


# Expression, Purification and Crystallisation of DNAse and Immunity Proteins in the S3 Pyocin of *Pseudomonas aeruginosa*

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# Abstract

*Pseudomonas aeruginosa* is an opportunistic pathogen known to cause several infections such as respiratory tract infections, urinary tract infections (UTI) and sepsis. It is often associated with nosocomial infections, due to its ability to form biofilms on medical equipment such as ventilators, catheters and cannulas. *P. aeruginosa* produces bacteriocins that kill related strains of bacteria and have potential implications as alternative antimicrobial therapy. One such bacteriocin is the S3 pyocin, which consists of 2 proteins, a a multi domain DNAse, and an immunity protein. Comparison of the DNase domains of S3 with other S type pyocins such as pyocin S1, S2, AP41 suggested that the DNase of S3 was atypical and did not have the characteristic zinc finger binding HNH motif. The DNase/Im encoding genes were cloned into the expression vector, pET21a and the DNAse/Immunity protein complex was purified using Ni-NTA and gel filtration chromatography. The DNAse was then separated from the Immunity protein by protein denaturation of the complex with guanidine and the DNAse activity of pyocin S3 was confirmed by its cleavage of plasmid DNA which was inhibited by the addition of the Immunity protein. Crystallisation trials were performed, and crystals were produced in a range of conditions. A selection of high quality, large crystals returned a resolution of 1.55-1.75Å from X-ray analysis via synchrotron but lack of phase data meant that the protein structure of the DNase/Im complex is still under investigation.

# Introduction

*Pseudomonas spp*. are a genus of Gram-negative, rod shaped, motile bacteria widely present in the environment, commonly inhabiting soil and fresh water. Certain species, such as *Pseudomonas aeruginosa* are considered opportunistic pathogens, due to their advanced virulence factors and antibiotic resistance mechanisms. They are often associated with nosocomial (hospital acquired) infections, commonly caused by biofilms formed on catheters, cannulas and ventilators. Biofilms are a community of a single, or several species of bacteria living within an extracellular matrix (Borgos et al., 2015). Biofilms act to protect the cells within from host immune systems or external harmful conditions such as change in pH, temperature, or presence of some antimicrobials (Stickler, 1999).

*Pseudomonas aeruginosa* is one of the most common organisms associated with nosocomial infections, and is largely treated through use of antibiotics (Levin et al., 1999). However, there is an increasing presence of drug-resistant *P. aeruginosa* infections, limiting the availability of treatments available for infection (Obritsch et al., 2005). A recent study by Barate and Ukesh (2012) demonstrated the ineffectiveness of common antibiotics such as ampicillin and penicillin-derivatives on *P. aeruginosa* urinary tract infections (UTIs), with only 8.6% of isolates studied being sensitive to ampicillin, a common antibiotic used to combat UTIs caused by *P. aeruginosa*. This resistance development shows a pressing need to research novel antimicrobials, to combat multi-drug resistant infections.

*P. aeruginosa* has several virulence factors which contribute to its pathogenicity, and explain its ability to cause different infections. Adhesins are one example virulence factors, which function to bind the bacterial cell to receptors on the human cell. *P. aeruginosa* can bind to galactose or mannose on human cells, through use of its fimbriae (Carnoy et al., 1994). A single cell can bind to human tissue and reproduce, forming a biofilm which in itself protects the bacterial infection from host defences. Invasins are proteases secreted by *P. aeruginosa* that act to permeate human cell membranes, or cleave collagen and other molecules on the surface of host cells. Invasins act either by killing the host cell, or by allowing the invasion of the host cell by the *Pseudomonas*, and is a common cause of deterioration in cystic fibrosis patients (Esen et al., 2001). This internalisation of *Pseudomonas* into the host cell is a further defence mechanism, by evading host immune responses (Lepanto et al., 2014). Last are toxins; Exoenzyme S and exotoxin A are two toxins produced by *P. aeruginosa* that are vital for its pathogenicity. Exoenzyme S reduces the ability for phagocytes to kill the bacterium, while exotoxin A prevents protein synthesis in the host cell, causing necrosis of host cells (Rocha et al., 2003; Morlon-Guyot et al., 2009).

As well as defence from host immune responses, *Pseudomonas* also produce bacteriocins to outcompete rival bacteria present (Garciaquintana, 1992). One such type of bacteriocin is pyocins, which are secreted proteins that attack rival cells (Parret & De Mot, 2002). Pyocins are a system of proteins that act to kill bacteria of the same, or different species without immunity to the pyocin, and they are present in 90% of *Pseudomonas aeruginosa* strains (Bakkal et al., 2010). Three types of pyocin have been extensively studied; R, F and S type pyocins (Duport et al., 1994). R and F type pyocins resemble bacteriophage tails in structure; the R-type pyocins have a hollow, rod-like, contractile structure, while the F-type pyocins

have a flexible, non-contractile rod structure, shown in figure 1.1 (Michel-Briand & Baysse, 2002). Both act to porate the cell membrane of susceptible cells, causing cell death (Nakayama et al., 2000). Pyocins are also known to aid in symbiotic infections; *P. aeruginosa* can commonly cause severe lung infections in conjunction with *Burkholdera cepacia*. It is known that an interaction between these two organisms is mediated by bacteriocin-like inhibitory activity, namely by the action of R, F and S type pyocins (Bakkal et al., 2010).

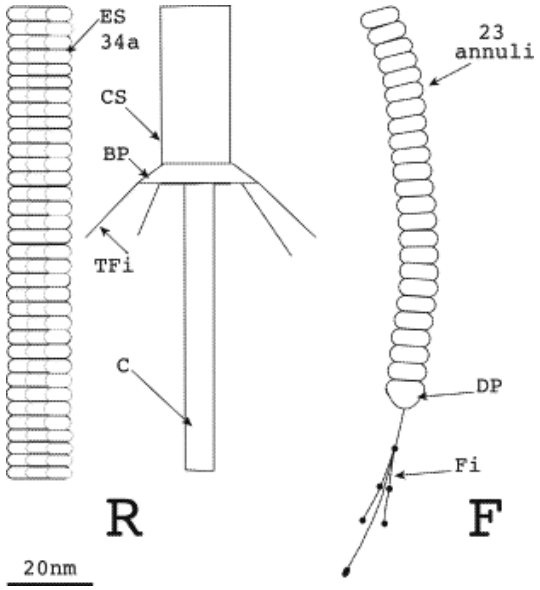



**FIGURE 1.1: Image showing the structures of R and F type pyocins (Michel-Briand & Baysse, 2002).** The R type pyocin structure resembles that of a phage tail, and is hollow, rod-like and contractile. The F type pyocin resembles a similar structure, but is non-contractile and flexible.

S-type pyocins are dissimilar in structure and function to R and F-type pyocins; they consist of two proteins, a DNAse and an Immunity protein (Duport et al., 1994). The DNAse is secreted by the host cell, and is uptaken into nearby cells. Those with the gene for the DNAse will have the immunity protein present in the cell, and will therefore survive. Meanwhile, cells without the immunity will be susceptible to the DNAse. The DNAse acts by binding to the enemy cells' DNA and catalyzes the cleavage of the phosphodiester bonds along the backbone of the DNA.

Baysse *et al*. (1999) investigated the uptake of the S3 pyocin DNAse into *Pseudomonas aeruginosa*, and found that only isolates with a type II pyoverdine, a membrane bound receptor protein, were susceptible to the DNAse.

They suggest this outer membrane protein is responsible for DNAse uptake, and found those without the type II pyoverdine, or with addition of type II ferripyoverdine, were unable to uptake DNAse and are therefore immune to its effects. Further to this, very little is known about the structure of proteins in the S3 pyocin. To date, the structure has not been solved and therefore the specific interactions between the DNAse and the immunity, or the DNAse and the DNA are not known.

Bacteriocins, such as pyocins, are a result of random mutation and evolution, and show a large diversity between different types of bacteriocins (Riley, 1998). The genes for pyocins are contained within the bacterial chromosome, and so are therefore passed onto further generations by vertical transmission. Bacteria without the bacteriocin gene are outcompeted, and so the prevalence of *Pseudomonas* with bacteriocin-producing ability increases.

This research seeks to begin investigation into the action and structure of the S3 DNAse and Immunity proteins, to give a clearer idea of their interaction with host and rival cells. The S3 DNAse and Immunity genes will be cloned into an overexpressing vector and transformed into *Escherichia coli* BL21 (DE3). The PCR used to amplify these genes prior to cloning will add a His-Tag to the end of the immunity gene, through use of an extended reverse primer, which will be used for later purification.

The proteins will be overexpressed within the cells, through use of the Lac operon, and induction with IPTG. The cells will be lysed by sonication, and the protein will then be purified from the lysate by use of a nickel sepharose flow column, hereon knows as a Ni-NTA column. The Ni-NTA column is filled with a Sepharose coated with a chelating ligand, which binds nickel ions. As proteins with poly-histidine residues are introduced to the column, they bind to the chelating ligand, as they have a high affinity to for the nickel ions. Proteins without the poly-histidine residues are washed through the column, as they do not bind. Imidazole is then added to the column in an increasing concentration gradient. The imidazole has higher affinity to the chelating ligand than the histidine residue on the protein, and therefore the protein is washed away. As these various washes are performed, fractions of the flow-through are collected and examined for UV absorbance, a peak in which suggests high protein content. It is known that the DNAse and the Immunity proteins form a tight complex, and so by addition of the His-Tag to the immunity gene by use of an extended reverse primer, both immunity and DNAse will be bound to the Ni-NTA column while other proteins will wash away, resulting in a pure protein complex.

Following this purification step, gel filtration chromatography will be used to separate the DNAse from other contaminant proteins still present following the Ni-NTA purification. Gel filtration chromatography is a size exclusion chromatography, where a protein sample is loaded into a column containing Sephadex and an equilibrated buffer. As the protein sample is pumped through the Sephadex, the smaller molecules are absorbed into the porous Sephadex while the larger molecules flow through the column. Therefore, larger molecules will flow through faster than smaller molecules. This purification step will provide a pure protein sample of the Immunity-DNAse complex. This will then be reloaded into the Ni-NTA

column, and a guanidine separation protocol will be used to separate the DNAse from the Immunity protein; as the proteins denature by the guanidine. The DNAse will separate from the immunity protein, which will remain in the column due to the His-Tag. This will give a pure DNase sample, which will be used for an activity assay. All purification steps will be carried out on the AKTAPrime system, in order to observe for UV absorbance spikes, representing protein presence in eluted fractions.

A DNAse activity assay will be performed, by exposing plasmid DNA to the purified DNAse protein, and incubating. This will confirm the DNAse function, and also confirm that the immunity-DNAse binding inactivates the DNAse, preventing DNA cleavage. Following this, the purified DNAse-Immunity complex, which is the product from the gel filtration chromatography, will be used in crystallisation trials. The protein will be exposed to precipitants using the 'sitting drop' method, and incubated in order to produce crystals, which will then be sent for X-ray diffraction in a synchrotron. While data regarding the crystals purity and resolution are returned immediately, solving the protein structure can require several months of analysis, and therefore will not be completed at the time of submission.

The S3 pyocin is dissimilar to other S-type pyocins, making it a noteworthy topic of research. Protein sequence comparison performed between the S3 pyocin and other S-type pyocins reveals very little similarity between them, most significantly the lack of an HNH motif in the S3 pyocin, which is present in all other pyocins (Michel-Briand and Baysse, 2002). A HNH motif is the catalytic centre of most pyocins, and is formed by two antiparallel $\beta$-strands, backed by an $\alpha$-helix with a divalent cation binding site between the two (Pingoud et al., 2005). The S3

pyocin is also dissimilar to other pyocins as the immunity protein is larger than the DNAse. The DNAse protein domain is 14.7kDa, while the immunity is larger, at 17.06kDa.

A study by Michel-Briand and Baysse (2002) demonstrated the potential of pyocins as antimicrobial agents in a medical setting. By constricting a hybrid pyocin using the receptor binding domain of the S1 Pyocin, and the DNAse, Immunity and translocation domains of the S3 pyocin, they were able to form a new pyocin, S1S3, which was found to be bactericidal against 87% of strains of *P. aeruginosa* tested against, collected from cystic fibrosis patients.

Pyocins, such as the S3 pyocin, are studied due to their potential as narrow-spectrum antimicrobials. They have potential uses in a medical setting, as an antibiotic or antimicrobial agent in devices commonly associated with *Pseudomonas* infections such as catheters, cannulas and intubation equipment, but also have uses in a commercial or domestic setting as an antimicrobial cleaning agent. Before the S3 pyocin can be considered for use in these applications, it is important to identify the proteins' structure, active sites and mode of action, in order to demonstrate its ability as an antimicrobial, but also to identify if the pyocin, specifically the DNAse component, can cause harm to human or animal cells. By crystallising the protein, the structure of the DNAse can be ascertained, which will identify its active sites and mode of action, as well as its interaction with the immunity protein, giving a clear understanding of its mechanism of action, and furthering the research into its use as an antimicrobial agent.

# Methods

## Strains and Media

The following experiments were conducted using standard strains and media. For transformation of plasmids and plasmid extraction, *Escherichia coli* strain JM109 was used, as it is capable of multiplying the plasmid, for a successful plasmid prep. For overexpression of the DNAse and Immunity proteins, *E. coli* strain BL21 (DE3) was used with pET vectors, utilising the Lac operon.

All growth on plates was conducted using standard Luria Broth agar, with 1mM ampicillin for antibiotic selection. All liquid cultures were grown in Luria Broth with 1mM ampicillin. In overexpression experiments, Luria Broth also contained 1mM IPTG.

## PCR, Mutagenesis and Transformation

The DNase and Immunity genes were amplified from a stock plasmid containing the S3 Pyocin, using primers YC16 and ImS3R (appendix 1). This PCR, and all further PCR (unless otherwise stated) was conducted using Q5 High Fidelity Polymerase, and the following conditions:

| Temperature (°C) | Time (s) | |
|---|---|---|
| 98 | 30 | |
| 98 | 10 | Repeat for 35 Cycles |
| 58 | 30 | |
| 72 | 30 | |
| 72 | 30 | |

**TABLE 2.1: PCR Conditions**

A table showing temperature and timing conditions for all Polymerase Chain Reaction procedures used.

The PCR product was run on a 1% agarose gel at 100v for 30-45 mins. Once a DNA fragment was observed on the gel, the QIAQuick PCR Cleanup Kit was used on the remaining PCR product and the gel rerun to ensure presence of band.

Stock plasmids 'G38A PNP69' and 'PNP69' (appendix 4) were digested using XhoI and NdeI. 1 μL of each restriction enzyme was added to 10 μL of plasmid DNA, 2μL of 10x Restriction enzyme buffer and 6 μL of $dH_2O$, and the solutions left to incubate at 37°C for 1h. The digest was then run on a 1% agarose gel, and the resultant plasmid band was excised from the gel and purified using the QIAQuick Gel Purification Kit. The PCR product was digested and purified in the same way, to make complementary sticky ends to the plasmid. A ligation of the PCR product was set up using 5μL of plasmid digest, 5μL of PCR product, 2μL of T4 DNA Ligase, 2μL of T4 buffer and 6μL of $dH_2O$. The ligation was incubated at 16°C, overnight.

Chemically competent *E. coli* JM109 cells were prepared using standard centrifugation methods and $CaCl_2$(NEB, 2017). The PCR-Plasmid ligation product was transformed into JM109 using heat shock transformation, and grown on ampicillin-containing LB plates. Single colonies were prepared in 10mL Ampicillin LB broth overnight, shaking at 37°C, and the Sigma Bacterial Plasmid Prep Kit was used on the cultures to extract the recombinant plasmid. The prepared plasmid was digested using XhoI and NdeI as before. This digest was run on a 1% agarose gel, and the DNase-Immunity gene fragment excised and purified using the QIAQuick Gel Purification Kit, before being ligated into pET21a (appendix 4), which was digested and purified as before. This ligation was transformed into chemically competent BL21 (DE3) *E. coli*. These cells were incubated, and single cells were used to prepare 10mL broth cultures as before, for plasmid prep. After running the purified plasmid on an agarose gel, the position

of gene on the gel, compared to the DNA ladder, appeared incorrect, so an SDS-PAGE gel was run to confirm gene expression. Two 10mL Ampicillin (100μg/mL) LB broths were set up and inoculated with the transformed BL21 (DE3) and incubated at 37°C, shaking at 200rpm for ~4 hours, until $OD_{600}$ = 0.4-0.6. One of the broths was then induced with IPTG to a final concentration of 1mM, and incubated for a further 4 hours. 10μL of each broth was then collected and mixed with 3μL SDS-PAGE Sample Loading Buffer (containing β-mercaptoethanol). Samples were then boiled at 100°C and run on a 12% SDS-PAGE gel at 200V for 30-45 min. An incomplete, single, overexpressing band was observed, suggesting a fault with the gene transformation.

Following this, the PCR product from the stock S3 plasmid was cloned into the Promega pGEM-T Easy vector (appendix 4). The gene was then digested from the plasmid using NdeI and XhoI as above, and cloned into pET21a. The same overexpression protocol was used, and an SDS-PAGE gel was prepared; the same band was observed.

Bioinformatic analysis of the DNA sequence (appendix 2) revealed an internal NdeI restriction site within the gene, suggesting the band observed on the SDS-PAGE gel was an incomplete protein complex. With this knowledge, mutagenesis primers were designed to remove the restriction site by replacing a single nucleotide without altering the amino acid sequence (appendix 3). Mutagenesis was performed using the primers S3_IntR and S3_IntF (appendix 1), and the NEB Q5 Site-Directed Mutagenesis Kit. This was performed on the transformed pGEM-T Easy vector, as Sanger sequencing showed the entire DNAse-Immunity gene was present.

Following mutagenesis, the genes were digested out as before, cloned into pET21a, and transformed into chemically competent *E. coli* BL21 (DE3). A final SDS-PAGE gel was run, and two bands were observed in the correct location on the protein standard, confirming two proteins of correct size were being overexpressed with IPTG (0.1mM). Using this strain, glycerol stocks were prepared by growing the *E. coli* in Ampicillin LB broth. At $OD_{600}$ 0.6-0.8, the sample was collected, mixed 1:1 with 50% glycerol, and snap frozen on dry ice before storing at -80°C.

## Protein Overexpression, Cell Harvest, Protein Purification and SDS

Following confirmation of protein overexpression, 2 500mL Ampicillin LB broth cultures were prepared, and at $OD_{600}$= 0.4-0.6 induced with IPTG (1mM). after being grown for a further 4h, the samples were centrifuged at 5000rpm for 30-45 min. The supernatant was removed and the pellet resuspended in 30mL 20mM Tris-HCl, 5mM $MgCl_2$, pH 7. The samples were then sonicated as below:

| Sonication strength (%) | Length of sonication | Cycle |
|---|---|---|
| 12 % | 6 min | 15s on, 15s off |
| 15% | 2 min | 15s on, 15s off |

**TABLE 2.2:** Sonication Conditions
A table showing times and strength of sonication used to lyse harvested cells.

The sample was kept on ice throughout sonication to prevent heat denaturation of the protein. The sample was then centrifuged at 14,000g for 20 min at 4°C to separate cell

debris from the protein suspension. NaCl was added to a concentration of 0.5mM to prevent the protein from precipitating.

Two buffers were prepared for use throughout the protein purification; buffer A (50mM $NaH_2PO_4$, 300mM NaCl and 10mM Imidazole), and buffer B (50mM $NaH_2PO_4$, 300mM NaCl and 500mM Imidazole).

Protein Purification was performed using a Nickel/NTA (Ni-NTA) column, as the reverse PCR primer (ImS3R) added a His tag to the immunity protein. A GE HisTrap HP was prepared by flushing through 20mL $dH_2O$ at 1.5mL/min, and 20mL of Buffer A at 1.5mL/min, using a peristaltic pump. The 30mL protein sample was then passed through the filter at 1.5mL/min. Following this, a further 20mL of Buffer A was passed through, to remove non-bound protein. The column was then loaded onto the AKTAPrime and the column again flushed with 20mL of Buffer A. A gradient was set up on the AKTAPrime to flush through 50mL of buffer in 2.5mL fractions, with the buffer percentage starting off at 100% Buffer A and 0% Buffer B, ending at 0% Buffer A and 100% Buffer B. Peaks in UV absorbance were observed in fractions 6-11, these fractions were run on an SDS-PAGE gel to locate the purified protein. The protein was found in high concentration in fractions 8-10; these were collected, mixed and placed in dialysis tubing in a dialysis buffer (50mM $NaH_2PO_4$, 100mM NaCl and 10mM Imidazole) overnight at 4°C.

A UV absorption at 280nm (Nanodrop) analysis was performed to calculate the protein concentration at this volume, shown to be 24μM. A higher concentration, above 70μM was required for later steps, so the protein sample was centrifuge filtered using a 3kDa centrifuge

filter. At a volume of 2mL, the concentration was increased to 90.1μM. this sample was then stored at -80°C. Following successful purification by this method, the process was repeated a further time, using 1L samples, to collect more protein.

Following this, a further purification step was performed to remove residual proteins present in the sample, observed on the SDS-PAGE gel after Ni-NTA purification; Gel filtration chromatography. An Amersham-Pharmica Superdex75 x16/60 gel filtration column was loaded onto the AKTAPrime system, and cleaned through with 250ml 8M urea, 250ml 20% ethanol and 250ml $dH_2O$. The column was then prepared by washing through with 400ml Sodium Phosphate buffer (50mM sodium phosphate, 150mM sodium chloride, pH7). The protein sample was then injected into the column, and the column continuously flushed through with the sodium phosphate buffer at 0.5mL/min, and 3mL fractions were collected. The flow through was observed for a peak in UV absorbance, and fractions showing a peak were collected and run on an SDS-PAGE gel, to confirm protein presence. Following confirmation by SDS-PAGE, the samples showing pure protein were mixed and centrifuge filtered to a volume of 2mL, showing a final concentration of 3.05mg/mL.

## DNAse Purification and Activity Assay

To confirm the overexpressing protein was a DNAse protein, it was separated from the Immunity protein using guanidine. The Ni-NTA column was prepared as above, and the 2mL of protein purified was thawed and loaded into the column as above. The column was then loaded into the AKTAPrime, and a gradient of Buffer A was used with a guanidine buffer (6M Guanidine, 20mM Tris, 50mM NaCl, pH8). As the guanidine concentration increased, fractions 3 and 4 were collected as they had a large UV Peak. These were run on a 16% SDS-PAGE gel

to confirm protein presence. The 2 fractions were mixed and put through 2 runs of dialysis in 2L of potassium buffer (50mM $K_2HPO_4$, 50mM$KH_2PO_4$, pH 7.5). The protein was analysed by Nanodrop, and the concentration increased to 30μM in 1mL by centrifuge filtration.

A DNAse assay was set up by mixing 10μL of colicin stock plasmid DNA, 5μL MgCl (20mM), and mixing these with the following:

| A | B | C | D |
|---|---|---|---|
| 20μL $dH_2O$ | 15μL $dH_2O$ | 10μL $dH_2O$ | 0μL $dH_2O$ |
| 0μL of DNAse | 5μL of DNAse | 10μL of DNAse | 20μL of DNAse |

**TABLE 2.3:** DNAse Activity Assay Conditions
A table showing volumes of DNAse and DNA used in DNAse activity assay experiments.

As well as these solutions, one sample was prepared using 10μL of plasmid DNA, 5μL $MgCl_2$ (20mM), and 10uL of the DNAse-Immunity purified complex, to confirm immunity activity. These solutions were incubated for 1h at 37°C, and then run on a 1% agarose gel and viewed under UV.

## Protein Crystallography

The purified DNAse-Immunity complex was centrifuge filtered to a final concentration of 18.37mg/ml, and a PCT Pre-Crystallization Test kit performed on the protein. Once successful, the protein was loaded into 4 96-well plates, using the sitting drop method, with various solutions from the following kits:

-PEGS II (Qiagen)

-BN150-1-36 (PACT Premier; Molecular Dimensions)

-BN178-1-47 (Morpheus; Molecular Dimensions)

-BN195-1-40 (JCSG Plus; Molecular Dimensions)

These plates were sealed with an adhesive sheet, and incubated at 20°C until crystals were visible. Crystals that did form were isolated and stored in a solution consisting of 70% mother liquor (the condition the crystal grew in) and either 30% glycerol or 30% ethylene glycol, as a cryoprotectant. Some crystals were exposed to heavy atom compounds to overcome the Phase problem; in this instance $HgBr_2$ and di-μ-iodobis(ethylenediamine) diplatinum(II) dinitrate, as deemed necessary by the crystallographers. These crystals were then sent to be examined by synchrotron radiation, and a resolution was returned.

# Results

## Cloning of the S3 Pyocin DNAse and Immunity Genes

Primers YC16 and ImS3R were designed according to the DNA sequence of the S3 Pyocin, and its predicted DNAse and Immunity domains. They were used to amplify a 859bp product from nucleotides 1903 to 2762 of the pyocin; the DNAse and Immunity domains in the S3 stock plasmid. Primer YC16 introduced an NdeI site at the 5' end of the amplicon, while primer ImS3R introduced 6 histidine residues, followed by an XhoI restriction site at the 3' end of the gene. This amplicon was then cloned into pET21a, to produce pCN1, and transformed into *E. coli* JM109. The resultant cells were grown and a plasmid prep performed, to extract pCN1. A digest of pCN1 was performed using NdeI and XhoI enzymes, and then ligated into pET21a to produce pCN2, and transformed into *E. coli* BL21 (DE3). This culture was grown and a plasmid prep performed to extract pCN2, which was run on an agarose gel, shown in Figure 5a. An SDS-PAGE protein gel was run showing both induced and uninduced samples of *E. coli* BL21 (DE3) with pCN2. As shown in Figure 3.1 only a single band is observed; two bands are expected, the DNAse and the Immunity proteins.

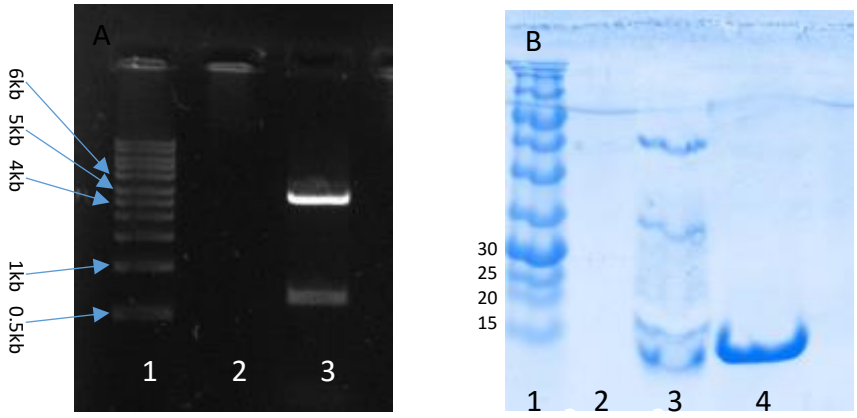



**FIGURE 3.1:** Gels of pCN2

a) Agarose gel showing digest of suspected DNAse and Immunity genes in pET21A (pCN2) (Lane 3). (Lane 2 is empty). DNA Ladder: NEB 1kb ladder

b) SDS-PAGE gel of uninduced (lane 3) and induced (lane 4) DNAse and Immunity in *E. coli* BL21(DE3). (Lane 2 is empty). Protein Marker: ThermoFisher PageRuler Protein Ladder

Following sequencing and bioinformatics, it was found there was an internal NdeI restriction site 72bp into the DNAse sequence, meaning that during the digestion and ligation steps, part of the sequence had been lost. Therefore, site directed mutagenesis was performed on the pCN1, which contained the complete sequence, to remove the internal NdeI site, and produced pCN3. Sequencing confirmed loss of the internal restriction site in pCN3, and therefore the plasmid was digested and the genes ligated into pET21a, to produce pCN4, which was then transformed into *E. coli* BL21(DE3). A further SDS-PAGE protein gel was run on uninduced and induced cultures of the mutant clone, shown in Figure 3.2.

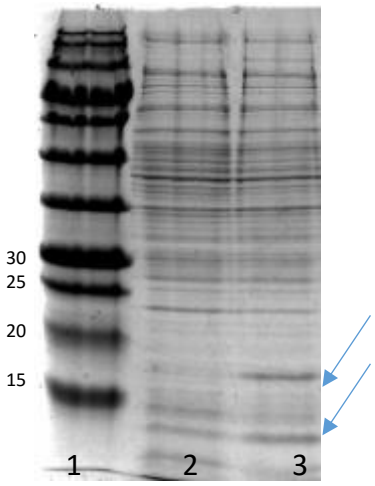



**FIGURE 3.2:** pCN4 Trial Overexpression

12% SDS-PAGE gel showing uninduced (Lane 2) and induced (Lane 3) DNAse and Immunity proteins. Two overexpressed bands are observed, the larger of the two being the immunity protein at 17.06kDa, and the smaller of the two being the DNAse, at 14.70kDa. Protein Marker: ThermoFisher PageRuler Protein Ladder

## DNAse and Immunity are Purified as a Complex

In order to purify the DNAse and Immunity proteins for activity assay and crystallography, protein overexpression was scaled up. 500mL cultures of *E. coli* BL21 (DE3) containing pCN4 were set up and protein overexpression was induced at $OD_{600}$=0.4-0.6 using IPTG to a final

concentration of 1mM. A 20 uL sample was taken of both uninduced and induced cultures was run on an SDS-PAGE gel, shown in Figure 3.3, to confirm overexpression activity.

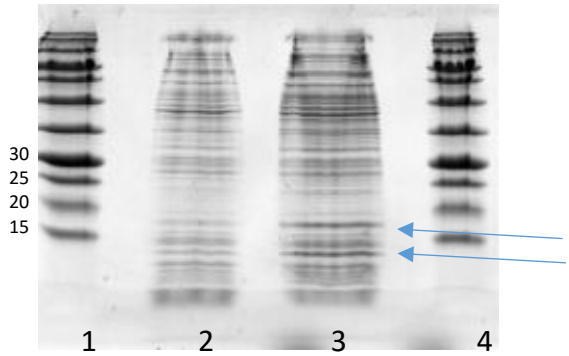



**FIGURE 3.3:** pCN4 Overexpression
AN SDS-PAGE gel showing uninduced (Lane 2) and induced (Lane 3) samples of BL21 (DE3) with pCN4 present. Two overexpressing bands are observed in the induced sample (at 17.06kDa and 17.70kDa). Protein Marker: ThermoFisher PageRuler Protein Ladder

Following confirmation of overexpression, the cell cultures were centrifuged and the resuspended cell pellets were sonicated to break the cell wall and release the cytoplasmic contents. The cell lysate was loaded into the Ni-NTA column, and the column washed with a buffer containing an increasing gradient of imidazole. The flow through was measured for peaks in absorbance at $A_{260nm}$. As shown in Figure 3.4a, there was a peak of absorbance in fractions 6-10. These fractions were then run on a 12% SDS-PAGE gel to determine which fractions contained the desired protein, as shown in Figure 3.4b.

The gel showed fractions 8-10 contained the desired protein in high concentrations. These fractions were dialysed in 100mM NaCl overnight to lower the salt level, and then centrifuge filtered to concentrate the solution to a final concentration of 2.2mg/mL (90.1uM) in approximately 2 ml.

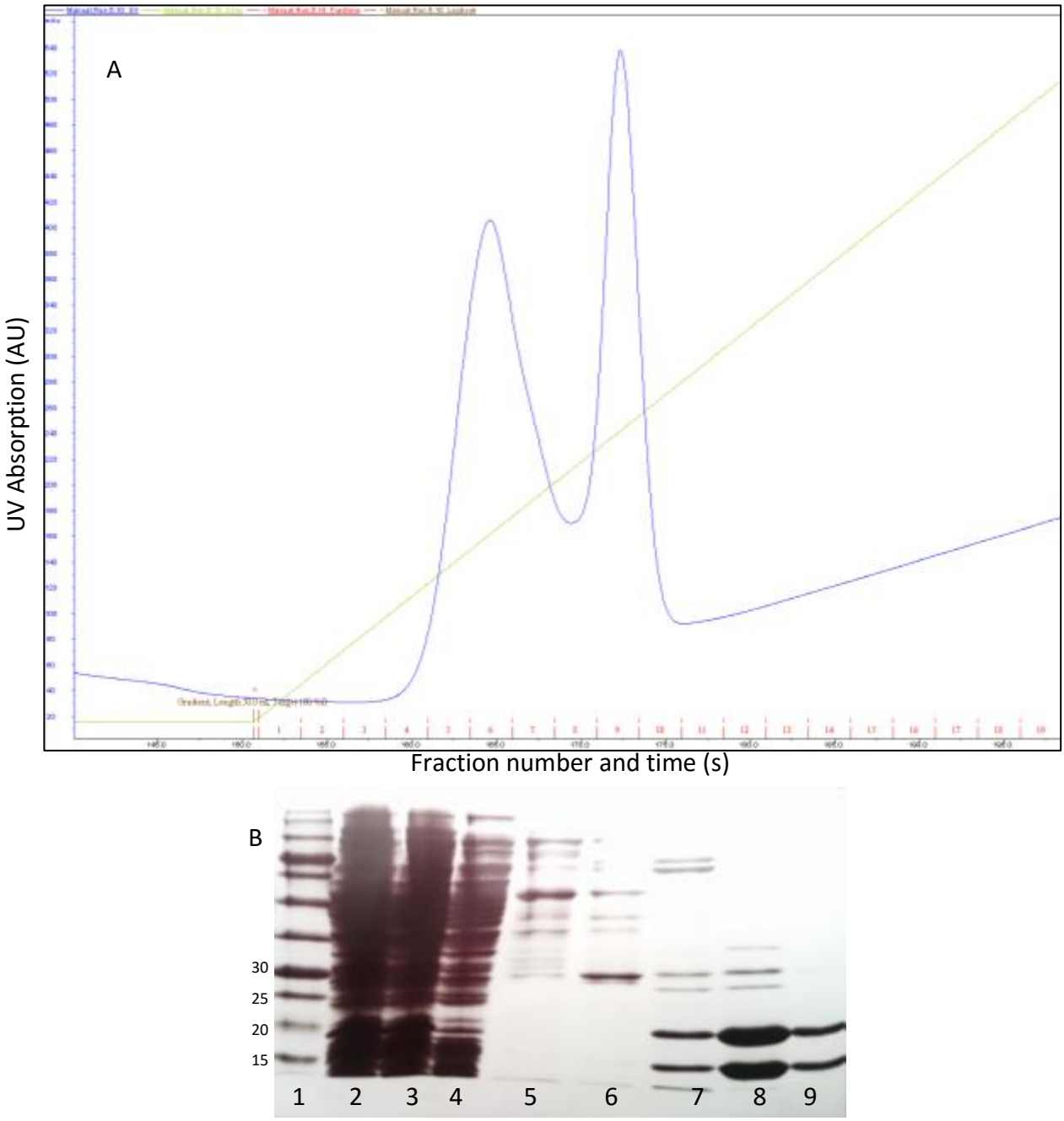



**FIGURE 3.4:** Ni-NTA Purification

a) Graph showing UV absorbance during Ni-NTA purification. The largest peak is observed in fractions 8-10. A smaller peak is observed in fractions 5-7.

b) SDS-PAGE gel showing fractions 8-10 (lanes 7-9), alongside the lysate content (lanes 2-3), and fractions 5-7 (lanes 4-6) where a smaller peak in UV absorbance was observed. Protein Marker: ThermoFisher PageRuler Protein Ladder

Gel filtration was then used to remove unwanted protein present in the sample after Ni-NTA purification. UV absorbance peaks were shown in samples 9-12 (Figure 3.5a); these samples were run on an SDS-Page gel to confirm presence and purity (Figure 3.5b). These samples were mixed and centrifuge filtered to a final concentration of 3.05 mg/mL.

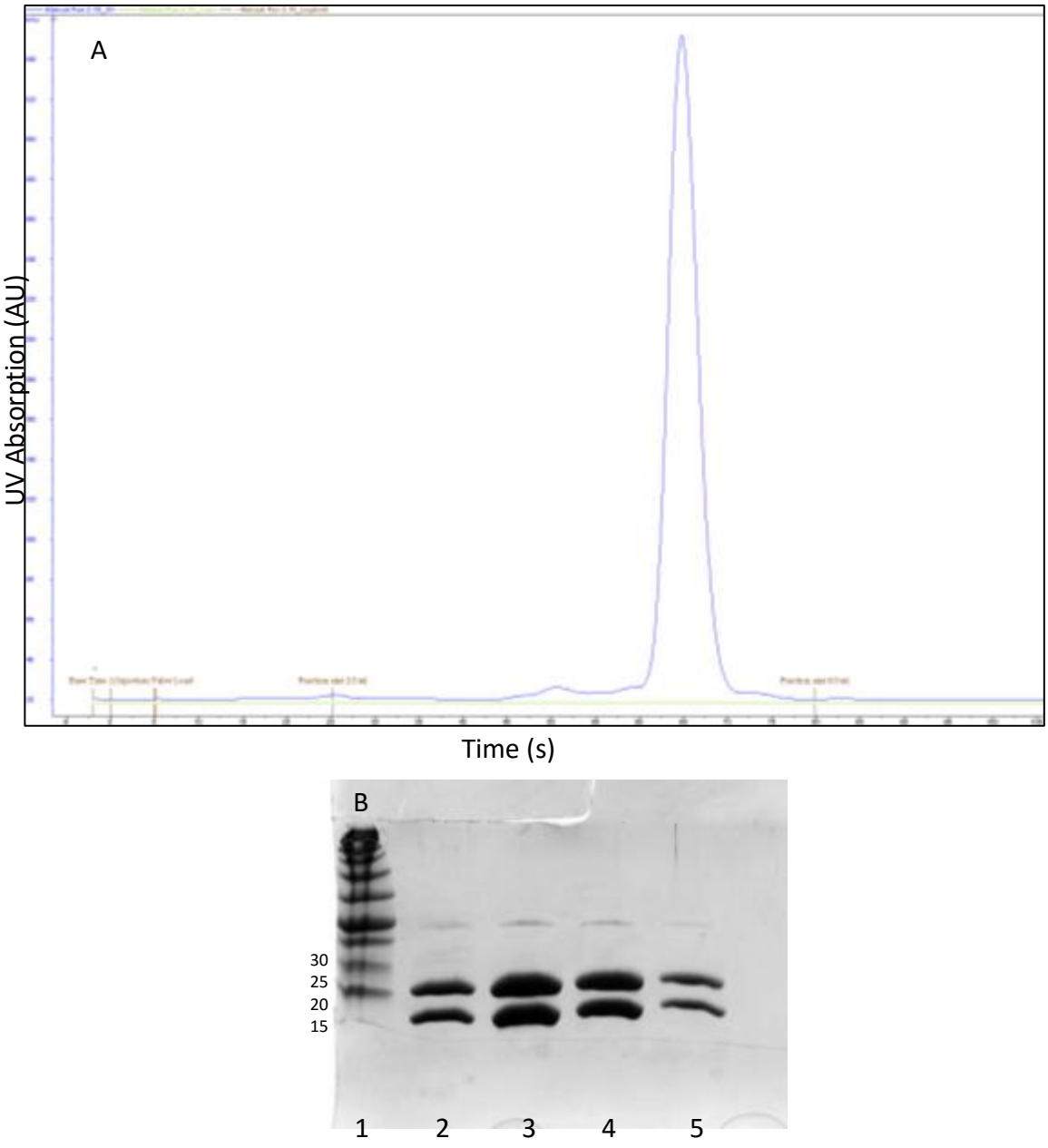



**FIGURE 3.5:** Gel Filtration Chromatography

a) A graph showing UV absorbance during Gel filtration chromatography. A single large peak is observed in fractions 9-12

b) An SDS-PAGE Gel showing fractions 9-12 (lanes 2-5) from Gel Filtration chromatography. Two large overexpressing bands are shown (DNase and Immunity protein), while other contaminant proteins have been removed. Protein Marker: ThermoFisher PageRuler Protein Ladder

## DNAse has Endonuclease Activity

Following successful purification of the DNAse and Immunity protein complex, the DNAse was separated from the immunity by using the Ni-NTA column. The protein complex was loaded onto the Ni-NTA column, and flushed through with an increasing gradient of guanidine. The guanidine denatures proteins present in the column, and due to the denaturing of the DNAse, it is washed through. The immunity, however, remains bound to the column because of the poly-histidine tag. A peak in UV absorbance was observed in fraction 4 (Figure 3.6a) Once complete, imidazole was also passed through, to remove remaining Immunity protein bound to the column. These samples were run on an SDS-PAGE gel (Figure 3.6b).

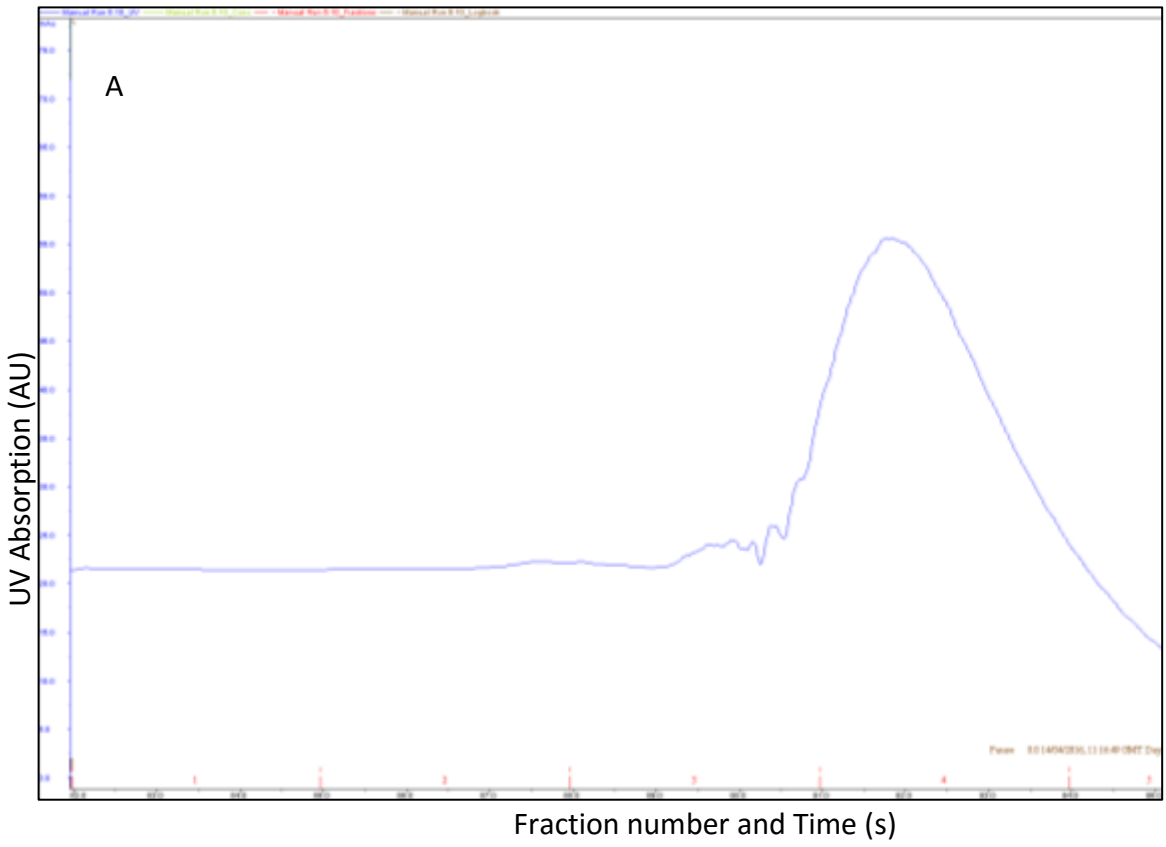
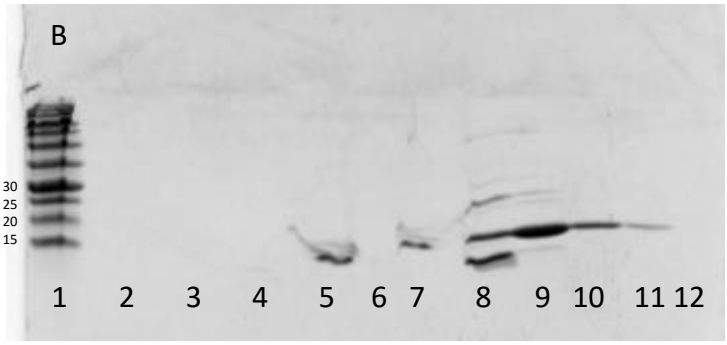



**FIGURE 3.6:** Guanadine Separation

a) A graph showing UV absorbance during guanidine gradient separation of DNAse. A peak in absorbance is seen in fraction 4.




b) An SDS-Page gel showing fraction 4 (Lane 5) alongside fractions collected from the imidazole wash-through following guanidine separation (lanes 7-11). (Lanes 2-4, 6 and 12 are empty). Protein Marker: ThermoFisher PageRuler Protein Ladder

As shown, DNAse was isolated from the immunity in one fraction, but much of the DNAse remained in the column after 6M Guanidine was passed through, due to the strong interaction between the two proteins. The fraction containing DNAse was dialysed in a potassium buffer to remove the guanidine, and then a DNAse assay was performed by mixing varying volumes of the DNAse with plasmid DNA, incubating, and running on an agarose gel to observe for DNA degradation. As shown in Figure 3.7, the DNAse showed strong endonuclease activity on the DNA, but when the immunity protein was present, no DNA damage occurred.

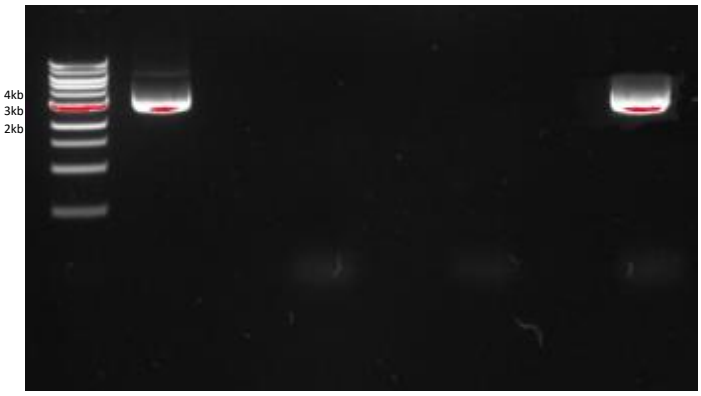



**FIGURE 3.7:** DNAse Activity Assay
An agarose gel showing pure plasmid DNA (Lane 2), DNA exposed to DNAse (lanes 3-6) and DNA exposed to the DNAse/immunity complex (Lane 8). As is shown, all DNA exposed to pure DNAse Was digested by endonuclease, while the sample with immunity present did not. (Lane 7 is empty). DNA Ladder: NEB 1kb ladder

## S3 Pyocin DNAse/Immunity complex Forms Large Crystals

The DNAse Immunity protein complex was set up in 96-well 'sitting drop' plates with crystallography precipitants, and sealed using adhesive tape. The plates were incubated at 20°C. Following 5 days of incubation, crystals were observed in 15 wells, a selection of which are shown in Figure 3.8.

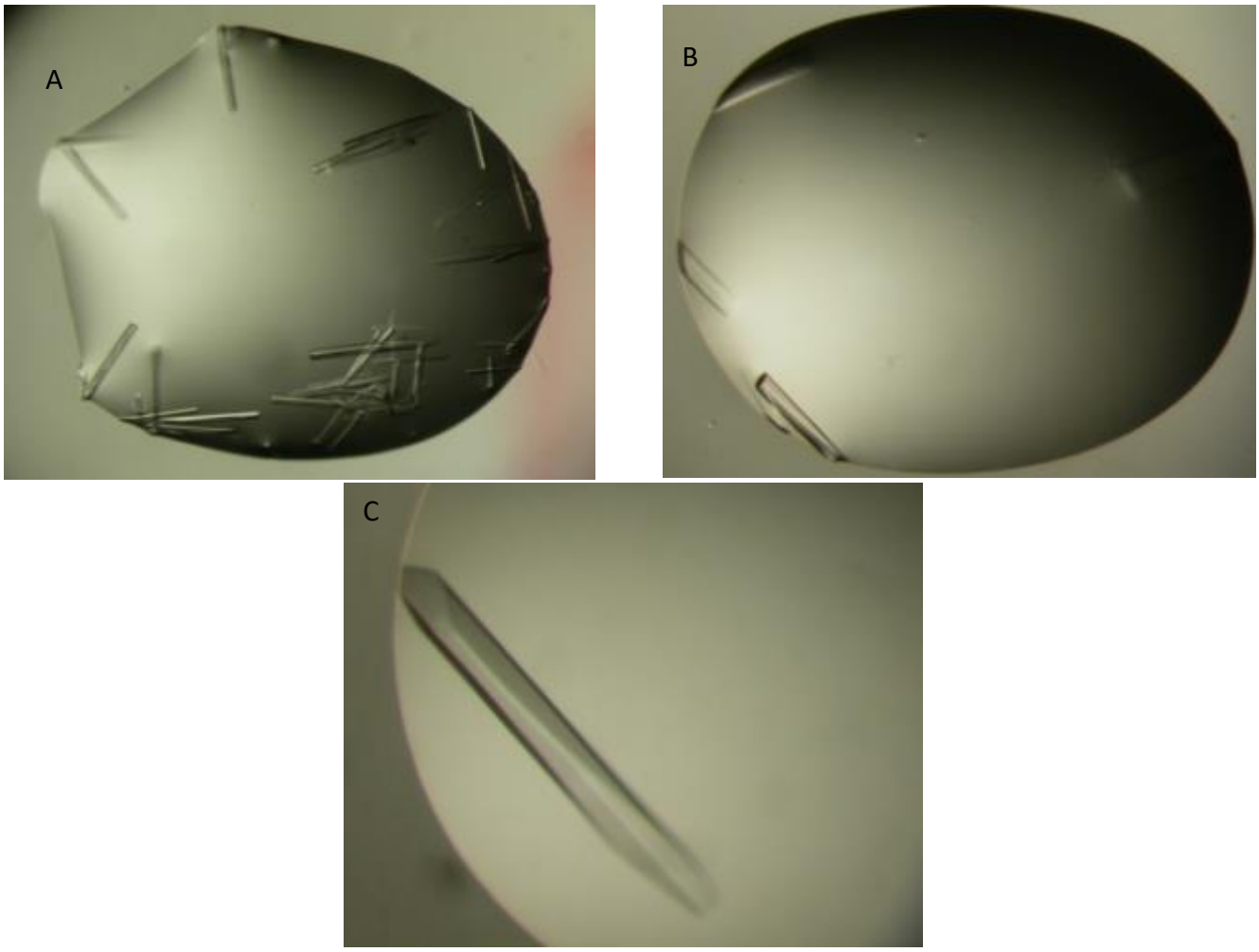



**FIGURE 3.8:** Protein Crystallisation

a) Small crystals formed in 0.2M trimethylamine N-oxide, 0.1mM Tris, pH 8.5, 20% polyethylene glycol (2K)
b) Crystals formed in 0.2M sodium fluoride, 0.1M Bis-Tris propane, pH 8.5, 20% polyethylene glycol (3.35k)
c) Large crystal formed in 0.2M Sodium acetate trihydrate, 0.1M Bis-Tris propane, pH 8.5, 20% polyethylene glycol (3.35k)

Several of the successful crystals were removed from the well, stored in a solution consisting of the mother liquor, as well as 30% glycerol, or 30% ethylene glycol, as a cryoprotectant. Some of the crystals were scanned by the synchrotron as they are, while some were soaked in heavy atom compounds to overcome the Phase problem. Data was collected from the synchrotron, and the resolution is shown below (Table 3.1).

| Condition name | Resolution(s) (Å) |
|---|---|
| H10 PACT (Soaked in $HgBr_2$) | 1.55-1.75 |
| H3 PACT (Soaked in di-μ-iodobis(ethylenediamine) diplatinum(II) dinitrate (PIP)) | 2.2 |
| D7 PACT (Soaked in di-μ-iodobis(ethylenediamine) diplatinum(II) dinitrate (PIP)) | 3-3.3 |
| H1 PACT (no heavy atom soaking) | 1.7 |
| H7 PACT (no heavy atom soaking) | 1.7 |
| H6 PACT (no heavy atom soaking) | 1.8 |

**TABLE 3.1:** Protein Crystal Resolutions
A table showing post-crystallisation treatment of some crystals, and the measured resolution from synchrotron analysis.

Crystal resolutions around 1Å are excellent quality crystals, but resolutions up to 3 are solvable; this shows that the protein crystals created are excellent quality, and can be solved for protein structure, though at the time of submission, the structure had not been solved.

## Discussion and Future Directions

The aim of this study was to purify and crystallise the S3 pyocin DNAse/Immunity protein complex for the purpose of determining the structure of the proteins by X-ray diffraction, and to perform a DNAse activity assay to ensure purification steps performed did not alter the function or configuration of the proteins, confirming the crystallography result was correct. The protocols carried out were designed with small scale purification as the main purpose, and with time constraints taken into account.

The initial PCR carried out, to amplify the stock S3 DNAse and Immunity domain, was performed using primers which placed restriction sites at the beginning and end of the sequence, to make cloning of the genes into plasmid vectors possible by digestion and ligation. It also added 6 histidine residues to the end of the immunity protein, to make Ni-NTA purification possible. Transformation of the S3 DNAse and immunity genes into an overexpressing vector was complicated by an internal restriction site within the DNAse sequence, but mutagenesis was used to remove this restriction site without altering the amino acid sequence of the protein, to ensure the purified protein was identical to that of a wildtype S3 pyocin. Final transformation into the pET21a vector was performed to utilise the Lac operon for overexpression, to produce large volumes of protein for crystallisation and the activity assay. *E. coli* BL21 (DE3) was the organism of choice for this procedure, because it is a T7 expression strain, making it compatible with the Lac operon within the pET21a.

Overexpression of the proteins within *E. coli* with pCN4 was successful, as shown by the overexpressing protein fragments shown in Figure 6. Following this, initial purification was carried out using the Ni-NTA purification system. This made use of the histidine residues added to the C-terminal of the immunity amino acid sequence. As the DNAse and immunity proteins bind strongly to each other, both remained bound to the Ni-NTA column while most contaminant proteins were removed.

Following Ni-NTA purification, SDS-Page gel analysis showed some contamination by other proteins within the DNAse/immunity sample. Therefore, a further purification step was included; gel filtration chromatography. This process purifies samples based on the molecular size, and as is demonstrated by the resultant SDS-PAGE gel, was successful at removing the remaining protein contaminants from the sample.

The AKTAPrime system was utilised during purification steps to monitor protein elution, and to make collecting fractions and altering Imidazole or guanidine concentration more accurate and reliable. The absorbance data from the AKTAPrime system gave clear and reliable data regarding protein presence in the fractions eluted. This made the process more accurate and efficient.

Separating the DNAse from the immunity protein was performed again using the Ni-NTA column. As is shown in Figure 10, by increasing the concentration of guanidine being passed through the column, the DNAse protein denatured, and therefore no longer bound to the immunity protein, and was washed out. The immunity proteins remain bound to the column due to its poly-histidine tag.

The DNAse assay was performed following dialysis of the purified DNAse, to ensure no guanidine was present in the sample which could alter the results of the assay. The assay confirmed that the DNAse had endonuclease activity on DNA, and also confirmed that the DNAse/immunity complex did not have endonuclease activity. This information ensured that the DNAse being used for crystallography had not been altered due to the purification steps, and therefore data returned from crystallography would reflect wild-type S3 pyocin structure accurately and reliably.

Pre-crystallisation trials were carried out to confirm the protein was able to be crystallised at its current concentration. Once successful, crystallisation trials were set up using standardised precipitants. Following a 5-day incubation at 20°C, crystals were observed in 15 conditions. The largest crystals were sent for examination by x-ray diffraction by use of a synchrotron. At the time of submission, the final model of protein structure is not yet complete. However, resolution of the crystals has been returned, and shows that in several conditions, the crystals produced are of excellent quality and purity. This resolution data means that a final protein structure should be possible to achieve.

Purification of the DNAse/Immunity protein complex, and solving of the protein structure are essential steps, but further research into these proteins is essential in determining its potential as an antimicrobial agent. Once the final protein structure is solved, comparisons between this and other similar bactereocins such as colicins can be made. This will provide insight into the active sites of the DNAse, and its mode of action. Following on from this research, further activity studies should be performed, initially on susceptible *Pseudomonas spp.* cultures, and if successful, further studies on tissue cultures infected with *Pseudomonas*

*spp*. would provide insight into its use as an antimicrobial agent in a medical setting.

Following on from successful live tissue activity assays, the DNAse protein can then be further researched using computer modelling and laboratory animal models, to demonstrate its ability to combat *Pseudomonas spp*. infection, and also to identify any side effects of the protein on the model. Following successful testing in laboratory models, the protein can then be escalated to human trials, hopefully demonstrating successful antimicrobial activity within a human model, without side effects.

Bacteriocins are currently being researched into for their potential as antimicrobial agents in other areas, such as food preservation. A study by Vijayakumar & Mutiana (2017) has demonstrated a 6-7 log decrease in *L. monocytogenes* in foods treated with bacteriocin-producing lactic acid bacteria. This shows the potential for bacteriocins as a novel antimicrobial agent, and provides promise for research into other bacteriocins such as the S3 pyocin. Further research into the S3 DNAse and other similar bacteriocins may help combat increasing antibiotic resistance, and current research, though in its early stages, is optimistic to this end.

## Conclusion

With the presence of multi-drug resistant bacteria becoming an increasing threat to human health, research into potential novel antimicrobials is a growing necessity. Narrow spectrum antimicrobial proteins, such as pyocins, are one of many potential solutions to the growing antibiotic resistance epidemic.

This research has been conducted to progress the potential application of the S3 pyocin as a novel antimicrobial. While its importance to human health has been stated, it has potential for use as cleaning agents in medical, commercial and domestic settings. Though the final image of the S3 DNAse and immunity structure is not complete at the time of submission, this research has advanced current knowledge of the S3 pyocin, and the final structure, which will soon be available, will progress the potential use of the S3 pyocin as an antimicrobial agent.

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# Appendices

## Appendix 1: Primer sequences

| Primer Name | Primer Sequence |
|---|---|
| Im3SR | 5’ - CCCATATGCCGGTTAAGCCGCTGGA |
| S3_IntF | 5’ - CCATCTTGACCACATGCCGGCTCAAG |
| S3_IntR | 5’ - TTTCCATCACGAGGTCTTCCTG |
| YC16 | 5’ - TTTCTTGTCCCCACCATGGAGCTCTC |

## Appendix 2: S3 DNAse and Immunity Sequence

### DNAse – 395bp

ATGCCGGTTAAGCCGCTGGAAGTGGACATTTATGGTAATTTTGCAGGAAGACCTCGTGATGGAAAC
CATCTTGACCACATGCCGGCTCAAGGTGCGTTGGCGACGAGTTTGAGAGCTATTTATCCAGACATCC
CCTATGGAGAAATTCGCAAATTAATGAAGAAAGGGGGGAGTGTTGCCATACCAGCTAGAGTGCATC
AGAGGTTCAGTGAAACCTACGGAGGACGTAACACCAAAGAAAAGCAACAGAAGGATGCCTCCGAC
TTGCGTGCAGCAGTCGATAGCAATTTCGATGCCGTCAAGAAAGGATTGCTAGAGGAGGGGTTCTCC
GAAGATGATATTGAGAGGGCGCGCGCAGAACTACACATGGTGAACAAAGAACAGGGGTGGTACTG

(Characters in red represent an internal NdeI restriction site, removed by site directed mutagenesis)

### Immunity – 486bp

ATGGAGAAGAAGCTGATCGTTAAATTAATTGATAGCATCGGAAAATCCCATGAGGAGATTGTTGGT
GCTGGATTGGTTAAGTCTGGAAAACTGGAGAGCCCCTACGAACATTCTGAACACCTAACGCTCTCTC
CAGAAAAAGGACTTGAACTAAACTTCCGAGCAGAAAACAAGATTCTTGAGAAGATTCATATTTCATT
AATTGACACTGTGGAGGGTTCTGCTGCTTACTCAGGGGAACTGCCTGAACCTCTAGTTAGAAAAATG
AGTAAAGAGCAAGTTCGTTCAGTGATGGGGAAACCATTTGAGACGAGAGAGTCTTTCAGAGTTCCT
GTGATTGGAATTCTTGGCGGCTGGGACTACTTTATGCACCCCCAGAAAGCCAATTTACGTATTGGGT
TCACATACACATCTGACTCGAATGTGTGCAATTTAACTTTCGCTCTTCTTGGNTCTAAACTCGAGCAC
CACCACCACCACCACTAG

## Appendix 3: S3 DNAse and Immunity Amino Acid Sequence

### DNAse – 14.7 kDa

MPVKPLEVDIYGNFAGRPRDGNHLDHMPAQGALATSLRAIYPDIPYGEIRKLMKKGGSVAIPARVHQRF

SETYGGRNTKEKQQKDASDLRAAVDSNFDAVKKGLLEEGFSEDDIERARAELHMVNKEQGWY

### Immunity – 17.06 kDa

MEKKLIVKLIDSIGKSHEEIVGAGLVKSGKLESPYEHSEHLTLSPEKGLELNFRAENKILEKIHISLIDTVEGSA

AYSGELPEPLVRKMSKEQVRSVMGKPFETRESFRVPVIGILGGWDYFMHPQKANLRIGFTYTSDSNVCN

LTFALLGSKLEHHHHHH

(Characters in red represent the histidine tag, added by primer ImS3R)

## Appendix 4: Plasmid Information

| Plasmid name | Plasmid information | Reference |
|---|---|---|
| pET21A | A high expression T7 vector with ampicillin resistance for selection | Merck Millipore http://www.merckmillipore.com/GB/en/product/pET-21a%28%2B%29-DNA---Novagen,EMD_BIO-69740 |
| pGEM-T | A linearised vector with multi-T tails for ligation and ampicillin resistance for selection | Promega: (https://www.promega.co.uk/resources/protocols/technical-manuals/0/pgem-t-and-pgem-t-easy-vector-systems-protocol/) |
| pCN1 | pNP69 with incomplete DNAse and immunity genes (due to internal restriction site | This Work |
| pCN2 | pET21a with incomplete DNAse and immunity genes (due to internal restriction site | This Work |
| pCN3 | pGEM-T with complete S3 DNAse and Immunity genes (following site directed mutagenesis) | This Work |
| pCN4 | pET21a with complete S3 DNAse and Immunity genes | This Work |

| | | |
|---|---|---|
| | (following site directed mutagenesis) | |
| pNP69 | A pET21a plasmid containing colicin genes between XhoI and NdeI restriction sites | Kindly provided by Dr. Christopher Penfold |