striegel, 2017).

The more sensitive technique isothermal titration calorimetry (ITC) was also conducted in an attempt to further determine whether PyoS3 interacts with TonB1 or TolAIII. An interaction between PyoS2 and TonB1 was observed by White *et al.* using ITC (White et al., 2017). ITC is a titration-based method used to assess the interaction affinity and enthalpy of binding interactions between proteins and or small molecules. ITC is performed at a constant temperature in solution with the only measurement being the thermal effects due to the absorption or release of heat that accompanies every reaction. The advantages of ITC are that a single experiment provides a thorough thermodynamic picture of protein interactions, as well as the stoichiometry of the interaction (Baranauskiene et al., 2019, Recht et al., 2016). The disadvantage of ITC is that large amounts of highly purified proteins and ligands are required. The proteins and ligands must be sufficiently soluble in aqueous solution and remain stable in solution under stirring for an hour at the studied temperature (Baranauskiene et al., 2019).

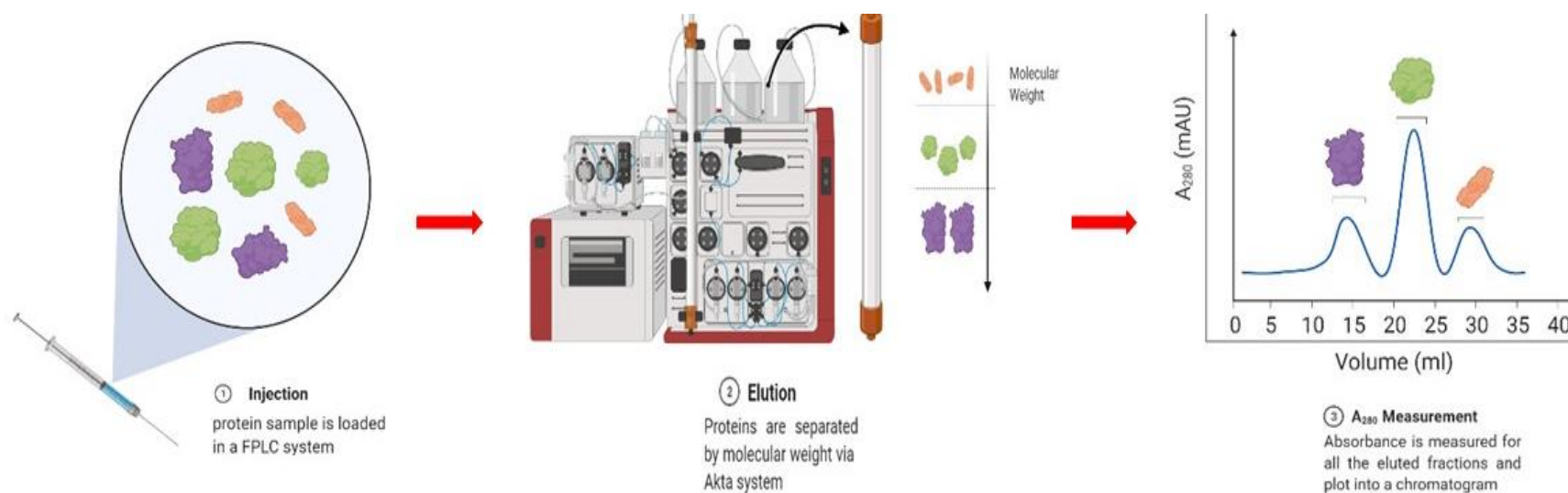


Figure 3.1: Illustration of Size-exclusion chromatography (SEC) analysis of protein purification and protein-protein interactions. Protein samples or protein complexes are injected into the FPLC system on the Akta Pure machine or AktaPrime machine. The column separates the protein or complexes by size; larger proteins elute first and smaller proteins elute later. Protein complex interactions are detected as changes in the spectra compared the spectra of individual non-complexed proteins. The graph shows the UV absorbance measurements ($A_{280\text{nm}}$) and the fractions eluted are plotted on the chromatogram.

3.2 Results

In order to generate sufficient quantities of purified proteins to test the interactions between PyoS3 and the receptors possibly involved in its uptake and import, genetic engineering was used to construct plasmids that overexpress PyoS3 R+T, TonB1 and TolAIII.

3.2.1 PCR amplification and Cloning of PyoS3 R+T and TonB1 into the pGEM-T easy vector and pET21a vector

DNA encoding the R+T domains of PyoS3 was amplified using a Mastercycler personal (Eppendorf®) PCR Cycler, and the resultant PCR reactions were separated on a 0.8% agarose gel to visualize the products. The expected fragment size of 2.0 kbp was obtained for the *PyoS3* R+T cDNA (Figure 3.2) and an amplified DNA fragment of the expected size of 0.8 kbp was obtained for *TonB* (Figure 3.3).

The DNA fragments encoding PyoS3 R+T and TonB1 DNA were extracted from the agarose gels, cloned into pGEM-T vector for blue-white screening, then cloned into the pET21a vector for expression. Restriction digestion of the recombinant plasmids encoding PyoS3 R+T and TonB1 was performed using *NdeI* and *XhoI*. A Mastercycler personal (Eppendorf®) PCR Cycler was used to control the temperature of the ligations more accurately than a water bath or heating block. DNA fragments of the expected sizes (2.0 kbp and 0.8 kbp) were obtained for the PyoS3 R+T and TonB1 plasmids, respectively (Figure 3.2 and Figure 3.3). The constructs were sent for sequencing to confirm the amplification and cloning were correct. The sequencing revealed correct amplification of the PyoS3 R+T and TonB1 cDNA fragments (see Appendix 1). Additionally, a pBAG1 plasmid expressing TolAIII, which had been cloned by MSc student Bashair AL-Gethami was sent for sequencing and confirmed to be correct (see Appendix 1).

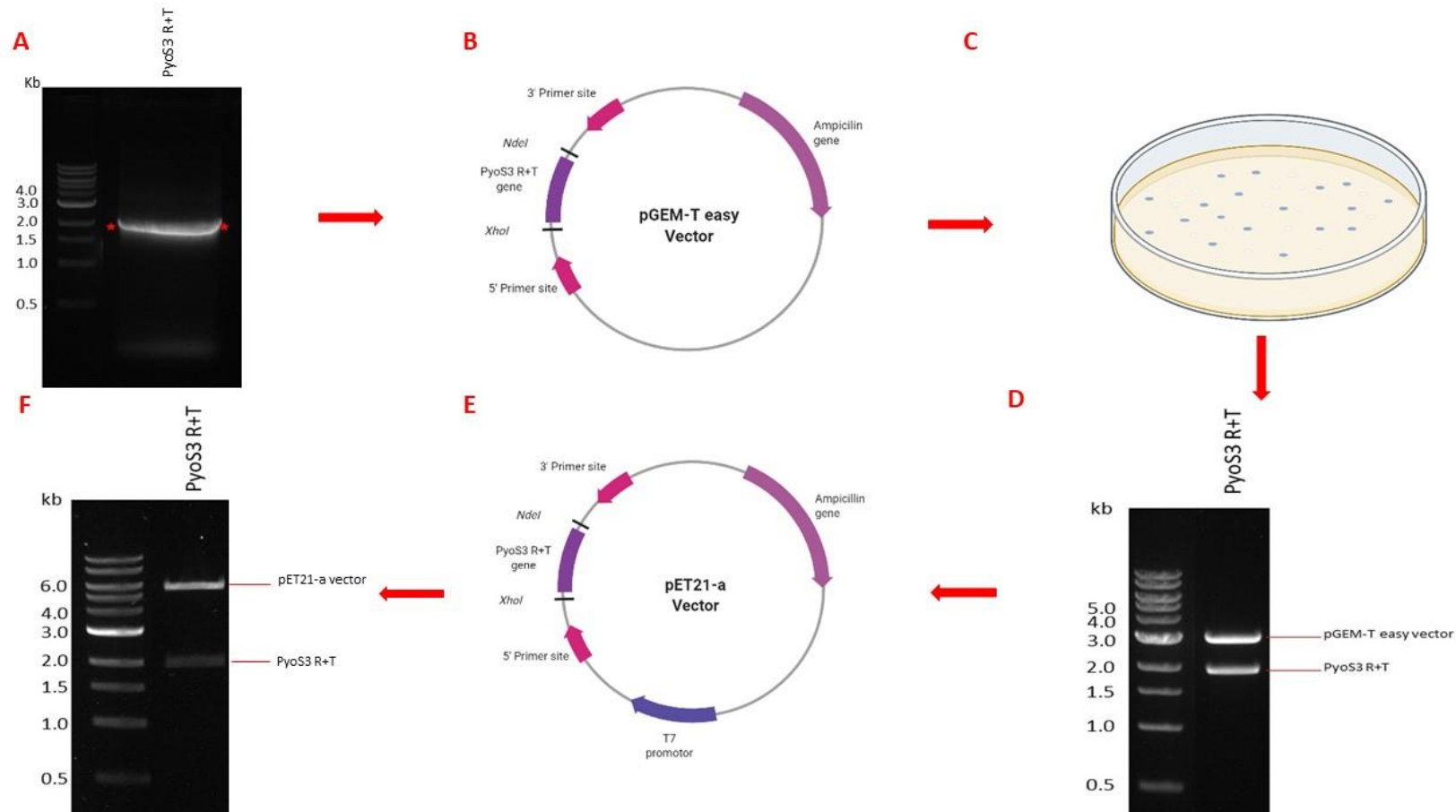


Figure 3.2: PyoS3 R+T amplification and cloning into a suitable vector for replication and expression. A: The cDNA encoding PyoS3 R+T was successfully amplified by PCR. Lane 1: Molecular weight markers of 100 kbp. Lane 2: PyoS3 R+T sample, the stars in red highlight the expected fragment size of 2.0 kbp. B: Diagram of the pGEMT-easy vector containing the DNA fragment of interest. C: Illustration of an Ampicillin + X-gal + IPTG plate. The white

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colonies confirmed insertion of the gene of interest into the vector. D: Restriction digestion with *NdeI* and *XhoI* verified the plasmid (pFATI) harbours the DNA encoding PyoS3 R+T . Lane 1: Molecular weight markers of 100 Kbp. Lane 2: Expected DNA fragment size of 2.0 kbp for PyoS3 R+T and the pGEM-T easy vector of 3.0 kbp. E: Diagram of the pET21a vector containing the gene of interest. F: Restriction digestion with *NdeI* and *XhoI* verified the plasmid (pFATI) harbours the DNA encoding PyoS3 R+T. Lane 1: Molecular weight markers of 100 kbp. Lane 2: Expected DNA fragments of 2.0 kbp for PyoS3 R+T and the pET21a plasmid of 6.0 kbp.

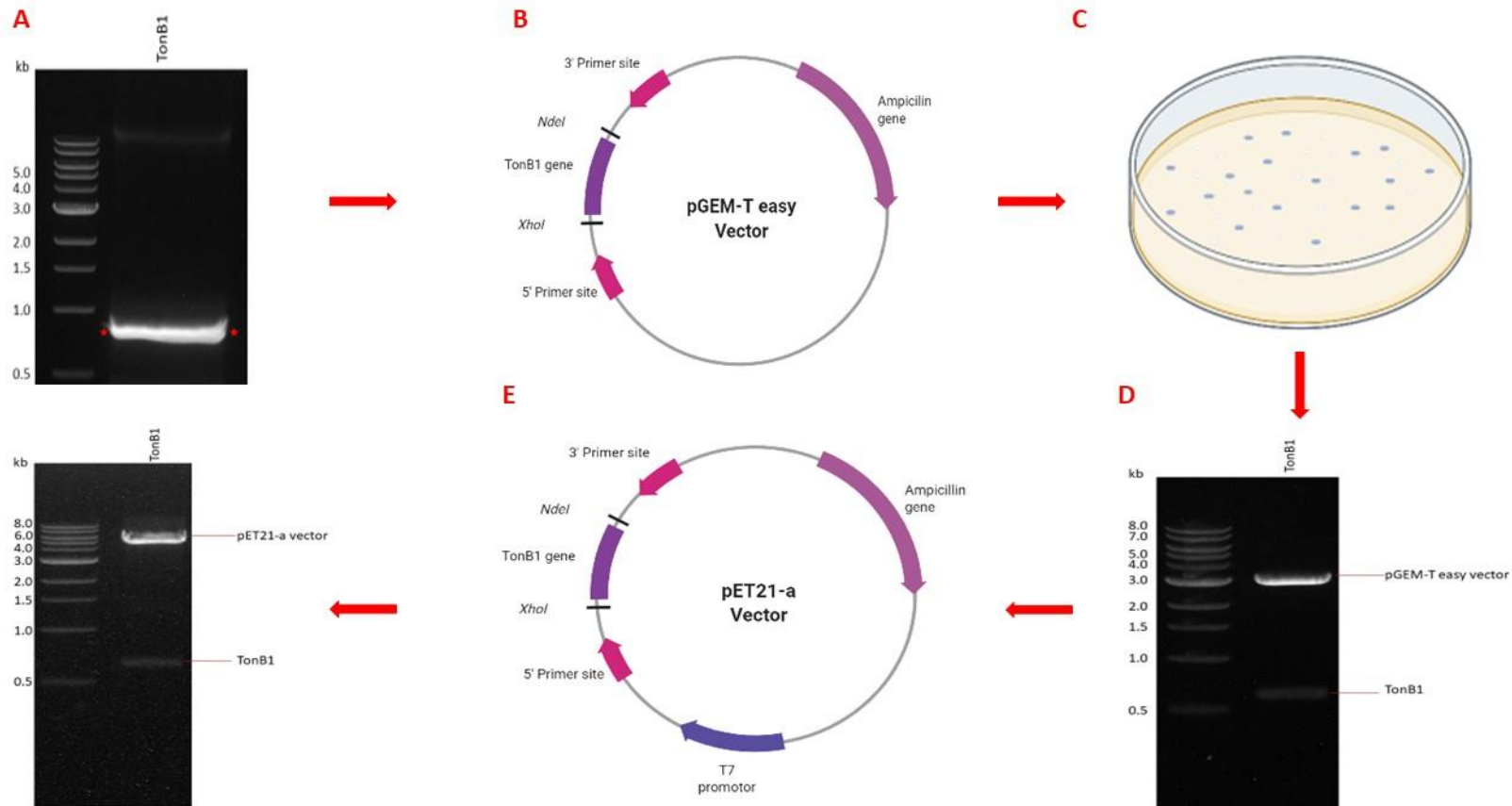


Figure 3.3: TonB1 amplification and cloning into a suitable vector for replication and expression. A: The cDNA encoding TonB1 was successfully amplified by PCR. Lane 1: Molecular weight markers of 100 kbp. lane 2: TonB1 sample, the stars in red highlight the expected fragment of 0.8 kbp. B: Diagram

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of the pGEMT-easy vector containing the inserted DNA fragment. C: Illustration of an Ampicillin + X-gal + IPTG plate. The white colonies confirmed insertion of the fragment of interest into the vector. D: Restriction digestion with *NdeI* and *XhoI* verified the plasmid (tFAI) harbours the cDNA TonB1. Lane 1: Molecular weight markers of 100 kbp. Lane 2: Expected DNA fragments of 0.8 kbp for TonB1 and the pGEM-T easy plasmid of 6.0 kbp. E: Diagram of the pET21a vector containing the gene of interest. F: Restriction digestion with *NdeI* and *XhoI* verified the plasmid (tFAII) harbours the DNA encoding TonB1. Lane 1: Molecular weight markers of 100 kbp. Lane 2: Expected DNA fragments of 0.8 kbp for TonB1 and the pET21a plasmid of 6.0 kbp.

3.2.2 Site-direct mutagenesis (SDM) of TonB1 DNA to remove the ochre (TAA) stop codon

After the sequence carried on the three plasmids pBAG1 (ToLAIII), tFAII (TonB1) and pFATII (PyoS3 R+T) recombinant plasmids, ochre (TAA) stop codon in TonB1 sequence was present. SDM was used to remove the stop codon. *E. coli* BL21(DE3) and *E. coli* ER2566 cells were used to grow the mutant TonB1 plasmid after the SDM but it was not successful and the stop codon still present (Figure 3.4).

TCTAGANTNTTTNGTTCTT**TAA**GAAGGAGATATACATATGCACCACCACCA
 S R X F X S L R R R Y T Y A P P P
 L X X X V L . E G D I H M H H H H
 I . X X X F F K K E I Y I C T T T

Figure 3.4: ochre stop codon in TonB1 sequence is present after SDM attempt to remove it.

3.2.3 Overexpression of PyoS3 R+T, TonB1 and TolAIII in BL21 (DE3) *E. coli*

Protein production from the plasmids constructed in this study (as detailed above) was analysed by inducing expression of the cloned genes with IPTG. Analysis of Coomassie-stained SDS PAGE gels showed PyoS3 R+T migrated at the expected protein size of 78 kDa. The expected protein size of 23 kDa was obtained for TonB1 and TolAIII was expressed at the expected size of 11 kDa (Figure 3.5).

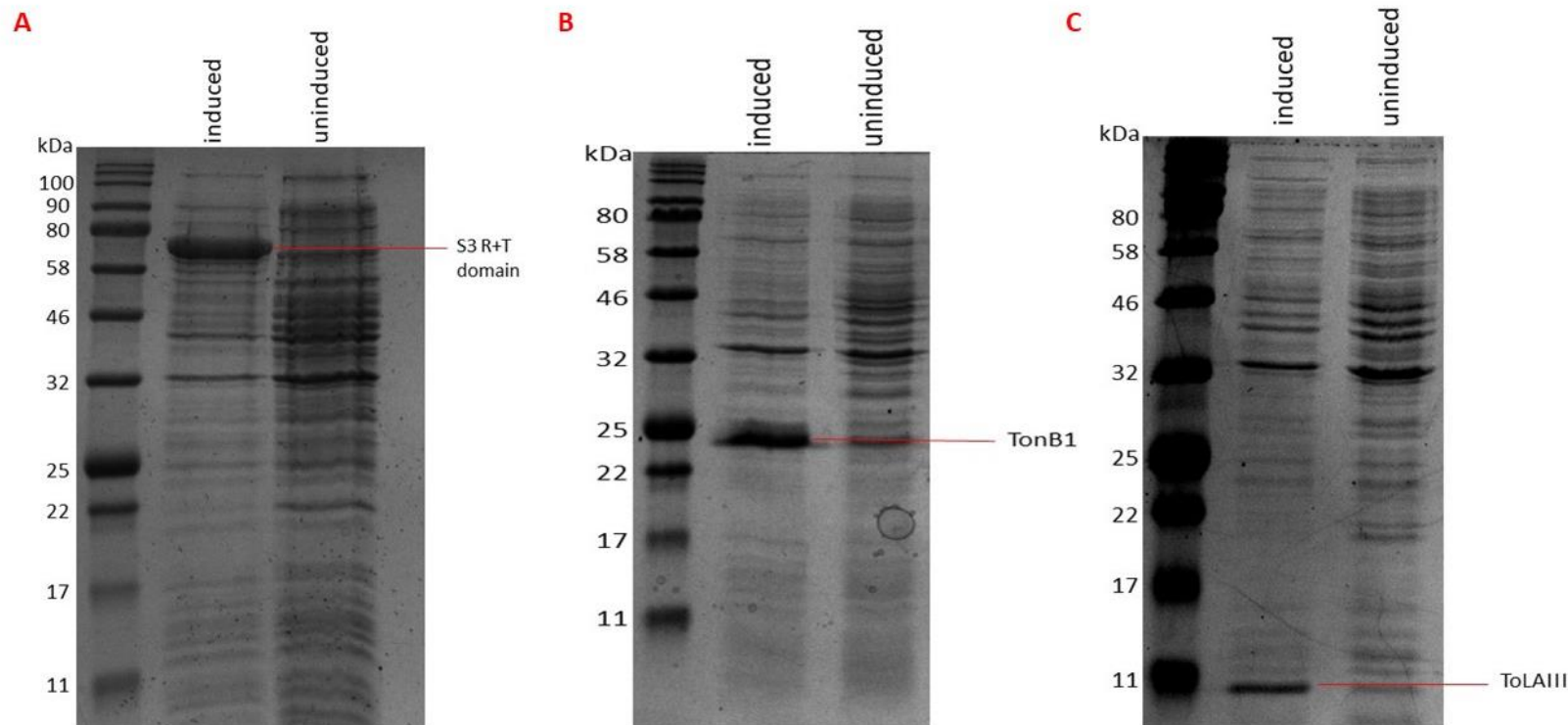


Figure 3.5: SDS-PAGE analysis of PyoS3 R+T, TonB1 and TolAIII protein expression. A: SDS-PAGE analysis of PyoS3 R+T expression. Lane 1: 11-245 kDa molecular marker. Lane 2: IPTG-induced protein sample, showing overexpression of PyoS3 R+T at the expected size of 78 kDa; Lane 3: uninduced protein sample. B: SDS-PAGE analysis of TonB1 expression. Lane 1: 11-245 kDa molecular marker. Lane 2: IPTG-induced protein sample, showing overexpression of the TonB1 at the expected size of 23 kDa; Lane 3: uninduced protein sample. C: SDS-PAGE analysis of TolAIII expression. Lane 1: 11-245 kDa molecular marker. Lane 2: IPTG-induced protein sample, showing overexpression of TolAIII protein at the expected size of 11 kDa; Lane 3: uninduced protein sample.

3.2.4 Metal chelation affinity chromatography purification of PyoS3 R+T, TonB1 and TolAIII

After each protein was over-produced *in vivo*, it was necessary to undertake purification steps to remove contaminants so that the interactions of the complexes could be assessed without non-specific interactions leading to inaccurate conclusions. His Trap HP columns and the AktaPrime system were used to purify the proteins since each had a His-tag engineered to their C-terminus during cloning. The eluted fractions of interest were collected and analysed via SDS-PAGE.

The AktaPrime system detects eluted proteins in real-time. The UV peaks shown on the graph correspond to eluted proteins; thus, fractions were collected as the peaks started to appear. Elution of the protein was initiated by the imidazole present in the purification running buffer (buffers A and B). The imidazole interacts with the nickel in the column with a higher affinity than the proteins, which displaces and elutes the bound proteins.

The first UV peak (Figure 3.6, blue line indicated by red arrow) detected during purification of PyoS3 R+T was a non-specific contaminant that washed out without binding to the column. Fractions 1-12 were collected at the start of the second UV peak (Figure 3.6), and only fractions F4–F6 were analysed via SDS-PAGE (Figure 3.7) because they correspond to the highest region of the second UV peak. SDS-PAGE (Figure 3.7) revealed that fractions F4 and F5 contained high levels of a protein with the expected molecular mass of PyoS3 R+T of 78 kDa (Figure 3.7).

The same strategy was applied during the purification of TonB1; the first peak (Figure 3.8) was most likely a non-specific contaminant, whilst the second peak was thought to be purified TonB1 (Figure 3.8). Fractions F1–F14 were collected and only fractions F1–F5 were assessed by SDS-PAGE (Figure 3.9). SDS-PAGE showed that F3–F5 contained a high level of a protein with the expected molecular weight of TonB1 (23 kDa), with F5 containing the highest quantity of this protein (Figure 3.9).

For TolAIII, the first UV peak (Figure 3.10) was a nonspecific contaminant being washed out of the column. Fractions were collected during the second and third UV peaks (F1–F11). SDS-PAGE (Figure 3.11) showed that F1–F4 contained high levels of a protein with the expected size for TolAIII (11 kDa), with F2 containing the highest quantity of this protein (Figure 3.11) .

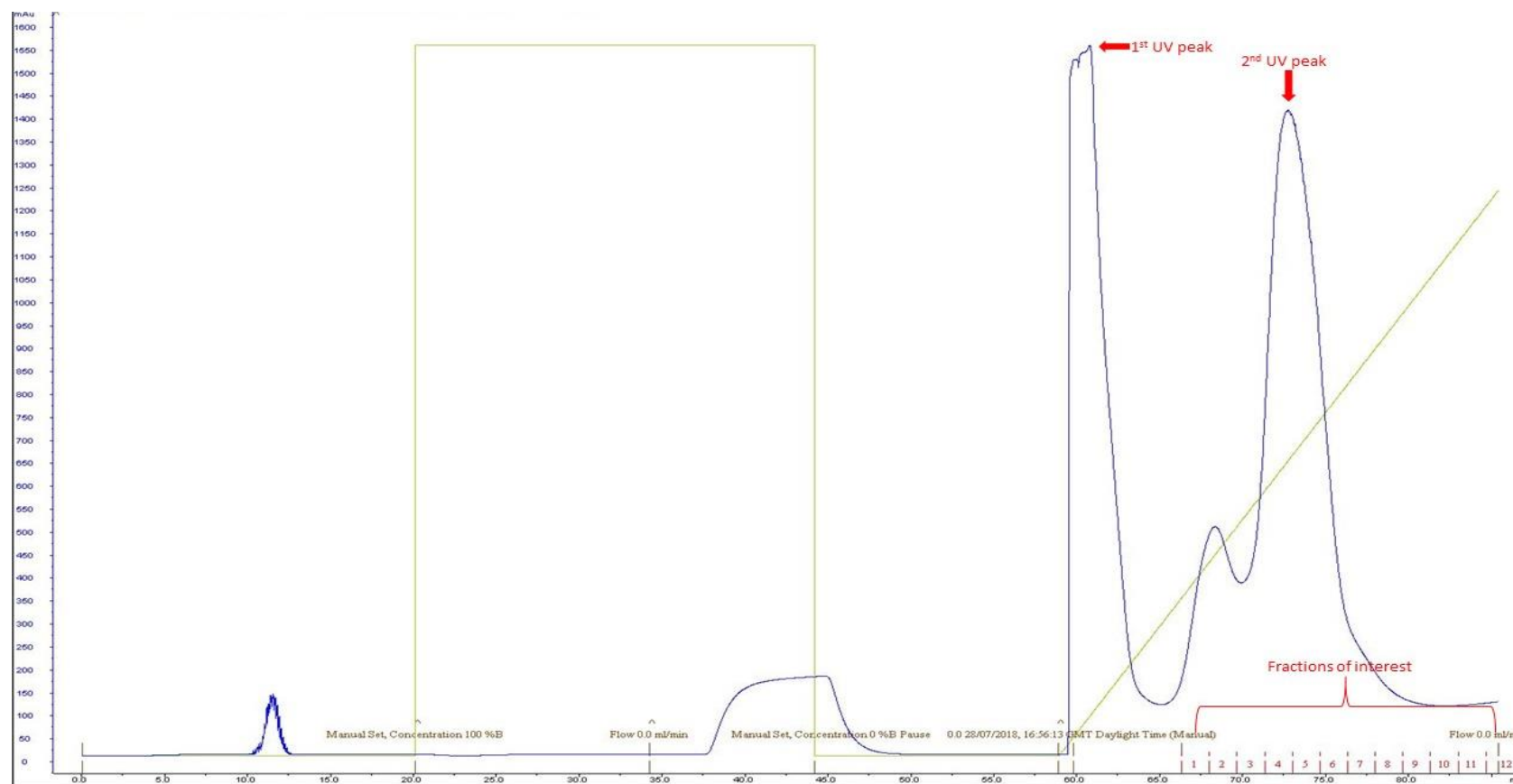


Figure 3.6: Purification of the PyoS3 R+T domain protein via a His Trap HP column and the AKTAPrime system. *E. coli* BL21(DE3) pFATII was induced with IPTG, sonicated, centrifuged and the soluble proteins in the lysate were applied to His Trap HP column. Buffer A was run through the column to wash unbound proteins through, and then bound proteins were eluted with Buffer B at the concentrations indicated by the green line. The y-axis on the affinity

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chromatography trace shows the UV absorbance (mAu) and the x -axis shows the volume (mL). The blue line shows the UV absorbance, the green line shows the concentration of buffer used for protein elution; the red numbers on the x -axis indicate the number of fractions of interest collected. The “1st UV peak” is the non-specific UV peak and the fractions of interest were collected during the “2nd UV peak”.

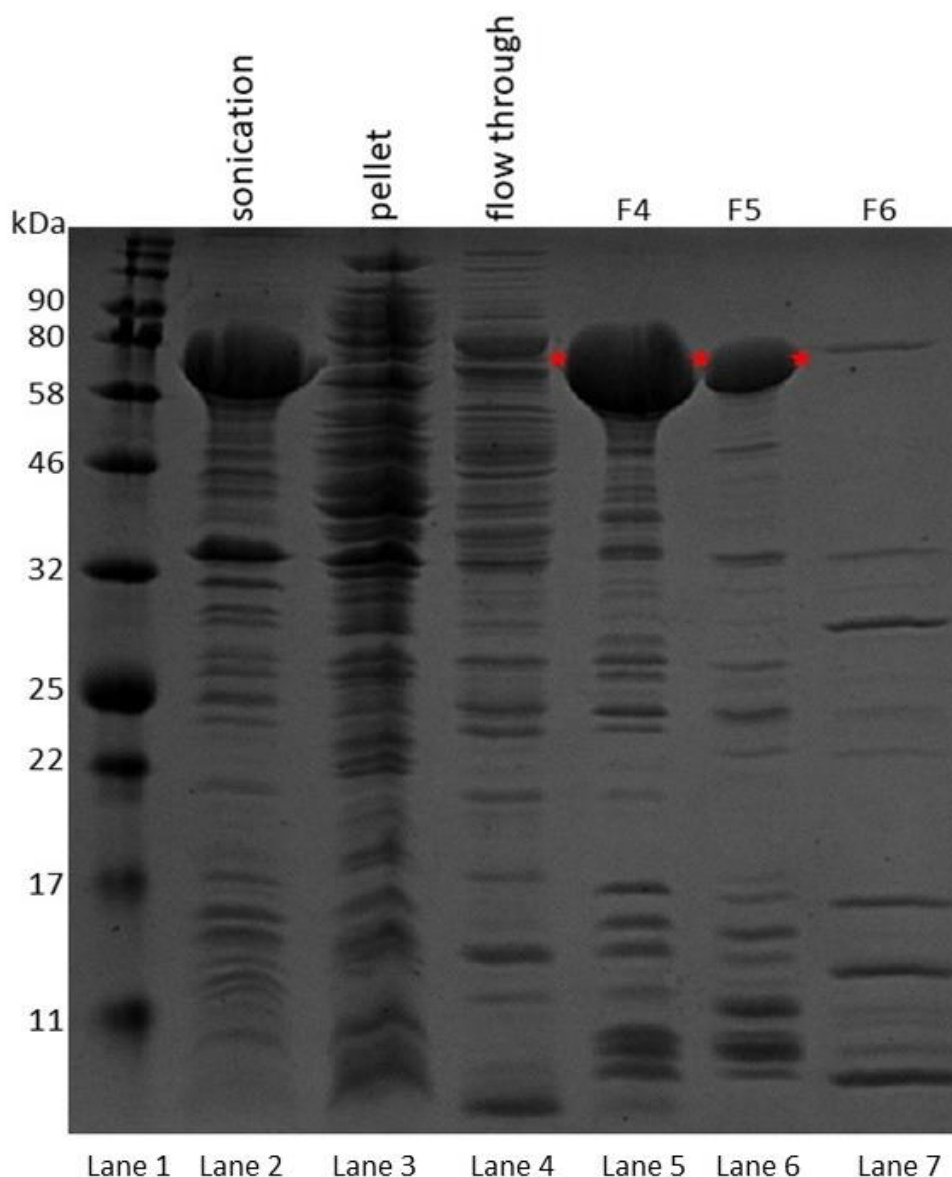


Figure 3.7: SDS-PAGE analysis of the fractions obtained during affinity chromatography purification of the PyoS3 R+T protein. Protein extracts containing PyoS3 R+T applied to and eluting from affinity chromatography (Figure 3.5) were loaded onto an SDS PAGE and stained with Coomassie blue. Lane 1: 11-245 kDa molecular marker (sizes indicated on the left). Lane 2: aliquot taken after sonication of IPTG induced *E. coli* strain pFATII. Lane 3: aliquot of the insoluble proteins in the lysate pelleted by centrifugation, and thus not applied to the affinity chromatography. Lane 4: aliquot taken from column flowthrough of buffer A (unbound soluble proteins). Lanes 5-7: fractions F4–F6 collected during elution with buffer B containing imidazole. F4 and F5 contain the PyoS3 R+T domain fragment with the expected size of 78 kDa.

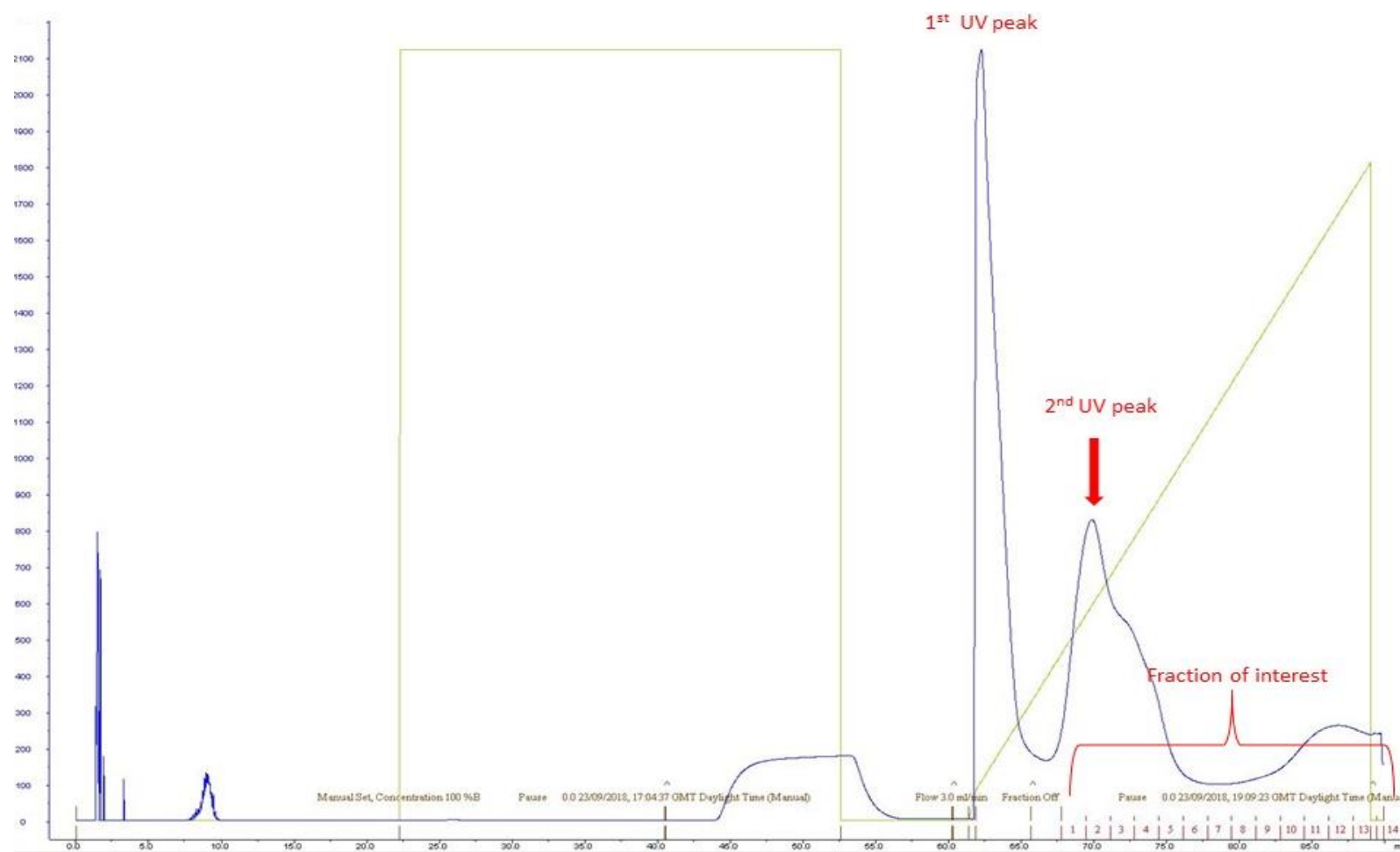


Figure 3.8: Purification of TonB1 protein via His Trap HP column and the AKTAPrime system. *E. coli* BL21(DE3) tFall was induced with IPTG, sonicated, centrifuged and the soluble proteins in the lysate were applied to His Trap HP column. Buffer A was run through the column to wash unbound proteins through,

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and then bound proteins were eluted with Buffer B at the concentrations indicated by the green line. The y -axis on the affinity chromatography trace shows the UV absorbance (mAu) and the x -axis shows the volume (mL). The blue line shows the UV absorbance, the green line shows the concentration of buffer used for protein elution; the red numbers on the x -axis indicate the number of fractions of interest collected. The “1st UV peak” is the non-specific UV peak and the fractions of interest were collected during the “2nd UV peak”.

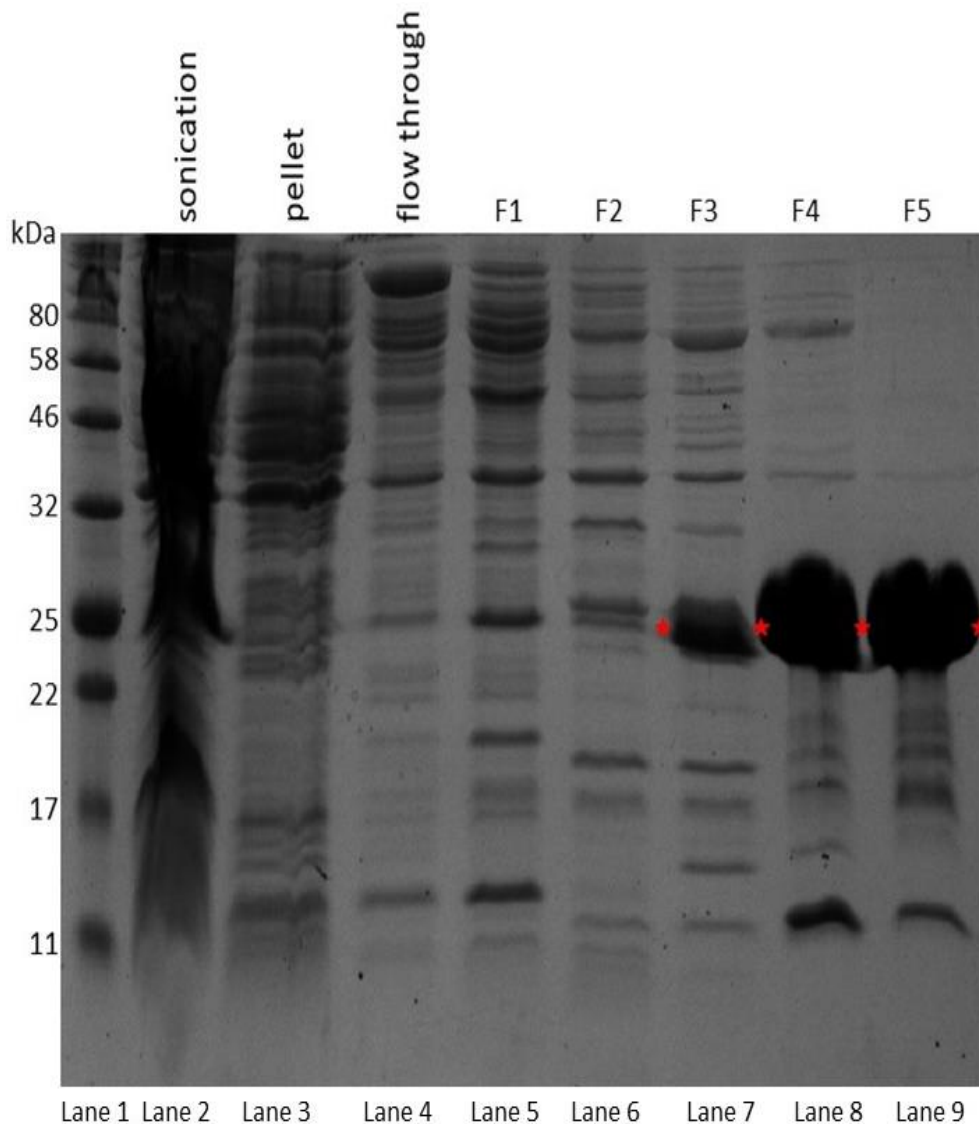


Figure 3.9: SDS-PAGE analysis of the fractions obtained during affinity chromatography purification of the TonB1 protein. Protein extracts containing TonB1 applied to and eluting from affinity chromatography (Figure 3.8) were loaded onto an SDS PAGE and stained with Coomassie blue. Lane 1: 11-245 kDa molecular marker (sizes indicated on the left). Lane 2: aliquot taken after sonication of IPTG induced *E. coli* strain tFAIL.; Lane 3: aliquot of the insoluble proteins in the lysate pelleted by centrifugation, and thus not applied to the affinity chromatography. Lane 4: aliquot taken from column flowthrough of buffer A (unbound soluble proteins). Lanes 5–9: fractions F1–F5 collected during elution with buffer B containing imidazole. F3–F5 contain TonB1 with the expected fragment size of 23 kDa, with the highest quantity of TonB1 in F5.

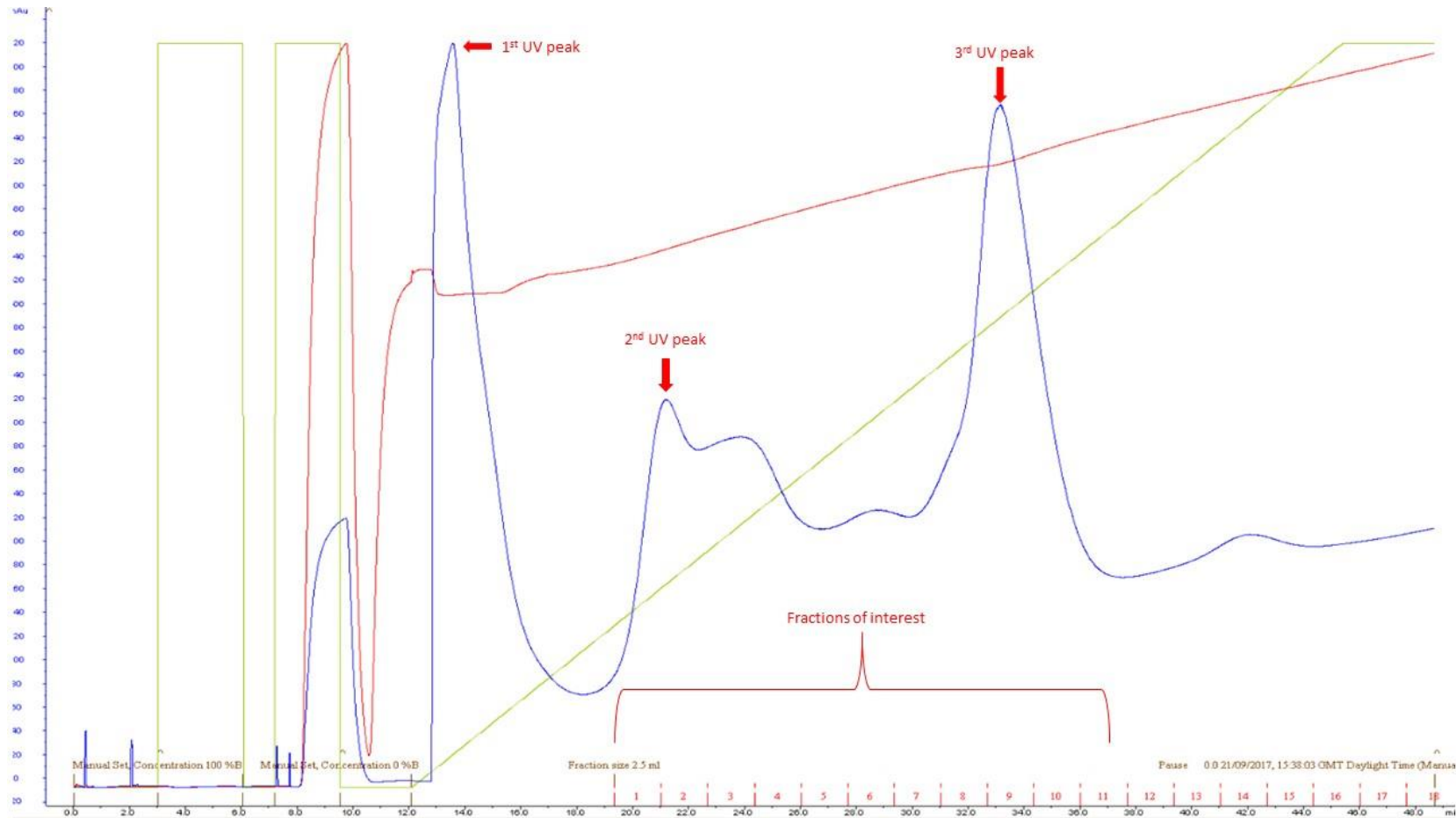


Figure 3.10: Purification of TolAIII protein via His Trap HP column and the AKTAPrime system. *E. coli* BL21(DE3) pBAG1 was induced with IPTG, sonicated, centrifuged and the soluble proteins in the lysate were applied to His Trap HP column. Buffer A was run through the column to wash unbound proteins through, and then bound proteins were eluted with Buffer B at the concentrations indicated by the green line. The y-axis on the affinity chromatography trace

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shows the UV absorbance (mAu) and the x-axis shows the volume (mL). The blue line shows the UV absorbance, the green line shows the concentration of buffer used for protein elution; the red numbers on the x-axis indicate the number of fractions of interest collected. The “1st UV peak” is the non-specific UV peak and the fractions of interest were collected during the “2nd UV peak”.

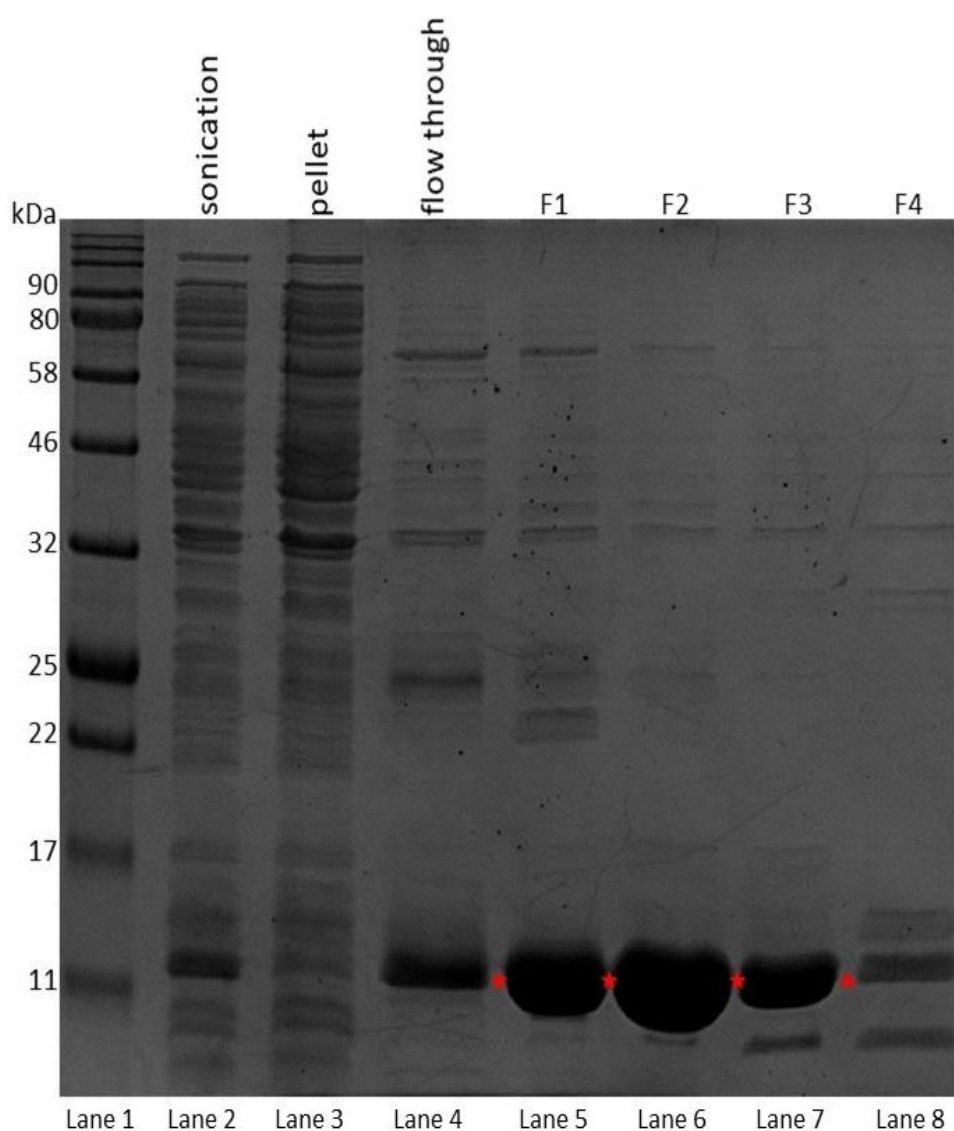


Figure 3.11: SDS-PAGE analysis of the fractions obtained during affinity chromatography purification of the TolAIII protein. Protein extracts containing *TonB1* applied to and eluting from affinity chromatography (Figure 3.10) were loaded onto an SDS PAGE and stained with Coomassie blue. Lane 1: 11–245 kDa molecular marker (sizes indicated on the left). Lane 2: aliquot taken after sonication of IPTG induced *E. coli* strain pBAG1. Lane 3: aliquot of the insoluble proteins in the lysate pelleted by centrifugation, and thus not applied to the affinity chromatography. Lane 4: aliquot taken from column flowthrough. Lanes 5–8: fractions F1–F4 collected during elution with buffer B containing imidazole. F1–F3 contain the TonB1 with the expected fragment size of 23 kDa, with the highest quantity of TonB1 in F2.

3.2.5 Size-exclusion chromatography (SEC) improved the purity of TonB1, PyoS3 R+T and TolAIII without significantly reducing the yield

After the affinity chromatography step to initially isolate each protein of interest for interaction studies (PyoS3 R+T, TonB1, and Tol AIII), SEC was used to remove the remaining contaminating proteins. PyoS3 R+T was purified on a Hiload 26/600 Superdex 75 pg size-exclusion chromatography column (Figure 3.12). Fractions F1–F13 were collected, but only fractions F9–F11 were analysed by SDS-PAGE because these fractions corresponded to the UV peak (Figure 3.12). SDS-PAGE analysis showed a protein in fractions F9–11 with the expected size of 78 kDa for PyoS3 R+T, with the highest amount of this protein in F10 (Figure 3.13). In addition, it can be seen that the contaminant that was visible with PyoS3 R+T protein in lane 5 (Figure 3.7) was not present in Lane 3 (Figure 3.13).

The same process was applied to TonB1; fractions F1–F13 were collected (Figure 3.14) and F1–F8 were analysed by SDS-PAGE (Figure 3.15). F5–F7 contained a protein of the expected size of 23 kDa for TonB1, with F7 containing the highest quantity of this protein (Figure 3.15). Furthermore, contaminants in lane 9 (Figure 3.9) now not evident in lane 8 (Figure 3.15). Similarly, F1–F14 were collected from SEC for TolAIII (Figure 3.16) and F10–F12 were analysed by SDS-PAGE (Figure 3.17). F10–F12 contained a protein of the expected size of 11 kDa, with F11 containing the highest level of this protein (Figure 3.17). Moreover, lane 6 (Figure 3.11) contaminants were not visible in lane 3 (Figure 3.17).

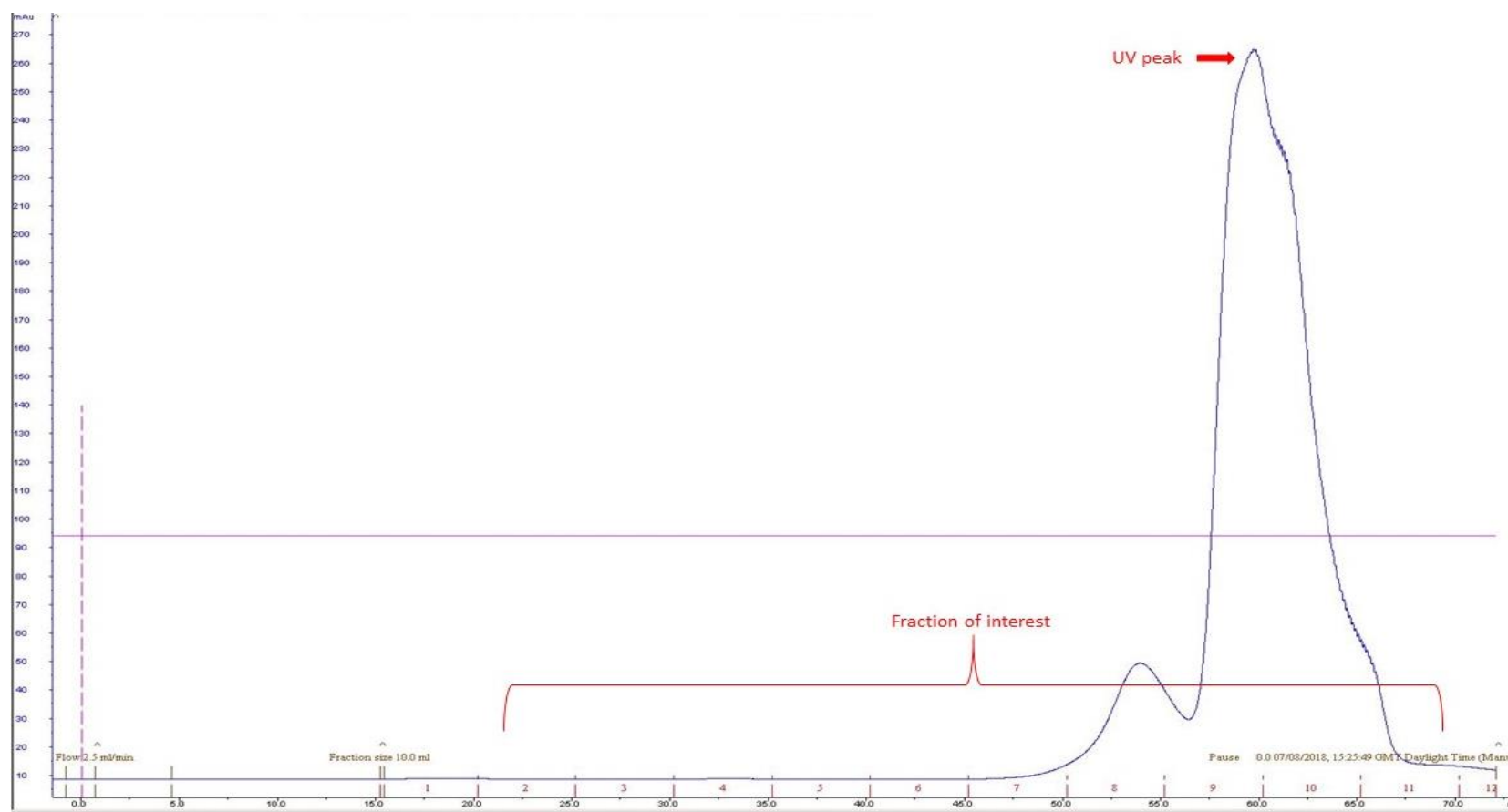


Figure 3.12: Purification of the PyoS3 R+T domain protein by size-exclusion chromatography on the Hiload 26/600 Superdex 75 pg column and AKTAPrime chromatography system. Fractions F4 and F5 eluted from affinity chromatography (Figure 3.6) containing PyoS3 R+T (lanes 5 and 6 in Figure

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3.7) were pooled and applied to SEC. The y -axis on the SEC chromatography trace shows the UV absorbance (mAu) and the x -axis shows the volume (mL). The blue line shows the UV absorbance; the red numbers on the x -axis indicate the number of fractions of interest collected. The fractions of interest were collected during the UV peak indicated by the red arrow.

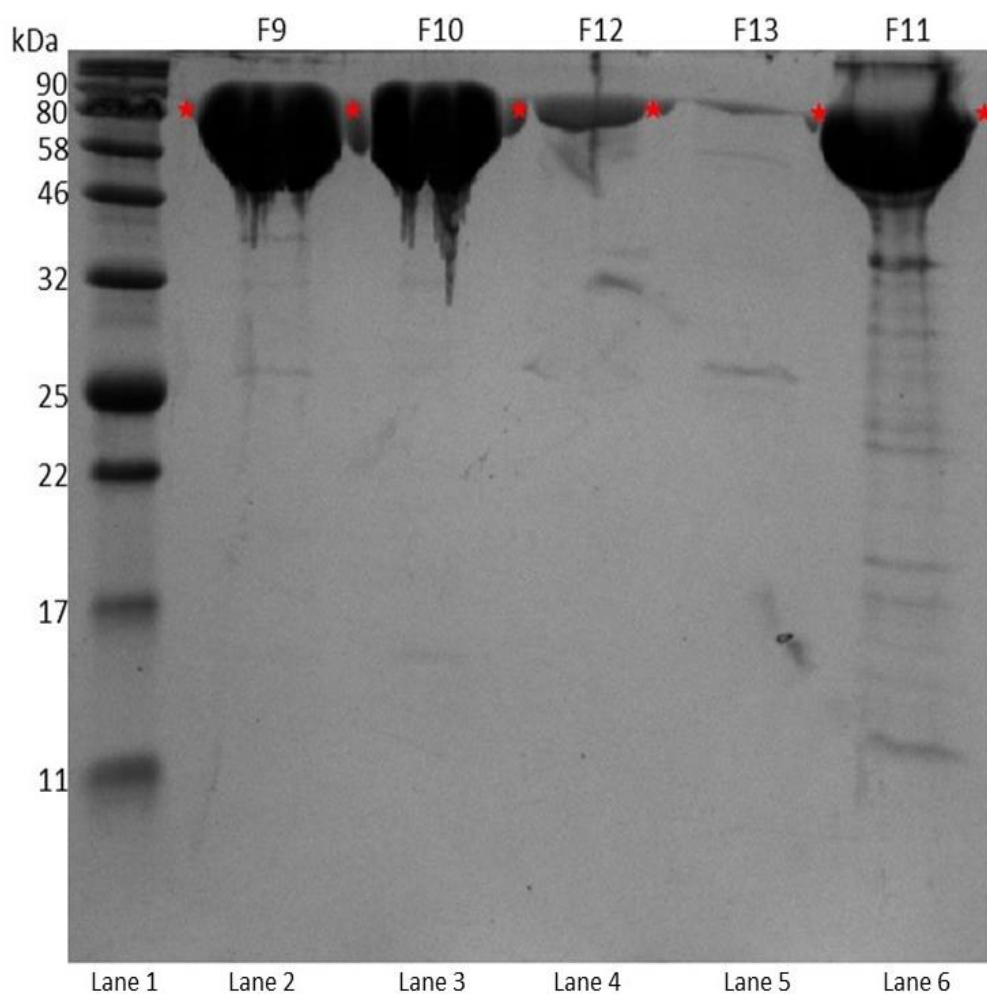


Figure 3.13: SDS-PAGE analysis of the fractions obtained during size-exclusion chromatography of the PyoS3 R+T protein. The indicated fractions from SEC of PyoS3 R+T (Figure 3.12) were applied to 10% SDS PAGE and visualised by Coomassie Blue staining. Lane 1: 11-245 kDa molecular marker. Lanes 2– 6: Fractions F9–F13. F9, F10 and F11 contain PyoS3 R+T at the expected size of 78 kDa; F10 contains the highest amount of PyoS3 R+T.

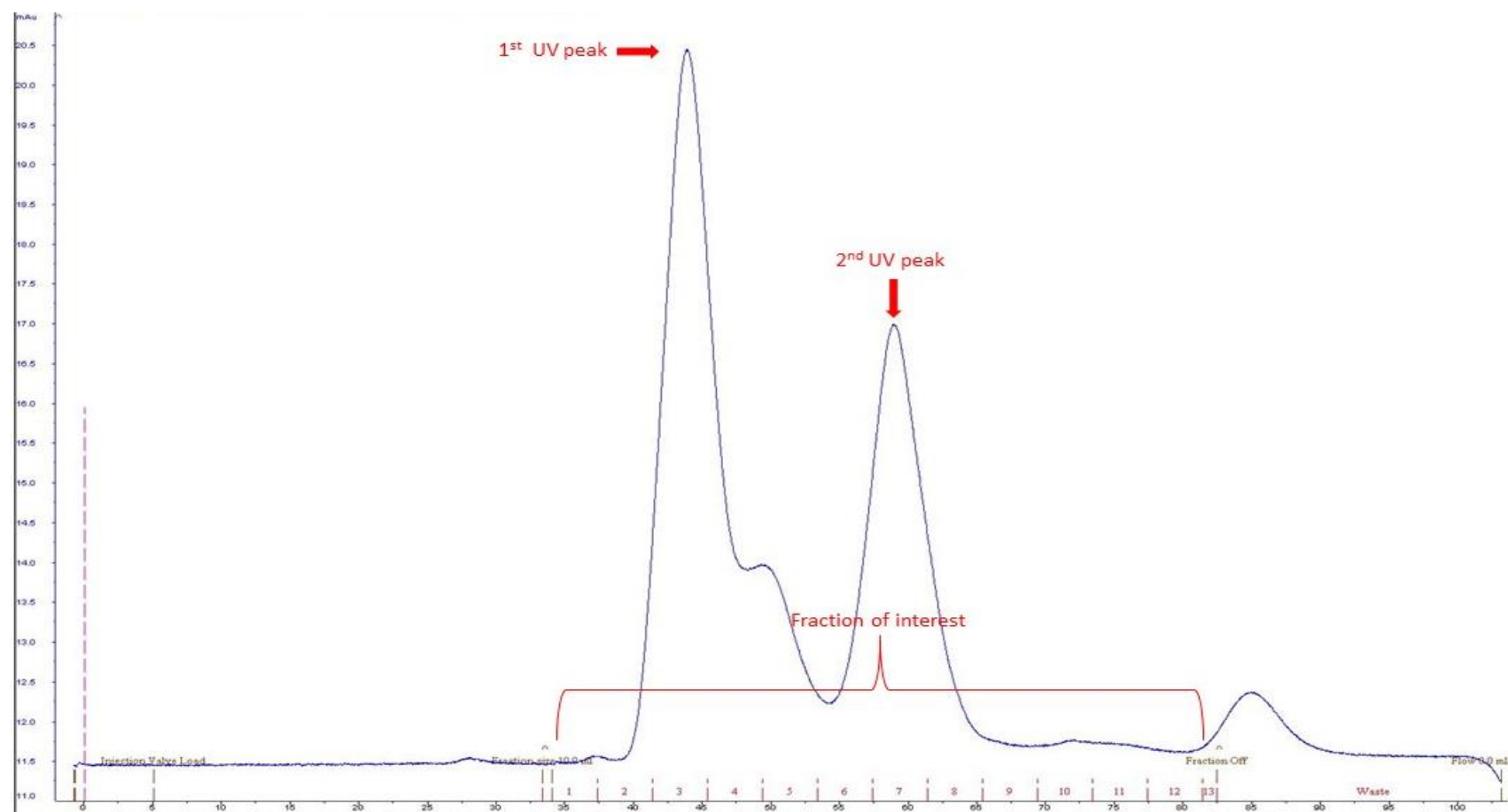


Figure 3.14: Purification of TonB1 by size-exclusion chromatography on the Hiload 26/600 Superdex 75 pg column and AKTAPrime chromatography system. Fractions F1-F5 eluted from affinity chromatography (Figure 3.8) containing TonB1 (lanes 5-9 in Figure 3.9) were pooled and applied to SEC. The y-

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axis on the affinity chromatography trace shows the UV absorbance (mAu) and the x-axis shows the volume (mL). The blue line shows the UV absorbance; the red numbers on the x-axis indicate the number of fractions of interest collected. The “1st UV peak” is the non-specific UV peak and the fractions of interest were collected during the UV peak indicated by the red arrow.

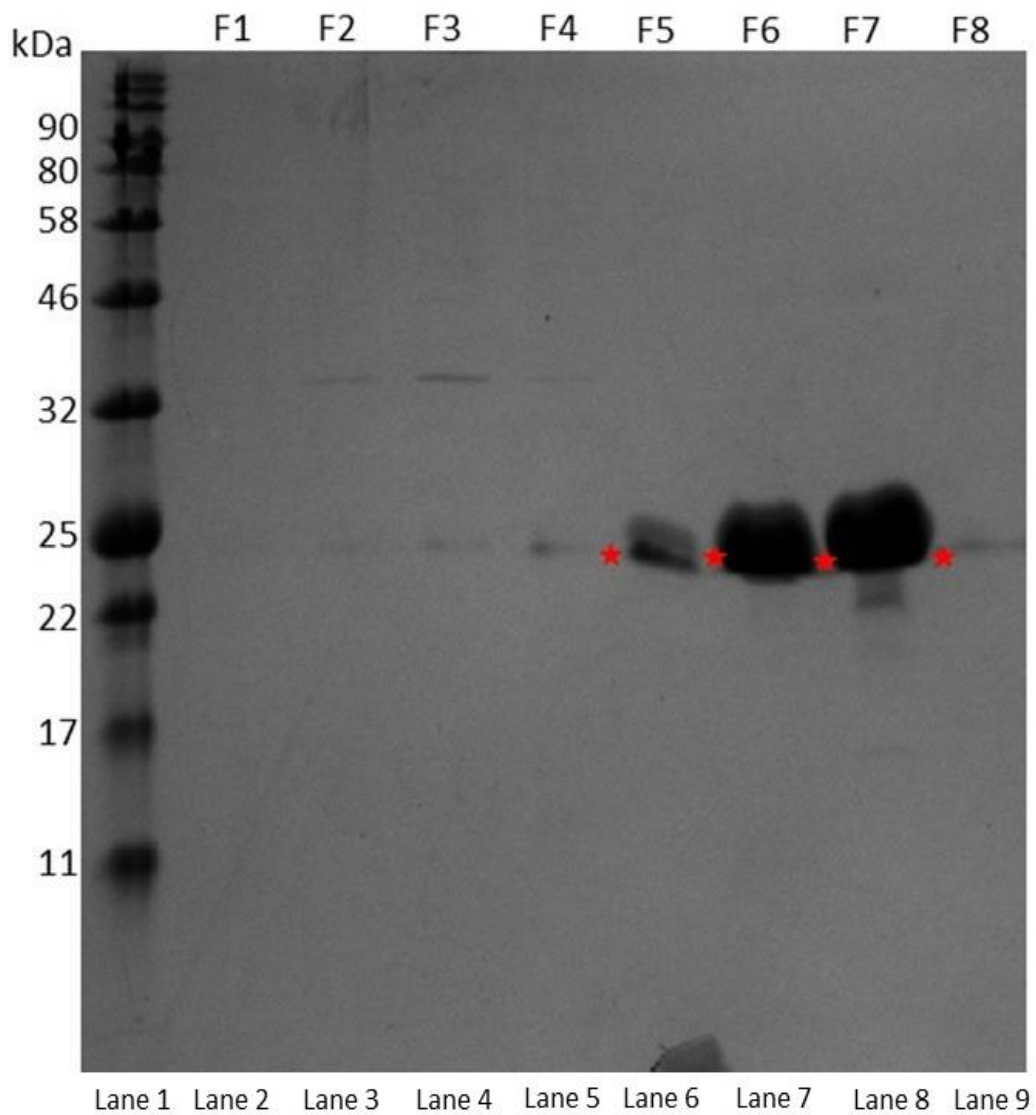


Figure 3.15: SDS-PAGE analysis of the fractions obtained during size-exclusion chromatography of the TonB1 protein. The indicated fractions from SEC of TonB1 (Figure 3.14) were applied to 10 % SDS PAGE and visualized by Coomassie Blue staining Lane 1: 11-245 kDa molecular marker. Lanes 2–9: F1–F8. F5–F7 contain TonB1 at the expected fragment size of 23 kDa; F7 contains the highest level of TonB1.

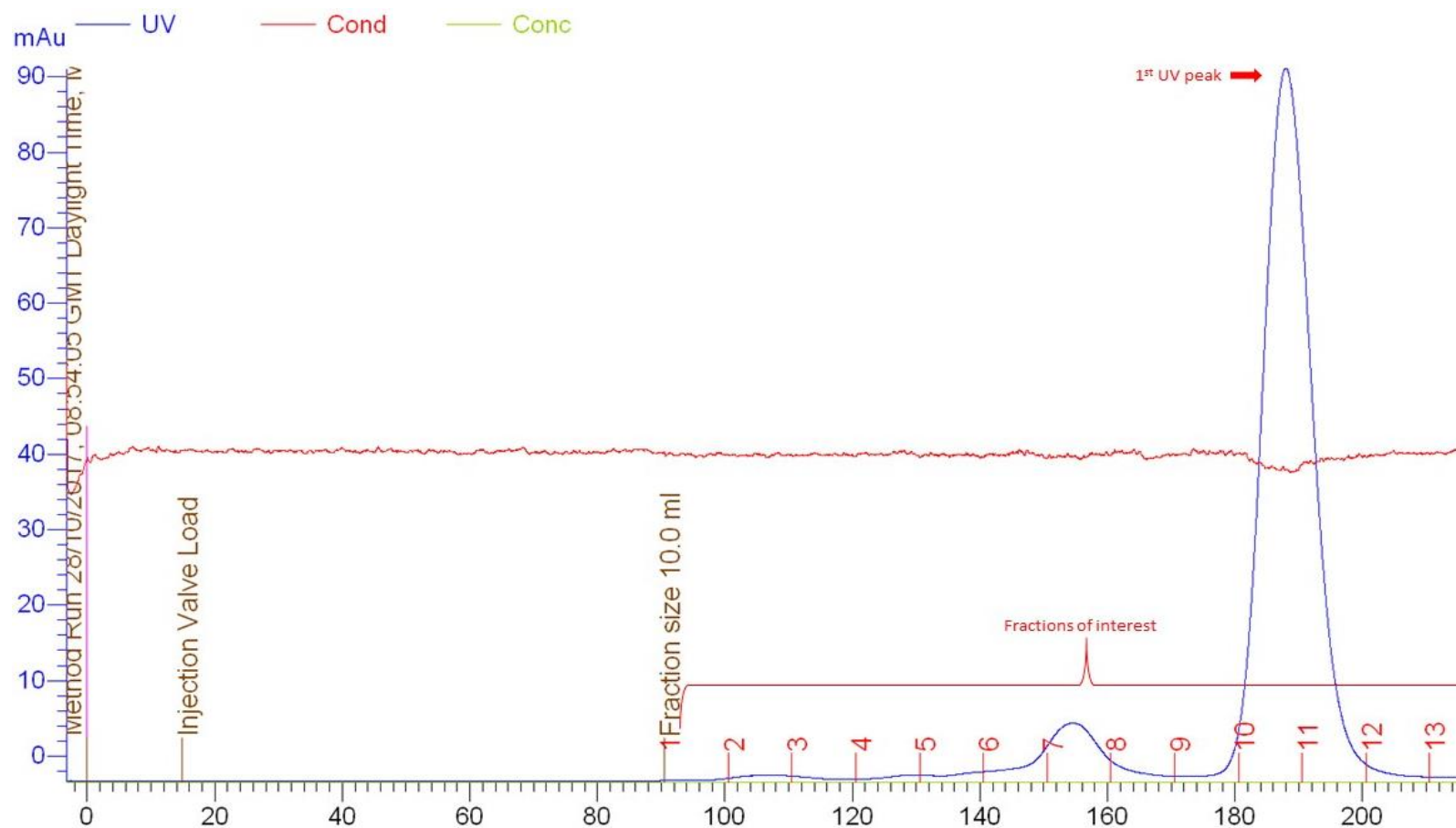


Figure 3.16: Purification of TolAIII by size-exclusion chromatography on the Hiload 26/600 Superdex 75 pg column and AKTAPrime chromatography system. Fractions F1-F4 eluted from affinity chromatography (Figure 3.10) containing TolAIII (lanes 5-8 in Figure 3.11) were pooled and applied to SEC. The

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y-axis on the affinity chromatography trace shows the UV absorbance (mAu) and the *x*-axis shows the volume (mL). The blue line shows the UV absorbance; the red numbers on the *x*-axis indicate the number of fractions of interest collected during the UV peak indicated by the red arrow.

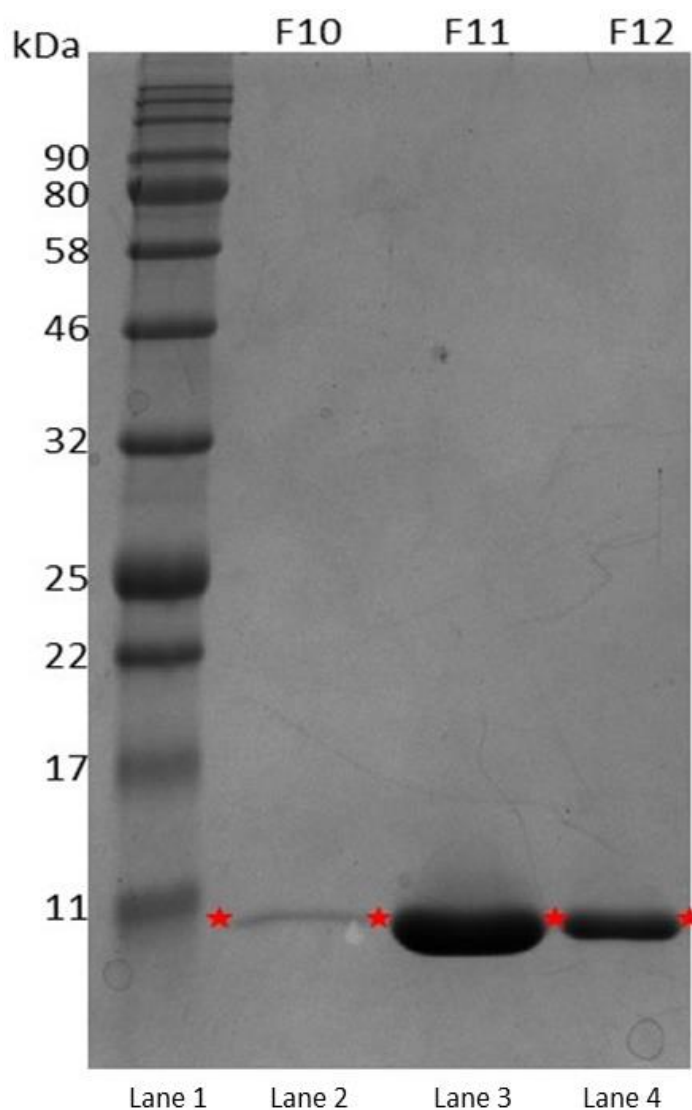


Figure 3.17: SDS-PAGE analysis of the fractions obtained during size-exclusion chromatography of TolAIII. The indicated fractions from SEC of TolAIII (Figure 3.16) were applied to 10 % SDS PAGE and visualised by Coomassie Blue staining. Lane 1: 11-245 kDa molecular marker. Lanes 2–4L F10-12; all fractions showed the expected fragment size of 23 kDa for TolAIII, and F11 contains the highest quantity of TolAIII.

3.2.6 Testing the interaction of PyoS3 with TonB1 and TolAIII via analytical gel filtration

Analytical gel filtration was performed to investigate whether PyoS3 R+T interacts with TonB1 or TolAIII. The purified PyoS3 R+T domain, TonB1 and TolAIII proteins (Figure 3.13, Figure 3.15 and Figure 3.17 respectively) were concentrated separately to 4.5 μ M using spin concentrators.

Firstly, the interaction between PyoS3 R+T and TonB1 was tested. PyoS3 R+T and TonB1 were mixed in an approximately 1:1 ratio and incubated (i) on ice for 1 hour, (ii) at room temperature for 30 min, and (iii) at 37 °C for 5 min. Then, the PyoS3 R+T and TonB1 proteins were run through the column separately as non-complexed controls, followed by the mixed proteins. The PyoS3 R+T/TonB1 mixtures of the first second and third incubation conditions generated two specific peaks that overlapped precisely with the peaks produced by the individual PyoS3 R+T and TonB1 proteins, indicating that PyoS3 R+T and TonB1 did not form a complex (see Appendix 2). However, the experiment was repeated with another incubation condition which was around 30-45 minutes in room temperature and both proteins mixed exactly in 1:1 ratio. The PyoS3 R+T/TonB1 mixtures showed a single peak that is eluting earlier than the UV peaks produced by individual PyoS3 R+T and TonB1 proteins, indicating that PyoS3 R+T and TonB1 did form a complex, which means an interaction between both PyoS3 R+T and TonB1 might have occurred (Figure 3.18).

The same processes were applied to test the interaction between PyoS3 R+T and TolAIII. The PyoS3 R+T and TolAIII mix contained two separate peaks that overlapped with the peaks of the individual PyoS3 R+T and TolAIII proteins, indicating that PyoS3 R+T and TolAIII did not interact (Figure 3.19).

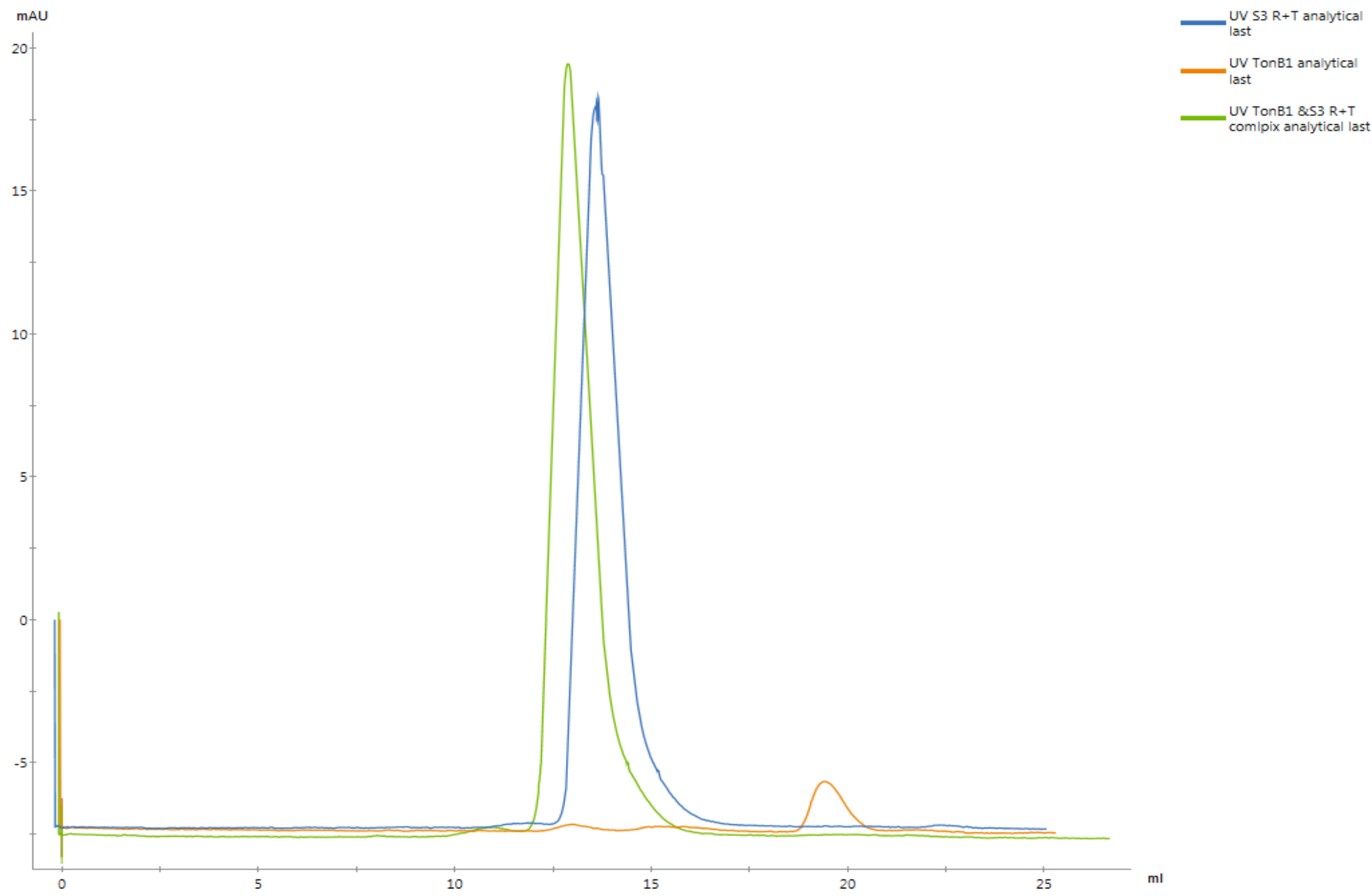


Figure 3.18: Analytical gel filtration of possible complex formation between PyoS3 R+T and TonB1. Superdex 75 Increase 5/150 GL column on the AktaPure system in 125 mM NaH_2PO_4 + 300 mM NaCl, pH 7. The y-axis shows the UV absorbance (mAu), the x-axis shows the volume (mL). The green, blue and orange lines indicate the UV absorbance of the proteins. The first UV peak to emerge is PyoS3 R+T and TonB1 complex (green) and the second UV peak is PyoS3 R+T (blue) and the third UV peak is TonB1 (orange). The complex (green) was evident following incubation of the proteins together at room temperature for 30 mins

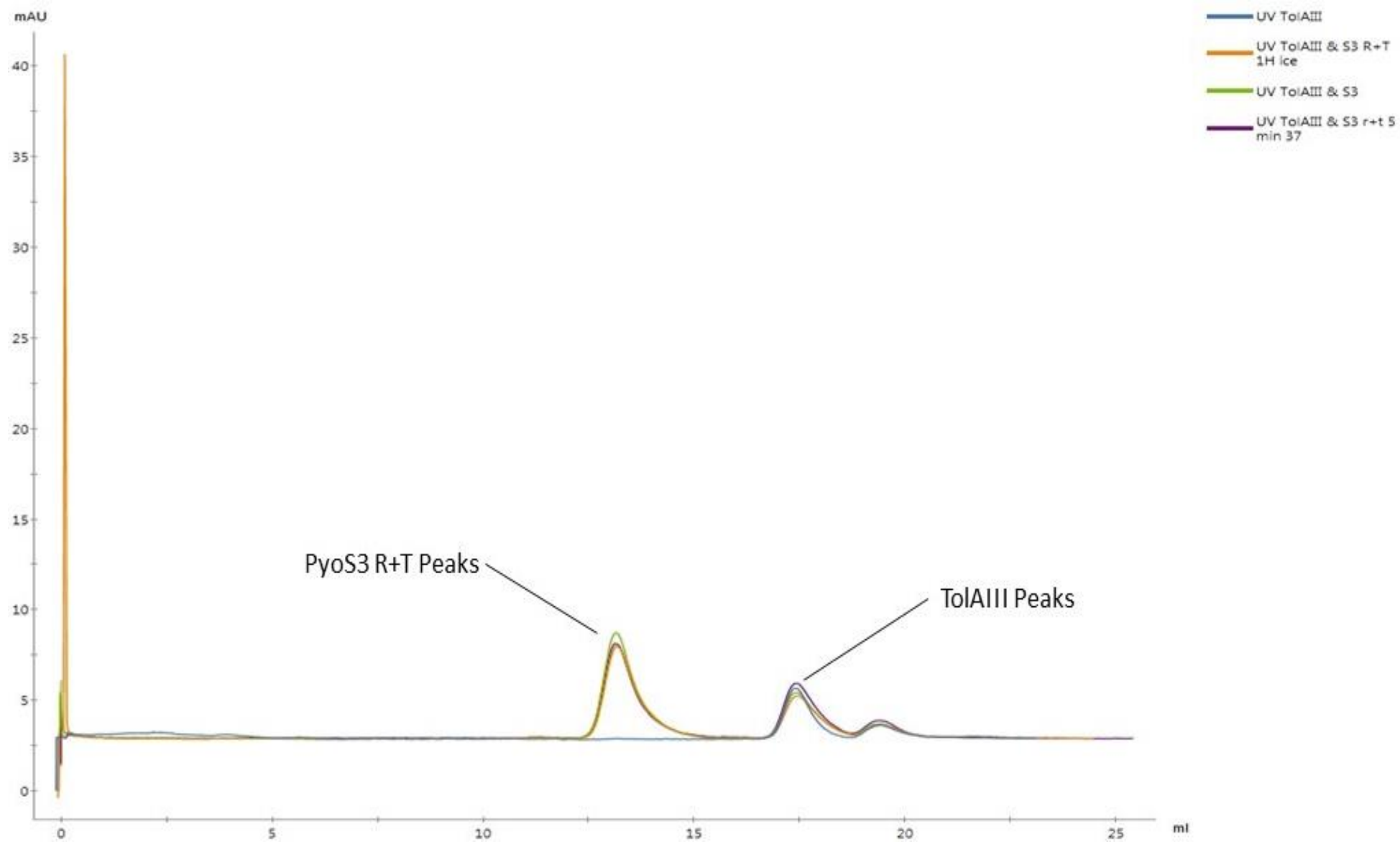


Figure 3.19: Analytical gel filtration to assess whether PyoS3 R+T and TolAIII form a complex. A Superdex 75 Increase 5/150 GL column and AktaPure system in 125 mM NaH_2PO_4 + 300 mM NaCl, pH 7. The y-axis shows the UV absorbance (mAu); the x-axis shows the volume (mL). The blue, orange, green and purple lines indicate the UV absorbance of the proteins. The first peak is PyoS3 R+T and the second peak is TolAIII. The PyoS3 R+T and TolAIII proteins were mixed together in three separate tubes, then each mixture incubated in three different conditions (1 hour on ice, 30 minutes room temperature and 5 minutes 37°C).

3.2.7 Testing the interaction of PyoS3 with TonB1 via ITC

ITC was also used in an attempt to provide further confirmation of TonB1 and PyoS3 R+T interaction. ITC is a more sensitive technique than analytical SEC for protein-protein interaction studies. Unfortunately, the ITC did not show interpretable results, thus it is not possible to conclude whether the interaction between PyoS3 R+T and TonB1 occurred in this experiment. It should be noted that on this occasion, there were a couple of technical issues before conducting the experiment, such as low protein concentration of both proteins, especially as TonB1 aggregated when concentrated and during the experiment (Figure 3.20).

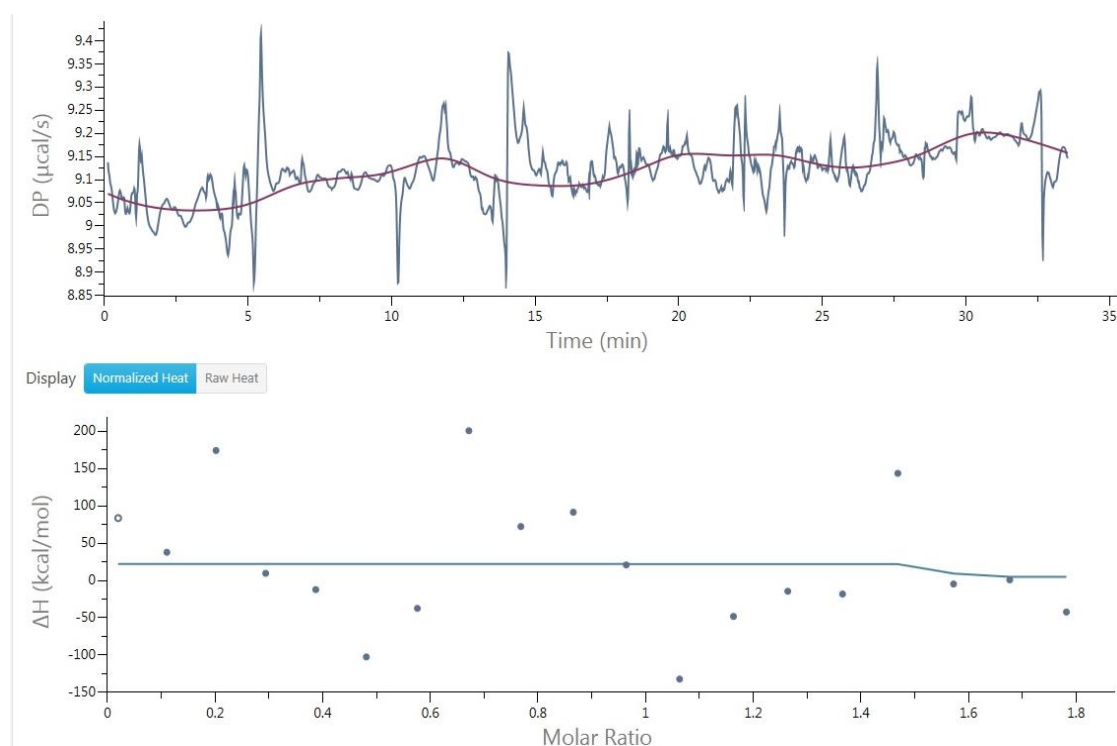


Figure 3.20: Preliminary test of the interaction between TonB1 and PyoS3 R+T using ITC.

PyoS3 R+T and TonB1 pure proteins were concentrated to the possible concentration which was $14\ \mu\text{M}$ for PyoS3 R+T and $4.5\ \mu\text{M}$ for TonB1. PyoS3 R+T was loaded into *** syringe and the TonB1 was loaded into the reservoir in the ITC machine. The top panel y-axis shows the DP (measured power differential between the reference and sample cells to maintain a zero temperature between the cells) and the x-axis shows the experimental run time. The clonometrics response is shown with 10 injections of PyoS3 R+T ($14\ \mu\text{M}$) into TonB1 ($4.5\ \mu\text{M}$) in the same

buffer (50 mM Na₂H₂PO₄, 150 mM NaCl, pH 7) at 37 °C. The bottom panel shows the injection heat.

3.2.8 Identification of the optimal buffer to prevent aggregation of TonB1

In preparation for further ITC experiments, buffer exchange was conducted to identify a buffer that would prevent aggregation of TonB1 protein when concentrated to more than 4.5 µM. As the molecular weight of TonB1 is 23 kDa, the protein was subjected to dialysis using 10 MWC high-molecular weight Spectra Por dialysis membrane (Fisherbrand™) overnight. Several buffers were tested (see Appendix 3). The only buffer that TonB1 was stable in and would not aggregate when concentrated to 50 mM was Na₂H₂PO₄, 150 mM NaCl + 5% glycerol + 1 mM DTT, pH 8. In addition, due to Covid-19 restriction, time constraints and mechanical breakdown of the calorimeter, it was not possible to repeat the ITC.

3.3 Discussion and Conclusion

Previous studies suggested that PyoS3 translocates across the outer membrane using the ferripyoverdine transporter type II (FpvAII) but did not determine the mechanism of translocation across the cell periplasmic space (Ghequire et al., 2018, Stilwell et al., 2018, Baysse et al., 1999). In contrast, PyoS2 was previously shown to bind to ferripyoverdine transporter type I (FpvAI) for uptake across the outer membrane of *Pseudomonas* (White et al., 2017). The same study went further to show that the N-terminus of PyoS2 interacts with TonB1 *in vitro* using ITC. Moreover, deletion of the PyoS2 β-hairpin abolished the interaction between PyoS2 and TonB1, indicating the importance of TonB1 for translocation of PyoS2 (White et al., 2017). Since S-type pyocins share sequence homology with E colicins, which use the TonB or Tol systems as a mode of cell envelope translocation, it is possible that PyoS3 could use either of these, and thus further investigation is required to verify the interactions it participates in. Colicin A is another example of a bacteriocin that uses the Tol system for translocation. To characterize the mode of translocation

and interactions of PyoS3 further, this study aimed to first identify whether PyoS3 uses TonB1 or TolAIII to translocate across the cell envelope.

The DNA encoding the PyoS3 R+T domains, TonB1 and TolAIII were cloned and inserted into pET21a vector for protein expression (Walker et al., 2001). TonB1 and TolAIII were successfully overproduced using our standard IPTG induction protocol (4 h incubation at 37 °C), and each protein migrated at the expected size. However, this protocol needed to be altered for PyoS3 R+T, possibly as the protein aggregated when incubated for 4 hours at 37 °C (Weng et al., 2006, Kaur et al., 2018). The optimal incubation time and temperature for PyoS3 R+T were 15 h at 17 °C, and the protein was produced successfully under these conditions. In addition, it was interesting that the TonB1 protein expression was successful as well, because the sequence analysis showed a stop codon ochre (TAA) that usually leads to a truncated protein. The successful expression of TonB1 might be due to the mutated tRAN, which might lead to the translation readthrough of the stop codon, that leads to a stop codon being read as a sense codon. These phenomena might be due to the present of background suppression of amber and opal stop codon in *E.coli* BL21(DE3) cells, which might be also the case of ochre stop codon (O'Donoghue et al., 2012). The stop codon tried to be removed by sit-direct mutagenesis, but it failed. This might be due to an error with primer design, the PCR conditions, or the concentration of template DNA.

Metal chelation affinity chromatography purification of the PyoS3 R+T, TonB1 and TolAIII proteins was successful. SDS-PAGE analysis confirmed that the three proteins were produced at the expected sizes, and that there was little contamination by other unwanted proteins. SEC was employed to further purify the proteins to 95 % purity. The proteins purified via SEC by size (Burgess, 2018) were concentrated to reach sufficient concentrations for interaction testing. PyoS3 R+T were concentrated to 25 µM and TolAIII was concentrated to 19 µM. However, the TonB1 protein aggregated when the concentration exceeded more than 4.5 µM.

Since TonB1 could not be concentrated sufficiently, analytical SEC was initially used to test if PyoS3 R+T interacted with either TonB1 or TolAIII. This technique was previously used to test the interaction between PyoS2 and FpvAI (White et al., 2017), and can detect interactions between proteins at low concentrations. Two separate peaks matching the individual proteins were detected in two of the mixtures incubated under two different conditions (1 hour on ice and 5 minutes at 37 °C), which indicates that PyoS3 R+T eluted separately from TonB1. However, the mixture incubated in the 30 minutes at room temperature condition showed a separate peak eluting faster than the individual peaks of PyoS3 R+T and TonB1 protein. Since this new peak was also the only one in the elution profile, this result suggests that PyoS3 R+T and TonB1 proteins formed a complex and indicates an interaction occurred between them. This suggests that PyoS3 might utilise TonB1 for translocation. This finding agrees with the literature, since (i) it is reported that PyoS3 uses FpvAIII which is a TonB-dependant receptor, and (ii) TonB translocate molecules such as ferripyoverdine and Pyocins (Baysse et al., 1999, White et al., 2017, Bonneau et al., 2020). Similar analytical SEC of PyoS3 R+T/TolAIII protein mixtures yielded two separate peaks matching the individual proteins following a range of incubations (30 minutes at room temperature, 1 hour on ice, 5 minutes at 37°C). This indicates that PyoS3 R+T eluted separately from TolAIII without participating in an incubation. These results suggest that PyoS3 R+T does not utilize TolAIII for translocation.

Attempts to use ITC to further prove if an interaction occurs between TonB1 and PyoS3 R+T did not detect any interaction and were inconclusive. This does not mean that the proteins do not interact. The lack of a detectable interaction may be due to the low concentration of TonB1 used in the experiment or because the buffer used in the experiment was not favourable for ITC. In addition, the purity of the TonB1 protein might not be sufficient or the protein might not be soluble in the buffer under stirring at the experimental temperature (Baranauskiene et al., 2019, Pierce et al., 1999).

In order to perform experiments such as ITC that require highly concentrated proteins, TonB1 purified by SEC was dialysed into different buffers with the aim of identifying conditions suitable to stabilise concentrated TonB1. The same

dialysis technique and buffer were used for PyoS3 R+T so that the buffer would be the same for both proteins during the interaction incubations. After testing several different buffers and conditions, it was found that TonB1 was soluble and did not aggregate when concentrated in 50 mM $\text{Na}_2\text{H}_2\text{PO}_4$, 150 mM NaCl + 5% glycerol + 1 mM DTT (pH 8). Unfortunately, further ITC experiments could not be performed due to the COVID-19 pandemic restrictions, and the proteins were stored at -80 °C for future analysis.

In follow-on work, it would be desirable to repeat the ITC experiments with stable proteins to further confirm the PyoS3 R+T and TonB1 interaction and validate the observations made by analytical SEC. It would be useful to combine this with truncated or modified versions of PyoS3 R+T to define the domains and residues involved in the interaction in higher resolution. In parallel, it would be interesting to incorporate FpvAII as a reported interaction partner of PyoS3 R+T. This would confirm the uptake of PyoS3 is through the pyoverdine uptake system, and further define the residues directing this interaction to establish if they are the shared or distinct from those involved in the interaction with TonB1

4 Chapter 4.

Random mutagenesis to identify novel genes involved in the uptake of pyocin S3.

4.1 Introduction

The previous chapter directly targeted a subset of the ferripyoverdine import machinery components as targets for pyocin S3 interaction since S-type pyocins had been shown to bind them to initiate cell uptake into the cells that they ultimately kill. There are a number of different types of pyoverdine (PvD) receptors, making a systematic analysis of them all ambitious. Previous studies have shown that the ferripyoverdine receptor FpvAI is utilized by Pyocin S2, whilst Pyocin S5 utilises the ferric pyochelin transporter FptA as the primary receptor for cell entry. In contrast, Pyocin G utilises the hemin transporter Hur as a receptor. All of these pyocins use TonB1 for translocation, with the exception of Pyocin AP41, which utilises the Tol system, but its receptor is unknown (Michel-Briand and Baysse, 2002). In addition, Pyocin AP41 shares 95% sequence homology of its first three domains with Pyocin S8 suggesting that it use the same receptor (Turano et al., 2020). White et al. (2017) recently described the uptake mechanism of Pyocin S2. pyocin S2 proline-rich region in the unstructured domain binds to the FpvAI plug domain. As a result, the FpvAI plug domain unfolds. Moreover, the N-terminus of Pyocin S2 binds to TonB1 in the periplasm through its TonB-box motif. Then the ExbB–ExbD–TonB1 complex induces transduction of the PMF from the cytoplasmic membrane into the periplasm, which energises the import of Pyocin S2 into the periplasm. A similar TonB-dependent FptA-mediated mechanism has been described for Pyocin S5 (White et al., 2017, Behrens et al., 2020a) (Michel-Briand and Baysse, 2002).

A previous study showed that PyoS3 uses the ferripyoverdine receptor FpvAI for target cell entry (Baysse et al., 1999). The subsequent pathway was not previously studied for PyoS3, although the data in the previous chapter suggests that PyoS3 utilises the TonB1 system for translocation. In this chapter, a random mutagenesis approach was conducted using a transposon (Tn) system to generate a transposon insertion library and identify the genes, and

thus the gene products, that are important or essential, for the uptake and translocation of PyoS3. This approach has the advantage of being unbiased, and high throughput.

In vivo transposon mutagenesis involves the introduction of heterologous DNA containing a transposon into recipient cells in combination with a gene encoding a transposase. The transposase subsequently stimulates transposition of the transposon into the genomic DNA of the target cell of interest. The transposase achieves this by removing the transposon from its original location, and inserting it into random locations in the genome of the target cells (Figure 4.1) (Levitan et al., 2020). By randomly jumping into the genome of the recipient cells, the transposon generates a set of random mutants (Monson et al., 2015). Transposon mutagenesis assures minimally biased insertion and achieves high coverage of the target genomic DNA to create a large transposon library (Sargison and Fitzgerald, 2021).

For the purposes of this study, the most appropriate recipient cell had to be identified first. It was necessary to select a strain of *P. aeruginosa* that was sensitive to PyoS3, and also amenable to accepting the plasmid bearing the transposon and transposase by conjugation from an *E. coli* donor. To do this, stab tests were conducted to determine the sensitivity of a set of *P. aeruginosa* clinical strains to PyoS3. The first step in this process required generating a plentiful supply of PyoS3. This was achieved by cloning the DNA encoding full-length PyoS3 with its cognate immunity protein (PyoS3-immS3) into the expression vector pET21a to produce soluble PyoS3. Two strains, PSA892 and PSA 971, were sensitive to PyoS3, but only the former was capable of receiving the donor plasmid following conjugation with *E. coli*. Transposon mutagenesis was applied to the *P. aeruginosa* clinical isolate PSA892, to generate a library of mutants. A spot testing screen using purified PyoS3 was developed and optimised with the aim of identifying transposon mutants of *P. aeruginosa* PSA 892 that had acquired resistance to killing. The genome of resistant mutants were sequenced to determine the locations in the genome where the transposons had inserted. Furthermore, a comprehensive genomic analysis was conducted to determine the putative association between PyoS3 and the mutated genes. Furthermore, as PyoS3 uses the pyoverdine (PvD)

receptor FpvAII (Baysse et al., 1999), which is part of the PvD system (Ringel and Brüser, 2018), PvD production was measured to identify transposon mutants in which PvD production was affected. In addition, genomic DNA of PyoS3-sensitive strains *PSA 892* and *PSA 971* was analysed to determine which PvD receptor each strain contains.

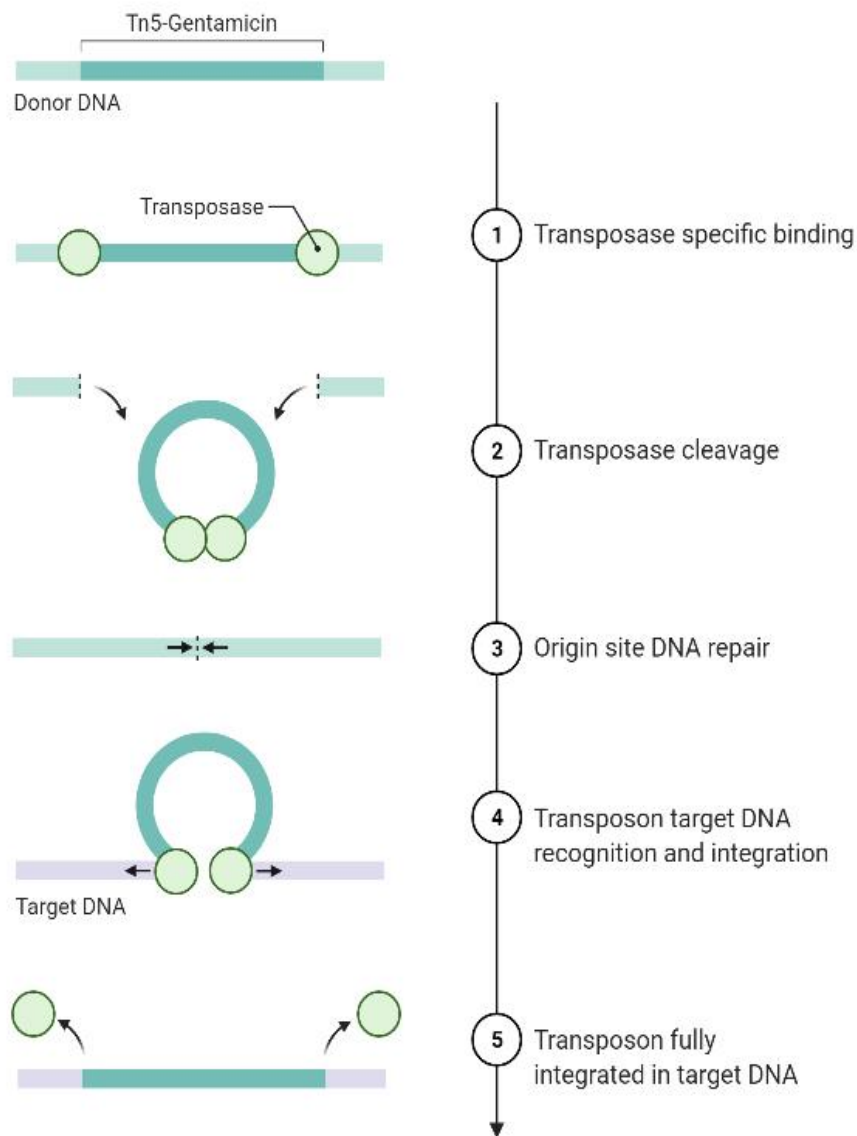


Figure 4.1: Tn5 transposon system mutagenesis. A bacterial donor cell that carries the Tn5 transposon DNA is conjugated into the target bacterial cells. The transposase cleaves and then inserts the transposon randomly into the genomic DNA of the target cells.

4.2 Results

To enable optimisation and high throughput screening of a large, random transposon library of *P. aeruginosa* mutants for strains deficient in proteins that PyoS3 interacts with, it was first necessary to generate a large quantity of PyoS3.

4.2.1 Cloning of pyoS3-immS3 into an overexpression plasmid

To produce soluble PyoS3 protein at a high level, a strain that overproduced the active form was required. This necessitated cloning the full length PyoS3 encoding gene with its cognate immunity protein to protect the producing cell from killing itself in the process. To do this, PCR was performed (see section 2.5) to amplify the DNA fragment encoding *PyoS3-immS3* from the pPyss3 plasmid. Agarose gel electrophoresis revealed the expected fragment size of 3.0 kbp for PyoS3-immS3 (Figure 4.2). The DNA encoding PyoS3-immS3 was extracted from the agarose gel, ligated into the pGEM-T easy vector for blue-white screening (pFAI), then cloned into the pET21a vector (pFAII) for overexpression. Restriction digests were conducted on the recombinant plasmid using *NdeI* and *XhoI* restriction enzymes to verify the insertion of the desired insertion. The restriction digestion reactions were separated on an agarose gel and the expected DNA fragment size of 3.0 kbp for the PyoS3-immS3 gene was obtained (Figure 4.2). The construct was sent for sequencing (GENEWIZ, UK), which confirmed PyoS3-immS3 had been correctly amplified and cloned into pET21a (see Appendix 1).

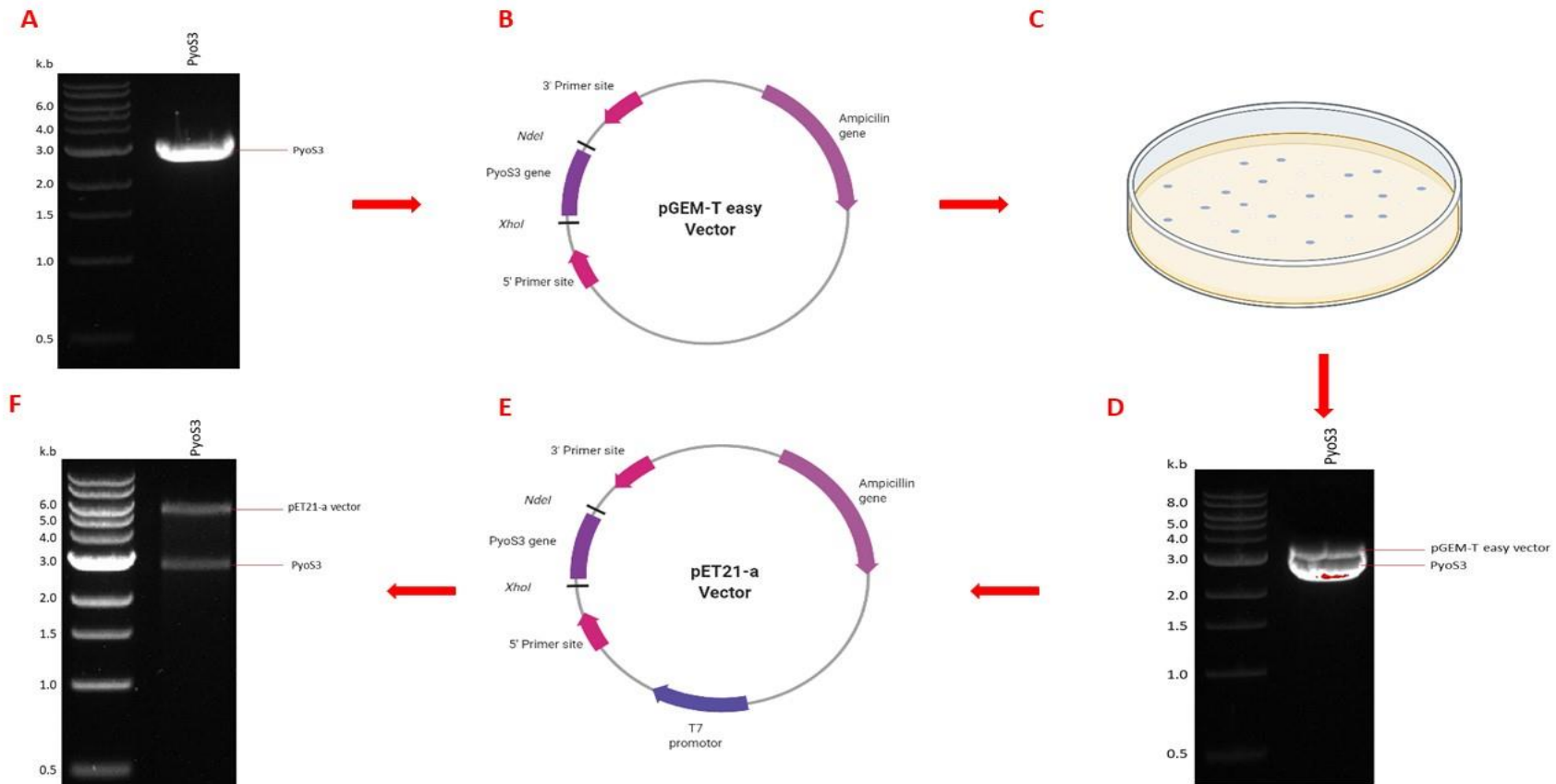


Figure 4.2: Amplification and cloning of PyoS3-immS3 into pET21. A: PyoS3-immS3 was amplified successfully via PCR. Lane 1: 100 kbp molecular weight markers. Lane 2: expected fragment size of 3.0 kbp for PyoS3-immS3 DNA. B: Diagram of the pGEM-T easy vector showing PyoS3 DNA insert (pFAI). C: LB

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plate containing IPTG + Ampicillin + X-gal. White colonies that indicated successful insertion of PyoS3-immS3 DNA into the pGEM-T easy vector. D: Restriction digestion using *NdeI* and *XhoI* verified the plasmid harboured the DNA encoding PyoS3-immS3. Lane 1: 100 kbp molecular weight markers. Lane 2: PyoS3-immS3 fragment with the expected size of 3.0 kbp and the pGEM-T easy vector of 3.0 kbp; the bands migrate together because of their similar size. E: Diagram of the pET21-a vector showing the PyoS3--immS3 DNA insert (pFAII). F: Restriction digestion using *NdeI* and *XhoI* verified the plasmid harbored the cDNA encoding PyoS3-immS3. Lane 1: 100 kbp molecular weight markers. Lane 2: Pet21-a vector of 6.0 kbp and PyoS3-immS3 with the expected size of 3.0 kbp.

4.2.2 Stab test to assess the sensitivity of the *PSA 971* and *PSA 892 P. aeruginosa* clinical strains to *PyoS3*

In order to find a suitable clinical isolate of *P. aeruginosa* for the transposon mutagenesis screen, the sensitivity to *PyoS3* of two clinical isolates suspected to have this phenotype by a previous screen undertaken by Siyu Wu was evaluated using a stab test experiment. Clinical isolates *PSA 971* and *PSA 892* PA were confirmed to be sensitive to *E. coli* BL21 (DE3) (pFA2) and were resistant to a control *E. coli* strain producing TonB1 (Figure 4.3), thus these strains were assessed for their suitability for transposon mutagenesis.

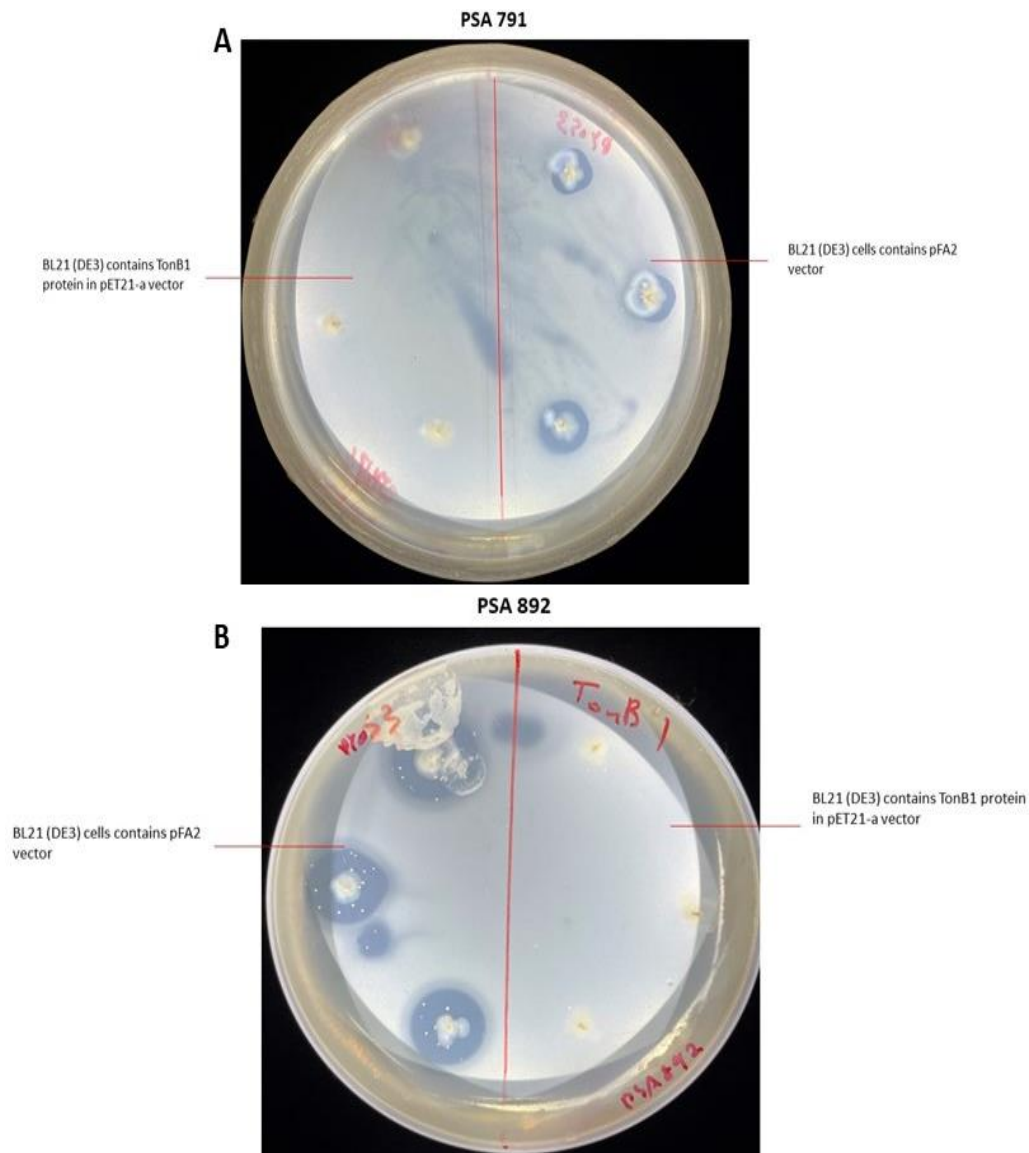


Figure 4.3: Stab test to confirm the sensitivity of the *P. aeruginosa* clinical strains *PSA 971* and *PSA 892* to *PyoS3*. Each clinical strain was separately seeded onto an agar plate. A:

P. aeruginosa PSA 971. B: *P. aeruginosa* PSA 892. Three stabs were then applied of either (i) *E. coli* BL21 (DE3) containing the pFA2 plasmid, which encodes active PyoS3-immS3 protein and would generate zones of growth inhibition, or (ii) the negative control *E. coli* BL21 (DE3) colonies that produce TonB1 protein from plasmid tFALL and would thus not generate zones of growth inhibition.

4.2.3 Over-production of active PyoS3-immS3 protein

The pFALL plasmid (section 4.2.1, Figure 4.2) produced high levels of PyoS3-immS3 protein when induced with IPTG. SDS-PAGE analysis revealed proteins of the expected sizes of 81 kDa for the large receptor-binding domain with its DNase protein and of 17 kDa for the small cognate immunity protein (ImmS3) (Figure 4.4).

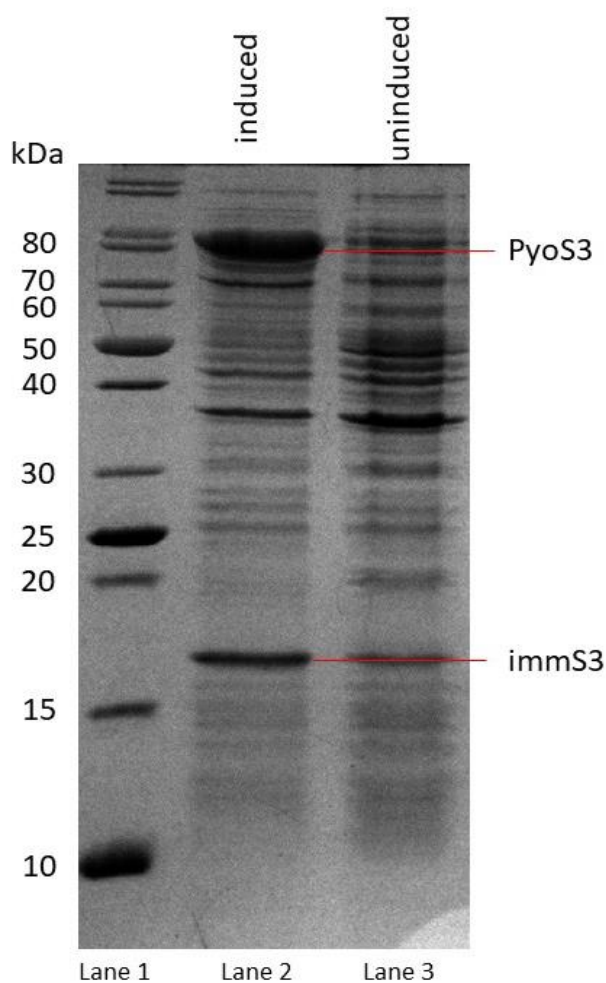


Figure 4.4: SDS-PAGE analysis confirming high level of PyoS3-ImmS3 protein production. *E. coli* BL21(DE3) carrying pFALL was grown with and without induction with IPTG

(Section 2.18) and whole cell aliquots applied to 10 % SDS-PAGE. Proteins were visualised by staining with Coomassie Blue (section 2.17). Lane 1: 10 – 245 kDa molecular markers. Lane 2: IPTG-induced protein. The expected protein sizes of 81 kDa for PyoS3 (large protein) and 17 kDa for the immS3 protein (small protein) were observed. Lane 3: uninduced sample.

4.2.4 Purification of PyoS3-immS3 by metal chelation affinity chromatography

Overproduced PyoS3-immS3 protein was purified by affinity chromatography to remove contaminants to prepare the protein for further studies. The protein fractions were collected and analysed using SDS-PAGE. The supernatant of the sonication step plus the flow through were also collected and analysed via SDS-PAGE. The analysis showed that PyoS3-immS3 protein was eluted in the fourth fraction, and the expected fragment sizes of 81 kDa for the large protein (receptor-binding domain and DNase) and 17 kDa for the small protein (immunity protein) were observed in the same fraction (Figure 4.5).

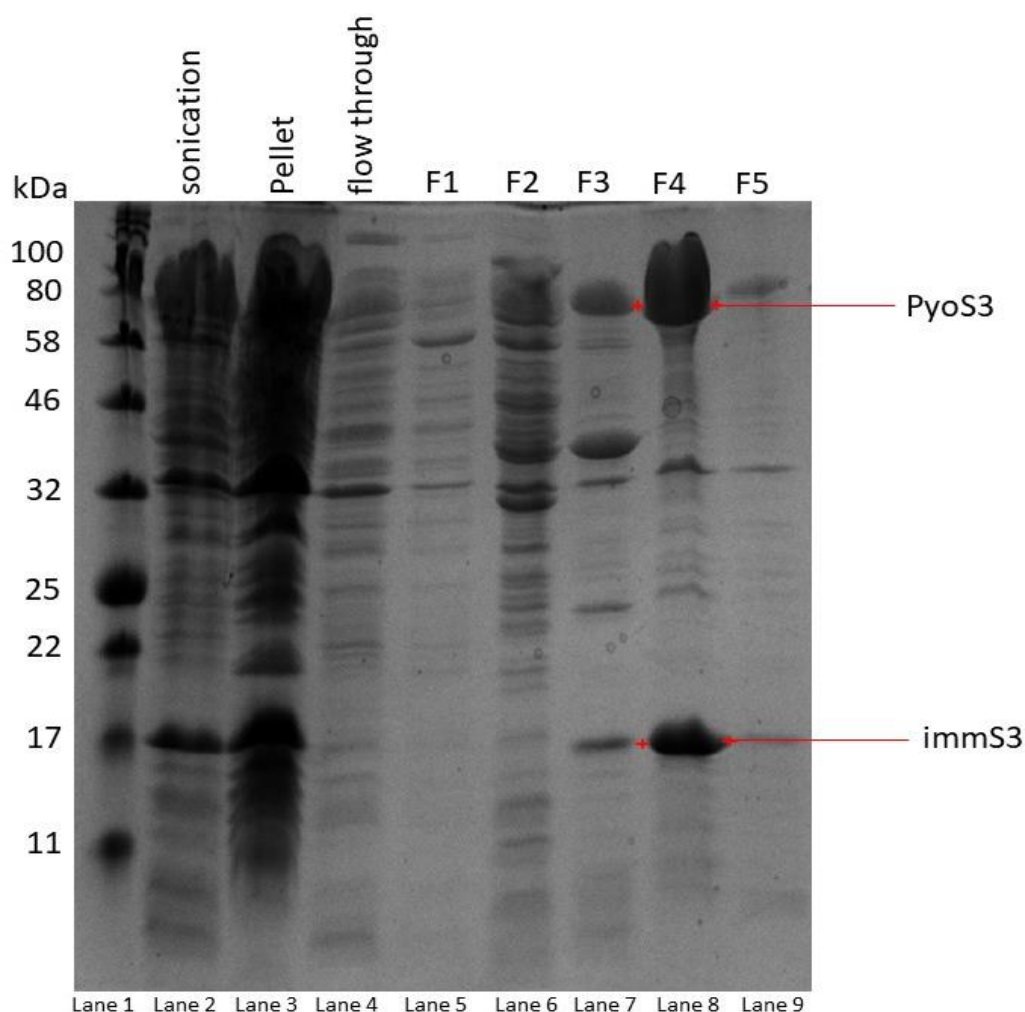


Figure 4.5: SDS-PAGE analysis of metal chelation affinity chromatography purification of PyoS3-immS3. Protein extracts containing PyoS3-immS3 applied to and eluting from affinity chromatography were loaded onto an SDS PAGE and stained with Coomassie blue (see section 2.17). Lane 1: 11 – 245 kDa molecular markers (sizes indicated on the left). Lane 2: Aliquot taken after sonication of IPTG induced *E. coli* strain pFAll (see section 2.18). Lane 3: aliquot of the insoluble proteins in the lysate pelleted by centrifugation, and thus not applied to the affinity chromatography .4: aliquot taken from column flowthrough of the elution buffer (unbound soluble proteins). Lanes 5-9: fractions F1–F5 collected during elution with elution buffer containing imidazole (see section 2.19). F3–F5 contain PyoS3 with the expected fragment sizes of 81 kDa for PyoS3 (large protein) and 17 kDa for immS3 (small protein) , with the highest protein amount is F4.

4.2.5 SEC enhanced the purity of PyoS3-immS3 and obtained a good yield of protein

SEC was used after affinity chromatography to further purify PyoS3-immS3 by removing further contaminants to obtain high-purity PyoS3-immS3. A Hiload

26/600 Superdex 75 pg SEC column was used to purify PyoS3-immS3 and an AKTApure machine was used to detect the eluted protein in real time. As shown in Figure 4.6, fractions (F21-F28) were collected; however, only fractions (F23-F26) were analysed via SDS-PAGE as they were collected during the high UV peak (Figure 4.6). F24–F26 contained proteins with the expected size of 81 kDa for the large protein (receptor-binding domain and DNase) and 17 kDa for the small immunity protein, with F25 being the most quantity (Figure 4.7).

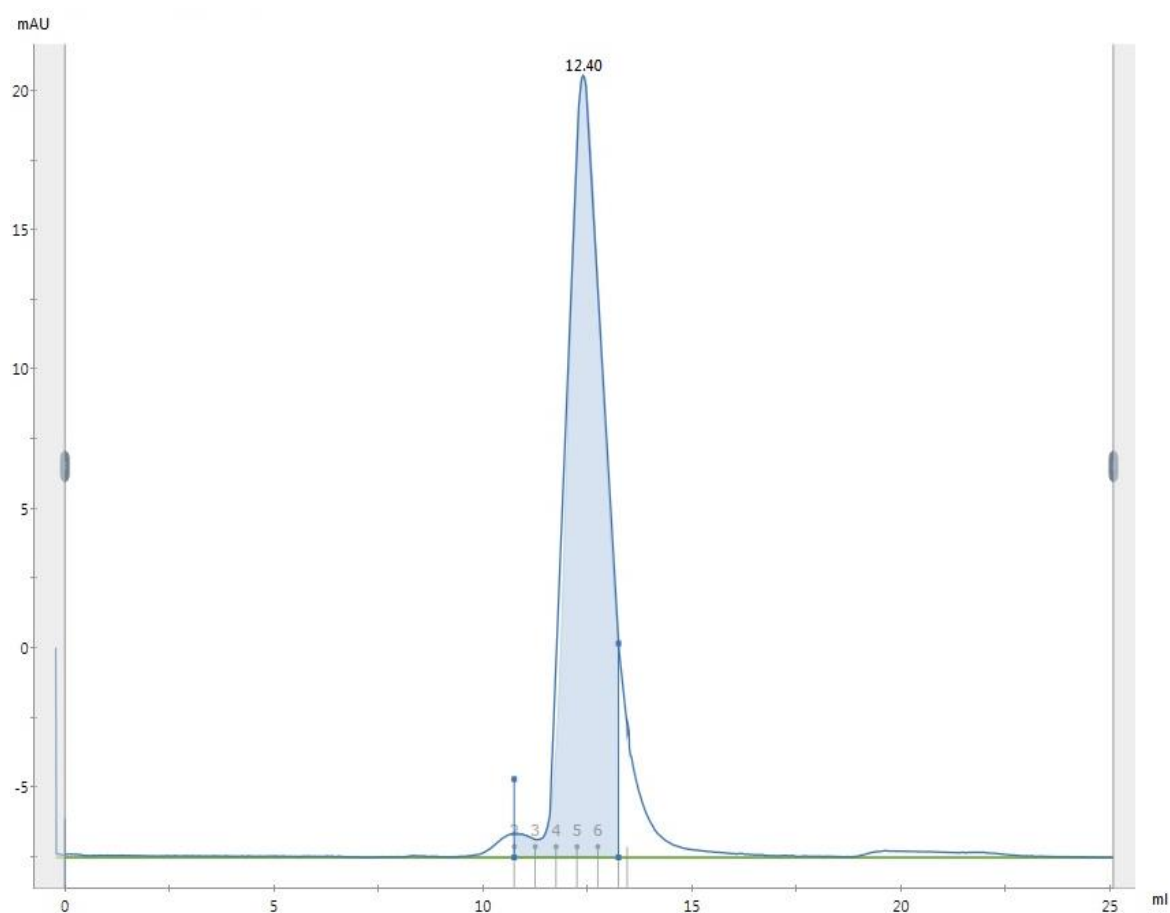


Figure 4.6: Purification of the PyoS3-immS3 protein by SEC on the Hiload 26/600 Superdex 75 pg column and AKTA Pure chromatography system. Fractions F4 eluted from affinity chromatography containing PyoS3-immS3 (lanes 8 Figure 4.5) were pooled and applied to SEC. The y-axis on the SEC trace shows the UV absorbance (mAu) and the x-axis shows the volume (mL). The blue line shows the UV absorbance, and the fractions of interest were collected during the UV peak.

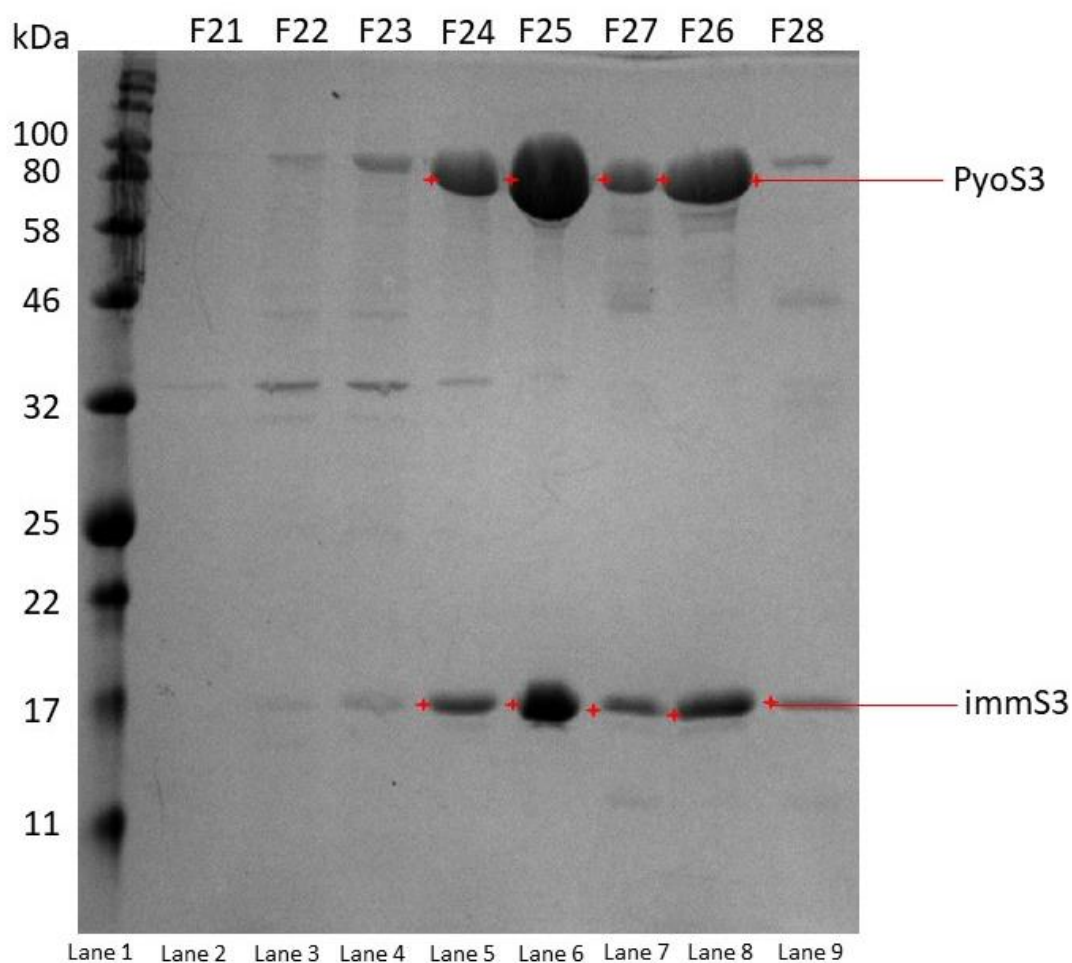


Figure 4.7: SDS-PAGE analysis to confirm improved purity of PyoS3-immS3 achieved by SEC. The indicated fractions from SEC of PyoS3-immS3 (Figure 4.6) were applied to 10% SDS PAGE and visualised by Coomassie Blue staining. Lane 1: 11 – 245 kDa molecular marker (sizes indicated on the left). Lanes 2-8: Fractions F21–F26, respectively, eluted from the Hiload 26/600 Superdex 75 pg SEC column by SEC elution buffer (see section 2.21). F24–F26 contain PyoS3 with the expected fragment sizes of 81 kDa for PyoS3 (large protein) and 17 kDa for immS3 (small protein), with the highest quantity of these proteins in F25.

4.2.6 Confirmation of the activity of purified PyoS3-immS3 protein against *P. aeruginosa* clinical strains PSA 971 and PSA 892

To test the activity of the purified PyoS3-immS3 protein produced from pFA2, samples of three ten-fold dilutions (undiluted, 1:10, 1:100, and 1:1000) of a 6.7 μ M preparation of the protein were dropped on top of the *P. aeruginosa* clinical strains PSA 971 and PSA 892. Inhibition zones were observed on plates

containing both PSA 971 and PSA 892, which indicates that both of these strains are sensitive to the purified PyoS3-immS3 (Figure 4.8).

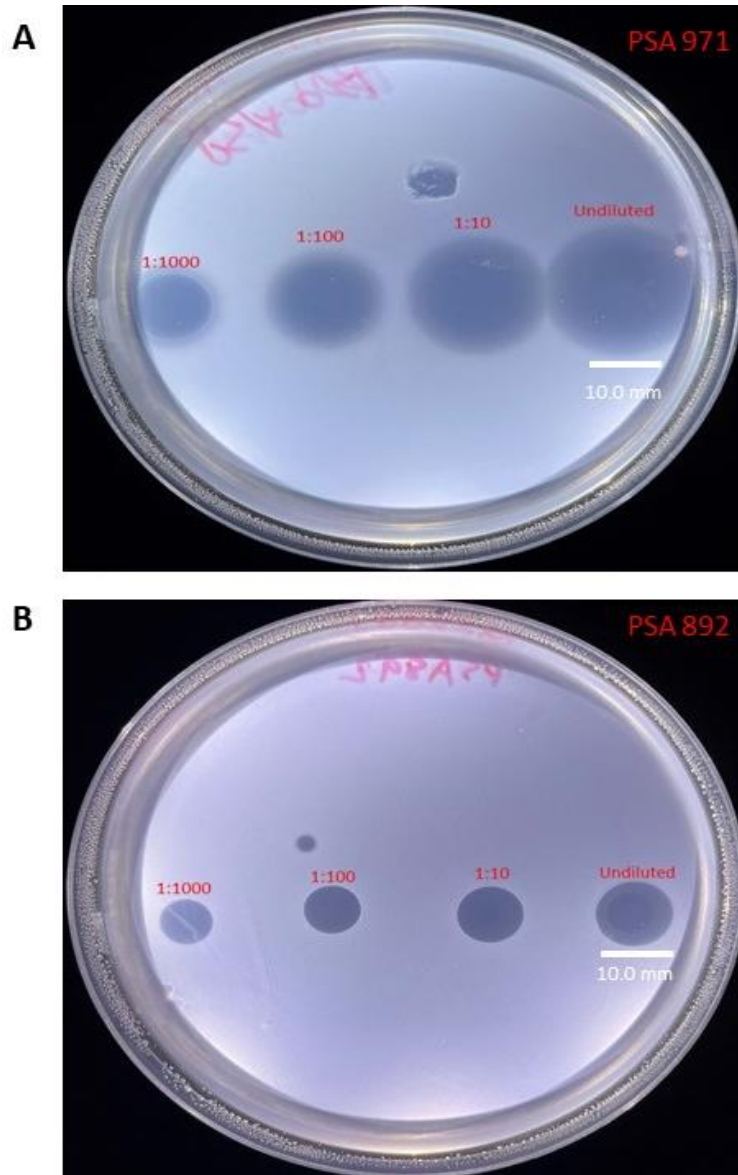


Figure 4.8: Spot test to confirm the sensitivity of *P. aeruginosa* clinical strains PSA 971 and PSA 892 to pure PyoS3-immS3. Undiluted purified PyoS3-immS3 and 1:10, 1:100 and 1:1000 dilutions of PyoS3-immS3 protein were dropped on the plates seeded with each clinical strain separately (see section 2.26). Concentration-dependent growth inhibition zones are shown for *P. aeruginosa* clinical strain PSA 971 (A) and PSA 892 (B). the scale bar of 10.0 mm was used on each undiluted inhibition zone of PSA 892 and PSA 971 strains. PSA 892 inhibition zone is 10.0 mm and inhibition of PSA 971 is ~20.0 mm.

4.2.7 PyoS3-sensitive clinical isolate *PSA 892* can accept plasmid bearing Tn5 following conjugation, but *PSA 971* cannot

The *PSA 971* and *PSA 892* clinical isolates were conjugated with an *E. coli* *S17-1* λ *pir* donor strain that contains the pME9978 transposon plasmid (Tn5 transposon). The conjugation with *PSA 971* did not produce any conjugants (data not shown). However, the conjugation with *PSA 892* produced multiple trans-conjugant colonies (Figure 4.9); thus, the transposon library was generated in *PSA 892*. A total of 4500 conjugate mutant colonies were isolated. Replica plates of each *PSA 892* conjugate mutant colony were generated to test the sensitivity of each colony against PyoS3-immS3.

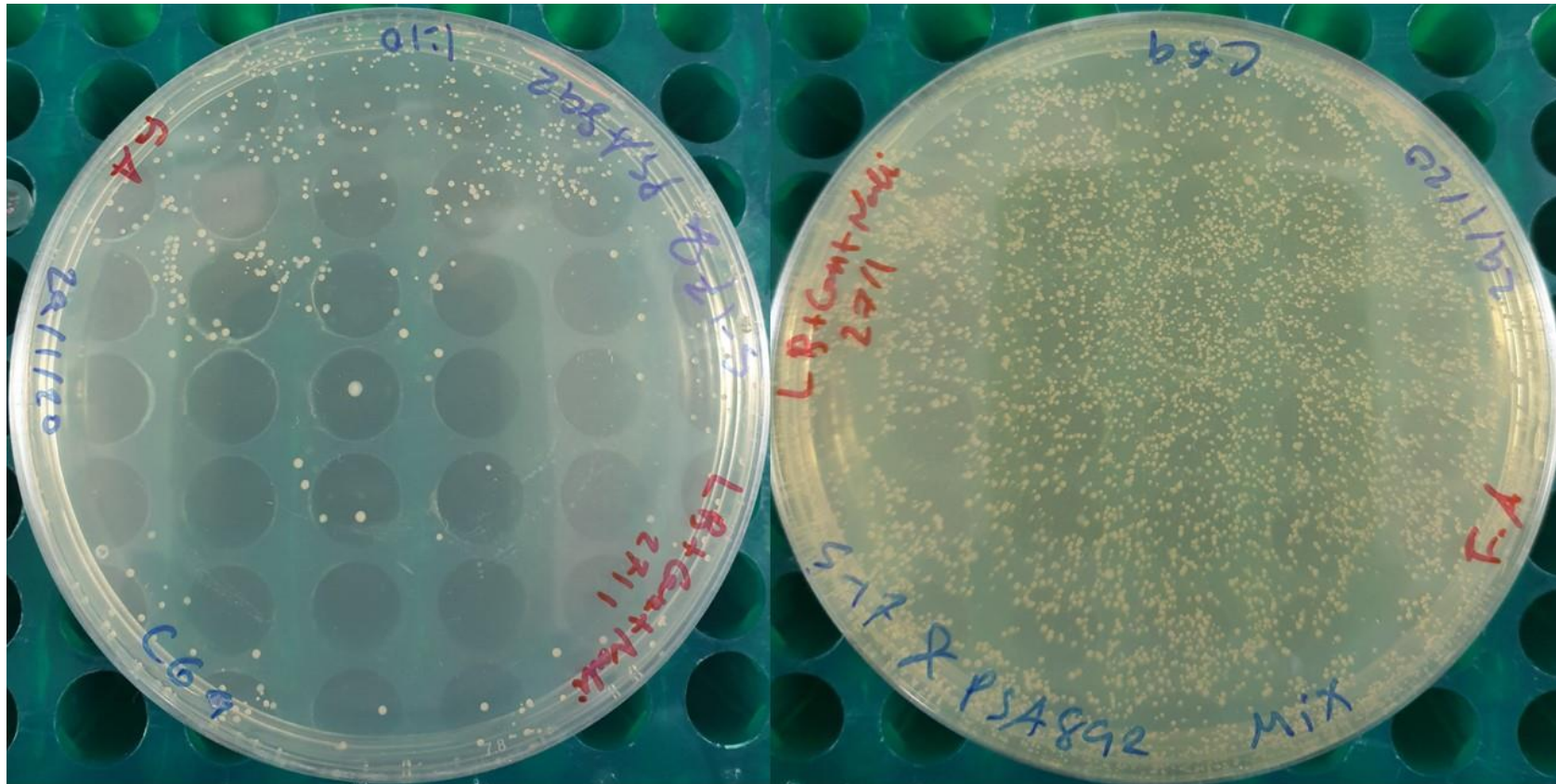


Figure 4.9: Plates of PSA 892 colonies after successful conjugation with *E. coli* S17-1 λ pir (pME9978). The PyoS3 sensitive clinical strain PSA 892 was conjugated with *E. coli* S17-1 λ pir (pME9978) to donate Tn5. The plates of the resultant colonies contain gentamicin, an antibiotic marker for the pME9978 transposon plasmid, and nalidixic acid to inhibit the growth of unwanted *E. coli* S17-1 λ pir cells after conjugation.

4.2.8 Screening of trans-conjugants of PSA 892 to identify Tn5 mutants resistant to PyoS3

4.2.8.1 Optimisation of PyoS3-immS3 sensitivity screen

To convert the routine PyoS3-immS3 sensitivity spot test (illustrated in Figure 4.8) to a high throughput screen amenable to assaying the sensitivity of a quantity of random mutants sufficient enough to yield a representative coverage of the 5.5-7 Mbp *P. aeruginosa* genome, it was adapted from a petri dish to a microtitre plate format. A set of optimisation experiments were undertaken using the PyoS3-sensitive clinical isolate (PSA 892) as positive control and PyoS3-resistant *P. aeruginosa* strain PA01 (Nottingham) to inoculate agar in separate wells in a 12-well microtitre plate. The finalised protocol for bacterial inoculation is described in section 2.27.2. Subsequently, 2 μ L of 6.7 μ M of purified PyoS3-immS3 (Figure 4.7) was dropped into each well, the plates were incubated overnight, and the resulting zones of inhibition were observed after 24 hours (Figure 4.10).

4.2.8.2 Identification of Tn5 mutants resistant to PyoS3

Conjugation of PSA 892 using the donor *E. coli* S17-1 λ pir donor strain containing the pME9978 transposon plasmid was performed to generate 4500 Tn5 mutants. The trans conjugant colonies were replica plated (section 2.27.2) and divided into nine batches. Each batch contained 500 mutants, so that no mismatching would occur during screening. Each PSA 892 transconjugant colony was subjected to sensitivity testing with PyoS3-immS3 (see 4.2.8.1) to identify colonies with mutations in genes that inhibited uptake of PyoS3 into *P. aeruginosa* cells through the absence of a zone of growth inhibition. Each PSA 892 Tn5 mutant was incubated separately in 12-well plates, alongside with the PSA 892 clinical strain as a positive control and *P. aeruginosa* PAO1 as a PyoS3 negative control strain on the same plate (Figure 4.10).

Five mutants were selected for further analysis based on their inhibition zone. One was contained within the first batch of mutants (mutant 44). Mutant 44 exhibited a smaller growth inhibition zone when PyoS3-immS3 was applied compared to the *PSA 892* positive control. From the fifth batch, mutant 107 was resistant to PyoS3-immS3, exhibiting no visible zone of growth inhibition (Figure 4.11). In the seventh batch, mutant 422 had similar growth inhibition zone compared to the *PSA 892* positive control. In the eighth batch, mutant 477 had a similar growth inhibition zone compared to the *PSA 892* positive control. Lastly in the ninth batch, mutant 362 had a larger growth inhibition zone than the *PSA 892* positive control.

As a further screen, *PSA 892* mutants 44, 107, 362, 422 and 477 were grown in Petri dishes and undiluted PyoS3-immS3 protein was spotted on top of each one. The growth inhibition zones of each *PSA 892* mutant were compared to that of the wild-type *PSA 892* clinical strain. The growth inhibition zone of undiluted PyoS3-immS3 on wild-type *PSA 892* was 10 mm. The growth inhibition zones were 6.0 mm for mutant 44, 14.0 mm for mutant 362, 10 mm for both mutants 422 and 477 (Figure 4.12). There was no growth inhibition zone for mutant 107, which confirmed that this mutant is fully resistant to PyoS3 (Figure 4.11).

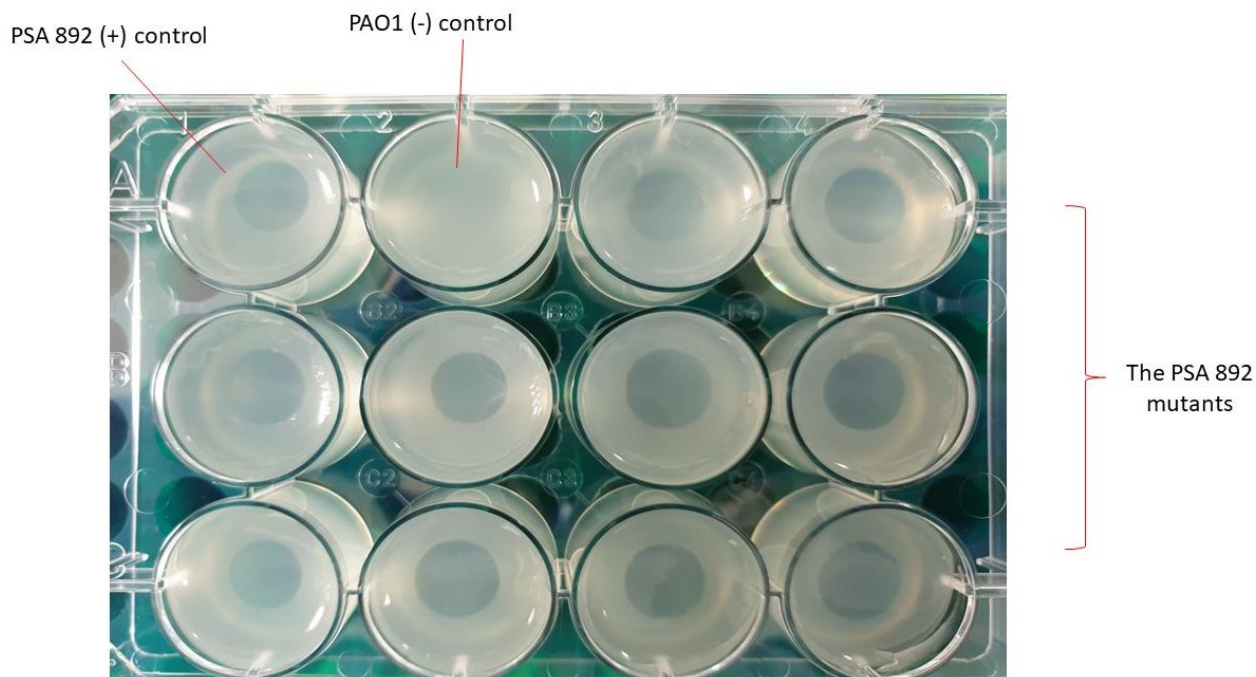


Figure 4.10: A typical assay plate demonstrating the high throughput PyoS3 sensitivity screen developed in this study. Agar in each of the 12 wells of a microtiter plate was separately inoculated with *P. aeruginosa*. Purified PyoS3-immS3 (Figure 4.8) was spotted on, and the plates were visually inspected following overnight incubation at 37°C (section 2.27.2). *PSA 892* was inoculated in one well as a positive control as evidenced by a clear zone of growth inhibition. *P. aeruginosa PAO1* was used as a negative control and lacked a zone of growth inhibition. The rest of the wells shown contain random Tn5 mutants of *PSA 892*. Mutants with smaller or larger inhibition zones than the positive control or resistant mutants were isolated for further analysis. This method was applied to screen all 4500 mutants of *PSA 892*.

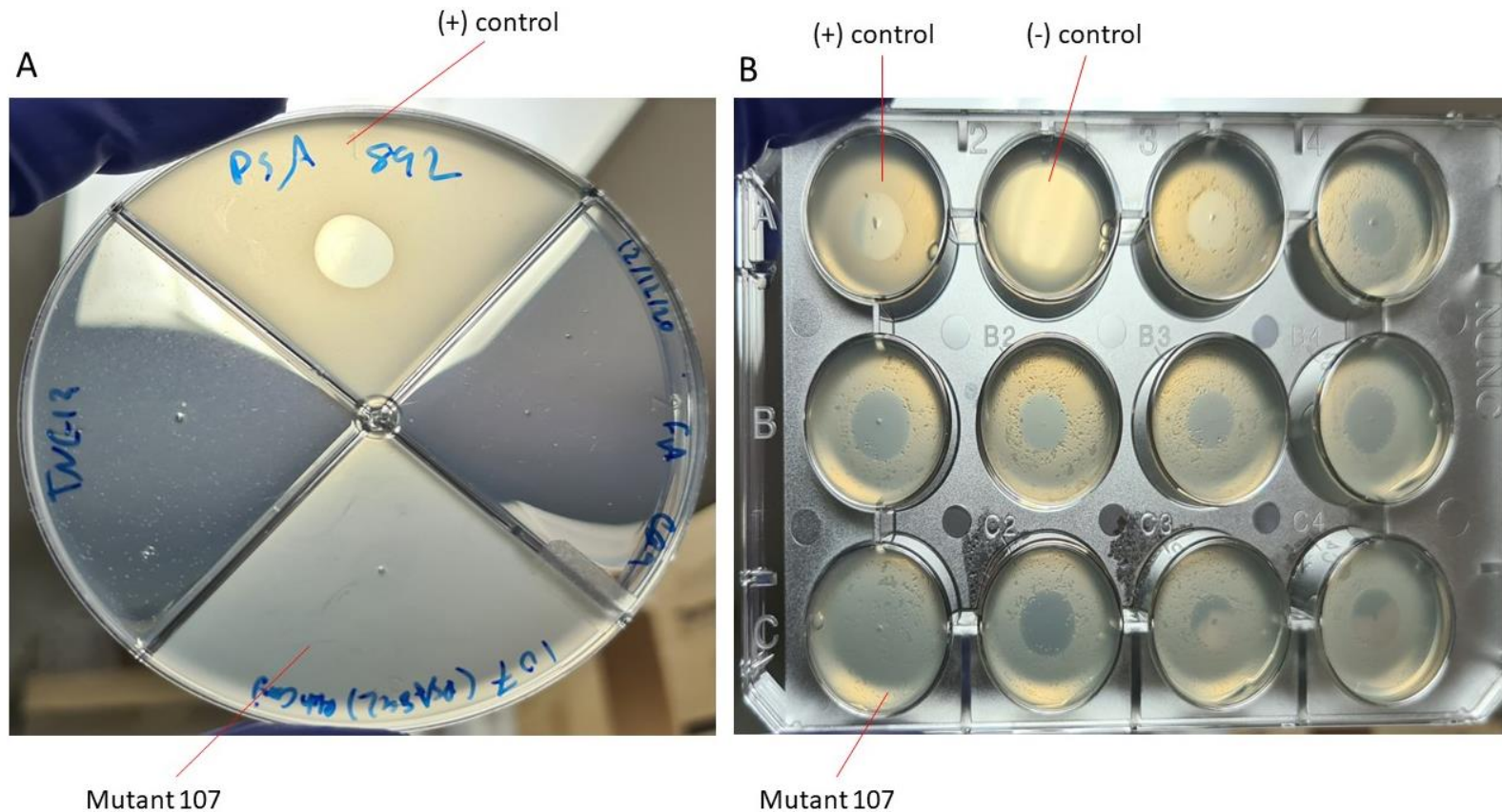


Figure 4.11: Demonstration of the resistance of PSA 892 mutant 107 to killing by PyoS3-immS3 in A; 12-well plates and B; four-compartment Petri dishes. PyoS3-immS3 pure protein was dropped on top of the sensitive clinical strain PSA 892 which shows an inhibition zone. Mutant 107 did not have a growth inhibition zone, which indicates that PSA 892 trans-conjugate mutant 107 was resistant to PyoS3-immS3.

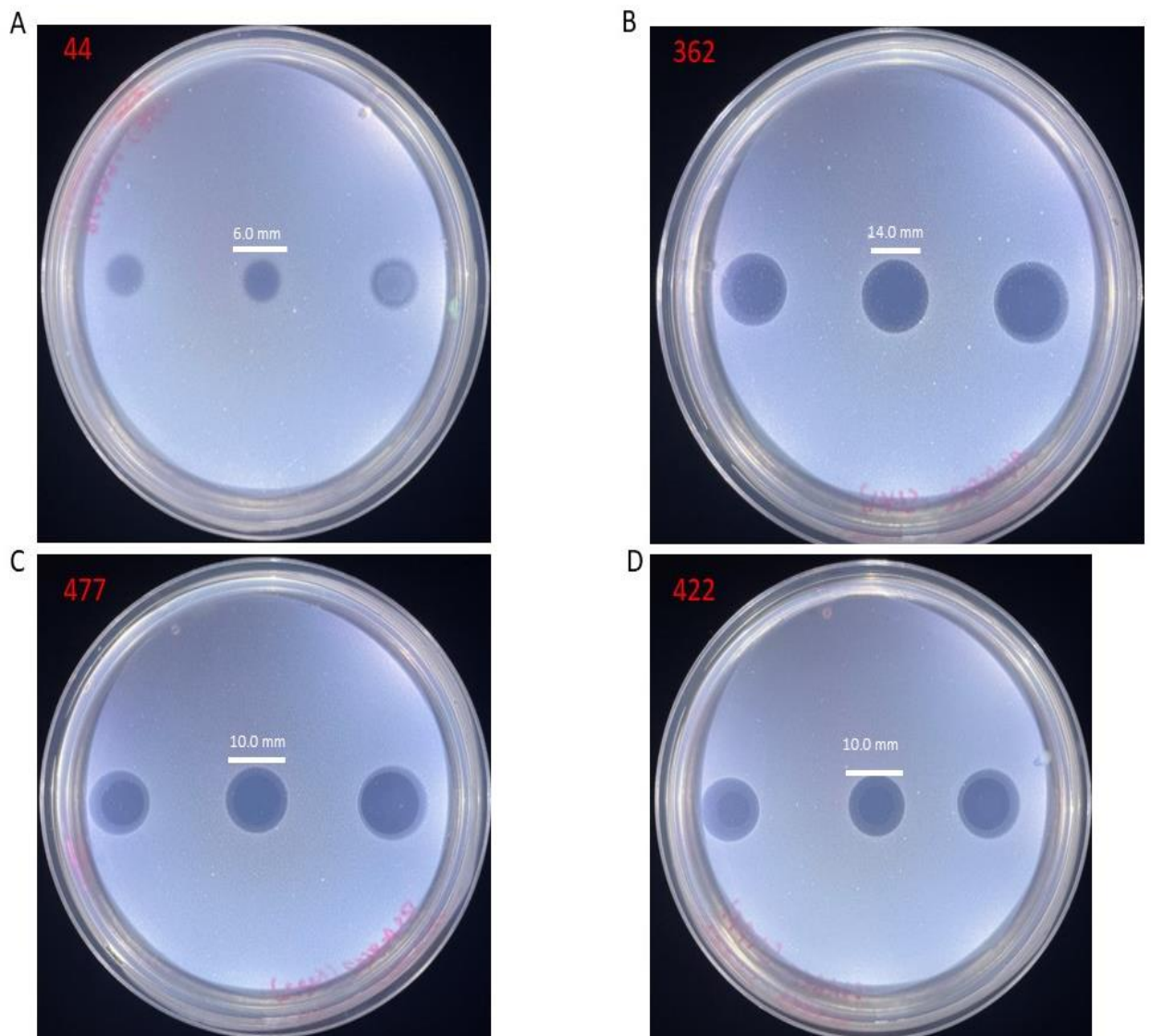


Figure 4.12: Confirmatory PyoS3-immS3 Spot tests confirm *PSA 892* transposon mutants 44, 362, 422 and 477 are more resistant to its growth inhibition. Triplicate spots of purified PyoS3-immS3 were applied to agar containing each mutant. The growth inhibition zone for the control strain *PSA 892* was 10 mm on a parallel plate (Figure 4.8) and the scale bar is 10.0 mm in each plate. A: The PyoS3- immS3 inhibition zone for mutant 44 was 6.0 mm. B: 14.0 mm for mutant 362. D and C: 10 mm for mutants 422 and 477.

4.2.9 Genomic sequencing of *PSA 892* and *PSA 971*

The genomes of the two PyoS3 sensitive strains available, *PSA 971* and *PSA 892* were sequenced via MicrobesNG, UK for three reasons. Firstly, in an

attempt to identify common features that might reveal why they are sensitive to PyoS3 when compared to resistant strains in available databases e.g., PAO1. Secondly, to identify if the *PSA* 892 and the transposon mutants ability to produce PvD were affected. Thirdly, as a baseline sequence for comparison to the site of insertion of Tn5 in the five mutants selected by their resistance to PyoS3. Analysis of the resulting sequences using the PATRIC bioinformatics tool (Davis et al., 2020) is presented in sections 4.2.10, 4.2.11 and 4.2.12.

4.2.10 Sequence analysis of the five Tn5 mutants of *PSA* 892 selected for further study identified five separate sites of transposon insertion.

Initially, attempts were made to PCR amplify the transposon insertion sites in mutants 44, 362, 107, 422 and 477. The *TPNRL17-1* forward and *TPNRL13-2* reverse primers were used to generate amplicons from the transposon insertions present in each *PSA* 892 mutant. However, the PCR was not successful (see Appendix 4) and because of the Covid-19-related shortage of PCR materials, another method was used to recover the plasmids (Larsen et al., 2002).

As an alternative, genomic DNA prepared from the 44, 107, 362, 422 and 477 mutants of *PSA* 892 were separately digested using *EcoRI*, which cuts the genomic DNA of the mutant but does not cut within the transposon. Then, ligase was used to circularise each transposon into a plasmid with its flanking genomic DNA. The plasmids were transformed into *E. coli DH5α* competent cells. The transposon-containing plasmids were extracted and sent to GENEWIZ, UK, for sequencing. The sequences of the transposon inserts were analysed via PATRIC BLAST (Davis et al., 2020) against the clinical strain *PSA* 892 and the reference strain *P. aeruginosa* PAO1. The *Pseudomonas* Database (Winsor et al., 2011) was used for further confirmation. The sequencing results are summarised in Table 4.1.

Table 4.1: Sequencing results of the PSA 892 Tn5 mutants selected for further study revealed each insertion had occurred at a different site in the genome. The transposon from each mutant was digested from the genome with flanking DNA and circularized into a plasmid. The plasmid sequences were analysed using PATRIC BLAST and NCBI protein BLAST (States and Gish, 1994, Davis et al., 2020) to identify the transposon insertion sites. The percentage identity and coverage of the query gene is shown for each.

Mutant plasmid	Gene identified	Accession number	Query coverage (%)	inhibition zone size
44	Transcriptional regulator LuxR family, VqsR	287.24992.con.0019	87	6.0 mm
362	Short-chain dehydrogenase reductase (SDR) family oxidoreductase	WP_034033786.1	93	14.0 mm
107	PvdM	287.24992.con.0037	94	-
422	Surface lipoprotein	287.24992.con.0009	90	10 mm
477	Carboxylic ester hydrolase	287.24992.con.0063	91	10 mm

4.2.11 *PSA 892* does not produce PvD

In mutant 107, the transposon sequence was located in *PvdM*, an enzyme that has a role in PvD production. To investigate this further, it was first determined whether *PSA 892* produces PvD and if the production of PvD was influenced by the transposon mutation in *PvdM*. The *PAO1* strain was used as a positive control as it produces PvD, hence its survivability in iron-limited media, and *P. aeruginosa PAO1 Tat* mutant was used as a negative control because it does not produce PvD, which means that it would not grow in iron-limited conditions (Soh et al., 2021). As shown in Figure 4.13, neither *PSA 892* nor the derived *PSA 892* transposon mutants (44, 362, 107, 442, 477), cell count was increased in the iron-limited conditions, which indicate that these mutants and *PSA 892* clinical strain dose not produced PvD.

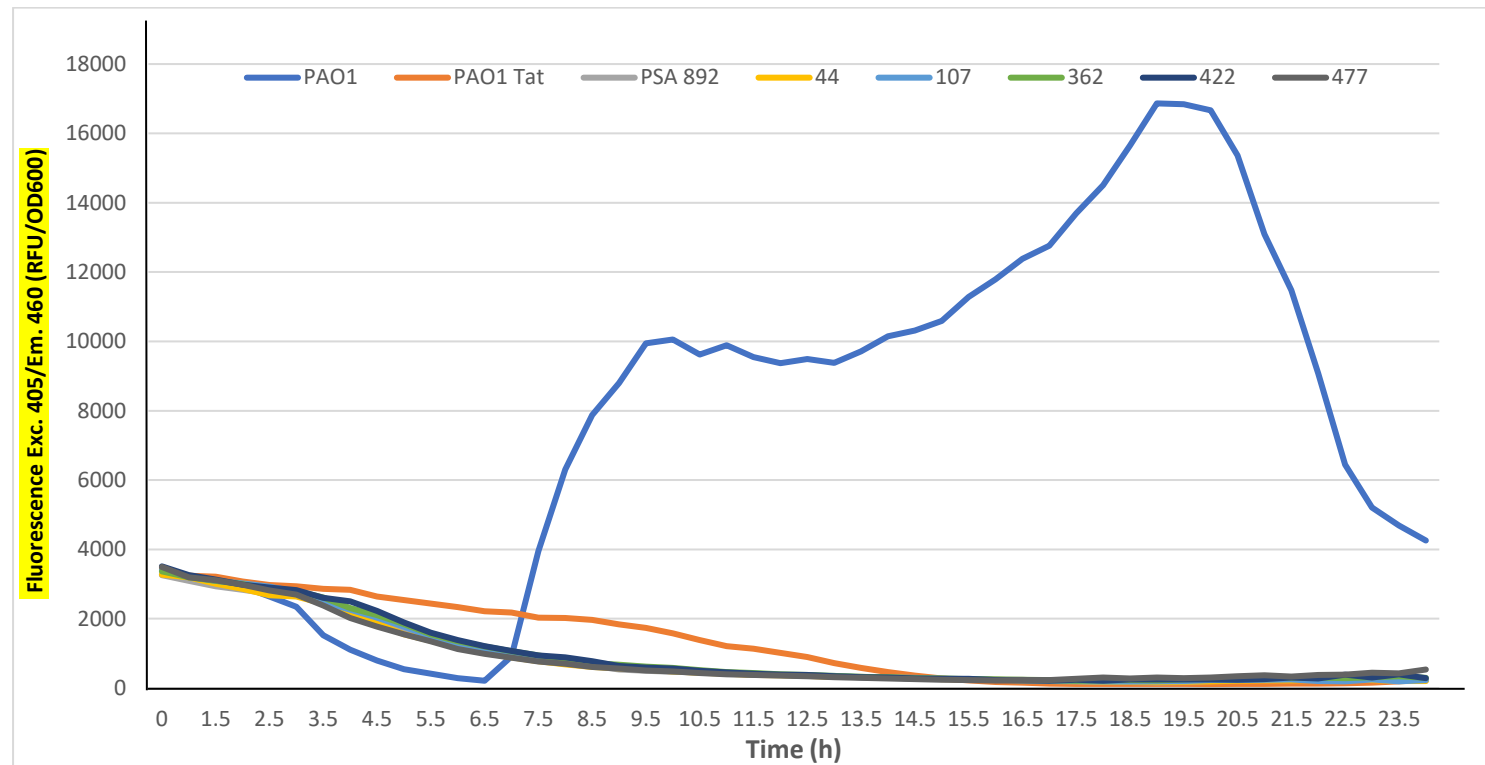


Figure 4.13: Pvd is not produced by *PSA 892* or the derived *PSA 892* mutants 44, 107, 362, 422 and 477.

The amount of Pvd produced by each strain was assessed as described in section 2.29. The x-axis indicates time of growth in hours; the samples were incubated in the TECAN plate reader for 24 hours. The y-axis indicates the Fluorescence Exc. 405/Em. 460 (RFU/OD600). The positive control *PAO1* strain

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produced PvD after 6.30 hours incubation; peak production was observed at 19:30-20.30 hours, then the production was declined. The negative control *PAO1* *Tat* mutant did not produce PvD. *PSA 892* and the derived *PSA 892* mutants (44,107,362, 422 and 477) did not produce PvD.

4.2.12 *In silico* analysis of PvD receptors and PvD biosynthesis proteins in the pyoS3-sensitive clinical isolates, PSA 892 and PSA 971:

Previous studies stated that FpvAll was the receptor of PyoS3 (Baysse et al., 1999). It would thus be expected that sensitive strains would have FpvAll on their surface. Based on Bodilis et al. FpvAll has two subclusters, FpvAlla and FpvAllb (Bodilis et al., 2009). The FpvAllb and FpvAlla sequences were aligned against the genomic sequences of *PSA 892* and *PSA 971* via PATRIC BLAST (Davis et al., 2020). Analysis of PvD receptor sequences revealed that the *PSA 892* genome contains the FpvAllb receptor subcluster with 100 % identity and query cover and the *PSA 971* genome contains the FpvAlla receptor subcluster 100 % identity and query cover (see Appendix 5). Thus *PSA 892* likely expresses FpvAllb and *PSA 971* likely expresses FpvAlla.

FpvB is an alternative receptor to FpvAI for PvDI (Bonneau et al., 2020). Interestingly, FpvB is present in the *PSA 892* and *PSA 971* PA strains as well as the *PACS2* and *C33791* PA strains with a homology of 100% and is found in all *P. aeruginosa* strains that express either FpvAI, FpvAll or FpvAllI (Bodilis et al., 2009).

The sequences of the PvD enzymes responsible for PvD biosynthesis were blasted against the *PSA 892* genome. As described before, biosynthesis of the PvD backbone starts at the cytoplasm with the non-ribosomal peptide synthetases (NRPSs) PvdL, PvdI, PvdJ and PvdD. In addition, other enzymes that play a role in PvD biosynthesis include PvdA, PvdF and PvdH, PvdE, PvdQ, PvdN, PvdO, PvdP, PvdG, PvdS and PvdM (Gasser et al., 2020, Ringel and Brüser, 2018, Schalk and Guillon, 2013). The Blast searches showed all of these enzymes are present in the reference strain *PAO1* and *PSA 892*, with the exception that PvdJ which is not present in *PSA 892*.

4.3 Discussion and Conclusion

This chapter aimed to use an unbiased approach to identify the genes/gene products that are involved in PyoS3 uptake and import. The genes encoding the full-length PyoS3 protein with its cognate immunity protein were successfully cloned, overexpressed and PyoS3-immS3 purified to identify PyoS3-sensitive *P. aeruginosa* strains. A representative transposon library of 4500 mutants was generated from the *P. aeruginosa* PyoS3-sensitive strain PSA 892. A high throughput screen was developed and optimised to identify mutants altered in their sensitivity to PyoS3. One PyoS3-resistant transposon mutant was generated in the *pvdM* gene, which encodes one of Pvd biosynthesis enzymes (Sugue et al., 2022). Pvd production assays showed the PyoS3-sensitive strain and newly generated Tn5 mutants did not produce Pvd, so assessing the impact of the mutation of pyoverdine production was limited. To compliment the transposon mutagenesis findings and shed light on the involvement of PvdM in PyoS3 translocation, genomic analysis was conducted to determine which Pvd receptors are present in the PyoS3-sensitive *P. aeruginosa* strains. It is likely that the Pvd receptor in PSA 892 is FpvAllb whereas for PSA 971 the Pvd receptor is likely FpvAlla (Bodilis et al., 2009).

The findings in this chapter rely on the purified protein used in the screening being PyoS3. Duport et al. first identified PyoS3 in the *P. aeruginosa* P12 strain, which was isolated from a patient with cystic fibrosis. The predicted size of the large killing protein of PyoS3 is 81 kDa and the smaller cognate immunity protein that protects the producing cell from suicide is 17 kDa (Duport et al., 1995). The PyoS3-immS3 proteins overproduced and purified in this study migrated at the same sizes. Thus, it was concluded that the protein used in this study was *bone fide* active PyoS3-immS3. Spot tests generated clear growth inhibition zones, indicating PyoS3-immS3 was active against the PSA 892 and PSA 971 clinical strains.

Previous studies screening a larger collection of clinical strains for sensitivity to PyoS3 had suggested a correlation with the Type 2 pyoverdine (PvDII) receptor FpvAll (Baysse et al., 1999). In agreement with this, *in silico* genome analysis of *P. aeruginosa* strains PSA 892 and PSA 971 showed that both have genes encoding FpvAll, although the subtypes are different with the PSA 892 genome

encoding the FpvAIIb receptor and the *PSA 971* genome encoding the FpvAIIa receptor. Interestingly, both strains also have the gene encoding FpvBI, which is an alternative to the pyoverdine type 1 (PvDI) receptor FpvAI. Thus, our analysis would support the suggestion of Baysse et al., who reported that PyoS3 utilises FpvAII for uptake (Baysse et al., 1999).

The Tn5 transposon mutagenesis conducted to identify genes responsible for the uptake and translocation of PyoS3 by identifying a loss PyoS3-sensitivity. *PSA 892* PyoS3-sensitive clinical strain and *PSA 971* PyoS3-sensitive clinical strain was used to generate trans-conjugate mutants, however only *PSA 892* produced conjugate mutants. Furthermore, When *PSA 971* and *PSA 892* strains were genetically analysed there was no clear feature detected that could be linked to alteration in the ability to accept plasmids by conjugation in *PSA 971* strain. However, the reason for *PSA 971* clinical strain transposon mutagenesis did not produce any conjugate might be that the strain has a mechanism that destroys foreign DNA such as the transposon DNA that enters the cell (Wheatley and MacLean, 2021). Therefore, further analysis will form part of a follow-on study to find the reason why transposon mutagenesis of *PSA 971* strain did not produce any conjugate mutants. Moreover, screening 4500 transposon mutants generated from *PSA 892* PyoS3-sensitive clinical strain was conducted. Despite time constraints limiting the transposon mutagenesis so that it was not entirely saturated, five interesting mutants were selected for further analysis. Each had a transposon inserted in a separate gene, indicating the random nature of the library. Moreover, *P. aeruginosa* consists of 5316 genes (Poulsen et al., 2019), for that reason if all genes were hit by the Tn5 experiment even once, 5316 trans-conjugate mutants should be generated. In our study, only 4500 trans-conjugate mutants were generated, thus not every gene of *P. aeruginosa* was hit. However, this does mean that the trans-conjugate mutants number in this study was not applicable as Other studio screened only 1500 mutants to reach its desired mutation in PvDII uptake (De Chial et al., 2003). Furthermore, more screening to cover all *P. aeruginosa* genes would be helpful by either continuing the Tn5 transposon experiment or maybe using other intensive method like Transposon Directed Insertion Sequencing (TraDIS) (Cain et al., 2020).

Two mutants, selected as interesting in the initial high throughput screen (Figure 4.12), did not show a growth inhibition zone different to the parent *PSA 892* (Figure 4.8), and are thus unlikely to play a substantial role in PyS3 translocation. Of these, the transposon in mutant 422 had inserted into a gene encoding the surface lipoprotein (*SLP*) gene. Homologous of *SLP* are structurally and functionally diverse, playing numerous roles in Gram-negative bacteria, such as nutrient acquisition, immune evasion, adhesion and cell signaling (Cole et al., 2021). The transposon insertion in mutant 477 affected the carboxylic ester hydrolase gene, which catalysis the hydrolysis of carboxylic esters.

Mutant 44 exhibited a consistently smaller growth inhibition zone in high throughput and petri dish PyoS3 sensitivity spot tests compared to the parent *PSA 892* (Figure 4.8 and Figure 4.12). Mutant 44 contained a transposon inserted in a transcriptional regulator of the LuxR family, the *VqsR* gene. LuxR-family transcriptional regulators play an important role in quorum-sensing (QS) systems in a wide variety of bacteria. QS involves the production of small chemical signal molecules that build up to a threshold concentration during growth and enable bacteria to link their population density with the control of gene expression via interacting with transcriptional regulators. A variety of genes are controlled by QS, including genes involved in virulence regulation, antibiotic biosynthesis, motility, and biofilm production. The LuxR family transcriptional regulators are controlled by interacting with *N*-acyl-homoserine lactones (AHLs), which are signalling molecules known as autoinducers (Tsai and Winans, 2010). *P. aeruginosa* has a complex hierarchical QS network. The two main AHL-interacting transcriptional regulators are LasR and RhIR. LasR interacts with a specific sequence motif in the promoter of genes it controls, a *las* box, when it is bound to its cognate autoinducer, 3-Oxo-C12-HS (*N*-(3-oxo-dodecanoyl)-L-homoserine lactone). *VqsR* possesses a *las* box in its promoter region and is regulated by LasR (Liang et al., 2012, Li et al., 2007). In a *VqsR* deficient mutant, the expression of approximately 200 genes is altered, and half of these overlap with the QS regulon (Liang et al., 2012). Several QS-controlled virulence factors were compromised, and AHL production was reduced in a

vqsR transposon insertion mutant (Juhas et al., 2004). Although VqsR did not directly regulate LasR or RhIR, it interacted with an inverted repeat motif in the promoter of *qscR*. QscR is a transcriptional reporter with homology to LasR/RhIR but lacking a cognate acyl-HSL synthase gene. QscR has been demonstrated to repress both *las* and *rhl* systems (Liang et al., 2012). The *vqsR* mutant also expressed lower levels of siderophore mRNA transcripts (Juhas et al., 2004). To date, no study has indicated a direct relationship between VqsR and the uptake of Pvd and pyocins. However, the VqsR mutation affects QS virulence and expression of the mRNA transcripts that produce siderophores. Thus, VqsR may affect the uptake of PyoS3 because PyoS3 utilises the same receptor as the siderophore PvdII (FpvAII).

It is worth mentioning that the PvdQ enzyme links Pvd production and QS, as the PvdQ enzyme is involved in the biosynthesis of molecules involved in QS (Ringel and Brüser, 2018). It has been suggested that PvdQ regulates the ratio between the two autoinducers found in *P. aeruginosa*, namely the AHL that interacts with LasR: 3-Oxo-C12-HSL (which is the substrate of PvdQ) and the short-chain autoinducer N-butanoyl-L-homoserine lactone (C4-HSL) that RhIR binds to in order to induce QS (Huang et al., 2003).

Interestingly, the transposon in mutant 362 was inserted in the SDR family oxidoreductase gene, which is also related to QS-controlled virulence. The SDR family oxidoreductase reduces QS-related virulence in *P. aeruginosa*. Expression of SDR significantly reduces the QS signalling molecule 3-oxo-C12-HSL, which decreases QS-controlled virulence factors via LasR (Bijtenhoorn et al., 2011). Transposon mutant 362 had a larger growth inhibition zone compared to the wild-type PSA 892 in the petri dish PyoS3 sensitivity spot test (Figure 4.8 and Figure 4.12). To our knowledge, no study has linked the uptake of pyocins and FpvA receptor production to SDR. However, knockout of SDR may increase virulence and lead to increased uptake of PyoS3, though further investigation needs to be conducted to confirm this hypothesis. Further study could include construction of a defined mutant and genetic complementation. The resultant strains could be phenotypically assayed for virulence production, QS signal molecule production and pyocin sensitivity. It would be interesting to

extend across all the S-type pyocins and utilize QS reporter gene fusions to build an in-depth mechanistic study.

The transposon insertion in mutant 107 was located in the *PvdM* gene. It is the most promising mutant to link to PyoS3 translocation as there was a complete absence of growth inhibition in both the high throughput and petri dish PyoS3 sensitivity spot tests (Figure 4.11). Until recently, there was little information on PvdM; its exact function was unknown, though it was known to play a role in Pvd biosynthesis and is a periplasmic enzyme (Schalk et al., 2020, Ringel and Brüser, 2018). However, Sugue et al. (2022) recently identified the function and the role of PvdM using the model organism *P. fluorescens*, which expresses similar PvdS to *P. aeruginosa* (Sugue et al., 2022). PvdM anchors to the cytoplasmic membrane facing towards the periplasm and is a dipeptidase that is structurally related to human renal dipeptidase. PvdM plays an important role in preventing the loss of ferribactin in the periplasm by leading ferribactin to PvdP for Pvd maturation before releasing it out of the cell (Sugue et al., 2022, Schalk et al., 2020). In addition, the *pvdM* gene is located in close proximity to TonB-dependant receptor-encoding genes, thus all PvdM homologs in the periplasm are linked with TonB-dependent uptake systems, which indicates that the PvdM homologs are functionally involved in the uptake of compounds such as PvdS or pyochelins (Sugue et al., 2022). This might explain why the transposon insertion in PvdM inhibited the uptake of PyoS3, as the literature indicates PyoS3 utilises FpvAII, which is a TonB-dependent receptor. Further investigation needs to be done to confirm this suggestion, though it is plausible that the PvdM mutation altered one of the periplasmic TonB proteins and thus inhibited the uptake of PyoS3.

Pvd production was assessed in the clinical strain PSA 892 and the transposon mutants 44, 107, 362, 422 and 477. However, PSA 892 and the mutant strains did not produce Pvd. Thus, the Pvd biosynthesis enzymes in PSA 892 and the transposon mutants were searched using PATRIC Blast. All of the enzymes were detected, except for the PvdJ enzyme. PvdJ is one of four non-ribosomal peptide synthetase (NRPS) enzymes involved in Pvd biosynthesis (Schalk et al., 2020). A study in *P. syringae* found that mutation of PvdJ inhibits Pvd production (Taguchi et al., 2010). As *P. aeruginosa* and *P. syringae* belong to

the *fluorescens Pseudomonas* group, the *PSA 892* clinical strain may not produce PvD as the PvdJ enzyme is missing. In addition, CF *P. aeruginosa* strains have a tendency to not produce PvD and it is the case of *PSA 892* that used in this study, which is a CF isolate (De Chial et al., 2003). Furthermore, the absent of PvD does not mean the PvD receptor are not produced, because it has been found that non-producing PvD *P. aeruginosa* strain uptake the PvD in iron limited environment when co-existed with PvD producing *P. aeruginosa* strains (De Vos et al., 2001).

In conclusion, the genes encoding PyoS3 and its cognate immunity protein were successfully cloned and overexpressed from the pFAI and pFAII plasmids and transposon mutagenesis was conducted. Due to Covid-19 restrictions and limited time, only five of the identified mutants were analysed. Mutants number 44 and 362 affected the transcriptional regulator LuxR family (VqsR) and SDR oxidoreductase, respectively; both genes are related to QS regulation. As the inhibition zone of PyoS3 was smaller for mutant 44 and larger for mutant 362, VqsR and SDR oxidoreductase may either directly or indirectly regulate the uptake of PyoS3. Further investigation, such as direct deletion of these genes, is necessary to confirm the involvement of these proteins in the translocation of PyoS3. Mutants 422 and 477 affected SLP and carboxylic ester hydrolase, respectively. However, these mutants had the same sizes of growth inhibition zone as the clinical strain *PSA 892*, thus these proteins do not affect the uptake of PyoS3. The final mutant 107, in which the transposon was inserted in the *pvdM* gene, was completely resistant to PyoS3. This finding is interesting and needs to be investigated further, as PvdM homologs have been associated with the TonB-dependent uptake systems and the FpvA receptors are TonB-dependant and used by PvD and Pyocins for their uptake into the cell. For instance, PvdM could be deleted in other strains that are sensitive to other Pyocins that utilise the TonB-dependent system to confirm the role of PvdM in the uptake of PyoS3.

5 Chapter 5.

Structural analysis of PyoS3

5.1 Introduction

X-Ray protein crystallography is the gold standard technique for establishing the three-dimensional structure of proteins. An X-ray beam is passed through the protein crystal and the diffraction pattern data is analysed to determine the structure of the protein (Drenth, 2007). According to the protein data bank, the structure of 191869 macromolecules have been published to date, and 90% of these structures were solved using X-ray crystallography (Berman et al., 2003). Solving protein structures by X-ray crystallography plays an important role in understanding the mechanisms of action of cellular components, including enzyme function, cellular signaling and cellular recognition, and also contributes to pharmaceutical structure-based drug discovery (Srivastava et al., 2018).

Macromolecular X-ray crystallography starts with placing the crystal onto a goniometer, which is rotated. A focused X-ray beam is passed through the crystal of interest and a specific pattern of diffracted X-rays is created by the atoms in the crystal. The Bragg equation is used to determine the number of diffraction images recorded, which represent the X-ray reflection of atoms within the crystal. Then, Fourier methods are used to convert the diffraction data into electron densities, which are used to build a 3D atomic model (Ameh, 2019, Rupp, 2009).

In order for crystallization to occur, the samples must be saturated under the crystallization conditions in the specific sample preparation buffer. Crystallization requires the solubility threshold of the sample to be crossed, so that repeat binding to other protein molecules becomes more energetically favourable than binding to solvent molecules (Gorrec, 2021). Thus, a diverse set of crystallization reagents with a wide variety of chemical compositions and temperatures are typically screened to determine which conditions will lead to the formation of a stable crystal for each protein (Rupp, 2015). The sitting drops vapour-diffusion approach is convenient and can be automated; thus, is thus currently the most widely used method. This process involves mixing the

macromolecular sample solution with the screened chemicals in single drops. Each drop is placed beside a reservoir that holds a much greater volume of the crystallisation screen. An airtight seal is used to seal the plate in order to allow osmolar equilibrium to occur between the samples containing the crystallization drop and the reservoir. As the concentration of solutes is higher in the reservoir than the sample drop, water will diffuse from the protein sample drop into the reservoir through the vapour phase in order to equilibrate the solute concentrations and osmolarity. Thus, the size of the sample drop will gradually decrease, which will increase the concentration of the sample and promote crystallization (Gorrec, 2021, McPherson and Gavira, 2014) (Figure 5.1).

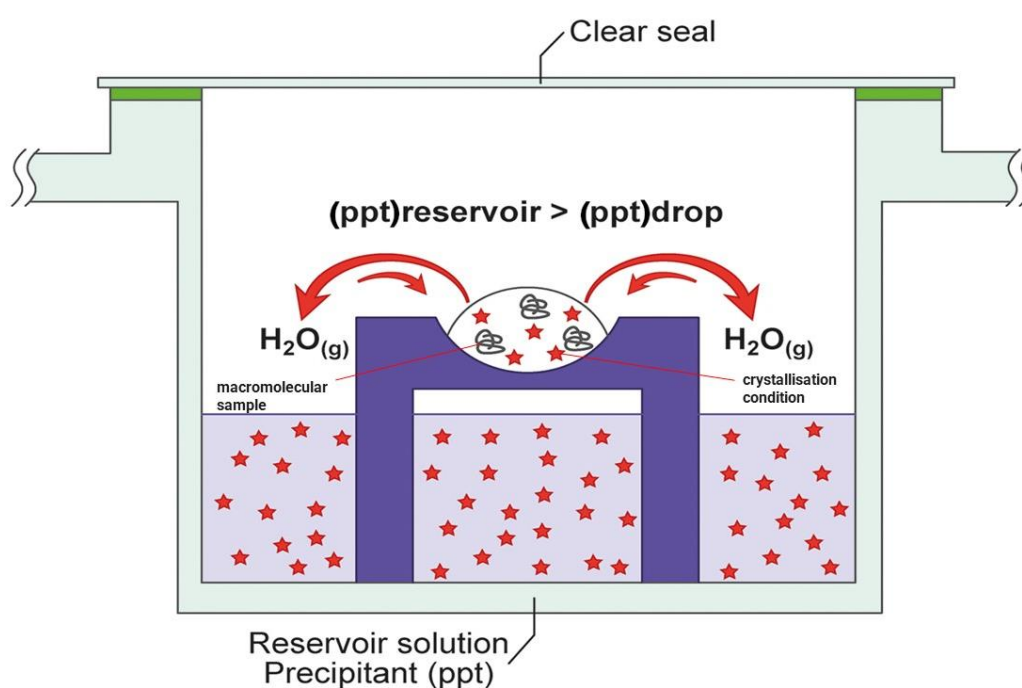


Figure 5.1: The sitting drops vapor-diffusion method. The drop contains an equal volume of the protein sample solution and the tested solution. The tested solution and protein are concentrated until equilibrium by evaporation of water in the drop into the chamber (Gorrec, 2021).

Structural information is available for several full-length bacteriocins, bacteriocin-receptor complexes and individual domains. The first complete crystal structure of a Gram-negative bacteriocin was published in 1997, which revealed the structure of colicin Ia is highly elongated and contains two remarkably long (~ 160 Å) α -helices (Figure 5.2)(Wiener et al., 1997).

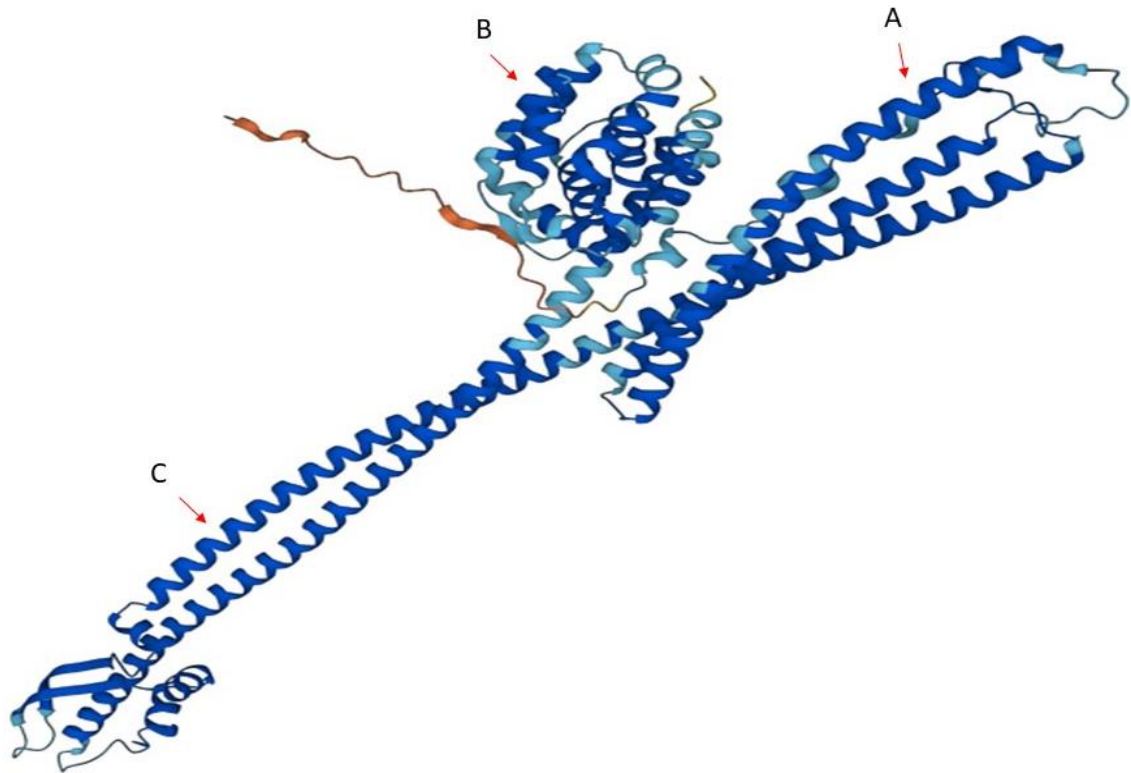


Figure 5.2: Crystal structure of Colicin Ia (Wiener et al., 1997). A indicates the translocation domain. B indicates the cytotoxic domain. C indicates the receptor binding domain.

Several full-length crystal structures of colicins N, E3, B, M, S4 and E9 were published in the following years (Vetter et al., 1998, Soelaiman et al., 2001, Hilsenbeck et al., 2004, Zeth et al., 2008, Arnold et al., 2009, Klein et al., 2016). The structures of many colicin nuclease-immunity complexes have been solved, including the colicin E9-Im9, colicin E3-Im3, colicin E7-Im7, colicin D-ImD, and colicin E5-Im5 complexes. These structures revealed a common inhibition mechanism. A nuclease is inhibited by the immunity protein when it binds at an exosite, which blocks substrate DNA binding. (Kleanthous et al., 1999, Carr et al., 2000, Sui et al., 2002, Graille et al., 2004, Luna-Chávez et al., 2006). A further goal of solving the structure of colicin catalytic-immunity proteins is to determine their protein folding landscapes, as well examine their protein-protein interactions in terms of the binding mechanisms and interaction specificity (Morton et al., 2007, Ferguson et al., 2001, Keeble and Kleanthous, 2005, Kortemme et al., 2004).

Unlike colicins, limited structural information is available for pyocins, especially the S-type pyocins. The molecular structures of some Pyocin S domains have been solved, such as the nuclease-immunity protein of Pyocin S1, S2 and AP41, as well as the Pyocin S2 N-terminal domains (Sano et al., 1993b, White et al., 2017). However, at present, only low-resolution structures of full-length pyocin S2 and AP41 have been obtained via small angle X-ray scattering (SAXS), which indicate an elongated morphology similar to some colicins (Klein et al., 2016). In addition, full-length crystal structures have only been recorded for Paem and L1; however, these pyocins are considered atypical pyocins because of their relatively smaller size (14 kDa for Paem and 28 kDa for L1) compared to other pyocins (mostly > 50 kDa) (Behrens et al., 2020c). However, PyoS3 has not yet been fully studied and there is little structural information known about this pyocin. Pyocin S2 N-terminal domain with FpvAI interaction was structurally solved by White et al. (Figure 5.3) (White et al., 2017). Five α -helices compose pyocin S2 N-terminal domain that also has a ~ 80 Å C-terminal helix extension from the interface and at the N terminus a short antiparallel β -hairpin. FpvAI plug domain was contacted via proline-rich region (PRR) consist of 11-amino acid. It has been found that binding between pyocin S2 N-terminal and FpvAI would decrease to $\sim 1,000$ -fold if the first 45 residues, including the PRR was deleted. In addition, it has been noticed that PRR resembles the shape of PvD and it occupies the same binding site (White et al., 2017).

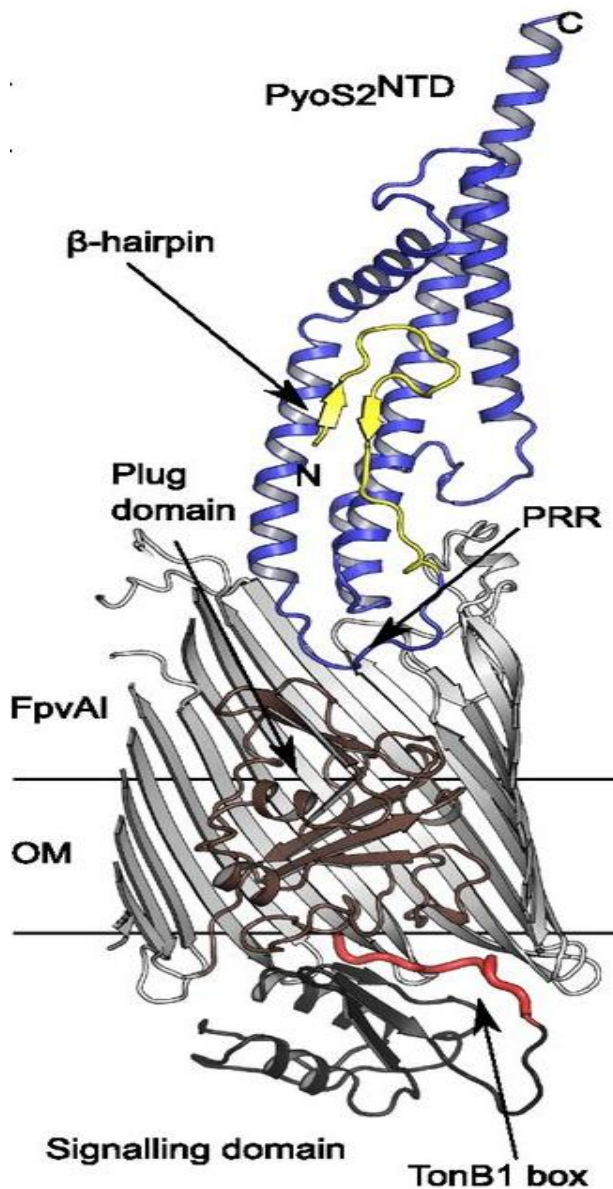


Figure 5.3: Pyocin S2 N-terminal binding to FpvAI crystal structure. PyoS2 N-terminal 2.8- Å crystal structure (blue) with FpvAI (gray) in a complex. Pyocin N-terminal β-hairpin colored in yellow. the FpvAI plug domain colored in brown. The signaling domains colored in black and the TonB1 box colored in red.

This chapter aimed to determine the crystal structure of purified PyoS3 to provide insight into how PyoS3 functions. Ultimately, structural information may help to provide an understanding of the mechanisms of uptake and cell killing and possibly identify novel therapeutic mechanisms that more broadly target infections. Since a relatively low percentage of *Pseudomonas* strains produce PyoS3, only a few strains of *Pseudomonas* are predicted to be resistant to the

killing activity of PyoS3. Recently, Dr Siyu Wu in our lab successfully solved the crystal structure of the PyoS3 DNase domain bound to the cognate Immunity protein. The approaches used by Dr Siyu Wu were used to inform the attempts to determine the crystal structure of full-length PyoS3 in this chapter.

5.2 Results

5.2.1 Overexpression and purification of full-length PyoS3

The genes encoding PyoS3 and its cognate immunity protein were cloned into pET21a to produce the pFA2 plasmid, as described in Chapter 2. The protein was expressed by IPTG induction. SDS-PAGE analysis (Figure 5.4) confirmed the proteins of the expected sizes of 81 kDa for the large protein (receptor-binding and DNase domains) and 17 kDa for the small protein (immunity protein) were produced. The Hexa-Histidine tag in the C-terminus of PyoS3 (as mentioned in Chapter 3) enabled purification of PyoS3 by nickel column affinity chromatography. The collected eluted fractions were assessed by SDS-PAGE to confirm the expected protein sizes (Figure 5.4). PyoS3 was further purified by size exclusion chromatography (SEC) using a Hiloal 26/600 Superdex 75 pg SEC column. Fractions 15–18 correlated with the largest UV peak and were collected. SDS-PAGE analysis confirmed the presence of pure PyoS3 (Figure 5.4). The protein fractions (F15–18) were concentrated as described in the material and methods section 2.22. The protein concentrations were measured using a NanoDrop spectrophotometer (ND-1000, ThermoFisher). UV absorbance indicated that concentration of PyoS3 was 3.1 mg/mL; the actual concentration after employing an extinction coefficient of $53000 \text{ M}^{-1} \text{ cm}^{-1}$ was determined to be 15 mg/mL.

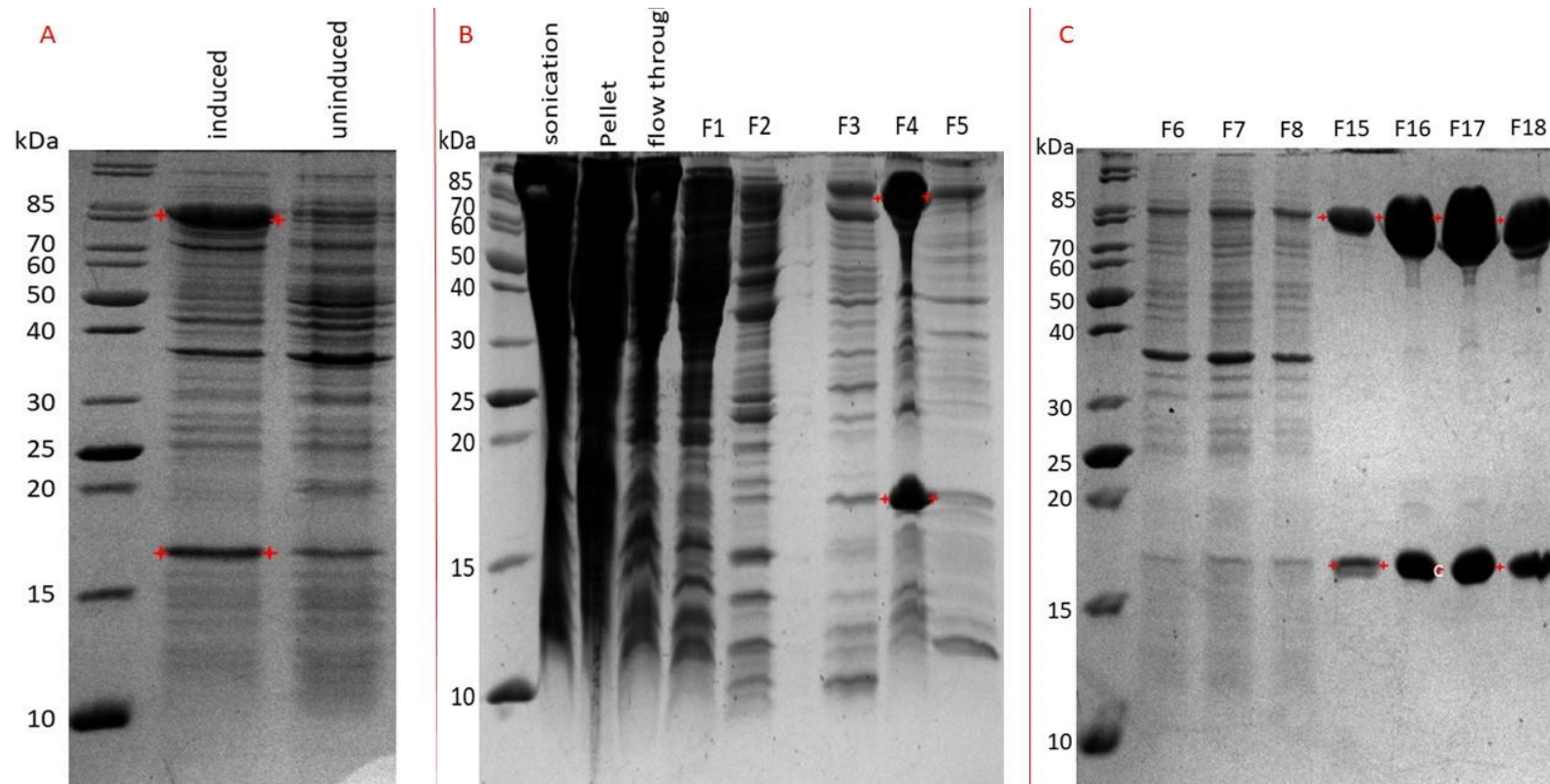


Figure 5.4: Expression and purification of PyoS3-immS3. A: SDS-PAGE of overexpression of PyoS3-immS3 indicated the expected protein sizes of 81 kDa for the large protein (PyoS3) and 17 kDa for the small protein (immS3; red asterisks). B: SDS-PAGE of affinity chromatography purification of PyoS3-immS3. A protein of the expected size is shown in fraction F4 with red asterisks showing the proteins of interest. C: SDS-PAGE analysis of further SEC purification of PyoS3 and immS3; F17 contained the highest concentrations of the proteins of interest.

5.2.2 Crystals formed in the crystallization screens

5.2.2.1 Initial crystal growth and inspection

Dr Siyu Wu at the University of Nottingham successfully crystallised the PyoS3 DNase-Immunity complex (unpublished data). The crystals formed in the presence of ZnCl_2 ; thus, in these experiments, zinc was included in the screens in the hope to form crystals of full-length PyoS3 protein. The 96-well crystallisation plates (SWISSCI, UK) allow two drops of liquid to be incubated against a single reservoir: one drop contains the protein sample and the screening solution, and the other drop contains the sample buffer without protein and the screening solution (control). The screens used in this experiment are described in section 2.30. After incubation in the 96-well plate at 20 °C for 2 weeks, visual inspection revealed that crystals formed in the Ligand Friendly Screen (LFS) A12, B12 and C12 wells, and PACT Premier C12 well. The crystals appeared to exhibit a geometric square-like shape (Figure 5.5).

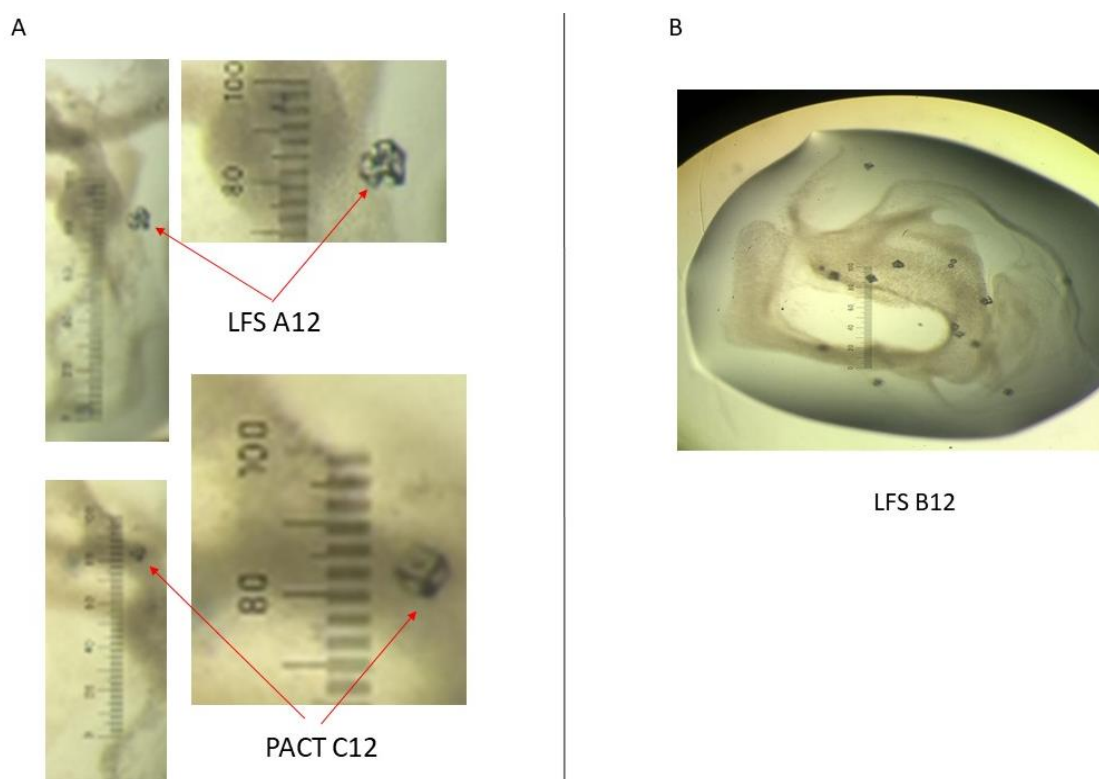


Figure 5.5: Preliminary inspection of the seven sparse matrix screens in which crystals formed. Four drops across two separate screens contained potential hits. Furthermore, the corresponding control drops (containing sample buffer, crystallisation mixture and no protein sample) did not contain crystals. The three crystal-containing drops formed similarly shaped crystals. Two more geometric crystals are shown in panel A: Ligand Friendly Screen (LFS) well A12 and PACT Premier (PACT) well C12. B: The entire well of LFS B12 is shown to indicate the crystal abundance and size.

5.2.2.2 UV microscopy detected protein crystals

The PyoS3-immS3 crystals were observed using a UV-Microscope JANSI UVEX p imaging system (SWISSCI, UK), with the help of crystallographer Dr Thomas Warwick, University of Nottingham. The crystals had a darker-than-background contrast, which indicated the characteristic UV absorption of protein crystals (Figure 5.6) (Daloglu et al., 2019). Moreover, the lack of crystal formation in the control drops suggested that the crystals were not ZnCl_2 .

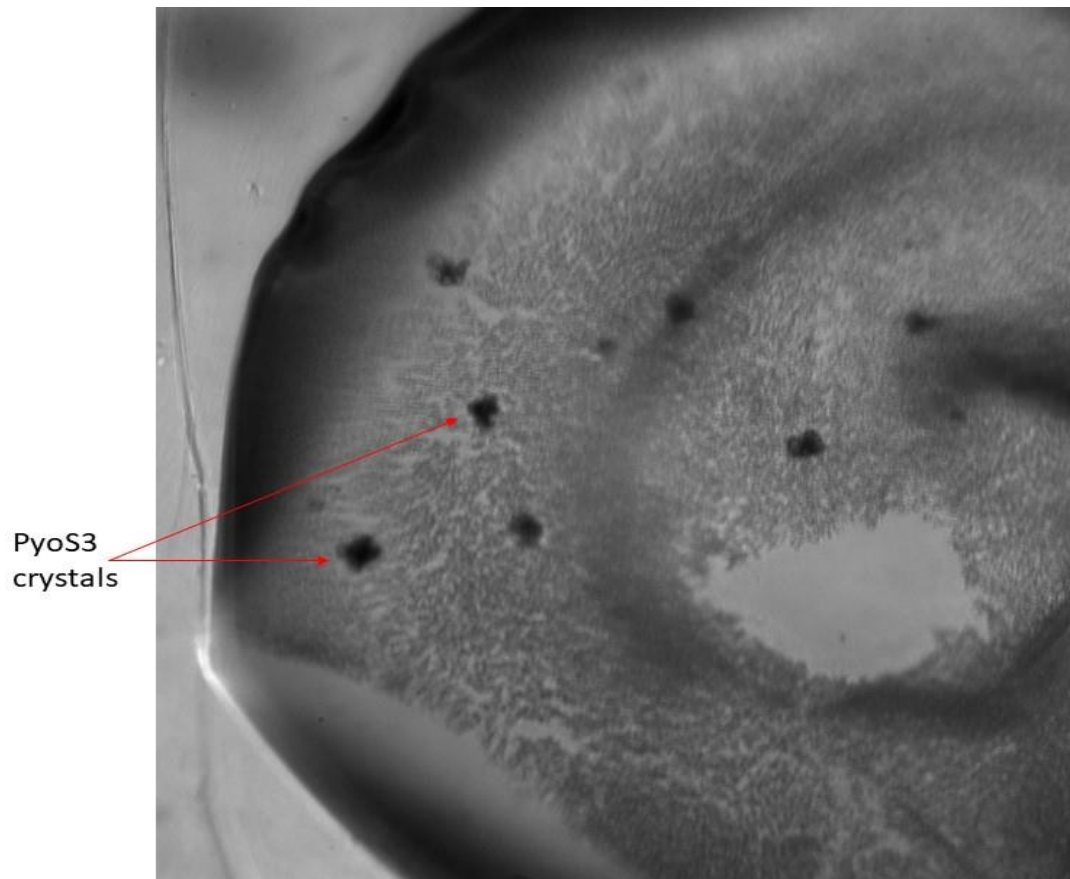


Figure 5.6: UV microscopy imaging to detect the UV absorption of potential PyoS3-immS3 protein crystals. The potential PyoS3-immS3 crystals appeared darker than the background, which correlates with UV absorption and indicated the crystals might be protein crystals.

5.2.3 X-diffraction analysis of PyoS3-immS3 crystals

The most promising PyoS3-immS3 crystals with potential for diffraction analysis formed in LFC well C12. This crystal was then crushed using an acupuncture needle and seeded with horsehair on top of a needle under conditions suitable for crystal formation by Dr. Thomas Warwick, University of Nottingham. Then, the crystal was cryo-protected using the same conditions as the LFC, with the addition of 30% v/v glycerol to prevent water crystals from forming when freezing the protein crystal. This best-grown crystal was frozen in liquid nitrogen and sent to the Synchrotron, Oxford, for X-ray diffraction analysis. Unfortunately, the diffraction patterns showed that the PyoS3 crystal was a salt crystal, not a protein. Protein crystals contain thousands of unique atoms; salt and chemical crystals contain fewer atoms and thus their chemical diffraction patterns typically have fewer spots than protein diffraction patterns (McPherson and Gavira, 2014). The data from the diffraction experiment is shown in Figure 5.7. Figure 5.8 shows the measurements of the crystal revealed three 90° angles and three equal sides, which correlates to a cubic-shaped crystal. A search of the Cambridge structural database for the experimental chemical structure of these diffraction measurements resulted in a hit with deposition number 1142518, identifier = DMAZCO (Figure 5.9), which is a false-positive imidazole crystal.

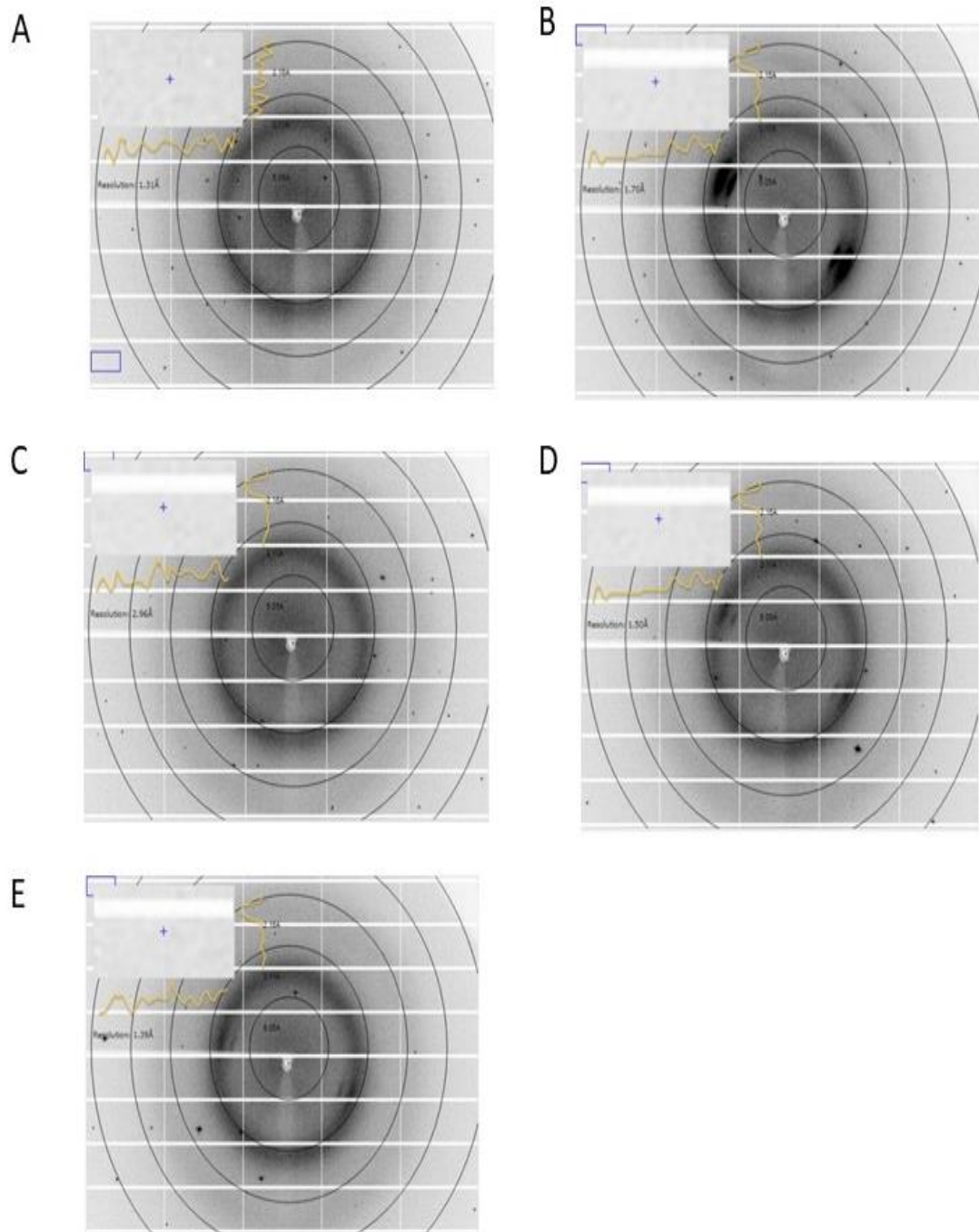


Figure 5.7: The five diffraction images corresponding to five unique crystal orientations.
A, B, C, D and E contain small numbers of diffraction spots, which indicates a salt crystal

Space Group	A	B	C	α	β	γ
P23	8.79	8.79	8.79	90.00	90.00	90.00

Figure 5.8: Crystal diffraction measurements. A, B and C are in Å and correspond to cell lengths, α , β and γ correspond to the angles between the cell edges and are in °. The three 90° angles and three equal sides describe a cube, which matches the morphology of the crystals.

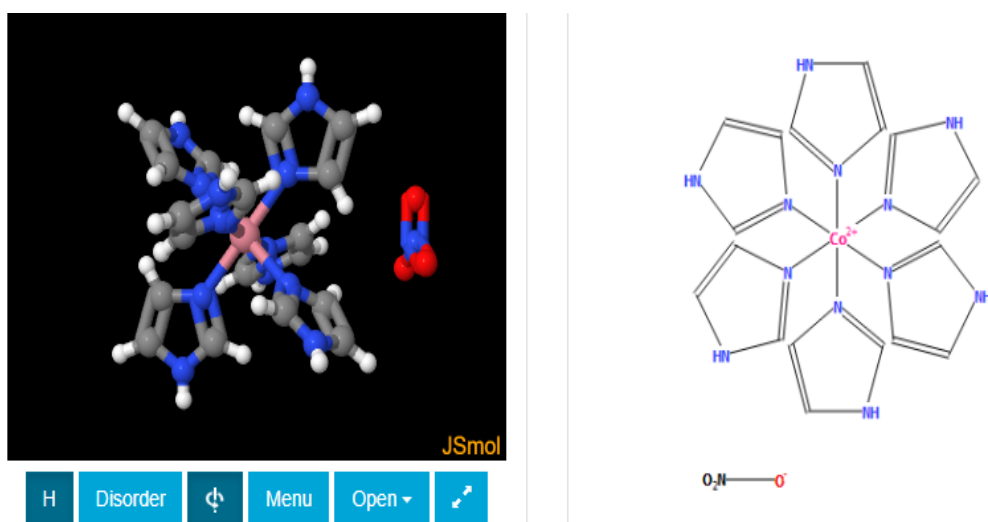


Figure 5.9: Search of the Cambridge structural database for experimental chemical structures revealed the crystal assessed in this study was CO₂ octahedrally coordinated to imidazole rings. A: The hit was Deposition number = 1142518, identifier = DMAZCO. B: Structure of CO₂ octahedrally coordinated to imidazole rings.

5.2.4 ZnCl₂ screens that generated crystals

The screens containing ZnCl₂ were performed based on the findings of Dr Siyu Wu, who successfully solved the structure of PyoS3 DNase-immunity crystals that grew in ZnCl₂ screens. However, the crystal that formed in this work was an imidazole salt. Thus, to minimise false positive results, follow-on screens were performed using only ZnCl₂ as a source of salt at different pH values with PEG as the precipitant (see section 2.30) to try to form PyoS3-immS3 crystals. Furthermore, pure PyoS3-immS3 was dropped on the screening solutions, which were incubated at 20 °C and regularly examined under a microscope to observe if crystals were growing. Excitingly, different irregular-shaped crystals were observed (Figure 5.10). However, due to Covid-19-related laboratory restrictions and the limited time in the laboratory, confirmation of these crystals by UV microscopy and X-ray diffraction could not be conducted during this study.

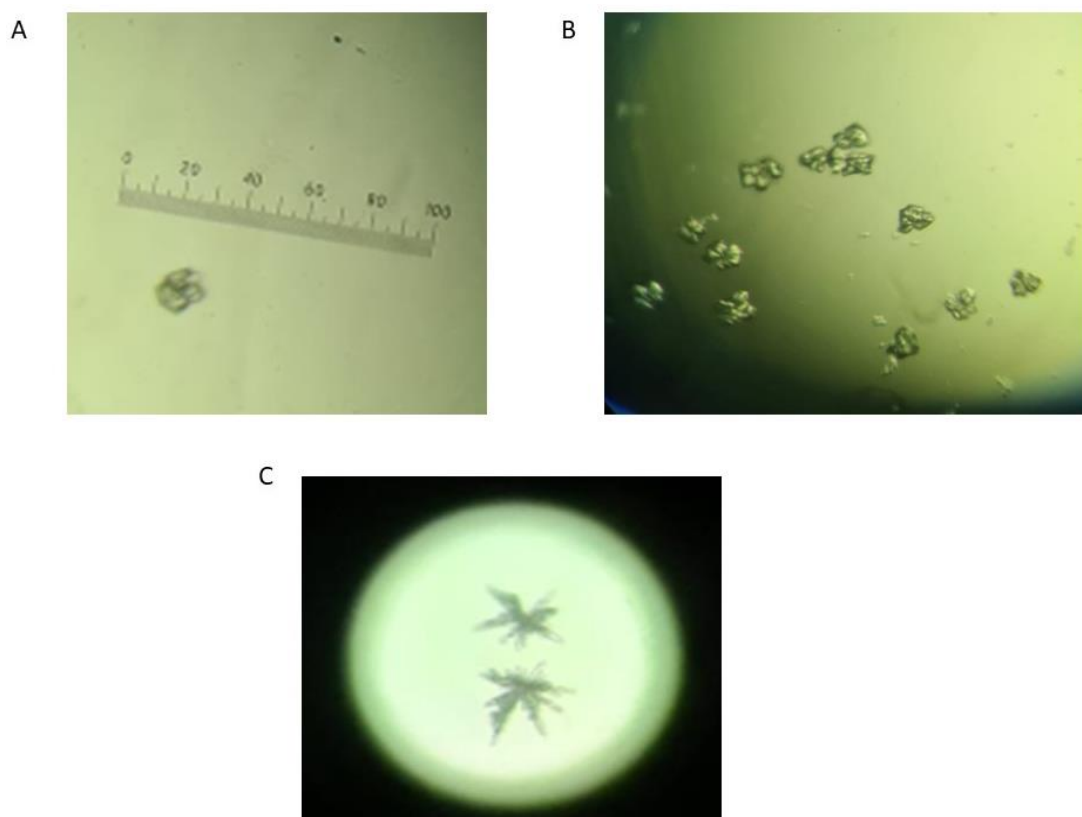


Figure 5.10: Inspection of crystals that grew in follow-on ZnCl₂ screens of purified PyoS3-immS3. Control drops (containing sample buffer, crystallisation mixture and no protein

sample) do not contain crystals. A. A crystal growing in ZnCl_2 at pH 6.5. B: Crystals growing in ZnCl_2 at pH 7.0. C: Crystals growing in ZnCl_2 at pH 8.5.

5.3 Discussion and Conclusion

The aim of this chapter was to crystallize full-length PyoS3 protein. Full-length PyoS3 was purified, then subjected to buffer exchange dialysis to change the buffer to Tris-base to remove other salts that may lead to formation of false positive crystals. After buffer exchange, the sitting drop vapour-diffusion approach was used to grow crystals and the chemical screens were performed based on Dr Siyu Wu's finding that the PyoS3 DNase-Immunity complex crystals grew in the presence of ZnCl_2 . The crystals had a unique cubic form and additionally, the absence of any crystals in the control drop indicated that the crystals could be a protein crystal. One suitable crystal was sent to the Synchrotron, Oxford, UK, for X-ray diffraction analysis; however, the diffraction data indicated the crystal was not a protein crystal.

It is important to distinguish protein crystals from salt crystals in protein crystallography. Generally, in contrast to salt crystals, protein crystals absorb UV and emit fluorescence when stimulated. As a result of strong UV absorption, protein crystals appear significantly darker than the background when viewed under the microscope. Proteins absorb UV due to the presence of tryptophan residues, which absorb UV and emit fluorescence (Daloglu et al., 2019). The UV microscope analysis showed that the crystals were distinctly darker than the background, which indicated the crystals were potentially protein crystals.

In X-ray diffraction, each image is a snapshot of an interference pattern caused by the scattering of X-rays by the atoms. Therefore, the number of spots in a diffraction frame corresponds to the number of atoms in the symmetrically unique portion of the crystal. Proteins contain more unique atoms than salts, thus chemical crystals create fewer diffraction spots than protein crystals (McPherson and Gavira, 2014). However, the crystal assessed in this study showed few diffraction spots, which means that the crystal is a salt crystal.

The measurements of the crystal were analysed in the Cambridge structural database, which revealed an imidazole structure. This result indicates that

imidazole was still present in the PyoS3-immS3 protein solution after the affinity chromatography purification step, since imidazole was used in that step and the histidine tag has high affinity for imidazole. This may explain the presence of imidazole, as the PyoS3-immS3 protein complex is histidine-tagged. Furthermore, this finding may also explain the UV absorption of the crystal in UV microscopy as imidazole is aromatic and may exhibit some UV fluorescence and absorption (Gopalakrishnan et al., 2021).

In conclusion, a false positive crystal was detected, even though a series of validation experiments were conducted. In future work, it is recommended to avoid inclusion of any chemicals that might hinder the crystallography experiment. Cleavage of the Histidine-tag from PyoS3 may help to obtain the protein in its native form when conducting crystallography, and may also minimise the presence of unwanted chemicals that the histidine tag has a high affinity for, such as imidazole (Hefti et al., 2001). A follow-on set of screening buffers only contained ZnCl_2 at different pH values and led to formation of some irregular crystals that are worth investigation, but further experiments could not be conducted due to Covid-19 restrictions at the time. In addition, different screening buffers should be tested, and more crystals should be examined in the hope of solving the crystal structure of full length of PyoS3.

6 General Discussion

Several factors contribute to the emergence of pathogens that are resistant to single antibiotics and, subsequently, to multiple antibiotics; these factors include the use of multiple broad-spectrum agents, globalization, and excessive use of antibiotics both in clinical settings and agriculture. In addition to spreading resistance across bacterial species, broad-spectrum antibiotics can also adversely affect the host microbiome. Therefore, common infectious diseases have become more difficult to treat, resulting in prolonged illnesses with higher mortality and morbidity rates (Weist and Högberg, 2014, Melander et al., 2018). To combat this issue, alternative methods for combating antibiotic-resistant pathogens must be developed as soon as possible. Among the alternative methods that have been investigated, antimicrobial peptides from a variety of sources, including bacteriocins, offer a promising strategy (Gradisteanu Pircalabioru et al., 2021). Bacteriocins have potential for use in the pharmaceutical and food industries instead of traditional antibiotics; one of their most important capabilities is their activity against biofilm-forming organisms (Mathur et al., 2018, Chandrakasan et al., 2019). The emergence of antibiotic resistance is associated with the formation of biofilms and biofilm-producing *P. aeruginosa* are often detected in patients with chronic infections, such as lung infections, wound infections, and rhinosinusitis (Duraismy et al., 2020, Thi et al., 2020). (Mojesky, 2021).

Furthermore, the combination of antimicrobials with bacteriocins may hold immense value. This strategy would reduce the risk of development of antimicrobial resistance in bacteria, since two different mechanisms of antimicrobial action are involved (Mathur et al., 2017). Therefore, the identification and characterisation of bacteriocins are important to enable the development of these alternative therapeutic options, since the effectiveness of traditional therapeutic options continues to become increasingly ineffective (Meade et al., 2020, Soltani et al., 2021). In addition, bacteriocins are produced by almost all bacterial species, such as the pyocins of *P. aeruginosa*, and target closely related species of bacteria, meaning they function as narrow-spectrum antibiotics (Mojesky, 2021). Narrow-spectrum antibiotics that do not promote cross-resistance in non-target pathogens and limit collateral damage to the host

microbiome may provide useful alternative therapeutic strategies in the fight against multidrug-resistant (MDR) infections (Melander et al., 2018).

This study aimed to establish if PyoS3 utilises TonB1 or TolAIII for translocation into the cells it targets and kills. This objective builds on the hypothesis that PyoS3 utilises FpvAII for translocation into prey bacteria (Baysse et al., 1999). FpvAII uses the TonB system for the import of pyoverdine II. TolAIII was included in this study as structural and functional similarity exists between pyocin S3 and colicin E9, a Group A colicin that uses the Tol system (Parret and De Mot, 2002, Cascales et al., 2007b). In addition, in an attempt to identify other important interaction partners of PyoS3, a transposon library was generated from a PyoS3-sensitive *P. aeruginosa* strain. Finally, to enable future fine-tuning of these interactions, full-length PyoS3 with its cognate immunity protein was purified with the aim of generating its crystal structure in order to shed light on the domain arrangement and structure of PyoS3.

6.1 TonB1 interacts with PyoS3

In Chapter 3, the interaction between PyoS3 and TonB1 was established via analytical size exclusion chromatography (SEC). First, the regions encoding the receptor (R) and translocation (T) domains, along with the cognate immunity protein, were cloned from the full-length PyoS3 encoding gene in the pPYSS-3 plasmid. TonB1 and TolAIII were also cloned, expressed, and purified. All of the proteins were concentrated for interaction studies; however, TonB1 aggregated when concentrated to more than 4.5 μ M. Thus, each protein was concentrated to 4.5 μ M then analytical SEC was employed for the interaction studies, as analytical SEC does not require high protein concentrations. A complex formed between PyoS3 R+T and TonB1 under one of the tested incubation conditions, which indicates that these proteins possibly interact (Figure 3.18). No interaction was observed between PyoS3 R+T and TolAIII, which indicates these proteins do not interact (Figure 3.19). This finding agrees with Baysse et al. 1999, who demonstrated that PyoS3 uses the Type II Pyoverdine (PvDII) receptor FpvAII as a receptor, which like other pyoverdine receptors such as FpvAI utilise TonB1 for translocation (Baysse et al., 1999, White et al., 2017). White et al. 2017 demonstrated that Pyocin S2 binds to TonB1, which agrees with findings of this study since both Pyocin S2 and

PyoS3 use the pyoverdine receptors FpvAI and FpvAII, respectively and both of them are TonB-dependent transporters (White et al., 2017). ITC, a more sensitive technique for analysis of protein-protein interactions (Baranauskiene et al., 2019, Recht et al., 2016) was used to further confirm this finding. Since the concentration of TonB1 protein was low and TonB1 started to aggregate as soon as it was injected into the ITC machine, the results of the current study were inconclusive (Figure 3.20). This might be due to the fact the concentration of TonB1 was too low or that the protein was insufficiently soluble when stirred at the temperatures tested in these experiments (Baranauskiene et al., 2019, Pierce et al., 1999). Pure TonB1 protein was dialysed against several buffers and conditions to find a suitable buffer that would stabilize the protein to repeat the ITC experiments. Only one buffer allowed TonB1 protein to remain stable when concentrated (50 mM Na₂H₂PO₄, 150 mM NaCl + 5% glycerol + 1 mM DTT, pH 8). Unfortunately, due to COVID-19 restrictions and the inaccessibility of the ITC machine, further experiments could not be conducted. The proteins have been stored at -80 °C for future analysis.

6.2 PvdM aids PyoS3-mediated killing of prey bacteria

Chapter 4 aimed to generate a transposon library from the *P. aeruginosa* 892 PyoS3-sensitive clinical strain. This approach enabled the generation of unbiased mutations (Sargison and Fitzgerald, 2021) in the genomic DNA of *P. aeruginosa* to identify genes involved in the uptake of PyoS3. Moreover, full-length PyoS3 with its cognate immunity protein was cloned from the pPYSS-3 plasmid for high-throughput sensitivity screening of the generated mutants. Active PyoS3 was successfully purified and behaved similarly to the previous descriptions by (Duport et al., 1995). Two *P. aeruginosa* clinical strains in our collection were found to be sensitive to PyoS3; however, only PSA 892 was used to generate a transposon library as conjugation of the transposon into PSA 971 was not successful. Following a screen of 4500 Tn5 mutants, one mutant demonstrated reproducible resistance to PyoS3 (mutant 107). Four other mutants were isolated as they exhibited growth inhibition zones when exposed to PyoS3 (mutants 44, 362, 422, 477).

Mutant 44 showed a smaller PyoS3 growth inhibition zone than the positive control PSA 892 strain and its transposon insertion was in the *VqsR* gene,

which encodes a LuxR family transcriptional regulator. Mutant 362 showed a larger inhibition zone than the positive control PSA 892 strain and its transposon insertion was in the gene encoding SDR family oxidoreductase. Both of these genes regulate quorum sensing (QS) in *P. aeruginosa* (Juhas et al., 2004, Liang et al., 2012, Tsai and Winans, 2010, Bijtenhoorn et al., 2011). Direct relationships between these proteins and PyoS3 have not yet been reported, thus further investigation is necessary to establish the links between VqsR and SDR and the uptake of PyoS3. The two other mutants (422 and 477) had transposon insertions in the surface lipoprotein (SLP) gene and carboxylic ester hydrolase gene, respectively. Both of these mutants exhibited the same size of growth inhibition zone as the positive control PSA 892 strain in a follow-up PyoS3 sensitivity screen; thus, no further analysis was conducted on these mutants as they did not affect the uptake of PyoS3. The mutant that was completely resistant to PyoS3 (107) had a transposon insertion in the gene encoding PvdM, which is a pyoverdine biosynthesis enzyme. PvdM homologs localise in the periplasm and have been linked to the TonB-dependent uptake system. PvdM homologs are also functionally involved in the uptake of compounds such as PVDs or pyochelin (Sugue et al., 2022). This might explain why the PvdM mutation halted the uptake of PyoS3, since PyoS3 utilises the TonB-dependent uptake system (FpvAII).

6.3 Progress towards solving the crystal structure of PyoS3

Dr Siyu Wu recently successfully crystallised the PyoS3 DNase domain in complex with its cognate immunity protein in our laboratory. The crystals were grown in the presence of ZnCl_2 . Thus, in this study, screens containing ZnCl_2 were performed in an attempt to crystallize full-length PyoS3-immS3. After confirming that the cubic-shaped crystals obtained (Figure 5.5) could be protein crystals via UV-microscopy (Figure 5.6), the crystals were subjected to X-ray diffraction analysis. However, the crystals were actually salt crystals. The measurements of the diffracted atoms were analysed via the Cambridge Structural Database and the crystals were identified as imidazole (Figure 5.9). This finding might explain why the UV microscopy confirmation generated a false positive result as imidazole strongly absorbs UV absorption, similarly to protein crystals (Gopalakrishnan et al., 2021).

6.4 Potential future work

The analytical SEC conducted in Chapter 3 provided evidence that PyoS3 binds to TonB1 protein. However, a more sensitive technique, such as ITC, needs to be used to confirm these findings. The dialysis buffer for TonB1 has already been optimized by finding a suitable buffer that TonB1 does not aggregate in. The protein can now be concentrated for ITC experiments. It would be interesting to extend this analysis by including FpVAI, FpvAII, and FpvAIII, as well as PvdM. In addition, variations could be incorporated in PyoS3 by replacing specific amino acids or altering the domains by genetic engineering.

Further transposon mutants of *PSA 892* that are resistant to PyoS3 should be generated to identify other genes involved in the uptake of PyoS3. It would be useful to saturate the genome with transposons; the 4500 mutants generated in this study do not include mutants in all genes as the *P. aeruginosa* genome contains > 5000 genes (Poulsen et al., 2019). However, it should be noted that this approach will not be able to detect genes that are essential for the survival of *P. aeruginosa*.

In addition, mutation of the VqsR gene and DR family oxidoreductase gene decreased and increased, respectively, the uptake of PyoS3 into *PSA 892*; these genes have previously been found to regulate QS. Thus, it would be interesting to measure QS signaling molecules (QSSM) in the transposon mutants to confirm the link between the mutations in these genes and QS.

Ongoing work in the laboratory to solve the crystal structure of PyoS3 could employ different commercial screens as well as ZnCl₂ screens, as described in Chapter 5. Ultimately, it would be interesting to generate the crystal structures of the complexes formed by PyoS3 R+T and TonB1 plus PyoS3 R+T and FpvAII to exactly determine how PyoS3 binds to the cell surface and translocates into cells, as previously determined for Pyocin S2 (White et al., 2017).

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Appendices:

Appendix 1

DNA Sequences of proteins used in this study.

PyoS3 R+T:

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CCAGGTCCCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
ATGGCTGATGCACCACCGCCACTGATTGTTAGATACTCAAGTCGCCCCG
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TACTAAGCAACTATCCGTTCCGTAACCCTTTCCGTGGTTTTACTCTGGCC
ATGGAGGCTGACTTCAAAAAGAGGAAAGAGAGTCTGGAAGCCGACGTTC
AGGCAGAGTTGGATACGGAAGCTGCCAAGTATCCGGCACTACCTGCCA
GCTTCACACCTGAGCAGTGGCTGGAAAGATCAACCGTAGTTGTCACCGG
GCTGCTAAATCGAAAGCGCCCTGAACTAGATAACGAATTGGATGTGGTT
CAGAACTCGGCTAGTCTGGATAAGCTGCAAGCAAGCTACAACACAATCA
TCCTGAATGATCAGATCAATAGTCTTCAAGAGCGCTTGAACAAGCTCAAT
GCTGAAACCGACCGCCGCCGGGCGGAGGCAGAAGCCCAGCGTCAGGC
AGAAGCGGACGCCAAACGCAAAGCGGAAGAAGCGGCACGCCTGGAAGC
CCAGCAGGTCACTCAACAGCTCGGTGCCGACTTCAACGCGATCACCCT
CCGACCGCCACCAAGGTCCAGCAACGTGTGAAAGCAGTGGATGCTAGT
CTGTCTCAGGTTTCCACCCAGGTCAGTGGTGCAGTCGCCTCGGCAACCC
AGGCGGTGCAGGTCAAGACAGCGCAAGCCCAGCAACAAGCCAACAGCC
AGATTTTCGTCCAGCCAGAACCTGATCAGCGCGGCATTTTCGGAATCAGAT
TGCCTTGGCAGCACAGGCCAGTGGAGAAGCCCAGGTCACGGCTTTCAA
CACCAAAGTCAAGCAGATCGTCAACGAAGCCACGGCGTTTTTCGACAGCA
CGAAAACAAGCGCTGGCTCAGTTTGCTGCCAATGCGGGCCGCCGAGGTC
GAAACCGAAGGTCAACAGGCTAACCGCCCAGGTCAAGGCCAGCCCCAC
CAAGGCGGCTTCGCAAGCTGCACAAGCAACCCTTCGACAATTCCCTGAA
AGCACCTAGGCCAGGTGAAACCCTTTGCCCGNAGAACGAAACAAGGCT
GCAGGCCAAAGCCAAGGAGGTTTCGGCACCGGTAACCAAAGGCCAAGG
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AAAAATTTTGGGGGGGAANNNTGGGGGGGGGGGGCCCCCTCCCCCCC
GGGTTTGGGGGCGGGTTTTTAAAATCGCCAA
```

TonB1:

NNNNNNNNNNNNNTCTAGANTNTTTNGTTTAACTTTAAGAAGGAGATAT
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GCCGGAACCGACGCCGCCGAAGATCGAGGAACCCAAGCCGGAGCCGC
CGAAGCCGAAGCCGGTGGAAAAACCCAAGCCCAAGCCGAAACCCAAGC
CCAAACCGGTGGAAAACGCCATTCCCAAGGCCAAGCCGAAGCCGGAAC
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CCAGCCCGTCCAGCGCCGCGCCGCCGCCGCCGCGCCTACCGTCGGGCAG
AGCACGCCCCGGCGCGCAGACTGCGCCCAGCGGCTCGTAAGGCCCGGC
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CGACATCCAGGTGCTGGAGTCGGTGCCGTCGCGCATGTTGACCGCGA
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GCTGCTAACAAAGCCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCT
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CCCTGTAGCGGCGCATTAAAGCGCGGCGGGTGTGGTGGTTACGCGCAGC
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TCCCTTCCTTTCTCGCCACGTTGCGCGGCTTTCCCCGTCAAGCTCTAAAT
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GACTCTTGTTCCAACTGGAACAACACTCAACCCTATCTCGGTCTATTCT
TTTGATTTATAAGGGATTTTGCCGATTTGCGCCTATTGGTTAAAAAATGAG
CTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGCTTACA
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NAANTNNTNCNNNNNNNTTNNAANGGNAANGTTTGNNTTTNCAACTTTN

CCGNNNCCCCNTTATTCCCTTTTTTGGGGNATTTTGCCTTCCGGTTTTNN
NNCCCCNAAAAACCNTGGGNAAANTAAAAANNCTTAAANNNATTTGG
GNNNCCNAANTGGNTTNNNTNCNG

TolAIII:

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GCCGACGAGGTGGGCAGCGAGGTCACCGGCAGTCTCGACGACCTGATC
GTCAACCTGGTGAGCCAGCAGTGGCGGCGTCCTCCATCGGCGCGTAAT
GGAATGAGCGTAGAAGTACTGATCGAAATGCTGCCGGACGGTACCATCA
CCAATGCCAGCGTCAGCCGTTTCGAGTGGCGACAAGCCTTTCGACAGTTC
GGCGGTGGCGGCGGTGCGCAACGTCGGCCGTATTCCCGAGATGCAGC
AATTGCCGCGGGCTACCTTCGACAGCCTGTATCGTCAGCGCCGCATCAT
CTTTAAACCGGAGGATTTGAGTCTGTGA

PyoS3-immS3:

TATGGCTGATGCACCACCGCCACTGATTGTTAGATACTCAAGTCGCCCC
GGGAAACCTGGCGGAGTTAGCATAGACGTATCACGAGACAGGCAGGAC
ATACTAAGCAACTATCCGTTTCGGTAACCCTTTCCGTGGTTTTACTCTGGC
CATGGAGGCTGACTTCAAAAAGAGGAAAGAGAGTCTGGAAGCCGACGTT
CAGGCAGAGTTGGATACGGAAGCTGCCAAGTATCCGGCACTACCTGCCA
GCTTCACACCTGAGCAGTGGCTGGAAAGATCAACCGTAGTTGTCACCGG
GCTGCTAAATCGAAAGCGCCCTGAACTAGATAACGAATTGGATGTGGTT
CAGAACTCGGCTAGTCTGGATAAGCTGCAAGCAAGCTACAACACAATCA
TCCTGAATGATCAGATCAATAGTCTTCAAGAGCGCTTGAACAAGCTCAAT
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AGAAGCGGACGCCAAACGCAAAGCGGAAGAAGCGGCACGCCTGGAAGC
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 CGAAAACAAGCGCTGGCTCAGTTTGCTGCCAATGCGGCCGCCGAGGTC
 GAAACCGAGGTCAACAGGCTAACCGCCCAGGTCAAGGCCAGCCCCACC
 AAGGCGGCTTCGCAAGCTGCACAAGCAACCCTTCGACAATTCACTGAAA
 GCACCTATGCCAAGGTGAACACCTTTGCCCTGCAGACCGAAGCAGCGCT
 GCAGGCCAAAGCCAAGGAGGTTTCGGCAGCGGTAGCCGAGGCACAGC
 GTCGGCGACCAACGATTTGCAAAAAACAGCCGATGTAGTACCAGGTGAG
 ATCATTGAGGCTGCGAACACCTTGCGGATGCCGGCCACCTCTACGCCGC
 TGGTTTCGGTGGCCGGGTTTGGTAGTGCGGCAGTGGAGACGGCTCGCC
 TGGCGGCTTCCCTAACGAATGCTGTCAATCGCTTGATTGAGATTGCTGGT
 AGTGGACCTGGCGCATACGTGGCCACCTTCGCGGTGCTATCGCTCTACT
 CCGACCAGGCTGGTAAAGACAGTGACAAGGTTCTGCGGGCGTTTCGCA
 ATGCATTGGCACTGGAGGCGTCCGCTCTGGGGCTTCCTGGCACGGCTG
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 GACTCACGAGTGCTGCACAAGAGAGCCCGAGTGGTAAATCGCAGATTGC
 CGCGATGCTGACCAACGGGGCAACTGTCCCCAAAGGCGTGCCTGTTCC
 GCGCCGGACCCTCAATACTGCGACAGGCCGGTATGAGGTGACGGTTCC
 CGCAAAGTCCACCGTGCCGAATACACCCCGCTGATCTTGACCTGGACC
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 GTACCGCAGCCGGCTCCGGTGTATGAGGGAGCAACGATTACCCAGTG
 CAGGCCGAACCGGAGTCCTATCCGGGAGTGCCGTTAGATCTGGACGAT
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 GCTAGAGTGCATCAGAGGTTGAGTGAACCTACGGAGGACGTAACACCA
 AAGAAAAGCAACAGAAGGATGCCTCCG-
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 GCTAGAGGAGGGGTTCTCCGAAGATGATATTGAGAGGGCGCGCGCAGA
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 TGATCGTTAAATTAATTGATAGCATCGGAAAATCCCATGAGGAGATTGTT
 GGTGCTGGATTGGTTAAGTCTGGAAAACCTGGAGAGCCCCTACGAACATT

CTGAACACCTAACGCTCTCTCCAGAAAAAGGACTTGAACTAACTTCCGA
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GAGGGTTCTGCTGCTTACTCAGGGGAACTGCCTGAACCTCTAGTTAGAA
AAATGAGTAAAGAGCAAGTTCGTTCAGTGATGGGGAAACCATTTGAGAC
GAGAGAGTCTTTCAGAGTTCCTGTGATTGGAATTCTTGGCGGCTGGGAC
TACTTTATGCACCCCCAGAAAGCCAATTTACGTATTGGGTTCACATACAC
ATCTGACTCGAATGTGTGCAATTTAACTTTCGCTCTTCTTGGTTCTAAA

Appendix 2

Analytical SEC to test the interaction of PyoS3 R+T with TonB1 and it showed a negative result in this experiment (Figure 0.1). The difference between this negative result and the positive result is that in this experiment the ratio between PyoS3 R+T/ TonB1 mixture was not exactly 1:1 as to the positive result in section 3.2.6 which was exactly 1:1. The incubation time for the complex that shown in the in the positive result in section 3.2.6 was different, it was 30-45 min room temperature. The incubation time in this experiment which was ((i) on ice for 1 hour, (ii) at room temperature for 30 min and (iii) at 37°C for 5 min).

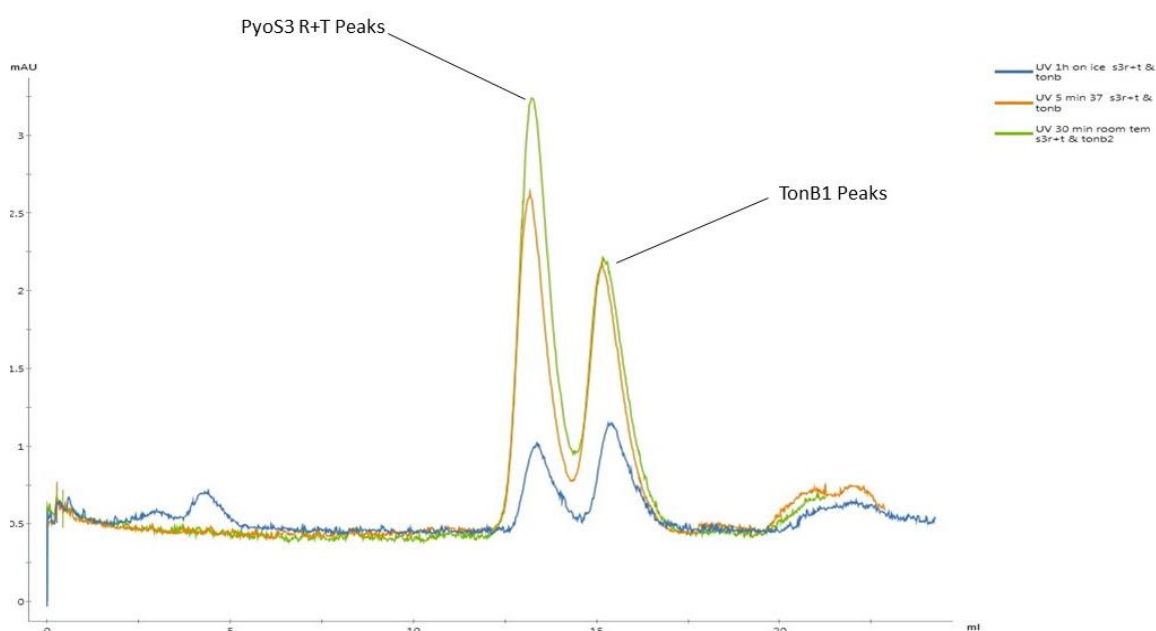


Figure 0.1: Analytical gel filtration of possible complex formation between PyoS3 R+T domain and TonB1. Superdex 75 Increase 5/150 GL column in 125mM NaH₂PO₄ + 300 mM NaCl pH 7 via Akta pure system was used. Y-axis showed the absorbance of the UV (mAu), X-axis shows the volume (ml). the blue, orange and green lines indicate the UV absorbance of the proteins. The first peaks to come into the graph were the PyoS3 R+T domain peaks and the second peaks were the TonB1 UV peaks. These separate peaks showed that there was no interaction between the two proteins otherwise it would appear as one peak.

Appendix 3

List of buffers used for TonB1 stability shown in Table 0.1. All the buffers used for dialysis has 50 mM $\text{Na}_2\text{H}_2\text{PO}_4$ and 150 mM NaCl since PyoS3 R+T is stable in this buffer. The changes were in how much glycerol and pH of the buffer. in addition, 1 mM DTT was added as reducing agent to help stabilise the protein in all the buffers used in dialysis.

Table 0.1: list buffers used for TonB1 buffer exchange dialysis. 50 mM $\text{Na}_2\text{H}_2\text{PO}_4$ and 150 mM NaCl plus 1 mM DTT were present in every buffer only the pH and the glycerol concentration were changed.

pH									
Glycerol %		3	4	5	6	7	8	9	10
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	+	-	-
	6	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-

Appendix 4

Transposon PCR to identify the transposon insertion in *PSA 892* PyoS3-sensitive strain trans-conjugate mutants was conducted, however as stated in section 4.2.10 this experiment did not yield result. The PCR experiment was repeated couple of times and two examples is shown in Figure 0.2 and Figure 0.3. In addition, as stated in section 4.2.10 the material was limited and due to Covid-19 restrictions and there was delays in orders, for that reason another approach was used to identify the insertion in *PSA 892* mutants and it is described in section 2.27.3.

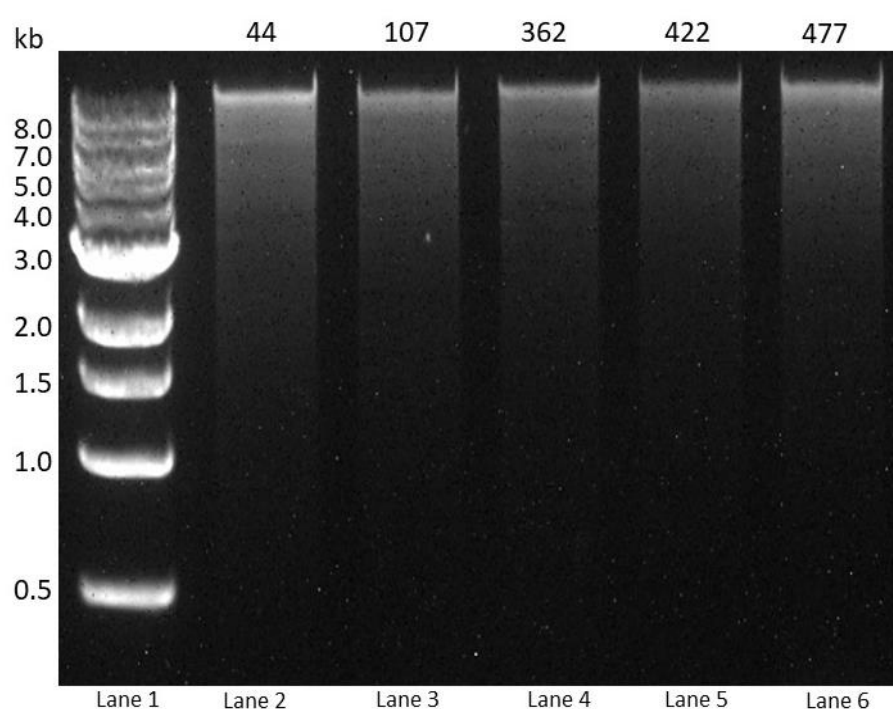


Figure 0.2: PCR run to identify the transposon insertion in *PSA 892* mutants. The first lane shows 1 kbp ladder. The second- sixth lane are mutants 44, 107, 362, 422 and 477 receptively and all of them shows smear that does not yield amplicon formed to sequence it.

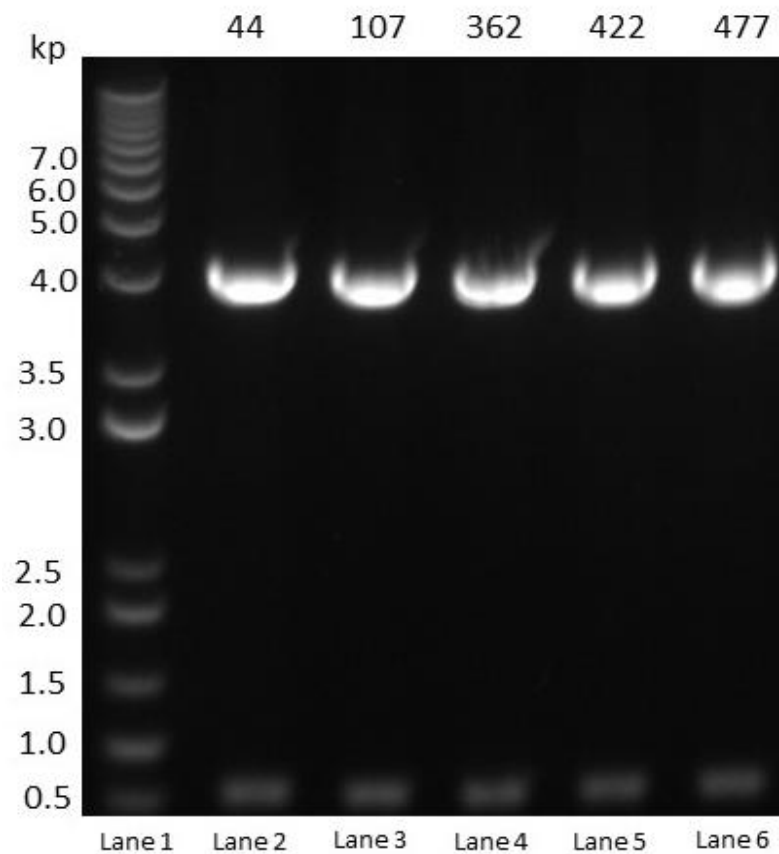


Figure 0.3: PCR run to identify the transposon insertion in *PSA 892* mutants. The first lane shows 1 kbp ladder. The second- sixth lane are mutants 44, 107, 362, 422 and 477 receptively and all of them shows primer dimers that does not yield amplicon formed to sequence it.

Appendix 5

BLAST PATRIC was used to determine the Pvd receptor that PSA 892 and PSA 971 contain (Davis et al., 2020). FpvAll which based on literature is utilised by PyoS3 as Pvd type 2 receptor and PyoS3 receptor (Baysse et al., 1999). There are two FpvAll subclusters, FpvAlla and FpvAllb (Bodilis et al., 2009). The BLAST PATRIC analysis showed that FpvAlla subcluster is present in PSA 971 and FpvAllb subcluster is present in PSA 892 with both having 100 % identity and c query cover (Figure 0.4).

A

Genome	Accession	Product	Identity (%)	Query cover (%)	Query Length	Sub Length	Score	E value
Pseudomonas aeruginosa PSA 892	287.11719.con.0037	assembly_contig_37	100	100	2382	60956	4378	0

B

Genome	Accession	Product	Identity (%)	Query cover (%)	Query Length	Sub Length	Score	E value
Pseudomonas aeruginosa PSA 971	287.26107.con.0009	assembly_contig_9	100	100	2382	221037	4344	0

Figure 0.4: PATRIC BLAST to detriment Fpvall receptors of PSA 892 and PSA 971. A: a table shows that FpvAllb sequence when BLASTed against PSA 892 genomic DNA resulted in a 100 % identity and query cover. B: similar to what has been done in A, a BLAST result of FpvAlla sequence against PSA 971 strain and it shows 100 % identity and query cover. This means that PSA 892 have FpvAllb receptor and PSA 971 have FpvAlla receptor.