

A structure-function
characterization of the novel pyocin
S3 DNase domain with its cognate
immunity protein

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the degree of Doctor of Philosophy

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Declaration

I hereby certify 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.

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Abstract

Pyocin S3 is an unorthodox S-type DNase pyocin produced by *Pseudomonas aeruginosa*. It is a multi-domain bacteriocin which comprises of an effector protein, which exerts killing activity, and an immunity component, which protects the pyocinogenic bacteria. The killing activity of pyocin S3 is due to a unique DNase domain which does not share sequence homology nor the characteristic H-N-H fold that defines the active site of all other Gram negative bacteriocin DNases such as the colicin E type family, pyocin S-type DNases and homing endonucleases. Due to its therapeutic potential as a novel narrow-spectrum antimicrobial, the DNA encoding the DNase-immunity domains of pyocin S3 produced by *P. aeruginosa* P12 was cloned into the expression vector, pET21a and purified following IPTG expression using metal chelate chromatography and gel filtration. The DNase was separated from its cognate Immunity protein by Guanidine-HCl denaturation. A final purification step was performed using gel filtration chromatography before the identification of DNase activity using *in vivo* cytotoxicity and *in vitro* endonuclease activities relative to the full length native pyocin S3. Further interrogation with isothermal titration calorimetry (ITC), inductively coupled plasma mass spectrometry (ICP-MS), circular dichroism (CD) and tryptophan fluorescence was conducted to provide an insight into the extent of structural integrity following refolding from Guanidine treatment and role of any divalent metal ion co-factor in the activity of pyocin S3 DNase similar to the role

of divalent metal ions in the H-N-H DNase activities of other pyocin and colicin proteins.

Metal dependence assays of the purified DNase domain in the presence or absence of divalent metal ions suggested that pyocin S3 is a metal-dependent DNase. A few first row transition metals, including Mg^{2+} , Mn^{2+} , Ni^{2+} , Ca^{2+} and Co^{2+} (metal ions listed from the most potent to less) could restore the activity of apo-pyocin S3 DNase in the absence of its cognate immunity protein to different extents.

X-ray crystallography revealed the structure of the purified DNase domain in complex with its cognate immunity protein. The structure is completely novel for a DNase/immunity complex, and resembles an inactivating octopus clasp by the larger immunity protein on its cognate DNase prey domain. Analysis of the X-ray structure of the DNase-Immunity protein complex combined with site directed mutagenesis of conserved residues identified two histidine residues (His26 and His66) and an aspartate residue (Asp25) as likely co-ordinating residues of a divalent metal ion within a novel 30-40 amino acid metal binding motif of a common DNA catalytic $\beta\beta\alpha$ fold.

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Statement of COVID impact

As a PhD student and a Mum of a lively toddler, I was hit by COVID hard physically and mentally. My immediate family is Chinese and thus I experienced early anxiety as the pandemic initially swept across China and I was uncertain how my family were.

Since the Covid restrictions have been placed in the UK in 2020, I wasn't able to perform any long-term experiment due to the limited capacity in the building and the labs. The pandemic restrictions included reduced capacity in the labs that necessitated working in shifts and there have been extreme delays in ordering and access to equipment with some not being able to be maintained in a timely manner. This meant that final experiments that were planned were not possible, and so it was necessary to write up the data that existed when the lockdown started. I also had to start home-teaching due to the school closure. I could only do some writing in the evenings till midnight or the next morning to catch up on my work as my husband was not able to help with childcare due to the change of his working hours during the lockdowns. The lack of sleep made me extremely exhausted and more stressed. Life was getting easier after the lockdowns in 2021. However, I caught the COVID three times before my thesis submission. I was very poorly for the last two times and the second one gave me long-term effects.

Doing a PhD was a lot harder than I ever expected. As a self-funded PhD student, I was forced to find ways to finance my studies for the extended

period due to the delays imposed by the pandemic. Luckily, the UoN COVID testing laboratories provided me an ideal opportunity for this, however, it further limited the amount of time I had available for writing my thesis and impaired my focus due to the additional expenditure energy it required. I got distracted from my PhD work constantly by all kinds of unexpected changes in my life, such as family issues and personal health issues including a long-term dental treatment involving travelling frequently to another city. Luckily, whenever I felt stressed or uncertain, my supervisors were always there looking after me and supporting me. I could not have made it without them.

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List of abbreviations

bp	base pair
CF	cystic fibrosis
CPA	common polysaccharide antigen
CV	column volume
DNA	Deoxyribose nucleic acid
DP	Different power
MDR	Multi-drug resistant(ce)
dH ₂ O	deionised water
EDTA	ethylenediaminetetraacetic acid
GST	glutathione S-transferase
H	hour(s)
His	histidine
ICP-MS	Inductively coupled plasma mass spectrometry
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl β -D-1-thiogalactopyranoside
kb	kilobases
kbp	Kilobase pairs
kDa	Kilo Dalton
LB	Luria Bertani
LPS	lipopolysaccharide
Mbp	million base pairs
mg	milligram
min	minutes
ml	millilitres
M	Molar
MWCo	Molecular Weight Cut-off
OD ₆₀₀	optical density at a wavelength of 600 nanometers
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction

PDB	Protein Databank
PMSF	phenylmethanesulfonyl fluoride
pmf	proton motive force
rpm	revolutions per minute
sec	seconds
Selenomethionine	SeMet
SDS	sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
TBE	Tris-boric acid-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
µg	microgram
µl	microliter
µM	micromolar
UV	Ultra violet
WHO	World Health Organisation
W/T	wild-type
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

Chapter 1. General Introduction

1.1 *Pseudomonas aeruginosa*

1.1.1 Characteristics of *P. aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, rod-shaped and monoflagellated bacterium which forms colonies with a pearlescent appearance. It is non-fermentative, catalase positive, oxidase positive, ammonia producer, and usually aerobic (Vasil 1986). It is a ubiquitous microorganism, which can grow well at 25°C to 37°C and survives under a variety of environmental conditions including anaerobically when nitrate, citrate and/or arginine are present. These conditions commonly feature in soil, water, plants and humans (Vasil 1986, Wu, Jin et al. 2015). Almost all the *P. aeruginosa* strains are motile due to a polar flagellum, which is also crucial for chemotaxis and adhesion and allows the bacterium to colonize a wide range of environment (Haiko and Westerlund-Wikström 2013, Jayaseelan, Ramaswamy et al. 2014). *P. aeruginosa* belongs in the *Pseudomonadaceae* family, which contains *P. alcaligenes*, *P. anguilliseptica*, *P. citronellolis*, *P. flavescens*, *P. jinjuensis*, *P. mendocina*, *P. nitroreducens*, *P. oleovorans*, *P. pseudoalcaligenes*, *P. resinovorans* and *P. straminea* (Jayaseelan, Ramaswamy et al. 2014). It can be easily distinguished from the other pseudomonads by its green coloured bacterial colony, which is caused by the blue-green phenazine pigment called pyocyanin. (Wu and Li 2015, Wu, Jin et al. 2015, Galdino, Branquinha et al. 2017). The guanine and cytosine content of the genome of *P. aeruginosa* can reach 65-67%

(Schmidt, Tümmler et al. 1996, Klockgether, Cramer et al. 2011). The genome is made up of a single circular chromosome and a variable number of plasmids. The core genome of *P. aeruginosa* strains consists of 5,109 protein-coding genes (Poulsen, Yang et al. 2019). It is the major part of the genome and highly conserved and has been defined in different ways including genes that can not be inactivated (ie are essential) or because of their distribution across many genomes (Whelan, Hall et al. 2021). The complete set of *P. aeruginosa* genes is named pangenome and consists core gene and accessory gene (Whelan, Hall et al. 2021). There are 321 core essential genes that have been identified using transposon insertion sequencing. They represent 6.6% of the genome and constitute a high-priority list of candidate targets for drug discovery (Poulsen, Yang et al. 2019). However, the essentiality of a gene can depend on both the genetic background and growth condition, which brings challenge to studies on determination of target essentiality. For instance, the pursuit of inhibitor of targets could either not be essential in the species or not essential in a subset of strains (Poulsen, Yang et al. 2019). The accessory genome, which consists of plasmids and of blocks of DNA ranging from a few hundred bases to over 200 kilo base pairs (kbp) integrating into the chromosome at various loci, accounts for the diversity of the genome (Klockgether, Cramer et al. 2011) and the variety of gene operons producing antibiotic resistance mechanisms (Jacoby 1986).

Chapter 1

1.1.2 *P. aeruginosa* infections

P. aeruginosa is primarily an opportunistic pathogen, and its ability to thrive in moist habitats and water resources provides it with many potential reservoirs of infection. It causes diseases in plants, animals, and humans (Mena and Gerba 2009) resulting in serious infections in immunocompromised patients with cancer, patients with severe burns and is notorious for causing chronic disease in the lungs of patients with genetic defects such as cystic fibrosis (CF) (Wu and Li 2015, Wu, Jin et al. 2015). Data set for 2012 from the UK Cystic Fibrosis Registry revealed that in the UK, *P. aeruginosa* is the leading cause of mortality in patients with cystic fibrosis, which is inherited, chronic, progressive disease found 1 in 2500 live births along with around 200-300 new diagnoses each year (UK Cystic Fibrosis Registry Annual data report 2012, <https://www.cysticfibrosis.org.uk/sites/default/files/2020-12/2012%20Registry%20Annual%20Data%20Report%20SUMMARY.pdf>). CF is one of the most common fatal genetic disorders caused by mutations in the gene encoding the CF transmembrane regulator. It is suggested that its characteristic blue-green pigment, pyocyanin, not only contributes to the persistence of this bacterium in the lungs of CF patient, but also disrupts many mammalian cell functions, such as cell respiration, epidermal cell growth and calcium homeostasis (Caldwell, Chen et al. 2009, Wu, Jin et al. 2015).

P. aeruginosa is one of the leading causes of nosocomial infections, resulting in a fatality rate up to 50% (DeRyke, Lodise et al. 2005). Of particular importance is the ability of *P. aeruginosa* to disrupt the lung

barrier and enter the bloodstream leading to bacteraemia. The mechanisms used by *P. aeruginosa* to breach both epithelial and endothelial barriers have been well studied (Berube, Rangel et al. 2016). The pathogenicity of *P. aeruginosa* is complex, and involves a wide range of virulence factors including quorum sensing, Type III (T3SS) and Type VI secretion systems (T6SS), flagella and type IV pili (Huang, Shao et al. 2019) (Figure 1.1). The regulatory network that *P. aeruginosa* has to control the production level and timing of these virulence factors is similarly complex, involving specific regulatory factors, hierarchical networks and control at different levels including metabolic, transcriptional and post-transcriptional (Balasubramanian, Schneper et al. 2013, Huang, Shao et al. 2019). Many of the virulence factors are secreted out of *P. aeruginosa*, where they are best placed to interact with hosts and scavenge from the environment. *P. aeruginosa* has multiple secretion machineries and one of the most important is the type III secretion system (T3SS), which is commonly found among *P. aeruginosa* clinical isolates (Berube, Rangel et al. 2016). The T3SS has been reported to contribute to pathogenesis of a few diseases in animal models, such as acute pneumonia, bacteraemia, burn infections and peritonitis (Hauser 2009). This secretion system is embedded in the inner membrane and responsible for injecting effector proteins into host cells (Hauser 2009). Another secretion system, T6SS, is one of the newest studied, which is relevant to interspecies interactions. It is a contractile injection system similar to bacteriophages that is located in the bacterial envelope. This system is present in more than 25% of all Gram-negative

bacteria. Similar to the T3SS, the T6SS is tightly regulated by the GacAS/Rsm pathway (Bingle, Bailey et al. 2008, Huang, Shao et al. 2019, Allsopp, Bernal et al. 2020).

P. aeruginosa is able to grow and coexist with a wider range of other microorganisms, including bacteria, fungi and viruses (Figure 1.2) and the interactions between these microbial communities can affect a patient's prognosis and worsen the outcome of the patient (Murray, Connell et al. 2014, Cendra and Torrents 2021). For instance, *P. aeruginosa*, together with *Staphylococcus aureus*, *Haemophilus influenza*, *Streptococcus pneumonia*, *Enterobacteriaceae*, *Moraxella catarrhalis* and *Acinetobacter baumannii*, was identified as one of the common co-infecting pathogens in Covid-19 patients in many regions in the world, causing exacerbation of illness and therapeutic complications (Contou, Claudinon et al. 2020, Hughes, Troise et al. 2020, Lansbury, Lim et al. 2020, Vaughn, Gandhi et al. 2020, Qu, Cai et al. 2021).

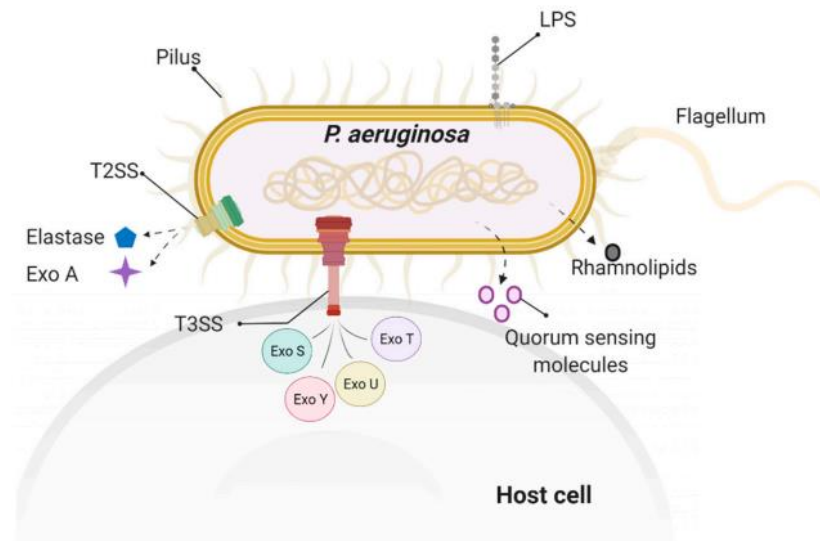


Figure 1.1 Virulence factors produced by *P. aeruginosa*.

P. aeruginosa possesses a full arsenal of virulence factors, including surface factors, such as flagellum, pilus and lipopolysaccharide (LPS); secreted factors, such as type III secretion system (T3SS) and rhamnolipid; and quorum-sensing factor (Wagener, Hu et al. 2021).

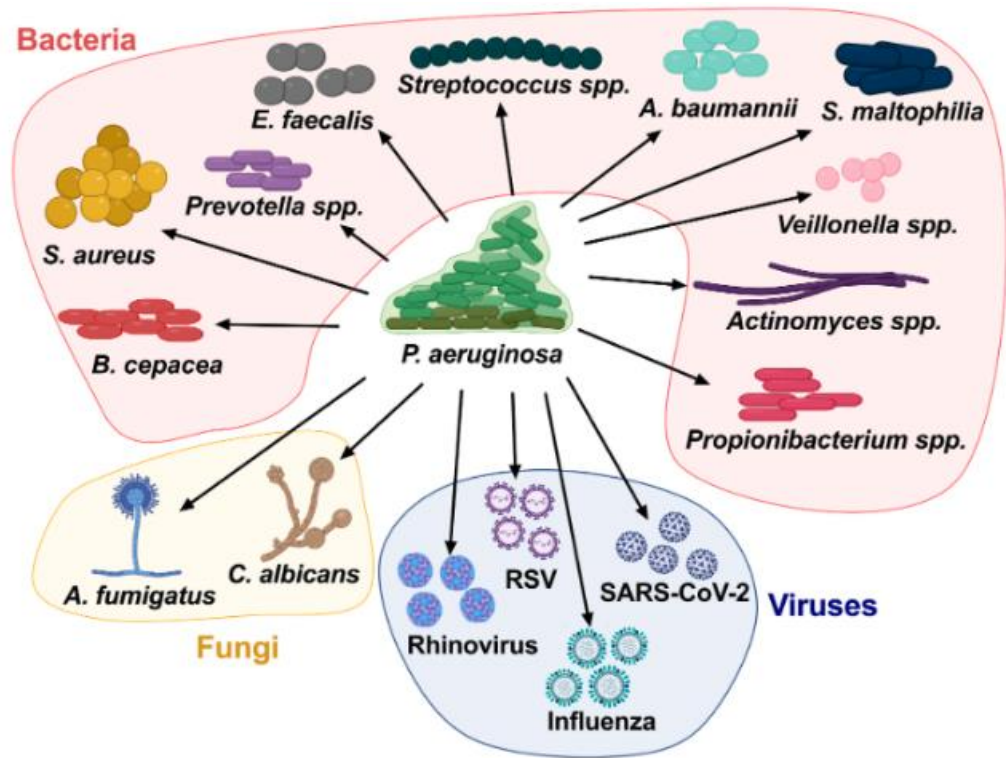


Figure 1.2 An outline of the clinically relevant microbial interactions of *P. aeruginosa*.

The outline of the clinically relevant microbial interactions of *P. aeruginosa* with a wide range of microbes, including bacteria, viruses and fungi (Cendra and Torrents 2021).

1.1.3. The current available treatment for *P. aeruginosa* infections

The current treatments for *P. aeruginosa* infections are: antibiotics, combination therapy (e.g. co-administration of anti-virulence and antibiotic options), and the direct inhalational therapy to the site of infection including inhaled antimicrobial agents (carbenicillin, gentamicin, cephaloridine, tobramycin, colistin, polymyxin B) and amikacin alone or in combination with parenteral antibiotics (Hancock and Speert 2000). Worryingly, treatment is getting more challenging due to the rise of multidrug-resistant strains and thus the conventional antibiotic therapies becoming increasingly ineffective. The main driving forces for the rising

prevalence of multidrug-resistant strains are overuse and misuse of antibiotics (Pang, Raudonis et al. 2019). The conventional antibiotics used to treat *P. aeruginosa* infections with success include aminoglycosides, semisynthetic penicillins, the third generation cephalosporins, quinolones and carbapenems. These are prone to be impacted by multidrug resistance (MDR) determinants. MDR is defined as simultaneous non-susceptibility to at least one antibiotic in at least 3 classes for which *P. aeruginosa* is generally expected to be susceptible (Tamma, Aitken et al. 2020). Moreover, *P. aeruginosa* infection has been classed as “difficult-to-treat” resistance (DTR) according to the guidance document established by the ‘Infectious Diseases Society of America’ (IDSA) in 2018. *P. aeruginosa* is DTR because it is non-susceptible to antibiotics that include piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, meropenem, imipenem-cilastatin, ciprofloxacin and levofloxacin. The recommended antibiotic treatment options and alternative treatment (if the first-line options not available or tolerated) for the “Difficult-to-Treat” *P. aeruginosa* have been outlined (Tamma, Aitken et al. 2020). The combination of (i) resistance to nearly all the currently available antibiotics, and (ii) prevalence in nosocomial settings, has led to *P. aeruginosa* being referred to as a ‘superbug’ (Hirsch and Tam 2010, Breijyeh, Jubeh et al. 2020).

Unfortunately, the development of new antibiotics is very slow while different combinations of antibiotic such as a combination of a semisynthetic penicillin and an aminoglycoside used for their possible synergistic effect is one of the strategies for improved treatment

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(Hancock and Speert 2000, Pang, Raudonis et al. 2019). The alternative therapeutic strategies for the treatment against *P. aeruginosa* infection are listed in Table 1.1 (Pang, Raudonis et al. 2019), however, due to the recurrence and chronicity of the infection, we can only hope to tackle it in the future with the continuous development and improvement of newer, efficient strategies for antimicrobial treatment (Cendra and Torrents 2021).

Therapeutic Strategy	Advantages	Disadvantages	References
Quorum sensing inhibition	Prevention or reduction of biofilm formation, decrease of bacterial virulence, low risk of development of bacterial resistance	Narrow spectrum, unintentional effect on beneficial bacteria	Reuter et al., 2016, Rasmussen and Givskov, 2006
Lectin inhibition	High stability, low risk of development of bacterial resistance	Narrow spectrum due to expression of more than one type of adhesin by bacteria	Krachler and Orth, 2013, Ofek et al., 2003
Iron chelation	Availability of FDA approved drugs, easy administration	Toxicity	Moreau Marquis et al., 2009, Chitambar, 2010
Phage therapy	Replication at infection site, high specificity to target bacteria without effects on commensal flora, less side-effects, bactericidal activity against antibiotic resistant bacteria, easy administration, delivery of antimicrobial agents to bacteria	Phage clearance after treatment, impurity of phage preparations, poor stability of phage preparations and lack of knowledge on the mode action of phages, bacterial resistance	Ly chatain, 2014, Sulakvelidze et al., 2001, Vandenheuvel et al., 2015
Vaccine strategy	Improvement of host immunity, prevention of infection	Low efficiency, no licensed vaccine	Priebe and Goldberg, 2014
Nanoparticles	High penetrability into bacterial membrane, disruption of biofilm formation, multiple antimicrobial mechanisms, good carriers of antibiotics	Toxicity	Wang et al., 2017, Elsaesser and Howard, 2012
Antimicrobial peptides	Broad spectrum activity, rapid killing kinetics, low levels of induced resistance, low toxicity to host	Hemolytic activity to host cells, reduced activity based on salt, serum, and pH sensitivity, susceptibility to proteolysis, high cost of production	Gordon et al., 2005, Hancock et al., 2016, Aoki and Ueda, 2013, Gordon et al., 2005
Electrochemical scaffold	Disruption of bacterial biofilms, increase of antibiotic penetration	Difficulty in implantation to clinical trials	Sultana et al., 2015

Table 1.1 Summary of alternative *P. aeruginosa* treatment strategies (Pang, Raudonis et al. 2019)

1.1.4. Multi-drug resistance; the influence of the pandemic and mechanisms in *P. aeruginosa*

Antibiotic resistance is one of the most serious threats that humans are facing nowadays (Breijyeh, Jubeh et al. 2020). The number of infections caused by multi-drug resistant Gram-negative bacteria has significantly increased (Exner, Bhattacharya et al. 2017). It is worsening and has been shown to be accelerated by COVID-19 (Hsu 2020). Data has revealed 72% of COVID-19 patients were receiving broad-spectrum antibiotics without showing any clinical need in the US (Strathdee, Davies et al. 2020). A systematic review showed that 7% of patients in hospital had a bacterial co-infection or secondary infection in a total of 3338 covid-19 patients and the prevalence was higher in the mixed ward/ICU (Langford, So et al. 2020). Notably, the use of antibiotics in combination with the drug hydroxychloroquine as therapies for COVID-19 was encouraged by some media and political leaders despite a lack of evidence (Hsu 2020). Another concern that has emerged during the pandemic that is relevant to *P. aeruginosa*, is the release of potential antimicrobial products from household disinfectants and hand sanitisers into water and soil environments that contain whole microecosystems that can have negative impact on the enrichment of multidrug resistance and resistance dissemination (Rezasoltani, Yadegar et al. 2020).

Unlike Gram-positive organisms, Gram-negative bacteria gain some antimicrobial resistance due to the structure of their cell envelope, which provides a barrier to antimicrobial penetration and can incorporate pumps that prevent the retention of chemical agents inside the bacterial

cell. The cell envelope consists of three layers including the outer membrane (OM), the peptidoglycan and the inner membrane (IM) (Figure 1.3) (Breijyeh, Jubeh et al. 2020). The major mechanisms used by *P. aeruginosa* to counter antibiotic attack can be classified into intrinsic resistance, acquired resistance and adaptive antibiotic resistance (Hirsch and Tam 2010). Intrinsic antibiotic resistance mechanisms include alterations to the permeability barrier of the outer membrane, alteration of the targeted drug binding sites, production of drug inhibition enzymes and induction of efflux mechanisms that pump antibiotics out of the cells.

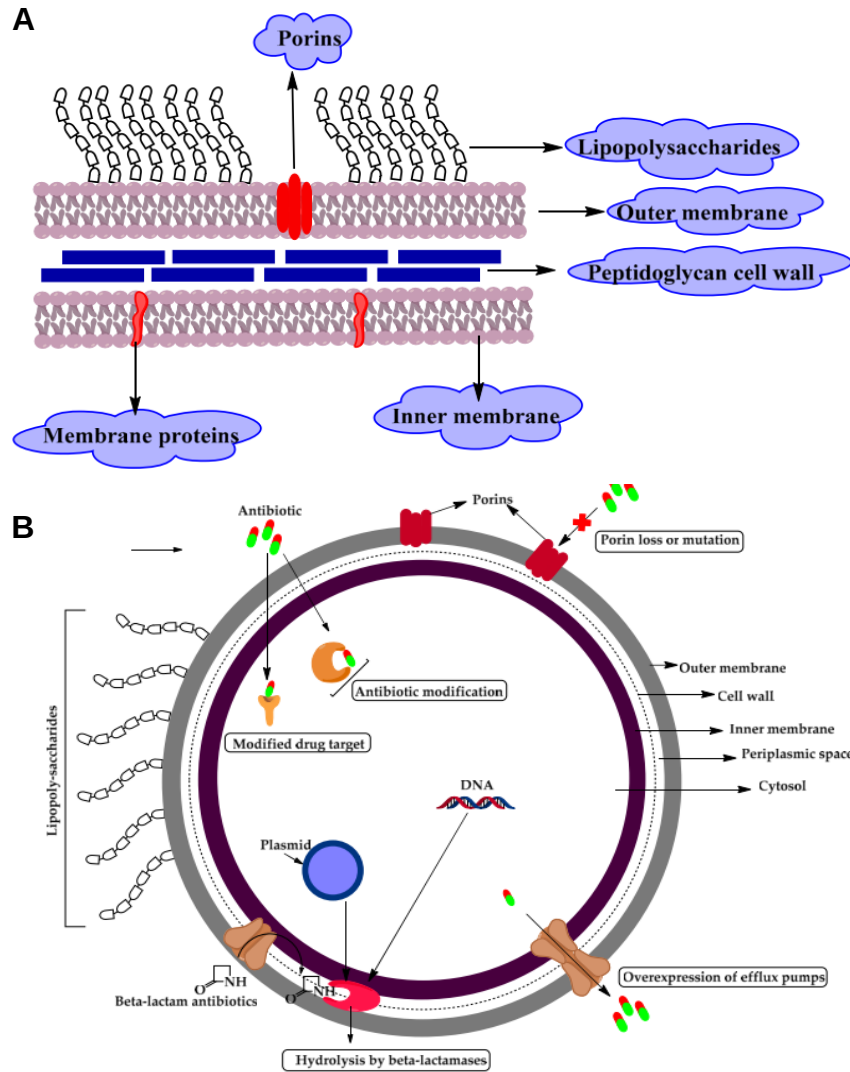


Figure 1.3 The structure of Gram-negative bacteria cell wall and the mechanism of their antibiotic resistance.

Panel A- The cell wall of Gram-negative bacteria consists of three layers: the outer membrane (OM), the peptidoglycan and the inner membrane (IM). Lipopolysaccharides (LPS), also known as endotoxin, binds to is located on the outside of the OM. Porins sit inside the OM allowing small molecules and small saccharides to pass through. Membrane proteins are inserted into the IM and are responsible for a variety of functions in the cell, ranging from metabolite exchange to cell signalling (Dalbey, Wang et al. 2011). Panel B- Increase of intrinsic resistance is caused by mutations in chromosomal genes resulting in the expression of antibiotic inactivating enzymes and non-enzymatic path. Acquired resistance is caused by transfer of mobile genetic elements, which carry resistance genes. Figure adapted from Breijyeh, Jubeh et al. 2020.

One mechanism of resistance that is well represented in *P. aeruginosa* is that conferred by efflux pumps, which has been found in some other microorganisms as well, such as *E. coli* and *Candida albicans* (*C. albicans*). Efflux pumps enable the *P. aeruginosa* to remove toxic substances, including antimicrobial agents, metabolites and quorum sensing signal molecules in order to regulate their internal environment. *P. aeruginosa* has at least 12 efflux pumps, among which, MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY have been widely studied (De Kievit, Parkins et al. 2001, Soto 2013). MexAB-OprM was reported as the only one that is constitutively expressed in wild-type, and therefore contributes to the intrinsic resistance of this organism (De Kievit, Parkins et al. 2001). The genes encoding those pumps are arranged as operons with 1). the first gene encoding a membrane fusion protein associating with the cytoplasmic membrane (MexA, MexC, MexE and MexX); 2). the second gene encoding the transporter (MexB, MexD, MexF and MexY), which export the substrate across the inner membrane; 3). the third gene encoding an outer membrane protein (OprM, OprJ and OprN), which facilitates passage of the substrate across the outer membrane (De Kievit, Parkins et al. 2001). Efflux pumps can be divided into six families: 1). The ATP-binding cassette superfamily; 2). the major facilitator superfamily; 3). The multidrug and toxic compound extrusion; 4). The small multidrug resistance family; 5). The resistance-nodulation-division superfamily; and 6). The drug metabolite transporter superfamily (Soto 2013). The major clinically relevant efflux systems in *P. aeruginosa* belong to the 5th superfamily, which is composed of a cytoplasmic

membrane pump, a periplasmic protein and an outer membrane protein channel.

P. aeruginosa bears a chromosomally encoded β -lactamase, which catalyses the hydrolysis of β -lactam antibiotics. This enzyme contributes to the intrinsic resistance against a number of β -lactams (Nikaido 1985). Having a fundamental level of intrinsic antibiotic resistance plus the ability to acquire additional resistance (either adaptive or acquired) means that, like other bacteria such as *Salmonella* and *E. coli* (Aslam, Wang et al. 2018), *P. aeruginosa* can evolve toward a higher level of antibiotic resistance. Exposure to antibiotics will select for chromosomal mutations that are associated with antibiotic resistance e.g. acquisition of an efflux pump encoding cassette or point mutation that enhances an intrinsic resistance mechanism. In the clinic this can lead to the failure of treatment of severe infections using nearly all available antipseudomonal agents. The mutations selected for include those resulting in overexpression of efflux pumps, reduction of antibiotic uptake, hyperproduction of β -lactamases and alteration of antibiotic targets (López-Causapé, Cabot et al. 2018). Interpatient spread of antimicrobial resistance mutations linked to the transmission of epidemic CF strains has already been demonstrated (López-Causapé, Sommer et al. 2017). Moreover, the mutation rate can increase under specific conditions, such as following exposure to DNA-damaging agents and during growth in biofilms, such as occurs in chronic infections like those in CF lungs (Tanimoto, Tomita et al. 2008, Breidenstein, de la Fuente-Nunez et al. 2011). The acquired resistance mechanisms include the processes of

horizontal transfer of resistance genes and mutational changes that lead to their introduction into strains (Miller, Zhu et al. 2009, Breidenstein, de la Fuente-Nunez et al. 2011, Pang, Raudonis et al. 2019). Adaptive resistance mechanisms are those induced through external/environmental stimuli, such as stress factor and the presence of certain antibiotic and it is unstable and reversible. They include biofilm-mediated resistance and persister cells in antibiotic resistance (Pang, Raudonis et al. 2019, Langendonk, Neill et al. 2021). The classification and mechanisms of different types of resistance in *P. aeruginosa* are listed in (Table 1.2).

Class of resistance	Stable	Inheritable	Dependency on environment	Mechanisms
Intrinsic	+	+	-	Low outer membrane permeability, β -lactamase production and efflux pump overexpression
Acquired	+	+	-	Horizontal transfer, mutations leading to reduced uptake and efflux pump overexpression
Adaptive	-	-	+	Gene expression changes including β -lactam and efflux pump overexpression owing to factors triggering expression of regulatory genes

Table 1.2 The characteristics of different types of resistance of *P. aeruginosa*.

Resistance with (+) or without (-) the property. Adapted from Table 1, (Breidenstein, de la Fuente-Nunez et al. 2011).

P. aeruginosa is associated with an ever-widening spectrum of infections causing very high morbidity and mortality, especially in intensive care units (ICU) (Kerr and Snelling 2009). The National Nosocomial Infections Surveillance system has revealed that from 1986 to 2003, *P. aeruginosa*

was found to be the second most common cause of pneumonia (18.1%), the third most common cause of urinary tract infection (16.3%) and the eighth most frequently isolated pathogen from the bloodstream (3.4%). An epidemiological study on isolated strains belonged to the serotype of *P. aeruginosa* P12 from 67 patients (with 64 of them in adult ICU) at a French hospital, found that those strains were resistant to almost all potential active antibiotics except colistin (Dubois, Arpin et al. 2001). A report in 2013 on antibiotic resistance threats in the United States showed that of 51,000 pseudomonas infections per year, 6,700 (13%) were multidrug-resistant (*P. aeruginosa*) causing 440 deaths. In 2017, the World Health Organization (WHO) listed *P. aeruginosa* as one of the antibiotic resistant bacteria in the top priority category (critical) which urgently call for new therapies (<https://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/>).

The continued emergence and spread of antimicrobial resistant strains makes it even more difficult to define a suitable therapy for these infections (Kerr and Snelling 2009), which have been associated with a greater number of more prolonged hospital stays and persistent infections (Kollef, Sherman et al. 1999, Ibrahim, Sherman et al. 2000). A global effort is required to develop new antibiotics or modify existing ones to fight MDR pathogens such as *P. aeruginosa* (Breijyeh, Jubeh et al. 2020).

1.2 Bacteriocins

The study of narrow-spectrum antagonistic proteins has gained lots of attention recently due to their potential to act as novel antimicrobials

without damaging non-target microbiota. They therefore offer the possibility to alleviate the increasing problem of multidrug resistance (Ghequire and De Mot 2014). Bacteriocins were first discovered by Gratia in 1925 (Gratia 1925) and are one of the three forms of protein-mediated bacterial antagonism identified. The other two are contact-dependent inhibition (CDI) and type VI secretion (Klein, Wojdyla et al. 2016). Bacteriocins are widely used as food preservatives (Ghraiiri, Chaftar et al. 2012) and treatment for malignant cancers nowadays (Lancaster, Wintermeyer et al. 2007). They are water-soluble ribosomally synthesized peptides or proteins produced by many bacteria that can kill the closely related strains or non-related bacteria (Yang, Lin et al. 2014). They are naturally produced by bacteria in response to environmental or competitive stress. The producing bacteria avoid harming themselves due to the protection offered by a specific cognate immunity protein (Kim, Tarr et al. 2014). It has been suggested that 99% of all bacteria may produce one or more bacteriocins (Klaenhammer 1988). Gram positive bacteria usually produce small peptide bacteriocins and the Gram negatives can produce both peptide and protein antibiotics (Kleanthous 2010).

Protein bacteriocins are large (~40-80 kDa) polypeptides consisting of multiple domains, that contain a cytotoxic domain with a narrow killing spectrum at their C terminus and an immunity domain responsible for protecting the producer cell from cell suicide (White, Joshi et al. 2017, Atanaskovic and Kleanthous 2019). Bacteriocins are divided into four classes due to their different biochemical and genetic characteristics.

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Class I and II bacteriocins are membrane-active, low molecular mass peptides of 3-10 kDa, whereas the class III bacteriocins are heat-labile with high molecular mass of over 30 kDa and class IV bacteriocins are complex peptides with lipid or carbohydrate moieties for their activity (Lee and Kim 2011). Before a bacteriocin can penetrate a cell envelope, it needs to bind to a specific receptor on the surface of the bacterium which defines its spectrum of activity (Atanaskovic and Kleanthous 2019). The receptor binding of bacterial surfaces, which allow the bacteriocins to pass through the membrane to perform cytotoxic activity, is a crucial part for the bactericidal mechanism of bacteriocins (Yang, Lin et al. 2014). To conduct cell killing, bacteriocins have to cross the multi-layered cell envelope of target cells by parasitizing host proteins involved in nutrient and metabolite trafficking to perform one of the actions: (i) forming pores in the inner membrane, (ii) acting as nucleases to degrade DNA or RNA, (iii) interfering with cell wall biosynthesis (Atanaskovic and Kleanthous 2019). Gram negative bacteriocins are divided into two groups: Group A and Group B (Figure 1.4). The former group utilises an uptake/import system consisting of periplasmic and IM proteins including Pal, TolA, TolB, TolR and TolQ, which is called the Tol-Pal system. The latter group utilizes the Ton system, which is made up of TonB, ExbB and ExbD proteins (Atanaskovic and Kleanthous 2019).

Though bacteriocins are safer and an environmentally friendly alternative to conventional broad-spectrum antibiotics because they leave the normal bacterial flora intact, it is probably necessary to use them in combinations rather than as single agents in order to enhance their ability

to kill target bacteria with different cell surface receptors and cell envelopes. Therefore, access to a wider range of bacteriocins will help with the development of novel antimicrobials to fight against the increasing drug resistant pathogen as well as to design preventative anti-infectives for use in agriculture (White, Joshi et al. 2017, Chai, Rooney et al. 2021). So far, colicins produced by *Escherichia coli* are the most extensively studied bacteriocins (Micenková, Bosák et al. 2019) followed by the well-studied S-type pyocins produced by *Pseudomonas aeruginosa* (Ghequire and De Mot 2014).

1.3 Colicin

E. coli releases colicins into the extracellular medium at times of nutrient or environmental stress (Kühlmann, Pommer et al. 2000). Colicins are capable of killing other *E. coli* strains as well as closely related bacteria, such as *Shigella* and *Salmonella* (Parret and De Mot 2002). All the well-characterized colicins are plasmid-encoded. The colicins are domain-containing proteins, apart from colicin J_s, which lacks identifiable domains (Smajs and Weinstock 2001, Micenková, Bosák et al. 2019). The release of colicins is mainly caused by the action of the bacteriocin-release factor, which is also called lysis protein, encoded by a lysis gene on the plasmid (Snijder and Dijkstra 2000). Colicins consist of three main domains including an N-terminal translocation domain, a central receptor binding domain and a C-terminal cytotoxic domain, which varies in size (Sharp, Bray et al. 2017). There has also been the suggestion of a conserved colicin pyocin domain (CCPD) adjacent to the cytotoxic domain (McCaughey, Josts et al. 2016).

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The plasmids containing colicin genes are found in 30% of *E. coli* strains in nature (Riley and Gordon 1992). As mentioned, the Group A colicins parasitize the Tol system (Figure 1.4A), while group B ones utilise the Ton system (Figure 1.4 B). Both Ton and Tol systems are coupled to the proton motive force (PMF) in the periplasm responsible for energy transduction for active transport or maintenance of outer envelope integrity for Ton and Tol systems, respectively (Housden, Loftus et al. 2005). For instance, the most well-studied translocation system of the Group A bacteriocin, colicin E9 recruits the outer membrane receptor BtuB, a porin translocator OmpF or OmpC and its periplasmic target TolB to pass through a porin channel. Then it engages the PMF-coupled Tol-Pal system, which is acting as an energy source (Housden, Loftus et al. 2005) (Figure 1.4 C). In contrast, the Group B bacteriocin, colicin B binds to the fifth loop of the outer membrane transporter, FepA, in the TonB system. It then utilises proteins, TonB, ExbB and ExbD of the TonB transport system to transduce the PMF to TonB dependent transporters to energize the translocation process (Figure 1.4 D) (Lazdunski, Bouveret et al. 1998, Devanathan and Postle 2007).

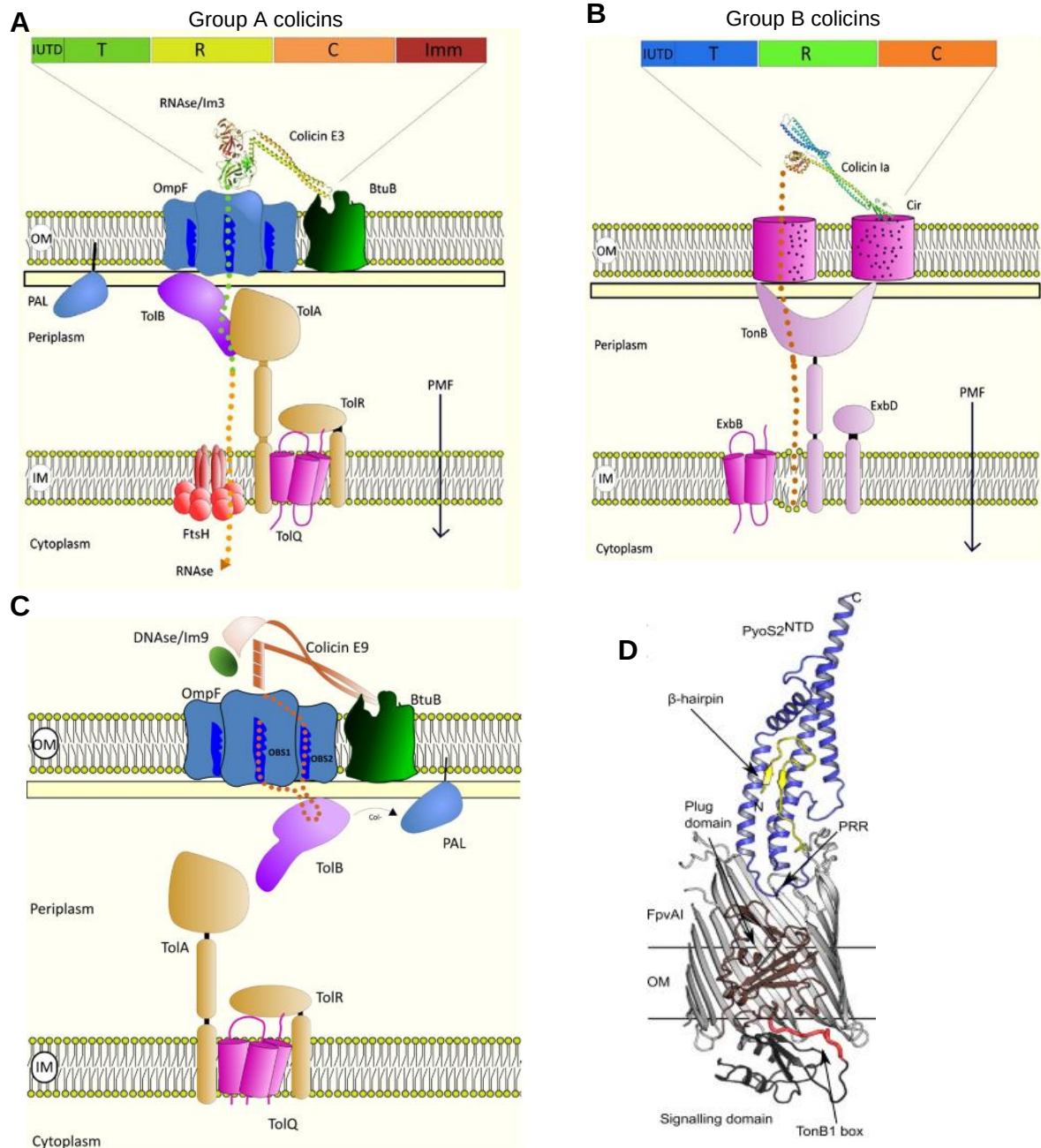


Figure 1.4 The Ton and Tol systems exploited by Group A and B bacteriocins.

Panels A-C (adapted from Fig 1 (Kim, Tarr et al. 2014)) represent the import pathway of group A, group B and colicin E9 bacteriocins. Panel D (adapted from Fig 1C (White, Joshi et al. 2017)) shows the crystal structure of pyocin S2 (blue) in complex with FpvA1 (grey). Colicins are categorized as group A and B, which parasitize the Tol system and Ton system, respectively. Colicins imported by both systems generally kill cells, either by depolarizing the inner membrane or by cleaving nucleic acids in the cytoplasm. The figure does not depict all colicin cytotoxic

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activities, or all of the proteins that have been implicated in colicin import. Panel A- The translocation paths of colicins into *Escherichia coli*. All colicins require an outer membrane receptor to localize the toxin to the cell surface, which allows the intrinsically unstructured translocation domain (IUTD) (green dots) to pass through the outer membrane to capture TolB. Then the N-terminus domain of colicin dissociates from TolB and interact with TolA and then with TolQ and/ or TolR before the translocation of C-terminal domain of colicin and formation of a pore in inner membrane. Panel B- Speculative model of import of Group B. The channel-forming colicin Ia uses outer membrane protein Cir as its primary receptor recruiting TonB machinery. The N-terminus domain of colicin interact with TonB by penetrating the outer membrane of target cell, energized by ExbB-ExbD-TonB complex. The N-terminus domain of colicin will then translocate to the cytoplasm. Panel C. Model of the colicin E9 translocon. The colicin E8 DNase-immunity complex recruits the translocator protein OmpF after binding to receptor protein BtuB. The two binding site of OmpF OBS1 and OBS2 occupy two of the three subunits of OmpF to hold TolB in place. Panel D. The crystal structure of the N-terminal domain of pyocin S2 in complex with FpvAI is solved by molecular replacement to a resolution of 2.8 Å. The N-terminal domain of pyocin S2 (blue) is composed of five α -helices and a β -hairpin (yellow). The primary receptor FpvAI for pyocin S2 and TonB1 box are in grey and red, respectively.

Until now, 25 different colicins have been identified (Micenková, Bosák et al. 2019). These colicins can be distinguished by their specificity to bind a particular cell surface receptor. For example, the well-known E group of DNase colicins. There are nine types of colicins in group E, Colicin E1- E9. Classification on their cytotoxic activity, e.g. pore-forming (E1), lipid II degradation, DNases (E2, E7, E8 and E9), tRNases and rRNases (E3, E5 and E6) (Garinot-Schneider, Pommer et al. 1996, Shushan and Kosloff 2021). Like E9, the colicins E2, E7 and E8 and the RNase colicins E3, E4 and E6 all bind the product of the *btuB* gene responsible for vitamin B12 transport in *E. coli* (Garinot-Schneider, Pommer et al. 1996, Shushan and Kosloff 2021).

Although some of the E colicins have similar physical properties and share the same receptor, they have different modes of action, such as colicin E2 and E3. The former causes DNA degradation, while the latter is responsible for the specific inhibition of protein synthesis in sensitive cells (Schaller and Nomura 1976). The N-terminal region of E group colicins is 3/4 size of the full-length colicin (60kDa), and is responsible for receptor binding and the translocation of C-terminal cytotoxic domain (Ohno-Iwashita and Imahori 1980). Even colicins with similar activities have subtly different features e.g. both colicin E7 (Ko, Liao et al. 1999) and E2 have non-specific DNase activity, but the latter one has activity against both single- and double-stranded DNA. In addition, a divalent metal ion in the active site of the nuclease is essential for its activity (Schaller and Nomura 1976). Whilst it has been reported that E2 and E7

use magnesium (Walker, Lancaster et al. 2004), the evidence is unconvincing.

The small co-expressed immunity protein of colicins binds tightly to the toxic protein to protect the producing strain from its own cytotoxic activity (Riley 1998). The sequence of the DNase domains of colicin E2, E8 and E9 are approximately 80% identical, while sequences of their immunity domains are approximately 50% identical (Ko, Liao et al. 1999). The binding affinities of the two cognate proteins was reported to be the strongest protein-protein interactions in the nature (Wallis, Leung et al. 1995) and the dissociation of the complexes requires unfolding of the protein, which happens during the cell mediated translocation process (Lazdunski, Bouveret et al. 2000). This high binding affinity has been exploited for overproduction and purification of the colicins. While other non-specific endonuclease could not overproduced without a tightly controlled expression system, the co-production of the DNase and immunity domains of colicins can be tolerated by heterologous hosts (Worrall and Connolly 1990, Garinot-Schneider, Pommer et al. 1996). The binding affinities of colicins for their cognate immunity proteins are 6-10 orders of magnitude higher than to other non-cognate immunity proteins (Shushan and Kosloff 2021). Critical to this is the ionisation state of the histidine residue in the active site since the removal of histidine by site-directed mutagenesis has a huge impact on the dissociation constant for the DNase-immunity complex (Schreiber and Fersht 1993). A 'dual recognition' system has been identified at the interface of the colicin and immunity protein. Conserved residues act as the binding-site

anchor and variable residues define the binding specificity (Parret and De Mot 2002). This thermodynamic interplay between conserved and variable sequences may underpin specificity in other protein-protein interactions (Kühlmann, Pommer et al. 2000). The inhibition of endonuclease activity by colicin E9 and E7 immunity proteins was studied by Kleanthous and Kühlmann et al., revealing that the immunity blocks not only the DNA, but also polyanion binding to the E9 DNase (Kleanthous, Kühlmann et al. 1999). The immunity proteins in DNase colicins are conserved in both sequence and structure, whilst their affinities for the cognate complex differ by ten orders of magnitude. It is possible that complex may be affected by pH, temperature and salt complex (Wallis, Moore et al. 1995, Kühlmann, Pommer et al. 2000).

The three-dimensional structures of colicins derived from X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy have contributed to the understanding of the principles of protein recognition during complex formation with the Immunity domain. For instance, colicin E7 DNase uses the peptide bonds (mainchains) to interact with the conserved residues of its cognate immunity and amino acid side chains to interact with the variable residues. Moreover, the binding specificity of the DNase and immunity protein of colicins is probably mediated predominantly by the immunity protein's variable arms. The immunity protein variable arms are parts of the structure that extend out from the main body of the protein, and which have variable sequences, lengths and relative positions. These variable arms

contribute to the tight binding between the two proteins (Ko, Liao et al. 1999).

Within the DNase domain is a metal-bound water molecule which likely serves as a nucleophilic hydroxide ion which attacks the phosphate atom of DNA, though the metal binding site is distant from the protein-protein interaction interface (Ko, Liao et al. 1999). The specific interaction between the C-terminal colicin DNase and its cognate immunity protein is used as a model system for the study of protein-protein interactions which was first demonstrated by Fredericq in 1957 (James, Kleanthous et al. 1996). Unlike the enzymes, such as proteases, ribonucleases and kinases, a surprising feature of colicin E9 is that the immunity protein does not bind directly to the active site of its cognate DNase protein. Moreover, the Colicin E9 immunity has a hyper efficient mechanism of inhibition, which folds within milliseconds and associates with the DNase at an exosite of the active site to form an inactive complex. It is speculated that the other toxic colicin enzyme systems function the same way (Kleanthous, Kühlmann et al. 1999).

1.4 Pyocin

A bacteriocin originating from *P. aeruginosa* was first described by Francois Jacob, who found an inducible compound released after a mutagenic agent was used as treatment against a *P. aeruginosa* strain. This compound was named as 'pyocin' (Jacob 1954). Unlike the plasmid-borne colicin genes, the pyocin genes are present in the chromosome (Sano and Kageyama 1984). More than 90% of strains isolated from the environment and clinical sources can produce pyocins. The synthesis of

pyocins is regulated such that only a few cells in a population are actively producing them (Duport, Baysse et al. 1995). The expression from pyocin encoding genes is regulated both positively by *priN* and negatively by *priR* regulatory genes via a RecA dependent pathway (Matsui, Sano et al. 1993) (Figure 1.5). DNA damaging agents, such as ultraviolet irradiation and mitomycin C activate the RecA protein, which inactivates the repressor *priR* gene causing the expression of the *priN* gene and allowing the synthesis of PriN. Pyocin genes are consequently expressed (Matsui, Sano et al. 1993). It was found that oxidative stress (such as hydrogen peroxide (H₂O₂)), and antibiotic response (such as ciprofloxacin) could also provoke the expression of pyocin genes (Brazas and Hancock 2005, Chang, Small et al. 2005).

Three types of pyocin have been identified: R-, F- and S- type, which have a diversity of killing and resistance mechanisms (Michel-Briand and Baysse 2002, Parret and De Mot 2002). The former two pyocins are produced by over 90% of clinical strains while the S-type pyocins are produced by over 70% of them (Neves, McCulloch et al. 2014). F- and R- pyocins (Figure 1.6) are insoluble high molecular weight bacteriophage tail-like proteins. They kill target cells by inserting and depolarizing the cell membrane and their killing component doesn't have a specified immunity to neutralize the toxin. While the S- pyocins are smaller, soluble and protease-sensitive proteins and like colicins, they have both killing and immunity genes (Michel-Briand and Baysse 2002, Parret and De Mot 2002). Up to now, S- type pyocins are classified into nine different types: AP41, S1, S2, S3, S4, S5, S6, SD2 and SD3

(McCaughey, Josts et al. 2016). The former four pyocins have DNase activity, while pyocin S4, S5 and S6 display tRNase, pore-forming and rRNase activity, respectively (Ghequire and De Mot 2014) (Table 1.3). A study in CF patients with chronic infections has shown that pyocin S2 is a more effective treatment against *P. aeruginosa* biofilms than aztreonam and tobramycin (Smith, Martin et al. 2012) indicating this kind of narrow, strain specific antimicrobial could be used as an improved alternative to commonly prescribed antibiotics (Scholl and Martin 2008).

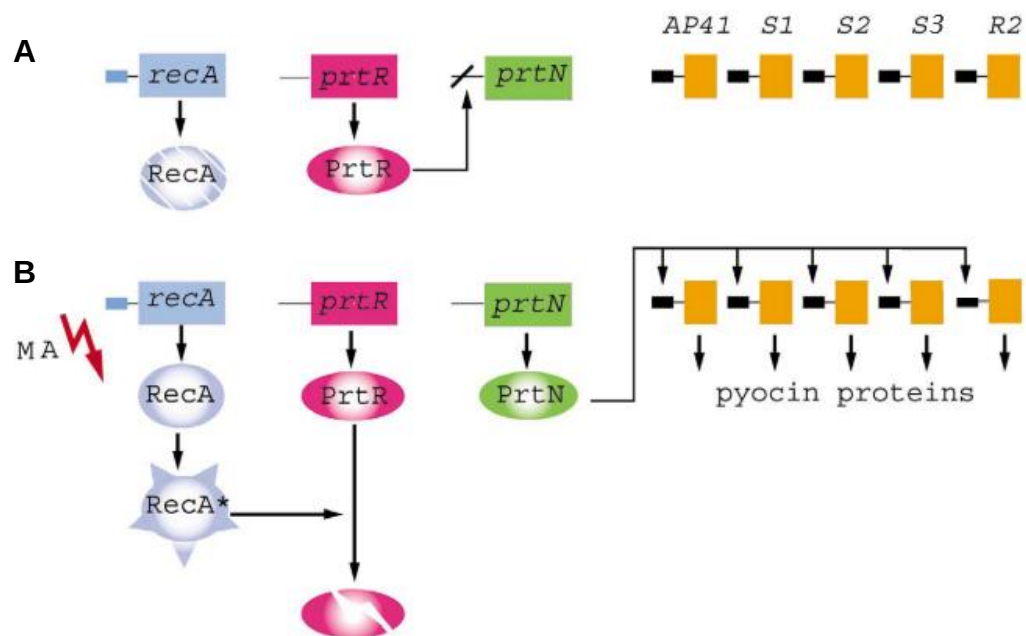


Figure 1.5 Regulation of pyocins genes.

Adapted from Fig 6. (Michel-Briand and Baysse 2002). Panel A- The expression of pyocin genes is negatively regulated by the repressor PrtR, which represses the expression of *prtN* gene. Panel B- Mutagenic Agent trigger the expression of RecA causing the cleavage and release of pyocin repressor PrtR from the promotor of *prtN*, hence the synthesis of PrtN resulting in the expression of pyocin genes.

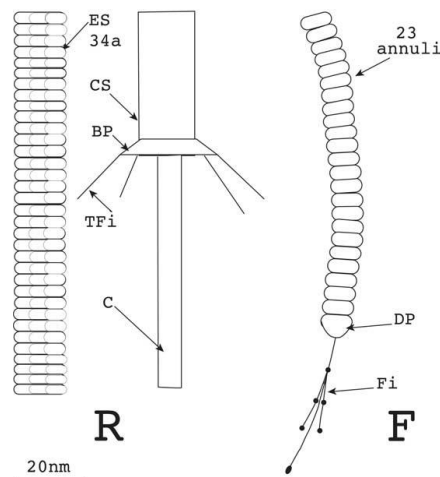


Figure 1.6 The schematic representation of pyocins.

The schematic representation bacteriophage tail-like pyocins: F- and R- pyocins (Ishii, Nishi et al. 1965).

Pyocin	Strain	Mode of action	Toxin		Immunity protein		
			Amino acids	Domain(s)*	Amino acids	Domain†	Receptor
AP41	PAF41-2	DNase (HNH)	777	PF06958; PF12639	90	PF01320	?
M1	JJ692	Lipid II degradation	289	PF14859	—	—	?
S1	NIH-H	DNase (HNH)	618	PF06958; PF12639	87	PF01320	? [‡]
S2	PAO1	DNase (HNH)	689	PF03515; PF06958; PF12639	87	PF01320	FpvAI
S3	P12	DNase	766	PF06958	153	—	FpvAI
S4	PAO1	tRNase	764	PF03515; PF06958; PF12106	112	PF11480	FpvAI
S5	PAO1	Pore-forming	498	PF01024	108	PF03526	FptA
S6	CF-PA39	rRNase	571	PF06958; PF09000	77	—	?
M4	BL01	Lipid II degradation	342	PF14859	—	—	?
S7	BWHP5A018	rRNase	642	PF06958; PF09000	77	—	?
S8	VRFP407	DNase (HNH)	772	PF06958; PF12639	85	PF01320	?
S9	BL04	DNase (HNH)	421	PF06958; PF12639	84	PF01320	?
S10	PABL056	DNase	517	PF05488; PF06958	157	—	?
S11	LESB58	tRNase	662	PF03515; PF06958; PF11429	90	PF09204	?
S12	PA7	tRNase	740	PF06958; PF11429	90	PF09204	?

Table 1.3 The characteristics of pyocins.

Adapted from Table 1. (Ghequire and De Mot 2014)

The killing activity of pyocins is initiated by absorption of pyocins onto specific outer membrane receptors of target cells (Ling, Saeidi et al. 2010). Pyocins utilize both the common polysaccharide antigen (CPA) of *P. aeruginosa* lipopolysaccharide (LPS) and a protein receptor for efficiently targeting the susceptible cells. The CPA can help the pyocins to efficiently locate their outer membrane receptors (McCaughey, Josts

et al. 2016). To date there are three outer membrane receptors identified as specific receptors to different pyocins: the type I ferripyoverdine transporter (FpvAI), the type II ferripyoverdine transporter (FpvAII) and the ferripyochelin transporter (FptA), which are responsible for the uptake of iron-siderophore complexes (McCaughey, Josts et al. 2016, Ghequire and Öztürk 2018). It has been reported that iron-transporting receptors are regulated by the concentrations of iron in the growth medium, which subsequently affect the sensitivity of susceptible bacteria to pyocins. The killing efficiency was lower when the bacteria were grown in an iron-rich medium due to the decreased capacity of these cells to absorb pyocin (Ohkawa, Shiga et al. 1980). The type I ferripyoverdine receptor FpvAI was proposed to be the cellular receptor for pyocin S2 (Sa) (Denayer, Matthijs et al. 2007), SD2 (McCaughey, Josts et al. 2016) and S4 (Elfarash, Wei et al. 2012), while the type II pyoverdine receptor FpvAII and the ferripyochelin receptor FptA were receptors for pyocins S3 and S5, respectively (Baysse, Meyer et al. 1999, Elfarash, Dingemans et al. 2014). Furthermore, an *in vitro* study on the characterization of pyocin SD2 indicated that only one copy of the receptor, FpvAI is required for full activity of pyocin SD2 to achieve protection against a lethal *P. aeruginosa* infection (McCaughey, Josts et al. 2016).

It is promising to find that the construction of hybrid pyocins can broaden the bactericidal activity of a pyocin. For example, the S5-S2b hybrid protein was constructed using the receptor binding domain of S5 and the killing domain of S2. It has a wider range of killing activity compared with the other soluble pyocins due to its receptor FptA, which is a more

widespread receptor amongst *P. aeruginosa* strains (Elfarash, Dingemans et al. 2014). This advantage is also demonstrated with a S1-S3 hybrid consisting of the receptor binding domain of S1 and translocation and killing domain of S3 (Michel-Briand and Baysse 2002). Those findings can add more potential to the new therapeutic approaches using ribosomally synthesised peptides for the treatment for *P. aeruginosa* infection.

Apart from the identification of outer membrane receptors, the pyocin translocation mechanism and the death of the non-immune cell are still poorly understood due to the lack of structural data. The majority of the understanding of pyocins is inferred from our knowledge on *E. coli* colicins, which have a very similar domain organization (Kim, Tarr et al. 2014, McCaughey, Josts et al. 2016, Behrens, Lowe et al. 2020). However, the mechanism of binding and translocation is unlikely to be exactly the same as it is for colicins due to the differences in size, structure and activity (McCaughey, Josts et al. 2016). It has been identified that colicins utilise the Tol-Pal or the TonB-ExbB-ExbD translocation system after gaining entry into target cells, the latter being responsible for the transport of ferrisiderophores (Lazdunski, Bouveret et al. 1998). It is speculated that, like colicins, the translocation of the soluble pyocins happens when the translocation domain interacts with the Tol or TonB-dependent translocation system (Behrens, Lowe et al. 2020). It has been reported that prior to binding to their high affinity receptors, colicins subsequently search for their translocators on the outer membrane landscape of the susceptible cells via lateral diffusion

(McCaughey, Josts et al. 2016). Behrens, Lowe et al. recently described how pyocin S5 recruits FptA and lipopolysaccharide (LPS) and the TonB1 energized uptake system in the outer and inner membrane, respectively to deliver its cytotoxic domain into targeted cells (Behrens, Lowe et al. 2020). For colicins, the immunity proteins release at the cell surface in a pmf-dependent step involving structural remodelling of the nuclease. The energy transduced from the cytoplasmic membrane to the outer membrane is shown to be the driving force for the dissociation of the immunity protein as well as for the translocation of the nuclease domain (Vankemmelbeke, Zhang et al. 2009, Farrance, Hann et al. 2013). However, the dissociation of immunity protein from pyocins is not yet well studied.

1.5 The H-N-H motif in DNase bacteriocin

The DNase domains of pyocin S1, S2, S8 and AP41, share structural and functional features with a subclass of the nuclease colicins of *E. coli* (E2, E7, E8 and E9), that all adopt a typical conserved H-N-H motif (Parret and De Mot 2002, Turano, Gomes et al. 2020). This motif, found in all life kingdoms of the prokaryotes, archaea and eukaryotes (Huang and Yuan 2007, Turano, Gomes et al. 2020), is composed of 32 amino acids located at the C-terminus of the killing domain responsible for catalysing random cleavages of the bacterial genome (Pommer, Cal et al. 2001, Walker, Georgiou et al. 2002, Joshi, Grinter et al. 2015). The H-N-H proteins are generally associated with DNA-binding proteins involved in endonuclease activity and adopt a $\beta\beta\alpha$ -Me fold bearing two antiparallel β -strands and one α -helix coordinated with a single divalent

metal cation, which is similar in topology to a zinc finger (Gorbalenya 1994, Kala, Cumby et al. 2014, Turano, Gomes et al. 2020). Interestingly, pyocin S3 has a completely different primary sequence that lacks the H-N-H DNase type structure (Parret and De Mot 2002, Ghequire and De Mot 2014, Joshi, Grinter et al. 2015) indicating that it is classified as a different type of DNase pyocin and may have an unusual co-ordinating association with its cognate immunity.

So far, it has been observed that the activity of all DNase bacteriocins are dependent on divalent metal ions binding to the active sites (Guérault, Picot et al. 2010). The DNase activity of pyocin S8 and AP41 was strongly induced by Ni^{2+} ion, whereas their activity were inhibited in the presence of Zn^{2+} ions, which was also observed in the metal dependence of the other H-N-H endonuclease (Maté and Kleanthous 2004, Turano, Gomes et al. 2020). Mn^{2+} and Mg^{2+} ions had moderate effects on the activity of pyocin S8 (Turano, Gomes et al. 2020). The H-N-H endonuclease colicins E9 and E7 were more active in the presence of Mg^{2+} ion and are largely inhibited with high concentrations of Zn^{2+} . Moreover, Ni^{2+} is the most potent divalent ion for colicin E7 but did not support the catalysis of colicin E9 (Pommer, Kühlmann et al. 1999, Ku, Liu et al. 2002, Walker, Georgiou et al. 2002). Maté and Kleanthous have rationalized that Zn^{2+} inhibited the activity of H-N-H endonuclease of colicin E9 due to a slightly different mechanism of metal-protein coordination. Three histidine residues involved in coordination of the Zn^{2+} resulted in a tighter binding, while the other 'active' transition metal ions were binding to the protein weakly via two histidine residues and a water

molecule serving as a protonating device for DNA hydrolysis (Maté and Kleanthous 2004). Nevertheless, up to now, there is no report on the structure of the pyocin S3 DNase and immunity proteins or their complex and there is very little literature available on the different metals that affect the DNA catalysis of the pyocin S3 DNase. The mechanism of inactivation of the pyocin S3 by the cognate immunity protein is also not yet characterized (Sano 1993, Duport, Baysse et al. 1995).

1.6 Aims and objectives

The world urgently needs new antimicrobials and new therapies, which can provide safety in use according to new regulations, good efficacy patterns and a low resistance profile, to overcome the increasing multi-drug resistant problem of *P. aeruginosa* infection (Bassetti, Merelli et al. 2013). DNase pyocins, which share basic cytotoxicity characteristics with colicins, have been studied using the well-known colicins as a model (Ghequire and De Mot 2014). Pyocin S3 was found as a special member of the S-type pyocin family, as it lacks sequence homology with corresponding domains of other DNase pyocins and doesn't appear to possess the common H-N-H motif in the active site of S-type pyocin DNases (Duport, Baysse et al. 1995).

The aims of this study are as follows:

- To find out the important residues involved in the pyocin S3 DNase activity and determine its active site.
- To solve the crystal structure of pyocin S3 DNase-immunity complex and investigate the interaction of the two high affinity protein partners.

- To identify the metal ions present in the active site of pyocin S3 DNase.

Chapter 2. Materials and Methods

2.1 Chemicals and kits

All the chemicals used in this study are listed in Table 2.1.

Media/ solutions	Component	Manufacture
LB Broth	Tryptone 10 g/L	Fisher BioReagents
	Yeast Extract 5 g/L	
	Sodium Chloride 10 g/L	
LB Agar	Tryptone 10 g/L	Fisher BioReagents
	Yeast Extract 5 g/L	
	Sodium Chloride 10 g/L	
	Agar 12 g/L	
SelenoMet™ medium Base	n/a	Molecular Dimensions Limited
SelenoMet™ Nutrient Mix	n/a	Molecular Dimensions Limited
250x Methionine Solution	n/a	Molecular Dimensions Limited
250x SelenoMethionine Solution	n/a	Molecular Dimensions Limited
Resuspension Buffer, pH 7	20 mM Tris-HCl	Fisher BioReagents
	5 mM MgCl ₂	Sigma Ultra
Low Imidazole Buffer, pH 7	50 mM NaH ₂ PO ₄ ·H ₂ O	AnalaR
	300 mM NaCl	Fisher Scientific
	10 mM Imidazole	Sigma-Aldrich
High Imidazole Buffer, pH 7	50 mM NaH ₂ PO ₄ ·H ₂ O	Sigma Ultra
	300 mM NaCl	AnalaR
	500 mM Imidazole	Fisher Scientific
Dialysis Buffer 1, pH 7	50 mM NaH ₂ PO ₄ ·H ₂ O	Sigma Ultra
	100 mM NaCl	AnalaR
	10 mM Imidazole	Fisher Scientific
100 mM EDTA	Ethylenediaminetetraacetic acid	FLUKA
50 mM NaOH	Sodium hydroxide	Fisher Scientific

0.5 M NiSO ₄	Nickel (II) sulphate hexahydrate	Sigma-Aldrich
Dialysis Buffer 2, pH 7.5	50 mM K ₂ HPO ₄	Fluka
	50 mM KH ₂ PO ₄	Sigma
Guanidine Buffer, pH 8.5/ pH 8.0	6 M Guanidine	VWR
	20 mM Tris-HCl	Fisher BioReagents
	50 mM NaCl	Fisher Scientific
Dialysis Buffer 2, pH 7.5	50 mM K ₂ HPO ₄	Fluka
	50 mM KH ₂ PO ₄	Sigma
* Sodium Phosphate Buffer, pH7	50 mM NaH ₂ PO ₄ ·H ₂ O	AnalaR
	150 mM NaCl	Fisher Scientific
*Tris-salt Buffer, pH 7	20 mM Tris-HCl	Fisher BioReagents
	150 mM NaCl	Fisher Scientific
*Storage Buffer (for Superdex 75 column)	0.2 M Sodium Acetate	Anhydrous
	20% (v/v) Ethanol	Fisher Scientific
25 mM MgSO ₄	n/a	Thermos Scientific
10X Buffer 0 with BSA	n/a	Thermos Scientific

Table 2.1 Media and solutions used in this study.

2.2 Bacterial strains and growth condition

2.2.1 Bacterial growth conditions

For liquid culture, bacteria were grown at 37°C with shaking at 200 rpm unless otherwise stated; agar plates were incubated at 37°C aerobically in a static incubator. Growth of bacteria was monitored using a spectrophotometer (Nanospec) by measuring optical density at 600 nm wavelength (OD₆₀₀).

Strains	Features	Reference or source
<i>Pseudomonas aeruginosa</i>		
CF971	Cystic Fibrosis (CF) clinical <i>P. aeruginosa</i> strain	An unknown epidemic strain. Isolated from cystic fibrosis patient sputum. Kindly donated by Dr. Matthew Hurley.
<i>Escherichia coli</i>		
DH5 α	a cloning strain; F ⁻ Φ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (<i>r_K</i> ⁻ , <i>m_K</i> ⁺) <i>phoA supE44</i> λ ⁻ <i>thi-1 gyrA96 relA1</i>	(Studier 1990)
BL21(DE3)	a general expression host; <i>fhuA2 [lon] ompT gal</i> (λ DE3) [<i>dcm</i>] Δ <i>hsdS</i> λ DE3 = λ <i>sBamHI</i> Δ <i>EcoRI-B</i> <i>int::(lacI::PlacUV5::T7 gene1)</i> <i>i21</i> Δ <i>nin5</i>	(Kwon, Kim et al. 2015)
B834(DE3)	a general expression; F ⁻ <i>ompT gal dcm lon hsdS_B</i> (<i>r_B</i> ⁻ <i>m_B</i> ⁻) λ (DE3 [<i>lacI lacUV5-T7 gene 1 ind1 sam7 nin5</i>])	Novagen

Table 2.2 Bacterial strains used in this study.

2.2.2 Long term storage of bacterial strains

Glycerol stocks of bacterial strains were prepared by gently mixing 1 ml of bacterial overnight culture with 250 μ l of sterile glycerol yielding a final concentration of 20% (v/v) glycerol before storage at -80°C.

2.3 Culture media and buffers

2.3.1 LB media

LB broth and agar (<https://www.thermofisher.com/uk/en/home/life-science/cell-culture/microbiological-culture/bacterial-growth-media/lb-broth-and-lb-agar.html>) were used for routine growth and protein overproduction unless otherwise indicated with the inclusion of appropriate antibiotics (see 2.3.3). For all the protein expression, IPTG

(isopropyl β -D-1-thiogalactopyranoside) was used at the final concentration of 1 mM. The protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added at the final concentration of 0.5 M during cell lysate sonication to minimise degradation of proteins of interest.

2.3.2 *SeMet medium*

Both methionine medium and SeMet medium containing 100 μ g/ml ampicillin were freshly prepared just before use following the manufacturer's instructions. The methionine auxotrophic *E. coli*. B834(DE3) was initially grown in LB broth supplemented with ampicillin and washed with sterilized 0.9% NaCl before being resuspended in 100 ml methionine medium containing ampicillin prior to aerobic incubation at 37°C with shaking overnight. The cell pellets of the overnight methionine culture were washed as before and transferred into 2 x 500 ml SeMet medium containing ampicillin to grow at 37°C until OD₆₀₀ reached to 0.6-0.8. (Li, Zhang et al. 2012).

2.3.3 *Antibiotics and X-gal*

100 μ g/ml ampicillin (MELFORD) was used to maintain plasmids in *E. coli* strains. Blue-white screening of pGEMT-Easy in DH5 α was achieved on plates containing 100 μ g/ml ampicillin and 50 μ g/ml 5-Bromo-4-Chloro-3-Indolyl β -D-Galactopyranoside (X-gal) (ThermoFisher).

2.4 DNA Manipulation

Plasmids	Features	Reference
pPYSS-3	Pyocin S3 genes in pBluescript KS+. pBluescript KS+ is an expression vector.	The plasmid was kindly donated by Pierre Cornelis (Pierre.Cornelis@vub.ac.be). (Duport, Baysse et al. 1995)
pGEMT-Easy	A cloning vector, containing T7 polymerase promoters flanking a multiple cloning region within the α -peptide coding region of the enzyme β -galactosidase.	Promega
pET21a	An expression vector, carrying an N- terminal T7 tag sequence plus a C-terminal His-tag sequence.	Novagen
pCN3	pGEMT-Easy with full-length DNase and full-length immunity genes minus internal <i>NdeI</i> restriction site	http://eprints.nottingham.ac.uk/42888/
pCN4	pET21a with full-length DNase and full-length immunity genes minus internal <i>NdeI</i> restriction site	http://eprints.nottingham.ac.uk/42888/

Table 2.3 Plasmids used in this study.

* All of the plasmids are ampicillin resistant.

2.4.1 DNA extraction

Plasmid DNA and DNA fragments cut from an agarose gel slice were extracted using GenElute™ Plasmid Miniprep Kit (Sigma) and Monarch DNA Gel Extraction Kit (Biolabs), respectively, following the manufacturer's recommended protocols. DNA was eluted with dH₂O.

2.4.2 DNA separation via gel electrophoresis and the DNA molecular weight marker

Gel electrophoresis using an agarose gel was used to examine the size and purity of DNA. DNA samples were mixed with 6x loading buffer to give a 1x final concentration, and loaded onto a 0.8-1% (w/v) agarose gels along with 1 kb/100 bp DNA molecular weight marker (NEB), which were put in 1x TAE buffer (<https://www.sigmaaldrich.com/GB/en/technical-documents/protocol/protein-biology/gel-electrophoresis/tae-and-tbe-running-buffers-recipe>) in a horizontal gel apparatus (Biorad). Electrophoresis was performed at 80 V for 45 min and the gels pre-stained by adding 10 µg/ml of SYBRSafe (before the gels set) were imaged using Ultraviolet (UV) Transilluminator GelDoc.

2.4.3 Genetic engineering of plasmids used in this study

2.4.3.1 Engineering to optimise *NdeI* site locations

The DNase and immunity genes of pyocin S3 from a stock plasmid of pPYS3-3 (Duport, Baysse et al. 1995) were amplified by PCR using forward primer YC16, designed to introduce an *NdeI* site at the start of the pyocin S3 gene and reverse primer ImS3R, designed to remove the natural stop codon by the introduction of a *XhoI* site at the end of the pyocin S3 immunity gene. The sequences of the primer pair are shown in Appendix 1. The PCR product was separated by agarose gel electrophoresis, purified using a Monarch DNA Gel Extraction Kit (Biolabs) and then ligated into the pGEMT-Easy vector (Promega). The resultant plasmid was named pCN3. An internal *NdeI* restriction site located within the DNase encoding region was removed by substituting

a synonymous CAT codon for CAC using site-directed mutagenesis using Q5 Site-Directed Mutagenesis Kit (Biolabs) and primers S3_IntF and S3_Int (Appendix 1). In this way, the plasmid pCN4 containing the full-length pyocin S3 DNase and the full-length immunity gene (the equivalent residue numbering for the full-length pyocin S3 are 662-791) was constructed after the removal of the sequence of the full length pyocin S3 genes encoding receptor binding domain and translocation domain. The plasmid pCN4 was constructed by the previous Master of Research student Chris Newman.

2.4.3.2 Pyocin S3 mutants

E. coli Asp25, His23, His26 and His66, Arg38, Arg50, Lys51, Lys54, Lys55 and Lys79 mutants were constructed by site-directed mutagenesis using Q5 Site-Directed Mutagenesis Kit (Biolabs) using primers listed in Appendix 1 and the template, pPYSS-3. Mutated DNA was transformed into *E. coli* DH5 α cells and the plasmids were extracted and sent to GENEWIZ® for DNA sequencing to confirm the desired substitutions prior to the subsequent sub-cloning into the pET21a vector for expression as described below.

2.4.4 DNA restriction digestion

All the restriction enzymes used were purchased from Thermos Scientific. Ten μ l digestion mix containing 1 μ l of 10x buffer Tango (with BSA), 1 μ l of each enzymes, *Nde*I and *Xho*I (10 U), and 7 μ l of plasmid was prepared following the manufacturer's recommendation. The mix was incubated at 37°C for 1 h in a water bath. Digestion products were viewed using agarose gel electrophoresis in section 2.4.2.

2.4.5 DNA ligation reaction

2.4.5.1 Ligation into cloning vector pGEMT-Easy

The mutated pyocin S3 DNase-immunity genes were ligated in to pGEMT-Easy vector (Promega) using recommended manufacturer's protocol. A 10 µl ligation mix consisted of 1 µl of 10x T4 ligase buffer, 1 µl of T4 ligase, 1 µl of pGEMT-Easy vector and 4 µl of purified DNA fragment of pyocin S3 DNase-immunity. The mix was incubated at 16°C overnight in a thermocycler.

2.4.5.2 Ligation into expression vector pET21a

Derivatives of pGEMT-Easy containing mutated S3 DNase-immunity encoding genes and the pET21a vector were digested in separated tubes using restriction enzymes *NdeI* and *XhoI*. The digestion products were analysed on a 1% agarose gel and the selected DNA fragment of pyocin S3 DNase-immunity was purified (section 2.5) prior to ligation to the pET21a vector. The ligation was achieved in a 20 µl ligation reaction comprising 9 µl of extracted vector and insert DNA mixed in the ratio 3:1, 2 µl of 10x T4 DNase ligase buffer and 1 µl of T4 DNA ligase. The reaction was incubated for at 16°C overnight in a thermocycler.

2.4.6 DNA Quantification and analysis

NanoDrop ND-1000 (Nanodrop Technologies) was used for measuring the quantity of DNA in solution. The actual DNA concentration was calculated by subtracting the blank (dH₂O, elution buffer) reading from the DNA solution reading. The wider spectra of readings at different wavelengths can be used to assess the DNA purity.

2.4.7 *E. coli* chemically competent cell preparation and transformation

2.4.7.1 *E. coli* chemically competent cells preparation

Chemically competent cells were prepared with 0.1 M CaCl₂ using a standard protocol (Sambrook, Fritsch et al. 1989). The required *E. coli* strain was freshly streaked from a glycerol stock onto an agar plate, and after overnight incubation was used to inoculate 5 ml of LB broth (no antibiotics) before being incubated at 37°C overnight with shaking at 200 rpm. A sub-culture was made by adding 0.5 ml of the above culture into a 250 ml flask containing 50 ml of LB broth and incubated at 37°C until the OD₆₀₀ reached to the range of 0.4-0.7. Forty ml of the sub-culture was centrifuged at 13,000 xg for 10 min at 4°C. The pellet was resuspended in 15 ml of pre-cooled 0.1 M CaCl₂ before being incubated on ice for 15 min. The resuspension was centrifuged again and the pellet was subsequently resuspended in 2 ml pre-cooled 0.1 M CaCl₂. The yielded competent cells were aliquoted in 200 µl aliquots into 1.5 ml Eppendorf's and stored on ice before use.

2.4.7.2 Transformation

Five µl of the pCN4 plasmid (Table 2.3) harbouring the full-length pyocin S3 DNase and immunity was mixed with 1 aliquot of the above freshly prepared competent cells and incubated on ice for 30 min prior to a 90 sec incubation at 42°C in a water bath. The mix was cooled on ice for 2 min and incubated in 800 µl LB broth at 37°C for 45 min with shaking at 200 rpm, and then 100-150 µl was plated on a LB agar containing 100 µg/ml ampicillin.

2.5 Pyocin S3 DNase-immunity expression and purification

2.5.1 *Expression of SeMet labelled pyocin S3 DNase-immunity in E. coli B834(DE3)*

To produce SeMet-labelled pyocin S3 DNase-immunity, the plasmid pCN4 was transformed into methionine auxotrophic *E. coli* B834 (DE3) cells. Two 5 ml of *E. coli* B834 (DE3) pCN4 overnight starter cultures were grown in LB broth supplemented with ampicillin at 37°C with shaking at 200 rpm. Following washing twice by resuspension into 5 ml of sterilized 0.9% NaCl they were incubated aerobically at 37°C with shaking for 30 mins. Cells were then centrifuged down at 6,000 $\times g$ for 10 min at room temperature and the cell pellets were resuspended in 2x 100 ml methionine medium containing ampicillin prior to aerobic incubation at 37°C with shaking overnight. The cell pellets of the overnight methionine culture were washed as before and transferred into 2x 500 ml SeMet medium containing ampicillin (Li, Zhang et al. 2012). Then, the culture was incubated aerobically at 37°C with shaking to an OD_{600} of the culture reached 0.6 to 0.8, at which point 1 mM IPTG was added and protein induction was performed for a further 4 h incubation in the same conditions. Both uninduced and induced protein samples were analysed by SDS-PAGE.

2.5.2 *Expression of native pyocin S3 DNase-immunity in E. coli BL21(DE3)*

To produce the pyocin S3 DNase-immunity complex for native structural study, a 1 L LB (supplemented with ampicillin) culture of *E. coli* BL21(DE3) pCN4 bacteria at an $OD_{600} = 0.7$ was induced for protein expression by addition of 1 mM IPTG. The cells were further incubated for 4 h at 37°C before harvesting the cells by centrifugation. The cells

were suspended in 35 ml of 20 mM Tris-HCl and 5 mM MgCl₂ buffer, pH 7 and stored at -80°C before use.

2.5.3 Cell lysis by sonication

Frozen cell pellets were thawed in cold water before 0.5 mM PMSF was added. The cells were then lysed on ice by sonication at 15 amplitude with 15 s plus on and 15 s plus off for a total active sonication time of 5 mins. Sixty µl of sample was collected as a whole cell extract after sonication for analysis on SDS-PAGE. The rest of cell lysis was centrifuged at 13,000 xg for 12 mins at 8°C. Supernatant as soluble proteins containing the pyocin S3 DNase-immunity was transferred into a new falcon tube and the pellet as insoluble proteins was resuspended with distilled water to the original sample volume. Both soluble and insoluble proteins were analysed on SDS-PAGE.

2.5.4 Preparation and use of a His-trap column

2.5.4.1 Preparation of liquid chromatography handling system for isolation of pyocin S3 DNase-immunity complex

The liquid chromatography handling system AKTApriime (GE healthcare) was used to dispense buffer over a concentration gradient. The system was washed with MilliQ water at a flow rate of 8 ml/min before use. Inlet B and Inlet A were primed with higher (50 mM NaH₂PO₄, 300 mM NaCl and 500 mM Imidazole) and lower (50 mM NaH₂PO₄, 300 mM NaCl and 10 mM Imidazole) concentration of imidazole buffer, respectively after the whole system was washed with lower imidazole buffer. Then, a HisTrap column loaded with cell lysis solution containing pyocin S3 DNase-immunity protein (Amersham, Biosciences) was attached to the system and the buffer was pumped through the column at a flow rate of

1.5 ml/min using a pump P-1 until a plateau of UV absorbance was observed.

2.5.4.2 Purification of pyocin S3 DNase-immunity complex using His-trap column

After cell lysis, the cell debris was removed by centrifugation at 13,000 xg as described. The lysate was then supplemented with 300 mM sodium chloride and 10 mM imidazole then subjected to nickel-affinity chromatography. In brief, the lysate was applied at 1.5 ml/min to a nickel-charged 5 ml HisTrap column (GE Healthcare) equilibrated with 50 mM NaH_2PO_4 , 300 mM NaCl and 10 mM Imidazole buffer, pH 7. The pyocin S3 DNase-immunity complex was eluted with a linear gradient of 10 mM to 500 mM imidazole. Collected fractions were analysed by SDS-PAGE and those containing the pyocin DNase-immunity complex were pooled and dialysed against 50 mM NaH_2PO_4 , 100 mM NaCl and 10 mM Imidazole, pH 7 at 4°C overnight.

2.5.4.3 Purification of pyocin S3 DNase using His-trap column

Pyocin S3 DNase domain was isolated from pyocin S3 DNase-immunity complex by guanidine denaturation during the nickel-affinity chromatography stage. In brief, a buffer of 6 M Guanidine, 20 mM Tris and 50 mM NaCl pH 8.0 was used to elute the DNase from the NTA-bound immunity protein by denaturation. The DNase was re-folded in 20 mM Tris-HCl, 100 mM NaCl and 10% glycerol buffer, pH 7.4 by dialysis. The immunity protein was then eluted from the his-trap column using an increasing imidazole gradient as mentioned above. Further purification of the DNase domain was performed using gel filtration chromatography as described for the complex (section 2.5.4.4). The concentration was

measured using a Nanodrop, employing an extinction coefficient of $12950 \text{ M}^{-1} \text{ cm}^{-1}$ for the DNase, and $11460 \text{ M}^{-1} \text{ cm}^{-1}$ for the his-tagged immunity protein. The final concentrated pyocin DNase was tested for nuclease activity and metal binding (see section 3.2.2 and 3.2.4).

2.5.4.4 Further protein purification by gel filtration chromatography

Further purification was performed by gel filtration chromatography using a Superdex 75 column (GE Healthcare) on a AKTApure system. All the buffer and water used for this experiment were filtered and degassed. The system was washed with at least two column volume (CV) of MilliQ water before it was equilibrated with the running buffer consisting of 50 mM Tris and 150 mM NaCl, pH 7 at a flow rate of 0.7 ml/min. Protein sample purified with a Histrap column was loaded carefully into a loop connected to the system using a syringe. Buffer was then pumped through the column at a flow rate of 2 ml/min until the complete UV absorbance peak of protein observed and a plateau of UV absorbance was shown.

2.6 Protein analysis and SDS-PAGE

2.6.1 Protein molecular weight marker

Protein samples were analysed by 15% Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a ladder, which is a standard broad range pre-stained colour protein ladder was used (11-245 kDa, NEB) as an indicator for molecular weight of protein bands on the gel.

2.6.2 *Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)*

A 15% SDS-PAGE gel was routinely used to investigate pyocin S3 DNase-immunity profiles unless stated otherwise. The separating gel (2 gels) contains 2.5 ml of separating buffer (1.5 M Tris-Cl pH 8.8 and 0.4% SDS), 5 ml of 30% acrylamide, 2.35 ml of water, 100 µl of 10% APS and 50 µl of TEMED. The stacking gel was made with 2 ml of stacking buffer (0.5 M Tris-Cl pH 6.8 and 0.4% SDS), 400 µl of 30% acrylamide, 1.45 ml of water, 50 µl of 10% APS and 5 µl of TEMED. The separating gel mixture was poured immediately into a gel cask after a gentle mix and 1 ml of isopropanol was added onto the top for gel setting. The isopropanol was removed after the separating gel had set and the stacking gel mixture was added. Gels were stored in cold room overnight set before use.

Sixty µl of protein samples were mixed with 20 µl of 2x sample loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 4% 2-mercaptoethanol, 0.2% bromophenol blue and 20% glycerol) before the samples were loaded on the gel. The protein marker mentioned above was run alongside the protein samples. The electrophoresis was carried out by an initial run at 150 V for 10 min until all the samples entered the separating gel and the second run at 200 v for 45 min until the loading dye reached to the bottom of the gel in 1x running buffer (500 ml of 10x running buffer stock contains 72.5 g glycine, 5 g SDS and 15 g Tris base) using a Bio-Rad power pack 1000. The gel was stained with Coomassie blue (1.2 g/L of Coomassie brilliant blue R250, 40% (v/v) of ethanol and 10% (v/v) of acetic acid) for 45 min at room temperature and washed 2 times with destain (50% (v/v)

of water, 40% (v/v) of methanol and 10% (v/v) of acetic acid) for 2h until bands were visible. The gel was imaged using Ultraviolet Transilluminator (GelDoc).

2.7 Dialysis

Nominal MWCO (Molecular Weight Cut-off) 3,500 Dialysis Tubing was used for all the dialysis process and all the dialysis process were conducted in cold room with gentle stirring. Viva Spin 500 concentrator with 3,000 MWCo was used for all the concentration process, unless otherwise stated.

2.8 Protein quantification

The concentration of proteins was determined by UV absorbance at 280 nm using a Nanodrop, employing an extinction coefficient of $24410 \text{ M}^{-1} \text{ cm}^{-1}$ for pyocin S3 DNase-immunity complex, $12950 \text{ M}^{-1} \text{ cm}^{-1}$ for the DNase, and $11460 \text{ M}^{-1} \text{ cm}^{-1}$ for immunity protein.

2.9 Crystal structure determination of pyocin S3 DNase-immunity complex

2.9.1 Sample preparation

Both native and SeMet pyocin S3 DNase-immunity complex were concentrated to 18 mg/ml, and crystallized in 0.2 M sodium fluoride, 0.1 M Bis-Tris propane, pH 8.5, 20% (w/v) PEG 3350 using the sitting drop method. The crystals were cryo-protected with the crystallisation solution with an additional 30% glycerol and cryo-cooled in liquid nitrogen.

2.9.2 The crystal structure of pyocin S3 DNase-immunity complex building

A lack of sufficiently homologous proteins to model the x-ray diffraction data to meant that the structure needed to be solved by experimental

phasing. The structure of the pyocin S3 DNase-immunity complex was determined by single anomalous dispersion (SAD) using SeMet substitution for both proteins. The SeMet crystals diffracted to 1.45 Å at peak wavelength and the native crystals diffracted to 1.8 Å. These data sets were integrated and scaled using XDS (Hendrickson, Horton et al. 1990) and Aimless (Evans and Murshudov 2013). There was one DNase-immunity complex per asymmetric unit with 48.2% solvent content. Seven out of the eight Se sites were found (The N-terminal SeMet was processed).

Autobuild (Terwilliger, Grosse-Kunstleve et al. 2008) was used to produce the initial model of the complex. The initial electron density map from SAD phasing showed clear electron density for most residues. The final model was built using iterative cycles of model building and refinement with COOT (Emsley, Lohkamp et al. 2010), REFMAC5 (Murshudov, Skubák et al. 2011) and Phenix refine (Adams, Afonine et al. 2010). To confirm that the SeMet structure was representative of the native structure, molecule replacement was used to solve the native data. The final models have R/R-free of 0.176/0.198 (native) and 0.174/0.194 (SeMet). The space group of both native and derivative crystals was $P 2_12_12_1$, and unit cell dimensions were $a = 42.92$ Å, $b = 43.69$ Å, $c = 160.25$ Å, $\alpha = \beta = \gamma = 90.0^\circ$ (native) and $a = 43.42$ Å, $b = 43.84$ Å, $c = 159.62$ Å, $\alpha = \beta = \gamma = 90.0^\circ$ (SeMet). Figures of protein structures were created using PyMOL (DeLano 2002).

2.10 Assays

2.10.1 *Pyocin S3 concentration and nuclease activity assay*

Purified DNase was concentrated using a freeze dryer (MicroModulyo, Thermo Savant) as described by Overcashier et al. (Overcashier, Brooks et al. 1997). Purified protein obtained from gel filtration was dialysed against 100 mM ammonium bicarbonate buffer, pH 7.8, so the protein would be in an aqueous solution of this buffer for lyophilization. The resulting protein powder was suspended in 500 µl of 20 mM Tris-HCl, 150 mM NaCl buffer, pH 7 and diluted to the required concentration with 20 mM Tris-HCl, 10% glycerol buffer, pH 8.0.

The nuclease activity analysis was conducted with 2 mg plasmid pGEMT-Easy as a substrate. The reaction buffer contained 5 mM MgSO₄ and 20 mM Tris-HCl, pH 8.0, and a total volume of 20 µl was incubated at 37°C for 30 min to 1 h. Samples were heated at 75°C for 15 min in a PCR block (MasterCycler personal, Eppendorf) to stop the reaction before being mixed with 2 µl of Gel Loading Dye (Biolabs), and analysed on a 0.8% agarose gel alongside 1 kb DNA ladder (Biolabs) at 80V for 45 min.

2.10.2 *Pyocin S3 DNase metal dependence assay*

The metal-dependence of the pyocin S3 DNase catalytic activity was detected using metal-free pyocin S3 DNase apo-protein, which was obtained by EDTA treatment. Purified DNase was dialysed against 10 mM EDTA using a 3,500 MWCo dialysis tubing for 4 h with shaking at 50 rpm at room temperature. And then, the EDTA treated DNase was subjected to 2 runs of dialysis against 20 mM Tris-HCl and 10% (w/v)

glycerol buffer, pH 7.4 with/without 1 μ M EDTA, which was treated with chelex 100 for metal removal. Metal dependence was undertaken with addition of 0.01 mM (figure not shown), 0.1 mM, 1 mM and 10 mM of MgCl_2 , ZnSO_4 , MnCl_2 , NiCl_2 , CaCl_2 , CoSO_4 solutions, which were made with MilliQ water. A substrate plasmid DNA, pGEMT-Easy, was treated with 1 mM EDTA for 15 min at 50 rpm on a shaker at room temperature for metal removal. Thereafter, 102.5 ng/ μ l (23.9 nM) of the metal-free plasmid DNA was incubated with 0.43 μ M of the resulting DNase for 60 min at 37°C in a static incubator. Results were viewed on a 0.8% agarose gel by mixing 10 μ l of the reaction with 2 μ l of Gel Loading Dye (Biolabs) before loaded on the gel alongside a 1 kb DNA ladder.

2.10.3 Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was performed by Dr. Simon Caulton using a MicroCal PEAQ-ITC and Malvern's software. The system was washed before and after each assay and a wash was performed between each run. EDTA (5 mM) treated protein was prepared freshly and dialysed in chelex100 (Solum form, Sigma-Aldrich) treated 20 mM Tris-HCl, 150 mM NaCl and 10% glycerol buffer, pH 7.4, which was also used to make the metal solution for buffer matching. Concentration of protein was measured using NanoDrop and calculated using its extinction coefficient. Metal solutions were prepared to a concentration of 10 times higher than the protein concentration, and then titrated with the protein solution during the ITC. 286 μ M of each metal solution was injected into 28.6 μ M of pyocin S3 DNase protein with 19 injections consisting of an initial 0.4 μ l injection and 18 x 2 μ l injections

at 25°C, which was in 20 mM Tris, 150 mM NaCl and 10% glycerol buffer, pH 7.4.

2.10.4 Inductively coupled plasma mass spectrometry (ICP-MS)

Multi-element analysis of protein solutions was undertaken using ICP-MS (Thermo-Fisher Scientific iCAP-Q and iCAP-TQ; Thermo Fisher Scientific, Bremen, Germany) by technician Saul Vazquez Reina in Dr. Scott Young's group (Division of Agricultural & Environmental Sciences, School of Biosciences, University of Nottingham). Protein samples were introduced (flow rate 1.2 mL min⁻¹) from an autosampler (Cetac ASX-520) incorporating an ASXpress™ rapid uptake module through a perfluoroalkoxy (PFA) Microflow PFA-ST nebuliser (Thermo Fisher Scientific, Bremen, Germany). Sample processing was undertaken using Qtegra™ software (Thermo-Fisher Scientific) utilizing external cross-calibration between pulse-counting and analogue detector modes when required.

2.10.5 Stab test assay

DNase activity was detected by stab test assay (Hockett and Baltrus 2017) using sensitive pyocin S3-treated indicator clinical strain *P. aeruginosa* 971 (PSA971). A single colony of *E. coli* DH5α pCN4 or its mutants were stabbed onto a fresh LB agar plate containing 100 µg/ml ampicillin. The plate was incubated at 37°C overnight before being exposed to a tissue paper soaked with ≥ 99.8% chloroform soaked on tissue paper for 15 min at room temperature in a fume hood. The plate was inverted and left in the fume hood without the lid on for 30 min, and then overlaid with 6 ml of warmed (~ 50°C) soft top agar containing 80 µl

of overnight of PSA971. Activity of pyocin S3 DNase was assessed by observation of a positive clear zone (active) or no inhibition zone (inactive) indicated on the plate after overnight incubation at 37°C.

2.10.6 Gel electrophoresis mobility shift assay

The 34 bp DNA fragment was labelled with Cyanine5.5 by PCR annealing using primers KEA1 and KEA3 (Appendix 1). The Cyanine5.5 fluorophore has an emission maximum at 705 nm and can be imaged using a Licor Odyssey BDI2_B60 (700 nm window). A few of initial trials using a 0.5x TBE buffer and 6% PAGE to assess if the pyocin S3 DNase and mutants could bind and reduce the mobility of 10 nM Cy5.5 labelled DNA. No obvious shift in DNA migration was observed for most gels, therefore, optimisations were performed, which would be described in Results.

2.10.7 Fluorescence polarization assay

The Tecan Spark (C69, BDI2) was used to measure fluorescence polarization of Bodipy530 labelled DNA (10 uM) in the presence and absence of pyocin DNases and commercial benzonase (a control nuclease, Millipore). The 34 bp DNA fragment was labelled with Bodipy530 by PCR annealing using primers KEA3 and KEA10 (Appendix 1). An Amersham Typhoon (Cy5 laser and IRshort) was used to image Bodipy530 which has an emission maximum of 550 nm (Cy3 laser and detection).

2.10.8 Analytical gel filtration chromatography assay

Wild type pyocin S3 DNase and mutants D25A, H26A and H66A proteins of 11 kDa, and a BSA (2.0 mg/ml) standard of 66 kDa were analysed in

this assay with/without addition of a piece of DNA, which is a ~200 bp (130 kDa) PCR product amplified from pET21a S3 pyocin DNase-immunity using primers T7F and H66Arev (Appendix 1). The PCR product was analysed by agarose gel electrophoresis, purified into 10 mM Tris-HCl buffer pH 8.0 using a Qiagen PCR purification kit, and quantified by Nanodrop prior to use.

The analytical Superdex75 10/300 GF column (GE Healthcare) in the AKTApure system was equilibrated using a filtered and degassed 50 mM sodium-phosphate pH 7.5, 100 mM NaCl, 1 mM EDTA buffer. A 100 μ l capillary was added to the AKTApure injection port and the program named 'SIYU WU S75_24ml 100uL INJ 1500 μ L fracs' was employed, to inject 100 μ l of sample, protein or protein-DNA mix to the column. The program controlled the injection and eluted 1.5 column volume (CV) using a flow rate of 0.7 ml/min, collecting 1.5 ml fractions. The injection capillary was loaded with 200 μ l sample just prior to beginning the program and rinsed with 3-4 ml of buffer between each chromatography run.

2.10.9 Circular dichroism assay

Experimental Circular Dichroism (CD), far UV measurements (180 to 250 nm) were recorded using a Chiralscan Plus Applied Photophysics Instrument (Bulheller and Hirst 2009) and performed by Dr. Jed Long (School of Chemistry, the University of Nottingham). A 350 μ l cuvette of 0.1 cm pathlength was employed. Five μ M of protein stocks used in the tryptophan fluorescence assay were diluted to 0.2 -1.0 mg/ml in 10 mM Tris, 2 mM MgSO₄ buffer, pH 7.4, following dialysis in this buffer. The

instrument lamp was purged of oxygen with nitrogen overnight prior to measurements and during operation at room temperature. Protein concentration was determined using the Bradford Assay (Bradford 1976) with BSA as the standard. The protein concentration corrected, pyocin S3 DNase-immunity complex, and DNase (and mutants) experimental spectra were reported as Molar Ellipticity (mdeg) vs wavelength (nm) plots after normalisation, at 208 nm, to their predicted pyocin S3 DNase-immunity complex and S3 pyocin DNase spectra, respectively. Normalisation was considered desirable to assist direct comparison of the spectra. To predict the far UV CD spectra of the pyocin S3 DNase-Immunity complex, isolated DNase enzyme and immunity protein, the atomic coordinates of these proteins from the crystal structure Protein Databank (PDB) file were submitted to the online tool PDB2CD (Mavridis and Janes 2016).

2.10.10 Tryptophan fluorescence assay

In brief, 5 μ M of each pyocin S3 to be analysed were prepared. Circular Dichroism (CD) was performed to confirm if the proteins were folded properly and appropriate to be used for the subsequence experiments. Concentration of those proteins were obtained based on their original quantification by UV absorbance spectroscopy using Nanodrop. The intrinsic fluorescence of tryptophan residues within pyocin DNase and mutants (D25A, H26A and H66A) proteins were measured in this assay using a Hitachi F2500 Fluorimeter with/ without titration of a ~200 bp DNA fragment generated by PCR, which was previously used for gel filtration chromatography. Fifty mM sodium-phosphate pH 7.5, 100 mM NaCl, 1

Chapter 2

mM EDTA buffer, which was also used for previous chromatography and a matching pair of ~125 ml quartz cuvettes were employed. The fluorimeter was operated at ambient temperature with excitation at 295 nm, emission scanned once at 1 nm/sec from 320-400 nm using instrument excitation and emission slit widths of 10 nm. Data was exported into Excel for manipulation.

In brief, the 5 μ M protein stocks were prepared based on protein quantification by UV absorbance spectroscopy using a Nanodrop. A Bradford Assay was subsequently performed following manufacturer's instructions prior to the tryptophan fluorescence to improve the accuracy of the concentrations used and ensure all the proteins analysed were present at 5.4 μ M. Moreover, the same volume of each protein was added to all the subsequent experiments to minimise technical error. The fluorescence of 120 μ l of buffer was measured first to obtain the background fluorescence reading. Then, ~ 37.5 μ l of one of the DNase proteins was added into the cuvette to achieve a protein concentration of 1 μ M. Two scans were performed and similar readings were expected prior to addition of 1 μ M and 2 μ M of DNA by gently mixing ~2.9 μ l of concentrated DNA (56 μ M) twice into the cuvette containing a total volume of ~157.5 μ l of protein solution. The tryptophan fluorescence was measured after each addition of DNA. Note, unlike gel filtration previously, there are no magnesium ions in this experiment with 1 mM EDTA present to reduce/prevent DNA cleavage.

2.11 Bioinformatics

2.11.1 DNA sequencing data

The correct DNA sequence including the DNA sequence of mutants were confirmed by sequencing of the recombinant plasmids. Samples were sequenced by Genewiz using the Sanger method with required primers (primer S3_seq_forward for the full-length pyocin DNase-immunity and mutants; primer T7 for the pET21a clones, Appendix 1).

2.11.2 The alignment for the amino acid sequence of pyocin S3 and other H-N-H bacteriocins

In brief, a discontinuous megablast, BLASTn search of the NCBI database, using a 900 bp region encoding the pyocin S3 DNase and immunity protein from UCB-PP-PA14 was initially used to identify dissimilar, non-*Pseudomonas aeruginosa* nucleotide sequences that encode sequential DNase and immunity proteins. For ease of identification, two additional bases were introduced into the query sequence so removing the frameshift between immunity (downstream) and the upstream DNase of the pyocin S3 DNase-immunity region. Further megablast, Blastn searches in specified organisms employing the initial hit sequences permitted expansion of the protein family. Duplicate identical sequences were subsequently removed prior to the Clustal Omega multi-sequence alignment of the DNase-immunity protein encoding region. In many instances the NCBI database failed to annotate immunity genes, presumably as a result of the large size of each complete pyocin gene and relatively small size of downstream immunity gene which are co-transcribed/translated out of frame of their pyocin.

2.11.3 Comparison of pyocin S3 DNase family and H-N-H DNase family topology

The alignment was performed by Dr. Philip Bardelang using the online tool, Jpred4 (<http://munk.cis.unimelb.edu.au/pro-origami/porun.shtml>).

This tool was employed to predict the secondary structure of the pyocin S3 DNase and immunity proteins from the multi-sequence alignment with the resulting predictions subsequently compared to the crystal structure.

Jpred4 successfully predicted the majority of secondary structure present in both proteins in the experimental complex.

Chapter 3. Pyocin S3 DNase requires a metal ion co-factor for its activity

3.1 Introduction

Duport *et al.* identified S3 pyocin as a DNase and predicted, from its sequence, that it had a novel structure (Duport, Baysse et al. 1995). However, the precise active site was not determined, and neither were the specific catalytic features of its activity. In this chapter, to define this further, the predicted pyocin S3 DNase domain has been overproduced and purified alone or with its cognate immunity protein. The primary aim was to demonstrate that the predicted pyocin S3 DNase domain cleaves DNA without the presence of its cognate immunity and demonstrate that the DNase endonuclease activity is abolished when the immunity protein is bound.

To separate a specific protein for further investigation from the rest of the proteins within the biological extract which contains it, a purification strategy is required. There is a wide range of approaches that can be employed to purify proteins. The choice of the most appropriate methodology depends on the properties of the target protein and its intended application. Several easy-to-use affinity tags have emerged that are ideally suited to protein purification and genetic engineering is generally employed to create a recombinant protein that is a fusion of the target protein and the desired affinity tag (Costa, Almeida et al. 2014). Subsequently, the tagged protein is applied to an affinity matrix which selectively binds the tagged protein, contaminating proteins and solutes

are washed away, and then the tagged protein is selectively eluted for onward study (Figure 3.1 A). This approach has the advantage of being targeted to the protein of interest and cuts down the amount of purification steps required to make the process faster. Column chromatography, which can be performed with an automated system, such as AKTApurify, is used to separate a large pool of proteins into many smaller pools, some of which will contain a high quantity of the protein of interest (<https://www.labome.com/method/Protein-Purification.html>). Depending on the proportion of contaminating proteins in the elution fraction with the protein of interest, it may be possible to characterize it directly or incorporate additional purification steps. One example of affinity tag driving purification involves fusion of the protein of interest to glutathione S-transferase (GST). GST binds to glutathione as the ligand and can be eluted with solutions of glutathione. Another example is the incorporation of a poly-histidine tag that has a strong interaction with the divalent metal ion Nickel via the histidine imidazole ring (Malhotra 2009) (Figure 3.1 B). The tagged proteins in such an immobilized metal affinity chromatography (IMAC), can be eluted with imidazole (Figure 3.1 A). In both cases, the affinity chromatography provides high selectivity, resolution and capacity in most protein purification schemes. It can also serve as a convenient concentration step because a large volume can be applied to the affinity resin, and elution can be achieved in a smaller volume (<https://www.bio-rad.com/en-uk/applications-technologies/affinity-chromatography?ID=LUSMJIDN>). Tagged proteins are frequently

Pyocin S3 DNase requires a metal ion co-factor for its activity overproduced in heterologous hosts where more options are available to manipulate the levels of production and optimise the conditions to favour protein folding and stability. One such host is *E. coli*, and the recombinant proteins which were overproduced, e.g., r-bacteriocins, are commonly purified using column chromatography that exploits their physiochemical based features, such as size exclusion (SEC) and ion exchange (IEX) chromatography. These techniques separate the target protein from similar polypeptides with minor differences in properties, such as size, charge and hydrophobicity (Costa, Almeida et al. 2014, Cui, Luo et al. 2021). SEC and IEX can also be used in conjunction with affinity tag purification to increase the purity or concentration of proteins.

In addition to the chromatography-based protein purification approaches summarized above, there are other techniques that can be incorporated into a protein purification pipeline (Labrou 2014). These include methods of precipitation or phase partitioning/fractionation that may have an advantage in some situations such as maintaining the functional integrity of the target protein, separating the target protein from a contaminant which shares many features such that it cannot be removed by other means or to simultaneously achieve buffer exchange or concentration. These include ammonium sulphate precipitation, solvent extraction and bacterial compartment fractionation or sucrose gradient fractionation of proteins in solution (Labrou 2014).

Obtaining a soluble protein from the purification is crucial to the success of sequential steps such as chromatography as spontaneous precipitation blocks the flow required in the process and alters the elution

profile leading to changes in the levels of co-eluting contaminating proteins. In addition, most proteins are most active in their soluble form since it corresponds to them having achieved their native structure and a functional active site. Moreover, the extent of protein solubility impacts on subsequent storage since it affects the quality of protein. Failing to maintain conditions that promote solubility of the target protein may cause unexpected protein unfolding, aggregation, degradation and loss of function. Purifying the protein as quickly as possible and under the most stabilizing conditions can also increase the chance of a successful purification scheme (<https://www.labome.com/method/Protein-Purification.html>). A very useful flowchart of strategies for obtaining soluble protein from *E. coli* is provided by Costa et al. and reproduced in Figure 3.1 panel C (Costa, Almeida et al. 2014).

In this study, a polyhistidine tag (His-tag, also called 6x His-tag) was selected, and combined with IMAC to achieve purification. The advantages of using a His-tag were its small size as it was predicted this would have minimal effect on the activity of the final protein, hence the His-tag would not need to be removed prior to structure and function characterization (<https://www.promega.co.uk/resources/guides/protein-analysis/protein-purification-methods/>). The His-tag was fused to the Immunity protein to enable capture of the pyocin S3 DNase-Immunity complex. A denaturation step involving Guanidine was required to separate the DNase domain from its cognate Immunity protein. Thus, TCA (Trichloroacetic acid) precipitation was employed to remove the guanidine that remained in protein fractions. This technique enables the

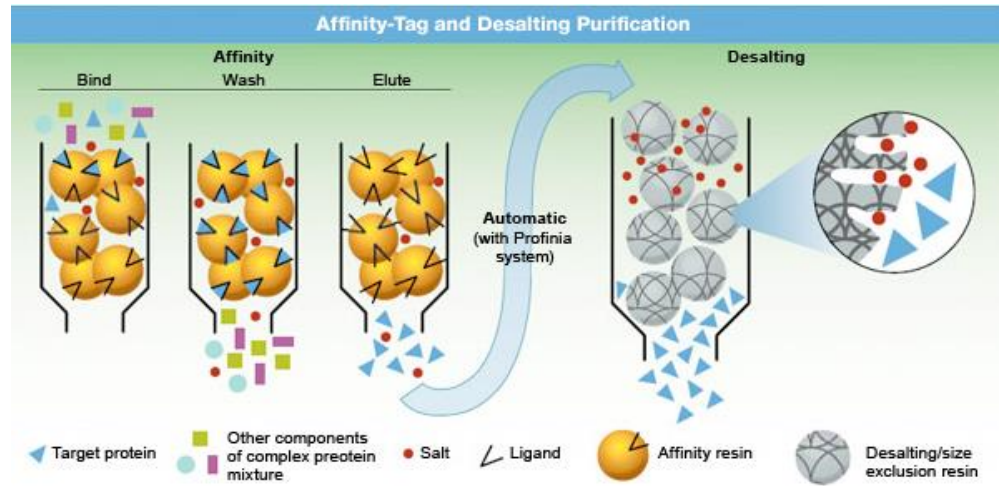
Pyocin S3 DNase requires a metal ion co-factor for its activity generation of a more accurate analysis of the size of the purified proteins using downstream SDS-PAGE (Koontz 2014), as protein migration would otherwise be distorted by the presence of guanidine. TCA achieves this by disrupting the hydrogen-bonded water molecules surrounding proteins, so that proteins became insoluble and can be recovered by centrifugation. Where downstream activity assays were planned, the Guanidine was removed by a gentler approach that facilitated simultaneous refolding of the denatured proteins (dialysis).

Metal ions, such as Ca^{2+} , Mn^{2+} and Zn^{2+} , are essential as cofactors for many enzymes which act on nucleic acids including DNases (Belkebir and Azeddoug 2013). Mg^{2+} was found to be the natural cofactor for the majority of them due to its high natural abundance (Maguire and Cowan 2002). It was reported as one of the most abundant elements on earth and the most abundant divalent cation within bacterial cells (Maguire and Cowan 2002). Therefore, some questions that needed to be addressed in this chapter included: 1). Is a metal ion essential for the endonuclease activity of pyocin S3 DNase protein? 2). Which metal ion is essential/preferred for pyocin S3 DNase activity? 3). Does the immunity protein inhibit the DNase killing activity by stripping the metal out of its active site? 4). Does the immunity protein cause structural change of the DNase protein to protect the producing cells from the lethal action of their own products? To answer the first couple of questions, the endonuclease activity and metal dependence of pyocin S3 DNase protein was assessed as below: the metal dependence of pyocin S3 DNase activity was demonstrated by the removal of bound metal ions from the purified

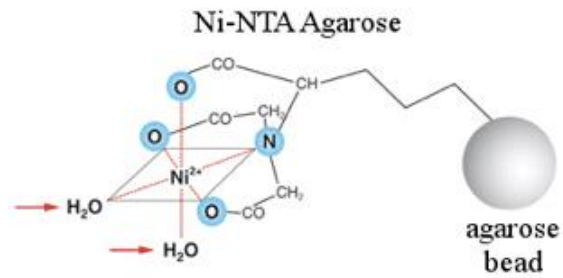
pyocin S3 DNase using 10 mM EDTA (Joshi, Grinter et al. 2015) before its endonuclease activity was subsequently re-examined by the titration of metal ion solutions, including Mg^{2+} , Zn^{2+} , Mn^{2+} , Ni^{2+} , Ca^{2+} , Co^{2+} back into the DNase active site. These metal ions have been found in the active site of several DNase bacteriocins and shown to be involved in DNA hydrolysis or to play some other significant role in DNA coordination (Pommer, Kühlmann et al. 1999, Pommer, Cal et al. 2001, Ku, Liu et al. 2002, Sui, Tsai et al. 2002, Maté and Kleanthous 2004). Isothermal titration calorimetry (ITC) was performed to further support the observation of metal dependence.

Pyocin S3 DNase requires a metal ion co-factor for its activity

A



B



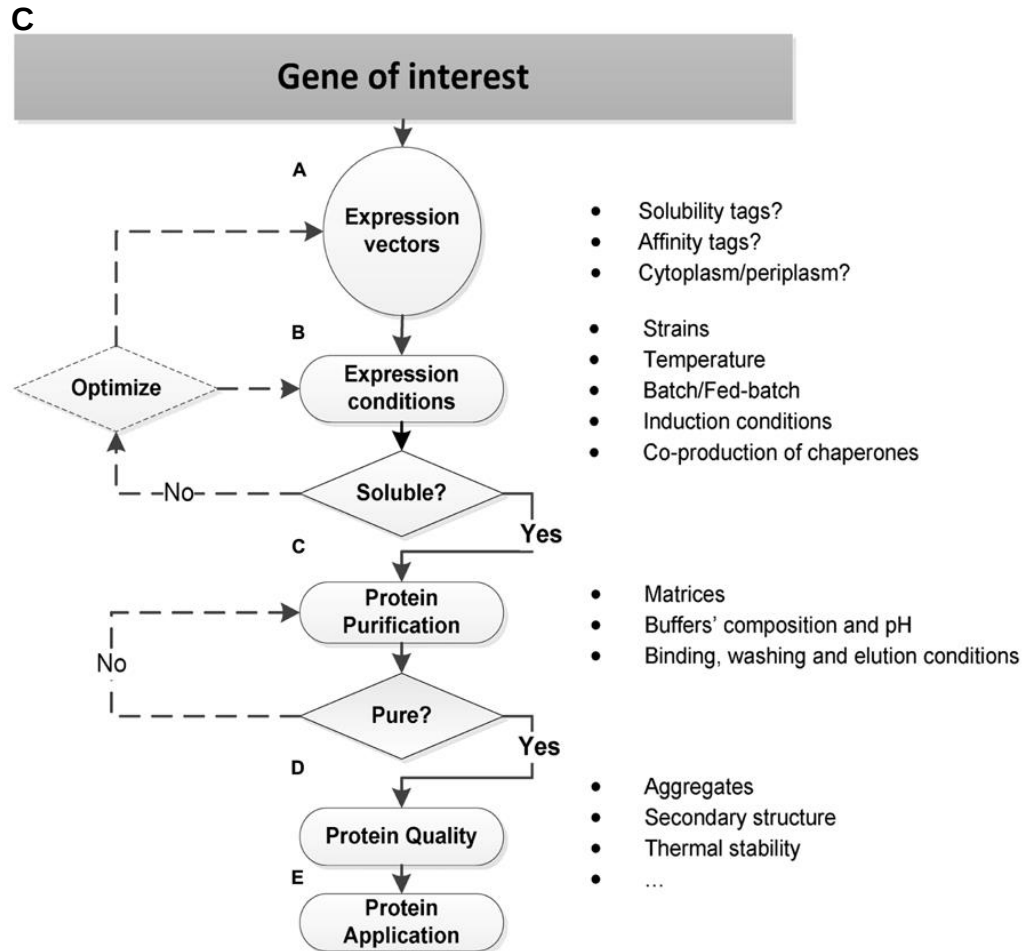


Figure 3.1 Schematic representation of affinity-tagged protein chromatography and how it might be incorporated into a successful protein purification strategy.

Panel A. Outline of the theory behind selective purification of a target protein using an affinity chromatography approach. A subsequent step of desalting is also illustrated for cases when it is necessary to remove the salts in the elution buffer from the purified protein of interest prior to downstream applications (<https://www.bio-rad.com/en-uk/applications-technologies/affinity-chromatography?ID=LUSMJIDN>). Panel B. Ni-NTA ligand covalently attached to a crosslinked agarose matrix for purification of polyhistidine-tagged proteins. Histidine residues can coordinate to the Ni^{2+} ion by replacing the bound water molecules (indicated by red arrows) (<https://www.labome.com/method/Protein-Purification.html>). Panel C. Strategies for isolating soluble proteins at high yield from *E. coli*. The protein purification strategy should be established at the beginning when selecting the expression vector (Costa, Almeida et al. 2014).

3.2 Results

3.2.1 Purification of the pyocin S3 DNase-immunity complex and separation of its two component parts

In order to isolate the pyocin S3 DNase-immunity protein complex, the first step was to insert the DNA encoding the truncated pyocin S3 DNase (with receptor binding and translocation domain removed) and the full-length immunity genes into the plasmid pGEMT-Easy. This was undertaken by Christopher Newman (<http://eprints.nottingham.ac.uk/id/eprint/42888>) and involved removing of an internal *Nde*I restriction site (CATATG) by substituting the CAT codon for histidine to CAC using primer S3_IntF and S3_Int (Appendix 1) by site directed mutagenesis. Thereafter, an *Nde*I site at the N-terminus of the DNase gene was introduced and the stop codon of the immunity gene was replaced with an *Xho*I site by PCR using primers YC16 and ImS3R (Appendix 1), which amplified a 859bp product from nucleotides 1903 to 2762 of the pyocin S3 stock plasmid. pPYSS-3 (Table 2.3). The pyocin S3 DNase-immunity genes were subsequently ligated into the expression vector pET21a alongside the 6x His-tag encoding DNA to fuse the His-tag to the N-terminus of the pyocin S3 immunity protein. Blue-white screening was performed to isolate the plasmids containing insertions. Thereafter, the sequence-verified over-expression plasmid (pCN4, Table 2.3) was transferred into an *E. coli* strain appropriate for protein overproduction (BL21(DE3)). The pET plasmid expression system is a powerful system commonly used for recombinant protein cloning and expression in *E. coli* (<https://research.fredhutch.org/content/dam/stripe/hahn/methods/biochem/pet.pdf>). The target gene is placed downstream of a promoter that is

under the strict control of the bacteriophage T7 RNA polymerase. The T7 RNA polymerase encoding gene is incorporated into the *E. coli* host strain such that it is induced with an analogue of lactose, IPTG. SDS PAGE analysis of whole cell extracts revealed high levels of two proteins (when IPTG was present) that had molecular weights corresponding to the pyocin S3 DNase domain and the immunity protein (Figure 3.2).

To separate the DNase-Immunity protein complex from other cytoplasmic proteins in the producing cell, a cell lysate containing the S3 DNase-Immunity complex was applied to a Ni-NTA column for affinity purification via the His-tagged immunity component. The UV absorbance trace (Panel A, Figure 3.3) revealed a peak (highlighted by red underlining) that was shown to contain the DNase-immunity protein complex by SDS-PAGE (Panel B, Figure 3.3). Fractions 11, 12 and 13 containing the DNase-immunity protein were pooled and dialysed against 50 mM NaH₂PO₄, 100 mM NaCl and 10 mM Imidazole buffer, pH 7. These fractions were of taken forward for study because they contained large quantities of the two proteins of interest, with low levels of contaminating proteins.

For further DNase separation from the Immunity protein, the pooled fractions containing the complex were reapplied to the Ni-NTA column. The DNase was subsequently dissociated from the immunity protein and eluted from the column using a buffer containing 6 M Guanidine, 20 mM Tris and 50 mM NaCl, pH 8.0. The remaining NTA-bound immunity protein was eluted with a linear gradient of 10 mM to 500 mM imidazole. The fractions of (i) pyocin S3 DNase-immunity complex, (ii) DNase alone,

Pyocin S3 DNase requires a metal ion co-factor for its activity and (iii) immunity protein were collected from the Ni-NTA column and applied to SDS-PAGE without (Panel A, Figure 3.4) or with (Panel B, Figure 3.4) TCA precipitation. The TCA precipitation step was included to remove traces of buffers that might otherwise distort the resolution of the SDS PAGE. Proteins of the expected size for pyocin S3 DNase (fraction G-F5 to HI-F2, Lane 7-14 Figure 3.4) and immunity (fraction HI-F5 and HI-F6, Lane 17 and 18, Figure 3.4) were evident. Panel B Figure 3.4 indicated that the guanidine had been successfully removed by TCA as the proteins were no longer migrating as distorted bands.

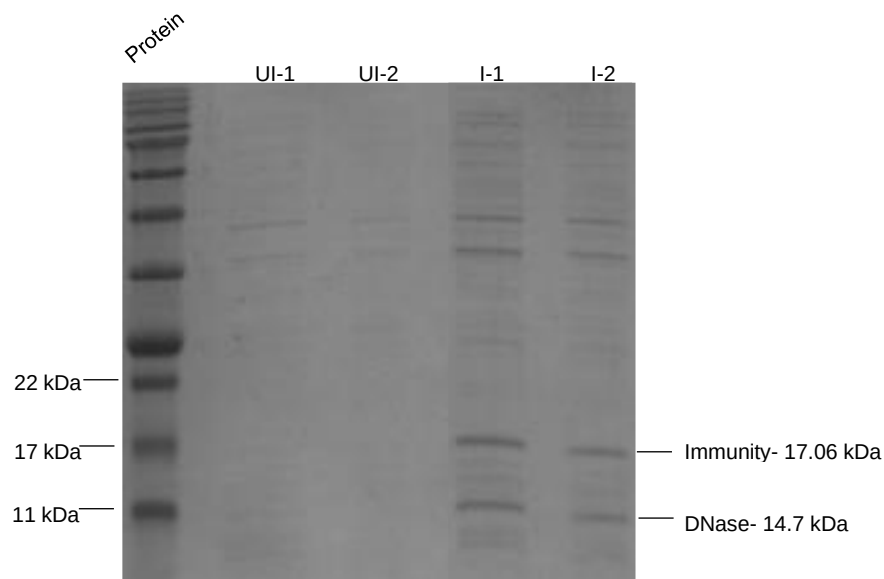
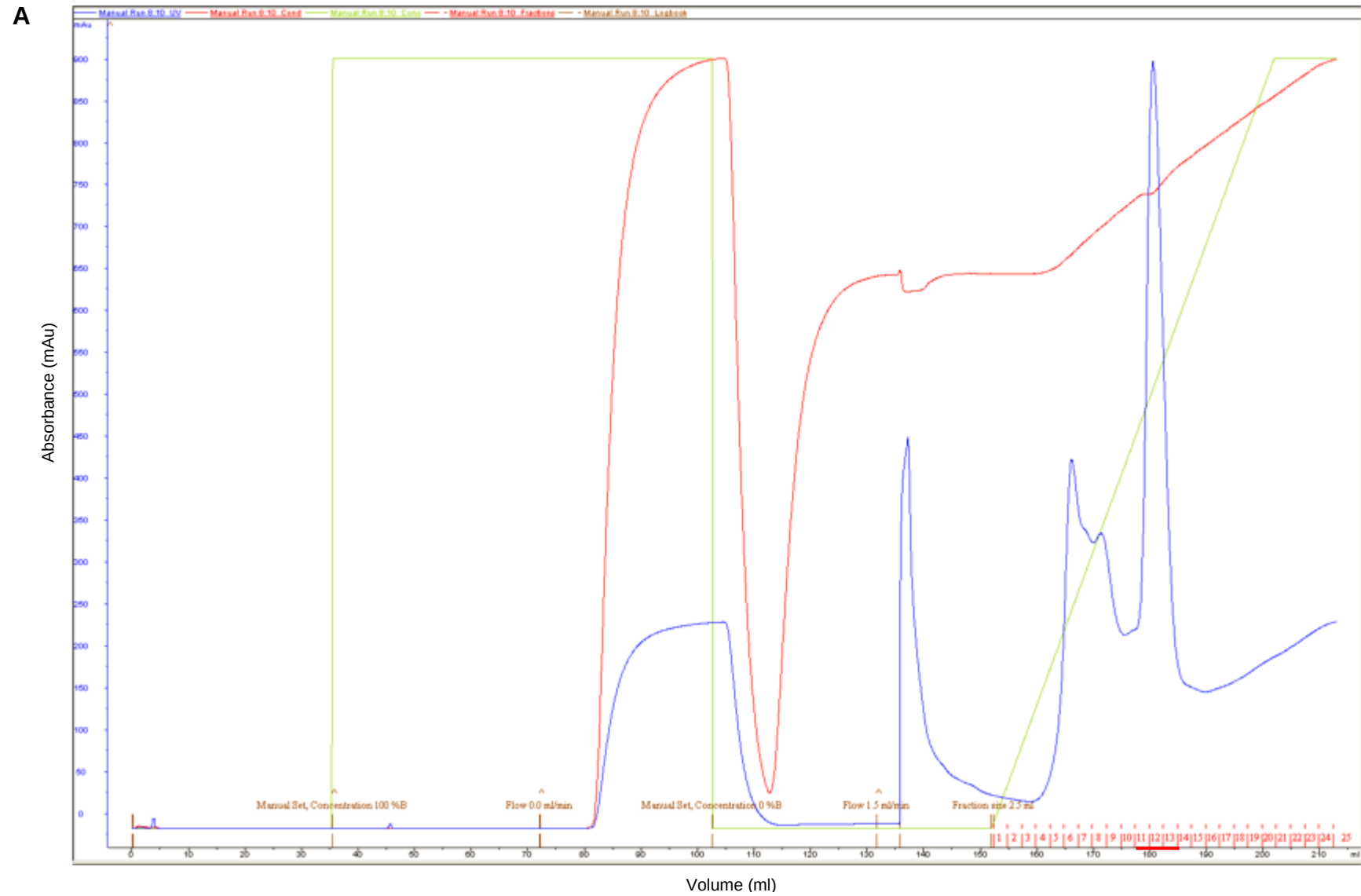


Figure 3.2 Pyocin S3 DNase domain can be overproduced with its cognate immunity protein.

Two parallel cultures of *E. coli* BL21 (DE3) containing the plasmid pCN4 were grown in LB medium at 37 °C with (I-1 and I-2) and without (UI-1, UI-2) induction with 1 mM IPTG (see 2.5.1 in Methods). 100 µl of culture were taken before and after induction and mixed with 20 µl of SDS-

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PAGE loading buffer and applied to a SDS-PAGE alongside marker proteins (protein ladder, sizes of relevant marker proteins indicated on the left). Proteins were visualised by Coomassie blue staining. The expected sizes of the DNase and Immunity proteins are indicated on the right.



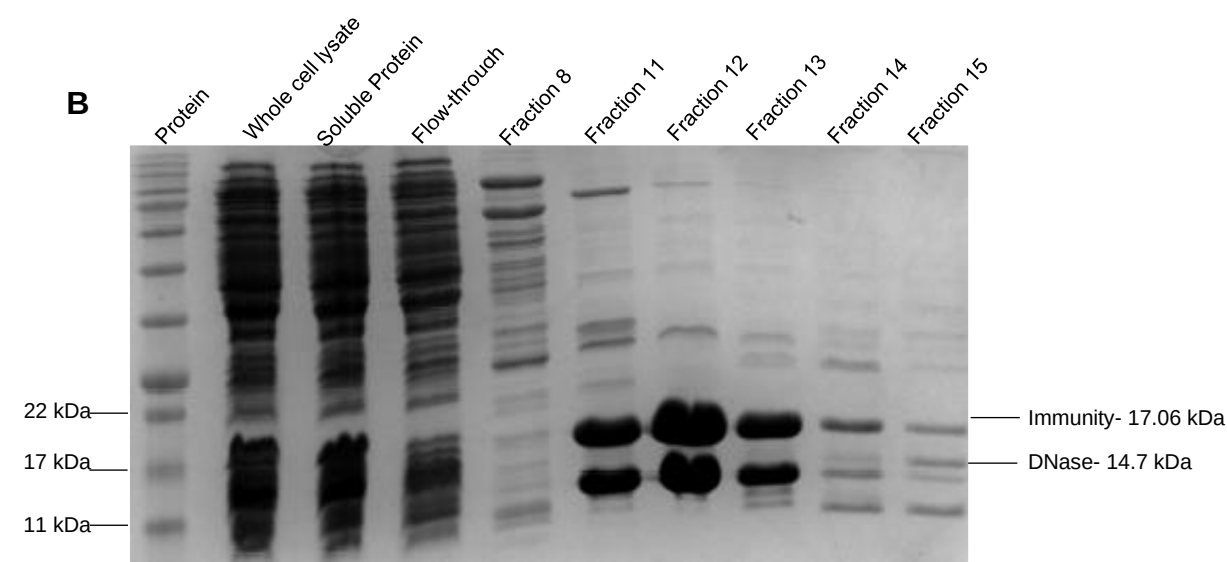
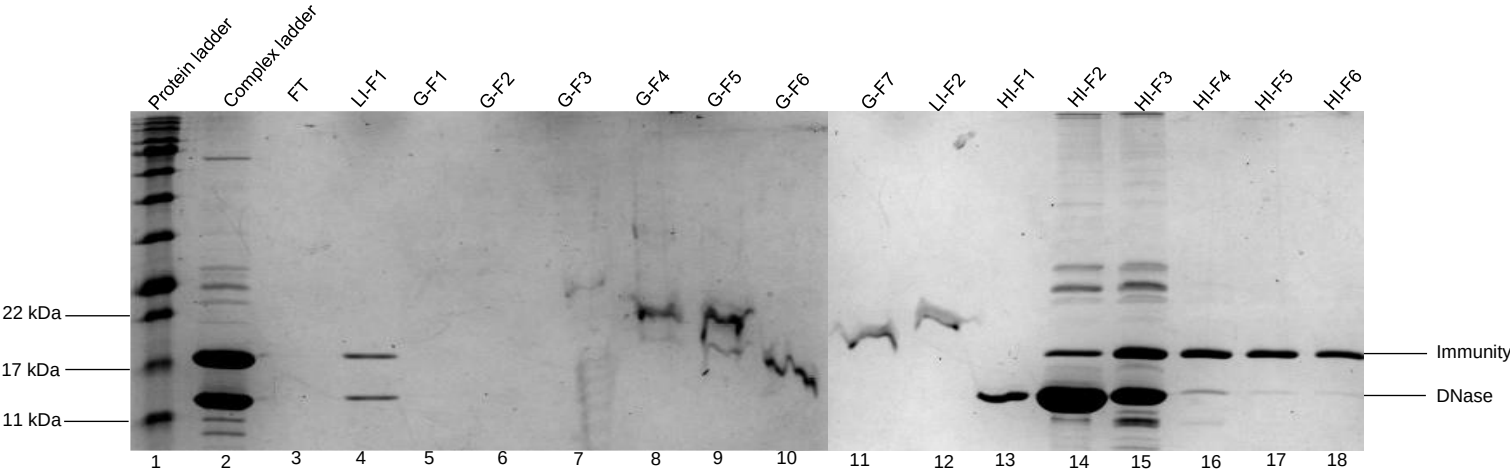


Figure 3.3 Purification of pyocin S3 DNase-immunity complex using a Ni-NTA column.

Panel A- Cell lysate containing pyocin S3 DNase-immunity complex protein was supplemented with 300 mM sodium chloride and 10 mM imidazole and the 5 ml Ni-NTA column was equilibrated with 50 mM NaH_2PO_4 , 300 mM NaCl and 10 mM Imidazole buffer, pH 7 before the lysate was loaded onto the column for purification. The purification was performed using an ÄKTAprimer™ PLUS system. The salt concentration trace mirrors the imidazole addition and approximately at 152 ml, high imidazole buffer was added to the system. Fractions (5ml) were collected as a linear gradient of 10 mM to 500 mM imidazole (fractions were numbered 1 to 25 in red) was applied until the UV absorbance indicated all proteins had been eluted by maintaining a steady low level. Red curve- conductivity trace, blue curve- UV absorbance trace and green line- salt concentration trace. The salt concentration trace mirrors the imidazole addition and approximately at 152 ml, high imidazole buffer was added to the system. Panel B- 60 μl of peak fractions containing DNase-Immunity complex protein were taken and mixed with 20 μl of SDS-PAGE loading buffer and applied to a SDS-PAGE running along with soluble protein collected from cell lysate and flow-through from the Ni-NTA column. Lane 1- Colour pre-stained protein standard; Lane 2- whole cell lysate; Lane 3- soluble protein; Lane 4- column flow-through; Lane 5- Fraction 8; Lane 6 to 10- Fractions 11 to 15 respectively. Coomassie blue was used to visualise proteins. The expected sizes of the DNase and Immunity proteins are indicated on the right in kDa, and the molecular weight of relevant marker proteins are shown on the left.

A



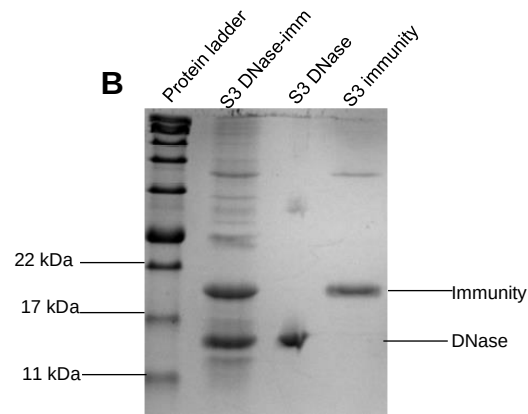


Figure 3.4 Separation of isolated pyocin S3 DNase from its cognate immunity protein.

Panel A- Fractions (11-13) containing purified DNase-Immunity complex protein from Fig 3.2 were dialysed against 50 mM Na_2HPO_4 , 100 mM NaCl and 10 mM imidazole buffer, pH 7 before being reloaded on the Ni-NTA column. Flow-through (FT) was collected. The DNase was denatured and eluted off the column using a total volume of 20 ml of buffer containing 6 M Guanidine (G), and 3 ml fractions were collected manually. Then, 20 ml of low imidazole buffer (LI) was passed through to elute all the remaining DNase before the immunity protein was eluted off by high imidazole buffer (HI). Fractions (3 ml) were collected manually and 60 μl of each fraction was taken and mixed with 20 μl of SDS-PAGE loading buffer to be analysed on a 15 % SDS-PAGE gel. Lane 1 and 2. Colour pre-stained protein standard ladder and complex ladder, respectively; Lane 3. FT, flow-through; Lane 4 and 12. LI-F1 and LI-F2, fraction collected from low imidazole wash; Lane 5- 11. G-F1 to G-F7, fraction 1 to fraction 7 collected from guanidine buffer wash; Fraction 13- 18. HI-F1 - HI-F6, fractions collected from high imidazole wash. Panel B- TCA precipitation of representative protein fractions analysed on a SDS-PAGE. Lane 1.

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Colour pre-stained protein standard ladder; Lane 2. pyocin S3 DNase-immunity complex protein; Lane 3. pyocin S3 DNase protein; Lane 4. pyocin S3 immunity protein.

Coomassie blue was used to stain the gel. The expected position of the DNase and Immunity proteins are indicated on the right.

3.2.2 DNase activity depends on the concentration of pyocin S3 DNase added to the reaction and the cognate immunity abolishes DNase activity.

To verify the purified DNase was active and that the immunity protein inhibited its function, the activity of the purified isolated DNase, isolated immunity protein and S3 DNase-immunity complex were assessed. To do this, an *in vitro* plasmid nicking assay was employed using a supercoiled pGEMT-Easy plasmid DNA as the substrate. Concentrations between 4.3 μ M and 0.43 nM of each protein were incubated with the plasmid DNA to provide an indication of the efficiency of endonuclease activity as reported by DNA degradation. Figure 3.5 shows that the most DNA degradation was observed with the highest concentration of pyocin S3 DNase since the DNA was no longer detectable (Lane 2, Figure 3.5). As the amount of DNase present in the incubation decreased from 4.3 μ M, firstly a smear and then discrete DNA fragments were visible (Lane 3, 4 and 5, Figure 3.5). This is consistent with the supercoiled plasmid being nicked to generate linear DNA plasmid that is then digested to nucleotides as the amount of DNase increases. To protect the producing cell from damage, it would be predicted that the presence of the pyocin S3 immunity protein would abolish the DNase activity by inhibition. The inhibition most likely occurs as a result of a direct physical interaction between the DNase and Immunity protein (Michel-Briand and Baysse 2002). Inhibition was demonstrated by incubating the DNase-Immunity complex with the plasmid DNA substrate (Lane 6- 9, Fig Figure 3.5). It is also clear that the pyocin S3 immunity alone did not show any endonuclease activity (Lane 10 and 11, Figure 3.5). There was no

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conversion of supercoiled DNA to whole linear DNA with any concentration of DNase-immunity complex (Lane 6-9, Figure 3.5) or isolated immunity protein (Lane 10 and 11, Figure 3.5). There was a trace of plasmid nicking visible at the highest concentration of the DNase-Immunity protein complex (Lane 6, Figure 3.5). Due to the DNase-immunity complex being in equilibrium with its component proteins, it is probable that some free DNase exists at this high concentration of the complex. This free DNase would presumably be able to digest the plasmid DNA at a minimal level.

To see if the DNase could interact with the cognate inhibitor after denaturation and separation, the isolated DNase was incubated with the isolated immunity at the same molar concentration. This incubation was performed in a buffer containing Tris and glycerol at room temperature for 10 min, prior to the incubation with the plasmid DNA substrate in the same reaction buffer. It was expected that similar results would be seen with the artificial mix of DNase and immunity protein as had been observed using the natural DNase-immunity complex. Unexpectedly, the DNase and immunity mix degraded plasmid DNA slightly less efficiently than the natural DNase (Lanes 12 and 13 compared to Lanes 2-4, Figure 3.5). A smear was observed in Lanes 12 and 13 indicating considerable DNA degradation suggesting that the isolated DNase and immunity proteins had minimal interactions when mixed. It is possible that the immunity protein becomes unstable after being separated from the DNase during its purification. Such fragility could lead to unfolding or

Pyocin S3 DNase requires a metal ion co-factor for its activity degradation of the immunity protein which would in turn result in the failure to inhibit DNase activity.

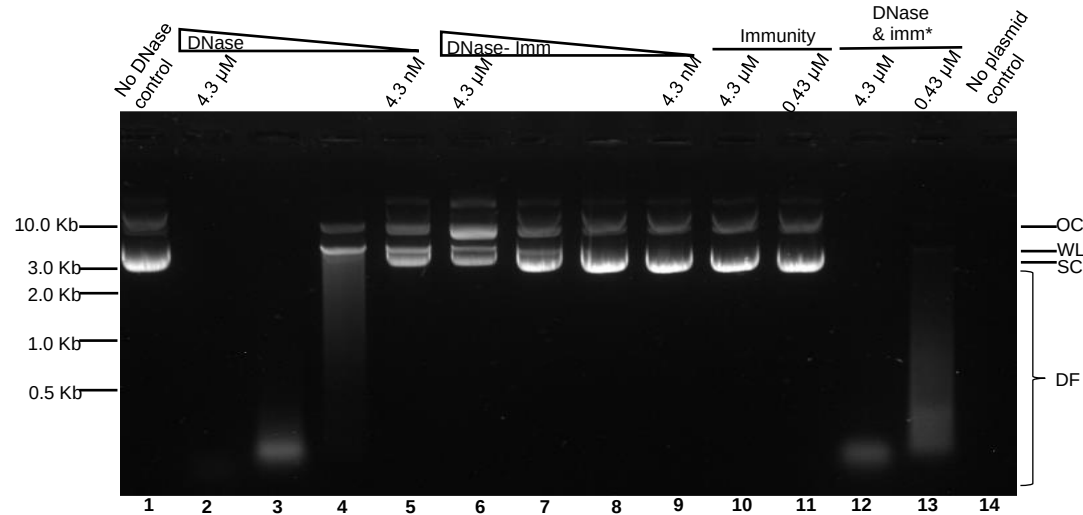


Figure 3.5 Plasmid nicking assay showing that pyocin S3 DNase is active in the absence of its cognate immunity protein.

Purified pyocin S3 DNase, immunity and their complex were subjected to 10-fold dilutions yielding concentrations 0.43 μM (1/ 10-fold) or to 4.3 nM (1/1000-fold) with buffer containing 20 mM Tris-HCl and 10% (v/v) glycerol. An aliquot (1 μl) of each dilution was incubated with 102.5 ng/ μl of plasmid DNA, pGEMT-Easy as a substrate, in 20 mM Tris-HCl, 5 mM MgSO_4 buffer, pH 7.4 for exactly 60 min at 37°C. Lane 1. negative control, no DNase was added to the reaction solution; Lane 2-5. 4.3 μM , 0.43 μM , 43 nM and 4.3 nM titrations of DNase to plasmid, respectively; Lane 6-9. 4.3 μM , 0.43 μM , 43 nM and 4.3 nM titration of DNase-immunity complex to plasmid respectively; Lane 10 and 11. 4.3 μM and 0.43 μM titrations of immunity to plasmid; Lane 12 and 13. titrations of pre-incubated equimolar DNase and immunity at 4.3 μM and 0.43 μM to plasmid, respectively;

Pyocin S3 DNase requires a metal ion co-factor for its activity

Lane 14. negative control, reaction solution without protein or DNA. Forms of plasmid DNA are indicated to the right of the panels: SC- supercoil; WL- whole linear DNA; DF- degraded fragments. * The pre-incubated DNase & immunity mix was incubated on a shaker at 50 rpm at room temperature for 10 min.

3.2.3 *DNase activity can be reduced by chelating metals*

DNase enzymes often incorporate a metal ion co-factor to enable them to catalyse the degradation of DNA. To investigate whether this was the case for pyocin S3 DNase, attempts were made to strip it of any bound metal ions, and then re-introduce specific metals to identify the cognate metal ion. The metal chelator most commonly used for protein purification and preparation (Mónico, Martínez-Senra et al. 2017), EDTA was added to make apo-S3 DNase (metal removed from the DNase protein). A Guanidine-EDTA treatment was performed to unfold the pyocin S3 DNase prior to metal removal, thus regenerating the native metal-binding site (Gupta, Dobritsa et al. 2002). Subsequently, a range of transition metals including Mg^{2+} were added. Initially, 10 mM and 50 mM EDTA treatments were performed for 15 min at room temperature prior to examination of pyocin S3 DNase activity in order to determine the optimal concentration of EDTA for metal removal. The incubation with the substrate DNA was performed for 30, 60 or 90 minutes (Figure 3.6). No DNase activity was observed after those treatments. Surprisingly, a smear was observed in the lane containing the highest concentration of DNase (292 μ M) with 10 mM EDTA treatment (Panel A Lane 3, 13 and 23, Figure 3.6). The smear became more prominent as the incubation time increased and was associated with less whole linear and open-coiled plasmid DNA being detected (Panel A Lane 13, 23, Figure 3.6). The increase in DNA degradation with time of incubation suggests cleavage was occurring and may be attributed to an insufficient

Pyocin S3 DNase requires a metal ion co-factor for its activity concentration of EDTA that was not able to collate all the divalent metal ions present in the protein.

Having identified the conditions required to remove metal ions from the DNase such that its activity was inhibited, the next step was to identify which metal ion the DNase bound. The most commonly found metal ion in DNases is Mg^{2+} . Thus initial experiments focussed on this metal, and revealed a reliance on Mg^{2+} for activity in the absence of the immunity protein, as a significant increase of degradation/cleavage on the substrate plasmid DNA was observed with addition of Mg^{2+} . The evidence for this includes the conversion of a smear (without Mg^{2+} , Lane 2 and 9, Figure 3.7) to a complete degradation (with Mg^{2+} , Lane 5 and 12, Figure 3.7) using the highest DNase concentration. This was underpinned at lower DNase concentrations where no cleavage (without Mg^{2+} , Lane 3, 4, 10 and 11, Figure 3.7) became a smear/ complete degradation with addition of Mg^{2+} (with Mg^{2+} , Lane 6, 7, 13 and 14, Figure 3.7). More DNA degradation was observed as incubation time increased (Lane 6, 7, 13 and 14, Figure 3.7). A concentration of 5 mM of metal (Mg^{2+}) addition was able to restore the DNase activity (Lane 5, 7, 12, and 14, Figure 3.7).

An EDTA titration assay (with 10 mM, 5 mM and 2 mM) was performed using 1.83 μ M, 9.1 nM and 0.46 nM pyocin S3 DNase to find out the optimal EDTA concentration to use for inhibiting DNase activity (Figure 3.8). Data demonstrated that 10 mM and 5 mM EDTA treatment could completely abolish the pyocin S3 DNase endonuclease activity. As observed previously, a small amount of nicking was observed at the

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highest concentration of DNase with 5 mM EDTA treatment (Lane 5 and 14, Figure 3.8); 2 mM EDTA per reaction can prevent up to 1.83 μ M DNA from nicking the substrate DNA (Lane 8 and 17, Fig Figure 3.8). There was no detectable increase of nicking observed as the incubation time increased from 60 min to 90 min. Therefore, it appeared that the optimal concentration of EDTA for metal removal was 10 mM. To determine if pyocin S3 DNase is only active when Mg^{2+} is present, or whether it can substitute in an alternative metal ion co-factor, the extent of DNase activity was assessed with a selection of different metals.

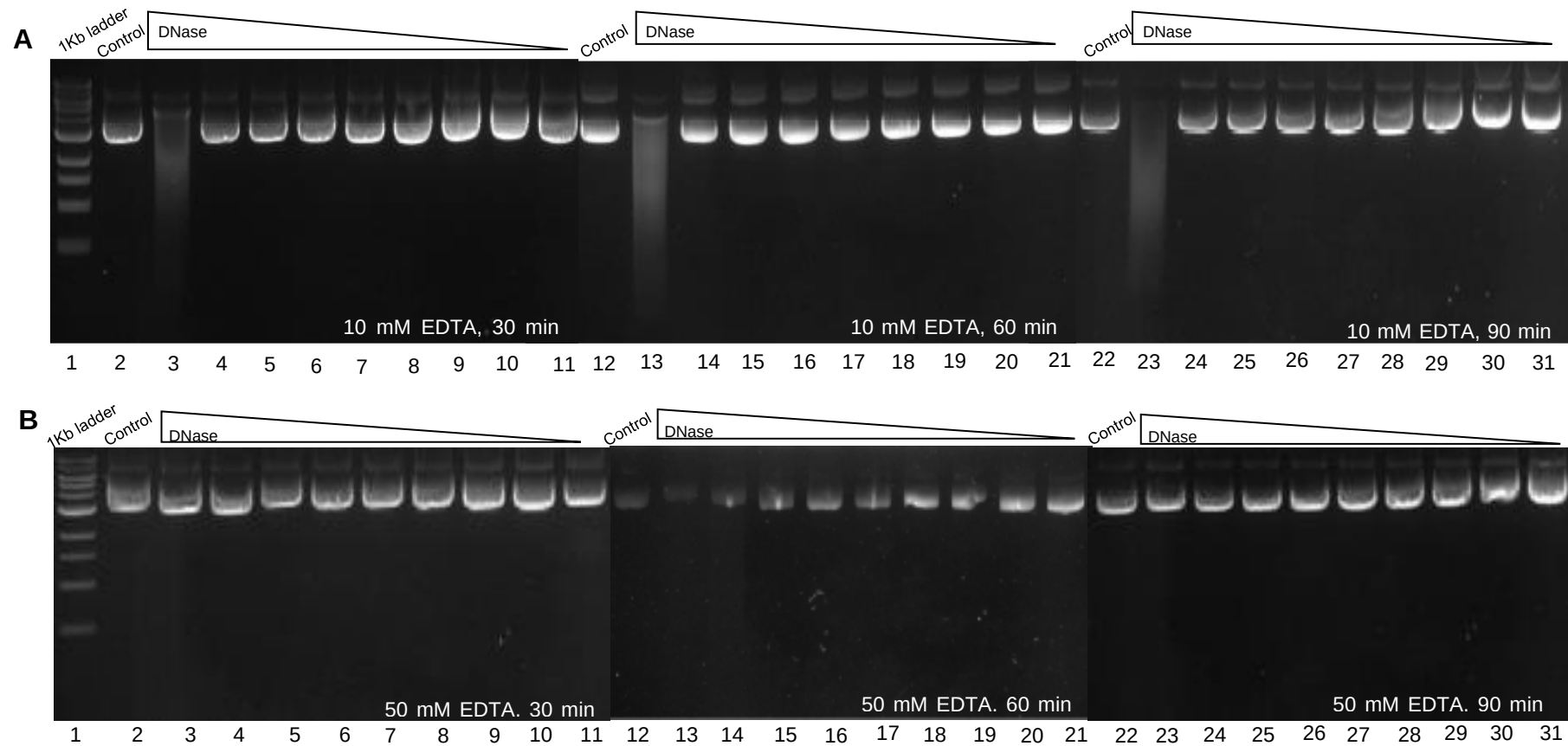


Figure 3.6 Titration of pyocin S3 DNase activity with either 10 mM or 50 mM EDTA treatment.

Purified pyocin S3 DNase was subjected to 1/16 dilution prior to 1/2 dilutions yielding concentrations 18.25 μM (1/16) to 0.15 μM (1/2048) with buffer containing 20 mM Tris and 10% glycerol. An aliquot of each dilution was incubated with 10 mM or 50 mM EDTA in a 20 mM Tris buffer, pH 8 for 15 min at room temperature before addition of 102.5 ng/ μl of plasmid DNA, pGEMT-Easy, as a substrate and further incubation for exactly 30 min, 60 min, 90 min and 120 min incubation at 37°C (figures for 120 min are not shown). Reactions are loaded onto a 0.8% agarose gel and viewed under UV. Panel A, B- DNase incubated with 10 mM and 50 mM EDTA, respectively. Lane 1. 1 Kb ladder; Lane 2, 12, 22. Control reaction with no DNase added with incubation of 30 min, 60 min and 90 min, respectively; Lane 3-11, 13-21, 23-31. Titration of purified pyocin S3 DNase at concentrations of 292 μM (neat), 913 nM (1/16), 456.5 nM (1/32), 228.3 nM (1/64), 114.3 nM (1/128), 57.3 nM (1/256), 29 nM (1/512), 14.5 nM (1/1024), 7.5 nM (1/2048) with incubation of 30 min, 60 min and 90 min at 37°C, respectively.

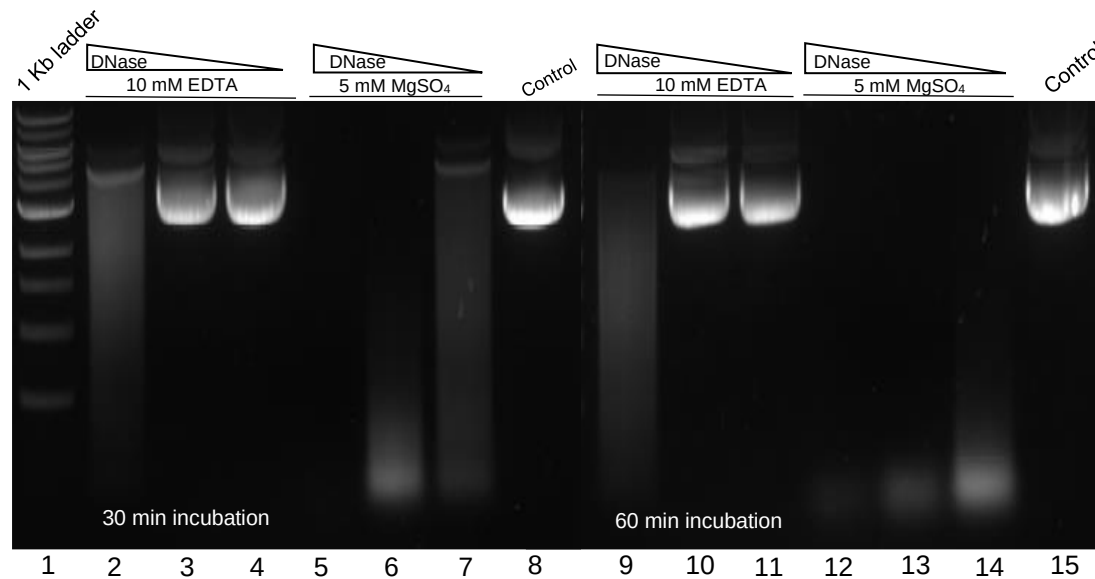


Figure 3.7 The reduced Pyocin S3 DNase activity resulting from treatment with 10 mM EDTA is restored by 5 mM Mg^{2+} .

Purified pyocin S3 DNase was subjected to 1/8 dilution and 1/16 dilutions yielding concentrations 36.5 μM (1/16) and 18.25 μM (1/16) with buffer containing 20 mM Tris and 10% glycerol. An aliquot of each dilution was incubated with 10 mM EDTA in 20 mM Tris buffer, pH 8 for 15 min at room temperature before addition of 102.5 ng/ μl of plasmid DNA, pGEMT-Easy as a substrate in 20 mM Tris buffer, pH 8.0 for exactly 30 min and 60 min incubation at 37°C. The same aliquot of the protein dilutions were incubated with the same concentration of plasmid DNA in the same buffer under the same condition mentioned above with addition of 5 mM $MgSO_4$. Reactions are loaded on a 0.8% agarose gel

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and viewed under UV. Lane 1. 1 Kb ladder; Lane 2-4 and 9-11. Titration of DNase in the concentration of 14.6 μM (neat), 1.83 μM (1/8) and 0.92 μM (1/16) incubated with 10 mM EDTA in 20 mM Tris buffer, pH 8.0 at 37°C for 30 min and 60 min, respectively; Lane 5-7 and 12-14. Titration of DNase in concentration of 14.6 μM (neat), 1.83 μM (1/8) and 0.92 μM (1/16) incubated in 20 mM Tris buffer, pH 8.0 containing 5 mM MgSO_4 at 37°C for 30 min and 60 min, respectively; Lane 8 and 15, control, no DNase added to the reaction incubated at 37°C for 30 min and 60 min, respectively.

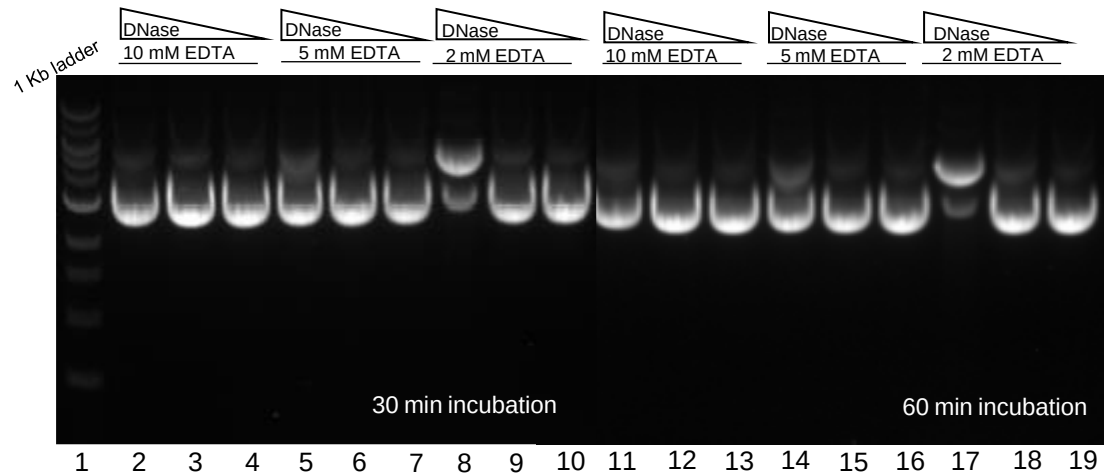


Figure 3.8 Titration identified 10 mM EDTA as optimal to abolish Pyocin S3 DNase activity.

Purified pyocin S3 DNase prepared in concentration of 292 μM (neat) was subjected to 1/8 dilution to 1/32 dilutions yielding concentrations 36.5 μM (1/8) to 9.13 μM (1/32) with buffer containing with buffer containing 20 mM Tris and 10% glycerol. An aliquot of each dilution was incubated with 10 mM, 5 mM and 2 mM EDTA in 20 mM Tris buffer, pH 8 for 15 min at room temperature before addition of 102.5 ng/ μl of plasmid DNA, pGEMT-Easy, as a substrate for exactly 30 min and 60 min. Reactions are loaded on a 0.8% agarose gel and viewed under UV. Lane 1. 1 Kb ladder; Lane 2-4 and 11-13. Titration of DNase in the concentration of 1.83 μM (1/8), 913 nM (1/16), 456 nM (1/32) with 10 mM EDTA treatment incubated at 37 $^{\circ}\text{C}$ for 30 min and 60 min, respectively; Lane 5-7 and 14-16. Titration of DNase in the concentration of 1.83 μM , (1/8), 913 nM (1/16), 456 nM (1/32) with 5 mM EDTA treatment incubated at 37 $^{\circ}\text{C}$ for 30 min and 60 min, respectively; Lane 8-10 and 17-19. Titration of DNase in the concentration of 1.83 μM , (1/8), 913 nM (1/16), 456 nM (1/32) with 2 mM EDTA treatment incubated at 37 $^{\circ}\text{C}$ for 30 min and 60 min, respectively.

3.2.4 Mg^{2+} , Mn^{2+} , Ni^{2+} , Zn^{2+} , Ca^{2+} and Co^{2+} can serve as co-factors for pyocin S3 DNase activity.

Working on the presumption that pyocin S3 DNase utilises a metal ion for DNA cleavage like the other DNase type bacteriocins, Mg^{2+} was shown to ameliorate DNase activity (Figure 3.7). To find out whether alternative metal ions could perform a similar function in the pyocin S3 DNase active site, apo-pyocin S3 DNase domain was prepared using Guanidine-EDTA treatment mentioned as above (Gupta, Dobritsa et al. 2002). Six divalent metals were subsequently introduced to test whether they could restore DNase activity in the initial assay. The metals included Mg^{2+} , Mn^{2+} , Ni^{2+} , Zn^{2+} , Ca^{2+} and Fe^{2+} (Figure 3.9). This approach to stripping off the bound metal ion from the pyocin S3 DNase and re-introducing alternative metals revealed a reliance on Mg^{2+} , Mn^{2+} and Ni^{2+} for activity in the absence of the immunity protein as the substrate plasmid DNA was completely degraded. Less DNase activity was detected in the presence of Zn^{2+} as conversion of the substrate plasmid DNA from supercoiled to linear with limited degradation was observed. With the addition of Fe^{2+} , a band at the top of the well with a smear going down to the bottom of the agarose gel was observed suggesting that the plasmid DNA did not migrate properly (Lane 5 Panel A, Figure 3.9). This is probably due to the oxidation of Fe^{2+} to Fe^{3+} during incubation in aqueous solution. The same result was observed with the addition of 2 mM of DTT (DL-Dithiothreitol) to the reaction buffer to stop Fe^{3+} turning into Fe^{2+} (Netto and Stadtman 1996) (data is not shown). Thus, the reason DNA did not migrate properly on the agarose gel upon addition of Fe^{2+} ion requires further investigation that was not pursued within the

Pyocin S3 DNase requires a metal ion co-factor for its activity current study. Because the possibility that the solution of substrate plasmid DNA contained bound metals was not taken into account in the above assay, a further assay using metal-free substrate plasmid was performed with/without addition of Mg^{2+} (Panel B, Figure 3.9). Addition of 1 mM Mg^{2+} ions could readily cause nicking of the substrate plasmid DNA by the EDTA inhibited pyocin S3 DNase, which was consistent with the previous data that was obtained from the plasmid nicking assay using non-metal-free substrate plasmid DNA (data is not shown). The Mg^{2+} ions was found to be the preferable metal for the DNase endonuclease activity, which was in line with observation on colicin E9 DNase (Pommer, Kühlmann et al. 1999).

A metal dependence assay on pyocin S3 DNase using titration of metal ions was performed to further investigate the relative efficiency of each metal. Lane 7-12, Figure 3.10 shows that the addition of 1 mM of each of the six metals tested was able to restore the activity of the EDTA-treated DNase. A high concentration of metal addition did not always support the DNase activity; in the presence of excess (10mM) cobalt and calcium ions the DNase failed to cleave DNA (Lanes 17 and 18 Figure 3.10). Similarly, addition of limited metal ion failed to restore endonuclease activity (Lanes 2-6 Fig Figure 3.10), with the exception of the addition of 0.1 mM magnesium ions (Lane 1 Fig Figure 3.10). It should be noted however that addition of 0.01 mM of magnesium ions failed to restore pyocin S3 DNase activity (data not shown). Our data therefore suggest that a magnesium Mg^{2+} ion is the preferred metal ion for pyocin S3 DNase endonuclease activity, however, it is likely *in vivo* that alternative

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metal ions such as those of zinc, nickel and manganese can, if present, can substitute for this ion.

Pyocin S3 DNase requires a metal ion co-factor for its activity

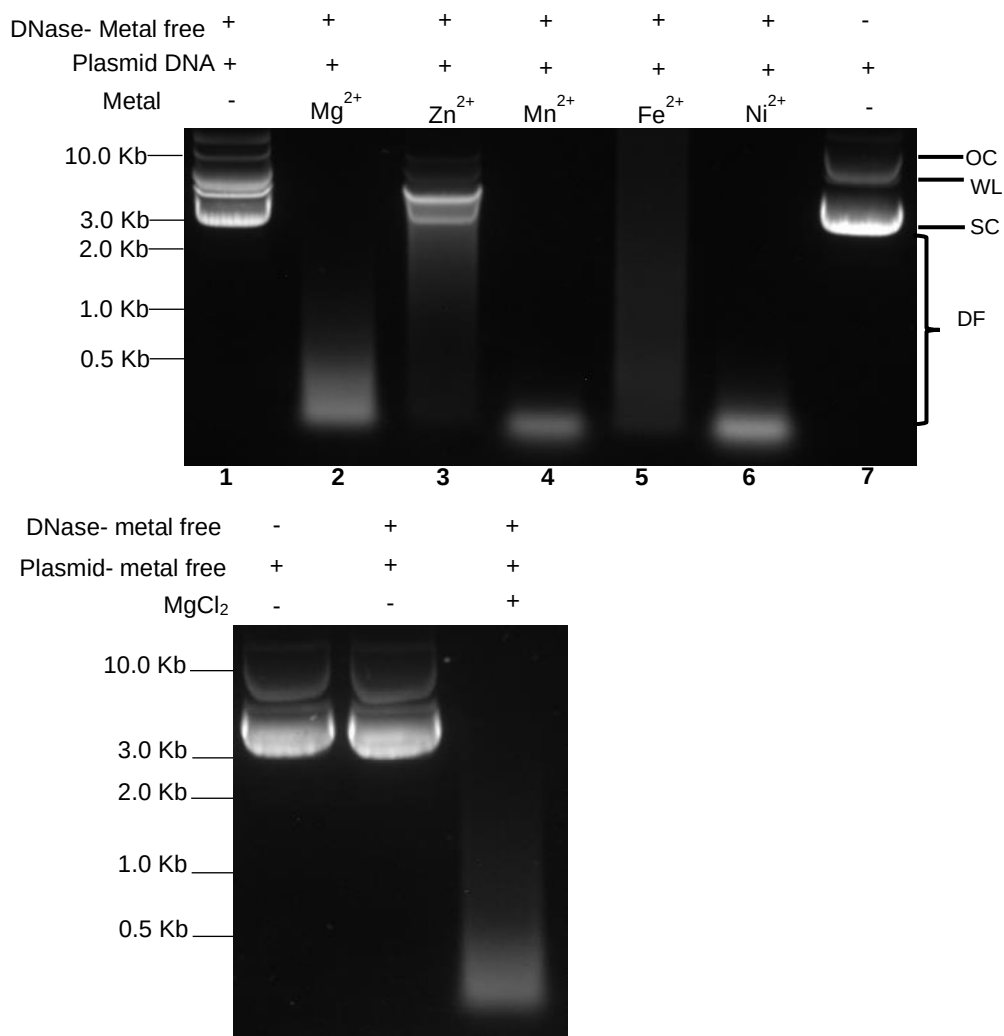


Figure 3.9 Mg^{2+} , Mn^{2+} and Ni^{2+} addition to EDTA-treated pyocin S3 restores DNase activity.

Panel A- Purified DNase was diluted with 20 mM Tris-HCl and 10% (v/v) glycerol buffer, pH 8.0 to required concentration. 1 μ l of each dilution and treated with 10 mM EDTA for 15 mins at room temperature was incubated with 102.5 ng/ μ l of plasmid DNA for 60 mins at 37°C, before being loaded on a 1% agarose gel and viewed under UV. Lane 1. negative control, no DNase was added to the reaction solution; Lane 2. neat, 14.6 μ M of DNase; Lane 3- 10. 913 nM, 456.5 nM, 228.3 nM, 114.3 nM, 57.3 nM, 29 nM, 14.5 nM, 7.5 nM of DNase, respectively. Panel B- Mg^{2+} , Mn^{2+} and Ni^{2+} to Guanidine-EDTA treated pyocin S3 restored DNase activity. A final concentration of 0.12 μ M of purified pyocin S3 DNase diluted with 20 mM Tris-HCl and 10% (v/v) glycerol buffer, pH 8.0 was treated with 20 mM Tris and 50 mM NaCl buffer, pH 8.0 containing 6 M Guanidine for 25 mins shaking at 60 rpm at room temperature, prior to 5 mM EDTA treatment

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for 15 mins at the same temperature. The treated DNase was then dialysed against 20 mM Tris and 10% glycerol buffer, pH 7.4 (metal-free) yielding a final concentration of 43 μ M. The metal-free DNase was then diluted with the dialysis buffer to required concentration and incubated with 102.5 ng/ μ l of plasmid DNA with addition of 1 mM of MgCl₂, ZnSO₄, MnCl₂, FeSO₄ and NiSO₄ for 60 mins at 37°C before being loaded on a 1% agarose gel and viewed under UV. Molecular weight marker sizes are indicated on the left in kb. The forms of plasmid DNA are indicated to the right of the panels: SC- supercoil; WL- whole linear DNA; DF- degraded fragments.

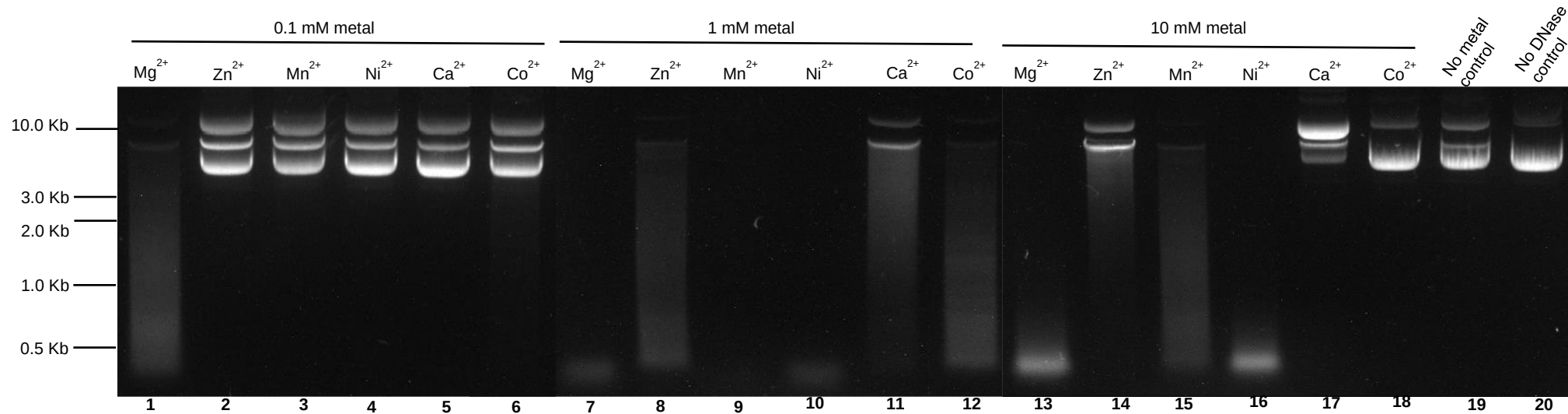


Figure 3.10 Mg²⁺ can restore pyocin S3 DNase activity at a lower concentration than other metals, suggesting it is the preferred co-factor.

Purified DNase was dialysed against 10 mM EDTA using a 3,500 MWco dialysis tubing for 4 h at 50 rpm on a shaker at room temperature and then subjected to 2 runs of dialysis against 20 mM Tris and 10% (w/v) glycerol buffer, pH 7.4 with/ without 1 μ M EDTA, which was treated with chelex 100 for metal removal. Metal dependence was undertaken with additional of 0.01 mM (figure is not shown), 0.1 mM, 1 mM and 10 mM of MgCl₂, ZnSO₄, MnCl₂, NiCl₂, CaCl₂, CoSO₄. The substrate plasmid DNA, pGEMT-Easy, was treated with 1 mM EDTA for 15 mins at 50 rpm on a shaker at room temperature, and 102.5 ng/ μ l of which was incubated with 0.43 μ M of the resulting DNase for 60 min at 37°C incubator. 10 μ l of each sample was mixed with 2 μ l of Gel Loading Dye (Biolabs) before applied to a 0.8% agarose gel alongside the 1 kb DNA ladder. Molecular weight marker

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sizes are indicated on the left in kb. Lane 1. addition of 0.1 mM Mg^{2+} ; Lane 2. addition of 0.1 mM Zn^{2+} ; Lane 3. addition of 0.1 mM Mn^{2+} ; Lane 4. addition of 0.1 mM Ni^{2+} ; Lane 5. addition of 0.1 mM Ca^{2+} ; Lane 6. addition of 0.1 mM Co^{2+} ; Lane 7. addition of 1 mM Mg^{2+} ; Lane 8. addition of 1 mM Zn^{2+} ; Lane 9. addition of 1 mM Mn^{2+} ; Lane 10. addition of 1 mM Ni^{2+} ; Lane 11. addition of 1 mM Ca^{2+} ; Lane 12. addition of 1 mM Co^{2+} ; Lane 13. addition of 10 mM Mg^{2+} ; Lane 14. addition of 10 mM Zn^{2+} ; Lane 15. addition of 10 mM Mn^{2+} ; Lane 16. addition of 10 mM Ni^{2+} ; Lane 17. addition of 10 mM Ca^{2+} ; Lane 18. addition of 10 mM Co^{2+} ; Lane 19. control, no metal was added to the reaction; Lane 20. control, no DNase was added to the reaction with 1 mM Mg^{2+}

3.2.5 *No detection of pyocin S3 DNase and metal ions (Mg^{2+} and Zn^{2+}) interaction using ITC*

Since the above metal dependence assay has shown distinct effects of metal ion addition on the pyocin S3 DNase activity, the stoichiometry and affinity of metal ions to pyocin S3 DNase was analysed using isothermal titration calorimetry (ITC) to provide further understanding of the protein-metal interaction by determining the energetics of binding. ITC is the most sensitive, quantitative, accurate and label-free method available currently to determine the thermodynamics of protein-protein or protein-metal binding/interaction. It is powerful as it monitors the heat change (either absorbed or released) when binding occurs during the gradual titration of metal ion into the protein solution of interest. Moreover, it measures binding parameters relevant to binding affinity and the interaction mechanism. These include binding constants (K_D), enthalpy (ΔH), Gibbs free energy (ΔG) and binding entropy (ΔS), plus binding stoichiometry (n). Measurements are undertaken simultaneously in a single experiment (Pierce, Raman et al. 1999, Recht, Nienaber et al. 2016, Prozeller, Morsbach et al. 2019).

According to the literature on colicin DNases (Pommer, Kühlmann et al. 1999, Pommer, Cal et al. 2001), both Mg^{2+} and Zn^{2+} ions have a higher chance of coordinating into the active site of DNase bacteriocins and being involved in catalysis of DNA hydrolysis. Therefore, $MgCl_2$ and $ZnSO_4$ solution were chosen to be the first ones to use for analysing the binding affinity of pyocin S3 DNase to metals using ITC. Typically, if ITC detects an interaction, each successive injection of co-factor generates a trough in the trace with a larger DP at its minimal point creating an

exponential plot over time (Quinn, Carpenter et al. 2016). Unfortunately, this pattern was not observed, indicating that there was no saturatable binding isotherm (Figure 3.11). It is possible that too much non-specific binding was occurring between the metal and the pyocin S3, causing the binding site of the DNase not to remain unsaturated.

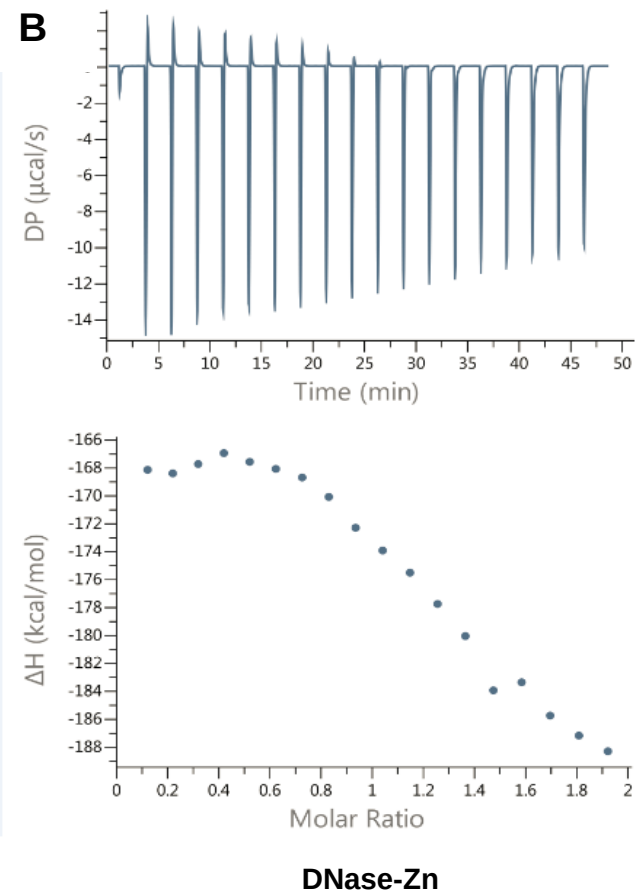
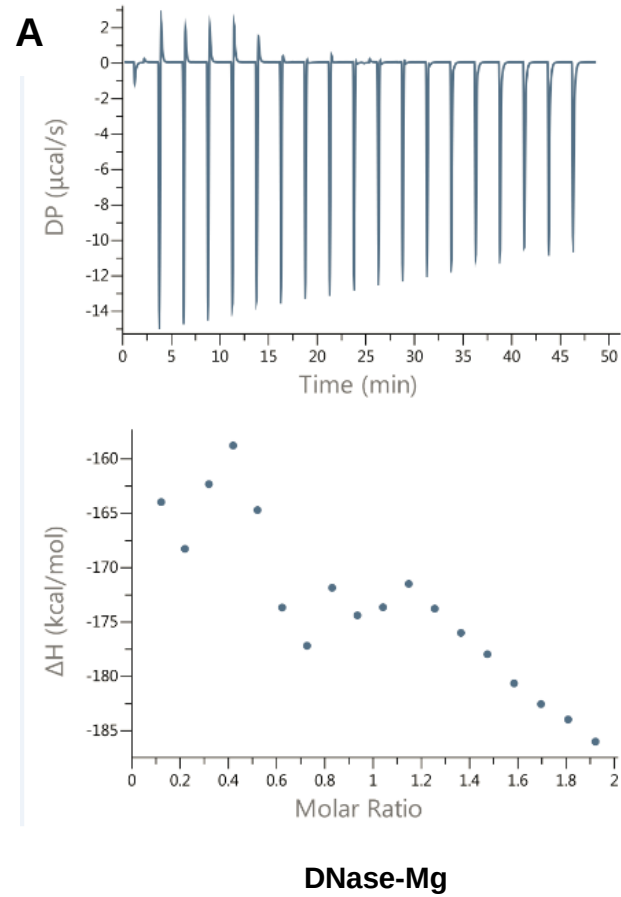


Figure 3.11 Monitoring magnesium and zinc ions binding to the pyocin S3 DNase by isothermal titration calorimetry was inconclusive.

Panel A and B- Isothermal titration calorimetry data showing the binding of magnesium and zinc to the pyocin S3 DNase, respectively. Top panels show the calorimetric response of 19 injections of metal solution (286 μM) to pyocin S3 DNase (28.6 μM) in 20 mM Tris, 150 mM NaCl and 10% glycerol buffer, pH 7.4 at 25°C. The injection consists of an initial 0.4 μl injection followed by 18x 2 μl injections. The bottom panels show integrated injection heats for the data above. Unfortunately, they cannot be fitted to a single, non-cooperative binding model to get a continuous line, which can represent the theoretical isotherm, due to no saturable binding isotherm being observed.

3.3 Discussion

3.3.1 *Pyocin S3 DNase carries endonuclease activity*

The DNase endonuclease activity of pyocin S3 was studied using the plasmid nicking assay and its efficiency was assessed by the extent of degradation of supercoiled pGEMT-Easy plasmid DNA as described by Shiokawa & Tanuma (Shiokawa and Tanuma 1999). It was demonstrated that, like the other DNase-type bacteriocins, pyocin S3 DNase carries DNA endonuclease activity in the absence of its cognate immunity protein and that the immunity protein protected the toxin producing strain by neutralization of the killing activity of the DNase (Figure 3.5) (Wallis, Leung et al. 1995, Joshi, Grinter et al. 2015). Figure 3.5 has revealed that pyocin S3 DNase was an efficient DNase since 4.3 nM of it was able to nick plasmid DNA that was present at a 5-fold higher concentration (23.9 nM) within a 60 min incubation.

Pommer et al. has reported that a metal ion, such as nickel or cobalt, was required for the DNase activity of colicin E9 and was involved in activating both the scissile phosphodiester and a water molecule for protonating the 3' oxygen (Pommer, Cal et al. 2001). We suspect that the presence of immunity altered the secondary structure of pyocin S3 DNase causing release of the crucial metal ion from the active site of the enzyme. Hence, the DNase could no longer cleave the plasmid DNA. The further investigation using x-ray crystallography will be demonstrated in chapter 5.

3.3.2 The endonuclease activity of pyocin S3 DNase is altered by the presence of different metal ions.

Since most DNases require the presence of a metal ion for their optimal activity and there is evidence suggesting that the nuclease activity of the DNase is highly dependent upon the metal added to the reaction (Moore 1981) and in most of the cases, transition metals result in a higher proportion of double-stranded cleavage (Price 1975, Campbell and Jackson 1980). Previous studies have suggested that a single transition metal ion, Zn^{2+} or Ni^{2+} was observed within the active site in the structure of colicin E7 (Ko, Liao et al. 1999) and E9 (Walker, Georgiou et al. 2002), respectively. Mg^{2+} or Ca^{2+} were also reported as an essential metal ion for the activity of the zinc-enzyme, colicin E7 (Pommer, Cal et al. 2001, Sui, Tsai et al. 2002). We have demonstrated here that pyocin S3 DNase is a metallo-protein, as EDTA-treated apo-DNase did not bear nuclease activity (Figure 3.8). However, the identity of the metal present in the DNase active site was initially unknown. To address this important question, the activity of EDTA treated pyocin S3 DNase with subsequent addition of different metal ions was investigated. Our data revealed a similar observation found on colicins (Sui, Tsai et al. 2002, Maté and Kleanthous 2004) that the apo-pyocin S3 DNase can utilize a few first-row transition metals for its activity in the absence of its cognate immunity protein. Mg^{2+} , Mn^{2+} , Ni^{2+} were the most potent divalent metal ions in restoring EDTA-treated pyocin S3 DNase activity, while Ca^{2+} and Co^{2+} were the least (Figure 3.10). On the other hand, pyocin S3 DNase had optimal activity with the presence of 1 mM of those divalent metal ions: Mg^{2+} , Mn^{2+} , Ni^{2+} and Co^{2+} , whilst showing very weak or no activity in the

Pyocin S3 DNase requires a metal ion co-factor for its activity presence of 10 mM of Zn^{2+} ions (Figure 3.10) as there was only a small amount of supercoiled plasmid DNA converted to linearised DNA. A similar metal dependent behaviour has been reported for pyocin AP41 DNase (Joshi, Grinter et al. 2015) and colicin E9 DNase (Pommer, Wallis et al. 1998). It is found that several Zn^{2+} -bound enzymes, such as colicin E7 DNase, metallo- β -lactamase and methionine aminopeptidase, were also inhibited by higher concentrations of Zn^{2+} ion (>1000 in molar ratio to enzyme) (Ku, Liu et al. 2002). It is consistent with the phenomenon observed in colicin E9 and pyocin S3 DNase in this study, in which a molar ratio of Zn^{2+} ion to enzyme was 10,000 (10 μM to 1 nM) (Pommer, Kühlmann et al. 1999) and 23256 (10 mM to 0.43 μM), respectively. The reason that Zn^{2+} ion has an inhibitory effect on colicin E9 DNase was elucidated by Maté and Kleanthous. The tight binding between Zn^{2+} ion and the three histidines stopped a water molecule replacing a histidine to serve as a protonating device for DNA hydrolysis (Maté and Kleanthous 2004), thus abolished the endonuclease activity. In addition, at high concentration of 10 mM, there was no nicking activity of pyocin S3 DNase observed in presence of Co^{2+} ion, as there was no conversion of supercoiled DNA to any other form of DNA; a higher proportion of double-strand breaks were shown in presence of Zn^{2+} ion than in Ca^{2+} ion, as there were more linearised DNA shown with Zn^{2+} and more open-circled DNA shown with Co^{2+} (Lane 14, 17 and 18, Figure 3.10).

Chapter 4. Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

4.1 Introduction

A $\beta\beta\alpha$ -Me fold has been observed in several H-N-H endonucleases, including the well-characterized colicin E7, E9 and three S-type DNase pyocins: S1, S2 and AP41 (Huang and Yuan 2007, Joshi, Grinter et al. 2015). The H-N-H motif of colicin E9 consists of residues His551, Asn566 and His575 (Pommer, Kühlmann et al. 1999). For colicin E7, the residues in the H-N-H motif are His545, Asn560 and His573 (Huang and Yuan 2007). The residues His739, Asn754 and His763 compose the H-N-H motif in pyocin AP41 (Walker, Georgiou et al. 2002). In addition, three histidine residues are involved in metal coordination together with some other ligand(s) in each of these H-N-H DNases. For example, His102, His127 and His131 are co-ordinated with a divalent metal ion and a phosphate ion in colicin E9 (Pommer, Kühlmann et al. 1999); His544, His569 and His573 with a divalent metal ion and phosphate ion in colicin E2 (Huang and Yuan 2007); and His738, His763 and His767 with a citrate anion and a water molecule in pyocin AP41 (Joshi, Grinter et al. 2015). In contrast to this, despite pyocin S3 being classified as a DNase and having demonstrable endonuclease activity (Duport, Baysse et al. 1995, Chapter 3), it lacks the conserved H-N-H motif in its primary sequence (Figure 4.1). This chapter sets out to investigate how pyocin S3 DNase manages to maintain the active site secondary structure

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding. without this typical motif and identify which residues could be involved in DNA catalysis or coordination.

Finding out the important residues involved in DNA binding or coordination in a DNA-binding protein such as pyocin S3, builds understanding of the mechanism of protein-DNA interaction, and furthermore contributes to the discovery and design of novel therapeutic drugs against *P. aeruginosa* infection. To do this, site directed mutagenesis of pyocin S3 was undertaken to make alanine substitutions of some conserved residues hypothesized to be key for DNase activity (Asp25, His23, His26 and His66) to identify the residues with critical roles. To monitor DNase activity or subsequent loss of activity resulting from the site directed mutation, the activity of full-length pyocin S3 was tested against 8 clinical *P. aeruginosa* strains, isolated from cystic fibrosis patients. If a strain is killed by pyocin S3, it can be incorporated into agar and a clear zone devoid of growing bacteria is visible around a stab of pyocin S3. Such an assay is higher throughput than the DNA-nicking assay used in Chapter 3, and thus ideally suited for the initial identification of inactive pyocin S3 mutants. To set up such a screen, it was necessary to select the most suitable sensitive strain to be used as an overlay for the *in vivo* stab test assay. Mutations that caused the loss of DNase activity would not generate a clear zone of growth inhibition on the stab test plate. This would suggest that the targeted residues may be involved in DNA catalysis or a related function, for example, coordinating of a metal ion required for catalysis and/or binding of DNA for catalysis. Thereafter, the plasmid nicking activity of these mutant pyocins was also

determined. As presented in Chapter 3, the *in vitro* plasmid nicking assay is performed essentially as described by Pommer et al. The extent of DNA cleavage is assessed by assessing the level of production of the reaction products (relaxed and linear DNA) as visualized on an agarose gel (Pommer, Wallis et al. 1998). This assay can distinguish between different DNA cleavage mechanisms of the DNase e.g. the introduction of a single phosphodiester bond break into one of the DNA strands, or the nicking of both strands after the enzyme and DNA interact (Campbell and Jackson 1980).

The primary questions addressed in this chapter were:

1. Do the mutated pyocin S3 residues have different roles in the active site of the DNase?
2. Are the residues involved in metal coordination or DNA binding?

To tackle the second question, a range of assays were utilized to find out the most feasible and suitable one for study. The assays included electrophoretic-mobility shift assay (EMSA), fluorescence polarization (FP) and analytical gel filtration chromatography. The advantages and disadvantages of each technique are discussed in this chapter.

4.2 Results

4.2.1 *Amino acid sequence alignment to identify potential active site residues in pyocin S3 DNase*

It was hypothesized that the colicins and pyocins that share DNase activity would have conserved amino acid residues in their active site. This is in contrast to the DNase-immunity binding site of different colicins/pyocins. Due to the specific recognition between the two cognate

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding. proteins, this is not conserved (Ku, Liu et al. 2002). The pairwise matching of sequences allows individual immunity proteins to specifically protect their producing cells from the killing activity of its cognate DNase. A set of *Pseudomonas* genomes were selected from publically accessible databases (NCBI, <https://www.ncbi.nlm.nih.gov/>) since they had a unique pair of pyocin S3 DNase – immunity genes/orthologues. Sequence alignments of $\beta\beta\alpha$ -Me DNase domains were constructed (Figure 4.1). Fifty two conserved residues (23 of them being identical) were evident and are indicated (Figure 4.1). The secondary structure elements to which these residues are predicted to contribute are also indicated. In the initial experiment, three of the residues shown as highly conserved in DNase domains of all the bacteriocins analysed were chosen for further investigation. Histidine and aspartic acid residues were selected since, in nucleases, they are often involved in the catalytic hydrolysis of phosphodiester bonds. These amino acids are strongly nucleophilic, and mutation of those residues would be predicted to abolish the nuclease activity, without affecting DNA binding (Gerlt 1993, Meiss, Scholz et al. 2001). Of the 4 identical histidine and aspartic acid residues, one was not selected since its spacing from the others suggested it was less likely to be part of the active site. The 3 residues chosen for mutagenesis were D25, H26 and H66 in the pyocin S3 DNase domain. Their equivalent positions in the full-length pyocin S3 are D683, H684 and H724, respectively. All three of these selected residues had been shown essential for nuclease activity of the PA14 pyocin S3 DNase (highlighted with red background in Figure 4.1). Following site directed

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mutagenesis to alanine, the *in vivo* and *in vitro* DNase activity assays were performed on the mutants to confirm the importance of the residues for DNase activity (see 4.2.2 onwards).

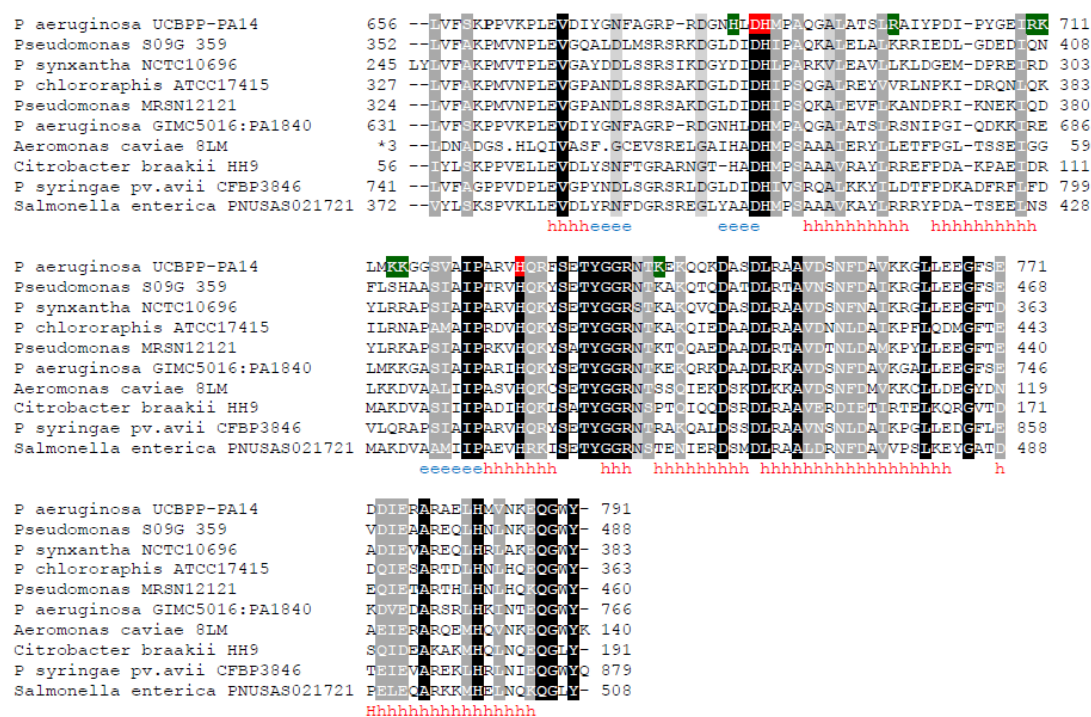


Figure 4.1 Primary sequence alignment of DNase domains identifies conserved residues.

The sequences of $\beta\beta\alpha$ -Me DNase domains from a selection of bacteriocins were kindly aligned by Dr. Phil Bardelang, to follow-on from similar alignments generated by myself. Conservation of residues is coloured as follows; white text on black background, identical; white text on grey background, highly similar; text with light grey background; similar. Residues shown essential for nuclease activity of the PA14 pyocin S3 DNase are highlighted with a red background. Alignments are annotated with secondary structure elements below the sequence; h, α -helix and e, β -strand. The Genbank accession numbers for the sequences are CP000438.1 (top; PA14), CP025263.1, LR590482.1, CP027714.1, CP010892.1, CP034430.1, CP024198.1, SJSF01000006.1, LIJ01000059.1 and EAN4680376.1 respectively.

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

4.2.2 *Site directed mutation of H23A, H26A and H66A abolishes in vivo killing of sensitive P. aeruginosa by pyocin S3 in stab tests.*

4.2.2.1 *Site directed mutagenesis of residues predicted to be important to pyocin S3 DNase activity.*

To identify whether the conserved histidines and aspartic acid residue identified in sequence alignments (Figure 4.1) were essential residues for DNase activity, site-directed mutagenesis was undertaken to substitute them for an alanine residue in full-length W/T pyocin S3. Alanine is non-bulky without a side chain chemistry but retains the β -carbon (the first atom of the sidechain in an amino acid). Substitution with alanine can remove all side chain atoms past the β -carbon so that the effect of the side chain of the targeted amino acid and the importance of that amino acid to the biological activity of the toxic protein can be evaluated (Garinot-Schneider, Pommer et al. 1996, Weiss, Watanabe et al. 2000). The three pyocin S3 site-directed mutants, D25A, H26A and H66A, were constructed using Q5 mutagenesis kit as described in Figure 4.2. Mutated DNA was transformed into *E. coli* DH5 α cells, and the plasmids were extracted and sent to GENEWIZ® for DNA sequencing to confirm the desired substitutions had occurred. Thereafter, examination of the biological activity of the w/t and mutant pyocin S3 DNase was undertaken using the *in vivo* stab test assay.

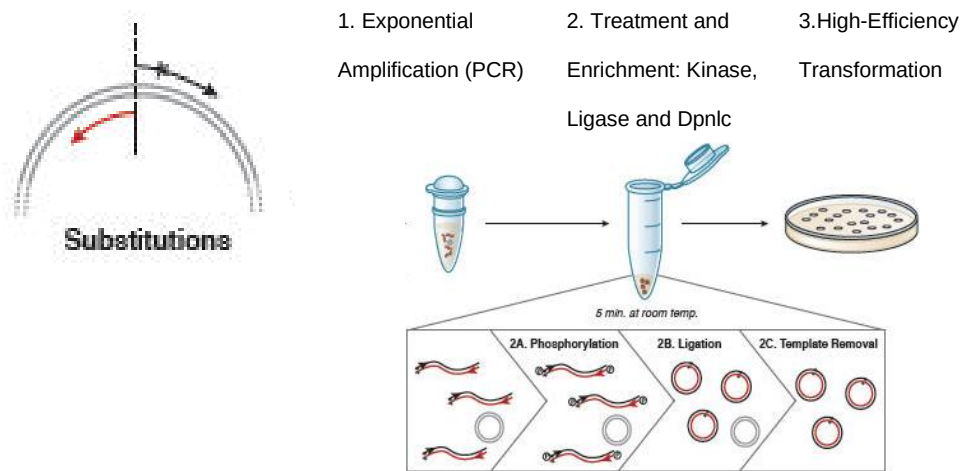


Figure 4.2 Schematic to outline the process of Q5 Site-Directed mutagenesis.

The Q5 Site-Directed mutagenesis kit enables efficient incorporation of substitutions into double stranded plasmid DNA. The first step is an exponential amplification using bespoke designed mutagenic primers (Appendix 1) and a master mix formulation of Q5 Hot Start High-Fidelity DNA Polymerase to create substitution. The second step involves incubation with a kinase-ligase-DpnI enzyme for rapid circularization of the PCR product and removal of the template DNA. The last step is a transformation into DH5 α chemically competent cells (adapted from Fig 2, Q5 Site-Directed Mutagenesis Kit Overview, Biolabs <https://international.neb.com/products/e0554-q5-site-directed-mutagenesis-kit#Product%20Information>).

4.2.2.2 Optimisation of the stab test to assess *in vivo* pyocin S3 activity.

In a stab assay, pyocin is released from the stabbed strain onto a sensitive strain that is growing within the overlay on an agar plate (Garinot-Schneider, Pommer et al. 1996). If killing of the sensitive strain occurs, a clear zone develops around the point of stabbing. The first step in developing a stab test is to select a pyocin S3-sensitive *P. aeruginosa* strain. To do this, 8 clinical *P. aeruginosa* strains isolated from cystic fibrosis patients were screened for sensitivity using *E. coli* producing full-length pyocin S3 (w/t) from plasmid pBluescribe KS+. Two of the *P. aeruginosa* strains were sensitive to the pyocin S3 (Figure 4.3).

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding. Increased sensitivity to pyocin S3 killing is indicated as a larger, clear zone of inhibition. *P. aeruginosa* strain 971 (PSA971) was thus chosen for subsequent stab test assays.

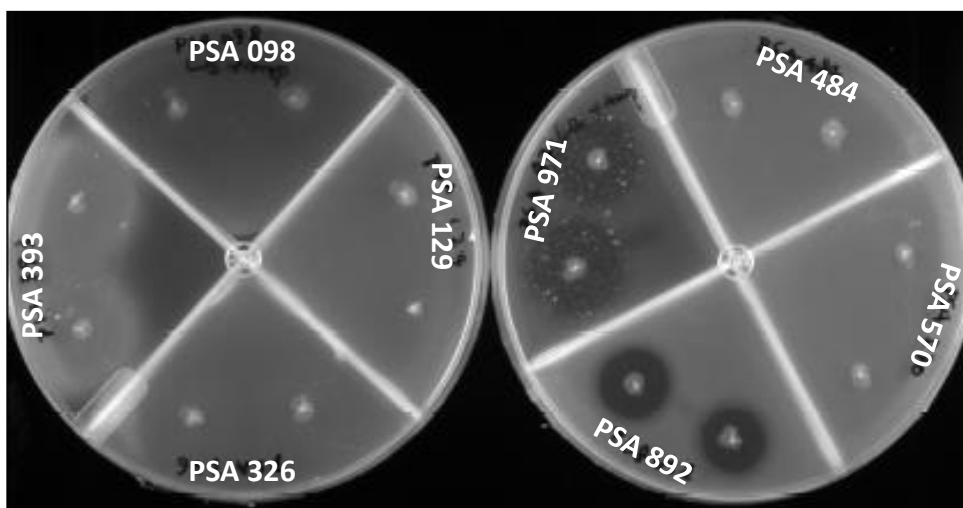


Figure 4.3 Two *P. aeruginosa* clinical strains are sensitive to killing by pyocin S3 DNase.

Single colonies of *E. coli* (pBluescribe KS+) producing full length wild type pyocin S3 DNase were stabbed onto fresh LB containing 100 µg/ml ampicillin in plates that were separated into 4 compartments. Two duplicate colonies were stabbed into each compartment. The plate was incubated at 37°C overnight before being exposed to chloroform vapour for 15 min at room temperature in a fume hood. The plate was inverted and left in the fume hood without a lid for 30 min, and then overlaid with 2 ml of warmed (~ 50°C) soft top agar inoculated with 27 µl of a single clinical strain per compartment. Plates were incubated at 37°C for overnight before imaged using GelDoc (Bio-Rad).

4.2.2.3 *Stab test screening reveals D25A, H26A and H66A mutants of pyocin S3 were inactive in vivo.*

A stab test was performed with all three pyocin mutants using *E. coli* producing the W/T pyocin S3 as a positive control. For each pyocin mutant (D25A, H26A and H66A), the sensitive strain PSA971 grew up to the stab, and no clear growth inhibition zone was observed on the agar

plate indicating that the killing activity of these mutants had been abolished (Figure 4.4). This is consistent with the previous sequence alignment data (Figure 4.1), suggesting D25, H26 and H66 were conserved amongst the pyocin S3 family and thus likely to be involved in catalysing DNA breakdown or a related function, for example, coordinating a metal ion required for catalysis and/or binding the DNA substrate.

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

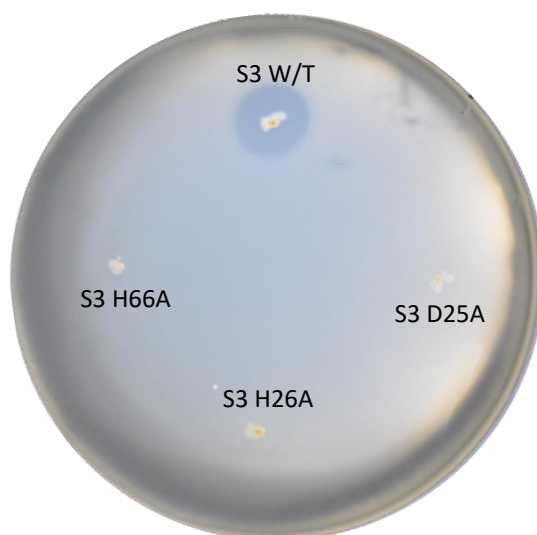
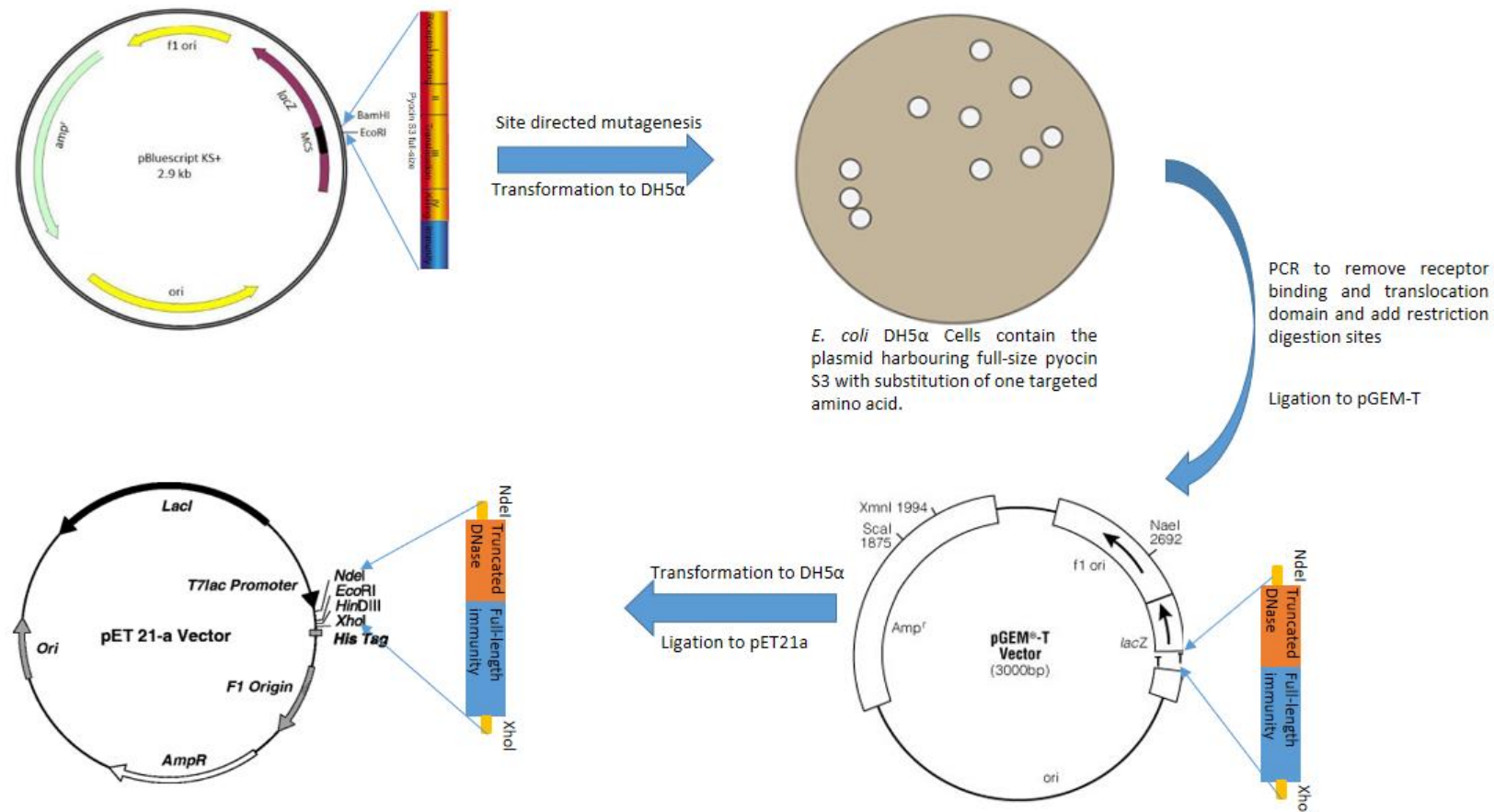


Figure 4.4 Full-length pyocin S3 DNase mutated in either D25A, H26A, or H66A does not kill the pyocin S3-sensitive clinical strain PSA971.

Alanine substitution of residues in predicted active site residues (D25A, H26A, H66A) were generated using Q5 site-directed mutagenesis kit (NEB, Figure 4.2). A single colony *E. coli* (pBluescribe KS+) producing W/T pyocin S3 DNase, *E. coli* (pBluescribe KS+) producing pyocin S3 D23A, *E. coli* (pBluescribe KS+) producing pyocin S3 H26A or *E. coli* (pBluescribe KS+) producing pyocin S3 H66A was stabbed onto plates as described in section 4.2.2.2 except that the soft top agar was inoculated with 80 μ l of clinical strain PSA971. Plates were incubated at 37°C for overnight before imaged in a dark room using a light box.



Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

Figure 4.5 A schematic to outline the site directed mutagenesis and downstream sub-cloning of pyocin S3.

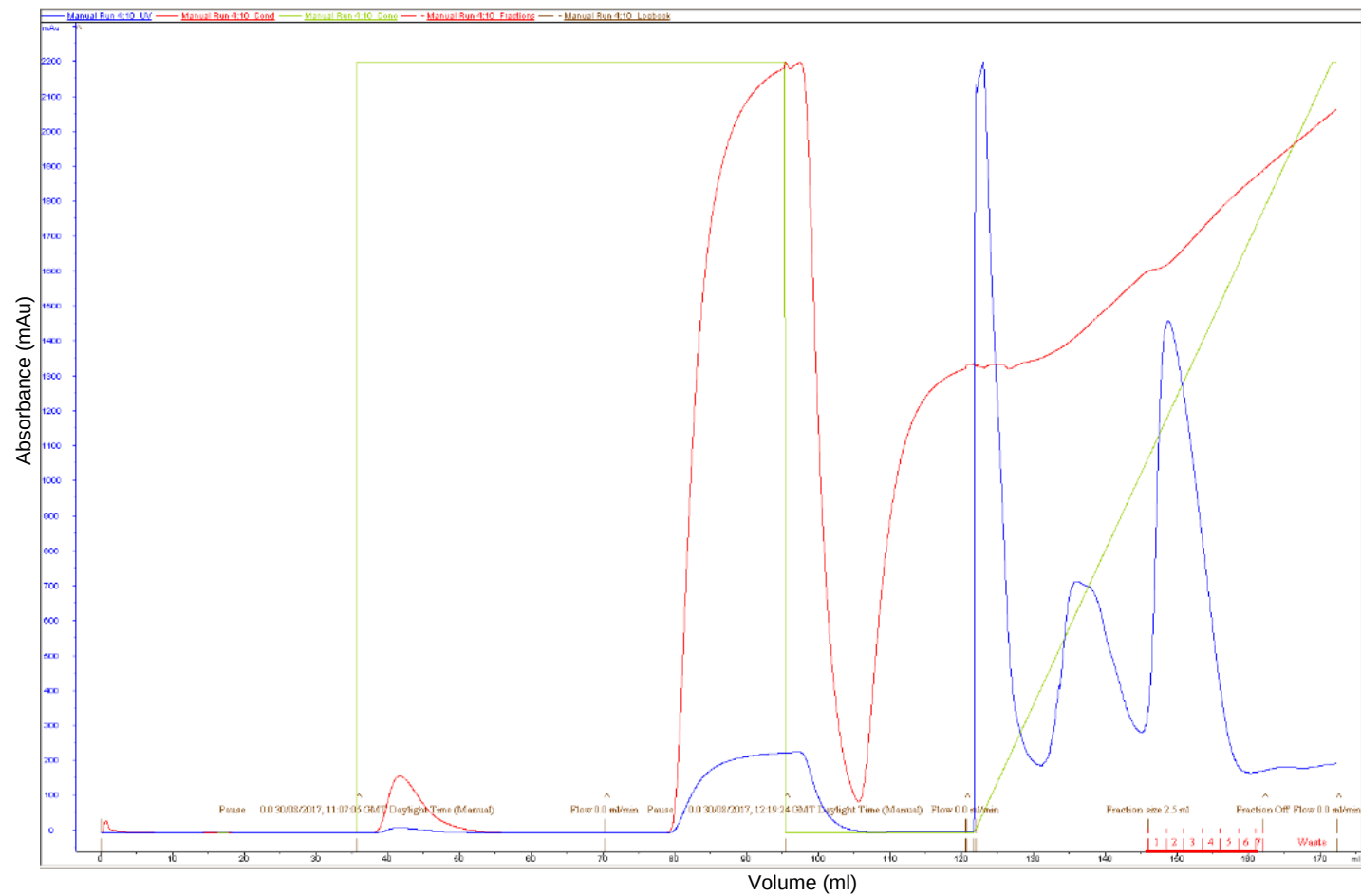
Alanine substitution of the targeted amino acid was achieved by mutagenesis of the encoding codon in the plasmid of pPYS3-3 containing the genes encoding the full size pyocin S3 DNase and immunity protein. Simultaneously, the internal *NdeI* restriction site was removed (described in 2.4.3.1). This was achieved by site directed mutagenesis (Q5 Site-Directed Mutagenesis Kit, NEB) using the primers name1 and name2 (Appendix 1). The resultant plasmid was transformed into *E. coli* DH5 α . Following sequence verification, the mutated plasmid was amplified by PCR using primers YC16 and ImS3R (Appendix 1). This introduced an *NdeI* site at the first codon of the pyocin S3 DNase gene and an *XhoI* site at the end of the immunity protein encoding gene. The PCR product was separated by agarose gel electrophoresis and purified using a Monarch DNA Gel Extraction Kit (Biolabs) prior to ligation into the pGEMT-Easy vector (Promega). This resulted in construction of plasmid pGEMT-Easy S3-*NdeI*, which encodes a truncated pyocin S3 DNase and the full-length immunity protein. Subsequent sub-cloning was accomplished by digestion at the introduced *NdeI* and *XhoI* sites and then ligation into the corresponding sites of the *E. coli* expression vector pET21a (Novagen) to yield pET pyocin S3, which encodes the mutant pyocin S3 DNase-immunity complex, the latter protein with a C-terminal hexa-histidine tag.

4.2.3 Over-production and purification for pyocin S3 mutant DNases

To further interrogate the role of the mutated pyocin S3 residues in the breakdown of DNA, it was necessary to overproduce and purify them for *in vitro* assays. To do this, the DNA encoding the mutant pyocin DNase domains was subcloned with the cognate immunity protein open reading into over expression plasmids. The same cloning strategy as described in section 3.2.1 for purification of the W/T pyocin DNase-immunity complex was followed, and is outlined in the schematic shown in Figure 4.5. With regards to overproduction and purification, all three mutant pyocin S3 DNase-Immunity complexes behaved similarly to the W/T (described in Chapter 3). Here, only the results for mutant D25A are presented as a representative sample, with the others being in Appendix 2 (H26A) and Appendix 3 (H66A). Pyocin S3 mutant D25A was overproduced from *E. coli* BL21 (DE3) grown in LB medium successfully because proteins of the expected size could be clearly observed after induction: pyocin S3 DNase (17.06 kDa) and S3 immunity (14.7 kDa) (Lane 3, Panel B, Figure 4.2.5.). The cell lysate containing the mutated S3 DNase-Immunity complex was applied to a Ni-NTA column for affinity purification via the His-tagged immunity component as previously. The UV absorbance trace (Panel A, Figure 4.6) revealed a peak highlighted by red underlining that was shown to contain the DNase-immunity protein complex by SDS-PAGE (Panel B, Figure 4.6). Fractions 1 to 6 containing the DNase-immunity protein were pooled and dialysed against 50 mM NaH₂PO₄, 100 mM NaCl and 10 mM Imidazole buffer, pH 7 for further DNase / immunity separation with a Ni-NTA column since they contained

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding. a large quantity of the protein complex with low levels of protein contaminants. After the separation of the DNase and immunity proteins using a buffer containing 6 M guanidine on a Ni-NTA column, the fractions of mutant pyocin S3 DNase-immunity complex protein, isolated mutant DNase protein and immunity protein collected from Ni-NTA purification with or without TCA precipitation were electrophoresed on SDS-PAGE to confirm the size of the proteins. Figure 4.7 (Panel A- all the fractions collected without TCA precipitation, Panel B- selected representative fractions with TCA precipitation) indicates that all the eluted proteins migrated at the expected size on the gel.

A



Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

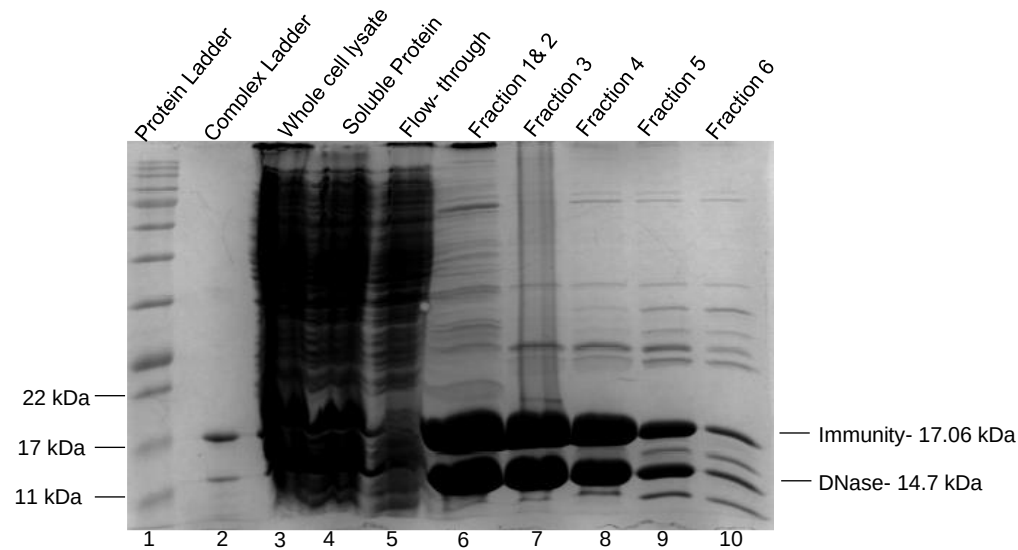


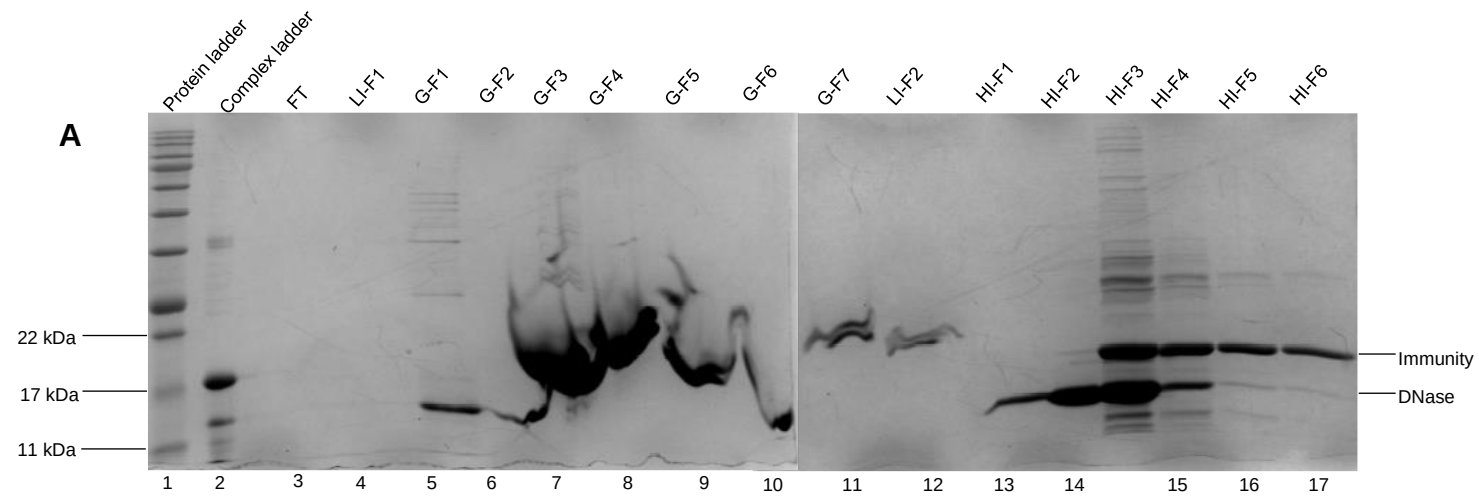
Figure 4.6 Purification of pyocin S3 mutant D25A.

Panel A- Cell lysate containing the mutant D25A of pyocin S3 DNase-immunity protein was loaded onto a 5 ml Ni-NTA column before purification using ÄKTAprimer™ PLUS system. The salt concentration trace mirrors the imidazole addition and approximately at 122 ml, high imidazole buffer was added to the system. 5 ml fractions of purified DNase protein were collected, which was eluted by high concentration of imidazole buffer, till all the protein samples came off the column, where the UV absorbance stayed low and flat (fractions were numbered 1 to 7 in red). Red curve- conductivity trace, blue curve- UV absorbance trace, green line- salt concentration, x axis- volume (ml), y axis- value of absorbance (mAu). Panel B- 60 µl of peak fractions containing DNase-Immunity complex protein were taken and mixed with 20 µl of SDS-PAGE loading buffer and applied to a

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SDS-PAGE running along with soluble protein collected from cell lysate and flow-through from the Ni-NTA column. Lane 1- Colour pre-stained protein standard; Lane 2- DNase-Immunity complex (complex ladder) prepared previously (data not shown); Lane 3- Whole cell lysate; Lane 4- soluble protein; Lane 5- column flow-through; Lane 6 to 10- Fraction 1& 2 to 6. Proteins were visualised using Coomassie blue stain. The expected sizes of the DNase and Immunity proteins are indicated on the right.

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.



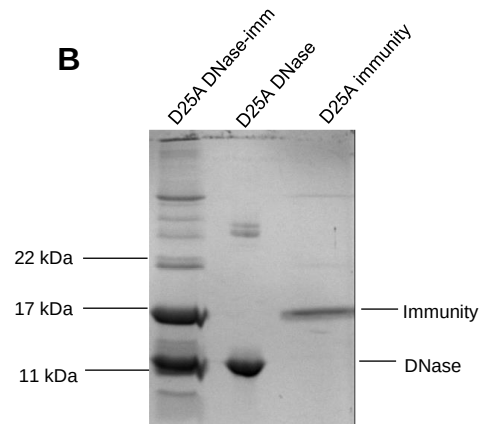


Figure 4.7 Separation of pyocin S3 mutant D25A DNase from cognate Immunity protein.

Panel A- Fractions containing purified DNase-Immunity complex protein from Fig 4.2.5 were dialysed against 50 mM Na₂HPO₄, 100 mM NaCl and 10 mM imidazole buffer, pH 7 before being reloaded onto the Ni-NTA column again for complex separation. Flow-through (FT) was collected. DNase was denatured and eluted off the column using total volume of 20 ml of buffer containing 6 M Guanidine and 3 ml fractions were collected manually using a P-1 pump. Then 20 ml of low imidazole buffer was passed through to push down all the remaining DNase before the immunity protein was eluted off by a high imidazole buffer. Three ml fractions were collected manually using P-1 pump and 60 µl of each fraction was taken and mixed with 20 µl of SDS-PAGE loading buffer to be analysed on a 15% SDS-PAGE gel. Lane 1 and 2. Colour pre-stained protein standard ladder and complex ladder, respectively; Lane 3. FT, flow-through; Lane 4 and 12. LI-F1 and LI-F2, fraction collected from low imidazole wash; Lane 5- 11. G-F1 to G-F7, fraction 1 fraction 7 collected from guanidine buffer wash; Fraction 13- 17. HI-F1 - HI-F6, fractions collected from high imidazole wash. Panel B- TCA precipitation of representative protein

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

fractions analysed on a SDS-PAGE. Lane 1. mutant D25A DNase-immunity complex protein; Lane 2. Mutant D25A DNase protein; Lane 3. mutant D25A immunity protein. Proteins were visualised using Coomassie blue stain. The expected sizes of the DNase and Immunity proteins are indicated on the right.

4.2.4 *Mutant DNases D25A, H26A and H66A showed no endonuclease activity in vitro*

To support the data obtained from the *in vivo* stab test (Figure 4.4), all the purified mutant DNases were assayed using the *in vitro* plasmid nicking/cleavage assay. The aim was to assess the ability of the mutant pyocin S3 DNases to cleave a supercoiled plasmid (pGEMT-Easy). Firstly, the concentrated W/T pyocin S3 DNase was diluted to prepare 2.3 μ M, 0.23 μ M and 2.3 nM nicking/cleavage reactions. Each mutant DNase was similarly diluted to create a nicking/cleavage reaction containing 2.3 μ M DNase (Figure 4.8). Incubated with 23.9 nM (102.5 ng/ μ l) plasmid DNA was performed for 60 min at 37°C before the DNA was visualized on an agarose gel. In lane 3 of Figure 4.2.7 it can be seen that 2.3 μ M of the W/T pyocin S3 DNase degraded the plasmid completely, while 2.3 μ M retained sufficient DNA endonuclease activity to nick the plasmid (Lane 3 and 2, Figure 4.8). At 23 nM the W/T pyocin S3 DNase did not show any DNase activity against the 23.9 nM plasmid DNA since it was indistinguishable from DNA on the gel looked similar to the control reaction lacking DNase (Lane 1 and 7, Figure 4.8). Although incubation with none of the mutants led to obvious DNA cleavage, the DNA fragment distribution observed only mirrored that of the negative control with mutant H26A (Lane 5 and Lane 7, Figure 4.8). The enhanced proportion of linear DNA observed with mutant H66A might be an indication of inefficient single-strand DNA cleavage by this mutant (Lane 6, Figure 4.8). The mobility shifted supercoiled DNA observed with mutant DNase D25A is difficult to explain at this stage, it is however unlikely to reflect DNA binding by mutant D25A to the plasmid which is

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding. present in approximately 100-fold (2.3 μ M: 23.9 nM) excess (Lane 4, Figure 4.8). At this point, it is tempting to speculate that the three residues D25, H26 and H66 are contained within the pyocin S3 DNase active site, and that altering them interferes with DNA cleavage.

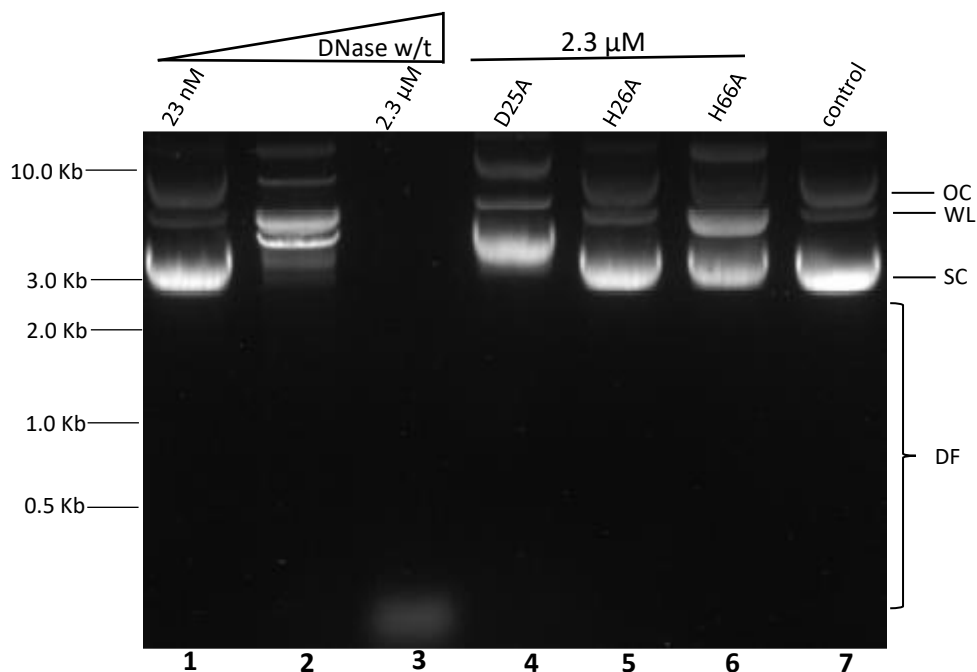


Figure 4.8 The three mutants D25A, H26A and H66A of pyocin S3 DNase in the proposed active site abolish pyocin S3 DNase activity.

Determination of the activity of full-length pyocin S3 DNase and its mutants *in vitro* using the plasmid nicking assay. Lanes 1-3. Increasing w/t DNase titrations of 23 nM, 0.23 μ M and 2.3 μ M, respectively; Lanes 4-6, Mutant DNase D25A, H26A and H66A titrations using 2.3 μ M enzyme, respectively; Lane 7. a negative control, plasmid without DNase added to the reaction. 10 μ l of each sample was mixed with 2 μ l of Gel Loading Dye (Biolabs) for analysis on a 0.8% agarose gel alongside the 1 kb DNA ladder at 80 V for 45 min. Individual forms of plasmid DNA are indicated to the right of the panels: sc- supercoil; wl- whole linear DNA; op- open coil; df- degraded fragments.

4.2.5 *Gel electrophoresis mobility shift assays suggest that the DNase mutant D25A interacts with DNA*

Having demonstrated that conversion of pyocin S3 residues D25, H26 and H66 to alanine prevents detection of DNA cleavage using *in vivo* (Figure 4.4) and *in vitro* assays (Figure 4.8), the next step was to demonstrate whether the mutant proteins retained the ability to interact with the substrate DNA. To do this, gel electrophoresis mobility shift assays (EMSA) were employed. EMSA is a simple and robust technique, making it the first tool of choice in the current study. EMSA is based on the observation that the mobility of a protein-DNA complex is much slower than free DNA in polyacrylamide gel electrophoresis (PAGE) under native conditions, as illustrated in Figure 4.9. (Hellman and Fried 2007, Seo, Lei et al. 2019). To serve as a substrate for the mutant pyocins, a 34 bp double stranded DNA molecule was synthesized by amplifying a commercial 5' cyanine 5.5 P3-DNA from Sigma. The fluorescence enabling detection of this DNA in EMAS has an emission maximum at 705 nm (Livak, Flood et al. 1995). In the initial assay, 0.5 μ M or 1 μ M pyocin S3 DNase w/t or its mutants were incubated with 10 nM of cyanine 5.5 labelled P3-DNA in a reaction buffer containing 5% glycerol, 20 mM Tris- HCl, 25 mM NaCl, 1 mM EDTA. The reactions were set in duplicate, either with or without 1.5 mM MgSO₄. The reactions were incubated on a shaker at 75 rpm for 20 min at 37°C. An aliquot (5 μ l) of the resulted reaction was loaded onto a 6% Tris-boric acid-EDTA (TBE) gel along with 1 μ l of 0.5% bromophenol blue on each side and run in TBE buffer at 200 V for 10 min prior to 120 V for 55 min in a cold room. Gels were analysed with the wavelength channel 700 nm and either a 2

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding. min or 10 min integration time using ODYSSEY, LI- COR (Figure 4.10).

In the EMSA, the gel mobility of the substrate DNA alone (Lane 1, Figure 4.10) or with either the H26A or the H66A pyocin S3 mutant was not visibly different. In contrast, with mutant D25A there was a slight shift towards a slower migration in the gel (Lane 2, 3 and 8, Figure 4.10). The change in mobility suggests an interaction between the DNA substrate and the D25A mutant pyocin. This is suggestive of residue D25 not being important for DNA binding or coordination by the pyocin S3 DNase as it is still able to occur following mutation.

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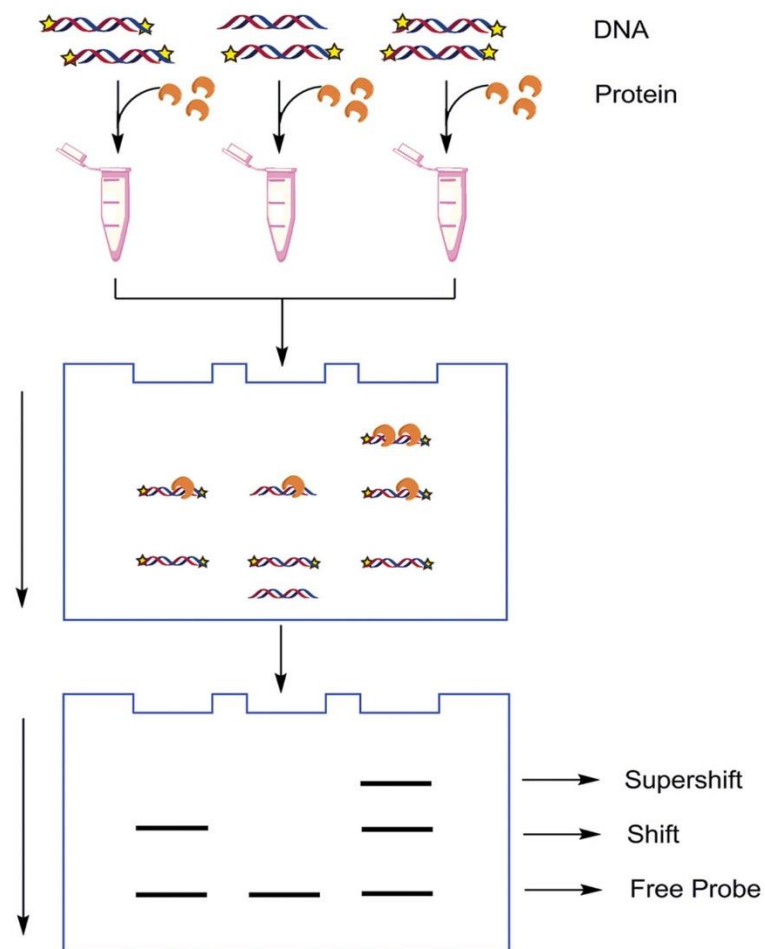


Figure 4.9 Principle of electrophoretic mobility shift assays (EMSA).

EMSA is based on the principle that the free (unbound) DNA probe migrates faster than DNA-protein complex when subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE). The mobility of the DNA is retarded when is bound with protein. Typically, the DNA is a short sequence and labelled to track its mobility during the electrophoresis. Figure adapted from Fig 3, Song, Zhang et al. 2015. (Hellman and Fried 2007, Song, Zhang et al. 2015).

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding. The data shown in Fig 4.2.9 indicates that a 2 min exposure time was adequate for the assay since the longer 10 min exposure time looked almost identical. Further refinements to the EMSA were investigated with the aim of increasing the sensitivity of the assay. The retardation of DNA migration due to interaction with a protein is enhanced as the pH of the gel is lowered (Carey 1988). As an example, successful EMSA was demonstrated by separating complexes in a 10% acrylamide gel using a circulated 10 mM NaH_2PO_4 buffer, pH 6.0 (Carey 1988). To simulate this, stocks of x2 reaction buffer containing 50 mM MgSO_4 and 1 M cyanine 5.5 labelled P3 DNA were prepared in 8% (v/v) glycerol, 50 mM NaCl, 2 mM EDTA, 0.5 mM CaCl_2 containing either 40 mM Na-phosphate buffer pH 6.25 or pH 7.0. An EMSA was performed with 50 nM DNA probe with and without 3.3 mM Mg^{2+} ions plus 100 nM pyocin S3 DNase (either W/T or one of the three mutants). BSA was also included to dilute the DNase and prevent it from aggregating in the well bound to DNA. To simplify the experiment, the buffer was not circulated. Circulating gel-running buffer during native PAGE involves removal of the “safety lid” and the use of electrodes only, with set up of a peristaltic pump to carefully exchange buffer between anode and cathode reservoirs at the same rate. In Panel A, Figure 4.11 some evidence for D25A shifting the DNA probe mobility is seen. It also appears that there has been some buffering failure as the DNA has not run straight through the gel despite stirring the anode buffer chamber during electrophoresis.

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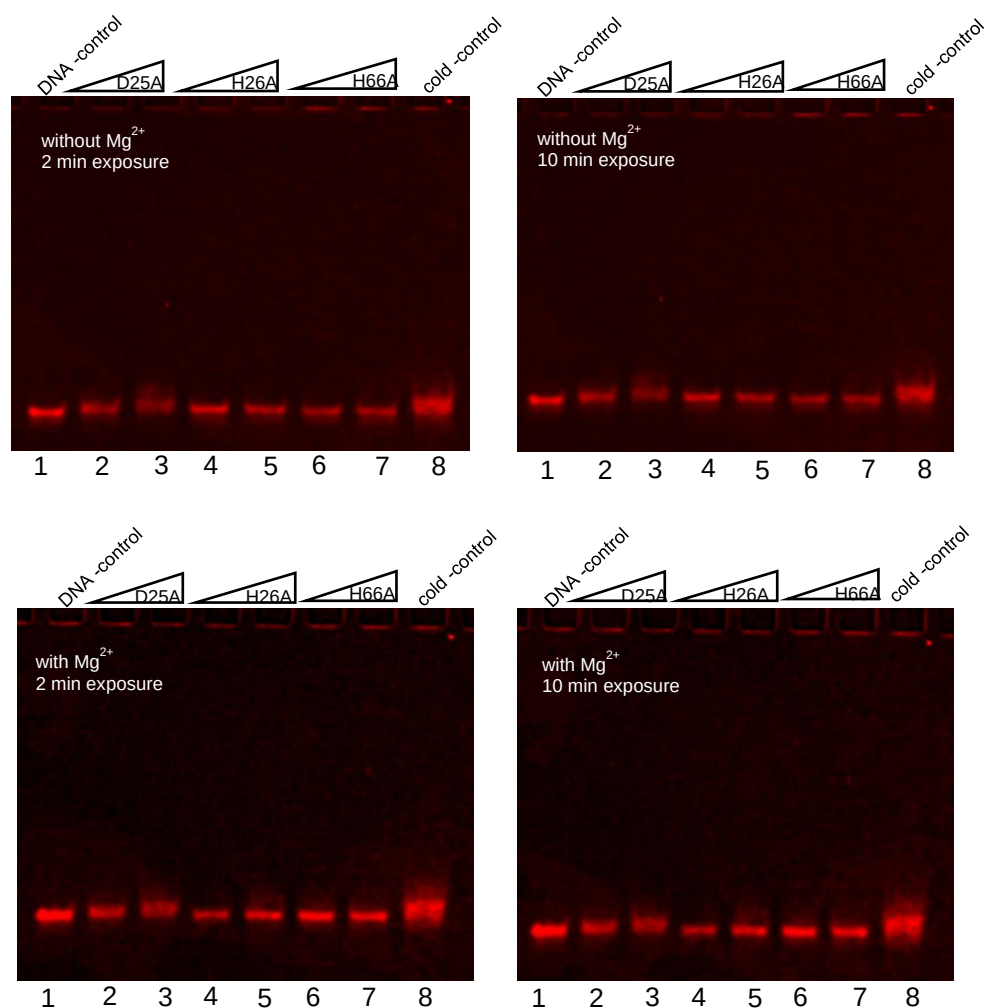


Figure 4.10 Electrophoretic mobility shift assay (EMSA) suggested pyocin S3 mutant D25A interacted with DNA.

10 nM of Cyanine 5.5 fluorophore labelled DNA substrate was incubated without (DNA- control) or with either 0.5 μM or 1 μM pyocin S3 mutants (D25A, H26A, or H66A) in a reaction buffer containing 5% (v/v) glycerol, 20 mM Tris-HCl buffer, pH 7.4, 25 mM NaCl, 1 mM EDTA with/without 1.5 mM MgSO_4 . The reactions were applied to a 6% TBE gel and run in $\times 0.5$ TBE at 220 V for 10 min and then 120 V for 55 min before being visualized with the Scanner Odyssey (Li-CoR Odyssey, Biosciences). Lane 1. Cyanine 5.5 labelled DNA control, no protein added to the reaction; Lane 2&3. 0.5 μM and 1 μM mutant D25A incubated with cyanine 5.5 labelled DNA, respectively; Lane 4&5. 0.5 μM and 1 μM mutant H26A incubated with cyanine 5.5 labelled DNA, respectively; Lane 6&7. 0.5 μM and 1 μM mutant H66A incubated with cyanine 5.5 labelled DNA, respectively; Lane 8. Cold incubation control, 1 μM D25A incubated with cyanine 5.5 labelled DNA on ice.

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding. To avoid circulating weak phosphate buffer, alternative EMSA buffers at lower pH have been used in the assay. For example, for *EcoR1* and *BamH1* restriction enzymes, separation of weak nonspecific DNA binding from specific DNA recognition has been achieved using osmolytes to stabilise protein-DNA complexes before they entered the gel. In addition, 20 mM HEPES buffer pH 6.7 was used to create mildly acidic EMSA reaction-gel-running (Sidorova and Rau 2001, Sidorova, Muradymov et al. 2005, Sidorova, Hung et al. 2010). To see if this approach was suitable for the analysis on pyocin S3-DNA interactions, an EMSA was performed in which 1 μ M pyocin S3 (either W/T or one of the 3 mutants) in 8% (v/v) glycerol, 40 mM HEPES buffer pH 6.7, 50 mM NaCl, 2 mM EDTA, 0.5 mM CaCl_2 was mixed with 50 nM P3 DNA probe with and without 3.3 mM Mg^{2+} ions. The results shown in panel B Figure 4.11 again fail to detect a mobility shift. As an improvement, the DNA migrated with a uniform line indicating that these buffer systems negated any need for buffer circularisation.

To test whether buffer circulation could improve the migration in gels using the approach utilized in Panel A (Figure 4.11), the pyocin DNA-binding reactions from Panel B Fig Figure 4.11(following storage at 4°C for 4 days) were separated through a gel using only electrodes and two P1 pumps to carefully circulate 10 mM phosphate, pH 7 buffer. The gel clearly showed that circulation of the buffer prevented distortion of the migration (Figure 4.11). Importantly, the W/T pyocin S3 generated a set of DNA fragments in the presence of Mg^{2+} ions that migrated differently to the untreated substrate DNA suggesting that in these conditions

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Pyocin S3 was able to bind the P3 DNA (Lane 7, Figure 4.12). Mutants H26A and H66A did not alter the migration of the substrate P3 DNA. In support of the previous observation (Figure 4.11), pyocin S3 mutant D25A produced a smear of DNA fragments migrating more slowly than the untreated P3 DNA substrate, suggesting a larger size that is most likely due to DNA-binding (Lane 2 & 3, Figure 4.12). The presence of Mg^{2+} did not appear to influence this interaction.

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

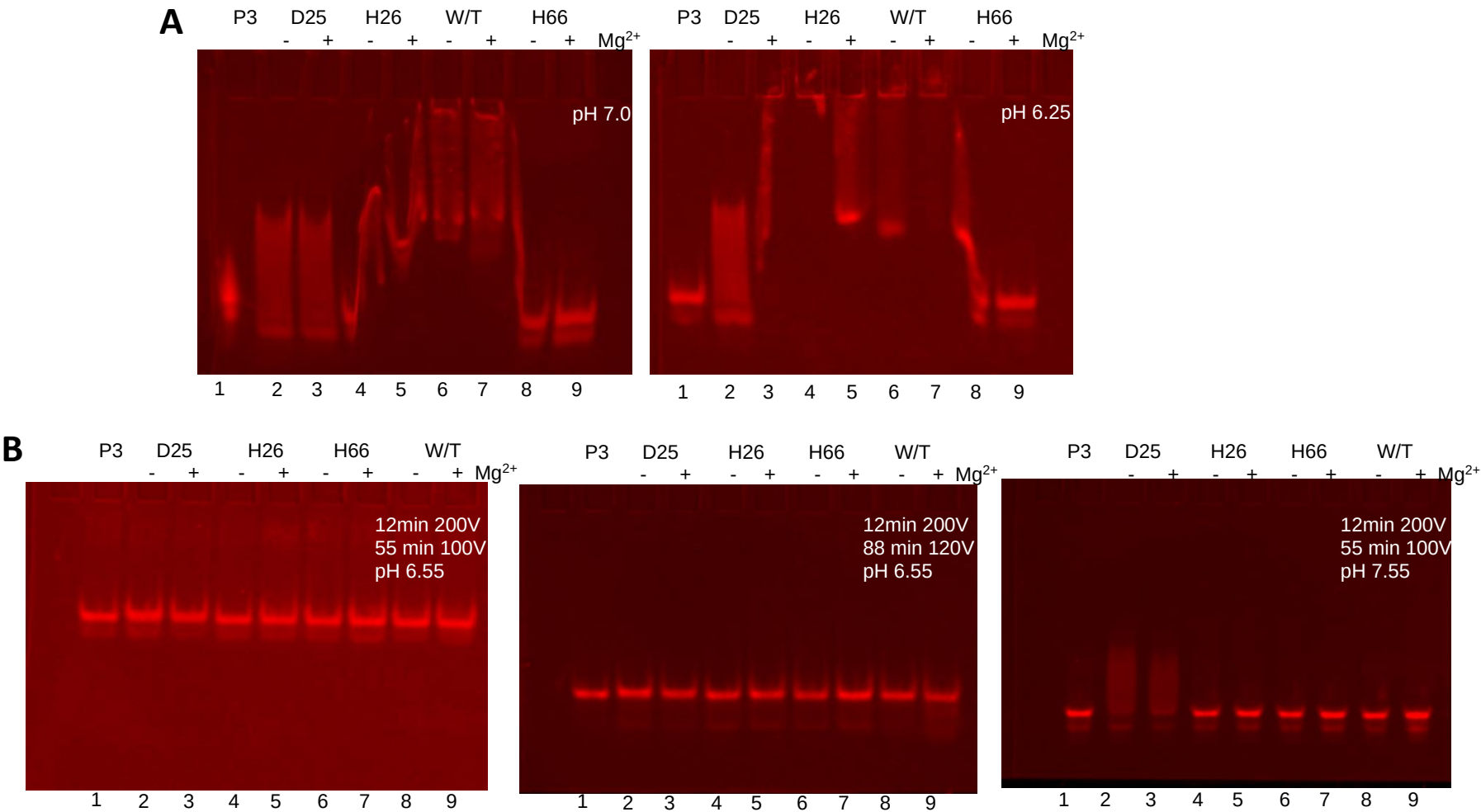


Figure 4.11 Electrophoretic mobility shift assay (EMSA) indicated D25A interacted with substrate DNA at pH 7.55.

Panel A. 50 nM of Cyanine 5.5 fluorophore labelled P3 DNA was incubated with 1 μ M pyocin S3 DNase or its mutants in reaction buffer containing 12%(v/v) glycerol, 40 mM sodium phosphate buffer, pH 7.0 (left) or pH 6.25 (right), 20 mM NaCl, 2 mM EDTA and 0.5 mM CaCl_2 with/without 3.3 mM MgSO_4 for 15 min. A 6% acrylamide gel was loaded with 6 μ l of each of the products and run in 10 mM sodium phosphate buffer of the same pH as the reaction buffer used. The gel was visualized with Scanner Odyssey (Li-CoR Odyssey, Biosciences). Lane 1. Cyanine 5.5 fluorophore labelled P3 DNA control, no protein added to the reaction; Lane 2 & 3, P3 DNA was incubated with mutant D25A in buffer without (-) and with (+) 3.3 mM Mg^{2+} , respectively; Lane 4 & 5, P3 DNA was incubated with mutant H26A in buffer without (-) and with (+) 3.3 mM Mg^{2+} , respectively; Lane 6 & 7, P3 DNA was incubated with W/T pyocin S3 DNase in buffer without (-) and with (+) 3.3 mM Mg^{2+} , respectively; Lane 8 & 9, P3 DNA was incubated with mutant H66A in buffer without (-) and with (+) 3.3 mM Mg^{2+} , respectively. Panel B. 50 nM of cyanine 5.5 fluorophore labelled P3 DNA was incubated with 1 μ M pyocin S3 DNase or its mutants in reaction buffer containing 8% (v/v) glycerol, 40 mM HEPES buffer, pH 6.5, 50 mM NaCl, 2 mM EDTA and 0.5 mM Ca^{2+} with/without 3.3 mM MgSO_4 . A 10% acrylamide gel was loaded with 6 μ l of each of the products and run in three different buffer conditions including 30 mM HEPES, pH 6.55; 20 mM HEPES, pH 6.55 and x1 TAE and 20 mM HEPES, pH 7.55 (from left to right) for 55 min, 88min and 55 min, respectively and visualized with Scanner Odyssey. Lane 1. Cyanine 5.5 fluorophore labelled P3 DNA control, no protein added to the reaction; Lane 2 & 3, P3 DNA was incubated with mutant D25A in buffer without (-) and with (+) 3.3 mM Mg^{2+} , respectively; Lane 4 & 5, P3 DNA was incubated with mutant H26A in buffer without (-) and with (+) 3.3 mM Mg^{2+} , respectively; Lane 6 & 7, P3 DNA was incubated with mutant H66A in buffer without (-) and with (+) 3.3 mM Mg^{2+} , respectively; Lane 8 & 9, P3 DNA was incubated with W/T pyocin S3 DNase in buffer without (-) and with (+) 3.3 mM Mg^{2+} , respectively.

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

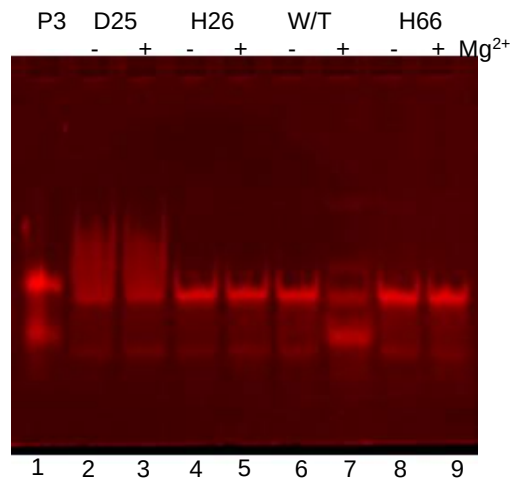


Figure 4.12 Electrophoretic mobility shift assay (EMSA) for pyocin S3 DNase and its mutants.

The EMSA was run in optimised conditions with buffer circulation supported the observation that mutant D25A interacts with the substrate DNA. The pyocin-DNase binding reactions from Fig 4.2.10B were used following storage for 4 days at 4°C. Reactions (6 µl) were run on a 9% bis/acrylamide gel in 10 mM phosphate buffer, pH 7.0 without/with 3.3 mM Mg²⁺. Lane 1. Cyanine 5.5 fluorophore labelled P3 DNA control, no protein added to the reaction; Lane 2 & 3. P3 DNA was incubated with mutant D25A in buffer without (-) and with (+) 3.3 mM Mg²⁺, respectively; Lane 4 & 5, P3 DNA was incubated with mutant H26A in buffer without (-) and with (+) 3.3 mM Mg²⁺, respectively; Lane 6 & 7, P3 DNA was incubated with w/t pyocin S3 DNase in buffer without (-) and with (+) 3.3 mM Mg²⁺, respectively; Lane 8 & 9, P3 DNA was incubated with mutant H66A in buffer without (-) and with (+) 3.3 mM Mg²⁺, respectively.

4.2.6 *Fluorescence Polarization did not give any clear indication of DNA cleavage by pyocin S3*

Fluorescence polarization (FP) is a powerful and sensitive non-radioactive technique for studying protein-DNA interaction. FP can be used to monitor the change of movement/rotation of a small fluorophore labelled DNA probe, which is excited by polarized light and has an emission at a certain wavelength. If the DNA binds to a much bigger molecule such as a protein, the DNA can no longer rotate rapidly, thus giving a higher FP signal due to the emitted light being in the same plane as the excitation light (Figure 4.13;(Lea and Simeonov 2011, Du 2015). The FP signal of a protein titration with a fixed concentration of DNA probe can be measured to quantify the interaction strength. FP has the following advantages over EMSA:

1. FP does not involve the separation of bound from free probe, therefore, it will not disturb the reaction equilibrium and can give a more accurate quantification, particularly for a protein-DNA interaction with low affinity.

2. FP does not require the protein-DNA complex to be a very different size from the free DNA probe in order to distinguish them. Assembly of protein-DNA can be detected relatively easily by FP.

3. The experimental conditions of FP are not as restrictive as those for EMSA, and therefore it is more flexible and rapid. EMSA is limited by the need to match the constituents of the interaction reaction buffer, electrophoresis running buffer and PAGE gel (Lundblad, Laurance et al. 1996).

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

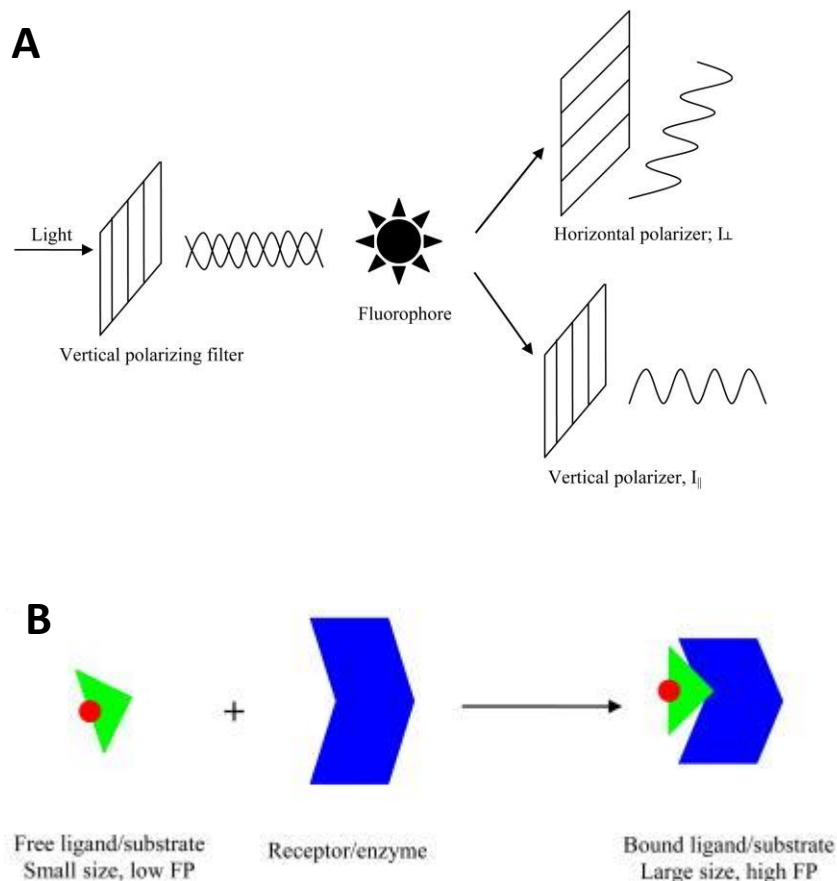


Figure 4.13 Schematic illustration of the basic principle of fluorescence polarization (FP).

Panel A. A fluorophore labelled substrate is excited by light, which is linearly polarized through an excitation polarizing filter. Then, the polarized fluorescence intensities will be measured by an emission polarizer parallel or perpendicular with respect to the plane of linearly polarized excitation light in order to determine the FP. Panel B. The rotation of free substrate is fast, showing a low FP value. When the free ligand/substrate is bound to enzyme (in blue), rotation of fluorophore becomes slow causing an emission increase of the FP value. Fluorophore and substrate are denoted by a red circle and green triangle, respectively (Lea and Simeonov 2011).

In the initial FP assay, the aim was to demonstrate that pyocin S3 DNase could digest bodipy labelled P3 substrate DNA (<https://www.thermofisher.com/uk/en/home/references/molecular-probes-the-handbook/fluorophores-and-their-amine-reactive-derivatives/bodipy-dye-series.html>) and reduce its FP signal as there

would be more free DNA moving faster. If this is successfully demonstrated, the next step would be to determine if it is dependent upon the presence of Mg^{2+} ions. To do this, the substrate DNA was resuspended at a final concentration of 100 nM in 10 mM sodium phosphate buffer pH 7.5, with either 1 mM EDTA or 10 mM Mg^{2+} ions. The FP stability of the DNA substrate was then measured over time in a 30 μ l reaction buffer. Subsequently, pyocin S3 DNase was added into a duplicate sample placed in a separate 384 microtitre plate well. FP measurements were taken over time and comparison made between mixtures with and without pyocin (Figure 4.14). In the presence of pyocin S3 DNase and Mg^{2+} a reduction in FP over time to a plateau (trend in red) was observed. However, the sample lacking the enzyme in the presence of EDTA had similar FP levels, although they did not reduce over time in such a pronounced way (trend in blue). This trend was unlike those observed with the mixture containing the DNase with the substrate in EDTA, or the substrate alone in Mg^{2+} (black and green). Both of these mixtures recorded FP values that remained fairly level and drifted subtly higher. The data was not suitable to draw any conclusions as to whether the pyocin DNase that had been purified could cleave DNA in a Mg^{2+} -dependent reaction. The raw data for this experiment is shown in Appendix 4.

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

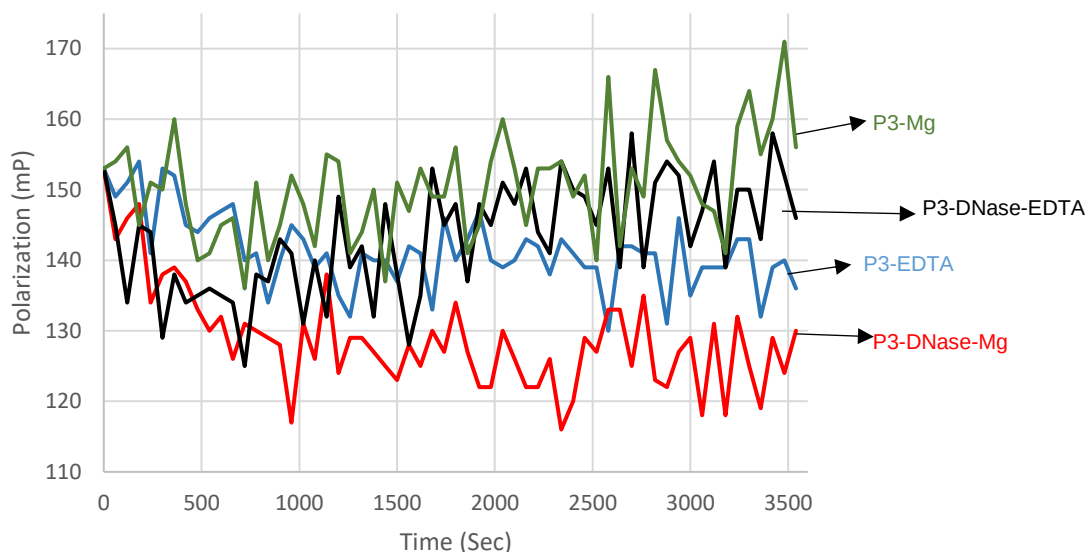


Figure 4.14 Fluorescence polarization assay of bodipy530 labelled P3 DNA with pyocin S3 DNase.

Fluorescence polarization assay of bodipy530 labelled P3 DNA with pyocin S3 DNase did not unequivocally indicate DNA cleavage. A 34 bp duplex P3 DNA template with bodipy530 at the free 5' end (100 nM) was suspended in 10 mM sodium phosphate buffer pH 7.5, with 1 mM EDTA or 10 mM Mg^{2+} ions, and its FP stability measured over time (blue and green lines, respectively). In parallel, the FP measurements of these DNA mixes with addition of pyocin S3 DNase (200 nM) were taken over time (red and black lines). Data was plotted by FP (mP) against time (s) and normalized to the same starting point.

To test the Tecan Spark instrument and the experimental set up, a commercial benzonase (Milipore) was used, which is capable of degrading all forms of DNA and RNA into small fragments with the optimal pH at around 8.0 to 9.2. The benzonase was diluted approximately 3000-fold into the bodipy labelled P3 substrate DNA fragment (100 nM), and analysed in the reaction conditions used previously (Figure 4.14). The FP measurement over time (Figure 4.15) confirmed benzonase cleaved DNA in the presence of Mg^{2+} ions (trend

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in yellow and in dark blue) more effectively than without Mg^{2+} ions (with EDTA, trend in grey). As expected, the more dilute benzonase (dark blue trend) did not continue cleavage to reach the same low FP end point as when the enzyme was present at 3-fold higher concentration (yellow trend). Omission of benzonase verified that the substrate DNA was relatively stable over time, although the amount of FP was different dependent on whether Mg^{2+} was present or absent (trend in light blue and in orange). The raw data for this experiment is show in Appendix 5.

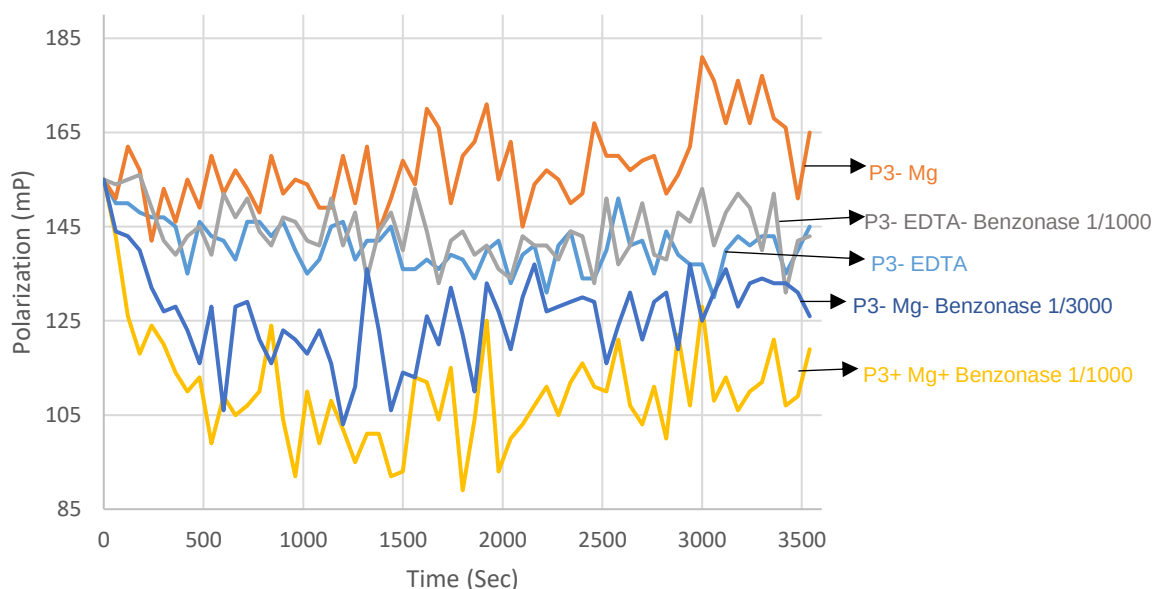


Figure 4.15 Fluorescence polarization assay of bodipy530 labelled P3 DNA verifies cleavage by benzonase is detectable.

A 34 bp duplex P3 DNA template with bodipy530 at the free 5' end (100 nM) was suspended in 10 mM sodium phosphate buffer pH 7.5, with 1 mM EDTA or 10 mM Mg^{2+} ions, and its FP stability measured over time. Serial dilution was performed on a commercial benzonase to ~3000 fold and added into a duplicate (30 μ l; 384 microtitre) plate well and FP measurements between the P3 DNA with benzonase were taken over time. Data was plotted by FP (mP) against time (s). Data was normalized to the starting point.

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding. Having shown that the DNase activity of benzonase can be demonstrated in the set-up used for FP monitoring, these reaction conditions were used for an additional analysis of pyocin S3 DNA cleavage. To do this, 100 nM bodipy labelled P3 DNA cleavage was directly compared between pyocin S3 DNase and benzonase. After FP measurements, aliquots were transferred from the microtitre plate onto a 0.5x TBE PAGE (Figure 4.16). On this occasion three identical pyocin cleavage reactions were set up in parallel, and two (E and G) showed a decrease in FP over time (panel A, Figure 4.16). The same reactions contained detectable DNA cleavage products on the PAGE (Panel B, Figure 4.16). The partial cleavage of P3 DNA visible in the PAGE provides a potential explanation for the mild influence on FP. The data for the unsuccessful pyocin cleavage reaction is not shown in Figure 4.16, but the full data set is presented in Appendix 6.

Whilst the reactions containing neat benzonase (H, I and J) displayed the most P3 DNA cleavage on the PAGE, it was unexpected to see that they showed very little or no reduction of FP over time (Figure 4.16. I and J are not shown in Panel A due to similar data obtained from the triplicates). The 1/1000 or 1/3000 dilutions of benzonase (K and L) contained smaller derivatives of the bodipy 530 labelled P3 DNA template and this was associated with a more drastic change in FP over time previously (Figure 4.15). Reaction controls incubated without DNase or nuclease appeared as uncut P3 DNA (B, Figure 4.16).

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Since the concentration of the benzonase control did not fully correlate with the data, a single experiment incorporating a more extensive serial dilution of benzonase from neat to 1/1000 dilution was performed. In this experiment, the FP was measured over time and the products inspected by PAGE (Figure 4.17). The PAGE clearly demonstrated that excess benzonase (neat) destroyed the substrate DNA. This was reflected in the measurement of a lower FP within minutes. Dilution of the benzonase from excess to below the substrate DNA concentration, resulted in more limited DNA cleavage. The FP data is difficult to interpret, although there was a general trend whereby FP was lower with more benzonase present. Extra time did not result in extra cleavage. This may represent poisoning of the benzonase by the fluorescent DNA substrate, or poor buffer conditions, which prevent substrate release from the enzyme.

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

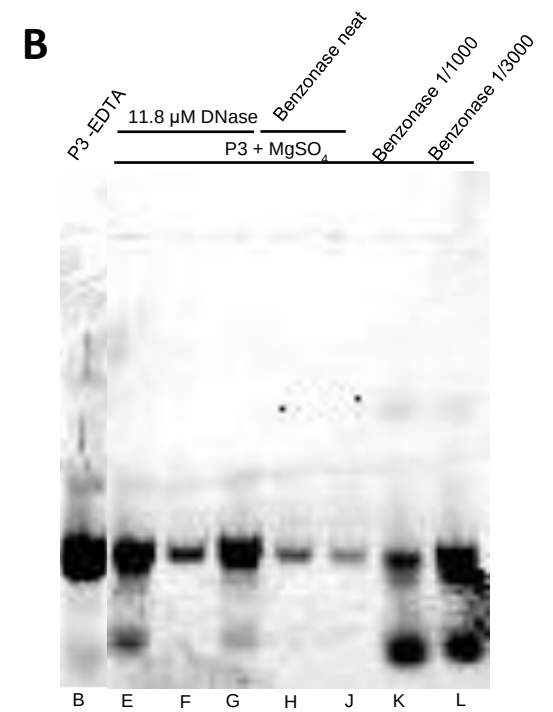
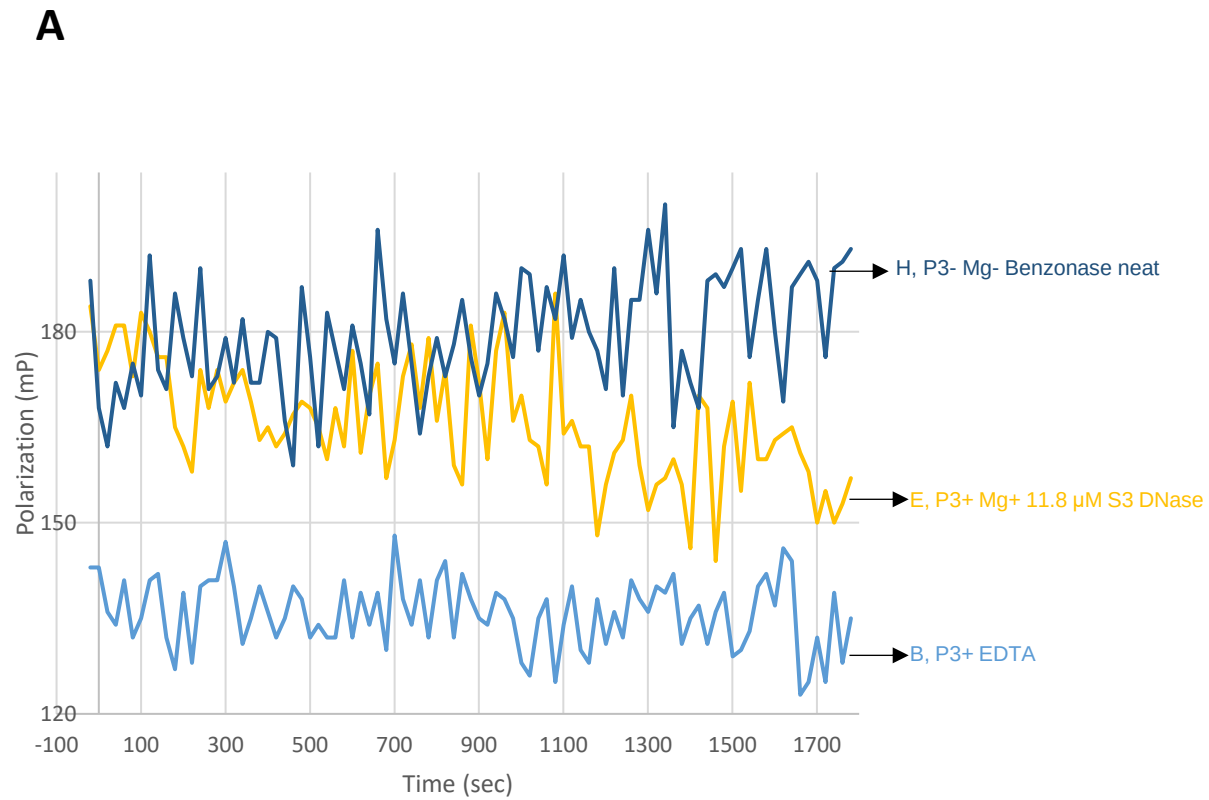


Figure 4.16 Bodipy-P3 DNA cleavage assay and EMSA analysis.

A 34 bp duplex P3 DNA template with bodipy530 at the free 5' end (100 nM) was suspended in 10 mM sodium phosphate buffer pH 7.5, with 1 mM EDTA and its FP stability measured over time. FP measurements between the P3 DNA with 11.8 μ M of pyocin S3 DNase and neat concentration of commercial benzonase were taken over time. Data was plotted by FP (mP) against time (s). After FP measurements (Panel A) samples of the DNase cleavage reactions were transferred from the microtitre plate onto a 0.5x TBE PAGE to be observed on EMSA (Panel B) along with the P3 DNA with 10 mM Mg^{2+} ions and 1/1000 or 1/3000 titration of benzonase from above run (Fig 4.2.14) as comparison.

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

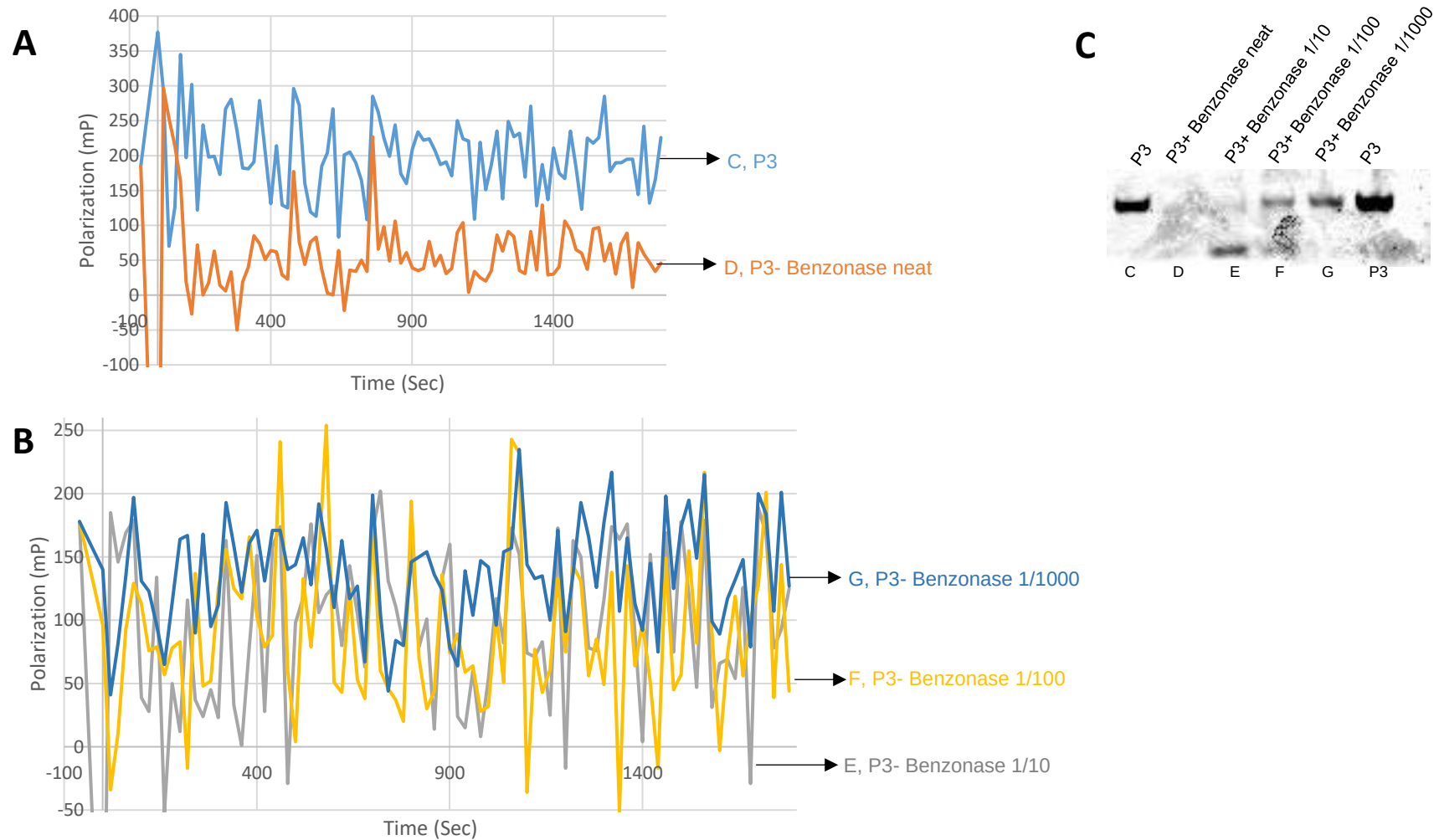


Figure 4.17 Bodipy-P3 DNA cleavage assay by Benzonase and EMSA analysis.

A 34 bp duplex P3 DNA template with bodipy530 at the free 5' end (100 nM) was suspended in 20 mM Tris buffer pH 8.0, 8% glycerol and 10 mM MgSO₄ buffer and its FP stability measured over time. FP measurements between the P3 DNA with titration (neat, 1/10, 1/100 and 1/1000) commercial benzonase were taken over time. Data was plotted by FP (mP) against time (s). After FP measurements (Panel A and B) samples of the DNase cleavage reactions were transferred from the microtitre plate onto a 0.5x TBE PAGE to be observed on EMSA (Panel C). Data is normalized to the same starting point.

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

4.2.7 Analytical gel filtration chromatography indicated potential DNA binding with the DNase mutant, H66A

Analytical gel filtration chromatography employs a size-exclusion column, also known as size-exclusion chromatography (SEC). It is a rapid and cost-effective technique compared to other techniques to analyse binding and understand protein-protein or protein-DNA interactions. It can be performed in any buffer system, which makes it fully compatible with preserving the stability of the protein being analysed (Mayer, Snyder et al. 2009, Hong, Koza et al. 2012, Bai 2015). The gel filtration column is filled with a porous matrix made up of cross-linked polymer in spherical particles. Differently sized molecules in a solution are separated in the column, and this can be monitored with sensitivity. Since small molecules can enter both big and small pores in the matrix particles, the route they take down the column is convoluted and takes longer than larger molecules. The length of elution time will correlate with size, with the smallest molecules taking the longest time. Large molecules that are too big to enter the matrix pores will be eluted from the column without entering the pores. In this way, the molecules will be eluted from the column in order of their size with the largest one coming first and smallest one coming last (Figure 4.18) (Bai 2015). The elution is monitored using UV absorbance over time, and for the purposes of this study, it was hypothesized that the pyocin-DNA complex would elute after a different time to the pyocin.

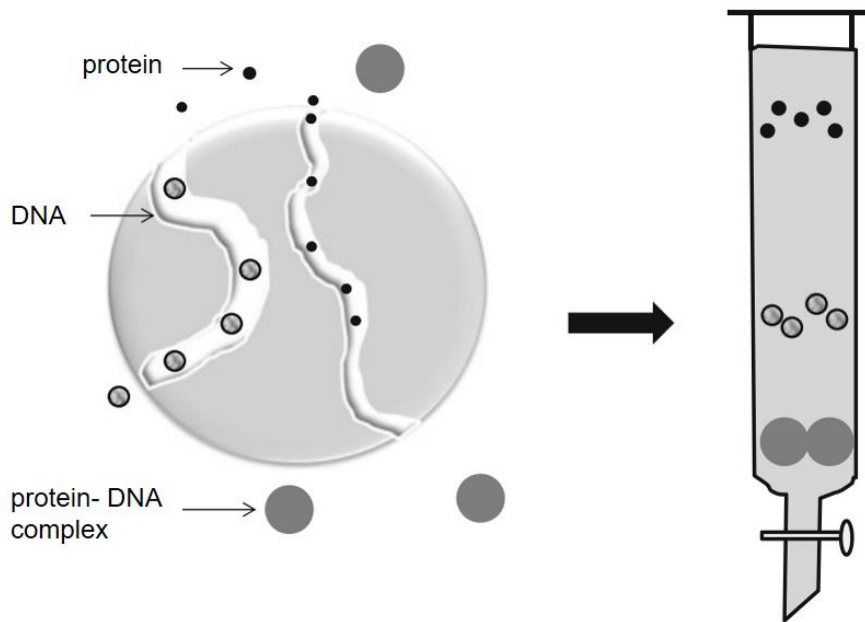
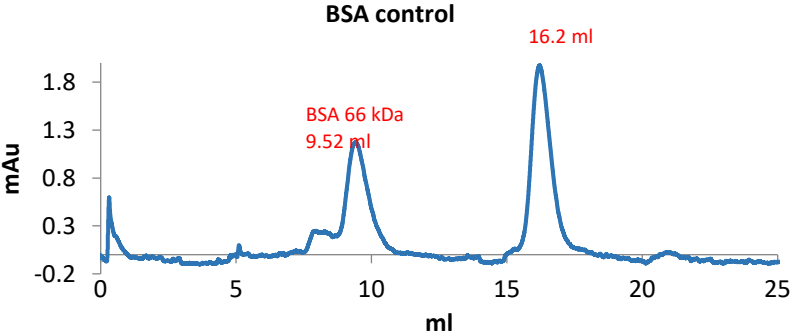


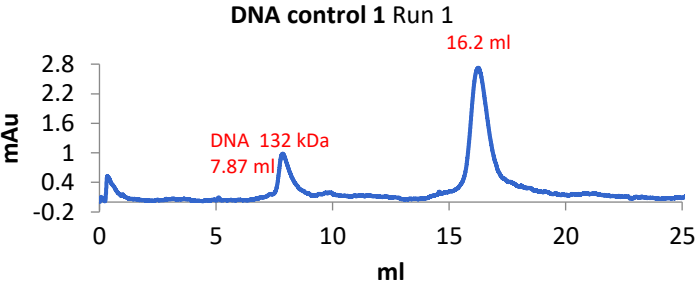
Figure 4.18 Schematic cartoon to illustrate the principle of analytical gel filtration.

The image on the left depicts a single particle of the SEC matrix with pale channels through it depicting pores of two different sizes. The darker grey-black circles represent differently sized molecules in the sample applied to SEC. The smallest molecules will fit into the majority of the matrix pores, delaying their progress through the SEC column (as illustrated on the right). A DNA-pyocin complex would be predicted to be larger than the pyocin alone, and thus expected to take less time to elute from the SEC column. Figure is adapted from Fig 1. Bai 2015 (Bai 2015).

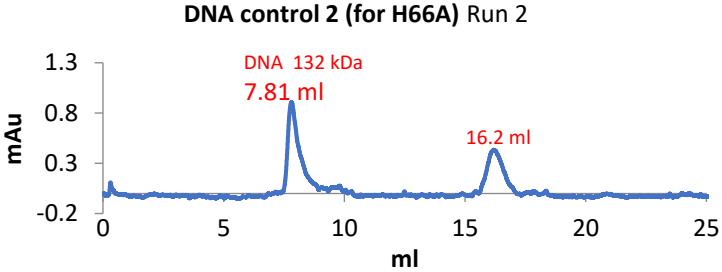
Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding. To show that the column was operational, that the analytical program was appropriate, and to judge the sensitivity of the UV recorder of the AKTApurime, a dilute BSA (66 kDa, 0.1 mg/ml) sample was first analysed as a control sample (Panel A, Figure 4.19). The program was operational, and BSA was observed eluting at 9.5 ml with an unknown contaminant eluting at 16.2 ml. Next, the elution time of unbound DNA was measured taking advantage of UV recording at 280 nm by the AKTApure also being able to detect DNA (albeit slightly less sensitively than protein). As shown in Panel B, Figure 4.19, 2 μ M DNA eluted at $\sim$ 7.8 ml, close to the column void volume which is consistent with it being large, $\sim$ 130 kDa (200 bp $\times$ 0.66, 1 bp is equivalent to 0.66 kDa). The sensitivity of DNA detection was appropriate for follow on experiments. The most likely scenario is that each molecule of pyocin S3 interacts with a single DNA molecule at a time, ie there is a stoichiometric interaction. Thus, detection of 2 μ M DNA would facilitate interrogation of binding to 2 μ M pyocin DNase. Considering the expected equilibrium binding constant for the protein-DNA interactions (K_{Deq}), this should be sufficient to detect physiologically relevant interactions. The unknown contaminant eluting at 16.3 ml was again detected.



B



C



Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

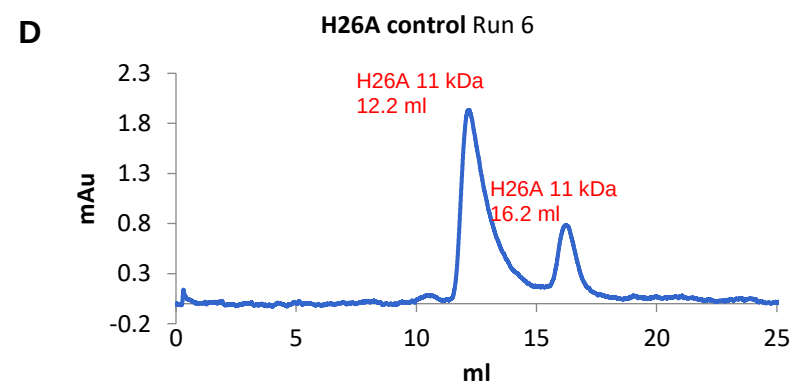
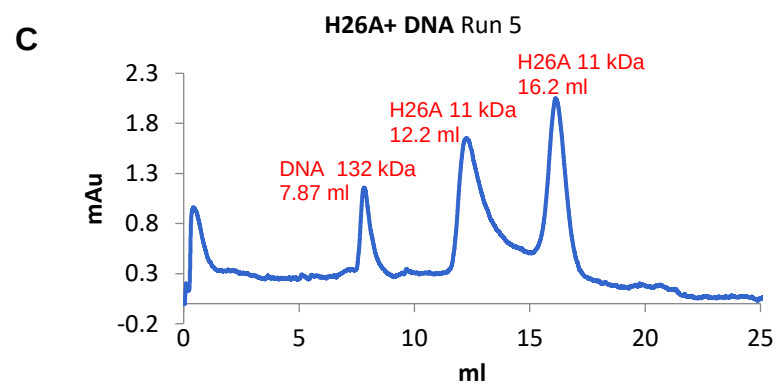
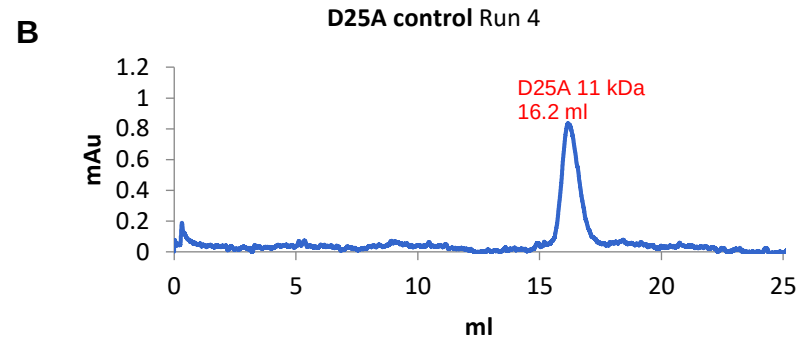
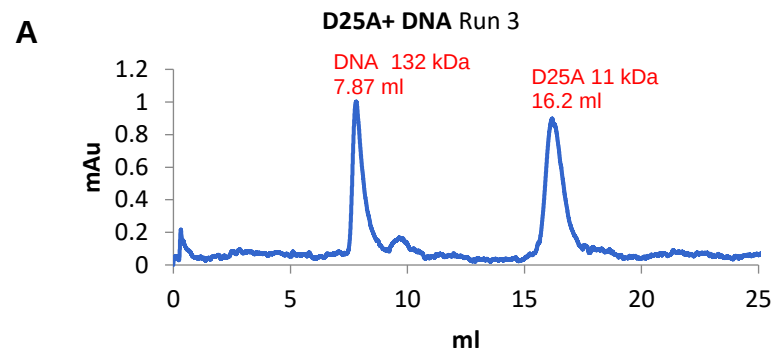
Figure 4.19 Analytical gel filtration controls: Protein (BSA) and DNA alone (DNA control).

The elution profile of 0.1 mg/ml BSA control is shown on the left. Two elution profiles of 2 mM 200 bp DNA controls are also shown on the right to illustrate the variation in the contaminant that eluted at 16.2 ml in all runs. The control profile that corresponds with analysis of pyocin S3 DNase W/T and the mutants D25A and H26A mutant is shown on the top (panel B). The DNA control elution profile for pyocin S3 DNase H66A mutant is at the bottom (panel C).

With the aim of detecting pyocin S3 interactions with DNA, each inactive mutant (D25A, H26A and H66A) was monitored through analytical gel filtration in the presence and absence of the DNA substrate. Figure 4.20 and Figure 4.21 show these paired gel filtration profiles. Since small differences in pipetting concentrated proteins may lead to differences in UV absorbance, steps were taken to minimise pipetting errors. Pyocin D25A mutant DNA-binding analysis was performed where one stock of 200 μ l pyocin DNase D25A was prepared at 4 μ M, divided into two equal halves, one then diluted with buffer to 2 μ M and the other diluted with DNA in buffer to obtain comparable 2 μ M pyocin and a 2 μ M pyocin 1:1 DNA mix. As shown in Panel B, Figure 4.20, mutant D25A eluted at 16.2 ml consistent with its small mass of 11 kDa. The prediction was that if pyocin binds the DNA there should be a new elution peak significantly earlier than free pyocin, a decrease in UV absorbance at 7.8 ml (unless it happens that the DNA-pyocin complex elutes at exactly the same volume), and an accompanying decrease in UV absorbance at 16.2 ml. In Run 3 (Panel A, Figure 4.20) the pyocin D25A mutant was incubated briefly with DNA in buffer with 2 mM Mg^{2+} ions prior to applying to the column. The elution peaks observed match those of the protein and the DNA run separately (both in intensity and timing), and thus provide no evidence for binding of mutant D25A to DNA. This was also true for pyocin DNase mutant H26A (Run 5 and 6, Figure 4.20). For the W/T pyocin DNase (Run 9 and 10, Panel C and D, Figure 4.21), the peak at 16.2 ml remained unchanged in the presence and absence of the DNA, which would suggest no interaction with the DNA had occurred.

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding. However, the peak representing the DNA shifted slightly from 7.8ml to 8.7 ml, and was much broader which could indicated that the DNA had been cleaved and was migrating differently through the column.

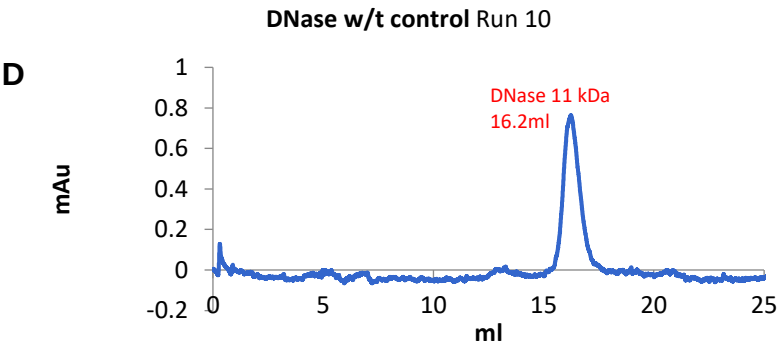
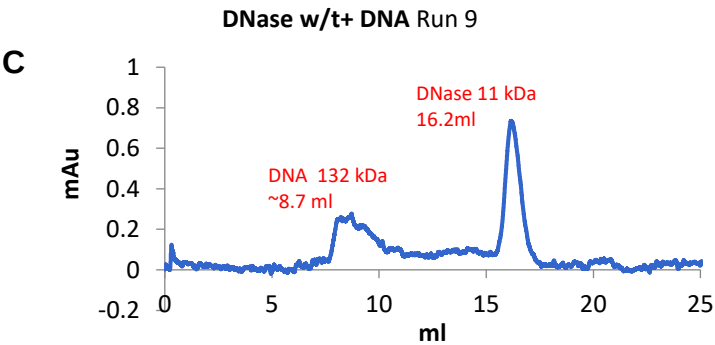
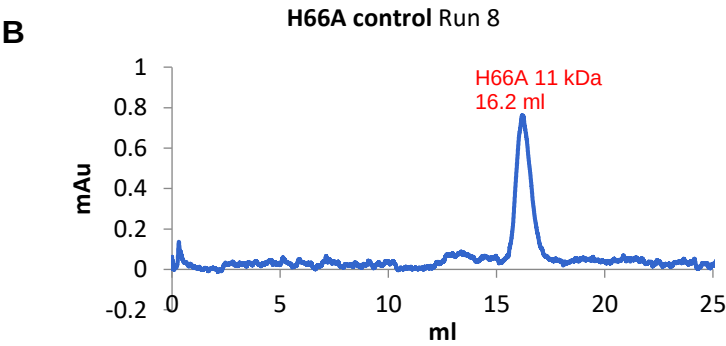
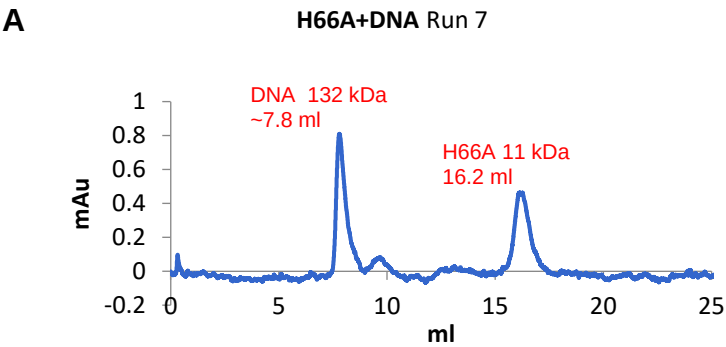
In contrast, the analytical gel filtration analysis of pyocin DNase mutant H66A suggested a potential DNA-binding interaction was occurring as a decrease in absorbance was evident for the 16.2ml peak in the presence of the DNA, see Penal A and B, Figure 4.21. In an attempt to verify this interaction, all the fractions collected from column were analysed by SDS-PAGE. The aim was to demonstrate the presence of pyocin H66A in the same fraction as would be expected to contain DNA (around 7.8ml or 9.5ml), however, no protein could be observed (image is not shown). It is most likely that the concentration of protein was below the level required for detection by the SDS-PAGE. One point to mention is that the DNA was employed for the H66A assay differed from that used for the other two mutants and W/T. Figure 4.21 DNA control 2 shows the elution of 2 μ M of this DNA alone, which interestingly only had a minor peak of the contaminant eluting at 16.2 ml.



Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

Figure 4.20 Analytical gel filtration of pyocin S3 DNase D25A and H26A mutants did not provide evidence of interaction with DNA.

Elution profiles of 2 mM pyocin S3 DNase mutant D25A incubated with 2 mM 200 bp DNA in a buffer containing 2 mM Mg^{2+} (Panel A), 2 mM pyocin S3 DNase D25A mutant (Panel B), 2 mM pyocin S3 DNase mutant H26A incubated with 2 mM 200 bp DNA in a buffer containing 2 mM Mg^{2+} (Panel C), and 2 mM pyocin S3 DNase H26A mutant (Panel D).



Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding.

Figure 4.21 Analytical gel filtration of pyocin S3 DNase H66A mutant and W/T indicates interaction with DNA.

Elution profiles of 2 mM pyocin S3 DNase mutant H66A incubated with 2 mM 200 bp DNA in a buffer containing 2 mM Mg^{2+} (Panel A), 2 mM pyocin S3 DNase H66A mutant (Panel B), 2 mM pyocin S3 DNase w/t incubated with 2 mM 200 bp DNA in a buffer containing 2 mM Mg^{2+} (Panel C), 2 mM pyocin S3 DNase w/t (Panel D), bottom left and bottom right, respectively.

4.3 Discussion

4.3.1 *Residue D25, H26 and H66 are in proposed pyocin S3 DNase active site*

In the previous chapter, it was demonstrated that pyocin S3 was active against double-stranded plasmid DNA and required a divalent metal ion for activity. Here, the aim was to identify the important residues for pyocin S3 DNase activity, and thereafter to find out the location of the DNase active site. The alignment of amino acid sequences of pyocin S3 with the other $\beta\beta\alpha$ -Me DNase showed conserved and non-conserved residues in pyocin S3 DNase domain (Figure 4.1). Highly conserved residues with indicative importance to DNA activity were identified as residues D25, H26 and H66 of the DNase domain. Thus site directed mutagenesis was performed to change these residues to alanine to produce the three mutants D25A, H26A and H66A which were investigated to the point of purification and preliminary characterisation of the DNase domains. This is in line with previous studies on colicin E9, which suggested that at least three of the residues identified in the DNase domain are potentially involved in either maintaining the overall fold of the enzyme or the mechanism of the endonuclease activity (Garinot-Schneider, Pommer et al. 1996).

The activity of full-length pyocin S3 DNase and its mutants (D25A, H26A and H66A) were assessed using an *in vivo* stab test assay that exploits the sensitivity of the clinical strain PSA971 to pyocin S3 (Figure 4.4). Pyocin S3 kills PSA971 and this is detected by a large clear zone on the plate. In contrast, the D25A, H26A and H66A mutants did not inhibit the growth of the susceptible strain, indicating that the mutations had

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding. Inactivated the nuclease. To further support this observation, the activities of the pyocin S3 DNase mutants were examined *in vitro* using the plasmid nicking assay employed in Chapter 3 (Figure 4.8). The W/T pyocin S3 DNase activity was consistent with the previous data (Figure 3.5) and cleaved the substrate plasmid DNA at 0.23 μ M whereas the mutants failed to do so. This observation is in line with the previous report on the critical role of histidine residues in DNase activity. The strongly nucleophilic histidine residues in DNase are likely to function as a general acid or base and are often involved in catalytic hydrolysis of phosphodiester bonds (Cheng, Hsueh et al. 2006). It is of note that the different pyocin S3 DNase mutants had slightly different effects on the mobility of the substrate DNA at this concentration. The observation of the mobility shifted supercoiled DNA band shown on mutant D25A (Figure 4.8) together with the ICP data discussed in Chapter 5.3.2, might be an indication of metal binding. It can probably be speculated that mutant D25A binds to a metal ion causing the shift/slower migration of DNA due to the positive charge of the metal ion. Finding that D25 is important is in line with previous reports on the role of an equivalent aspartic acid residue in other H-N-H DNases, such as Endo VII, Hpy991, Cas9 and I-Hmul. In the listed enzymes, this residue, which is placed next to the first important histidine residue in the $\beta\beta\alpha$ -Me fold is likely to be involved in DNase activity as a metal-binding residue (Wu, Lin et al. 2020). Only H26A generated DNA fragments that resembled those generated by the wild type pyocin S3 DNase domain at a 10-fold lower concentration (23 nM) or by the negative control (lacking the addition of

any DNase). Together, this data suggests that the three residues H25, H26 and H66 are likely to be in the active site of pyocin S3 DNase as alanine substitution of those residues abolished both biological and enzymatic activity. Three ways that mutants could silence DNase activity was interpreted by Garinot-Schneider et al. 1996 for colicin E9: (i) mutations may affect an interaction with the receptor binding domain or translocation domain; (ii) mutations may be involved in DNA catalysis or metal binding directly; (iv) mutations cause a structural change of the pyocin S3 killing domain, thereby abolishing DNase activity (Garinot-Schneider, Pommer et al. 1996). Therefore, further study on protein-protein and protein-DNA interaction was performed to find out the mode of action of pyocin S3 DNase.

4.3.2 EMSA and analytical gel filtration assay showed that mutant D25A and H66A had the potential to bind DNA, respectively

In this chapter, three different DNA binding assays were used to analyse the DNA binding properties of pyocin S3 DNase W/T and mutants (D25A, H26A and H66A). Firstly, the classical protein-DNA binding assay - electrophoretic mobility shift assay (EMSA) was performed using cyanine 5.5 labelled DNA. Buffer failure resulted in samples loaded in the gel away from anode / cathode not migrating down the gel properly (Panel A, Figure 4.11). Figure 4.12 has suggested that circulation of buffer was probably essential for the weak buffer system, such as sodium phosphate buffer, which is weaker compared to Tris-HCl buffer. No clear shift of the free DNA in the gel for mutant H26A and H66A was observed, while a smear was observed for mutant D25A indicating the mutant may shift the migration of DNA (Figure 4.12). This result is in line with the data

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding. obtained from a previous plasmid nicking assay (Lane 4, Figure 4.8), in which the migration of supercoiled DNA was shifted by mutant D25A indicating the mutant may be involved in DNA binding. The data has raised a question: was mutant D25A smearing because it had one less negative charge due to the alteration of aspartic acid for alanine, thus predisposing it to a shift? It is observed on Figure 4.12 that the free P3 DNA probe actually runs slower than when any pyocin or mutant is present. Although theoretically possible it was considered an artefact as no example was reported in the literature previously.

Fluorescence polarization (FP) was performed next using bodipy labelled DNA and commercial benzonase as a control to support the data obtained from the above EMSA. Both of the two assays used fluorophore labelled DNA to distinguish bound and unbound proteins, but followed this with different ways of detecting the signal/ monitoring the DNase binding activity. It would be expected that both of the assays would show the same results, however, it was not possible to demonstrate the pyocin S3 DNase digesting Bodipy labelled DNA resulting in a decrease of FP as a control (Figure 4.15). The difficulties that were encountered are thus discussed with respect to the EMSA and FP experiments:

1. The cyanine 5.5 is better for EMSA, while bodipy530 is better for FP. Therefore, no direct comparison using the same fluorophore labelled DNA could be made for those experiments.
2. A TBE gel containing EDTA is approximately pH 8.0, which is commonly used for EMSA. However, no obvious shift of DNA probe

could be observed. It is probably due to the PI of the pyocin S3 DNase protein, which is around pH 8.0 and the protein is not holding any charge at its PI. Hence, the protein-DNA complex could not migrate to the positively charged side when bound.

3. To solve the above problem, a NaH_2PO_4 , pH 6.0 gel was employed for EMSA and the same buffer was used to match the gel. It was hypothesized that more binding would be observed in NaH_2PO_4 , pH 6.0 buffer, which contained more Na^{2+} and PO_4^{4-} ions than a Tris buffer. It is also found that the NaH_2PO_4 , pH 6.0 buffer gave much less noise in the background for FP compared to the Tris buffer. However, the commercial DNase, Benzonase works better at pH 8.0.

4. A small amount of EDTA was required in the reaction to protect DNA. However, the EDTA may precipitate in NaH_2PO_4 , pH 6.0 buffer, as it can be only dissolved at pH 8.0, though there was no visible precipitation observed in the reaction tubes. Moreover, the EDTA may also inactive the DNase activity by stripping metal from its active site.

5. A small amount of glycerol in the reaction was required to make the DNase last longer during the assay. The glycerol in the reaction for FP can also help load the samples directly on a gel for EMSA afterwards for comparison/ further investigation. However, the glycerol can slow down the DNA movement by making the buffer more viscous, resulting in a higher FP at the starting point.

6. The temperature in the Tecan for the FP assay rose with time. The DNA molecule moves faster at higher temperature. However, the

Pyocin S3 DNase mutants (D25A, H26A and H66A) lack endonuclease activity, suggesting these residues may be involved in DNA binding. temperature of the Tecan may not have a big effect on the data. The first two techniques to study the protein-DNA interaction mentioned both provide high sensitivity, however both of them need the specific fluorescent label, which limited the application of the techniques. Compared to those fluorescent assays, analytical gel filtration assay is non-destructive and dynamic (Ma, Yang et al. 2018). The analytical gel filtration profile for H26A is very different from the others and interestingly because it revealed that this pyocin mutant, even when present at a diluted concentration of 2 μ M in a physiological buffer is predominantly a dimer (75%) and not monomeric according to the ratio of absorbance elution peaks at 12.2 ml (dimer) and 16.2 ml (monomer). This maybe one reason this mutant does not bind DNA in EMSA and GF assays. And it may also support the data obtained from Lane 5, Figure 4.8, in which H26A has shown exactly the same DNA migration distribution as the plasmid DNA control (no addition of DNase) in the plasmid nicking assay indicating the mutant H26A may be not involved in DNA binding. Similarly, the decrease in absorbance for the mutant H66A with DNA in the gel filtration may indicate that this mutant has the ability to bind DNA consistent with the observation of a slightly shifted supercoiled plasmid DNA from the plasmid nicking assay with this mutant (Lane 6, Figure 4.8). The data for pyocin S3 DNase W/T confirmed that the buffer conditions used in the experiments permitted binding and cleavage of DNA (Run 11 and Run 12, Figure 4.21). Failure to catch pyocin DNase in the act eluting earlier in its gel filtration run is consistent with our

findings in previous EMSA and FP assays. Therefore, probably a more appropriate method is required for the study of protein-DNA interaction.

Chapter 5. The crystal structure of pyocin S3 DNase-immunity complex

5.1. Introduction

Characterising the interactions between individual residues at the interface of the DNase/Immunity proteins is crucial for understanding the protein-protein interaction. In some cases, two proteins can interact, and this can lead to inactivation of the active site of one because the interaction occurs at an overlapping place, such as the cytotoxic tRNase CdiA^{E479} and CdiA^{E1026b} with their cognate immunity protein from *Burkholderia pseudomallei*. The CdiA^{E479} and CdiA^{E1026b} share no significant sequence or structural homology and neither do their immunity proteins, and each immunity protein inactivates with its cognate toxin by binding within the nuclease active site cleft to prevent access to tRNA (Johnson, Gucinski et al. 2016). In other cases, the inhibitory protein may interact at a distant position in the active protein (exo-site), such as Colicin E3 and its cognate immunity. The two proteins interact at a distant position in the active protein (Carr, Walker et al. 2000). Thus, knowing which residues interact in two interacting proteins can inform their functional relationship. Interfaces of permanent complexes (e.g., the homodimer cytochrome C') are more closely packed and contain fewer intermolecular hydrogen bonds compared to interfaces of non-obligatory complexes (e.g., the enzyme-inhibitor complex) (Jones and Thornton 1996). From the evolutionary perspective, both types of the complexes have presumably evolved over time to optimize the interface to suit their

biological function. For example, the function may require the evolution of strong binding (Jones and Thornton 1996). The most detailed way of achieving a thorough understanding of protein-protein interactions comes from an analysis of the X-ray crystal structure. This is particularly insightful when the structures of both proteins are available in their monomeric and also their polymeric complex forms (Schreiber and Fersht 1995). This chapter sought to solve the structure of pyocin S3 DNase-immunity complex first, as the complex protein is supposed to be more stable than the separated proteins, and hence is more likely to crystallise. The molecular structure of the three pyocins, pyocin S1, S2 and AP41 exists, and indicates that they originated from a common ancestor of the colicin E2 group (Sano, Matsui et al. 1993). Characterization revealed that they all adopt the typical H-N-H motif, which is involved in both protein folding, DNA binding and DNase catalysis simultaneously (Pommer, Cal et al. 2001, Maté and Kleanthous 2004). The crystal structure of pyocins S2 and AP41 were determined with their cognate Immunity protein to investigate the ultra-high-affinity protein-protein interaction between them. This revealed that both adopt the typical $\beta\beta\alpha$ -Me DNase fold with conserved water molecules at the DNase-immunity interface (Joshi, Grinter et al. 2015). The $\beta\beta\alpha$ -Me fold has been previously observed in a number of H-N-H endonucleases (Huang and Yuan 2007). Some water molecules were strictly conserved in colicin E2, E7 and E9 (Joshi, Grinter et al. 2015). In H-N-H bacteriocins, the $\beta\beta\alpha$ -Me fold consists of two antiparallel β -strands linked to a C-terminal α -helix with a divalent metal ion in the centre, which plays

The crystal structure of pyocin S3 DNase-immunity complex a role as a cofactor for DNA hydrolysis (Huang and Yuan 2007, Wu, Lin et al. 2020). Metal ions are essential for the activity of many enzymes (Campbell and Jackson 1980). For instance, a Zn^{2+} ion was found binding to three conserved histidine's in the active site of many DNase bacteriocins, such as colicins E2, E7, E8 and E9 and pyocins S1 and AP41 (Ko, Liao et al. 1999). Different divalent metal ions can serve as the cofactor for the bacteriocins E2, E7, E8 and E9 and pyocins S1 and AP41. For example, Ni^{2+} in pyocin AP41 (Joshi, Grinter et al. 2015), Mg^{2+} in colicin E9 (Pommer, Kühlmann et al. 1999) and Zn^{2+} in E7 (Sui, Tsai et al. 2002). Pyocin S3, which is predicted to lack the classical H-N-H endonuclease motif of homing endonucleases, is still not yet well-studied at the structural level, and there is no report on the mode of action of this non-H-N-H DNase pyocin (Michel-Briand and Baysse 2002).

In this chapter, the first crystal structure of pyocin S3 DNase-immunity complex was solved and is described in detail. Although the pyocin S3 DNase-immunity complex had been successfully overproduced, purified and crystallized using native conditions by a previous student, Chris Newman (as part of his MRes degree), the crystal structure couldn't be solved using molecular replacement even with a very good x-ray diffraction pattern (Table 5.4). This was due to the lack of similarity with any known protein structures in the Protein Databank (PDB). A major bottleneck in studying some proteins, is that the structures of these proteins are under-represented in the PDB. This is often caused by the difficulties associated with producing proteins in their native state in sufficient quantities for analysis (Berntsson, Alia Oktaviani et al. 2009).

This challenge has been in part alleviated thanks to the selenomethionine (SeMet) incorporation method, which was first exploited by Hendrickson in 1990 (Hendrickson, Horton et al. 1990). Selenium is a heavier atom than the ones (carbon, nitrogen and oxygen) found in proteins, which absorbs and re-emits X-rays with element-specific resonance. Subsequently, crystallographers can determine the phase using the resonant diffraction data. Results from a large number of experiments has proved that the biological substitution of SeMet for Met residues dose not disturb the 3D structure of the labelled proteins. Therefore, SeMet incorporation has been used commonly in protein crystallography nowadays (Strub, Hoh et al. 2003) and has become a standard method for the phase determination in protein crystallography (Jones, van Heyningen et al. 1999, Larsson and Jones 2007).

In this study, the SeMet incorporation method was employed to solve the structure of the pyocin S3-Immunity protein complex, so that the native data could be solved using molecular replacement with the SeMet to resolve the structure. The structure of pyocin S3 DNase-immunity complex revealed a novel octopus fold of the immunity protein around the DNase that protects producing bacteria against the S3 pyocin DNase defence weapon during production. Surprisingly, there was no metal ion observed in the structure of the complex. Moreover, it is interesting that the secondary structure of pyocin S3 DNase domain revealed a $\beta\beta\alpha$ fold, which is very similar to the $\beta\beta\alpha$ -Me fold of AP41 despite the S3 not having a recognised H-N-H domain. A question is raised: is the $\beta\beta\alpha$ fold in

The crystal structure of pyocin S3 DNase-immunity complex pyocin S3 playing a similar role as the $\beta\beta\alpha$ -Me fold in the N-H-N motif of the other DNases?

To solve the structure of the pyocin S3 DNase without its cognate immunity bound would help with answering those questions. To protect the producing cell from the predicted DNase activity, the pyocin S3 DNase domain was overproduced alongside its cognate immunity protein as described in Chapter 3 since the immunity protein is predicted to inhibit the hydrolysis of DNA catalysed by the DNase. A buffer containing 6 M Guanidine was used to separate the DNase protein from the immunity protein by denaturing the protein complex (Garinot-Schneider, Pommer et al. 1996, Joshi, Grinter et al. 2015). Then different methods for protein concentration were employed to find the most suitable and efficient way to obtain a high yield of purified pyocin S3 DNase. Those methods included spin column protein concentration using a Viva spin 500 concentration (Filipek, de Araujo et al. 2017), a continuous concentration by permosmosis using polyethylene glycol (PEG) 20,000 (Poison 1973) and lyophilisation using a freeze dryer (Garidel and Presser 2019). All the three protein concentration methods successfully increased the concentration of the pyocin S3 with various efficiencies. However, the final concentration of purified DNase was only increased to 1.07 mg/ml due to a large amount of protein loss during each step of the purification process and the initial DNase crystallization wasn't successful. Thereafter, a series of protein purification optimization approaches were explored using different purification strategies to get a high yield of protein. Finally, a significantly higher protein yield was

obtained by separating the pyocin S3 DNase and immunity protein while washing the loaded cell lysate containing the protein complex using Guanidine buffer on a Ni-NTA column rather than separation of protein complex after purification. Then, the pyocin S3 DNase and immunity protein were purified using a 5 ml Heparin column and step gradient, respectively before putting it down in the crystallization trays. Though neither of the two proteins were crystallized, the lessons learnt from the protein purification optimization will be valuable for future investigations. In parallel, more mutants (R38A, R50A, K51A, K54A, K55A and K79A) were made in the predicted DNA binding site using the same method described in 4.2.2 to obtain *in vivo* evidence to support the interpretation of the crystal structure with regard to DNase function.

Studies on protein-DNA interactions within several H-N-H DNases have shown that the $\beta\beta\alpha$ -Me motif not only can coordinate with DNA binding, but also be involved in DNA cleavage (Chevalier and Stoddard 2001, Doudeva, Huang et al. 2006). The crystal structure of the pyocin S3-immunity complex revealed that residues D25, H26 and H66 are all located in the $\beta\beta\alpha$ fold of the DNase. This finding renewed the motivation to investigate the role of the $\beta\beta\alpha$ fold in pyocin S3 by looking at how the substitution of those residues with alanine residues affect DNA binding. Therefore, a renewed attempt was made to investigate the protein-DNA interaction. The secondary structure of the pyocin S3 DNase and mutants used in the protein-DNA interaction assays (see chapter 4) were confirmed using both online and experimental circular dichroism (CD), showing all the proteins were folded. The pyocin S3 DNase w/t and

The crystal structure of pyocin S3 DNase-immunity complex selected mutants were also analysed by inductively coupled plasma-mass spectrometry (ICP) to detect the presence of metal(s) in a small volume of the protein solutions.

5.2. Results

5.2.1. Engineering of optimal strain for incorporation of selenomethionine (SeMet) into Pyocin S3 DNase-Immunity protein complex

An appropriate overproduction system existed to enable the generation of sufficient quantities of the pyocin S3/Immunity protein complex for high resolution X-ray diffraction (as described in Chapter 3). To enable incorporation of selenomethionine (SeMet) during the protein overproduction, the plasmid (pCN4, Table 2.3) was transformed into methionine auxotrophic *E. coli* B834(DE3) competent cells.

5.2.2. Overproduction and purification of pyocin S3 DNase-immunity protein complex incorporating selenomethionine (SeMet)

Overproduction of the expected proteins was verified in SeMet medium (Figure 5.1) which clearly shows pyocin S3 DNase (14.7 kDa) and immunity protein (17.06 kDa) after induction using IPTG. As a hexahistidine tag had been added to the C-terminal of the Immunity protein during the cloning process, the next step was purification by metal chelate chromatography of the DNase/Immunity protein complex. To do this, a cell lysate containing the S3 DNase-immunity complex produced in SeMet medium was applied to a Ni-NTA column. The UV absorbance trace (Panel A, Figure 5.2) revealed a peak highlighted by red underlining that was shown to contain the DNase-immunity protein complex by SDS-PAGE (Panel B, Figure 5.2). Fractions 10, 11 and 12 were pooled for further purification via gel filtration chromatography as they contained

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large quantities of the two proteins of interest with low levels of contaminating proteins.

The crystal structure of pyocin S3 DNase-immunity complex

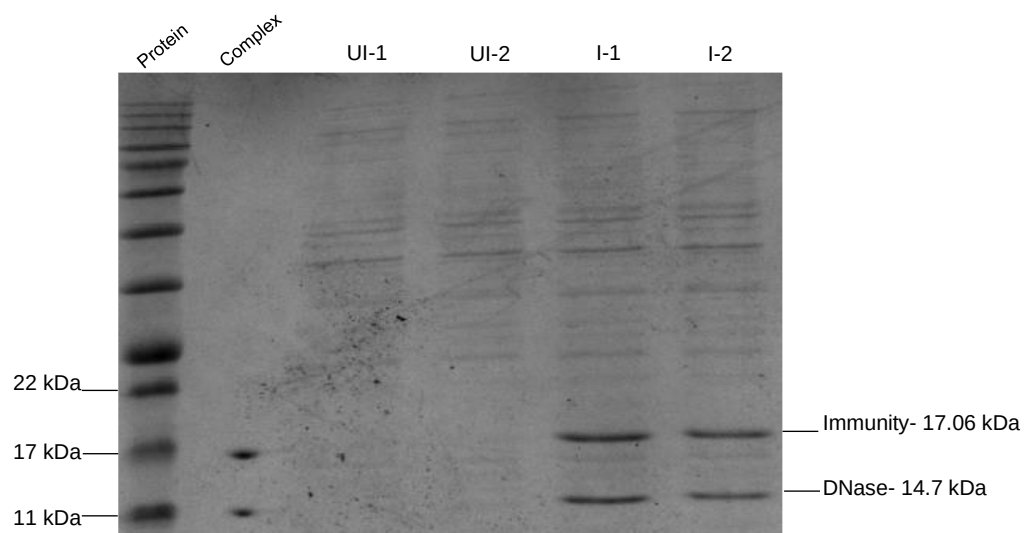


Figure 5.1 Pyocin S3 DNase-immunity complex in *E. coli* B834(ED3) over-produced in SeMet Medium.

Two parallel cultures of *E. coli* B834 (DE3) containing the S3 pyocin DNase-immunity genes on plasmid pCN4 was grown in SeMet Medium at 37°C with (I-1 and I-2) and without (UI-1, UI-2) induction with 1 mM IPTG (see methods). 100 µl of culture were taken before and after induction and mixed with 20 µl of SDS-PAGE loading buffer and applied to an 15% SDS-PAGE along with the complex protein before induction and a protein ladder (Colour prestained protein standard, Broad range (11-245 kDa, NEB). S3 pyocin DNase-Immunity complex (complex ladder) prepared previously (data not shown). Proteins were visualized by Coomassie blue staining. The expected sizes of the DNase and Immunity proteins are indicated on the right.

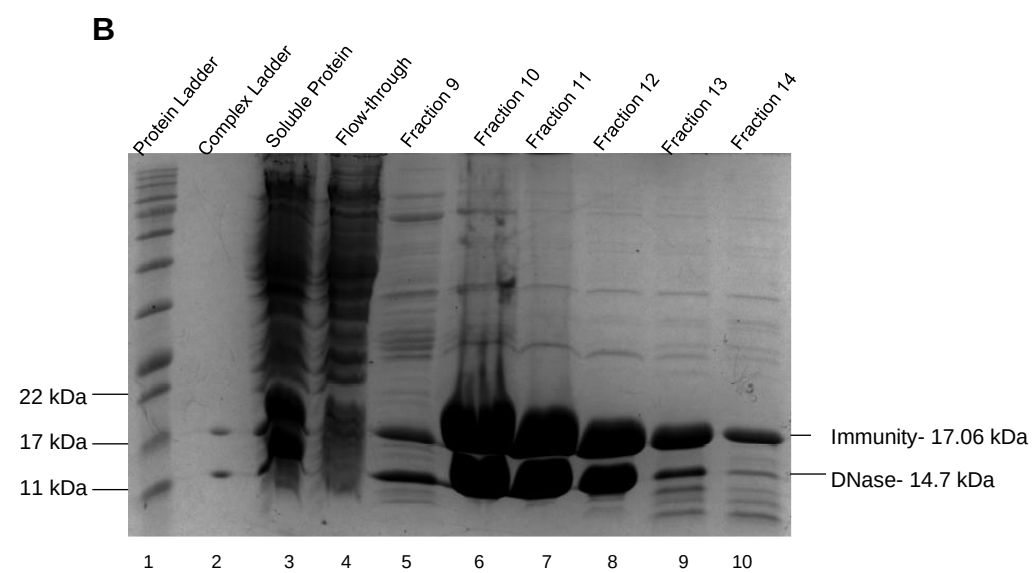
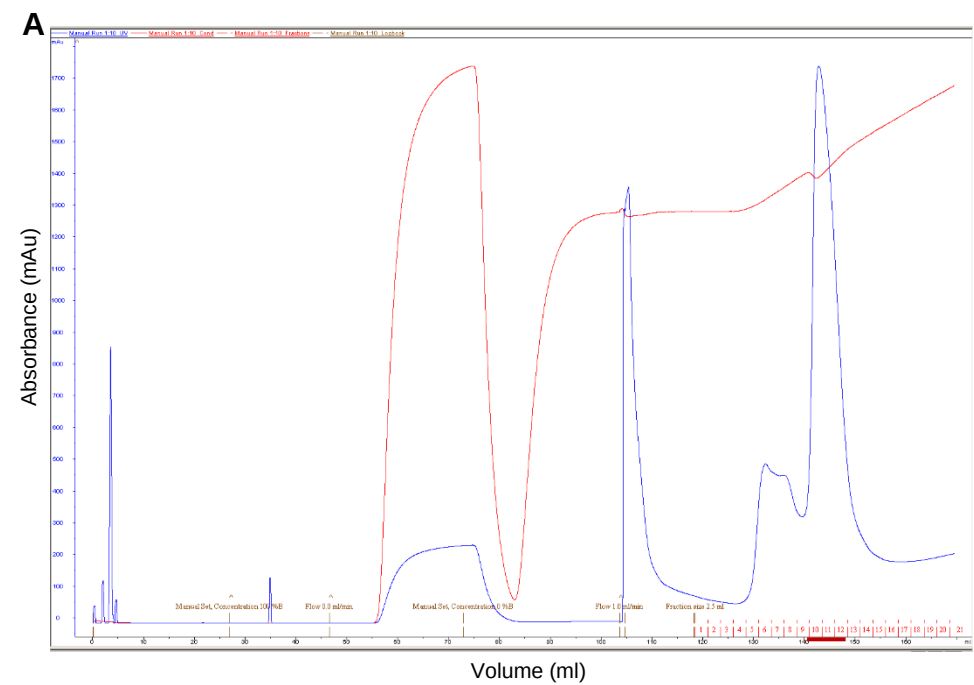


Figure 5.2 Purification of SeMet pyocin S3 DNase-immunity protein complex using a Ni-NTA column.

Panel A- Cell lysate containing the pyocin S3 DNase-immunity complex protein was loaded onto a 5 ml Ni-NTA column before purification using ÄKTAprimer™ PLUS system. 5 ml fractions of purified complex protein were collected, which was eluted by high concentration of imidazole buffer, till all the protein samples came off the column, where the UV absorbance stayed low and flat (fractions were numbered 10 to 14 in red). Red curve- conductivity trace, blue curve- UV absorbance trace, x axis- volume (ml), y axis- value of absorbance (mAu). Panel B- The soluble protein was separated by being centrifuging down the lysed protein complex cell at 13,000 xg for 12 mins and run through Ni-NTA column (flow-through was collected) before the proteins were purified and fractionated by ÄKTAprimer™ PLUS system. 60 µl of peak fractions containing DNase-Immunity complex protein were taken and mixed with 20 µl of SDS-PAGE loading buffer and applied to a SDS-PAGE running along with soluble protein collected from cell lysate and flow-through from the Ni-NTA column. Lane 1- Colour pre-stained protein standard; Lane 2- DNase-Immunity complex (complex ladder) prepared previously (data not shown); Lane 3- soluble protein; Lane 4- column flow-through; Lane 5 to 10- Fraction 9 to 14. Proteins were visualised using Coomassie blue stain. The expected sizes of the DNase and Immunity proteins are indicated on the right.

Subsequent protein complex purification was performed using a Superdex 75 column prior to concentration using a 5,000 MWCo vivaspin concentrator. The absorbance trace from gel filtration (Panel A, Figure 5.3) revealed two peaks, one (underlined in red) that SDS-PAGE (Panel B, Figure 5.3) revealed contained proteins of the sizes of the DNase and Immunity proteins (fraction 17-22). The other peak (underlined in yellow, fraction 23-26) was also subjected to SDS-PAGE, revealing primarily a protein the size of the Immunity protein. The protein profiles and peak sizes were indicative of large quantities of pure protein, the latter being confirmed by quantification by UV absorbance at 280 nm (Table 5.1) using a Nano drop spectrophotometer (ND-1000, ThermoFisher). The actual concentration of each pyocin S3 DNase-immunity protein fraction was calculated by the UV absorbance employing an extinction coefficient of $24410 \text{ M}^{-1} \text{ cm}^{-1}$. Distilled water was used as blank and 2 μl of each sample was measured. The UV absorbance reading was consistent with the protein levels detected by SDS PAGE. Fractions 18, 19 and 20 had the highest UV absorbance reading and the highest levels of the DNase-Immunity protein complex (Lanes 4-5, Figure 5.3).

The crystal structure of pyocin S3 DNase-immunity complex

Sample	UV absorbance reading (mg/ ml)	Actual Concentration (mg/ ml)
Fraction 17	0.16	0.215
Fraction 18	2.16	2.90
Fraction 19	3.72	5
Fraction 20	1.31	1.76
Fraction 21	0.14	0.19

Table 5.1 The UV absorbance and concentration of fractionated SeMet pyocin S3 DNase-Immunity protein complex.

* The actual concentration of each pyocin S3 DNase-immunity protein fractions was calculated by the UV absorbance employing an extinction coefficient of $24410 \text{ M}^{-1} \text{ cm}^{-1}$.

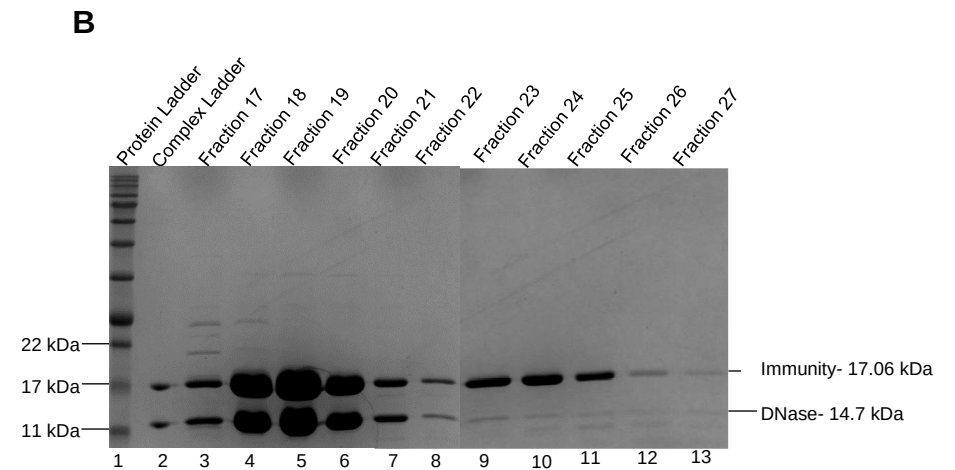
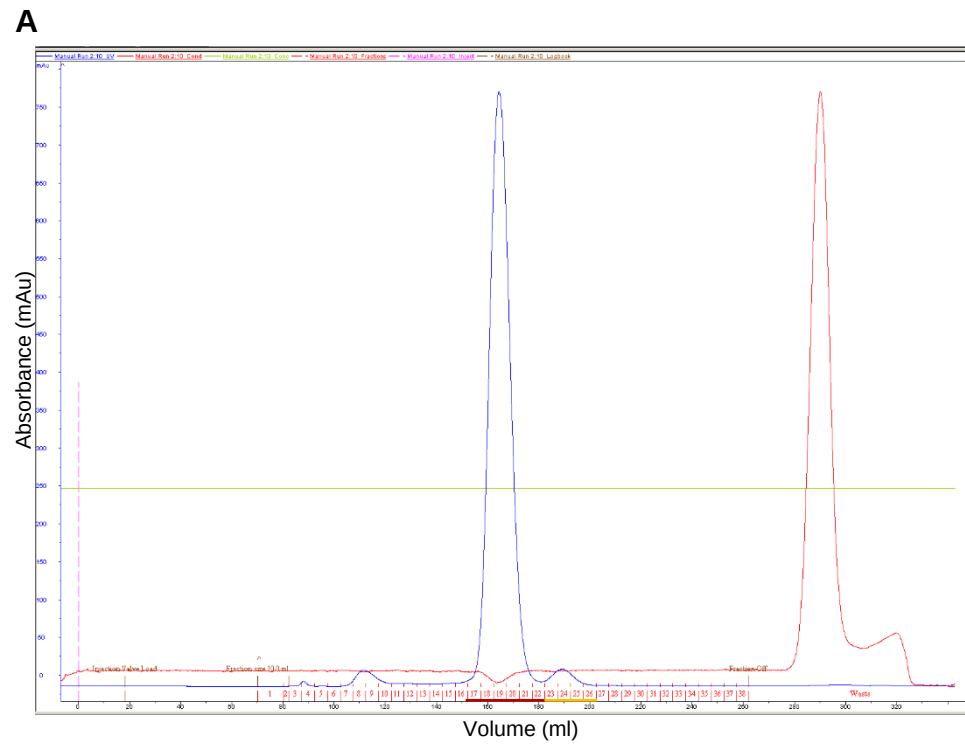


Figure 5.3 Purification of SeMet pyocin S3 DNase-immunity protein complex by gel filtration.

Panel A- UV profile of pyocin S3 DNase-immunity complex further using gel filtration chromatography. The purified DNase-Immunity pool of fractions collected from Ni-NTA column was injected into 2x 5 ml loops before loaded in to ÄKTAprimer™ PLUS system and eluted by Tris-Salt buffer. 10 ml fractions were collected firstly, then 5 ml fractions were collected before the DNase-Immunity protein were eluted off till all the protein samples had gone through the column, where the UV absorbance stayed low and flat (fractions were numbered 1 to 38 in red). Red curve- absorbance trace, blue curve- UV absorbance trace, x axis- volume (ml), y axis- value of absorbance (mAu). Panel B- SDS-PAGE gel of fractions collected during gel filtration chromatography for pyocin S3 DNase-immunity complex purification. 60 µl of peak fractions containing DNase-Immunity complex were taken and mixed with 20 µl of SDS-PAGE loading buffer and applied to an SDS-PAGE. Lane 1- Colour pre-stained protein standard; Lane 2- DNase-Immunity complex (complex ladder) prepared previously (data not shown); Lane 3 to 13- Fraction 17 to 27. Proteins were visualised using Coomassie blue stain. The expected sizes of the DNase and Immunity proteins are indicated on the right.

5.2.3. Crystallization of pyocin S3 DNase-immunity complex

Macromolecular crystallization includes crystallization of proteins, nucleic acids and larger macromolecular. Crystallization is a process of separation of an ordered crystalline phase from a metastable solution and the growth of crystal, which is largely empirical in nature, requires patience, perseverance and intuition (Drenth 2001, Rupp 2015). The aim is to exceed the limitation of the protein solubility by adjusting the solution conditions in order to produce crystals, since a protein can only stay in solution up to a certain concentration. The protein will move to a new phase/ no longer remain solvated after this point, as there is insufficient solvent to maintain full hydration of the protein (shown as 'super saturated' state in Panel A, Figure 5.4) (Asherie 2004). Since certain conditions such as the chemical composition and the temperature necessary for successful formation of a stable crystal is usually unknown, the protein needs to be screened against a chemically diverse set of crystallization reagents (Rupp 2015). The sitting-drops vapour-diffusion method is one of the techniques commonly used for achieving protein supersaturation performed using multichambered trays (Panel B, Figure 5.4). The drop on the elevated platform consisting of two solutions in equal parts, one part being the stock protein solution and the other part being the reservoir solution, is sealed in a chamber with the precipitant solution. Subsequently, water vapour diffuses out of the drop until the osmolarity of the drop and the precipitant are equal. This means that both the protein and precipitant are slowly concentrated until equilibrium, ideally in the labile zone (also known as nucleation zone) of the phase

The crystal structure of pyocin S3 DNase-immunity complex diagram where protein is normally crystallized (Panel A, Figure 5.4) (Asherie 2004).

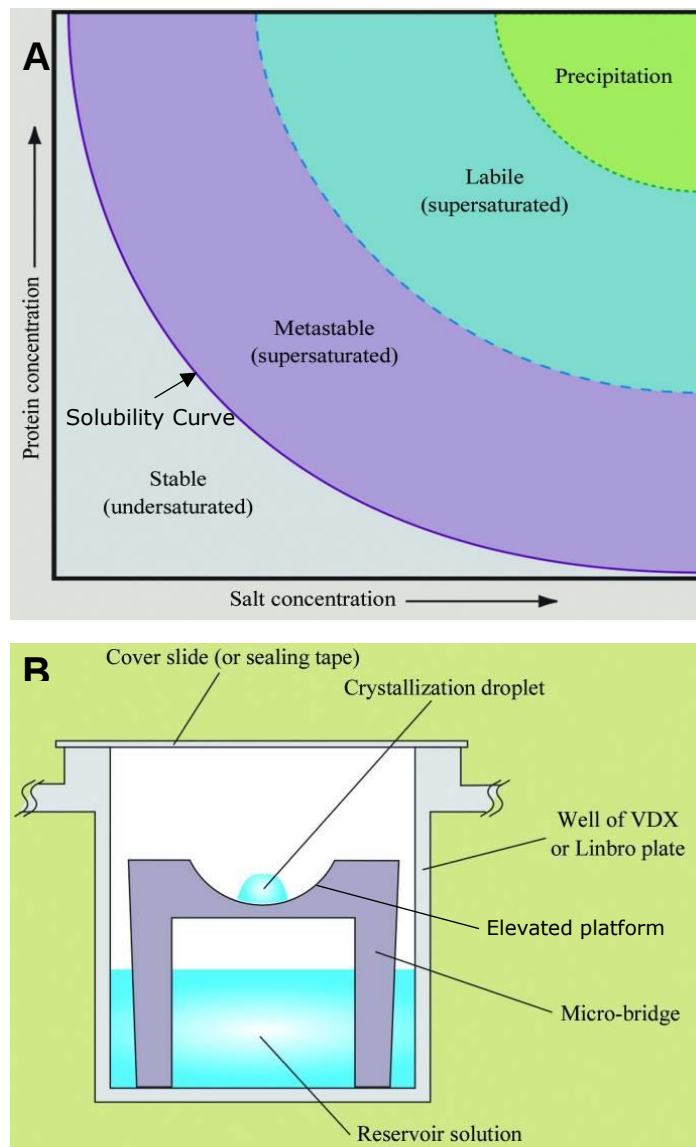


Figure 5.4 The principle of protein crystallization.

Panel A- A phase diagram for protein crystallization. The solubility of a protein varies with the solution conditions and the solubility diagram is divided into two regions: undersaturation and supersaturation region. The latter one is further divided into metastable and labile regions. In the metastable region no nucleation will occur. In the labile zone, the supersaturation is large enough that spontaneous nucleation will be observed. In the precipitation region, the aggregates and precipitates form faster than the crystals, which is unfavourable for crystal formation. Panel B- The sitting drop vapor-diffusion method. Equal volumes of precipitant solution are present in the drop. Evaporation of water in the drop into the chamber, followed by diffusion across the chamber into the well and both the precipitant solution and protein will be concentrated until equilibrium. Adapted from Fig 3, MacPherson and Gavira 2014. (McPherson and Gavira 2014).

Figure 5.3 together with Table 5.1 reveal the high levels of pure DNase and Immunity protein present in fractions 19 and 20. Consequently fractions 19 and 20 were selected for the next step and pooled. Subsequently, dialysis against tris-salt buffer (see material and methods) was performed, and the proteins were concentrated using a 5,000 MWCo concentrator that yielded 1.5 ml of the protein at a concentration of 17.94 mg/ ml for crystallographic trials. Based on the crystallization conditions previously used for the native pyocin S3 DNase-immunity protein complex, two concentrations of the protein complex solution were prepared: 17.94 mg/ ml and 8.97 mg/ ml. Three crystallization screens were prepared in 3x 96-well plates using the sitting drop method and incubated at 20°C. The three screens were: PEGs II, PACT and JSCG+. Surprisingly, as soon as the pooled protein was introduced into the solution conditions, crystals formed within 5 mins. Those conditions are listed as Table 5.2. More crystals were observed after 72 h incubation under the conditions listed as Table 5.3. It was verified that the crystals were not salt crystal by comparing the images of crystals formed by the two concentrations in the same condition, which were very different. Table 5.2 and Table 5.3 reveal that the protein complex crystallized at a high pH around pH 9.0 in the presence of PEG. Images of the crystals (Figure 5.5) showed that most of the crystals were a long rod shape with a dip down the middle and they were quite large.

Having successfully grown crystals, the following processes were performed by the crystallographer Dr. Simon Caulton: A good, formed

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crystal (Figure 5.5) was crushed with an acupuncture needle and seeded with a horse hair into the condition best suited to crystal formation. When the crystal was fished, it was cryo-protected in the same solution highlighted in yellow in Table 5.2 with 30% (v/v) glycerol to allow the liquid to freeze without crystals of water forming. The best crystal formed in this condition was frozen by liquid nitrogen before it was sent to the Synchrotron, Oxford to obtain X-ray diffraction from the crystal. The returned structural data was computationally analysed by Dr. Simon Caulton to construct the atomic structure of the pyocin S3-Immunity protein complex.

The crystal structure of pyocin S3 DNase-immunity complex

Screens	Tube No.	Conditions
PEGs II	B 4	0.1 M Tris, pH 8.5, 30% (w/v) PEG 1000
	D 10	0.2 M sodium acetate, 0.1 M Tris, pH 8.5, 30% (w/v) PEG 4000
PACT	B 6	0.1 M MIB buffer, pH9.0, 25% (w/v) PEG 1500
	C 6	0.1 M PCTP buffer, pH 9.0, 25% (w/v) PEG 1500
	D 6	0.1 M MMT buffer, pH 9.0, 25% (w/v) PEG 1500
	H 1	0.2 M sodium fluoride, 0.1 M Bis Tris propane, pH 8.5, 20% (w/v) PEG3350
	H 3	0.2 M sodium iodide, 0.1 M Bis Tris propane, pH 8.5, 20% (w/v) PEG3350
	H 4	0.2 M potassium thiocyanate, 0.1 M Bis Tris propane, pH 8.5, 20% (w/v) PEG3350
	H 5	0.2 M sodium nitrate, 0.1 M Bis Tris propane, pH 8.5, 20% (w/v) PEG3350
	H 7	0.2 M sodium acetate, 0.1 M Bis Tris propane, pH 8.5, 20% (w/v) PEG3350
	H 10	0.02 M sodium/potassium phosphate, 0.1 M Bis Tris propane, pH 8.5, 20% (w/v) PEG3350

Table 5.2 Details of conditions under which crystals formed within 5 mins after screens were set up.

Screens	Tube No.	Conditions
PEGs II	C 8	0.2 M Sodium acetate, 0.1 M Tris, pH 8.5, 16% (w/v) PEG 4000
	G 12	1.0 M Lithium chloride, 0.1 M Sodium acetate, 30% (w/v) PEG 6000
PACT	D 9	0.2 M lithium chloride, 0.1 M Tris, pH 8.0, 20% (w/v) PEG 6000
	F 7	0.2 M sodium acetate, 0.1 M Bis Tris propane, pH 6.5, 20% (w/v) PEG3350
	H 2	0.2 M sodium bromide, 0.1 M Bis Tris propane, pH 8.5, 20% (w/v) PEG3350
	H 6	0.2 M sodium formate, 0.1 M Bis Tris propane, pH 8.5, 20% (w/v) PEG3350
JCSG+	A 4	0.02 M Calcium chloride, 0.1 M Sodium acetate, pH 4.6, 30% (w/v) MPD
	B 1	0.8 M Ammonium sulfate, 0.1 M Citric acid, pH 3.5 (final pH 4)
	C 3	0.2 M Ammonium nitrate, 20% (w/v) PEG 3350

Table 5.3 Details of conditions under which crystals formed after 72 h incubation.



Figure 5.5 Crystals of pyocin S3 DNase-Immunity protein complex formed within 5 mins of setting up crystallographic screens.

Crystals spontaneously formed under the conditions listed on Table 5 and 6. A representative crystal is shown as indicated by the red arrow. One such crystal from the H4 PACT condition was crushed with an acupuncture needle and seeded with a horse hair into the H1 PACT condition. Crystals were successfully reproduced and cryoprotected in 0.2 M sodium fluoride, 0.1 M Bis Tris propane, pH 8.5, 20% (w/v) PEG3350, 30% (v/v) glycerol. Before being subjected to X-ray diffraction.

5.2.4. Structure of pyocin S3 DNase-immunity indicating a novel octopus structure without an H-N-H motif or bound metal ion

The first aim was to determine the crystal structure of pyocin S3 DNase-immunity protein complex to investigate the interactions between the two high affinity proteins. Crystals of SeMet-labelled pyocin S3 DNase-immunity protein grew within minutes, diffracted X-rays to 1.45 Å resolution and were found to belong to the $P 2_12_12_1$ space group (Table 5.4 X-ray diffraction and structure refinement statistics (data provided by crystallographer Dr. Simon Caulton)). The structure was solved using SAD phasing and refined to a final $R_{\text{work}}/R_{\text{free}} = 0.18/0.20$. To confirm that the native structure was the same as the SeMet structure, the native

structure was solved by molecular replacement using the SeMet structure as a search model. The native structure was solved and refined to a $R_{\text{work}}/R_{\text{free}} = 0.19/0.22$ and showed that the structures were almost identical to the SeMet version (the SeMet DNase Met1 was processed and immunity Cys144 was oxidised). Data collection and model statistics for native and SeMet structures are shown in Table 5.4 X-ray diffraction and structure refinement statistics (data provided by crystallographer Dr. Simon Caulton). It is noteworthy that there was no metal observed in the structure of the pyocin S3 DNase-immunity protein complex (Panel A and B, Figure 5.7); however, further structural or biochemical information might be required to confirm this. Panel A and C in Figure 5.6 reveal that the pyocin S3 DNase fold consists of a three anti-parallel-strand β -sheet and eight α -helices in the order $\beta 1$ -H1- $\beta 2$ -H2-H3- $\beta 3$ -H4-H5-H6-H7-H8. Helices 7 and 8 at the C-terminus form a long anti-parallel helical pair that act as a base for the rest of the protein to pack against. The small anti-parallel β -sheet and helix 1 pack primarily on top of helix 8, and helices 4, 5 and 6 pack against helix 7. In conjunction with helix 2 and 3, which form a double helical prong that extends out from the centre of the protein, they create a flat face with a pocketed centre. The opposite face is formed by helices 7 and 8 and create a domed face opposite the pocket. The double helical prong extends from the rest of the protein, to act as a docking site for the immunity protein (Panel C, Figure 5.6). Observing the electrostatic surface potential of the DNase (Panel E, Figure 5.6), the pocketed face has a bullseye appearance, with basic residues found around the outside of the pocket, then a layer of

The crystal structure of pyocin S3 DNase-immunity complex uncharged residues and finally a central negative charge at the deepest part of the pocket, mediated primarily by Asp25.

Pyocin S3 immunity domain comprises three α -helices packed on to a nine anti-parallel-stranded β -sheet in the sequence H1-H2- β 1- β 2- β 3-H3- β 4- β 5- β 6- β 7- β 8- β 9 (Panel B and D in Figure 5.6). β -strands 1-4 and 7-9 form a large anti-parallel β -sheet. The first three strands are contiguous, whereas a large loop and helix separate the fourth strand, which is found on the opposite end of the sheet, proceeding inwards to β -strand 9. β -strands 5 and 6 are located in a loop and form a small hairpin sheet with one another. All three helices pack against one face of this β -sheet; and are not involved in the interaction of the immunity protein with the DNase. The immunity protein adopts an octopus-like grasp that emerges from the β -sheet and locks onto the DNase protein (Panel B, Figure 5.7). It grasps on to the double helical prong that projects from the DNase. This docking feature fits into the curvature of the large β -sheet of the immunity protein. The three grasping loops together with residues form the face of the beta sheet providing the stabilising interaction (Panel C-F, Figure 5.7). Grasping loop 1 (Panel C, Figure 5.7) makes a number of interactions with the double helical prong on the flat, pocketed face of the DNase subunit. These include hydrophobic packing of immunity residues Y35 and P34 with DNase residue Y46; hydrogen bonding of DNase N77's sidechain and backbone with E36 and H37, respectively, and DNase residue Q82 carbonyl H-bonding with immunity residue H37. Finally, immunity E39 further interacts with K79 on DNase helix 6, forming a salt bridge. Grasping loop 2 (Panel D, Figure 5.7) also provides

a diverse array of interactions with the DNase. It contributes multiple electrostatic interactions via immunity residues E72 and D69, which interact with DNase residues K54 and K51. In addition, DNase L52 forms hydrophobic interactions with immunity V71, and DNase K51 also H-bonds with immunity S74. Grasping loop 3 of immunity protein (Panel E, Figure 5.7) is primarily hydrophobic in nature, forming a complex network of hydrophobic packing interactions. These include residues F108, V110, P111, V112, I113 and L116 with residues of the DNase I40, Y41, L52, W130 and the alkyl chain of K55. In addition, the backbone nitrogen of DNase V112 forms a H-bond with the carbonyl of Q128. The beta sheet itself (Panel F, Figure 5.7) forms hydrophobic, electrostatic and H-bond interactions with the helical prong of the DNase. Immunity H40 H-bonds with DNase Y46 hydroxyl group; E50 forms H-bonds from both sidechain oxygens with the backbone nitrogen atoms of DNase Y46 and G47; K62 H-bonds with the carbonyl of P42; DNase Y41 and D43 H-bond with immunity N144; and immunity R105 H-bonds with the carbonyl of DNase I40. Hydrophobic packing can be seen between DNase P42 and immunity W119 and a salt bridge is seen centrally between DNase D43 and both immunity R131 and K62. The high-resolution structure also shows a high number of waters that form H-bond networks between the two proteins.

The crystal structure of pyocin S3 DNase-immunity complex

	Selenomet	Native
Resolution range	22.18 - 1.45 (1.502 - 1.45)	33.82 - 1.8 (1.864 - 1.8)
Space group	P 2 ₁ 2 ₁ 2 ₁	P 2 ₁ 2 ₁ 2 ₁
Unit cell	43.42 43.84 159.62 90 90 90	42.92 43.69 160.25 90 90 90
Unique reflections	54771 (5159)	28802 (2829)
Completeness (%)	99.40 (94.99)	99.84 (99.58)
Wilson B-factor	20.37	23.44
Reflections used in refinement	54771 (5159)	28803 (2829)
Reflections used for R-free	2674 (280)	1437 (151)
R-work	0.1801 (0.2891)	0.1865 (0.2452)
R-free	0.1994 (0.3249)	0.2160 (0.3080)
Number of non-hydrogen atoms	2682	2479
macromolecules	2344	2300
ligands	8	7
solvent	330	172
Protein residues	283	282
RMS(bonds)	0.006	0.006
RMS(angles)	0.86	0.83
Ramachandran favored (%)	98.56	98.56
Ramachandran allowed (%)	1.08	1.08
Ramachandran outliers (%)	0.36	0.36
Rotamer outliers (%)	0.39	0.81
Clashscore	3.18	5.80
Average B-factor	26.43	30.10
macromolecules	24.86	29.35
ligands	49.01	43.62
solvent	37.06	39.64

Table 5.4 X-ray diffraction and structure refinement statistics (data provided by crystallographer Dr. Simon Caulton)

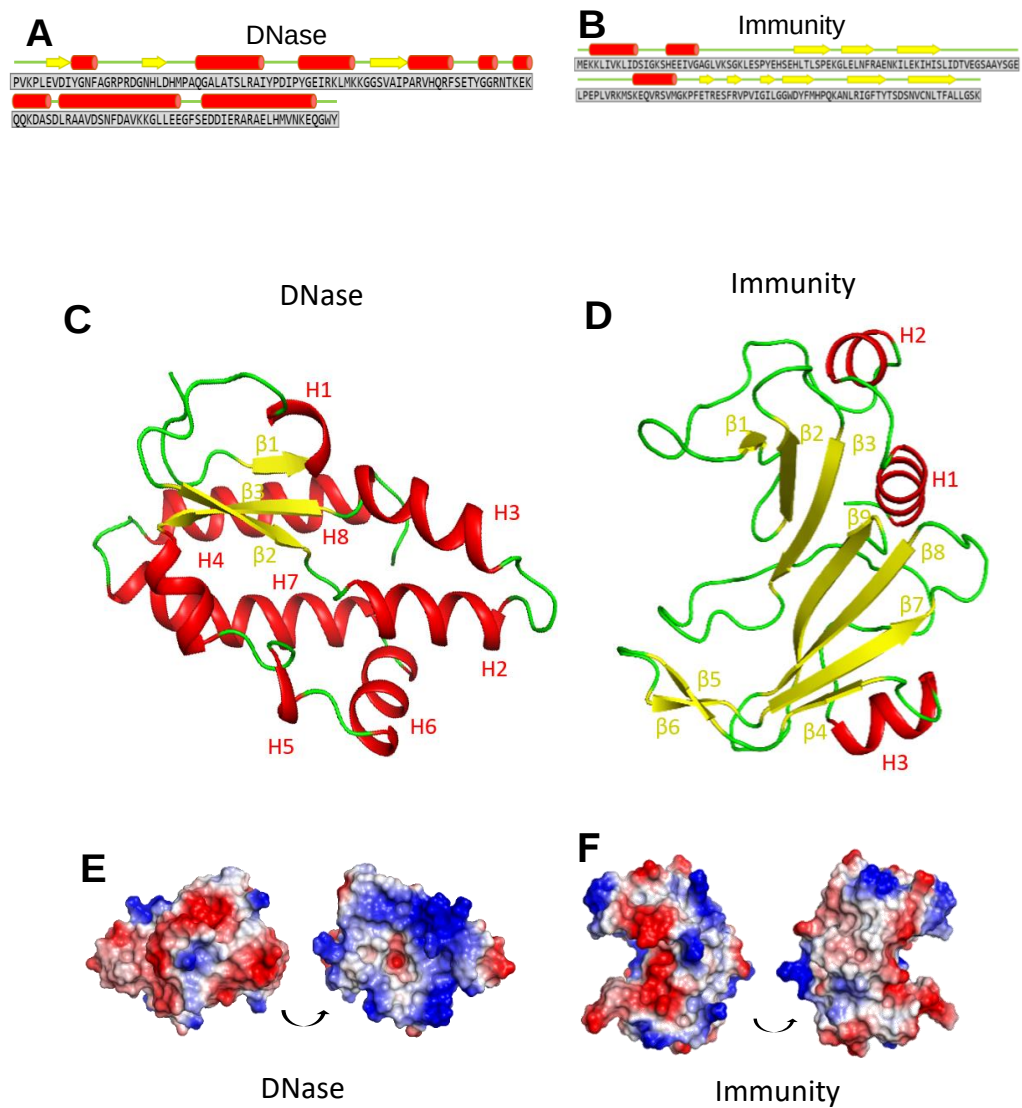


Figure 5.6 Structure of pyocin S3 DNase and its cognate immunity protein shown in cartoon representation.

Panel A and B- The sequences of the pyocin S3 DNase domain and immunity domain, respectively. α -helices and β -sheets are represented by red cylinders and yellow arrows, respectively; Panel C and D- The secondary structure elements of the pyocin S3 DNase domain and immunity domain, respectively; Panel E and F- Electrostatics surface of DNase domain and immunity domain, respectively. Red, blue and white symbolize negative, positive and neutral charge, respectively. Figures were generated using PyMOL.

The crystal structure of pyocin S3 DNase-immunity complex

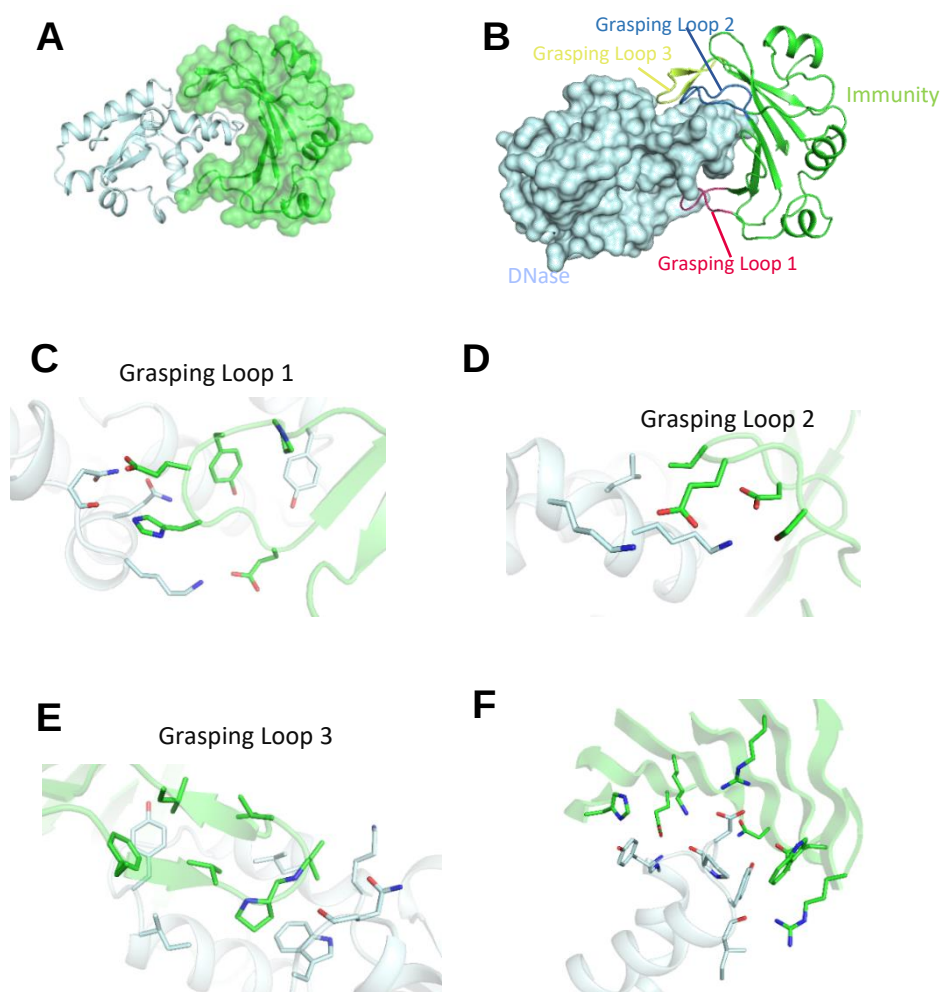


Figure 5.7 Structure and surface analysis of pyocin S3 DNase-immunity protein complex shown in cartoon representation.

The three loops in the immunity together with residues from the face of the beta sheet providing the stabilising interaction. Panel A- Surface of pyocin S3 immunity domain grasping its cognate DNase. DNase domain in light blue and Immunity protein in green; Panel B- The three grasping loops of immunity domain onto the DNase are indicated in different colour in the cartoon representation. Pyocin S3 immunity domain shown in cartoon representation and DNase domain represented as a light blue surface; Panel C, D, E and F- Interactions between pyocin S3 DNase and the three grasping loop 1, 2 and 3 and the face of β -sheet of the immunity domain. Cartoon representation of pyocin S3 DNase is in transparent green and immunity is in transparent grey, and selected side chains shown as sticks with oxygen atoms coloured in red and nitrogen atoms coloured in blue.

5.2.5. The predicted active site and DNA binding site in the crystal structure of pyocin S3 DNase

Panel A, Figure 5.8 indicates the proposed active site of the DNase, which was determined basing on the information obtained from previous stab test (Figure 4.3), plasmid nicking assay (Figure 4.8) and the crystal structure of pyocin S3 DNase-immunity complex (Figure 5.6). The three mutants D25A, H26A and H66A found to be conserved among pyocin family proteins caused a loss of DNase activity were indicated on the figure (Figure 5.8 A). Meanwhile, residues R38, R50, K51, K54, K55 and K79 would be suggested to be potentially involved in DNA binding based on the crystal structure of pyocin S3 DNase-immunity complex (Figure 5.8 A). It was mentioned previously that residue R50, K51, K54 and K55 formed a positively charged patch at the end of helix (Panel C and E, Figure 5.6). This area and the helix plug are very close. It may be that the double helix plug that goes into the immunity protein also binds into the groove of DNA. Since a DNA-binding protein will most likely have a pocket of positive charges, which helps it to bind DNA since DNA is covered in negative charges from the phosphates. Moreover, a pocket of negative charge on one protein and a bump of positive charge on the other (vice versa) might indicate a possible interaction site. However, there was no data to support this hypothesis. Therefore, alanine substitution was made to those residues proposed to be in the DNA binding site, as well as to a residue that had the potential to be located in DNase active site (His23). To do this, site directed mutagenesis was performed as descried in section 4.2.2. Mutated DNA was transformed into *E. coli* DH5 α cells and the plasmids were extracted and sent to

GENEWIZ® for DNA sequencing to confirm the desired substitutions. Thereafter, their biological activity was assessed using the *in vivo* stab test assay as described in section 4.2.2. It was found that all these mutants retained the ability to kill the susceptible *P. aeruginosa* indicator strain, suggesting that residues H23, R38, R50, K51, K54, K55 and K79 were not essential for DNA catalysis, metal coordination or high affinity DNA binding. In order to support this observation, a further *in vitro* stab test assay using the purified DNase domain of these mutants was performed (Figure 5.8 B).

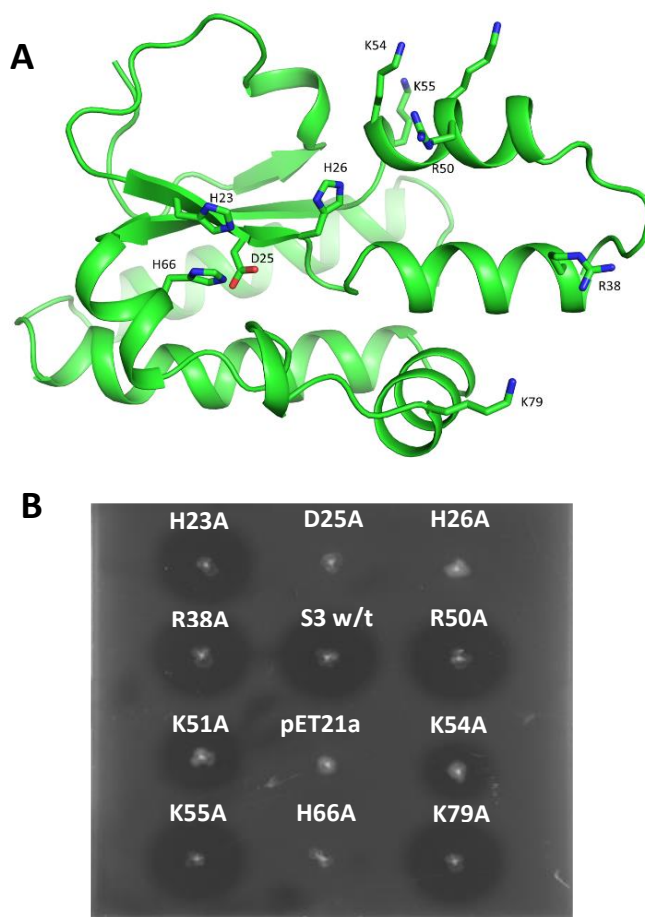


Figure 5.8 Alanine substitution of residues predicted from the crystal structure to be in the active site or DNA binding site of pyocin S3 retain activity *in vivo*.

Panel A- A cartoon representation of predicted pyocin S3 DNase active site and DNA binding site with mutated residues shown. Oxygen atoms are coloured red and nitrogen atoms blue; Panel B- Determination of the activity of full-length pyocin S3 DNase mutants in the predicted DNA binding site against sensitive clinical strain PSA971 using the *in vivo* stab test assay. Alanine substitution of residues in predicted DNA binding site (R38A, R50A, K51A, K54A, K55A and K79A) were generated using Q5 site-directed mutagenesis kit (NEB). A single colony producing either pyocin S3 DNase or a mutant was stabbed onto a fresh LB plates containing 100 µg/ml ampicillin. A colony of *E. coli* DH5α pET21a plasmid was included as a negative control. The plate was incubated at 37°C overnight before exposed to chloroform vapour for 15 min at room temperature in a fume hood. The plate was inverted and left in the fume hood without a lid for 30 min, and then overlaid with 6 ml of warmed (~ 50°C) soft top agar inoculated with 80 µl of clinical strain PSA971. Plates were incubated at 37°C for overnight before imaged using GelDoc (Bio-Rad).

5.2.6. The secondary structure of pyocin S3 DNase bears a $\beta\beta\alpha$ fold

As mentioned in the introduction, the $\beta\beta\alpha$ -Me fold observed in a number of H-N-H endonucleases was not only found to coordinate with DNA, but was also involved in DNA cleavage (Chevalier and Stoddard 2001, Doudeva, Huang et al. 2006). As pyocin S3, lacks the conserved H-N-H motif in its primary sequence (Figure 4.1) it is of interest to investigate how the pyocin S3 DNase forms an active site in the absence of the H-N-H fold. Figure 5.9 shows the alignment of the secondary structure of pyocin S3 DNase domain with the $\beta\beta\alpha$ -Me fold in the representative H-N-H DNase- pyocin AP41. Residues 63-69 of pyocin S3 DNase form a helix that matches a helix of pyocin AP41. Moreover, residues 22-26 and 57-61 of pyocin S3 DNase match the β -strand 1 and 2 of pyocin AP41, respectively. When the $\beta\beta\alpha$ fold of pyocin S3 and AP41 are overlaid, residue His26 and His76 of pyocin S3 DNase matches His739 and His763 of pyocin AP41, respectively. However, His23 of pyocin S3 DNase does not align, and His767 and His737 of pyocin AP41 do not align. In place of His737 of AP41, Asp25 of pyocin S3 is found, which looks like it could easily coordinate a metal ion found in that region.

Another alignment of pyocin S3 and other H-N-H family pyocins was made using the online tool Pro-origami (<http://munk.cis.unimelb.edu.au/pro-origami/porun.shtml>) (Stivala, Wybrow et al. 2011) by entering the PDB file of the crystal structure of pyocin S3 DNase to support the above data for the primary structure alignment (Figure 4.1, Materials and Methods 2.11.3) and the crystal structure alignment (Figure 5.9). The topology comparison of pyocin S3

DNase with the H-N-H family of DNases indicates that the $\beta\beta\alpha$ fold motif of the pyocin S3 DNase is a variation of that of the H-N-H DNase family with different active site residues; catalytic residues are shown labelled with a red dot (Figure 5.10). Two essential residues, His26 and Asp25, for catalysis reside in the first β -strand and two, H66 and S70 residues, in the helix of the motif. Helices 7+8 in the S3 topology map are postulated to become one larger helix once immunity has released. Immunity grasps the two helices in the loop between the β -strands to silence activity; helices 4 and 5 in the S3 topology map. By comparison the linear 30 residue H-N-H motif has short helices in a much smaller loop between β -strands with structurally conserved non-catalytic residues in the loop, those labelled with a blue dot, (G111 and N118) and just preceding (E100) the H-N-H fold are not present in the S3 DNase fold. Unlike for pyocin S3, the immunity protein for the H-N-H DNase family bind the loop preceding the H-N-H motif to silence its activity.

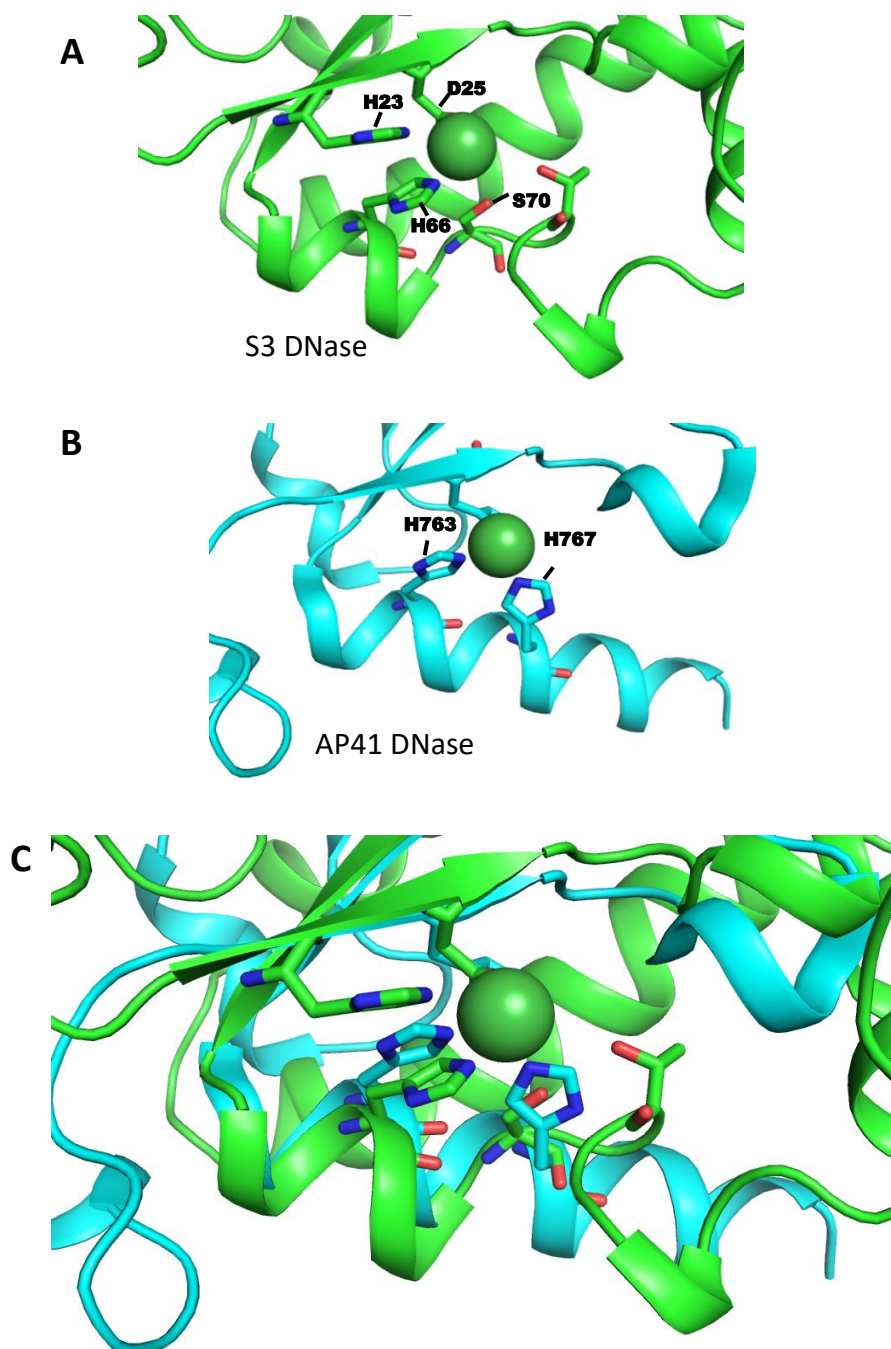


Figure 5.9 The alignment of the predicted metal binding site of pyocin S3 DNase with the $\beta\beta\alpha$ -Me fold in H-N-H motif (pyocin AP41)

Panel A- The predicted active site of pyocin S3 DNase bears a $\beta\beta\alpha$ fold with no metal bound.
 Panel B- The metal binding site of pyocin AP41. Panel C- The predicted metal binding site of pyocin S3 is coloured in green and the typical conserved $\beta\beta\alpha$ -Me fold in H-N-H motif of pyocin AP41 is coloured in blue. Metal molecule is shown as green sphere.

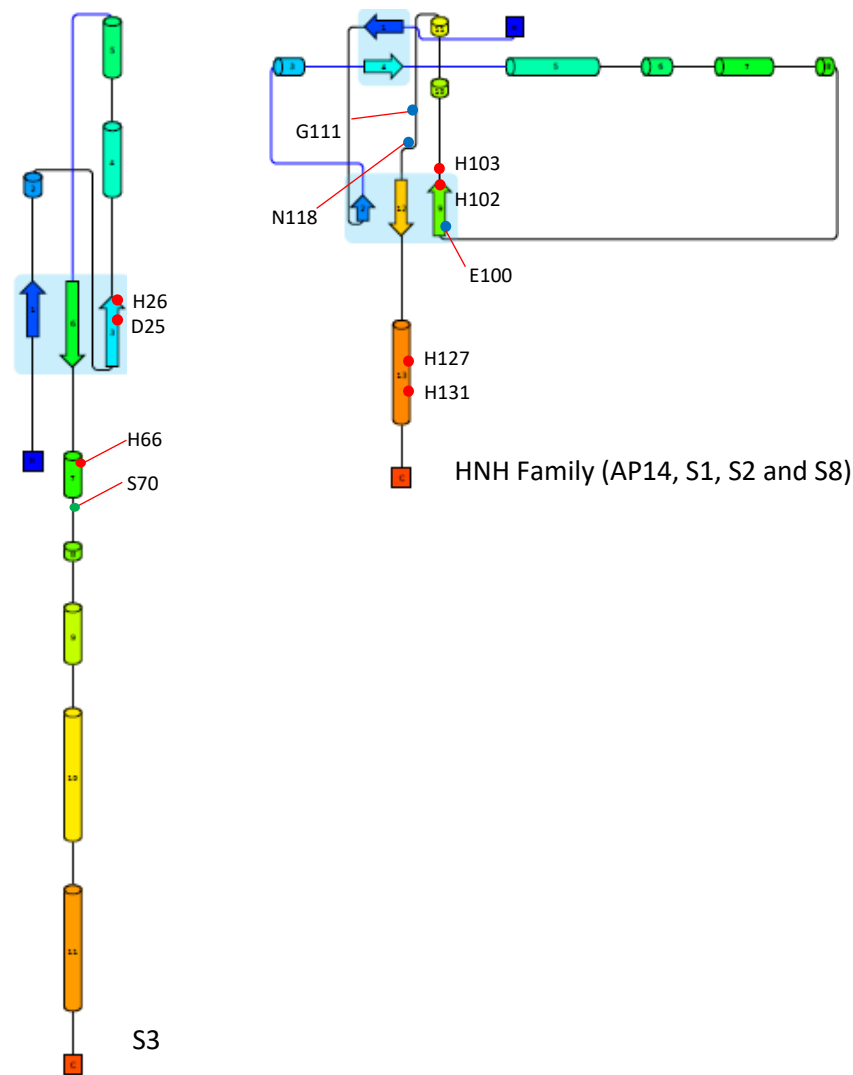


Figure 5.10 The predicted active site of pyocin S3 DNase.

Comparison of pyocin S3 DNase family and H-N-H DNase family topology. Catalytic residues and non-catalytic residues are shown labelling a red dot and a blue dot, respectively. This topology map was drawn using the pro-origami online tool (Stivala, Wybrow et al. 2011).

5.2.7. Mg^{2+} and Ca^{2+} were detected in DNases (including mutant D25A, H26A and H66A) and DNase-immunity protein complex using ICP-MS

To investigate the metal binding dependence of pyocin S3 DNase, Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was

The crystal structure of pyocin S3 DNase-immunity complex performed. ICP-MS quantifies the concentration of metal elements soluble in HNO_3 that are present in small amounts of protein samples. ICP-MS was used to analyse W/T pyocin S3 DNase (with and without EDTA treatment) plus mutants D25A, H26A and H66A. Magnesium was the most abundant metal detected in each protein analysed except the DNase-Immunity protein complex (Figure 5.11), calcium was the second most abundant metal detected in all except the DNase-Immunity protein complex, where it was the predominant metal detected. Zinc was the third most abundant metal detected metal in all of the proteins, and in each case manganese was found in higher levels than cobalt. Nickel was detected at levels in excess of those of manganese which likely represents contamination from the nickel-charged affinity resin used for the his-tagged protein purification. As expected, a large amount of selenium ions was found only in the DNase-Immunity protein complex sample, as the complex protein was labelled with selenium for crystallography trials through the use of selenomethionine (SeMet) medium for growth during overproduction.

The amount of magnesium and calcium detected in the protein solutions of mutants H26A and H66A were much lower (approximately 3-fold) than they were in the DNase W/T protein. Mutant D25A contained higher levels of magnesium and calcium than the other two mutants, but these levels were also lower than the amounts of magnesium and calcium detected in W/T DNase (by approximately a half). The amount of magnesium detected in the DNase-immunity protein complex was considerably lower (25-fold) than it was in DNase w/t protein solution,

which is consistent with the lack of metal observed in the crystal structure (Figure 5.6). Unexpectedly, the EDTA treated DNase contained high levels of magnesium and calcium, suggesting that the treatment had not successfully chelated these metals, or that residual EDTA-metal complexes remained in the solution.

A

	Mg (mol/L)	Ca (mol/L)	Mn (mol/L)	Ni (mol/L)	Zn (mol/L)	Co (mol/L)	Se (mol/L)
W/T DNase- EDTA treated	14.3467538	14.010104	0.01682681	0.0483468	0.1778331	0.0010151	0.0001406
W/T DNase	17.9892839	8.5239074	0.00614524	0.0249821	0.0620543	0.0004205	0.0001053
D25A	7.05159496	3.7245151	0.00652977	0.0098812	0.0773256	0.0001013	5.135E-05
H26A	4.70426087	1.4086774	0.00341587	0.0109164	0.0154457	0.0001128	4.594E-05
H66A	4.3056262	2.6619723	0.00746361	0.0143281	0.0408197	8.023E-05	5.899E-05
DNase-Imm complex	0.73777365	5.3056315	0.00716119	0.0202136	0.1064459	0.0001062	4.0897774

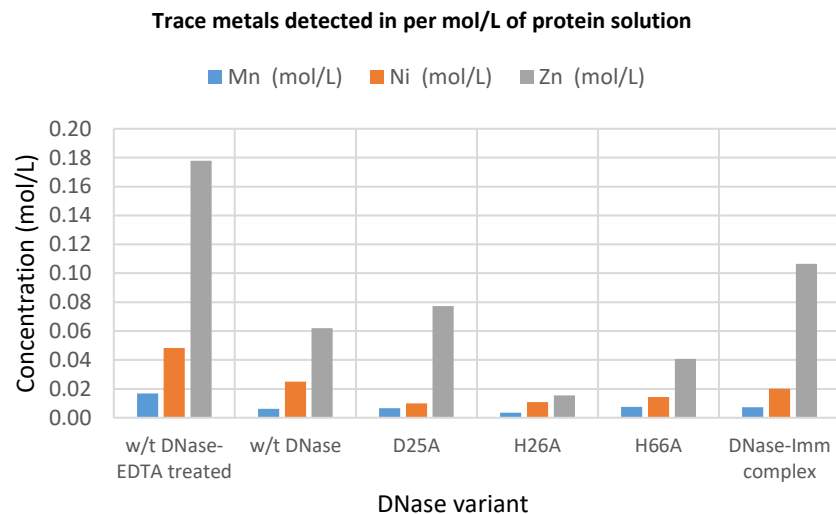
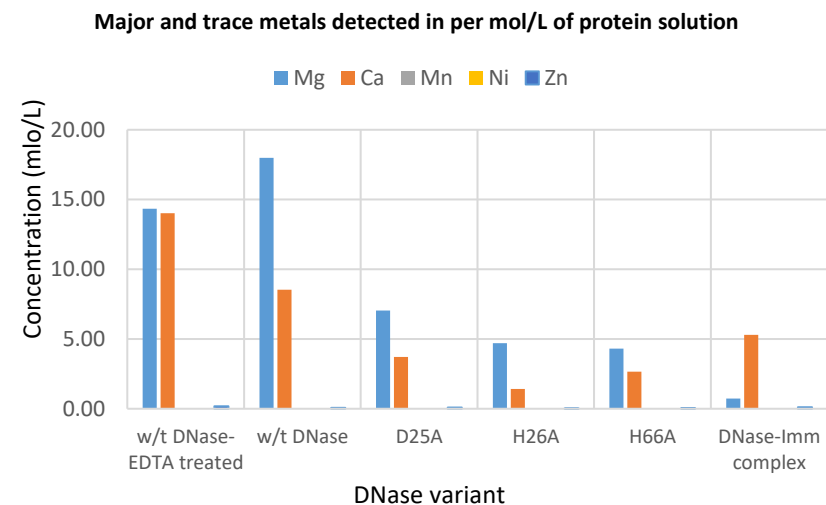
B**C**

Figure 5.11 Distribution of major and trace metal elements in S3 pyocin and S3 DNase.

Aliquots of 50 μL of pyocin S3 preparations at concentrations of 5×10^{-5} mol/L except for the w/t DNase protein, which was at the lower concentration of 3×10^{-5} mol/L (no EDTA treated) and 2.8×10^{-5} mol/L (EDTA treated), respectively, were subjected to ICP-MS (see section 2.10.4 for method). Panel A shows the concentration of each metal detected per 1 mL. Panel B displays the same data, but since only the major elements are visible on this scale, the trace metal elements are also displayed in Panel C. The proteins analysed were free DNase (W/T plus mutants D25A, H26A and H66A). The free W/T DNase was analysed with and without EDTA treatment (as described in section 2.10.2). The DNase-Immunity protein complex was also analysed.

5.2.8. Determination of Protein-DNA interactions using a Tryptophan fluorescence assay

Tryptophan, known to emit intrinsic fluorescence which can be measured by fluorescence spectroscopy, is a large hydrophobic amino acid normally partially or fully buried in the protein hydrophobic site or bound to a ligand via hydrophobic interaction (Ghisaidoobe and Chung 2014). Tryptophan is very sensitive to the surrounding environment and can be selectively excited at 295 nm wavelength, where only little absorption occurs by other residues. Tryptophan fluorescence is commonly used for monitoring the change in proteins/protein folding because of the tendency of indole rings to donate electrons while in the excited state. Local structure and dynamics interferes with this, and so it can be measured to investigate protein binding and interactions (Möller and Denicola 2002). For example, structural changes in the vicinity of tryptophan residues caused by ligand interactions or protein conformational changes would result in a change of fluorescence intensity and a waveshift in the emission spectrum (Vivian and Callis 2001, Ghisaidoobe and Chung 2014). Increase of hydrophobicity results in shift in tryptophan emission spectrum toward lower wavelengths/higher energy (blue shift) (Möller and Denicola 2002).

Tryptophan fluorescence measurement was undertaken to detect whether there was any fluorescence change due to the interaction of pyocin S3 DNase protein and DNA. Formation of a complex between the protein and the DNA will result in quenched tryptophan fluorescence. The extent of the quenching would depend on the proportion of protein

compared to the concentration of quencher, in this experiment, DNA (Carpenter, Oliver et al. 2001).

The crystal structure (Panel C, Figure 5.6 and Panel A, Figure 5.7) showed that mutations D25A and H26A are located at the end of the second β -sheet strand of the 3-strand antiparallel β -sheet of the DNase, where β -strands 2 and 3 (central strand) are separated by the two large α -helices captured by immunity in the complex. Mutation H66A is located away from the Immunity protein, and opposite β -strand 1 in the α -helix immediately after β -strand 3. See cartoon ribbon diagram, Figure 5.12, where the two large α -helices captured by immunity protein in the complex are shown in white, as are mutated residues of the DNase (shown in sticks). Immunity protein, not shown, would be located around the bottom left. The sole tryptophan, W129 is indicated by the red stick, other light absorbing tyrosine residues are shown as orange sticks and the N-terminus is highlighted in blue. W129 is therefore closest to H26, but located on the opposite site of the two large α -helices captured by immunity protein in the complex, which in the Immunity protein's absence is postulated to access DNA during DNA cleavage. In brief, the tryptophan appears in a good position to report a DNA binding complex, but is located away from the actual cleavage site. Therefore, the aim of this experiment was to compare native tryptophan fluorescence of each pyocin S3 DNase mutant relative to W/T, and measure any change in native tryptophan fluorescence when titrated with a stoichiometric or small excess of DNA at a physiological concentration. The question

The crystal structure of pyocin S3 DNase-immunity complex being posed was 'Could each mutant be shown to have similar native tryptophan fluorescence to W/T and respond to DNA in the same way?'

Since the tryptophan fluorescence is very sensitive, the Bradford assay was performed to determine the concentration of the proteins analysed. Firstly, a BSA solution served as a standard to produce a standard curve (Panel A, Figure 5.12), DNase stock solutions which had been estimated using a NanoDrop (ND-1000, ThermoFisher) to have concentrations of: W/T- 51 μM , D25A- 48.2 μM , H26A- 70.3 μM and H66A- 52.5 μM were diluted 1/10. The diluent buffer was 50 mM Na-Phosphate, 100 mM NaCl and 1 mM EDTA buffer, pH 7.5, which was used in previous analytical gel filtration experiments (see section 4.2.7). The concentration of the diluted solutions was calculated using the BSA standard curve as 4.2 μM , 7.4 μM , 6.2 μM and 8.8 μM for W/T, D25A, H26A and H66A, respectively. The background tryptophan fluorescence of the buffer was measured a number of times to determine reproducibility of the set-up (Panel B, Figure 5.12). The fluorescence intensity curves are not identical, but the differences are minor. The peak at 331 nm is the Raman scattering peak of water observed when exciting at 295 nm. Buffer only spectra are subtracted from protein and protein-DNA complex spectra. There was an insignificant background fluorescence of DNA at 1 μM (Panel C, Figure 5.12). Analysis of the DNase W/T and mutants (Panel D, Figure 5.12) showed that a maximum fluorescence emission at 336 nm (± 3 nm) which is consistent with W129 being located in a predominantly hydrophobic environment of the folded DNase. The spectra highlight that W129 in each mutant has a different quantum yield (emission efficiency).

The emission of the W/T is least efficient, D25A is slightly more efficient than W/T, H66A is twice as efficient and H26A is approximately 3-fold more efficient. This trend was consistent with data produced on separate occasions (data not shown). Overall, this assay was repeated 4 times, and variation was shown between the experiments indicating limited reproducibility. This issue has been reported previously (<https://www.researchgate.net/post/How-to-avoid-fluctuations-in-tryptophan-protein-fluorescence>). The most reliable/representative data set is presented here.

The DNases were next incubated with DNA at 1 μ M or 2 μ M and the tryptophan fluorescence was measured (Figure 5.13 and Panel A, Figure 5.11). The addition of a single or double dose of 2 μ M was compared. In the assay, proteins (~37.5 ml) were diluted into buffer (120 ml), gently shaken for 5 min, excited at 295 nm and emission measured. DNA was then added (2.9 μ l), the cuvette gently mixed again by shaking, and a scan performed prior to further DNA addition and repeat mixing and measurements. Fluorescence emission scans from 320-400 nm, that were corrected for buffer Raman scattering are shown (Figure 5.13). The spectra show that the addition of DNA shifts the fluorescence emission maximum.

As shown previously (Figure 5.12), pyocin DNase mutant H66A has the largest red shift in its peak of fluorescence emission of ~4 nm (from 338 up to 342nm), and mutant H26A also has a red shift, but only of 1 nm to 337 nm (Figure 5.12). The spectra suggest that mutant D25A is most like W/T with respect to fluorescence of residue W129, since the

The crystal structure of pyocin S3 DNase-immunity complex fluorescence intensity rises to 38% with 1 μM DNA (197% for W/T) and to 67% with 2 μM DNA addition (174% for W/T). There is also a 1 nm blue shift with 2 μM DNA addition to 337 nm for D25A (compared to 336 nm for W/T) (Figure 5.13 and Panel A, Figure 5.11). Both mutants H26A and H66A showed a fluorescence intensity drop with 1 μM and 2 μM DNA addition. Mutant H66A experienced more obvious fluorescence quenching, since there was a further drop with extra addition of DNA. The drop of fluorescence remained the same with 1 μM and 2 μM DNA addition to mutant H26A.

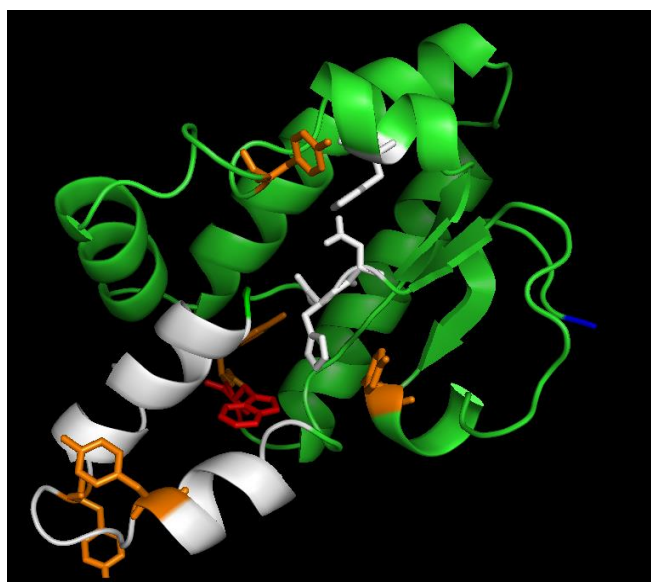


Figure 5.12 Relative localization of tryptophan and tyrosin residues in pyocin S3 DNase.

The two large α -helices captured by immunity in the complex are shown in white, as are mutated residues of the DNase (shown in white stick). Immunity, not shown, would be located around the bottom left. The sole tryptophan, W129 is shown in red sticks, other light absorbing tyrosine residues are shown in orange sticks and the N-terminus is highlighted in blue.

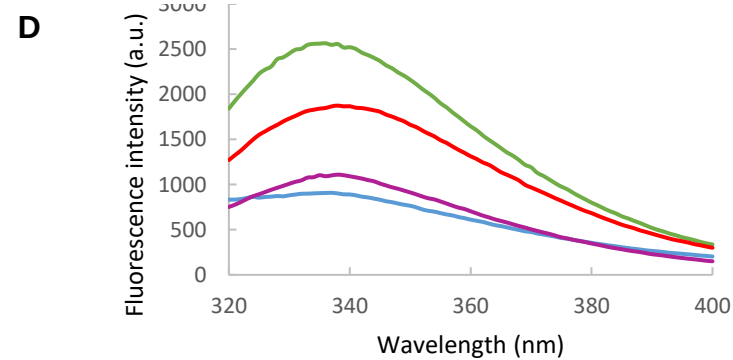
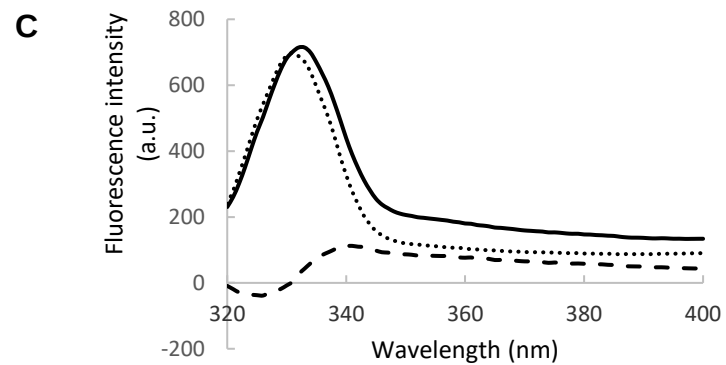
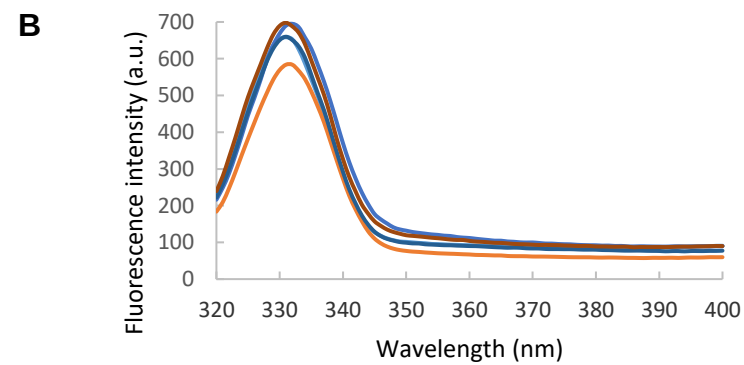
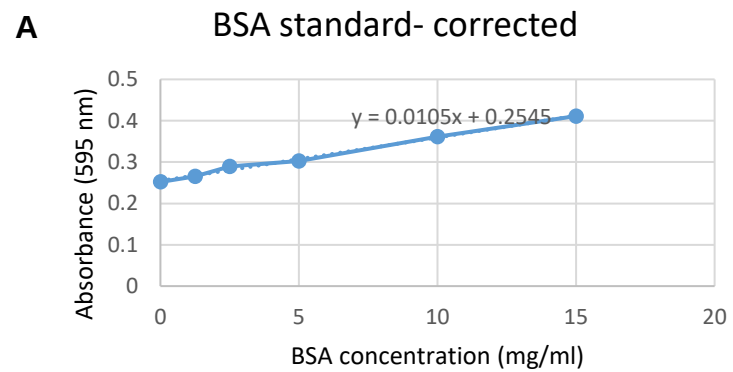
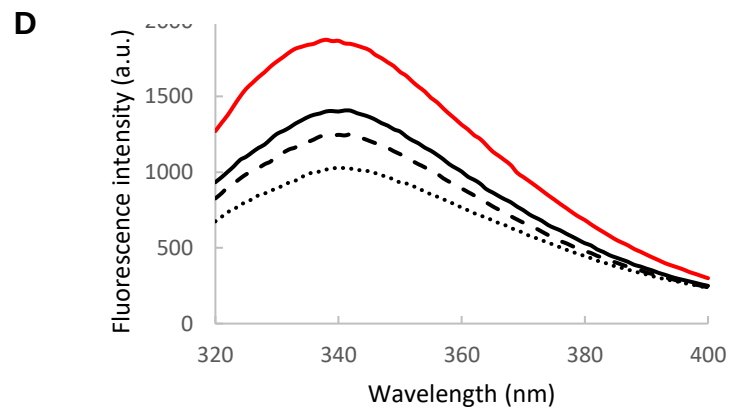
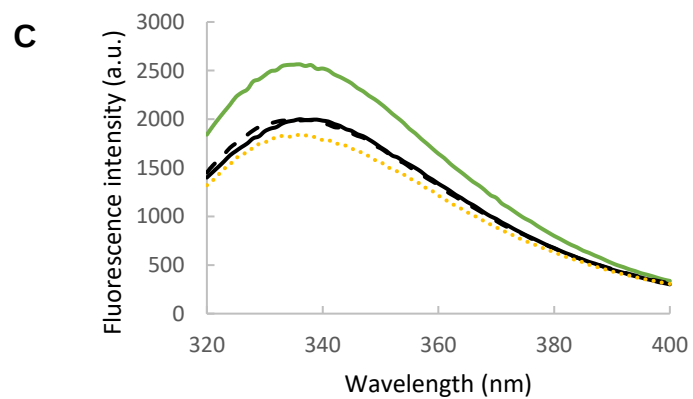
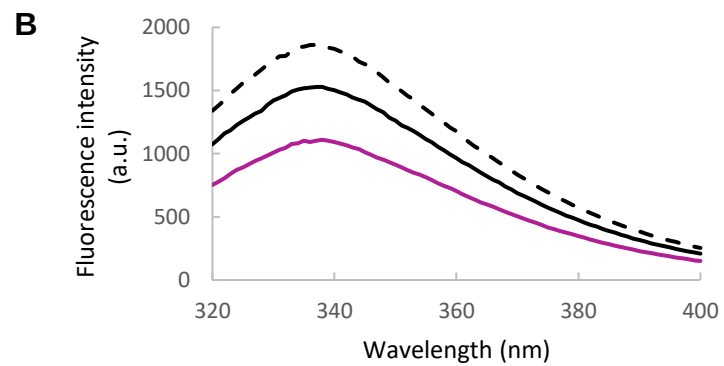
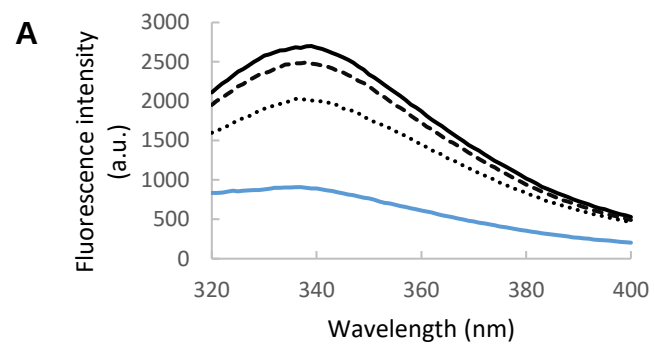


Figure 5.13 Determination of pyocin S3 DNases and DNA interactions by tryptophan fluorescence.

Panel A- BSA standard curve Bradford Assay measurements were plotted and the relationship between absorbance and BSA concentration described as a line of best fit equation. Panel B- Reproducibility of background buffer fluorescence. Excitation at 295 nm yields a Raman scattering peak for the buffer centred at 331 nm. The Raman Scattering peak for 5 experiments using two different cuvettes is shown; Panel C- Comparison of buffer and 1 μ M DNA in buffer. Excitation at 295 nm yields a Raman scattering peak for the buffer (dotted line). DNA in buffer (line) with Raman scattering subtracted (dashed line) yielded a negligible increase in background fluorescence/scatter. Panel D- Comparison of tryptophan fluorescence emission of 1 μ M pyocin DNase w/t (blue) and mutants D25A (magenta), H26A (green) and H66A (red).



E

			FI reading			Change of Trend (%)		
			1 uM DNA	2 uM DNA		1 uM DNA	2 uM DNA	
Pyocin	conc [uM]	FI [AU]	1st reading	2nd reading		1st reading	2nd reading	
W/T	1	908.8	2700.8	2491.8	2028.9	197.1831 up	174.1857 up	123.2504 up
D25A	1	1110	1529	1861.6	n/a	37.74775 up	67.71171 up	n/a n/a
H26A	1	2565	2000	1990.4	1841	-22.0273 drop	-22.4016 drop	-28.2261 drop
H66A	1	1874.5	1407.7	1250	1028.6	-24.9026 drop	-33.3156 drop	-45.1267 drop

Figure 5.14 The tryptophan fluorescence of pyocin DNase alters in the presence of DNA.

In the assay, proteins (~37.5 ml) were diluted into buffer (120 ml), gently shaken for 5 min, excited at 295 nm and emission measured. DNA was then added (2.9 µl), the cuvette gently mixed again by shaking, and a fluorescence scan performed (1uM DNA). A further addition of DNA brought the concentration up to 2uM and two separate fluorescence scans were recorded (1st reading and 2nd reading). Fluorescence emission scans from 320-400 nm, corrected for buffer Raman scattering are shown in the figure. Tryptophan fluorescence emission is shown for 1 µM pyocin DNase (blue line, Panel A) and mutants D25A (magenta line, Panel B), H26A (green line, Panel C) and H66A (red line, Panel D) alone or in the presence of 1 mM (black line) or 2 mM DNA addition (dashed black line) or with repeated addition of DNA (dotted line). Changes in pyocin DNase tryptophan fluorescence emission with DNA are shown in table (Panel E). The peak of fluorescence Intensity (FI) is shown in arbitrary units (AU) prior to and following DNA addition. The % change is also indicated alongside whether the trend was increase (up) or decrease (drop).

5.2.9. Circular dichroism showed all three alanine mutants (D25A, H26A and H66A) of pyocin S3 DNase have nearly identical secondary structure to the W/T DNase

To confirm that the loss of activity in mutant pyocin S3 DNases was not due to significant alterations in protein folding, the secondary structure of the purified pyocin S3 DNases was determined using circular dichroism (CD) (Kelly, Jess et al. 2005). CD is a very useful tool for evaluating the secondary structure and folding properties of proteins in solution. It can determine whether a purified protein is folded, as well as if a mutation has changed the conformation or stability of a protein (Greenfield 2006). In brief, CD measures the difference of the absorption of left-handed and right-handed circularly polarized light, when a beam of light is passed through a protein solution. Different secondary structural elements have their own specific CD spectra. For instance, proteins with α -helical structure only have negative bands at 222 nm and 208 nm and a positive band at 193 nm, while proteins with β -sheet structure only display negative bands at 218 nm and positive bands at 195 nm. Disordered proteins have very low ellipticity above 210 nm and negative bands near 195 nm (Figure 5.14) (Greenfield 2006).

An online CD prediction for the DNase using the atomic coordinates from the crystal structure was firstly performed to compare with the experimental data (Panel B, Figure 5.15, Materials and Methods). Since CD spectra comparisons are highly dependent upon very accurate protein quantification, the raw spectra was standardised with the Bradford assay concentration quantifications for each preparation. The CD spectra of all the DNases exhibit a positive peak at 191 nm and two

The crystal structure of pyocin S3 DNase-immunity complex negative peaks at 208 nm and 222 nm, all of which are sensitive to conformational alterations in protein structure (Kelly, Jess et al. 2005). It was considered desirable to normalise the CD spectra to the online prediction of the S3 pyocin DNase to assist direct comparison of all spectra. Normalisation of each spectra data comprised baseline correction at 250 nm followed by normalisation at 208 nm, as this represents the wavelength at which CD originates exclusively from an α -helical signal. Experimental data were approximately 1.4-fold lower than the predicted DNase circular dichroism at 208 nm, an experimental artefact postulated to originate from using BSA as the Bradford assay standard. After accounting for this difference in signal magnitude between the structure-derived (predicted) and experimental spectra, data sets are all very similar, however, the predicted data miss the slight signal increase around 215 nm (Panel B, Figure 5.15). The spectra data shown in Panel A and B in Figure 5.15 reveal that all mutants have near identical secondary structure, and that no, or negligible change in secondary structure has occurred as a result of the introduction of each of the mutations. Hence, the mutants used in the previous plasmid nicking assay (Figure 4.8) and tryptophan fluorescence assay (Figure 5.13) were not unfolded.

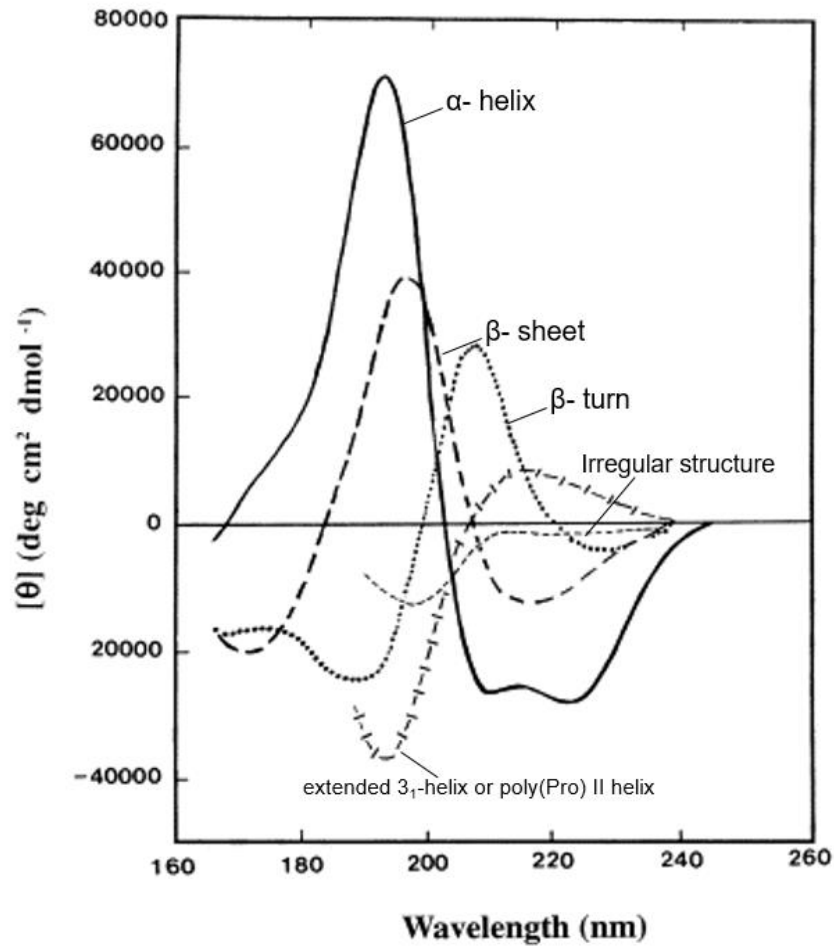


Figure 5.15 Circular dichroism (CD) spectra of proteins with representative secondary structures.

CD spectra of protein with pure α - helix, pure anti- parallel β - sheet, type I β - turn, extended 3_1 - helix or poly (Pro) II helix and irregular structure are shown in solid line, long dashed line, dotted line, cross dashed line and short dashed line, respectively. Figure adapted from Fig 3, Kelly, Jess et al. (Kelly, Jess et al. 2005).

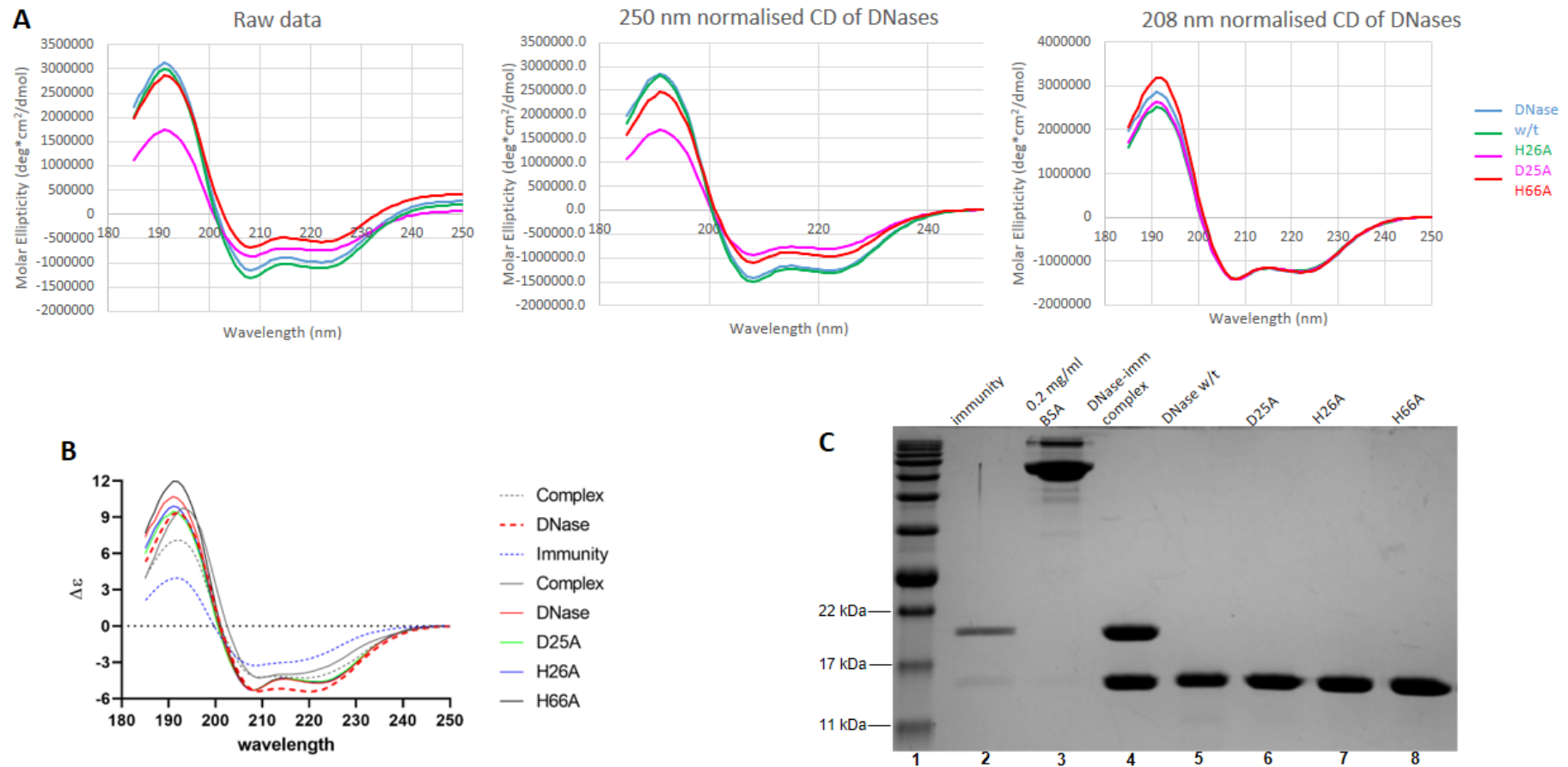


Figure 5.16 Circular Dichroism (CD) spectra reveals similar secondary structure of pyocin S3 DNase w/t and mutants.

Panel A- Experimental CD spectra for pyocin DNases (DNase w/t in blue, D25A in magenta, H26A in green and H66A in red) are plotted together as molar ellipticity (mdeg) versus wavelength (nm). Protein concentration was used to correct spectra (Bradford assay data). A baseline corrected spectra at 250 nm (top middle) is shown adjacent to 208 nm (top right) "signal adjusted or normalised" spectra. Panel B- Purified proteins were diluted to 0.2- 1.0 mg/ ml in 10 mM Tris, 2 mM MgSO₄ buffer, pH 7.4, and were measured for CD at room temperature. Molar circular dichroism ($\Delta\epsilon$) versus wavelength (nm) far-UV experimental measurements for the pyocin S3 DNase-immunity complex (grey), DNase (red) and DNase mutants D25A (green), H26A (blue) and H66 (black) are shown alongside predicted CD spectra for the pyocin S3 DNase-immunity complex (grey dashed), DNase (red dashed) and immunity protein (blue dashed). The later were obtained using the atomic coordinates of proteins from the pyocin S3 DNase-immunity complex acquired in this work and the online prediction tool PDB2CD (Mavridis and Janes 2016). Repeat attempts to measure CD for the immunity protein failed (data not shown). Panel C- The purified protein samples used on CD spectra were confirmed by SDS-PAGE on a 15% gel. Lane 1. Colour protein standard ladder; Lane 2. Immunity; Lane 3. 0.2 mg/ml BSA; Lane 4. DNase-immunity complex; Lane 5. DNase w/t; Lane 6. D25A; Lane 7. H26A; Lane 8. H66A). Proteins were visualized using Coomassie blue staining, sizes of selected marker proteins are indicated in kDa on the left.

5.3. Discussion

5.3.1. *Pyocin S3 DNase-Immunity complex adopts a novel octopus structure and the DNase domain reveals a $\beta\beta\alpha$ fold*

The SeMet labelled pyocin S3 DNase-immunity complex folded in the same way as the native one, and the structures were almost identical Table 5.4. The advantage of the SeMet labelling was that there was no reliance on the existence of a similar structure in the PDB with which to solve the structure. The pyocin S3 DNase and immunity protein form a complex with an interface area of 1219 Å². Observing the B-factors, flexible regions of both proteins can be seen, whereby the most flexible regions are those far from the interface of the two proteins. In addition, the DNA-binding region was also found to be rigid in structure. Submitting both proteins to the Dali server showed that the pyocin S3 DNase is unique, and had no similar homologous structures to the other proteins or the H-N-H family of DNase, such as pyocin S1, S2 (Sano and Kageyama 1993, Sano, Matsui et al. 1993) and AP41 (Joshi, Grinter et al. 2015) or colicins ColE2 (Schaller and Nomura 1976), ColE7 (Chak, Kuo et al. 1991), ColE8 (Toba, Masaki et al. 1988) and ColE9 (Wallis, Moore et al. 1992), and is therefore a novel fold. The immunity protein, however, has homologous structures in the Protein Data Bank (PDB). The β -sheet is relatively conserved with regards to structure and its topology, as are the helices that pack against the opposite side to its DNase binding pocket. Interestingly, although some of these structures only feature a homologous protein of similar fold, some contain N- and C-terminal extensions that reside within the binding pocket that the DNase was found. The structures of these subdomains are diverse, and

none utilise the double helix plug that was observed in the DNase. The existence of these structures showed that the fold observed for the immunity protein is commonly used to grasp other proteins, and as evidenced by the low sequence homology, the proteins can be diverse to allow specific binding to their targets. This structure of the DNase-immunity complex represents the only case in the PDB where a separate protein is grasped by this fold.

As a $\beta\beta\alpha$ -Me fold is observed in all the H-N-H motif-containing structures (Huang and Yuan 2007), an alignment of the $\beta\beta\alpha$ fold of pyocin S3 DNase and the typical H-N-H endonuclease pyocin AP41 was performed (Fig 5.9). It is perceivable that in pyocin S3 DNase domain, the metal ion is 'moved up' the helix slightly and coordinated by the two histidines (His26 and His66) and aspartic acid (Asp25), that may bind with them. Due to the absence of metal ion in the crystal structure of the pyocin S3 DNase-immunity complex, it is speculated that the binding of the immunity protein to the DNase domain resulted in dissociation of metal ion, hence, the DNase activity was inhibited. However, how the pyocin S3 DNase maintains the active site secondary structure without the typical H-N-H motif is still not clear.

5.3.2 ICP data revealed that pyocin S3 DNase preferentially utilised a magnesium ion for its lethal activity

The previous metal dependence data showed that under the same conditions, i.e., a low concentration of Mg^{2+} , the activity of apo-pyocin S3 was readily re-activated causing nicking of the substrate plasmid, whereas other metals ions failed to do so (Lane 1-6, Figure 3.10). This

The crystal structure of pyocin S3 DNase-immunity complex observation is further supported by the ICP data. Panel A, Fig 5.11 suggested that more magnesium was found in the protein samples than any other metals, despite a much lower concentration of it being present in pyocin S3 DNase-immunity complex protein. Together, this implies that pyocin S3 DNase has optimal activity in the presence of Mg^{2+} . This preference for Mg^{2+} has also been identified for some other H-N-H enzymes, such as colicin E7, E9 DNase, I-Cmoel and LtrA (Drouin, Lucas et al. 2000, Ku, Liu et al. 2002, San Filippo and Lambowitz 2002, Maté and Kleanthous 2004). The important role of magnesium ions in catalysis of DNA hydrolysis has been reported. This metal ion could have three functions acting as either bases or acids: 1). Activating a water molecule to attack a scissile phosphate, 2). stabilizing a pentavalent phosphorous transition state, or 3) forming a bond with a water molecule to donate a proton to the hydroxyl leaving group (Sui, Tsai et al. 2002, Maté and Kleanthous 2004). Much smaller amounts of magnesium ions were detected in pyocin S3 DNase-immunity complex protein, which is consistent with the proposed inhibition of pyocin S3 activity by immunity stripping the metal ion from its active site as supported by the crystal structure of pyocin S3 DNase-immunity protein complex (no metal ion observed).

The concentration of magnesium and calcium detected in the protein solution of mutants H26A and H66A were much lower than they were in the DNase w/t protein, which may suggest that both residues were involved in metal binding or coordination (Panel A, Figure 5.11). Interestingly, the equivalent residue (H66) in $\beta\beta\alpha$ -Me nucleases has also

been reported as a metal-ion-binding residue, which was more likely coordinating to the Mg^{2+} ion (Wu, Lin et al. 2020). The concentration of magnesium and calcium was higher in mutant D25A than in the other two mutants, but was approximately 1/2 the amount in W/T DNase. It is possible that residue D25 could play a different role from the other two residues (H26 and H66) and it may still be involved in the metal coordination.

During the data analysis process, attempts were made to subtract the reading of metal ions in buffer blank (without the presence of protein) from protein samples to get a more accurate value for the concentration of metal ions present in the protein samples. However, it was found that the readings for some of the metals in the diluent (buffer blank) were actually higher than they were in protein samples. This is probably due to the very small volume (50 μ l) of protein sample diluted into the much bigger volume of diluent (5 ml of 2% nitric acid). Therefore, the buffer readings were not subtracted from sample readings for the data analysis.

5.3.3. Mutant H66A exhibited quenched fluorescence in tryptophan fluorescence assay with addition of DNA

In the tryptophan fluorescence experiment, the protocol was optimised by quantifying the proteins using the Bradford Assay and diluting all the proteins to the same concentration before tryptophan fluorescence was measured. An accurate 1 μ M protein and 1 μ M DNA were added together to the cuvette followed by an additional introduction of DNA. Very similar quantum yield (emission efficiency), and lambda max (environment for tryptophan) was observed for W/T pyocin DNase and mutant D25A

The crystal structure of pyocin S3 DNase-immunity complex (Figure 5.12), showing W129 present in the W/T pyocin DNase and mutant D25A was very similar. But the W129 fluorescence in mutants H66A and H26A was approximately 2-fold and 3-fold more efficient than the W/T, respectively. The complete data set suggested that mutant D25A is most like W/T with respect to fluorescence of residue W129 during DNA binding. It should be noted that the pyocin S3 DNase W/T W129 exhibited higher sensitivity to DNA addition showing the most fluorescence intensity change (increase) and slightly more shift (red) (Figure 5.13). The reason why the fluorescence intensity of both W/T and mutant D25A increased rather than being quenching with DNA addition is hard to explain. The D25A mutant required more DNA to yield the increase in W129 fluorescence observed for W/T DNA-binding, and excess DNA added to the wild type pyocin DNase resulted in a further increase in W129 (Panel B, Figure 5.13).

The H26A and H66A mutants experienced a fluorescence drop with the addition of DNA. H66A exhibited further fluorescence drop with further addition of DNA. Again, based on this change in W129 fluorescence H26A and H66A certainly respond to the presence of DNA, but what this means in light of a failure to detect any protein-DNase complex using EMSA and fluorescence polarisation is difficult to understand. Unlike the mutant H66A, the fluorescence intensity of H26A stayed the same with only 1 nm red shift with extra addition of DNA. This probably reflects oligomerization observed for H26A in the previous analytical gel filtration assay (Figure 4.20), leading to an increased population of pyocin DNase H26A with essentially W/T conformation, but decreased quenching of

W129. This observation would also be consistent with CD spectra of W/T pyocin DNase matching that of the mutants (Figure 5.14). The considerable fluorescence quenching of H66A indicates potential protein-DNA binding and is consistent with the observation in the analytical gel filtration assays (Figure 4.21) that the UV absorbance decreased with the addition of DNA, leading to a further drop of fluorescence intensity with higher concentration of the quencher (DNA). Those data may also match with the result for plasmid nicking assay for pyocin S3 DNase and mutants that a thick shifted band observed above the supercoiled DNA band (Lane 7, Figure 4.8) indicating the potential protein-DNA binding.

5.3.4. The mutations had a negligible effect on the secondary structure of the DNase

The comparison of the circular dichroism spectra between pyocin S3 DNase wild type and three of the mutants, which are confirmed to be in the predicted DNase activity site, implies that the alanine mutations of D25, H26 and H66 retain a fold similar to that of the wild type (Figure 5.14). It also gives evidence of the correct folding of proteins used in the previous DNase activity *in vitro* assay (Figure 4.8) and tryptophan fluorescence assay (Figure 5.13), as the same proteins were used on both of those tests. Further investigation is required to identify a better assay/ optimization for techniques to investigate the protein-DNA interactions occurring. Currently there remains a lack of strong evidence for pyocin S3 DNase binding to DNA from the four assays conducted (EMSA, fluorescence polarization, analytical gel filtration and tryptophan fluorescence).

Chapter 6. General Discussion

Clinical pathogens evolve by developing antibiotic resistance to enable their progeny to survive (Duraismy, Balakrishnan et al. 2020). New types of therapeutic molecules and strategies are urgently required to deal with the increasing number of infections caused by those antibiotic resistant bacteria, which have become a global threat to public health (Soltani, Hammami et al. 2020). Bacteriocins as a form of antimicrobial peptides (AMPs) are one of the most promising natural substances to use instead of traditional antibiotics for both the food and pharmaceutical industries and have been spotlighted due to their potential activity against organisms producing biofilms (Al-Mahrous and Upton 2011, Mansour, de la Fuente-Núñez et al. 2015, McCaughey, Josts et al. 2016, McCaughey, Josts et al. 2016, Chandrakasan, Rodríguez-Hernández et al. 2019). To be able to combat biofilms is important since these bacterial communities are associated with the emergence of antibiotic resistance (Balakrishnan, Ibrahim et al. 2020) and are implicated as a cause of 80% of severe and chronic microbial diseases (Duraismy, Balakrishnan et al. 2020). Notably, the combination of bacteriocin with other antimicrobials could enhance the killing effect of each individual component and reduce the development of antibiotic resistance in bacteria (Duraismy, Balakrishnan et al. 2020). Therefore, the identification and characterization of bacteriocins is of great importance to find suitable substitutes for antibiotics (Soltani, Hammami et al. 2020).

The aim of this study was to characterize and obtain structural information about one of the DNase bacteriocins, pyocin S3. Pyocin S3 was first identified in 1995 by Duport and was found to be unique due to a lack of the conserved H-N-H motif in its predicted DNase domain (Duport, Baysse et al. 1995). The other known non- H-N-H bacteriocin so far is carocin D produced by *P. carotovorum* subsp. *Carotovorum* identified and characterized by Roh et al. in 2010. Carocin D may have arisen from a chimera of colicin E and pyocin (Fig 6.1) (Roh, Park et al. 2010).

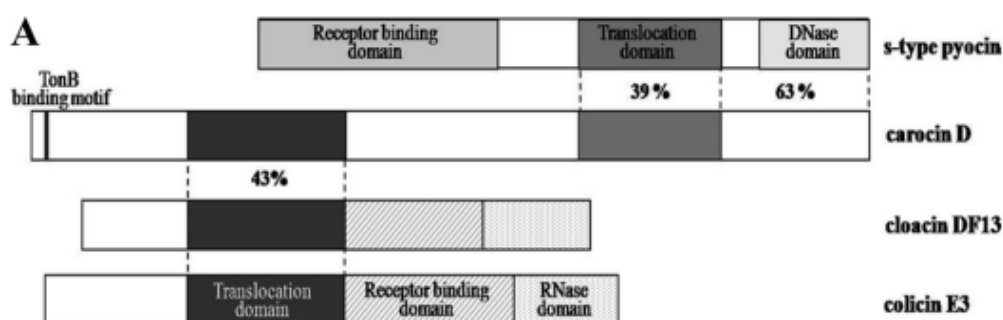


Figure 6.1 Alignment of the regions of S-type pyocin, carocin D, cloacin and colicin E3.

The positions of organization of functional domains with the percentage of identical residues are shown (Roh, Park et al. 2010).

6.1 The metal co-factor for pyocin S3 DNase activity

The DNase assays described in Chapter 3 confirmed the findings of Duport et al. 1995, that demonstrated the loss of pyocin S3 immunity protein enabled the whole pyocin to show strong DNase activity (Duport,

Baysse et al. 1995). The follow-on assays (also presented in Chapter 3) further investigated the mechanism of the activity of pyocin S3 DNase. This was achieved by separating the DNase domain from the full-length active pyocin through removal of the translocation domain and reception binding domain prior to examining its DNase activity *in vitro* using the plasmid nicking assay and plasmid pGEMT-Easy as a substrate. It was confirmed that the pyocin S3 DNase had endonuclease activity which was abolished by its cognate immunity protein, presumably to protect the producing host cell from its own toxin as occurs for other DNase bacteriocins (Kolade, Carr et al. 2002). There has been limited literature published describing features of pyocin S3 since the report that pyocin S3 targets the ferripyoverdine type II receptor of *P. aeruginosa* as the initial interaction partner during uptake and import into sensitive bacterial cells (Baysse, Meyer et al. 1999). At the beginning of this study, it remained uncertain whether pyocin S3 was indeed a non-H-N-H DNase that required metal ion binding for activity. This was the case despite the metal binding specificity of the H-N-H/ $\beta\beta\alpha$ -Me motif in colicins having already been widely studied. Different catalytic strategies of the H-N-H motif exist that are dependent on the metal that is bound. Specifically, the mechanism of DNA hydrolysis of colicin DNase is different with the presence of transition metals or Mg^{2+} (Pommer, Cal et al. 2001). To understand more about how pyocin S3 behaves, three different assays were employed in this thesis to detect the metal in the pyocin S3 DNase. The DNase activity *in vitro* was examined by combining a plasmid nicking assay with the addition of different metal ions to apo-DNase. In addition,

the analytical techniques of ITC and ICP-MS were utilized. The first glimpse of how Mg^{2+} ions and other metal ions affect the ability of pyocin S3 DNase to cleave dsDNA *in vitro* was provided by plasmid nicking assays. As has been demonstrated for H-N-H/ $\beta\beta\alpha$ -Me endonucleases, it was confirmed that the non-H-N-H pyocin S3 uses Mg^{2+} as a co-factor to cleave DNA. Other metals (Mn^{2+} , Ni^{2+} and Co^{2+}) can substitute for Mg^{2+} , but cleavage is less efficient. Notably, there was an optimal concentration of metal to support the pyocin S3 DNase activity (Figure 3.10). This is not the first observation of higher metal concentrations being deleterious to DNase activity as it was also observed for pyocin AP41 and colicin E9 (Pommer, Wallis et al. 1998, Joshi, Grinter et al. 2015). Further investigation is required to fully elucidate the contribution of metal to the catalytic mechanism of DNA cleavage by pyocin S3, most crucially perhaps being the precise coordination of the metal within the active site of the enzyme.

6.2 Proposed active site of pyocin S3 DNase

Chapter 4, sought to identify the catalytic residues in pyocin S3 DNase using site directed mutagenesis. This approach allows examination of the involvement of key residues in pyocin S3-dependent bacterial killing, metal ion binding and catalysis of DNA cleavage. To investigate the contribution of a disruption in DNA binding to the loss of DNase activity of those pyocin S3 DNase mutants, three different assays were used: gel electrophoresis mobility shift assay, fluorescence polarization and analytical gel filtration. There was some evidence indicating that mutant D25A had the potential to participate in DNA binding. This is in line with

the hypothesis that D25 is involved in metal ion coordination because it is adjacent to H26, which is postulated to constitute part of the pyocin S3 DNase motif. The hypothesis is based on the role of the equivalent amino acid in H-N-H nucleases (Wu, Lin et al. 2020).

6.3 The $\beta\beta\alpha$ -Me motif in the active site of pyocin S3 DNase

In Chapter 5, the crystal structure of pyocin S3 DNase-immunity protein complex is presented. It is important to note that the translocation and receptor binding domains of pyocin S3 were not present. The structure adopts an $\beta\beta\alpha$ -Me motif like many other H-N-H/ $\beta\beta\alpha$ -Me DNases suggesting a closer relationship than was expected (Figure 6.2).

The immunity protein binding site in the structure is in line with speculation in the literature that exosite binding of colicins by their cognate immunity protein is a selective advantage to enzymatic colicin-producing bacteria, since it allows extensive sequence variability to evolve at the DNase-immunity protein interfaces (Kleanthous and Walker 2001).

The predicted metal binding site in pyocin S3 DNase envisioned a tight coordination of the three residues (D25, H23 and H66) and a metal ion (Figure 5.9). Contrary to expectations, however, a metal ion was absent in the crystal structure of pyocin S3 DNase-immunity protein complex. This could be caused by the strong interaction of the immunity protein stripping the metal from the DNase protein. More data need to be collected to illustrate a structural basis for understanding how the pyocin S3 DNase utilises the metal cofactor for its endonuclease activity, though some enzymological data and metal dependence information of pyocin

S3 DNase activity *in vitro* is provided in Chapter 3. One of the solutions is to solve the crystal structure of the pyocin S3 DNase without its cognate immunity, that is expected to have its physiological metal bond within the protein structure.

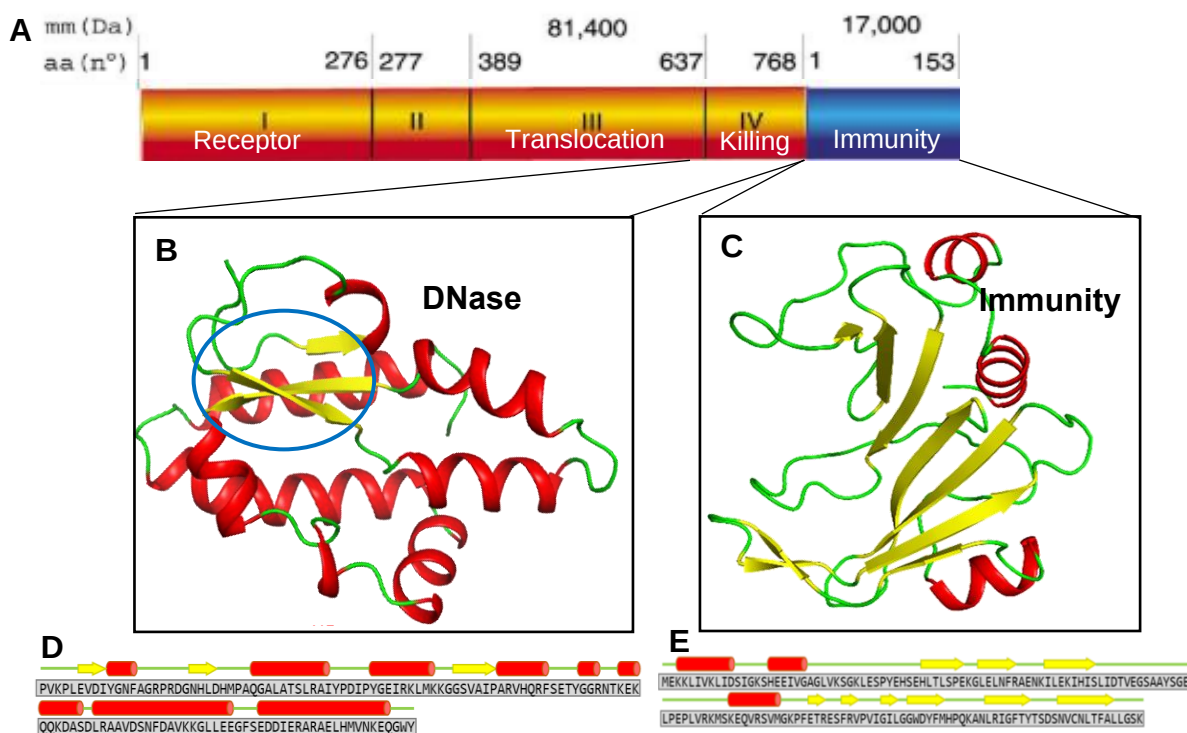


Figure 6.2 The organization of pyocin S3 and the crystal structure pyocin S3 DNase-immunity complex obtained from X-ray crystallography.

Panel A- The pyocin S3 is a complex of two proteins: the DNase protein and the immunity protein. The DNase protein comprises three functional domains arranged from the N-terminus to the C-terminus as follows: 1). a receptor binding domain, 2). a translocation domain, and 3). a killing domain. Molecular mass (mm) (Dalton) and amino acid (aa) which constitutes the different domains are indicated. Panel B & C- The secondary structure elements of the pyocin S3 DNase domain and immunity domain, respectively. The predicted DNase activity site is circled in blue. The secondary structure of proteins is indicated in yellow (β sheet) and red (α -helix). Panel D and E- The sequence of the pyocin S3 DNase domain and immunity domain, respectively. α -helices and β -sheets are represented by red cylinders and yellow arrows, respectively.

6.4 Future Directions

Chapter 3 presented convincing evidence that Mg^{2+} is the primary metal co-factor for used by pyocin S3 DNase to catalyse the cleavage of DNA *in vitro*. However, further data is required to unequivocally prove which metal ion is most physiologically relevant. Whilst progress was made to apply the techniques of ITC and ICP-MS towards this end, the data generated was inconclusive (Figure 3.11 and Figure 5.11). Optimization of ICP-MS could be performed using higher concentrations and volumes of pyocins S3 (in HNO_3) to minimize the background noise. It would be expected that this would generate a more accurate and clear-cut set of evidence.

Further optimization of the EMSA assays, described in Chapter 4 to investigate the DNase-DNA binding, is also required. Modifications could incorporate new versions of the pyocin S3 DNase W/T and mutants with an enhanced positive charge that would be predicted to show a more obvious shift in gel migration. For example, a serine and two lysine residues could be included in the following way. A primer could be designed to add an *NdeI* (Met), plus a Serine (S) and a pair of lysine (K) residues.

Future work that could also be carried out would be to solve the crystal structure of the pyocin S3 DNase protein or the pyocin DNase domain in complex with dsDNA as previously described for Colicin E9 (Maté and Kleanthous 2004). To do this, a new protein purification strategy would be needed to purify the pyocin S3 DNase its cognate immunity protein in order to get a higher yield of protein to meet the crystallization

requirement for appropriate crystallisation of a protein solution. This new strategy could involve separating the pyocin S3 DNase and immunity protein while washing the loaded cell lysate containing the protein complex using Guanidine buffer on a Ni-NTA column. Then, purify the pyocin S3 DNase and immunity protein using a 5 ml Heparin column and step gradient, respectively.

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(!!! INVALID CITATION !!! (Pommer, Kühlmann et al. 1999)).

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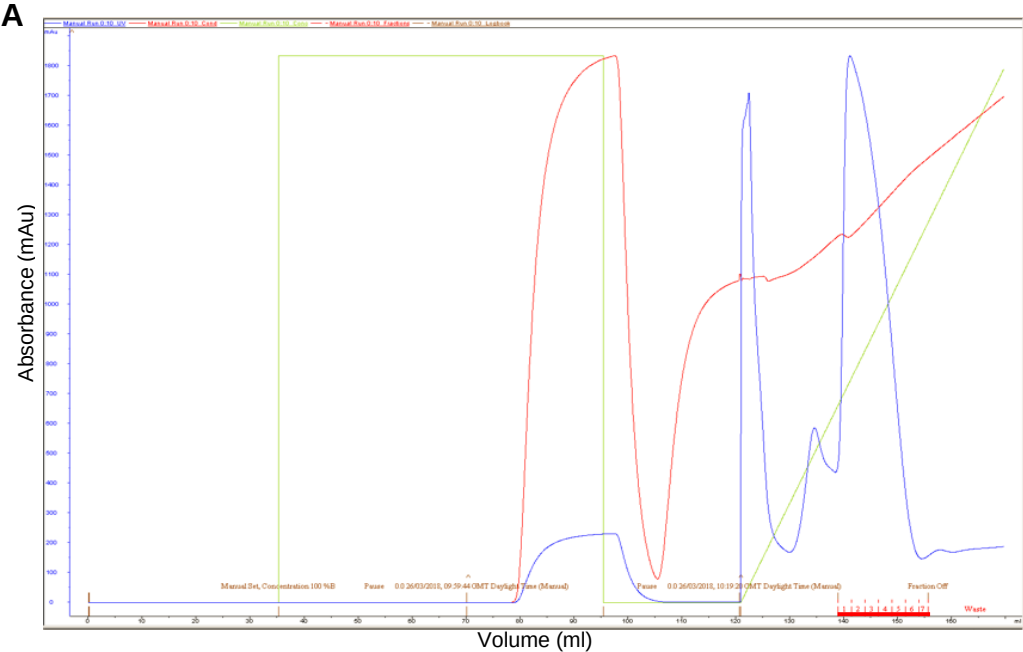
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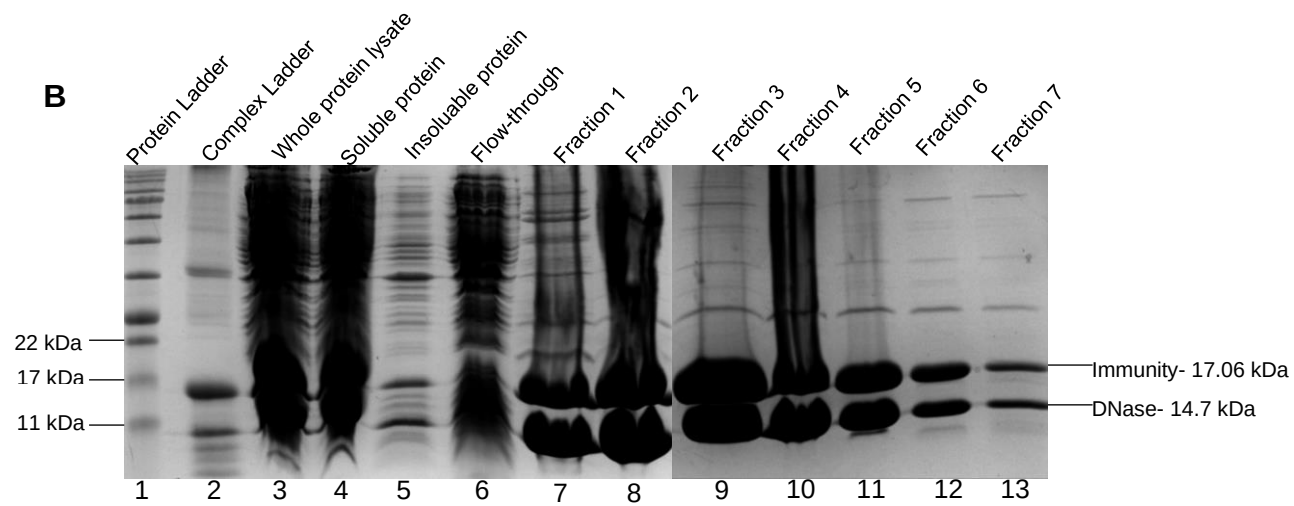
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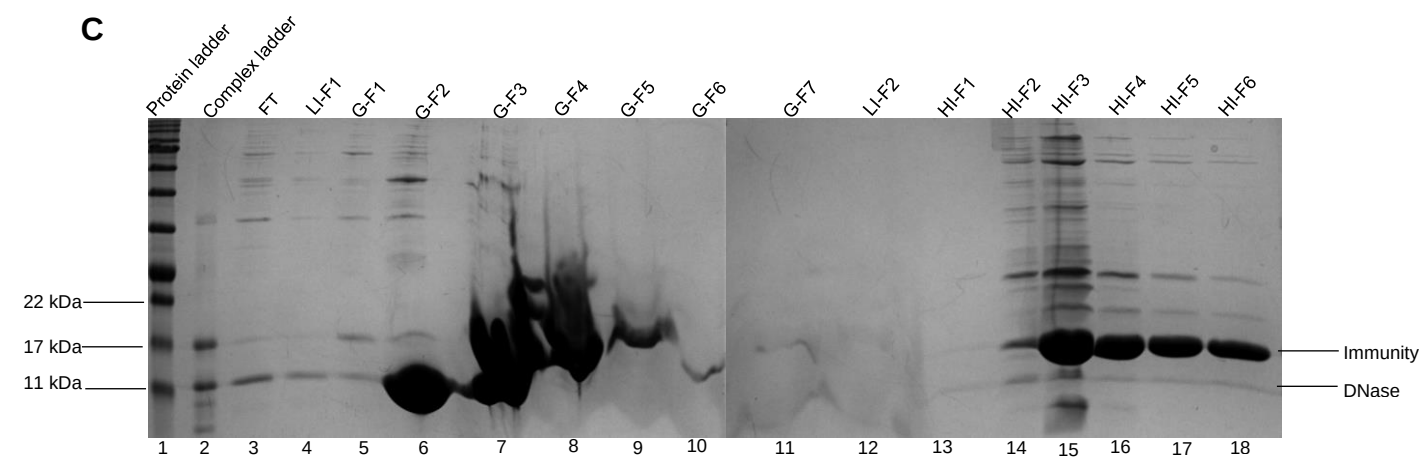
Appendix 1. Primer sequences.

Name of primer	Sequence (5'-3')	Useage
Seq DNase S3-Forward	CCGTTAGATCTGGACGATCT	For sequencing to confirm the present of mutant after Q5 mutagenesis.
YC16 Forward	CCC <u>CATATG</u> CCGGTTAAGC	1. remove the receptor and translocation domains from the full-size pyocin S3 in pBluescript for further cloning (to pGEMT and then pET21a) 2. add start condon and NdeI site (CAT ATG) to the N-terminal of the pyocin S3 Dnase-Imm gene 3. to knock out the stop codon (TAG) and add XhoI site (CTC GAG) to the C-terminal of that gene.
ImS3 Reverse	ATCTCGAGTTTAGAACCAAGAAGAGC	
pyocin S3 E7A_F	TAAGCCGCTGGCAGTGGACGTTTATG	Forward/reverse primer to make mutagenesis by substituting the Glutamic Acid7 residue on pyocin S3-NdeI (in pBluescript) with Alanine.
pyocin S3 E7A_R	ACCGGCGGCTTGGAAGG	
PyocinS3 DNase H23A Forward	TGATGGAAACGCTCTTGAACACATGCC	Forward/reverse primer to make mutagenesis by substituting the Histide23 residue on pyocin S3-NdeI (in pBluescript) with Alanine.
PyocinS3 DNase H23A Rev	CGAGGTCTTCCTGCAAAATTAC	
Q5SDM D25A_NdeI_F	AAACCATCTTGCCACATGCCGGATC	Forward/reverse primer to make mutagenesis by substituting the Asprtic Acid25 residue on pyocin S3-NdeI (in pBluescript) with Alanine.
Q5SDM D25A_NdeI_R	CCATCACGAGGTCTTCCT	
PyocinS3 DNase H26A Forward	CCATCTTGACGCCATGCCGGCTC	Forward/reverse primer to make mutagenesis by substituting the Histide26 residue on pyocin S3-NdeI (in pBluescript) with Alanine.
PyocinS3 DNase H26A Rev	TTTCCATCACGAGGTCTTC	
PyocinS3 DNase R38A Forward	GACGAGTTTGGCAGCTATTATCCAGACATCCC	Forward/reverse primer to make mutagenesis by substituting the Arginine38 residue on pyocin S3-NdeI (in pBluescript) with Alanine.
PyocinS3 DNase R38A Rev	GCCAACGCACCTTGAGCC	
PyocinS3 DNase R50A Forward	TGGAGAAATTGCCAAATT AATGAAGAAAGG	Forward/reverse primer to make mutagenesis by substituting the Arginine50 residue on pyocin S3-NdeI (in pBluescript) with Alanine.
PyocinS3 DNase R50A Rev	TAGGGGATGTCTGGATAAATAG	
PyocinS3 DNase K51A Forward	AGAAATTCGCGCATTAATGAAGAAAGGGG	Forward/reverse primer to make mutagenesis by substituting the Lysine51 residue on pyocin S3-NdeI (in pBluescript) with Alanine.
PyocinS3 DNase K51A Rev	CCATAGGGGATGTCTGGA	

PyocinS3 DNase K54A Forward	CAAATTAATGGCGAAAGG GGGGAG	Forward/reverse primer to make mutagenesis by substituting the Lysine54 residue on pyocin S3-NdeI (in pBluescript) with Alanine.
PyocinS3 DNase K54A Rev	CGAATTTCTCCATAGGGG	
PyocinS3 DNase K55A Forward	ATTAATGAAGGCAGGGG GGAGTG	Forward/reverse primer to make mutagenesis by substituting the Lysine55 residue on pyocin S3-NdeI (in pBluescript) with Alanine.
PyocinS3 DNase K55A Rev	TTGCGAATTTCTCCATAG	
PyocinS3 DNase H66A Forward	AGCTAGAGTGGCTCAGA GGTTCTGTGAAAC	Forward/reverse primer to make mutagenesis by substituting the Histidine66 residue on pyocin S3-NdeI (in pBluescript) with Alanine.
PyocinS3 DNase H66A Rev	GGTATGGCAACACTCCC C	
PyocinS3 DNase K79A Forward	ACGTAACACCGCAGAAAA GCAACAG	Forward/reverse primer to make mutagenesis by substituting the Lysine79 residue on pyocin S3-NdeI (in pBluescript) with Alanine.
PyocinS3 DNase K79A Rev	CCTCCGTAGGTTTCACTG	
Primer T7F (GENEWIZ)	TAATACGACTCACTATAG GG	Forward/reverse primer for sequencing to check the success of site directed mutagenesis.
Primer T7R/Term (GENEWIZ)	GCTAGTTATTGCTCAGCG G	
S3_IntF	CCATCTTGACCACATGCC GGCTCAAG	Forward/reverse primer for removing the internal <i>NdeI</i> restriction site from the pyocin S3.
S3_IntR	TTTCCATCACGAGGTCTT CCTG	
KEA1	5'[Cyanine5.5]TTCTTAACT AGTCGTTTTTTATTCTTAA CTGTAA	Forward primer to label a 34 bp DNA fragment with cyanine5.5 fluorophore.
KEA3	3'AAGAATTGATCAGCAAA AAATAAGAATTGACATT	Reverse primer to label a 34 bp DNA fragment with cyanine5.5/bodipy530 fluorophore.
KEA10	5'[BO530]TTCTTAACTAGT CGTTTTTTATTCTTAACTG TAA	Forward primer to label a 34 bp DNA fragment with bodipy530 fluorophore.

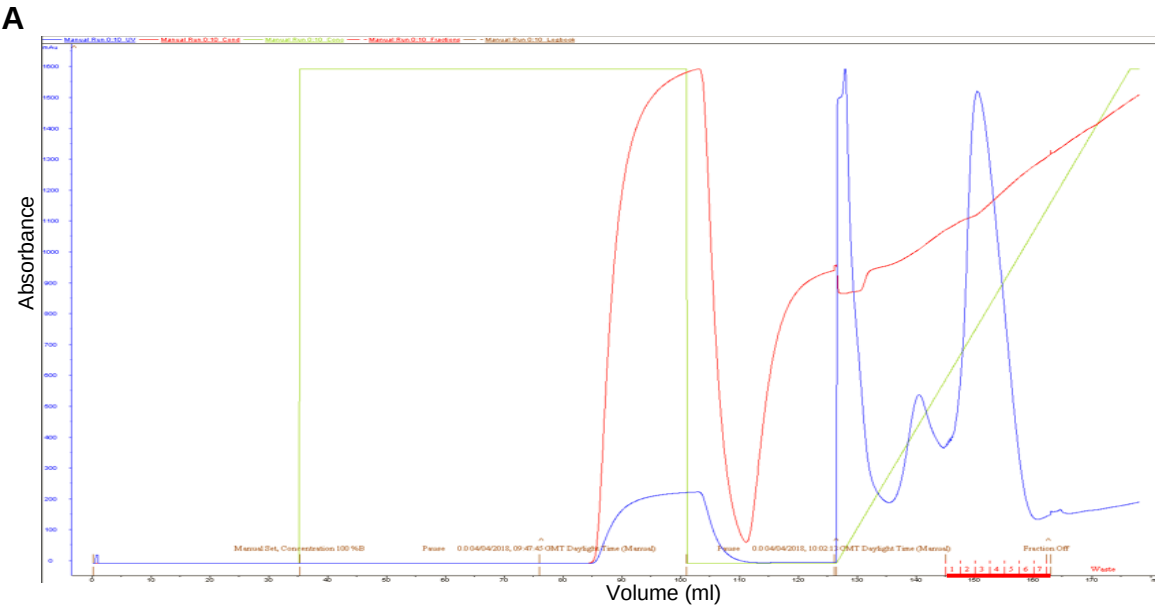


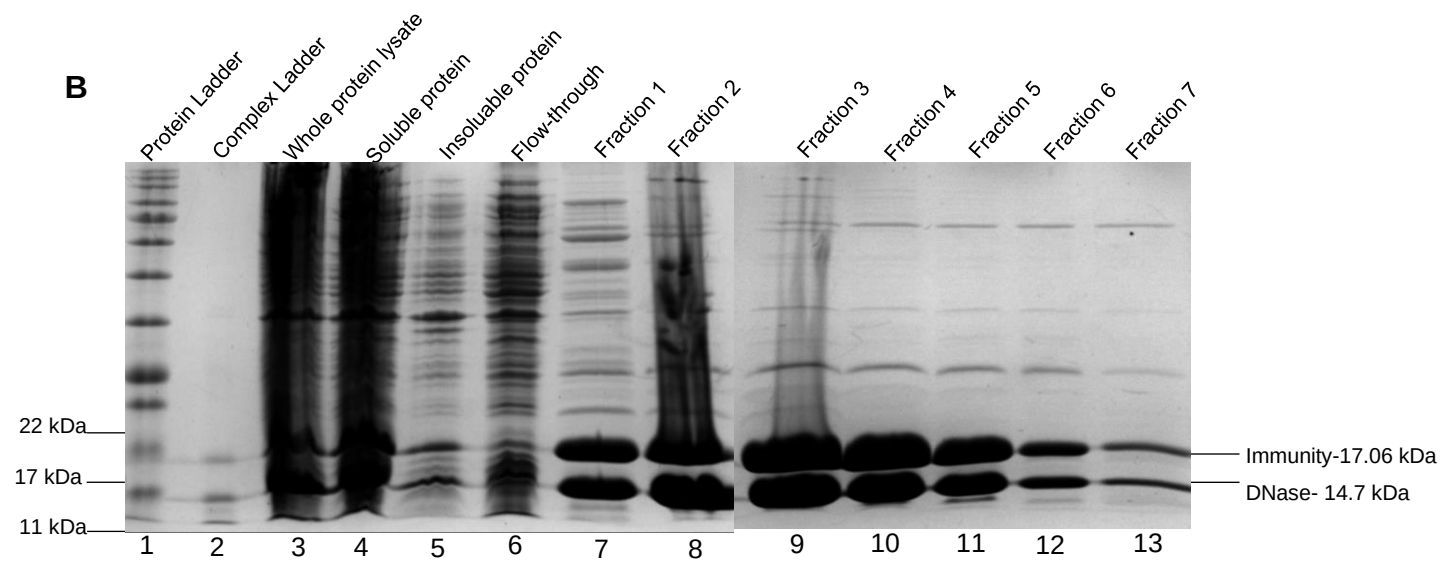


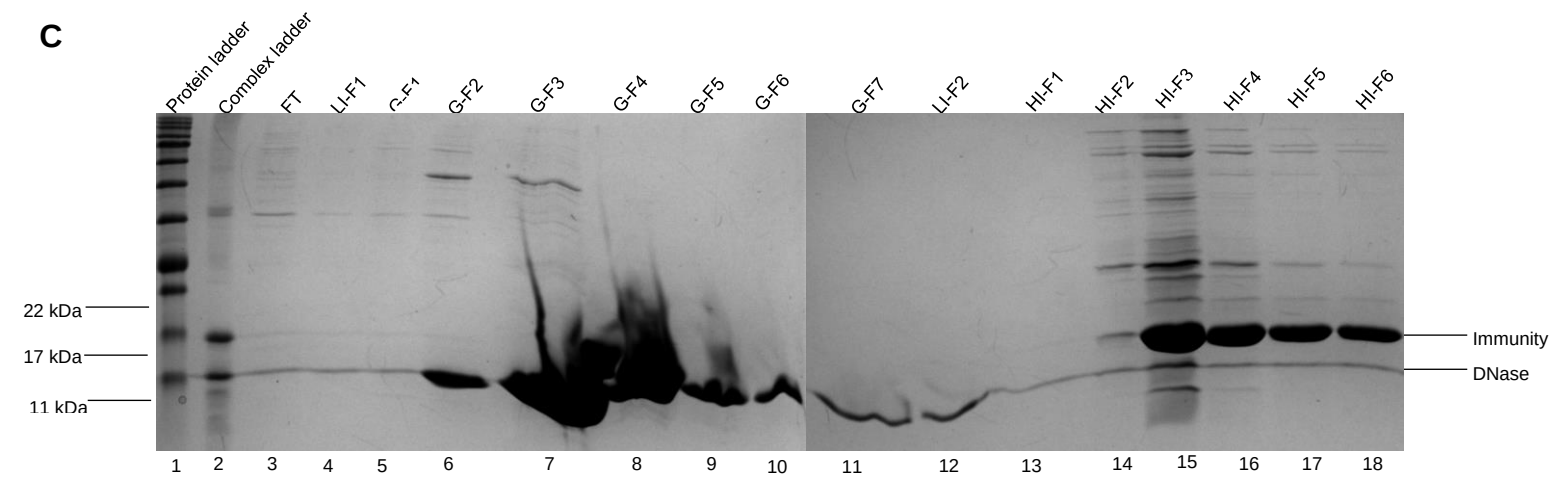


Appendix 2. Purification for H26A mutant of pyocin S3 using a Ni-NTA column.

Panel A- Cell lysate containing the mutant H26A of pyocin S3 DNase-immunity protein was supplemented with 300 mM sodium chloride and 10 mM imidazole and the 5 ml Ni-NTA column was equilibrated with 50 mM NaH_2PO_4 , 300 mM NaCl and 10 mM Imidazole buffer, pH 7 before the lysate was loaded onto the column for purification. The purification was performed using an ÄKTAprimer™ PLUS system. The salt concentration trace mirrors the imidazole addition and approximately at 120 ml, high imidazole buffer was added to the system. Fractions (5ml) were collected as a linear gradient of 10 mM to 500 mM imidazole (fractions were numbered 1 to 7 in red) was applied until the UV absorbance indicated all proteins had been eluted by maintaining a steady low level. Red curve- conductivity trace, blue curve- UV absorbance trace and green curve- salt concentration curve. Panel B- 60 μl of peak fractions containing H26A mutant of pyocin S3 DNase-Immunity complex protein were taken and mixed with 20 μl of SDS-PAGE loading buffer and applied to a SDS-PAGE running along with soluble protein collected from cell lysate and flow-through from the Ni-NTA column. Lane 1 and 2. colour pre-stained protein standard ladder and complex ladder, respectively; Lane 3. whole cell lysate; Lane 4. soluble protein; Lane 5. insoluble protein; Lane 6. flow-through (FT); Lane 7-13. Fraction 1 to 7. Coomassie blue was used to visualize proteins. The expected sizes of the DNase and Immunity proteins are indicated on the right in kDa. Panel C- Fractions (1-7) containing purified H26A mutant DNase-Immunity complex protein from the above panel B were dialysed against 50 mM Na_2HPO_4 , 100 mM NaCl and 10 mM imidazole buffer, pH 7 before being reloaded on to the Ni-NTA column. Flow-through (FT) was collected. The H26A mutant DNase was denatured and eluted off the column using total volume of 20 ml of buffer containing 6 M Guanidine (G) and 3 ml fractions were collected manually. Then, 20 ml of low imidazole buffer (LI) was passed through to push down all the remaining DNase before the immunity protein was eluted off by a high imidazole buffer (HI). Fractions (3 ml) were collected manually and 60 μl of each fraction was taken and mixed with 20 μl of SDS-PAGE loading buffer to be analysed on a 15% SDS-PAGE gel. Lane 1 and 2. Colour pre-stained protein standard ladder and complex ladder, respectively; Lane 3. FT, flow-through; Lane 4 and 12. LI-F1 and LI-F2, fraction collected from low imidazole wash; Lane 5- 11. G-F1 to G-F7, fraction 1 fraction 7 collected from guanidine buffer wash; Fraction 13- 18. HI-F1 - HI-F6, fractions collected from high imidazole wash. Coomassie blue was used to stain the gel. The expected position of the DNase and Immunity proteins are indicated on the right.



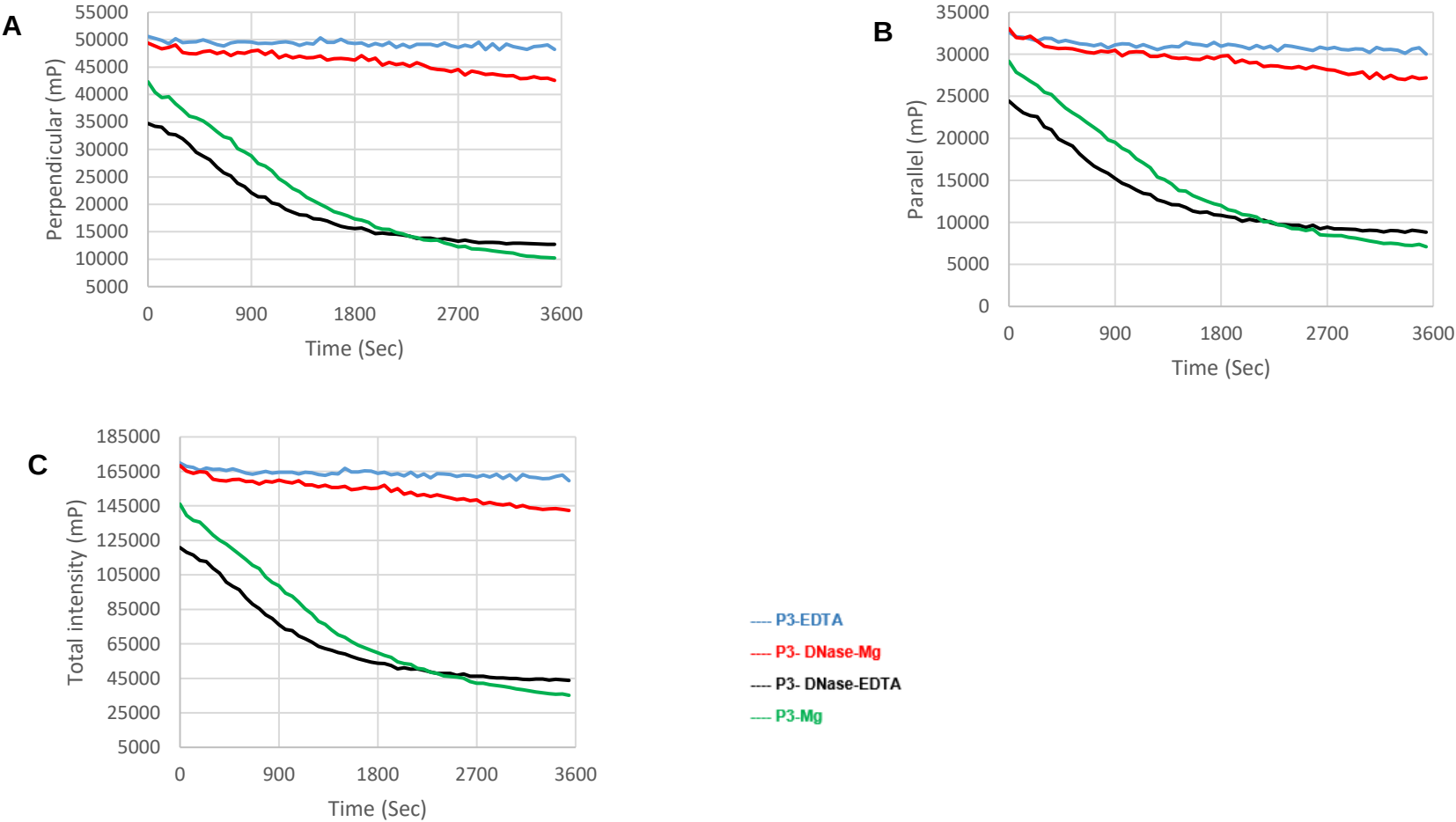




Appendix 3 Purification for H66A mutant of pyocin S3 using a Ni-NTA column.

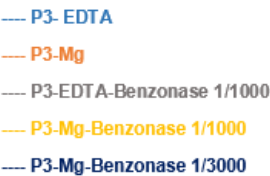
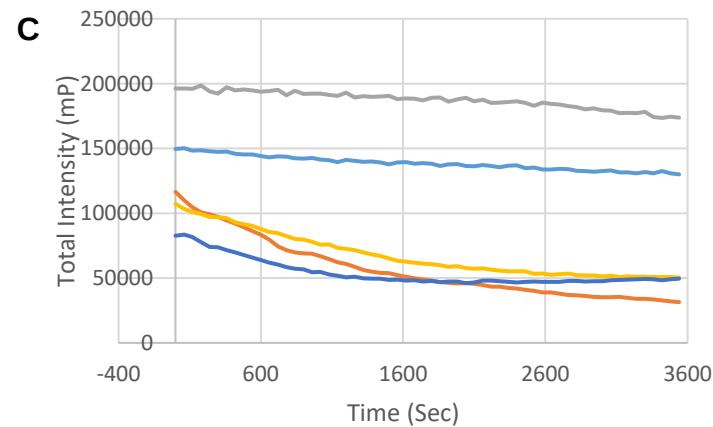
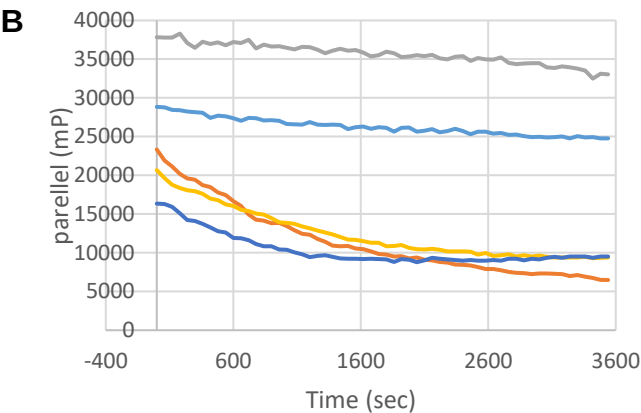
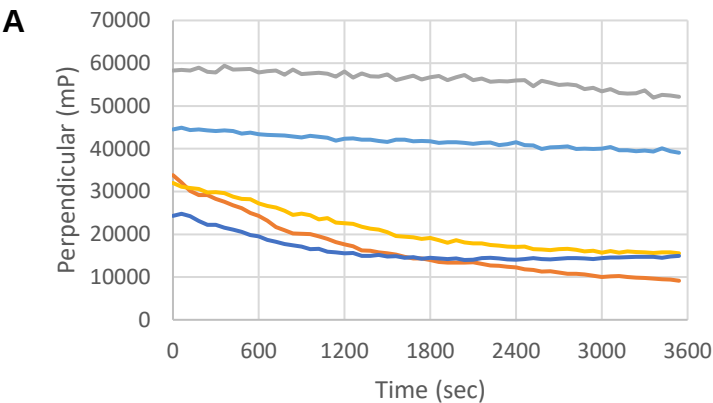
Panel A- Cell lysate containing the mutant H66A of pyocin S3 DNase-immunity protein was supplemented with 300 mM sodium chloride and 10 mM imidazole and the 5 ml Ni-NTA column was equilibrated with 50 mM NaH_2PO_4 , 300 mM NaCl and 10 mM Imidazole buffer, pH 7 before the lysate was loaded onto the column for purification. The purification was performed using an ÄKTAprimer™ PLUS system. The salt concentration trace mirrors the imidazole addition and approximately at 126 ml, high imidazole buffer was added to the system. Fractions (5ml) were collected as a linear gradient of 10 mM to 500 mM imidazole (fractions were numbered 1 to 7 in red) was applied until the UV absorbance indicated all proteins had been eluted by maintaining a steady low level. Red curve- conductivity trace, blue curve- UV absorbance trace, green curve- salt concentration trace.

Panel B- 60 μl of peak fractions containing H66A mutant of pyocin S3 DNase-Immunity complex protein were taken and mixed with 20 μl of SDS-PAGE loading buffer and applied to a SDS-PAGE running along with soluble protein collected from cell lysate and flow-through from the Ni-NTA column. Lane 1 and 2. colour pre-stained protein standard ladder and complex ladder, respectively; Lane 3. whole cell lysate; Lane 4. soluble protein; Lane 5. insoluble protein; Lane 6. flow-through (FT); Lane 7-13. Fraction 1 to 7. Coomassie blue was used to visualize proteins. The expected sizes of the DNase and Immunity proteins are indicated on the right in kDa. Panel C- Fractions (1-7) containing purified H66A mutant DNase-Immunity complex protein from the above panel B were dialysed against 50 mM Na_2HPO_4 , 100 mM NaCl and 10 mM imidazole buffer, pH 7 before being reloaded on to the Ni-NTA column. Flow-through (FT) was collected. The H626A mutant DNase was denatured and eluted off the column using total volume of 20 ml of buffer containing 6 M Guanidine (G) and 3 ml fractions were collected manually. Then, 20 ml of low imidazole buffer (LI) was passed through to push down all the remaining DNase before the immunity protein was eluted off by a high imidazole buffer (HI). Fractions (3 ml) were collected manually and 60 μl of each fraction was taken and mixed with 20 μl of SDS-PAGE loading buffer to be analysed on a 15% SDS-PAGE gel. Lane 1 and 2. Colour pre-stained protein standard ladder and complex ladder, respectively; Lane 3. FT, flow-through; Lane 4 and 12. LI-F1 and LI-F2, fraction collected from low imidazole wash; Lane 5- 11. G-F1 to G-F7, fraction 1 fraction 7 collected from guanidine buffer wash; Fraction 13- 18. HI-F1 - HI-F6, fractions collected from high imidazole wash. Coomassie blue was used to stain the gel. The expected position of the DNase and Immunity proteins are indicated on the right.



Appendix 4 The raw data for fluorescence polarization assay of bodipy530 labelled P3 DNA with pyocin S3 DNase (Figure 4.14).

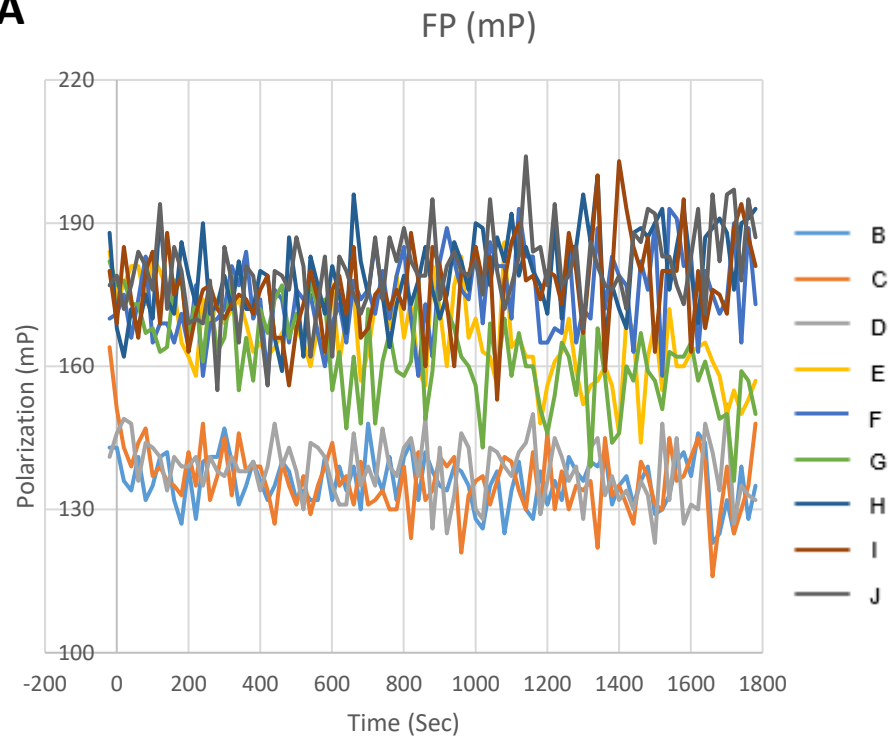
A 34 bp duplex P3 DNA template with bodipy530 at the free 5' end (100 nM) was suspended in 10 mM sodium phosphate buffer pH 7.5, with 1 mM EDTA or 10 mM Mg^{2+} ions, and its FP stability measured over time (blue and green lines, respectively). In parallel, the FP measurements of these DNA mixes with addition of pyocin S3 DNase (200 nM) were taken over time (red and black lines). Panel A, B and C, the perpendicular, the parallel components and the total intensity emitted from the P3 DNA, respectively. Data was plotted as FP (mP) against time (s).



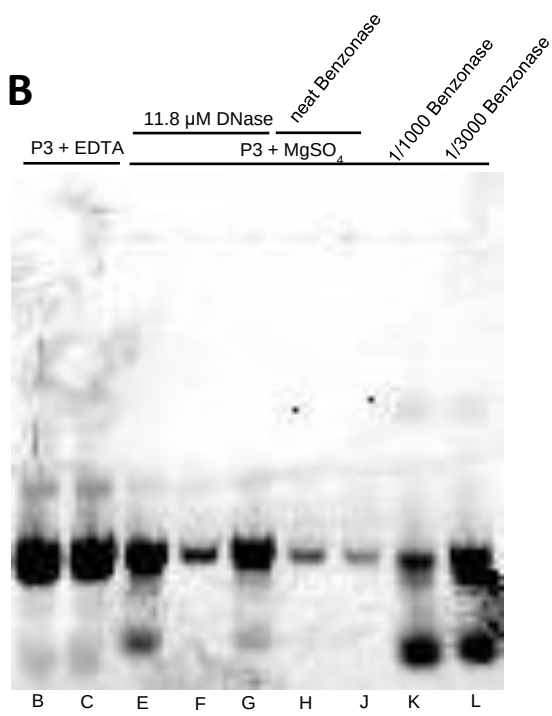
Appendix 5 The raw data for fluorescence polarization assay of bodipy530 labelled P3 DNA (Figure 4.15).

A 34 bp duplex P3 DNA template with bodipy530 at the free 5' end (100 nM) was suspended in 10 mM sodium phosphate buffer pH 7.5, with 1 mM EDTA or 10 mM Mg^{2+} ions, and its FP measured over time. Commercial benzonase was diluted either 1000 or 3000 fold and added into duplicate (30 μ l; 384 microtitre) plate wells and FP measurements of the P3 DNA with benzonase were taken over time. Panel A, B and C, the perpendicular, the parallel components and the total intensity emitted from the P3 DNA, respectively. Data was plotted as FP (mP) against time (s).

A



B



Appendix 6 The full data for Bodipy-P3 DNA cleavage assay and EMSA analysis (Figure 4.16).

A 34 bp duplex P3 DNA template with bodipy530 at the free 5' end (100 nM) was suspended in 10 mM sodium phosphate buffer pH 7.5, with 1 mM EDTA and its FP stability measured over time. FP measurements between the P3 DNA with 11.8 μ M of pyocin S3 DNase and neat concentration of commercial benzonase were taken over time. Data was plotted as FP (mP) against time (s). After FP measurements (Panel A) samples of the DNase cleavage reactions were transferred from the microtitre plate onto a 0.5x TBE PAGE to be observed on EMSA (Panel B) along with the P3 DNA with 10 mM Mg^{2+} ions and 1/1000 or 1/3000 titration of benzonase (from Fig 4.2.14) as comparison. Each sample (labelled on top of the panel B) was coded with a letter at the bottom of the panel that match the label on panel A.