Investigation of the antimicrobial properties of the R-type pyocins of *Pseudomonas aeruginosa* and the role of *lasR* in their expression.

Olubukola Oluyombo

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Declaration

Unless otherwise stated, the study presented in this thesis is my original work and has not been submitted wholly or in part for the award of a different degree in the University of Nottingham or any other institution or establishment.

Olubukola Oluyombo

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen responsible for a number of different human infections, and is the leading cause of mortality in cystic fibrosis (CF) patients. *P. aeruginosa* infections are difficult to treat due to a number of antibiotic resistance mechanisms and its propensity to form multicellular biofilms. It also uses a complex quorum sensing (QS) signalling mechanism to regulate virulence, but mutants in a key QS regulator (LasR) are often prevalent in CF clinical strains. Different strains of *P. aeruginosa* compete to dominate infections and one way they achieve this is to produce chromosomally encoded bacteriocins, called pyocins. The major classes of pyocins are the soluble (S-types) and the tailocins (R- and F-types).

This study investigated the distribution of six S-type and three major groups of R-type pyocins in CF clinical isolates and their roles during strain interactions. Competition assays between strain pairs in both planktonic and biofilm modes of growth were performed between clinical strains and corresponding R-pyocin deletion mutants.

Each clinical strain produced one R-pyocin but the distribution of S-pyocins was random. R-pyocins were central to strain dominance as evidenced by the reversal of competitive advantage in null-R-pyocin mutants both in planktonic and biofilm states. R-pyocins also demonstrated novel anti-biofilm activities. Genomic analysis of the most competitive strain (A026) showed that it is a *lasR* mutant, phylogenetically related to Liverpool Epidemic Strains (LES). Promoter fusion assays to study R-pyocin gene expression showed an increase in the expression of R-pyocin genes in a *lasR* mutant of PAO1 compared to the wild type. This LasR-linked pyocin expression was RecA-independent. Overall these findings establish the crucial role of R pyocins in *P. aeruginosa* strain competition and a link between QS and R-pyocins.

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OLUYOMBO, Olubukola (Bukky).

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Abbreviations

CF – Cystic Fibrosis
CFTR – Cystic Fibrosis Transmembrane Regulator
LES – Liverpool Epidemic strain
AHL – Acyl homoserine lactone
PQS – Pseudomonas Quinolone signal
HHQ- 2-heptyl-3-hydroxi-4-quinolone
T3SS – Type 3 Secretion System
T6SS – Type 6 Secretion system
IROMPs – Iron regulated outer membrane proteins
RGP – Regions of genomic plasticity
MDR – Multi-drug resistant(ce)
PMN - Polymorphonuclear
EPS – Extracellular polymeric substances
Pel – associated with **Pel**licle formation
Psl – Polysaccharide synthesis locus
ROS – Reactive oxygen species
tRNA – transfer ribonucleic acid
rRNA – ribosomal ribonucleic acid
RBD – Receptor binding domain
BPAR – Base plate attachment region
ACT – Artemis Comparative Tool
CFU – Colony forming unit
INDEL – Insertions and deletions
SNP – Single nucleotide polymorphism
BRIG – Blast Ring Image Generator
MMC – Mitomycin C
SCC – Silver carbine complexes

Chapter 1

General Introduction

1.1. The genus *Pseudomonas*

Members of this genus are Gram negative, non-spore forming bacteria. They are aerobic, oxidase positive and motile with the aid of polar flagella. Since the genus was first described by Migula in 1894 (Migula, 1894) several characteristics have been used to describe the organisms that are classified into it. Various classifications have led to the inclusion of new species and the exclusion of others. Taxonomy criteria have been constantly reviewed since the first classification was published in 1923 (Bergey *et al.*, 1923). New criteria led to the re-classification of some old members into new genera e.g. *Acidovorax spp., Stenotrophomonas spp., Burkholderia spp.* etc. and the taxonomy is constantly evolving. In addition to the widely popular genotypic classification based on the 16S rRNA gene sequence (Anzai *et al.*, 2000), other highly specific sequences like the housekeeping genes (*recA, gyrB, rpoB*) (Hilario *et al.*, 2004) and the 16S-23S rRNA intergenic spacer (Gurtler & Stanisich, 1996) have been instrumental in discriminating among close relatives. However, phenotypic properties like cell morphology, flagella type, pigment production, antibiotic resistance and production have formed the bases for identification in many clinical isolates.

Members of this genus have been noted to be successful across extremes of climates and habitats. Being ubiquitous in existence, they are found free-living in the environment e.g. soil (*P. duriflava, P. putida, P. aeruginosa*), or causing infections in plants (*P. syringae*), animals (*P. simiae* in monkeys) and humans (*P. aeruginosa*) while some are non-pathogenic (*P. fluorescens*).

1.2. *Pseudomonas aeruginosa*

Members of the *Pseudomonas aeruginosa* species have a single polar flagellum and are ubiquitous in nature having been isolated from soil, plants and water especially sewage water contaminated by human and animal wastes. (Hardalo & Edberg, 1997). Though widespread in nature, it does not often infect humans with normal immune functions. It causes opportunistic infections in a background of compromised immunity and has gained relevance as a nosocomial pathogen. The mortality rate of patients with *P. aeruginosa* bacteraemia over two-weeks admission period is as high as 22.2% (Kim *et al.*, 2014b) and it is one of the leading organisms in multi-drug resistance (MDR) (Livermore, 2001). Increased prevalence is recorded in burn wounds (Lyczak *et al.*, 2000) and cystic fibrosis lung infections (Lambiase *et al.*, 2006) and it is easily transmitted among patients in intensive care and/or with invasive therapeutic interventions (Boyer *et al.*, 2011).

P. aeruginosa has also been isolated from infections involving other systems of the body e.g. they cause urinary tract infections, meningitis, pneumonia, otitis externa, otitis media, bacteraemia, gastroenteritis etc.

1.2.1 Molecular Biology of *P. aeruginosa*

The genome size of the PAO1 prototype strain is approximately 6.3 million base pairs (Mbp) (Stover et al., 2000). Further analysis of this strain has helped in the understanding of the genetic makeup underpinning some traits that have made *P. aeruginosa* particularly successful in survival, antimicrobial resistance, virulence factor production and biofilm formation.

The constituent genes in the genome have been broadly divided into the core and accessory genes.

About 90% of the genes in *P. aeruginosa* genome are conserved being represented in many strains (Wolfgang *et al.*, 2003). Most of these house-keeping genes, being central to the metabolic functions are common to most members across many habitats. These set of genes constitute the core genome. The core genome encodes some traits that have made *P. aeruginosa* particularly successful for its survival, antimicrobial resistance, virulence and biofilm formation. Some virulence factors are outlined in Table 1.1. Some of these ensure survival in the host while others either promote competition or co-operation in the micro-ecology. Some characterised mechanisms of antibiotic resistance encoded by the genome include efflux pumps (Sun *et al.*, 2014) and reduced permeability to beta-lactam drugs (Lakaye *et al.*, 2002).

Another set of genes constitute the accessory genome. the accessory genome is not widely distributed and is only present in some members of the species. This genome does not follow a random distribution but tends to form a cluster within specific loci (Kung *et al.*, 2010). What determines these loci is a function of the degree of plasticity present in some regions of the genome. these 'regions of genomic plasticity' (RGP) vary in sizes and are referred to as genomic islands or islets when they are greater or less than 10 kb respectively (Mathee *et al.*, 2008). The RGPs tend to serve as hot spots for the incorporation of accessory genes, and the transfer RNAs (tRNAs) at these sites have been discovered to serve integration functions for the entry of these horizontally acquired genes (Williams, 2002). Other hot spots include integrases and transposases.

The variation of the accessory genome amongst closely related strains suggests that they encode gene products that are specific for the strain from which they are derived e.g. for special niche-based functions, virulence and adaptation (Kung *et al.*, 2010). The core genome of *P. aeruginosa* normally has a high %G+C content (~67%) but the accessory genome has a much less %G+C value suggesting that they are sets of genes acquired from other species (Kung *et al.*, 2010). Since they occur in the genome, accessory genes are also exposed to selective pressures such as DNA mutations that alter their genetic make-up over time (Kung *et al.*, 2010). Although they are horizontally acquired, they can be vertically transmitted due to integration into the genome.

The versatility of the nutrients that *P. aeruginosa* can utilise for growth allows it to adapt to a wide range of habitats and environmental niches and contributes to the perpetuation of its nosocomial spread (Hota *et al.*, 2009). Nearly 200 of its cytoplasmic membrane transport systems are employed in importing nutrients to maintain this versatility (Stover *et al.*, 2000). Furthermore, about 0.3% of its genome codes for proteins that confer its intrinsic antibiotic resistance while approximately 10% of its genes are arranged in various pathogenicity islands which encode virulence factors important in colonisation and survival (Mesaros *et al.*, 2007). It is also able to acquire resistance (R)-plasmids encoding resistance to various antibiotics by a single horizontal gene transfer (Shahid *et al.*, 2003).

Virulence factors	*Functions*	*Ref*
Lipopolysaccharides (LPS)	Cytokine production Endotoxin Anti-phagocytosis	(Rocchetta *et al.*, 1999)
Outer membrane vesicle factors (haemolysin, Cif, protease, quinolines)	IL-8 stimulation Bactericidal effect	(Kadurugamuwa & Beveridge, 1995)
Flagella	Motility Biofilm formation	(Feldman *et al.*, 1998)
Type IV Pili	Adherence to respiratory epithelial cells	(Hahn, 1997)
Rhamnolipids	Detergent effect causing surfactant hydrolysis	(Davey *et al.*, 2003)
Toxins	Exotoxin A: Inhibition of host cell protein synthesis	(Jorgensen *et al.*, 2005)
	Exotoxin S: Anti-phagocytosis	(Ganesan *et al.*, 1999)
	Exotoxin U: Cytotoxic	(FinckBarbancon *et al.*, 1997)
	Phospholipase C: Digestion of phospholipid surfactant	(Terada *et al.*, 1999)
Alkaline protease	Degrades fibrin	(Suter, 1994)
Extracellular polymeric substances (Alginate, Pel, Psl)	Anti-phagocytosis Biofilm formation	(Pier *et al.*, 2001)
Pyocins	Bactericidal against closely related strains	(Michel-Briand & Baysse, 2002)
Pyocyanin **Pyoverdine**	Bactericidal action against other bacteria	(Lau *et al.*, 2004)
Siderophore (pyoverdine)	Regulates production of exotoxin A, endoprotease and pyoverdine itself	(Lamont *et al.*, 2002)
Phenazines **Lipopeptides**	Antimicrobial activity against other bacteria and eukaryotes e.g. fungi	(Gross & Loper, 2009)

Table 1.1: Selected virulence factors of *P. aeruginosa*

1.3. Clinical Importance of *P. aeruginosa*

The opportunistic nature of *P. aeruginosa* has earned it special attention as a nosocomial pathogen. It is seldom part of the normal body flora especially the gut and its isolation in an infection is an indication of a breach in the normal muco-cutaneous barriers or immune defence (Sadikot *et al.*, 2005). This usually occurs in hospitalised patients and the immunocompromised. Nathwani *et al.,* 2014 conducted a systematic review and meta-analysis of the clinical outcomes of multi-drug resistant (MDR) and susceptible strains of hospital-acquired *P. aeruginosa*. The respective mortality rates of these strains were 34% and 22%. The *Pseudomonas spp.* was also reported to be responsible for 28% of organisms causing infections in an ICU being linked closely with procedures like endotracheal intubation and mechanical ventilation (Gupta *et al.*, 2016).

Many factors that prevail in the hospital setting are instrumental to encouraging the spread and perpetuation of *P. aeruginosa* infections. One of these factors includes multiple antibiotic therapy especially in ICUs, which encourages selection of resistant strains (Marwick & Davey, 2009, Radji *et al.*, 2011). These strains are often MDR against many of the front line classes of antibiotics which was highlighted in a study conducted by Obritsch *et al.,* 2014. The study showed that resistance to carbapenems, fluoroquinolones, macrolides and cephalosporins in ICU patients rose from 4% to 14% within a 10-year period (Obritsch *et al.*, 2004). Other factors that contribute to the nosocomial success of *P. aeruginosa* include therapeutic interventions that breach the natural anatomical barriers e.g. urinary catheterisation, intubation, surgeries (Donlan, 2001).

1.4. *P. aeruginosa* clinical infections

1.4.1. Chronic Lung Infections in Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive inherited disease with an incidence of 1/3500 in Europe (Southern *et al.*, 2007). It is a disorder of the CF conductance regulator (CFTR) gene on the long arm of chromosome 7 at position q31.2, with various well documented mutations (Rommens *et al.*, 1989). These mutations have been grouped into six major classes (O'Reilly & Elphick, 2013). Class I mutations affect the biosynthesis of CFTR due to the presence of a premature stop codon within the gene e.g. *Trp1282X* (<2%) and *Gly542X* (<3%). Class II mutations affect CFTR maturation due to the deletion of a codon e.g. *Phe508del*, a deletion of phenylalanine at position 508 is the commonest mutation, accounting for 72% of CF population in Non-Hispanic Caucasians (Watson *et al.*, 2004). Class III mutations are substitution mutations which affect the chloride channel gating/regulation system e.g. *Gly551Asp* (<3%). Class IV mutations affect chloride ion conductance across the pores of the channels e.g. *Arg117His* (<1%). Class V mutations affect the stability of the mRNA (a milder clinical presentation, <1%) and Class VI mutations affect CFTR stability e.g. *Glu1412X.*

CFTR disorder causes a malfunction of the cyclic adenosine monophosphate (c-AMP) which co-ordinates the activity of the chloride channel. The channel pumps chloride ions from the intracellular to the extracellular space (Kreda *et al.*, 2012, Folkesson *et al.*, 2012). Some hypotheses have been postulated to explain the sputum dehydration associated with CFTR deficiency. The first proposes that water transport is partially under the control of chloride ions and thus in CFTR deficiency there is reduced water content in the secreted extracellular mucus because of reduced content of chloride ions in the extracellular space. Another known function of the CFTR is the

inhibition of sodium reabsorption. As the passive transport of water across cell membrane barriers is linked with sodium ion absorption, sodium and water reabsorption goes uninhibited causing abnormally thick (viscid) mucus in the extracellular space (Lubamba *et al.*, 2012, Rubenstein, 2006). The central pathology is the production of thick mucus and plugging of secretory glands.

Many systems with secretory functions are affected e.g. digestive, reproductive, skin and respiratory. Prenatal detection can be made from the digestive system of the foetus where ultrasonography shows an echogenic bowel which is confirmed by DNA analysis (Scotet *et al.*, 2002). After birth patients show progressive worsening of intestinal malabsorption and obstructive biliary disease (Westaby, 2006). The reproductive system of the males is more adversely affected. Almost all males present with sterility due to absent or obliterated vasa deferentia and epididymis (Sokol, 2001). High salt content in the sweat of CF patients is due to inspissated (dehydrated) secretions of the eccrine sweat gland (LeGrys, 2001). In the respiratory system, there are thick mucus plugs due to depletion of airway surface moisture which contributes to defective mucociliary clearance of the airway (Matsui *et al.*, 1998).

Although CF is a multi-system disorder, lung pathologies are the most studied because most deaths are due to pulmonary infections. At the onset of respiratory tract colonisation in childhood, different organisms are often isolated with many of the early colonisers being *Staphylococcus aureus, Haemophilus influenza*e and *P. aeruginosa* (Starner & McCray, 2005). These infections may be treated or cleared in early childhood but they do become recurrent and chronic with strains of *P. aeruginosa* becoming the dominant pathogen by teenage years (Starner &

McCray, 2005). There is associated significant morbidity and chronic chest infections caused by *P. aeruginosa* is the leading cause of mortality in CF patients (Lambiase *et al.*, 2006).

The adaptation of *P. aeruginosa* to the CF lung is particularly striking. The properties of biofilm formation, mucoidy, hypermutability and resistance all contribute to its success. Dehydrated viscous sputum of CF is rich in nutrients for microbial growth and impaired ciliary clearance encourages the persistence of infections. Chronic inflammation is the hallmark of *P. aeruginosa* CF lung infection. The inflammatory changes are sustained by the persistence of leucocytes and their products e.g. proteases, reactive oxygen species (ROS), myeloperoxidases etc. (Ciofu *et al.*, 2005). Abundant protease production (e.g. elastase) overwhelms the anti-protease mechanisms of the lungs causing extensive weakening of the respiratory support structures and elasticity leading to a condition called bronchiectasis (Kettle *et al.*, 2004). The transition from non-mucoid to a mucoid variant and establishment of a biofilm community of bacterial cells marks the beginning of a steep decline of lung functions and takes an average of about 10.9 years (Li *et al.*, 2005) (Figure 1.1).

Eradication of mucoid *P. aeruginosa* infections from the lower respiratory system after the establishment of a biofilm has been reported as almost impossible, but some success has been recorded (Troxler *et al.*, 2012, McPherson *et al.*, 2010). However, there is a window of opportunity which most eradication measures try to target for an improved success rate. This exists before chronic inflammatory changes and biofilm development (Figure 1.1) and is carried out in children. In a study by Hansen *et al*, the efficacy of this measure was sustained for 15 years (Hansen *et al.*, 2008) and Douglas *et al*

also reported a high eradication rate of 88.5% (Douglas *et al.*, 2009).

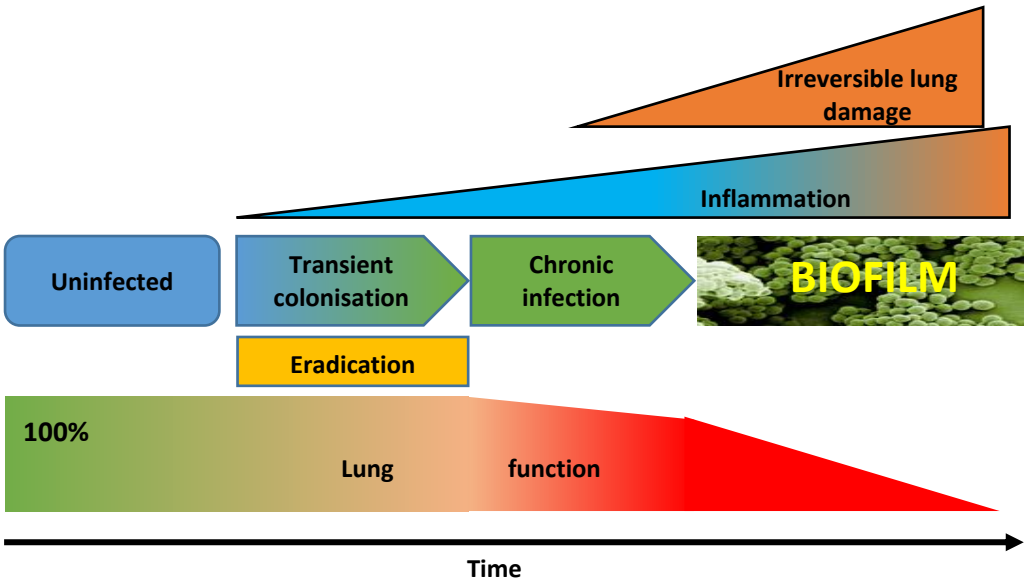


Fig 1.1: Progression of P. aeruginosa lung infection in cystic fibrosis shows the best time of treatment to achieve eradication is before the onset of chronic inflammation and establishment of biofilm (Starner & McCray, 2005).

Antibiotic therapies in chronic lung infections of CF patients include inhalation of nebulised tobramycin or colistin and oral ciprofloxacin (Taccetti *et al.*, 2012), powder forms of tobramycin (Geller *et al.*, 2007) and colistin (Westerman *et al.*, 2007) and nebulised aztreonam lysine (Oermann *et al.*, 2010) with some improvement in lung functions. A proposed method of cycling of antibiotics (e.g. oral ciprofloxacin and inhaled colistin) to prevent development of resistance was shown to have no significant effect on the initial and chronic infections

in *P. aeruginosa* naïve children (Tramper-Stranders *et al.*, 2010). Silver carbine complexes (SCCs) are broad spectrum antimicrobial agents with a novel mechanism of action that have been proven efficacious in many multi-drug resistant organisms including *P. aeruginosa* isolated from CF lungs (Kascatan-Nebioglu *et al.*, 2007). To facilitate delivery of these SCCs to infection sites, they have been modified by encapsulating them in dextran nanoparticles (Ornelas-Megiatto *et al.*, 2012).

Gene therapy for the uptake of the normal CFTR gene would have appropriately been the umbrella cure regardless of the mutation type. However, such therapy has undergone many trials without success because the cells that successfully take up the gene lose the expression over time (Oakland *et al.*, 2012). Mutation-specific therapies have however been successful. The first licensed drug in the US and Europe is Ivacaftor used in patients with class III substitution mutations affecting the chloride channel gating/regulation system. It is an oral drug used in age groups above six years. It increases the time period that the CFTR channels remain open. Studies have shown that it improves lung function, reduces respiratory exacerbations, reduces salt concentration in sweat and clinical trials have shown a weight gain of over 2.7 kg more in the treated individuals compared with the placebo group (Ramsey *et al.*, 2011).

1.4.2. Chronic wound infections

Some conditions which can cause chronic wounds include diabetic foot ulcers, pressure sores, venous ulcers and burn wounds. Many bacterial species are involved in chronic wound infections but *P. aeruginosa* is one of the leading organisms

isolated from such wounds accounting for more than half of the chronic wound isolates (Gjodsbol *et al.*, 2006).

A wound is said to be 'colonised' when the normal process of wound healing is not delayed by the presence of micro-organisms (usually of limited number). However, there exists a less well defined demarcation between when we can call a wound 'colonised' or 'infected' since the former usually leads to the latter. An objective and quantifiable bacterial count which has been proposed is 10^5 bacterial cells per gram of tissue (10^5 cells/g), below or above which a wound can be defined as colonised or infected respectively (Robson *et al.*, 1968, Bendy *et al.*, 1964).

P. aeruginosa infected wounds have been noted to cover a larger surface area than those caused by other organisms (Gjodsbol *et al.*, 2006, Madsen *et al.*, 1996). The wide surface area of devitalised/damaged tissue presented by burn wounds particularly encourages *P. aeruginosa* to fester, worsening the prognosis. Wound biofilms occur as micro-colonies which are embedded in host tissues and surrounded by host cells such as the polymorphonuclear (PMN) cells (Kirketerp-Moller *et al.*, 2008, Malic *et al.*, 2009). These isolated pockets of biofilms (micro-colonies) produce rhamnolipids which are quorum sensing regulated and these rhamnolipids directly kill the PMN cells (Jensen *et al.*, 2007). Isolated micro-colonies embedded at various depths in chronic wounds frustrate antibiotic eradication of such wound infections. These antibiotics are only effective against the planktonic forms while the cells embedded in the micro-colonies perpetuate the infection. The measures for eradication of infections in chronic wounds are mainly surgical, involving extensive debridement of the wound to remove devitalised tissues and surgical removal of such

tissues or in extreme scenarios an amputation when limbs are involved and the infection is too extensive.

1.4.3. Other *P. aeruginosa* infections

Virtually all body sites can be infected by *P. aeruginosa*. Some of the common clinical infections include urinary tract infections, bacteraemia, pneumonia, endocarditis, meningitis, otitis externa, otitis media, osteomyelitis, gastritis etc. A common denominator in such infections is an underlying condition causing immunosuppression either pathologically or iatrogenically i.e. health care associated (Zavascki *et al.*, 2006).

Cheong *et al* studied some predictors of community-onset *P. aeruginosa* bacteraemia and reported that 78.3% of the study population had either a haematological malignancy or a solid tumour (Cheong *et al.*, 2008). Presentation with neutropenia (47.7%) and prior use of immunosuppressant in the preceding 30-days period (46.2%) were also prominent predictors of infection. The mortality rate of *P. aeruginosa* infections was significantly higher compared to that of *E. coli* and inappropriate initial antibiotic therapy was an independent risk factor in the high mortality recorded (Cheong *et al.*, 2008).

In another study that focused on hospitalised patients, the most prevalent sites of infection were wound (39%), respiratory tract (22%) and urinary tract (18%) with blood accounting for only 8.5%. Significant independent risk factors included admission from a chronic care facility and invasive devices while isolation of MDR strains were significantly associated with ICU admissions and exposure to increased number of antibiotics (Aloush *et al.*, 2006).

1.5. *P. aeruginosa* biofilms in clinical infections

An important factor during *P. aeruginosa* infection is its ability to form biofilms. Biofilms are aggregates of bacterial cells that are encased in a self-produced matrix and they are often attached to solid surfaces. The major components of a biofilm include extracellular polymeric substances (EPS), microbial cells, proteins, glycoproteins, glycolipids and extracellular DNA (Flemming *et al.*, 2007).

Bacterial cells in a biofilm state are physiologically more complex than their planktonic counterparts and differ from the planktonic cells from which they are derived (Hall-Stoodley *et al.*, 2004). The biofilm population of *Pseudomonas aeruginosa* in the CF lung is phenotypically different from those in the environment or any other class of immunocompromised host (Speert *et al.*, 2002). Biofilm properties of adherence, mucoidy, hypermutability and resistance in the context of clinical infections are further discussed below.

1.5.1. Adherence

Generally, the first stage in the establishment of a biofilm is the attachment to an abiotic or biotic interphase. This is achieved with the aid of both flagella and type IV pili which are used for swimming and twitching motilities respectively (Klausen *et al.*, 2003). Mutants of the type IV pili (O'Toole & Kolter, 1998) or non-flagellated strains (Sauer *et al.*, 2002) for example, are not able to form typical mature biofilms. Initial stages of attachment are reversible; however, as the cell population grows, this becomes irreversible being further strengthened by cell aggregation as cells adhere in micro-colonies. Mature biofilms are formed when cells secrete EPS which forms part of the structural matrix that ensures the

embedding of the biofilm (Rasamiravaka *et al.*, 2015). The EPS majorly comprises Alginate, Pel (for **pel**licle formation) and Psl (**p**olysaccharide **s**ynthesis **l**ocus) polysaccharides (Ghafoor *et al.*, 2011) whose roles are outlined (with other components of the matrix) in Table 1.2.

Matrix component	***Function***	***Ref***
Pel/Psl	Early stages of biofilm development / scaffolding	(Colvin *et al.*, 2011, Yang *et al.*, 2011, Ma *et al.*, 2007)
Alginate	Linear unbranched polymer Structural stability of biofilms Retention of water and nutrients	(Sutherland, 2001)
eDNA	For initial stage of cell-to cell contact Nutrient reservoir for cells during starvation	(Allesen-Holm *et al.*, 2006), (Finkel & Kolter, 2001)
Flagella/ Type IV pili/ Cup fimbriae	Irreversible stage of cell-to-interphase attachment. Type IV pili-Psl fibre matrix complex Microcolony formation	(O'Toole & Kolter, 1998), (Wang *et al.*, 2013), (Kulasekara *et al.*, 2005)
Lectins	LecA for biofilm formation LecB binds to carbohydrate residues	(Dubern & Diggle, 2008), (Tielker *et al.*, 2005)

Table 1.2: Some components of the biofilm matrix formed by *P. aeruginosa*

1.5.2. Motility

The source of carbon in a biofilm has been described as a factor that can affect motility of the participating cells. Pioneering

cells in a biofilm tend to become immotile when glucose is the carbon source thus allowing the cells to pile up in a stalk (Klausen *et al.*, 2003). However, citrate keeps all the cells motile thus giving a more flat architecture to the biofilm (O'Toole & Kolter, 1998).

Loss of motility by shedding off the flagella in CF chronic lung infections is one of the peculiar characteristics of the disease. In chronic infections such as observed in the CF lung, biofilm formation is central to the continued existence of the organism and may actually be triggered by unfavourable conditions like antimicrobial therapy (Ciofu *et al.*, 2005). It is not clear at what stage in the infection process these cells become sessile but the presence of immotile non-flagellated cells in CF lungs is an indication of chronicity (Wright *et al.*, 2013, Folkesson *et al.*, 2012).

1.5.3. Mucoidy

Mucoidy is common in CF lung infections although many isolates are non-mucoid. Studies of the genetic basis of mucoidy conversion reveals the inactivation of the *mucA* gene, and to a lesser extent the *mucB* and *mucD* genes (Deretic *et al.*, 1995). *mucA* inactivation induces the over-expression of alginate (Ramsey & Wozniak, 2005) leading to a mucoid colony phenotype (Qiu *et al.*, 2007, Hassett *et al.*, 2009).

Alginate is an EPS which is made up of mannuronate and guluronate monomers. Its synthesis is complex, involving twelve genes in the alginate operon (Hentzer *et al.*, 2001). Other important EPS constituents are Pel/Psl. Pel was first identified in association with pellicle formation in a static liquid biofilm (Friedman & Kolter, 2004). The *pel* locus has seven genes for the biosynthesis and export of the Pel polysaccharide (Franklin *et al.*, 2011). The Psl polysaccharide are pentasaccharide

repeating subunits made up of D-mannose, L-rhamnose and D-glucose units. It is encoded by the polysaccharide synthesis locus (*psl*) formed by twelve genes (Byrd *et al.*, 2009).

The relative proportions of the alginate and the Pel/Psl polysaccharides determine the extent of mucoidy displayed by different biofilms. Therefore non-mucoid biofilms usually produce less alginate in their matrix (Ma *et al.*, 2009). Alginate confers stability on the biofilm, protects the member cells and contributes to water and nutrient retention (Sutherland, 2001). These properties are desirable in CF lung infections where resilience is key to survival in an environment of host immune defence mechanisms and antibiotic insults.

Mucoid phenotypes are difficult to eradicate and they contribute more to antibiotic resistance. The persistence of cells for longer in the airway predisposes infected lungs to more tissue damage and persistence of infections. Alginate also protects the bacterial cells by scavenging for the free radicals released from the neutrophils (Simpson *et al.*, 1989).

1.5.4. Hypermutability

In a chronic CF lung infection, hypermutable strains predominate. These phenotypes are over a thousand times more predominant than the non-mutator variants (Rodriguez-Rojas *et al.*, 2012). Defects in some anti-mutator genes e.g. *mutS, mutL, uvrD* are most frequently involved (Macia *et al.*, 2005, Oliver *et al.*, 2000). Other inactivated non-mutator genes reported are *mutY, mutT* and *mutM* (Ciofu *et al.,* 2010).

The extreme selective environment in the CF lung promotes the evolution of these hypermutable strains. An inflammatory response from the host induces the stimulation of reactive oxygen species (ROS). These ROS are lethal to the bacteria and are able to kill a considerable number of them, but they also

induce oxidative stress on the genome of the surviving population causing mutations (Ciofu *et al.*, 2005).

Another property contributing to the evolution of hypermutable strains is antibiotic pressure. Sub-inhibitory and sub-lethal doses of antibiotics promote the selection for these strains (Wright *et al.*, 2013, Macia *et al.*, 2005). Several genes undergo different mutation types to evolve a mixed prevailing population that is better fitted for the CF lung micro-environment causing intra-clonal diversification (Jorth *et al.*, 2015).

1.5.5. Resistance

Cells in a biofilm are about a thousand times more resistant to antibiotics than their planktonic counterparts (Mah *et al.*, 2003). The spatial distribution of cells in biofilm ensures that some cells are not accessible to antimicrobials and host immune clearance. These 'persister' cells survive several bouts of antimicrobial insults acquiring resistance genes and mutations down many generations to ensure the continued existence of established infections (Folsom *et al.*, 2010).

Persister cells are a dormant population of cells in a biofilm that are usually isolated from deeper layers that are shielded from antimicrobial and immunological clearance. Persister cells of chronic infections (called high persister – *hip*) mutants are survivors of prolonged antibiotic therapy and have acquired several mutations which they vertically pass on when favourable conditions interrupt their dormancy (Lewis, 2012). Furthermore, since many antibiotics act better on actively dividing cells, a state of dormancy confers a reduced metabolic rate and reduces the sensitivity of cells to antibiotics.

Resistance mechanisms in biofilms can also be by horizontal gene transfer. Biofilm architecture establishes close proximity

of cells and encourages horizontal exchanges of virulence traits which fortify the growing microecology (Madsen *et al.*, 2012).

Although *P. aeruginosa* is known to be an obligate aerobe, the spatial distribution and prevailing pathology in the CF lung usually gives rise to altered CF phenotypes. These so called CF phenotypes are microaerophilic/ anaerobic and are able to survive in the anoxic and hypoxic niches of the CF airway by fermentation/denitrification of arginine (Hassett *et al.*, 2009). Most conventional methods of selecting anti-pseudomonal antibiotics use susceptibility testing against isolates grown in aerobic conditions to mimic the typical aerobic nature of *P. aeruginosa*. These methods cannot replicate the biophysical environment of the CF lungs which harbour a predominant population of non-typical anaerobic / microaerophilic CF phenotypes. Thus, the resistance of isolates in clinical infections is in discordance with the susceptibility profiles at the bench.

1.6. Biofilm models

Although the biofilm phenotype has contributed significantly to the resistance of infections caused by organisms that persist predominantly in biofilms, the technical constraints in the development of biofilms in conditions that closely replicate what occurs in clinical infections is also a major setback (Moreau-Marquis *et al.*, 2008). The Centre for Disease Control and Prevention (CDC) estimated the prevalence of biofilms in clinical infections at 65% (Potera, 1999). The high prevalence in human infections has generated interest in the study of the special features of biofilms. This warranted the development

of various biofilm models which have emerged over the years with creative modifications to accommodate variations of environmental conditions that closely mimic clinical infections. A general limitation for these models is that they do not perfectly reproduce the host environment. However, they have contributed immensely to our understanding of the physiology of biofilms. Many models have been developed and they are majorly classified into *in vitro, ex vivo* and *in vivo* models (Lebeaux *et al.*, 2013).

1.6.1. *In vitro* models

These are the most common. They are easy to set up and use, cheap, and can be easily modified for high throughput analysis. The over simplified nature and inability to incorporate host factors are the major set-backs. Three groups of *in vitro* models are described. These are static models, dynamic systems and microcosms.

a) Static models

Static models are very useful in quantifying the mass of a biofilm and are easily stained with crystal violet or congo red for this purpose or with XTT reduction assay for viable cell count (Lebeaux *et al.*, 2013). It has found applicability in the study of early events of biofilm formation such as adherence and micro-colony formation (Merritt *et al.*, 2005). The models are easy to set up using common laboratory equipment and following simple protocols. However, the static nature of these models means there is no exchange of medium and formation of mature biofilms may be delayed.

One example of a static model is the colony biofilm in which biofilms are grown in colonies on semipermeable membranes placed on agar plates. The membranes can be transferred to different agars containing different constituents e.g. varied

carbon source, antibiotics etc. It is especially useful for antibiotic sensitivity assessment since changes in the colony population is solely attributed to cell death and not detachment or migration (Walters *et al.*, 2003). Other examples are micro-titre plate models in which biofilms attach to the walls of the wells (Christensen *et al.*, 1985), and Calgary Biofilm Device in which the cover of the well plates have detachable pegs on which biofilms are cultivated (Ceri *et al.*, 1999). The Kadouri Drip-fed biofilm model is mid-way between static and dynamic systems. It incorporates inflow and outflow channels to the well lids for a very slow exchange of medium and can be used for genomic and proteomic studies (Jurgens *et al.*, 2008).

b) Dynamic systems

Dynamic systems mimic a continuous culture procedure in which there is a constant exchange of spent medium for a fresh one in a one-way laminar flow. Dynamic systems allow the regulation of the prevailing conditions to closely mimic *in vivo* physiological conditions e.g. the shear force of the media can be controlled for generating healthy and mature biofilms. The models also allow high resolution microscopic visualisation and analysis at single cell level, and other analytical profiling such as microarray and proteomics. Dynamic systems are usually employed in the study of antibiotics resistance and anti-biofilm properties of novel antimicrobial agents.

One example of a dynamic system is the flow cell apparatus or Robbins Device (named after the inventor), which was later renamed the Modified Robbins Device (McCoy *et al.*, 1981). It essentially bears retractable plugs onto which coupons made of different materials may be inserted to cultivate biofilms. Peristaltic pumps also ensure the flow of micro-organisms, antibiotics, nutrients and other parameters which can be varied accordingly. Other examples are the CDC Biofilm Reactors

which regulate the shear force generated through a magnetic stir bar with modifications for applications with *in vivo* models (Williams *et al.*, 2011), drip flow reactors which allow biofilm development at the solid-liquid and solid-air interphases (Schwartz *et al.*, 2010) and the BioFlux® Device by Fluxion Biosciences which allows individual shear force regulation of the well channels via a pneumatic pump. The BioFlux® Device has an added advantage of a high throughput analysis and single cell studies within the biofilm (Benoit *et al.*, 2010).

c) Microcosms

Microcosms are developed to mimic closely the *in situ* environment. The addition of some essential material(s) that make(s) a particular habitat unique can be used to transform a model into a microcosm in order to study biofilms in prevailing environments that closely replicate physiologic conditions. Essentially, both static and dynamic biofilm models may be modified into microcosms e.g. in Zürich Oral Biofilm-model, the study of dental biofilms in microcosms required the addition of saliva and hydroxyapatite to the medium (Guggenheim *et al.*, 2004) while the addition of human endothelial cells provided the exact receptors to study the mechanisms of adherence of *C. albicans* to endothelium (Grubb *et al.*, 2009) and the addition of HeLa cells in co-culture with enterohaemorrhagic *E. coli* (EHEC) was used to mimic infections in the gut (Kim *et al.*, 2010).

1.6.2. *Ex vivo* models

Ex vivo models are animal tissues or organs which are suspended in artificial growth media for the purpose of experimentation under the specific conditions the organ or tissue affords. *Ex vivo* models are in between *in vivo* and *in vitro* models. The models are usually tissues or organs taken from vertebrates for experiments which may have otherwise been

disapproved using *in vivo* methods for ethical reasons. They can also be used to measure time points in the course of an experiment especially for image generation and analyses involving special manoeuvres. Examples of tissues that are usually involved in these model systems are teeth (Huang *et al.*, 2008), heart valves (Chuang-Smith *et al.*, 2010) and vaginal mucosa (Harriott *et al.*, 2010).

1.6.3. *In vivo* models

Simplification of experimental procedure and analysis is provided by *in vitro* biofilm models. However, the true test of experimental hypothesis and applicability to target (usually higher) organisms is important and are not achievable in *in vitro* models. Thus, *in vivo* models are used to answer such pertinent questions like the relevance of such experimental procedures to human or animal therapy and host-microbe interaction. Due to the ethical hurdles involved in the use of higher mammals in experimentation, *in vivo* models are usually limited to non-mammals e.g. plants (Schlaich, 2011), insects (Kounatidis & Ligoxygakis, 2012) worms (Marsh & May, 2012) and fish (Kanther & Rawls, 2010), and site-specific models in lower mammals e.g. mice (Clarke *et al.*, 1992, Bragonzi, 2010, Hoffmann *et al.*, 2007).

1.7. Co-operation and competition in *P. aeruginosa*

Two properties of cooperation and competition are exhibited by *P. aeruginosa*. In terms of cooperation, Quorum Sensing (QS) is a major strategy used by *P. aeruginosa* to harmonise the behaviour of the participating members of a strain for the collective good. However, the presence of competitors that share the same habitat and resources necessitates the need for

the defence of the community. To a large extent, the pyocins are important in killing competitors and preserving the niche.

1.7.1. Quorum sensing

Quorum Sensing (QS) refers to a process of intercellular communication whereby bacterial cells produce, detect and respond to diffusible signal molecules (autoinducers) for coordinated specific behaviours. The concentration of signals is proportional to cell population density and at a threshold concentration, bacterial behaviour and gene expression are altered in a concerted fashion. Some QS controlled processes are biofilm formation, virulence factor production, antibiotic production and sporulation (Williams & Camara, 2009).

The first autoinducer signals described in *P. aeruginosa* belong to the acyl-homoserine lactone (AHL) family (Salmond *et al.*, 1995). The AHL system has two main circuits: Las and Rhl. The Las system is made up of the *lasI* and *lasR* genes while the Rhl system contains the *rhlI* and *rhlR* genes. The Las system hierarchically co-ordinates the Rhl system and both systems regulate the expression of hundreds of genes (Wagner *et al.*, 2003). Autoinducers secreted by the LasI/LasR and RhlI/RhlR systems are 3-oxo-C12-homoserine lactone (3O-C12-HSL) and butanoyl homoserine lactone (C4-HSL) respectively. A second class of autoinducers involved in QS belong to the alkylquinolone family (Heeb *et al.*, 2011).

In the AHL system, *lasI* gene product (LasI) is an AHL synthase which synthesises 3O-C12-HSL while the *lasR* gene product (LasR) is a transcriptional regulator. LasR detects and binds to 3O-C12-HSL for its own activation (Scotet *et al.*, 2002). The LasR/3O-C12-HSL complex induces the transcription of target genes involved in virulence e.g. production of elastase, exotoxin A, proteases etc. (Gambello & Iglewski, 1991,

Gambello *et al.*, 1993). The complex also has two other targets – *lasI* for a feed forward autoinduction (Seed *et al.*, 1995) and *rhlI* for induction of the second (Rhl) circuit (Latifi *et al.*, 1996). In the Rhl system, the *rhlI* product – RhlI, an AHL synthase – produces C4-HSL which binds to RhlR. The RhlR/C4-HSL complex activates target genes involved in virulence e.g. protease, siderophore and pyocyanin production and also loops back to *rhlI* for autoinduction (Schuster *et al.*, 2003). The absolute hierarchical control of the Las system over the Rhl system has been queried because some *lasR*⁻ mutants were detected to retain the ability to express pyocyanin (Dekimpe & Deziel, 2009).

In the *Pseudomonas* Quinolone Signal (PQS) system, the *pqsA-D* genes express PqsA, PqsB, PqsC, PqsD, which produce 2-heptyl-3-hydroxi-4-quinolone (also called HHQ) while *pqsH* produces PqsH which converts HHQ to PQS (Deziel *et al.*, 2004). PQS binds to PqsR, the product of *pqsR*. PQS is however non-essential because pqsH mutants retain the ability to fully express PqsR-related functions (Xiao *et al.*, 2006). The PqsR-HHQ complex causes the auto-induction of *pqsA-D* (Diggle *et al.*, 2007b). The PQS system is intricately linked to the AHL system – *pqsABCD* and *pqsR* are repressed by the RhlR/C4-HSL complex whilst the LasR/3O-C12 complex induces the expression of *pqsR* and *pqsH* (Gallagher *et al.*, 2002). Conversely, the PqsR-HHQ in turn activates *rhlR* (Diggle *et al.*, 2007b).

PqsE is essential for the production of exoproducts like pyocyanin, elastase and rhamnolipids (Gallagher *et al.*, 2002, Diggle *et al.*, 2003). PQS is able to form iron III complexes to bind extracellular ferric ions (Fe^{3+}) acting more like an iron trapping device to reduce the concentration of iron in the extracellular environment. This enhances the production of

iron-chelating siderophores like pyoverdin and pyochelin (Diggle *et al.*, 2007b).

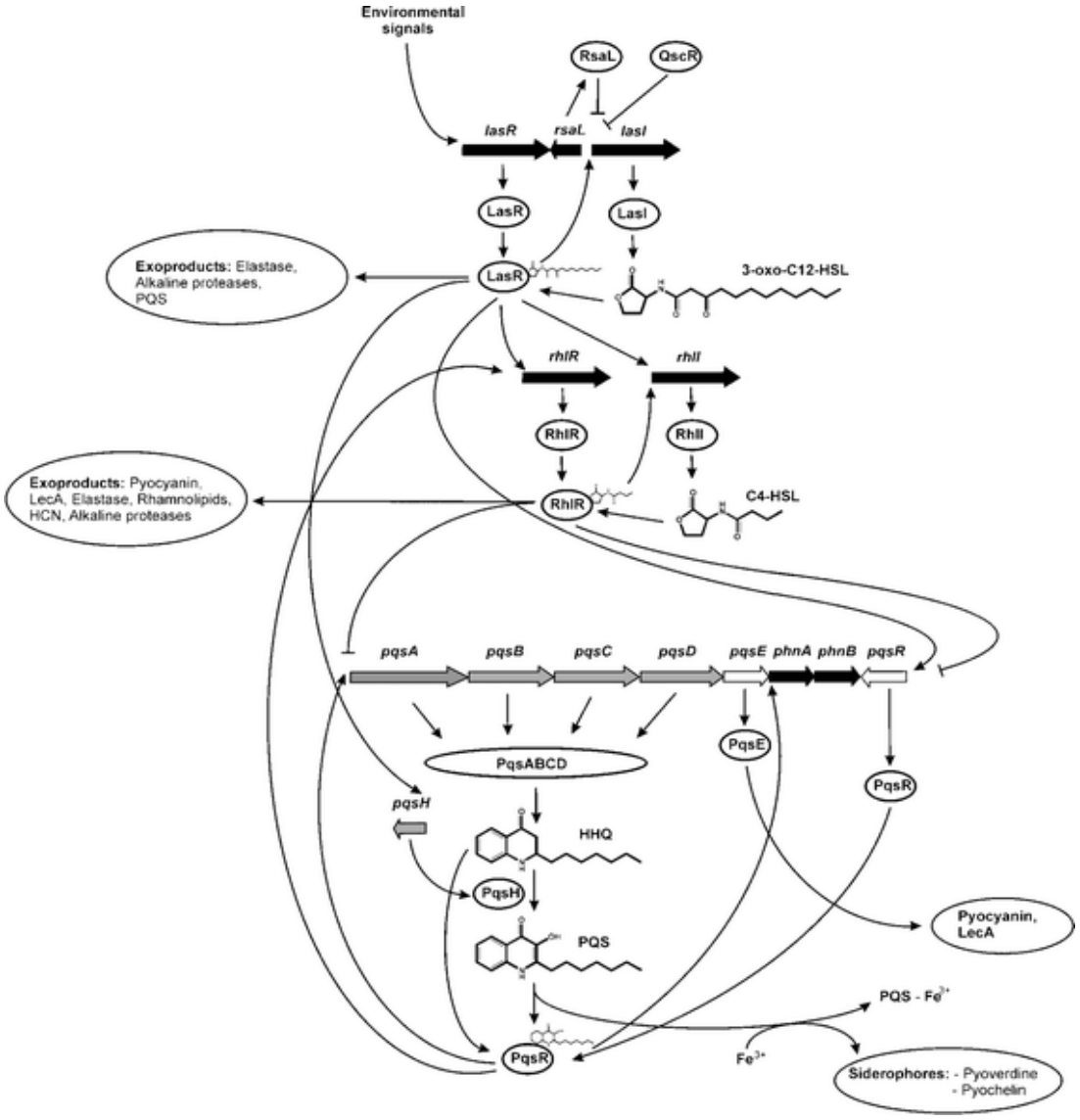


Fig. 1.2: Quorum Sensing signalling pathway of *P. aeruginosa* (Dubern & Diggle, 2008). There is an extensive network between the acyl-homoserine lactone (AHL) family and the '*Pseudomonas* Quinolone Signal' (PQS) system. The AHL family consists of LasI/LasR and RhlI/RhlR systems while the PQS system has the *pqsABCDE* operon, *pqsE, pqsH* and *pqsR*. LasR/3O-C12-HSL induces RhlI, PqsR and PqsH expression while RhlR/C4-HSL represses PqsR and PqsA-D expression. PqsE is essential for pyocyanin and lectin production. When released from the cell, PQS has the ability to trap Fe^{3+} in complexes thus causing a low extracellular iron concentration; and induction of iron-chelating siderophores.

1.7.2. Pyocins – The Bacteriocins of *P. aeruginosa*

Bacteriocins – a brief overview

Bacteriocins are a diverse family of functional, ribosomally synthesised toxins that are produced by an abundant variety of micro-organisms. The first bacteriocin discovered was colicin in 1925 by Gratia (Gratia, 1925) and its narrow specificity based on its recognition by receptors present on sensitive cells, was demonstrated in 1946 (Gratia & Fredericq, 1946). Since then, many bacteria (both Gram negative and Gram positive) and also Archaea have been reported to produce substances with similar characteristics leading to the common name of 'bacteriocins' in 1953 (Jacob *et al.*, 1953). Individual bacteriocin names have been coined which usually reflects the species from which the bacteriocin was identified e.g. colicins and pesticins from *E. coli* and *Y. pestis* respectively, and the pyocins of *P. aeruginosa* which was originally named after the species *Pseudomonas pyocera* before its name was changed to *P. aeruginosa* (Kim *et al.,* 2014a).

Although, *P. aeruginosa* produces antimicrobial agents like phenazines and lipopeptides against other bacteria and fungi (Gross & Loper, 2009), its survival in a niche is usually dependent on its ability to combat close competitors with phylogenetic relatedness, that share the same habitat, nutrients and metabolites. Therefore, specific pyocin genes or gene operons are expressed during environmental stresses such as DNA damage from a neighbouring strains to kill related strains with specific antibacterial functions (Ghequire & De Mot, 2014). The mode of killing of the pyocins is the single hit mechanism whereby one pyocin molecule effects the killing of a non-immune cell irrespective of the number of pyocin molecules adsorbed on the surface of the cell (Michel-Briand &

Baysse, 2002). The pyocins are broadly divided into the soluble pyocins (S-pyocins) and the protease resistant tailocins (R- and F-pyocins).

Pyocin evolution

The S-pyocins are closely related genetically, structurally and functionally to the colicins of *E. coli.* The genetic arrangement of the colicins consist of the toxin, an immunity gene with or without a lysis gene depending on their mechanism of action i.e. nuclease or pore forming (Riley & Wertz, 2002, Riley, 1998). The S-pyocins of *P. aeruginosa* also bear this close link between their toxin and the immunity gene but in contrast, they do not have an associated lysis gene although it has been suggested that the lytic system of the R-F pyocin locus (PA0614 and PA0629) are also shared by the S-pyocins (Nakayama *et al.*, 2000). Another difference is that colicins are borne on plasmids while S-pyocin genes are present on the genome of the producing cells (Michel-Briand & Baysse, 2002).

Structurally, the colicins are arranged in domains. The colicin protein consists of the translocation, receptor recognition, and killing domains from the N- to the C-termini, respectively. In the pyocin protein however, there is a swap between the translocation and receptor recognition domains and a fourth domain with yet unknown functions may also exist between them (Riley & Wertz, 2002). In both pyocins and the nuclease colicins, an immunity protein is cognate to the killing domain and the interaction of these two domains protect the cell from killing itself (Michel-Briand & Baysse, 2002).

Functionally, both the nuclease and pore-forming modes of action are reported in the colicins and pyocins. Colicin M and the pyocin PaeM display a lipid II hydrolase activity.

The R- and F- pyocins (the tailocins) are usually called defective phages because although they resemble bacteriophages, they lack the head region (capsid) which bears the genetic elements and thus have lost the propensity to self-replicate in a susceptible cell (Kageyama, 1985). They are essentially phage tails which have evolutionarily specialised as bacteriocins with their encoding genes integrated into the *P. aeruginosa* genome (Nakayama *et al.*, 2000). The R- and F-pyocins are related to the P2 and λ phages respectively (Nakayama *et al.*, 2000).

The evolution of the tailocins have been traced to bacteriophages that infect bacteria by punching a hole using their tail fibres. These bacteriophages belong to the *Caudovirales* order of viruses. This order is further divided into three families which are – *Siphoviridae, Myoviridae* and *Podoviridae*. Viruses belonging to these three families are non-enveloped and have double stranded linear DNA. The *Siphoviridae* family members have non-contractile tails and are classified to the lambda phage super group which is evolutionarily related to the F-pyocins.

The R-pyocins are related to the phages of the *Myoviridae* family which have a neck region made up of two concentric rings (outer contractile and central tube) and a tail which has helical symmetry (Fig. 1.3b). The lysis gene cassette (holin-like genes) present in the R- and F-pyocin loci are also similar to those in bacteriophages (Nakayama *et al.*, 2000)

Structures similar to the tailocins

The type VI secretion system (T6SS) of *P. aeruginosa* bears a close resemblance to the phage-like pyocins being often referred to as an inverted phage (Leiman *et al.*, 2009) (Fig. 1.3).

It is the most recently discovered of the many protein secretory apparatus of bacterium (Cascales & Cambillau, 2012).

Some structural components of the type VI secretion apparatus include the haemolysin co-regulated proteins (Hcp) which are arranged as stacked rings of hexamers (Mougous *et al.*, 2006) (Fig. 1.3a). This tube bears a close resemblance to the inner tubular structure of the R-pyocins through which intracellular ions escape to the exterior after pore formation (Fig. 1.3b).

The Hcp tube protein is incorporated in the larger proteins, VipA and VipB which constitute what looks like the contracted sheath of the T4 phages (Leiman *et al.*, 2009) and they effect the passage of the Hcp tube from the cell membrane to the exterior. This is much like the contractile outer sheath of the R-pyocins which shrinks to introduce the inner tube into the non-immune cell.

The VgrG protein forms a trimer capping the Hcp tube. Being too narrow to allow the passage of proteins, it disintegrates after piercing the target cell thereby allowing the injection of effector proteins (Silverman *et al.*, 2012). VgrG can also be viewed as a carrier located at the tip of the T6SS apparatus with effector protein connected to it via. This VgrG cap does not have a counterpart in the R-pyocins. Other components of the T6SS are the base plate complex proteins DotU and IcmF which is also represented in the R-pyocins. ATPase acts as the motor for protein translocation since the VipA/VipB proteins are ATPase-dependent.

The T6SS is different from the R-pyocins in many respects. Although they both exhibit a similar mechanism of action which involve the formation of a pore channel, the T6SS forms part of the living cell while R-pyocins are released after lysis of a dead cell. Also, effector proteins are injected into the target

cell through the T6SS apparatus but the R-pyocins kill sensitive cells by causing a communicating channel between the high-pressure intracellular compartment and the exterior that allows the escape of intracellular ions causing membrane depolarisation which kills the cell. Furthermore, whereas tail fibres attached to the base plate of R-pyocins function for receptor binding on sensitive cells, the base plate of the T6SS remain as part of the producing cell thus the name 'inverted phages'.

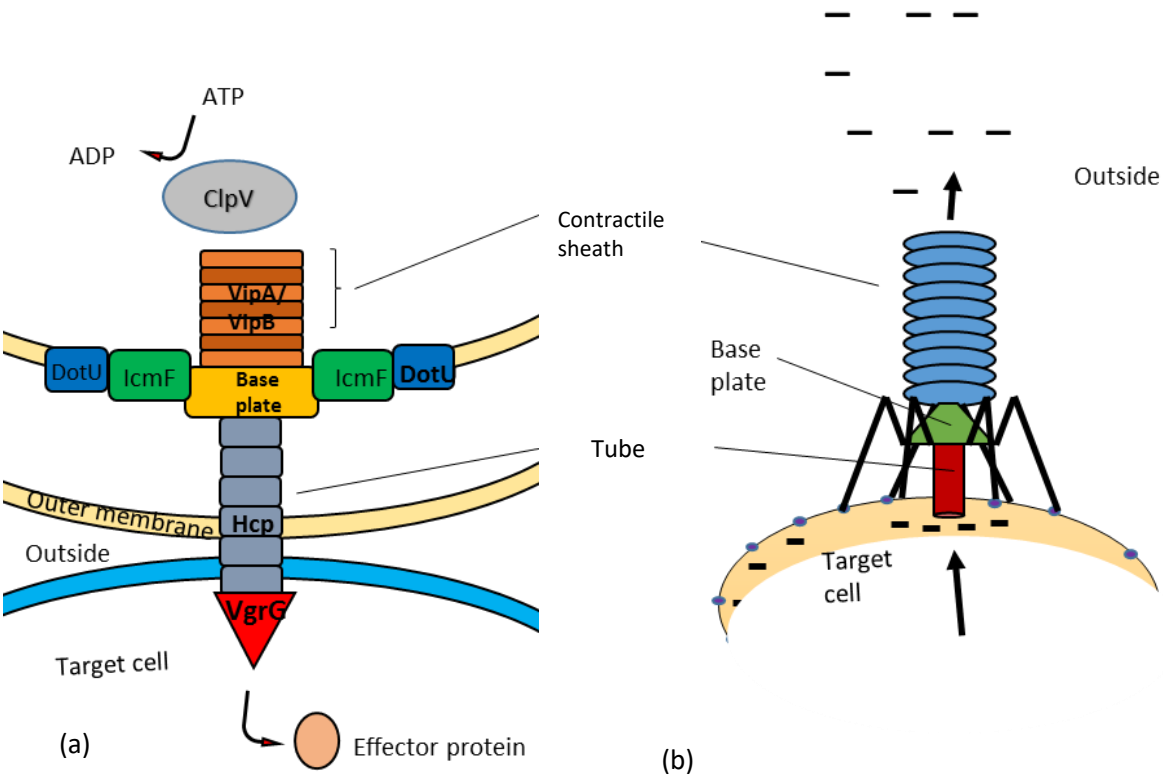


Fig. 1.3: Comparing the structures of the type VI secretion system (T6SS) apparatus (a) and the R-pyocin (b). Structures common to both include the base plate, contractile sheath and the tube. While the T6SS apparatus is part of a living cell, ATP-dependent and is used for the transport of proteins from one cell to another via the Hcp communicating tube bridge, the R-pyocin is released after the lysis of a dead cell, energy-independent and its tube connects the intracellular compartment of the target cell to the exterior causing the escape of intracellular ions and killing the cell by membrane depolarisation.

The type III secretion system (T3SS) also looks like the phage-like pyocins (Salmond & Reeves, 1993). They are assembled into what is commonly referred to as injectisomes. Their basal body is anchored on the cell membrane while the needle complex projects beyond the cell surface. It shares similarities in structure and assembly with the flagella (Pallen & Gophna, 2007, Gophna *et al.*, 2003). Much like the T6SS, they are intricately linked to the living cell from where they effect protein secretion.

Regulation of pyocin production

Pyocin production is induced by DNA damaging agents like ultraviolet (UV) radiation and mitomycin C via a RecA dependent pathway (Matsui *et al.*, 1993). These mutagenic agents trigger the expression and activation of RecA protein. Activated RecA induces the auto-cleavage of PrtR (a repressor of the *prtN* gene), leading to the expression of PrtN. PrtN binds to the P box in the promoter sequence of S-, R- and F-pyocins causing the expression of these pyocin proteins. PrtN also induces the expression of the lysis cassette genes which code for some enzymes including the holin-like and chitinase-like enzymes. The expression of these enzymes ensures the lysis of the cell and release of the pyocin molecules after they are synthesised (Fig. 1.4).

The RecA-mediated regulation of pyocins is part of a global SOS response in *P. aeruginosa*. Other RecA regulated genes are *lexA* and PA0960 (Figure 1.4). Activated RecA causes the de-repression of LexA on the LexA regulon; expression of genes in this regulon initiate the repair process after DNA damage. LexA repression is however not involved in pyocin biosynthesis

(Matsui *et al.*, 1993). PA0960 is believed to activate a set of genes whose functions are yet to be determined.

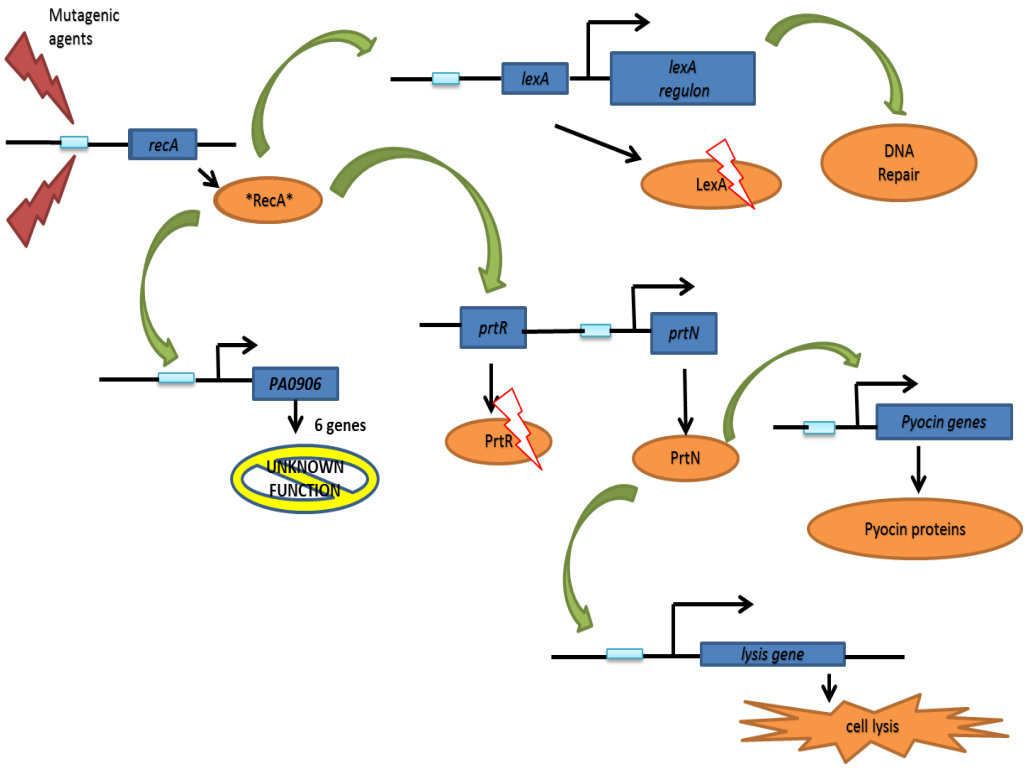


Fig. 1.4: Regulation of pyocin production shows a RecA-dependent pathway. DNA damage by mutagenic agents and the release of single stranded DNA induce the expression of RecA. Activated RecA (*RecA*) regulates the expression of three genes (*lexA, prtR* and *PA0906*). Expression and autocleavage of LexA and PrtR are involved in the DNA repair (SOS response) and pyocin expression respectively. Induction of the lysis genes *(PA0614* and *PA0629*) ensure the release of the expressed pyocin proteins. The functions of the six genes regulated by the expression of *PA0906* is yet unknown.

1.7.2.1 The S-pyocins

The S-pyocins are soluble, heat labile, proteins encoded on the chromosome. Sub-classes described so far are S1 (Ito *et al.*, 1970), S2 (Ohkawa & Kageyama, 1973), S3 (Duport *et al.*, 1995), S4 (Elfarash *et al.*, 2012), S5 (Ling *et al.*, 2010), S6 (Dingemans *et al.*, 2016), AP41 (Holloway *et al.*, 1973), PaeM (Barreteau *et al.*, 2009), SD1, SD2 and SD3 (McCaughey *et al.*, 2016)

A second protein known as the immunity protein is required to protect the producing cell from the lethal effects of the toxin. In the case of the nuclease type pyocins, the toxin + immunity protein form a binary complex. The domain architecture of the toxin – from the amino to the carboxyl terminals – are for receptor binding, translocation and enzymatic action respectively. A fourth domain of unknown function has been reported between the domains for binding and translocation (Michel-Briand & Baysse, 2002) (Fig. 1.5). Although the function is yet to be defined, it has been reported that this domain is indispensable in the killing mechanism (Sano *et al.*, 1993a) although this is disputable because the same domain is absent in the fully functional S2-pyocin (Michel-Briand & Baysse, 2002). The lethal pyocins do not kill the producing strain because the immunity protein confers protection by the interaction of its amino- terminal with the cognate carboxyl-terminal of the enzymatic domain of the toxin (Michel-Briand & Baysse, 2002, Joshi *et al.*, 2015).

The enzymatic domain of the different S type nuclease pyocins have different mechanisms of action. Pyocins S1, S2 (Seo & Galloway, 1990, Sano, 1993) and AP41 (Duport *et al.*, 1995) have a DNase activity using a conserved HNH motif (Joshi *et al.*, 2015); S3 also has a DNase activity but does not have the HNH motif (Parret & De Mot, 2002). S4 (Elfarash *et al.*, 2012) and SD2 (McCaughey *et al.*, 2016) have tRNase activity while S6 has an

rRNase activity (Dingemans *et al.*, 2016). The S5 pyocin has a pore-forming activity thus causing membrane damage and leakage of intracellular molecules (Ling *et al.*, 2010). Pyocin PaeM has a narrow spectrum of activity, it acts in the periplasm by blocking peptidoglycan synthesis and its action is bacteriostatic (Barreteau *et al.*, 2009).

The nuclease type S-pyocins are released as a protein complex consisting of the structural and immunity components in equimolar ratio (Sano & Kageyama, 1981). The transcription of the two proteins are in tandem and the Shine-Dalgarno complex of the immunity protein are present in the coding sequence of the enzymatic domain (Sano *et al.*, 1993b). In contrast however, the immunity gene of pyocin S5 is transcribed in the reverse orientation (Stover *et al.*, 2000). Some strains have 'orphan' immunity genes which are not linked to a structural gene but confer immunity against the corresponding enzymatic domain (Denayer *et al.*, 2007, Elfarash *et al.*, 2014, Dingemans *et al.*, 2016).

In iron-poor conditions, *P. aeruginosa* secretes siderophores (pyoverdine and pyochelin) which scavenge for and bind iron (Fe^{3+}). Iron-siderophore complexes (ferripyoverdine and ferripyochelin) are taken up into the cells by iron-regulated outer-membrane proteins (IROMPs) e.g FpvA and FptA. These IROMPs are also utilised by S-pyocins to gain entry into cells (Ohkawa *et al.*, 1980). Three types of ferripyoverdin receptors are known (FpvAI, FpvAII and FpvAIII). Type I receptors (FpvAI) are employed by pyocins S2 and S4 (Elfarash *et al.*, 2012) while the Type II receptors (FpvAII) are used by S3 pyocins (Baysse *et al.*, 1999); the ferripyochelin receptor (FptA) is utilised by pyocin S5 (Elfarash *et al.*, 2014).

Following the interaction with receptors, the pyocins are translocated across the cell membrane in order to get to their target sites. The use of ferrisiderophore receptors by S-pyocins have suggested the involvement of the same translocation mechanism used for iron uptake. Therefore, although the mechanism of S-pyocin translocation is not fully understood, the TonB system utilised in iron uptake is a possibility (Cornelis & Dingemans, 2013) (Figure 1.6). The proposed translocation pathway of the AP41 pyocin is via the Tol machinery encoded by three operons (*orf1-tolQRA, tolB* and *oprL-orf2*) (Duan *et al.*, 2000) (Figure 1.6). Complementation of *tolQRA* gene into a *tol* mutant restored its sensitivity to AP41 pyocin (Dennis *et al.*, 1996); however the AP41 receptor is yet unknown.

The TonB-dependent or Tol-dependent translocation systems are a group of interacting proteins that are involved with maintaining cell integrity and transducing energy generated by the proton motif force (PMF) from the inner membrane to the cell envelope necessary for substrate uptake (Lloubes *et al.*, 2012). In addition to an outer membrane receptor such as BtuB, Cir, FepA or FhuA, the TonB-dependent system consists of three inner membrane proteins, TonB, ExbB and ExbD whilst the Tol-dependent system consists of five interacting proteins, TolA, TolB, TolQ, TolR and Pal (Jakes & Cramer, 2012) (Figure 1.6). ExbB and ExbD, and TolQ and TolR are functionally analogous because mutations in one set of these proteins can be compensated by expression of the other (Jakes & Cramer, 2012, Witty *et al.*, 2002).

Fig. 1.5: Domain organisation of the structural and immunity proteins of the S-pyocin. From the N- to the C-terminals, domain I is the receptor binding domain, domain II does no specific function, domain III is the translocation domain whilst domain IV is the killing domain (e.g. a DNAse). The immunity protein (imm) is cognate to domain IV and interacts with it to prevent the lethal activity of the killing domain against the producing strain.

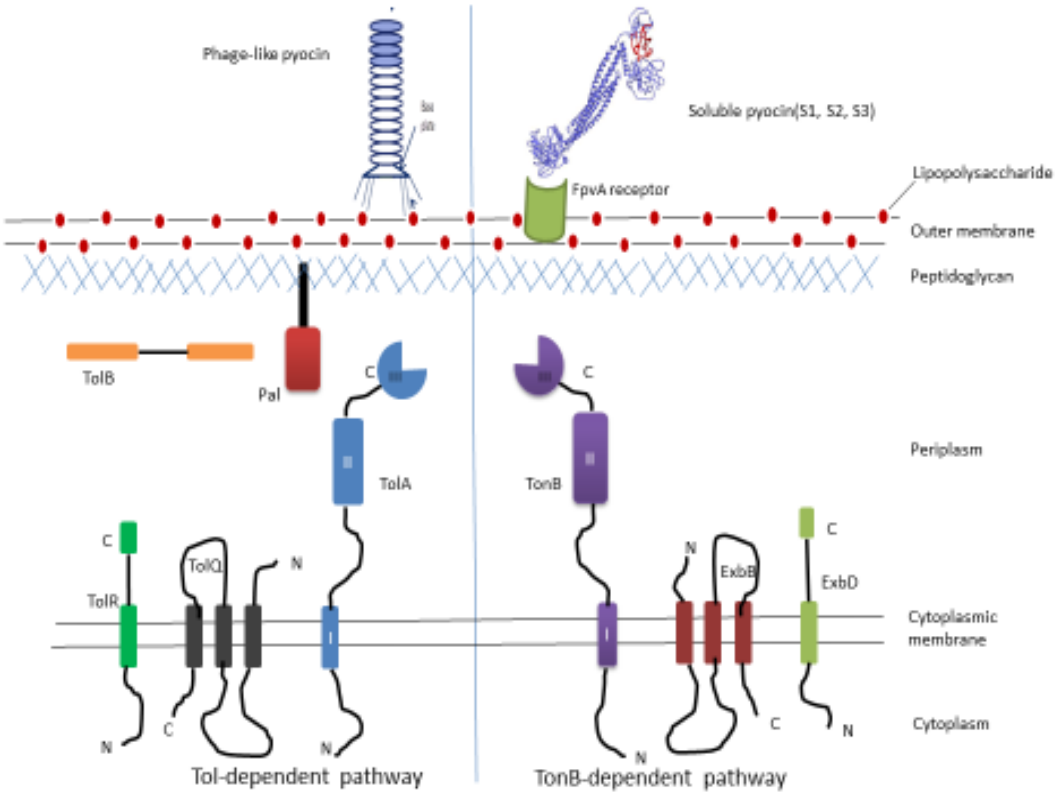


Fig. 1.6: Tol-dependent and TonB-dependent proposed pathways of pyocin translocation into the active site. Tol proteins are TolQRA, TolB and Pal while the inner membrane proteins of the TonB system are ExbB, ExbD and TonB. Supposedly, the TonB pathway is utilised by the S-pyocin while the tailocins (and AP41) employ the Tol pathway.

1.7.2.2 The Tailocins

a) R-F pyocin locus

The R2 and F2 pyocin genes of PAO1 are sequentially arranged between the genes for tryptophan biosynthesis (*trpE* and *trpG*), forming a cluster (Bakkal *et al.*, 2010). The R2-F2 pyocin gene locus of PAO1 strain spans genes PA0615 to PA0648 with lysis gene cassettes interposed between them (Chang *et al.*, 2005) (Fig. 1.7) – the locus of R2 are PA0615-PA0628 and that of F2 are PA0633-PA0648. Genes PA0614 and PA0629 are the lysis genes used in a study by Penterman *et al* (Penterman *et al.*, 2014). Nakayama *et al* studied different strains of *P. aeruginosa* which produced various classes of the R- and F-type pyocins and demonstrated that all R- and F-type pyocin classes have this same sequential arrangement between the *trpE* and *trpG* genes (Nakayama *et al.*, 2000) (Figure 1.7).

Some strains *of P. aeruginosa* have been characterised which lack either the R- or the F-pyocin locus. LESB58 strain for example lacks the F-pyocin locus (Winstanley *et al.*, 2009) whilst PL14 strain lacks the R-pyocin locus (Nakayama *et al.*, 2000) and PA14 strain possesses a defective F-pyocin (Heo *et al.*, 2007)

The loci for the S-type pyocin genes do not have a permanent predictable location on the genome unlike the R- and F-pyocin loci. They are usually scattered throughout the genome and an organism is able to produce more than one class of the S-type pyocin e.g. the PAO1 strain produces S2, S4 and S5 pyocins from genes PA1150, PA3866 and PA0985 respectively (Winsor *et al.*, 2011). However, the S-type pyocin expression is still under the regulation of the *recA, prtN* and *prtR* regulatory genes (Michel-Briand & Baysse, 2002, Matsui *et al.*, 1993).

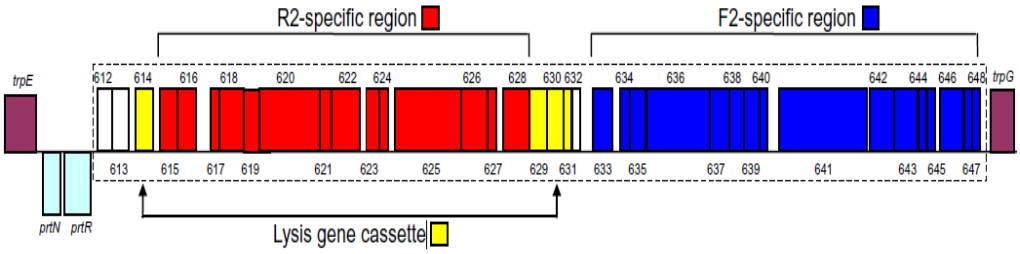


Fig. 1.7: Genetic organisation of the R/F pyocin locus between genes for tryptophan biosynthesis *trpE* and *trpG* (Chang *et al.*, 2005, Michel-Briand & Baysse, 2002, Nakayama *et al.*, 2000). In the R2/F2 locus of PAO1 genome represented above the R2-specific pyocin operon is genes PA0615-PA0628 while F2-pyocin operon is genes PA0633-PA0648. Lysis gene cassettes exist upstream of each operon and in this case they are recognised as genes PA0614 and PA0629 (yellow boxes). Regulatory genes *prtN* and *prtR* (light blue boxes) are located upstream of the R/F locus.

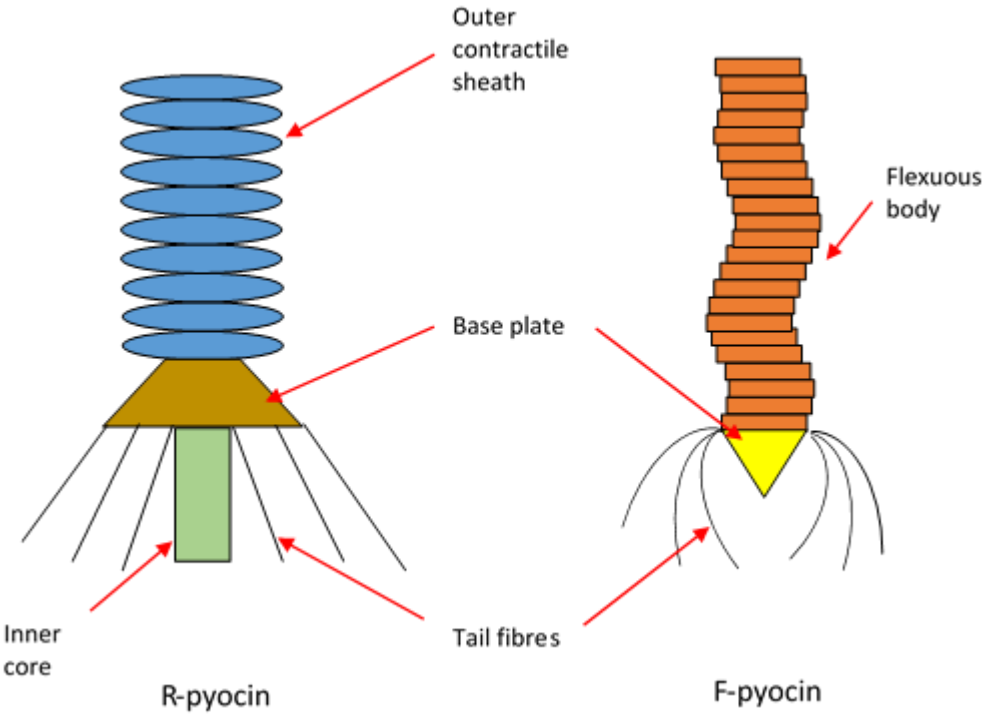


Fig. 1.8: Schematic representation of the structures of the tailocins (R- and F-pyocins). They resemble the tail region of bacteriophages, hence the name tailocins. They lack the head region containing the genetic materials but attach to sensitive cells by using tail fibres. The R-pyocin has a rigid structure and two concentric rings, the inner tube penetrates the cell, forming a pore for the escape of intracellular ions. The F-pyocin has a flexuous body and a base plate connecting the body to the tail fibres.

b) F-pyocins

The F-pyocin was first described by Takeya *et al* in 1967 (Takeya *et al.*, 1967). This high molecular weight bacteriocin of *P. aeruginosa* was reportedly produced by a strain which also produced an R-pyocin (Govan, 1974a). Much like the R-type pyocins, it could be isolated by ultracentrifugation and is also protease resistant (Michel-Briand & Baysse, 2002). However, it has no serological cross reactivity with the antisera of the R-type pyocins (Kuroda & Kageyama, 1981) but shows cross reactivity with phage KF1 (Kuroda & Kagiyama, 1983).

F-pyocins are rod-like flexuous particles resembling the members of the lambda (λ) phage family (Nakayama *et al.*, 2000). They are non-contractile and possess tail fibres which confer receptor specificity (Kuroda & Kageyama, 1979, Kuroda & Kageyama, 1981) (Fig. 1.8). Unlike a bacteriophage, they lack the genetic determinants and ability for autonomous replication (Kuroda & Kagiyama, 1983). The lipopolysaccharide component of the cell envelope of susceptible cells is the receptor for the tail fibres and they effect killing by cytoplasmic membrane depolarisation (Kuroda & Kagiyama, 1983).

F-type pyocins described so far include F1 and F2 (Kuroda & Kageyama, 1979), F3 (Kuroda & Kageyama, 1981), pyocin 28 (Takeya *et al.*, 1967), pyocin 430f (Govan, 1974b).

c) R-pyocins

The R-pyocins are particulate high molecular weight bacteriocins of *P. aeruginosa* first described by Francois Jacob in 1954 (Jacob, 1954). They are rigid rod-like contractile particles that lack DNA and ability for self-replication

(Nakayama *et al.*, 2000) (Fig. 1.8). R-type pyocins are about 1-2 x 10^7 Da in weight and have dimensions 120 nm by 15 nm in length and width, respectively (Kageyama *et al.*, 1964). Like the F-pyocins, they can be isolated by ultracentrifugation at ≥100,000 x *g*, are protease resistant and can be neutralised by their specific antisera (Nakayama *et al.*, 2000).

On average, 200 R-pyocin molecules are produced by a cell after induction (Shinomiy.T, 1972). One or two of these are adsorbed on the surface of a susceptible cell. In contrast, 200 - 300 molecules of S-pyocins are adsorbed on a single cell which is subsequently killed by only one molecule by the single hit mechanism (Michel-Briand & Baysse, 2002). This makes the R-pyocins a more effective weapon of bacterial warfare. Furthermore, unlike the S-pyocins with a restricted spectrum to other *Pseudomonas spp,*, the R-pyocins cover a wider scope involving other Gram negative organisms including member strains of *Neisseriae* (Morse *et al.*, 1976), *Haemophilus* (Phillips *et al.*, 1990) and *Campylobacter spp.* (Blackwell *et al.*, 1982).

Structurally, an R-type pyocin molecule consists of a double hollow tube with an inner core and an outer contractile sheath. Attached to the distal end of the tube is the base plate from which tail fibres emerge. The tail fibres are six in number and are symmetrically arranged on the base plate (Michel-Briand & Baysse, 2002). These tail fibres are divided into two ends: The N-terminal end which attaches to the base plate termed the base plate attachment region (BPAR) and the C-terminal free end which is the receptor binding domain (RBD). The BPAR is more conserved than the RBD (Tetart *et al.*, 1998). Specificity of the spectrum of susceptible strains is determined by the more variable RBD (Scholl & Williams, 2010). Genetic engineering involving the RBD has been used to extend the spectrum of antimicrobial activity of the R-pyocins to *Yersinia*

pestis (Williams *et al.*, 2008) and *E. coli* O157:H7 (Ritchie *et al.*, 2011).

The tail fibre protein Prf15 is encoded by the *prf15* gene (gene PA0620 on the R2-pyocin locus of PAO1 strain) while its cognate chaperone protein Prf16 is encoded by the adjacent *prf16* gene (gene PA0621 of PAO1) (Williams *et al.*, 2008). The chaperone protein ensures the proper folding of the tail fibre protein and the coordinated assembly of the fibres on the base plate without necessarily forming part of the bacteriocin. Both Prf15 and Prf16 proteins are believed to have co-evolved from a common parent (Scholl & Williams, 2010).

The tail fibres bind to the LPS of susceptible cells using their RBDs (Scholl & Williams, 2010). Contraction of the sheath brings the core in contact with the cell which traverses the entire cell envelope to come in contact with the cell membrane. Penetration of the cell membrane causes loss of the intracellular negative charge through the hollow core. This depolarisation causes disruption of the cell membrane potential, loss of the cell integrity, arrest of nucleic acid and protein synthesis and eventual cell death (Uratani & Hoshino, 1984).

Based on target spectra, five classes of R-type pyocins (R1 to R5) have been identified (Williams *et al.*, 2008). The class R5 exhibits the widest spectrum of activity encompassing the spectra of R1, R2, R3, and R4. The receptor specificities of R1 to R4 appear to diverge into two family branches from a common R5 root. R1 occupies a branch and exhibits no relatedness to the other three. The scope of R2 covers the spectra of R4 and R3 while that of R4 encompasses the spectra of R3. It follows that any *P. aeruginosa* strain that is susceptible to any of R1, R2, R3 or R4 pyocins is also susceptible to R5

pyocin. However, some strains are susceptible only to R5 pyocin while some are resistant to all classes of R-type pyocins (Scholl *et al.*, 2009).

With R2-pyocin (produced by PAO1) as a reference point, R3 and R4 pyocins show closer amino acid sequence identity than R1 and R5. The diversity in the tail fibre protein PRF15 is more pronounced from the amino acid 430 to the terminus while 1-429 are more conserved. The cognate chaperone protein PRF16 shows a wider diversity with R1 and R5 showing only 32% and 35% homology to the R2 chaperone protein, respectively (Scholl & Williams, 2010) (Table 1.3).

The lipopolysaccharide (LPS) was identified early as the receptor for R-pyocins (Ikeda & Egami, 1969). The possibility of sharing common LPS receptors with other bacteria has been proposed as the reason why other organisms are sensitive to R-pyocins including *Haemophilus influenzae* (Phillips *et al.*, 1990), *Neisseria gonorrhoeae* (Morse *et al.*, 1976) and *Haemophilus ducreyi* (Campagnari *et al.*, 1994). The involvement of the LPS core was reported by Meadow *et al.* (Meadow & Wells, 1978). However, more specifically, some sugar residues in the outer core of the LPS have been identified as receptors for R1, R2 and R5 pyocins – these are L-Rhamnose (R1) and two separate residues of α-Glucose (R2 and R5) (Kohler *et al.*, 2010).

R-pyocin class	% *prf15*	%*prf15* (aa 1-429)	%*prf15* (aa 430-end)	% *prf16*
R1	82	99	52	32
R2	100	100	100	100
R3	99	99	98	98
R4	98	99	99	99
R5	83	97	58	35

Table 1.3: Percentage amino acid sequence homology of *prf15* (*PA0620* in PAO1 prototype) and *prf16* (*PA0621* in PAO1) in pyocins R1 to R5 using R2 as reference. For the *prf15* (tail fibre) gene, the divergence of homology is more pronounced from amino acid 430 to the end while 1-429 are more conserved. The homology of *prf16* (chaperone) gene of R1 and R5 are even more divergent from R2 showing only 32% and 35% homology for R1 and R5 respectively (Scholl & Williams, 2010).

1.7.3. Lectin-like bacteriocins

These are protein antibiotics first isolated in a plant strain *P. putida* BW11M1 with the ability to kill other plant species of *Pseudomonas* (Parret *et al.*, 2003). They contain two carbohydrate domains belonging to the monocot mannose-binding lectin (MMBL) family. Unlike the S-pyocins they lack the active cytotoxic domain and the protective immunity protein. They are usually genus specific killing strains that are limited to the *Pseudomonas* genus.

A similar bacteriocin found in *P. aeruginosa* is pyocin L1 It was isolated from a CF patient. It utilises the common polysaccharide antigen (CPA) of the LPS as a receptor using the D-rhamnose ligands (McCaughey *et al.*, 2014). The wide spread inclusion of D-rhamnose in the O-antigen of the *Pseudomonas* strains could explain the genus-specific activity of these lectin-like bacteriocins (McCaughey *et al.*, 2014).

1.8. Objectives of the study

Working with 24 clinical *P. aeruginosa* strains isolated from lung infections of cystic fibrosis patients, this study aims to investigate the pyocin profile of the major sub-classes of the S- and R-pyocins in these isolates, their role in competition and R-pyocin function as an anti-biofilm agent. Furthermore, it will seek an understanding of the genetic bases of pyocin resistance and also probe a possible link between quorum sensing (QS) and pyocin genes by constructing transcriptional bioreporters to study the expression of R-pyocin genes in wild type and QS-deficient *(lasR* mutants) *P. aeruginosa* strains.

Chapter 2

Materials and Methods

2.1 Chemicals and kits

All chemicals used were supplied by Sigma Aldrich™ (UK) unless otherwise stated.

2.2 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are outlined in tables 2.1.

2.3 Culture conditions, media and buffers

2.3.1 LB media

E. coli and *P. aeruginosa* strains were routinely cultured in Luria Bertani media – broth and agar, at 37 °C overnight. For the generation of mutants, *P. aeruginosa* strains were cultured in LB broth at 42 °C overnight. The media LB (5 g Oxoid yeast extract, 10 g Oxoid bacto peptone, 10 g Oxoid sodium chloride per litre) was autoclaved at 121 °C at 15 psi for 20 min before use (Sambrook & Russel, 2001)

2.3.2 Minimal media M9

For the generation of biofilms on beads, *P. aeruginosa* strains were cultured in minimal media M9 at 37 °C over 24 h shaking at 80 rpm. The minimal media M9 was made from autoclaved 10 x basal salt solution (68 g/L Na_2HPO_4, 30 g/L KH_2PO_4 and 5 g/L NaCl), supplements (1M NH_4Cl, 1M $CaCl_2$ and 1M $MgSO_4.7H_2O$ autoclaved separately) and 100 x citric acid (10 g in 50 ml) as the carbon source. The final composition of the constituents in M9 media was: 10% of the 10 x basal salt solution, 1% 1M NH_4Cl, 0.01% 1M $CaCl_2$, 0.1% 1M $MgSO_4.7H_2O$ and 1% of the 100 x citrate which was added through a membrane filter after cooling down the autoclaved components.

2.3.3 G medium

The G medium was used to cultivate *P. aeruginosa* strains for R-pyocin expression and purification. The strains were cultured at 37 °C and shaken at 200 rpm until they achieved log phase (3-4 h). The G medium composed of 20 g/L sodium glutamate, 5 g/L glucose, 2.23 g/L Na_2HPO_4, 500 mg/L yeast extract, 250 mg/L KH_2PO_4 and 100 mg/L $MgSO_4.7H_2O$.

2.3.4 TN50 buffer

TN50 buffer was used to keep purified R-pyocins in a stable state after purification (stable for 60 days at 4 °C) (Scholl & Martin, 2008). The constituents were 50 mM NaCl and 10 mM Tris HCl adjusted to pH 7.5.

2.4 Antibiotics

Antibiotics were used to maintain plasmids in *E. coli* strains, DH5α and S17-1 and also to select for strains containing the desired plasmids. The lactose phenotypes of pGEM-T were selected in DH5α with 100 µg/ml ampicillin and 40 µg/ml X-gal; others were tetracycline 5 µg/ml (*E. coli*), 200 µg/ml (*P. aeruginosa*) and gentamicin 10 µg/ml (*E. coli*), 100 µg/ml (*P. aeruginosa*).

***E. coli* strains**		
DH5α	*F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1*	(Sambrook & Russel, 2001)
S17-1 *λpir*	*galU galK rpsL(Str^{R}) endA1 nupG thi pro hsdR hsdM^{+} recA* (RP4-2 Tc::Mu Km::Tn7) λpir	(Simon *et al.*, 1983)
S17-1 *λpir* mini-CTX-*lux*	S17-1 λpir containing the promoterless mini-CTX-*lux* plasmid, Tet^{R}	This study
S17-1 *λpir* mini-CTX-pkan::*lux*	S17-1 *λpir* containing mini-CTX *lux* having kanamycin promoter, Tet^{R}	This study
S17-1 *λpir* mini-CTX-*recAp*::*lux*	S17-1 *λpir* containing mini-CTX-lux having the *recA* (*PA3617*) promoter, Tet^{R}	This study
S17-1 *λpir* mini-CTX-*PA0615p*::*lux*	S17-1 *λpir* containing mini-CTX-lux with promoter of the first R2 pyocin gene (*PA0615*) in the R-pyocin operon, Tet^{R}	This study
S17-1 *λpir lasR*::pUCP18	S17-1 *λpir* containing pUCP18 with the *lasR* gene, Tet^{R}	This study
***P. aeruginosa* strains**		
PAO1-N	Wild type (Nottingham strain)	Thanks to Dr. Steve Diggle
Δ*lasR*-N	*lasR* insertional mutant of PAO1-N, Gm^{R}	Thanks to Dr. Steve Diggle
PAO1-N mini-CTX-*pkan::lux*	PAO1-N having mini-CTX-lux with kanamycin reporter, Tet^{R}	This study
PAO1-N mini-CTX-*recAp*::*lux*	PAO1-N having mini-CTX-lux with *recA* (PA3617) promoter, Tet^{R}	This study
PAO1-N mini-CTX-PA0615p::*lux*	PAO1-N having mini-CTX-lux with promoter of the first R2 pyocin gene (PA0615) in the operon, Tet^{R}	This study
Δ*lasR*-N mini-CTX-*pkan::lux*	Δ*lasR*-N having mini-CTX-lux with kanamycin reporter, Gm^{R}, Tet^{R}	This study
Δ*lasR*-N mini-CTX-*recAp*::*lux*	Δ*lasR*-N having mini-CTX-lux with *recA* (PA3617) promoter, Gm^{R}, Tet^{R}	This study
Δ*lasR*-N mini-CTX-PA0615p::*lux*	Δ*lasR*-N having mini-CTX-lux with promoter of the first R2 pyocin gene (PA0615) in the operon, Tet^{R}	This study

Plasmids		
pGEM-T Easy	lacZ (α-fragment) ColE1 *ori; Amp*^{R}	Promega corporation
pME3087	Suicide vector; colE1-replicon, IncP-1, *mob*$^{+}$*; Tc*^{R}	(Voisard *et al.*, 1994)
pOO1	pME3087 vector carrying the R-pyocin gene deletion construct *(ΔPA0620-*PA0621), *Hind*III/*Bam*HI, *Tet*^{R}	This study
pME6032-ptac::EGFP	Shuttle vector between *E. coli* and *P. aeruginosa* containing *lacI*^{q}*-Ptac:EGFP*	Thanks to Dr F. Dafhnis-calas
pME6032-ptac::mCherry	Shuttle vector between *E. coli* and *P. aeruginosa* containing *lacI*^{q}*-Ptac:mCherry*	Thanks to Dr F. Dafhnis-calas
p-mini-CTX-*lux*	Contains modified *lux* gene cluster (*lux CDABE*) from *Xenorhabdus luminescens* in mini-CTX1 vector, *Tet*^{R}	Thanks to Dr Steve Higgins
p-mini-CTX-pkan::*lux*	mini-CTX-*lux* with kanamycin reporter, *Tet*^{R}	Thanks to Diana Palenzuela
pOO2	p-mini-CTX-*lux* vector transcriptionally fused with promoter sequence of PA0615 gene (PA0615*::lux*), *Tet*^{R}	This study
pOO3	p-mini-CTX-*lux* vector transcriptionally fused with promoter sequence of *recA* gene (*recAp::lux*), *Tet*^{R}	This study
pUCP18	*E. coli-P. aeruginosa* multicopy shuttle vector, *Cbn*^{R}	Thanks to Dr S. Diggle
pOO4	*lasR* overexpression clone under transcriptional control of native promoter in pUCP18, *Cbn*^{R}	This study

Primers			
Gene amplification **Pyocin genes**			
Name	***Sequence***	***Size (bp)***	***Ref***
S1-F	TTCAACTCTACAACTGTCACG	518	This study
S1-R	TTCCATTTCCCTGTCGAGG		
S2-F	TTCGATGGTTATTACACATGTGC	770	This study
S2-R	AAGGCATTGTTTGCAGTCTGC		
S3-F	TGAATGGAGAAGAAGCTGATCG	320	This study
S3-R	TCTCTCGTCTCAAATGGTTTCC		
S4-F	AGAAGGCAATGGGAAGATGTG	236	This study
S4-R	AAGCATCTTCCTCTGTACTCTC		
S5-F	ATACGAGGTTCCCCCTATCG	415	This study
S5-R	AACAAGCTGCTGAAAAGGGTAC		
AP41-F	AATTGTCGATGGCGAACTGG	870	This study
AP41-R	ATTGAAACACTGCCGACATCG		
R1-F	ATGATTTTTTTCCATGCCGCCACG	441	This study
R1-R	TCAGGGGGTGATGAGCGATTGG		
R2-F	ATGCCGATGCTTCGATTAC	257	This study
R2-R	AAACCTCTCGCAAGGAGG		
R5-F	TGGAATCGTCAACCGCTCGCTG	140	This study
R5-R	TGGTGCTGACGCTGACATCTGC		
Promoter sequences			
N-*recAp*-H3-F	TACAAGCTTTGAAGTCCTCGCGAAGTCAG	165	*recA* gene promoter (This study)
N-*recAp*-ER1-R	TATGAATTCTGTTCTCGTCCATCCGCGCCTG		
*PA0615*p-H3-F	TATAAGCTTGGGTACTGATCCTGCTCG	515	R2 pyocin gene *PA0615* promoter (this study)
*PA0615*p-ER1-R	TATGAATTCATCGCGCTCGGGCAGTAGC		
Deletion mutant generation *R-pyocin mutation*			
L-0619H3	TCAAGCTTAGCCGACTGCTGCCGCCAAAC	454	This study
R-0619	AGCCGATGCCGAACGGTCAGGTGTCTGCTC		
L-0622	CGTTCGGCATCGGCTCGTCTATCTACCTGG	637	This study
R-0622BHI	ATGGATCCCCACAGGCGATAGCCATCGTCG		
Genetic complementation *lasR* gene complementation			
LasR-pUCP18-ER-F	TATGAATTCATGGCCTTGGTTGACGGTTTTC TTGAGC	742	This study
LasR-XbaI-R	ACATTCTAGAGCGTCAGAGAGTAATAAGAC CCAAATTAACG		

Table 2.1: Strains, plasmids and primers used in this study. Underlined sequences in the primers are restriction sites.

2.5 DNA manipulations

2.5.1 DNA extraction

Genomic DNA and high copy plasmids were routinely extracted using Sigma Genomic DNA and plasmid miniprep kits respectively following the manufacturer's recommended protocols. For the plasmid kit, HPLC grade H_2O (Fisher Scientific, UK) was used to elute the DNA.

Low copy plasmids were recovered using Qiaprep midiprep kit (Qiagen, Germany) according to manufacturer's recommended protocols.

DNA was extracted from agarose gel slices using Qiagen Qiaquick Gel Extraction Kit (Qiagen, Germany) following manufacturer's instructions. DNA elution was done with 30 µl of the elution buffer.

2.5.2 DNA quantification

To quantify the DNA in solution, the NanoDrop® ND-1000 (Nanodrop Technologies) was used. Using 2-3 µl, the relative DNA concentration between the blank (elution buffer) and DNA solution was recorded. Wider spectra of readings at different wavelengths can also be used to assess purity.

2.5.3 Restriction enzyme digestion

All restriction enzymes used were purchased from New England Biolabs (NEB, UK). The 20 µl digestion mix was prepared using manufacturer's recommended 10 x buffer. This was incubated at 37 °C for 1 h in a water bath. Digestion products were viewed using agarose gel electrophoresis.

2.5.4 Agarose gel electrophoresis for DNA analysis and purification

Agarose gel electrophoresis was used to assess DNA purity and size and to analyse digestion products. DNA samples were loaded on 0.8-1 % (w/v) agarose gels using 6 x loading buffers. The electrophoresis was run in a horizontal gel apparatus (Biorad, UK) using 1 x TAE buffer which was also used to prepare the gel. Electrophoresis was conducted at 80 V over 30-50 min. Ultraviolet (UV) Transilluminator GelDoc was used to visualise the DNA fragments pre-stained by adding 10 µg/ml of SYBR®Safe to the gel before setting.

DNA molecular weight markers

The sizes of the DNA bands on agarose gels were measured by comparing them 1.5 µg of 100 bp and/or 1 kb DNA molecular weight markers (NEB, UK) run alongside the DNA during electrophoresis.

2.5.5 DNA amplification - Polymerase Chain Reaction (PCR)

a) Primers design and synthesis

The primers used in this study are listed in Table 2.1. Gene specific primers were designed using the *P. aeruginosa* genome database (http://www.pseudomonas.com). Various manipulations made to the 5' end included restriction site insertion for ease of cloning and 12-16 complementary base insertion to aid in the construction of deletion mutants by the method of splicing of overlapping extensions. The primer oligonucleotides were synthesised by Sigma-Aldrich®, UK.

b) PCR conditions

A PCR volume of 50 µl comprised 2.5 µl each of the primers (10 mM) and the template, 10 µl each of buffer and GC enhancer, 1 µl of dNTPs (10 mM), 0.5 µl of Q5 high fidelity polymerase and 21 µl of deionised water. PCR conditions were 98 ˚C for 30 s, 30

cycles of 98 ˚C for 10 s, 58 ˚C for 30 s, 72 ˚C for 30 s/kilobase followed by a final extension at 72 ˚C for 2 min. The annealing temperature was optimised for each primer and ranged between 58 ˚C and 65 ˚C.

2.5.6 A-tailing of PCR amplicons

Blunt-ended amplicons were fixed with an A-tail to facilitate ligation into pGEM®-T Easy cloning vector. The A-tailing mix was 1-7 µl of gel purified amplicon (0.5-1 µg), 1 µl of 2 mM dATP, 1µl of Taq polymerase buffer, 5 units of Taq polymerase (1 µl) and deionised water to a final volume of 10 µl. The reaction was incubated at 70 ˚C for 30 min and used for p-GEMT ligation after cooling.

2.5.7 Ligation reactions

a) Ligation into pGEM®-T Easy vector

The A-tailed amplicon was ligated into pGEM®-T Easy cloning vector using the System I cloning protocol of the manufacturer (Promega, UK). A 30 µl ligation mix consisted of 1 µl pGEM-T vector, 15 µl of 2 x T4 ligase buffer, 3 µl T4 ligase and 11 µl of the A-tailed amplicon. This was incubated at 4 ˚C overnight.

b) Ligation into definitive vector

Using the enzymes whose restriction sites are encoded on the primers, the pGEM®-T clones and their respective definitive vectors (e.g. pME3087, p-mini-CTX lux, pUCP18) were digested. The digestion products were run on the agarose gel and purified as stated in sections 2.5.4 and 2.5.1. Ligation into the vectors was achieved in a 30 µl ligation reaction comprising 24 µl of the extracted vector and insert DNA mixed in the ratio 1:3, 3 µl of T4 ligase buffer and 3 µl of T4 ligase. The reaction was incubated at 16 ˚C overnight in a thermocycler.

2.5.8 DNA sequencing

a) DNA sequencing data

The right nucleotide sequence and absence of point mutations were confirmed by sequencing the recombinant plasmids. Samples were sequenced by Source Bioscience using the Sangers method and the ABI Basecaller system.

b) Whole genome sequencing data

The whole genomes of A018 and A026 were sequenced using the Illumina sequencing outfit.

c) DNA data analysis

The Artemis and Artemis Comparative Tool (ACT) software packages designed the Wellcome Trust Sanger Institute was used in the genomic analysis. The NCBI Sequence Alignment Tool (Clustal Omega) was also used to assess gene alignment and phylogeny.

2.6 Strain manipulations

2.6.1 Transformation

Electro-competent *P. aeruginosa* and *E. coli* cells were prepared and transformed with the different plasmids used in this study.

a) Preparation of electro-competent *E. coli* cells

In a 500 ml flask, 100 ml fresh culture of 1:100 dilutions from overnight culture of *E. coli* cells was prepared. The culture was incubated at 37 ˚C shaking at 220 rpm until mid-exponential growth phase (3-4 h). Cells were immediately cooled on ice and then harvested by centrifugation at 4000 x *g*, 4 ˚C for 15 min. The cells were serially washed twice in decreasing volumes of ice cold dH_2O (100 ml and 50 ml) centrifuging at the same conditions as stated above. Final washing was done using 4 ml of 10% glycerol at 4 ˚C. Cells were suspended in 1 ml 10%

glycerol and aliquoted into 50 µl volumes. The vials were snap-frozen in liquid nitrogen and stored at -80˚C.

b) Preparation of electro-competent *P. aeruginosa* cells

Overnight cultures of *P. aeruginosa* cells (20 ml) were set up in a fresh culture medium (1:1000 dilution) and incubated at 42 ˚C shaking at 220 rpm until mid-exponential phase (4-5 h). Cells were immediately cooled on ice and then harvested by centrifugation at 4000 x *g*, 4 ˚C for 15 min. The cells were serially washed twice in decreasing volumes of ice cold 300 mM sucrose (20 ml and 10 ml) centrifuging at the same conditions. Harvested cells were washed once in 10% glycerol and subsequently suspended in 10% glycerol in aliquots and stored frozen at -80 ˚C.

c) Electroporation of electro-competent cells (*E. coli* and *P. aeruginosa*)

Electro-competent *E. coli* and *P. aeruginosa* cells were used, with pulse settings of 25 µF, 200 Ω and 2.5 kV for 2 mm gap cuvettes; and 25 µF, 200 Ω and 1.8 kV for the 1 mm cuvettes. Within 30 s of applying the pulse, 1 ml of SOC outgrowth medium was added to the cells and incubated at 37 ˚C with shaking for 1 h. Transformed cells were selected on appropriate selective media.

2.6.2 Conjugation

P. aeruginosa isolates were cultured in LB broth without antibiotics and incubated with shaking (220 rpm) overnight at 42 ˚C to weaken the restriction system. S17-1 *E. coli* cells containing the respective plasmids were grown in LB broth at 37 ˚C, 220 rpm using the appropriate antibiotics. One millilitre of the *P. aeruginosa* isolate was harvested in an Eppendorf tube and washed with fresh LB broth. To the harvested cells was added 1 ml of the S17-1 *E. coli* donor cells. After centrifuging, the resulting pellet was re-suspended in 1 ml fresh LB broth to

wash the cells and mix the two isolates together. The resulting pellets were re-suspended in 50 µl of fresh LB broth. This suspension was spotted on dry, pre-warmed LB agar plate to facilitate uptake. The plates were incubated face up at 37 °C for 5 h after which they were gently scraped off the agar surface and re-suspended in 1 ml fresh LB broth. Volumes 100 µl and 200 µl were plated out on selective *Pseudomonas* isolation agar (PIA) containing appropriate antibiotics; plates were incubated at 37 °C. Donor (S17-1 *E. coli*) and recipients (*P. aeruginosa* strains) were also inoculated on same selective media as controls. Colonies of *P. aeruginosa* trans-conjugants appeared after 24-48 h.

2.6.3 *P. aeruginosa* deletion mutant generation

A 2-stage PCR was used to generate a deletion mutant gene construct for the R pyocin gene deleting genes PA0620 and PA0621. The first stage generated two amplicons of the flanking upstream (PA0619) and downstream (PA0622) sequences to the gene of interest. The two primer pairs for the respective genes were L-0619H3/R-0619 and L-0622/R-0622BHI. In the second PCR, the two amplicons of the first stage were mixed in equimolar proportions to splice the complementary overlapping extensions. The two end primers bearing the restriction sites were used in the second PCR. The amplicon from the second PCR was A-tailed and ligated into pGEM-T vector and finally into pME3087 suicide vector using the respective restriction enzymes. The resulting plasmid, pOO1 was used to transform *E. coli* S17-1 cells.

Conjugation between the wild type *P. aeruginosa* and the donor *E. coli* S17-1 strains was carried out as outlined in section 2.6.2. Trans-conjugant *P. aeruginosa* were selected on LB agar plates containing Tet 150 µg/ml and Nalidixic acid 15 µg/ml. Colonies

appeared after 48 h and were further purified on fresh selective media.

In order to enrich for unmarked mutants which have undergone the double cross-over event, some colonies were cultured overnight without antibiotics. Starting with 1:100 dilutions, fresh culture was grown at 37 °C, 220 rpm till OD_{600} 0.5-0.7. Serially at 1 h interval, tetracycline and carbenicillin were added to a final concentration of 20 µg/ml and 2000 µg/ml respectively. Cells were further incubated for 5 h, washed, and the enrichment process was repeated. At the end of the second enrichment, the cells were re-suspended in 1 ml LB broth, 100µl each of ten-fold dilution (10^{-3} and 10^{-4}) were plated out on LB agar without antibiotics. The plates were incubated overnight at 37 °C. Individual colonies were tested for loss of tetracycline resistance by replica plating on LB agar with and without 15µg/ml tetracycline. The mutant candidates were confirmed by PCR using the end primers.

2.6.4. Genetic complementation of *lasR* into ΔlasR-N mutant strain

The whole *lasR* gene was amplified using the primers LasR-pUCP18-ER-F and LasR-XbaI-R. The 742 bp amplicon was A-tailed and ligated into pGEM-T vector. The amplified gene was subsequently transferred into pUCP18 plasmid by digesting the pGEM-T recombinant plasmid and pUCP18 with *Eco*RI and *Xba*I, and ligating the digestion products. The resultant plasmid – pOO4 has the *lasR* gene under the transcriptional control of a native promoter in pUCP18. pOO4 was used to transform the ΔlasR-N mutant by electroporation. The complemented lasR-N mutant (lasR+) clones were selected for and maintained on LB agar containing 300 µg/ml carbenicillin.

2.6.5 R-pyocin expression

a) R-pyocin expression and purification

From an overnight culture of *P. aeruginosa*, a starter 1:100 culture was grown to log phase (OD_{600} ~0.250) in G-medium. Mitomycin C was added to a final concentration of 3 µg/ml. The culture was incubated for a further 3 h; cell debris was removed by centrifuging at 17,000 x *g* for 1 h at 4°C. To the supernatant was added 4 M $(NH_4)_2SO_4$ titrated at a rate of 1 ml/min with stirring at 4 °C (65 ml to every 100 ml supernatant). The suspension was stored at 4 °C overnight, and then centrifuged at 22,000 x *g* for 1 h to harvest the pellets containing the R-pyocins. Pellets were re-suspended in TN50 buffer. Ultracentrifugation at 65,000 x *g*, 4 °C for 1 h further concentrated the R-pyocins. Pellets were re-suspended in 2 ml TN50 buffer.

b) Comparative R-pyocin expression in PAO1-N and ΔlasR-N strains.

The same protocol as stated in a) above was adopted for the expression and purification of R-pyocins from PAO1-N and ΔlasR-N. In addition, the starting inoculum and the culture before the addition of mitomycin C were adjusted to comparable OD_{600} values. Also, the final R-pyocin pellets were suspended in equal volumes of TN50 buffer and equal volumes of the suspension were assessed using SDS-PAGE. To obtain clear bands the extracts were diluted accordingly at the same ratio.

2.6.6. Reporter gene constructs

a) GFP and m-Cherry labelling of strains

The *P. aeruginosa* strains labelled with GFP and mCherry were made competent using the method stated in section 2.6.1.b. Plasmids pME6032-ptac::EGFP and pME6032-ptac::mCherry were used to transform the strains by electroporation. Colonies were selected on Tet 25 µg/ml LB agar plates and the ones that glowed under the fluorescent camera were chosen. Stocks were stored at -80 ˚C in 25% glycerol.

b) Generation of mini-CTX *lux* transcriptional fusion strains.

The promoter regions for the first pyocin gene PA0615 in the R-pyocin operon (PA0615p) and that of the *recA* gene (*recAp*) were identified as the sequence upstream of the genes of interest. The promoter properties of these sequences were interrogated using BPROM software (Softberry, Inc., Mount Kisco, NY). These promoter sequences were amplified using the primer pairs PA0615p-H3-F/PA0615pER1-R and N-RecAp-H3-F/N-recAp-ER1-R respectively. The amplicons were ligated into pGEM-T Easy vector after A-tailing. Using the corresponding enzymes, *Hind*III and *Eco*RI, the promoter sequences were liberated from the recombinant pGEM-T clones and ligated into p-mini-CTX-lux digested with the same enzymes to give plasmids pOO2 and pOO3 respectively. These plasmids were used to transform *E. coli* S17-1 strains thus constituting the donor strains. The plasmids were each transferred into recipient strains PAO1-N and ΔlasR-N by conjugating with the donor strains. *P. aeruginosa* transconjugants were selected for on Pseudomonas Isolation Agar (PIA) containing Tet 25 µg/ml being confirmed by their excitation under light camera

(Hamamatsu Photonics K.K. Model C2741-3OH, Software: Wasabi version 1.5).

2.7 Assays

2.7.1 Biological activity assay (Spot test)

Five millilitres of cooled soft top (0.4%) agar was inoculated with 2 µl of overnight cultures of *P. aeruginosa* isolates (adjusted to OD_{600} = 0.5 ± 0.02) and poured on LB agar plates to produce an overlay. Overnight cell cultures were vortexed with equal volume of chloroform and centrifuged to obtain cell free extracts from the supernatant. Individual 7 µl drops from the supernatant were spotted on the overlay indicator strains and incubated at 37°C overnight. Clear zones of growth inhibition indicate pyocin lysis of sensitive strains. The pyocins were further treated with a protease by incubating with 100 µg/ml trypsin at 37°C for 30 min. Treated pyocins were used for a repeat spot test.

2.7.2 Study of dynamics of pyocin production during growth

A fresh culture of each of the clinical isolates was incubated with shaking at 37 °C using a 1:100 dilution of the overnight culture. Every hour 300 µl volumes were taken for pyocin extraction respectively. Soft top agar overlays of the clinical isolates were prepared and the serially extracted pyocins were used to generate a gradient of pyocin activity on the sensitive indicator strain.

2.7.3 Competition assay

A standard 6-well plate with transwell polyester membrane having pore size 0.4 µm (Corning®) was used to study cell-free interaction of two populations of isolates placed on either side of the membrane. Start cultures of either isolate was adjusted to OD_{600} = 0.05 ± 0.01 and isolate pairs were set up in triplicates with 2.5 ml volume in each well. Plates were incubated with

gentle shaking (60 rpm) at 37˚C. Every hour, 50 µl samples were taken from either well and washed twice with PBS. The cells were stained by incubating at 37˚C for 30 min with Baclight® LIVE/DEAD stain. The two populations of cells were stained and viewed using the confocal microscope. The first set of confocal studies was done using cells from the wells with little disturbance to view their native state while in the second set, the cells were diluted out to aid cell counting in many fields. Controls included null-R mutants of either strain paired up with wild type of either strain and unpaired free growing wild type of either strain. Percentage live cells were calculated in different fields over time.

2.7.4 Bioluminescence assay

Pseudomonas aeruginosa transconjugants bearing the promoter::*lux* fusion construct were selected for, on PIA plate containing Tet 25 µg/ml. Some colonies that fluoresced under the light camera (Hamamatsu Photonics, K.K. Model C2741-3OH, Software: Wasabi version 1.5) were individually cultured in LB broth overnight and their growth patterns were compared with that of the wild type to ensure there was no effect of the conjugation process on growth. As controls, PAO1-N strain bearing the constitutive reporter having a kanamycin cassette was also transcriptionally fused with the lux reporter used to transform all the strains (*pkan::lux* strains). Overnight cultures of selected colonies and controls were adjusted to the same OD_{600} values (0.05). Using the 96-well microtitre plate pre-sterilised for 10 min under UV radiation, triplicate 250 µl volumes of each culture was analysed for absorbance (OD_{600}) and relative luminescence unit (RLU) in the TECAN reader System (Infinite ® 200 pro) at 37 ˚C for 18 – 24 h. For induction, mitomycin C was added to separate wells at a final concentration of 0.25 µg/ml.

Data were analysed using TECAN Sorter Macro Excel file and then the data were assessed using GraphPad Prism 7 software.

2.8 Biofilm models and studies

2.8.1 Bead biofilm studies

Overnight bacterial cultures were grown in M9 broth. Starting with 1:100 dilution of culture, sterile beads were suspended in 100 ml of fresh M9 culture in a 500 ml flask. Shaking at 80 rpm, 37 ˚C, biofilms were cultivated on the beads for full 24 h. The beads (hollow cylinder shapes) had approximate total surface area of 2.6 cm^2. After 24 h, the beads were harvested every hour and gently rinsed twice in PBS. They were then either suspended in 1 ml PBS and vortexed for CFU count or in 1 ml purified pyocin for 1 h. Pyocin treated beads were further rinsed twice in PBS and thereafter suspended in 1 ml PBS and vortexed for CFU count. Controls included treatment with pyocin extracted from null-R mutants of the isolates.

2.8.2 BioFlux® microfluidic biofilm studies

Biofilms from mixed and mono-cultures were studied in the microfluidic system using BioFlux 2000 (Fluxion Biosciences Inc., CA, USA). The system comprised twin wells (inflow and outflow) connected by microchannels for cultivating biofilms. The channels were primed with 100 µl of LB medium from the inflow well with a share setting of 2 dyne/cm^2 for 3 min. Thereafter, fresh cultures at mid-log growth phase adjusted to OD_{600} = 0.05 was used to seed the microchannels from the outflow well by applying a back pressure of 0.5 dyne/cm^2 for 2 s. Cell adhesion to the channels was enhanced by incubating the set-up on the heating plate at 37 ˚C for 30 min without flow. Approximately 1.2 ml of 10 % LB medium was added to the inflow well at 0.25 dyne/cm^2 and 37 ˚C for 15 h. Green fluorescence protein (GFP) and mCherry tagged strains were

used to seed the microchannels. Mono-cultures consisted of A026_GFP, A018_mCherry, A026_mCherry, A018_GFP, P013_mCherry and P013_GFP; while the mixed cultures were A026_GFP/A018_mCherry, A026_mCherry/A018_GFP, A026ΔR _mCherry/A018_GFP, A026ΔR_GFP/A018_mCherry, A026_mCherry/A018ΔR _GFP, A026_GFP/A018ΔR _mCherry, A026ΔR_mCherry/A018ΔR_GFP, A026ΔR_GFP/A018ΔR_mCherry, A018_mCherry/P013_GFP, and A018_GFP/P013_mCherry. After 15 h, biofilms were viewed using confocal laser scanning microscope (Zeiss® LSM 700).

Using the microfluidic system to study R-pyocin activity – A dynamic flow of pyocin treatment was studied on biofilms cultured in BioFlux 2000 microfluidic system. Fifteen-hour biofilms were cultivated and stained simultaneously by adding the Baclight® LIVE/DEAD stain to the 10% LB broth used to cultivate the biofilm. After 15 h, same stain was added to the purified R-pyocin for treatment and staining. Maintaining same temperature and pressure conditions, the biofilm treatment and staining proceeded for 6 h. Controls included pyocin extract from null-R-pyocin mutants and untreated biofilms.

2.8.3 Chronic wound model biofilm studies

Supplemented collagen matrices for cultivation of chronic wound biofilm model were prepared in 400 µl volumes per well using polystyrene 24-well microtitre plate. The components of the matrix were pre-chilled on ice. Each 400 µl well matrix was made up of 80 µl of 10 mg/ml rat-tail collagen type I (BD Bioscience), 40 µl 0.1% v/v acetic acid, 240 µl simulated wound fluid SWF (50% peptone water + 50% foetal bovine serum) and 40 µl 0.1 M NaOH mixed in that order. The plate was incubated at 37 ˚C for 1 h to allow for polymerisation of the collagen matrix.

Fresh bacterial cultures were grown to mid-log phase (4-5 h). After diluting cultures to OD_{600} 0.05 ± 0.01, 100 µl volumes were pipetted on the surfaces of the matrix to inoculate the polymerised collagen and the plate was incubated at 37°C for 24 h. Unabsorbed bacterial suspension was aspirated from the surfaces and replaced with 100 µl of purified R-pyocin followed by incubation for a 1 h, 2 h, 3 h or hourly intermittent treatment over three hours. The hourly intermittent treatment entailed inoculation of the matrices with 100 µl R-pyocins for one hour, aspiration of the spent pyocins and re-inoculation with fresh R-pyocins for another one hour in that order for three treatments. Controls included pyocin extracts from R-pyocin mutants and no pyocin treatments. After the end of the experiment, unabsorbed pyocin was aspirated and the matrices were dissolved using 600 µl of 0.5 mg/ml collagenase in PBS. The collagenase treated wells were further incubated for 1 h at 37°C. The dissolved collagen was homogenised and washed twice in PBS to harvest the cells for CFU count.

2.9 Protein analysis and SDS PAGE

R-pyocin molecules that have been previously expressed, purified and suspended in TN50 buffer were used. The R-pyocin suspension was mixed with 100 µl of 1 x sample buffer (50mM Tris HCl, pH 6.8, 100mM DTT, 2% (w/v), SDS, 0.1% (w/v), bromophenol blue and 10% glycerol. The mixture was boiled (at 100 °C) for 5 min to denature the proteins before loading onto the gel.

The polyacrylamide gel was made up of 12% separating gel and 5% stacking gel (Table 2.2). the electrophoresis was carried out using a Bio-Rad power pack 1000 at 100V/gel for the stacking gel run and later increased to 150 V/gel when the samples entered the separating gel. This voltage was maintained until the loading buffer was noticed at the end of the gel. A protein

marker was run alongside the samples in order to evaluate the molecular weights of the proteins as they separate into bands. The gel was stained with Coomassie blue (0.4g of Coomassie blue R350 in 200 ml of 400 v/v isopropanol) for one hour and thereafter washed 3 times with Destain solution (10% isopropanol). The gel picture was taken using Ultraviolet (UV) Transilluminator GelDoc.

	Stacking gel	**Separating gel**
Buffer (ml)	Buffer B (0.520 ml) (0.5 ml Tris pH 6.8, 04% SDS)	Buffer A (2.000 ml) (1.5 M Tris pH 8.8 + 0.4%)
Acrylamide (30%) (ml)	0.400	3.200
Water (ml)	1.080	2.720
10% APS (ml)	0.004	0.028
TEMED (ml)	0.004	0.028
Total (ml)	**2.000**	**8.000**

Table 2.2: Composition of the Stacking and Separating polyacrylamide gels used in this study.

Chapter 3

Pyocin profile and biological interaction of planktonic cultures of clinical isolates

3.1. Introduction

Over 90% of known strains of *P. aeruginosa* are able to produce pyocins (Michel-Briand & Baysse, 2002, Riley, 1998). These ribosomally synthesised bacteriocins possess a narrow spectrum of antimicrobial activity against closely related strains. Combating genetically related strains is an important strategy in survival because available nutrients and space in an ecological niche are more likely to be shared and competed for among related strains.

Many producing strains are able to produce more than one type of pyocin (Riley, 1998). Some laboratory prototype strains have had their pyocin types identified e.g. PAO1 produces S2, S4, S5, R2 and F2 pyocins www.pseudomonas.com (Winsor *et al.*, 2016). The full annotation of the genes of these laboratory strains and predictability of the outcomes of their genetic manipulations have encouraged their use in many studies. However, laboratory growth conditions and passaging strains over many generations create strains that are not representative of isolates that have evolved during clinical infections. Some pyocin studies have focused on clinical strains (Poh *et al.*, 1988, Kohler *et al.*, 2010, Naz *et al.*, 2015, Dingemans *et al.*, 2016). Whilst Naz *et al.*, 2015 and Dingemans *et al.*, 2016 focused on detailed studies of novel pyocin subtypes, Kohler *et al.,* 2010 classified the R-pyocin subtypes in 47 clinical isolates and their susceptibility to the three main classes of R-subtypes – R1, R2 and R5.

In this chapter, the two major classes of pyocins (soluble pyocins and tailocins) were investigated in 24 clinical isolates

to understand how their presence regulate interactions among different strains and the genetic basis of pyocin resistance. The *P. aeruginosa* isolates were collected from lung infections of cystic fibrosis (CF) patients by Dr. Shanika Crusz (Shanika Crusz Ph.D. Thesis, 2010). They were labelled A (adult) and P (paediatric) strains, each with an associated identification number. Twenty-four strains were randomly selected from this strain collection and were made up of 14 strains from adult patients and 10 from paediatric patients. The age at CF diagnosis for all the patients was less than 6 years. The CF genotypes most represented was *Phe508del* in nine patients whilst one patient each had *Gly542X* and *3659delC* genotypes; the rest of the CF genotypes were not identified.

Furthermore, in this chapter, the genes of six subclasses of the S-pyocins (S1, S2, S3, S4, S5 and AP41) and three subclasses of the R-pyocins (R1, R2 and R5) were amplified and used to produce a profile of the pyocin content per strain. The biological activity of each strain against the others was conducted using an all-by-all spot test assay in which each of the strains was used both as indicator and test strains. The protease sensitivity nature of the S-type pyocins was used to classify the various interactions; this entailed repeating the spot test assays with trypsin-treated pyocins. The pyocin types that were responsible for a particular trend of reciprocal inhibition in which either member of a competing pair produced a lethal pyocin against the other was noticed to be protease resistant. Nine pairs exhibiting such trend were discovered, with strain A026 being a competing member of eight of such pairs. The pyocins of null-R pyocin mutants generated in five of these reciprocal pair members showed loss of this reciprocity. Competition assays using planktonic cells of both wild type (WT) and null-R mutants (ΔR) of these

competitors in different combinations in transwell membrane plates experiments confirmed the central role of the R-type pyocins in strain competition and survival.

3.2. Results

3.2.1. Pyocin subtypes are widely distributed in CF clinical isolates with S5 and R1 being the most prevalent.

The genes encoding soluble pyocins S1, S2, S3, S4, S5 and AP41; and R-pyocins R1, R2 and R5 were amplified using the primers listed in Table 2.1. For the S-pyocins, both the structural and immunity gene sequences for each pyocin subtype were aligned with those of the other subtypes in order to design highly specific primers from the more disparate sequences. The gene sequences of the immunity proteins, DNase and translocation domains of pyocins S1 and S2 bear striking homologies. Therefore, their primers were designed from the gene sequence of the receptor binding domain. Furthermore, the immunity gene sequences of pyocins S3 and S4, and the structural genes of S5 and AP41 showed high specificity and were used for the primer designs. Separate PCR reactions were conducted for each S-pyocin subclass.

In case of the R-pyocins, R2, R3 and R4 have at least 98% homology in their sequences and are very difficult to differentiate with any certainty (Scholl & Williams, 2010), thus they were all grouped into the R2 subtype (Kohler *et al.*, 2010). The genes *prf15* and *prf16* (PA0620 and PA0621 in PAO1 strain) www.pseudomonas.com (Winsor *et al.*, 2016) code for the phage tail and a chaperone protein and are more specific in distinguishing the various subtypes (Scholl & Williams, 2010). They were used to design the specific primers for R1 and R5. For the amplification of the R-pyocin gene of each isolate, a multiplex PCR was conducted. This incorporated all the three specific primer pairs (R1, R2 and R5) with the genomic DNA of each isolate in a single reaction mix (Fig. 3.1).

From the 24 isolates, the most prevalent soluble pyocin gene amplified in 20 isolates was S5, (83.3%) whilst the least was S1 which was identified in four isolates (16.7%). For the R-pyocins, only one isolate had the R5 subclass (4.2%) while R1 was the most represented from 15 isolates (62.5%) (Figure 3.1 and Table 3.1).

	A007	A010	A014	A017	A018	A019	A024	A026	A031	A032	A033	A034	A035	A037	P003	P004	P006	P009	P010	P013	P015	P016	P018	P020
S1			√		√										√					√				
S2	√	√	√	√	√	√	√		√		√	√	√	√	√	√	√		√	√				√
S3					√				√			√	√		√	√	√		√	√				√
S4	√	√	√		√	√	√		√			√	√	√	√	√	√		√	√			√	√
S5	√	√	√	√	√	√	√		√	√		√	√	√	√	√	√	√	√	√			√	√
AP 41	√	√	√	√			√		√		√	√	√	√	√	√	√	√	√			√		√
R1		√				√		√	√		√	√	√	√		√	√	√			√	√	√	√
R2	√		√		√		√			√					√				√	√				
R5				√																				

Table 3.1: Distribution of S- and R-pyocin subclasses in the twenty-four clinical isolates studied. The blue row shows the twenty-four clinical isolates; green rows are for the S-pyocins tested (S1, S2, S3, S4, S5 and AP41) while the orange rows are for the R-pyocin groups (R1, R2 and R5). Key: √ =presence of the concerned from PCR, empty boxes = absence of the amplified genes.

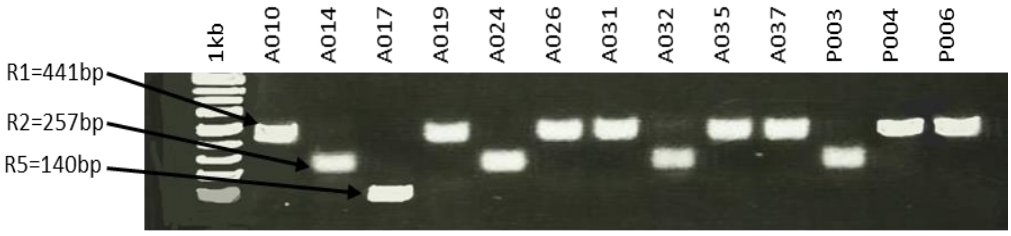


Fig. 3.1: Multiplex PCR of the R-pyocin gene groups (R1, R2 and R5) of some isolates. Each PCR mix contained the three primer pairs and the genome template of each isolate with other PCR components. Only one PCR amplicon was generated from each of the multiplex reactions.

3.2.2. Both soluble and phage-like pyocins contribute to the biological activity of the isolates.

In order to study the biological effect of the killing properties of each isolate on one another, pairwise spot tests of all the isolates was conducted. Each strain was used both as a pyocin producing test strain and also as an indicator overlay on agar plates. Biological killing on agar plates showed as zones of inhibition of bacterial growth while non-concentric clearance indicated strain resistance or tolerance. This generated a profile of interactions showing an all-against-all activity of the isolates. Figure 3.2 shows the interaction of the pyocin-producing strains and the indicator strains with the arrow heads pointing to the indicator strains. The R-pyocin subclasses of each strain are shown in colour codes: R1 (green), R2 (blue) and R5 (yellow). The red arrows link strain pairs which show reciprocated pyocin activities and also retained such activities after protease (trypsin) treatment. Other interactions were random (shown as black arrows). Although some

interactions in black arrows were retained after pyocin treatment, they did not follow any particular trend. Some isolates were only susceptible to the tested strains and did not show any pyocin activities (A037, P016, P009, P018, A019 and A010), some had pyocin activities but were resistant to tested pyocins (P004, A031, A032) whilst one isolate neither displayed pyocin activity nor sensitivity (A034).

Since both soluble and phage-like pyocins are represented in various combinations in the isolates, it is possible that the killing effects seen from the biological activity assays was due to soluble, phage-like or both pyocin types. In order to ascertain the contributing pyocin, the protease sensitivity properties of the S-pyocins was utilised. Therefore, the spot tests were repeated using cell free pyocin extracts that had been treated with trypsin.

Following trypsin treatment, some activities were unaffected whilst others were totally or partially lost. It was clear that some activities were due to S-type pyocins. For example, the pyocin activities that were totally lost were entirely S-pyocin effects whilst some that displayed a partial loss with reduced inhibition zone circumference after trypsin treatment were due to both soluble pyocins and tailocins. Others were due to the tailocins (R- or F-type pyocins) only, showing no appreciable change in the intensity and circumference of the zones of inhibition.

It was interesting that the activities of strains involved in the reciprocal killing were unaffected by the trypsin treatment suggesting that those activities were totally due to effect of tailocin(s). Analysis of the sub-type of R-pyocins present in these strains that displayed reciprocated killing (from PCR results) showed differences in each pair. Strain A026 for

example, has the R1 subtype while the other eight strains (A007, A014, A018, A024, A032, P003, P013 and P020) that showed reciprocated killing against it were all R2 producers (Fig. 3.2). Another pair – A014 and A033 – also showed reciprocated activity and their R pyocin subtypes were R2 and R1 respectively (Fig. 3.2).

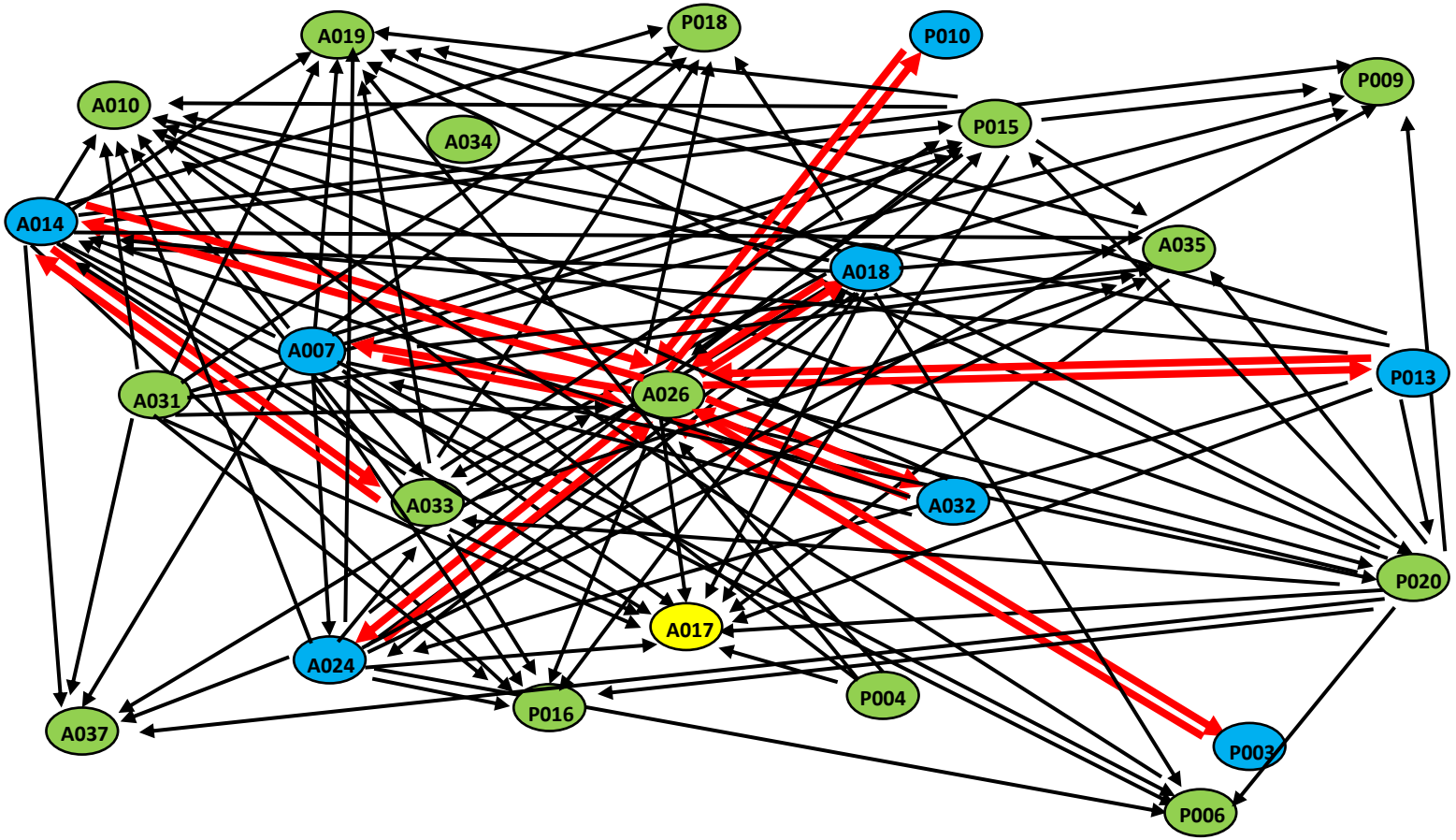


Fig. 3.2: A profile of the biological interactions of the 24 clinical strains using a spot test assay of biological activity. Arrows show the interaction of the pyocin-producing strains and the indicator strains with the arrow heads pointing to the latter. The isolates in green are R1-pyocin producers while the blue and yellow isolates are R2 and R5-pyocin producers respectively. The red arrows show pyocin activities which are reciprocated and retained after protease (trypsin) treatment. Other interactions were random (black arrows) and some of these were also retained after protease treatment though they did not follow any particular order. Some isolates were only susceptible to the tested strains and did not show any pyocin activities (A037, P016, P009, P018, A019 and A010), some had pyocin activities but were resistant to tested pyocins (P004, A031, A032), one isolate neither displayed pyocin activity nor sensitivity (A034) while others showed both pyocin activity and sensitivity.

3.2.3. Generation of R-pyocin mutants by allelic exchange showed that reciprocated pyocin activity is mediated by the R-pyocins.

In the reciprocated pairs where biological activity of strains remained following protease treatment, it was presumed that this activity was not due to any of the soluble pyocin subtypes, but rather a tailocin effect. Thus, mutations in the R-pyocin operon were engineered to verify this. Mutants having the tail fibre and chaperone genes of the R-pyocin (genes PA0620 and PA0621 in PAO1 strain) deleted were produced. This was achieved by making a gene construct in a plasmid suicide shuttle vector compatible with *P. aeruginosa*.

Flanking genes PA0619 and PA0622 were amplified separately using the primer pairs L-0619H3/R-0619 and L-0622/R-0622BHI (P1/P2 and P3/P4 in Fig. 3.4A). Some modifications made to these primers include the insertion of *Hind*III and *Bam*HI restriction sites in P1 and P4 respectively, and designing the 15 bases on the 5' end of P2 to be complementary to the 15 bases on the 5' end of P3. A second stage (convergent) PCR utilised both amplicons from the first PCR reactions as a fusion template using end primers P1 and P4 (Fig. 3.4A). The resulting second stage amplicon was cloned into the suicide vector pME3087 and named pOO1 (Fig. 3.4B).

Plasmid pOO1 was delivered into *P. aeruginosa* strains with the aid of the S17-1 *E. coli* carrier strain (Fig. 3.4C). Single copy chromosomal integration of the recombinant plasmid into *P. aeruginosa* genome ensured the generation of unmarked mutants by allelic exchange from a double cross over event. Some cross over events were however incomplete i.e. single, and thus generated meroploids which retained the antibiotic marker. Both unmarked mutants and meroploids existed in

approximately equal populations (Fig. 3.4D). The meroploids were selected against, by the enrichment method (section 2.6.3). Candidate null-R mutants were confirmed by tetracycline sensitivity and PCR (Fig. 3.4E).

Null-R mutants were engineered in 5 out of the 9 strains that showed pair-wise killing – these 5 strains were A014, A018, A026, P003 and P013. The null-R mutants were used to repeat the spot tests. The reciprocal killings noticed in the first spot tests were all abolished, confirming the role of the R-pyocins in reciprocal killing. The biological activities of strains A014, A018, P003 and P013 (and their null-R pyocin mutants) against strain A026 are shown in Fig.3.3.

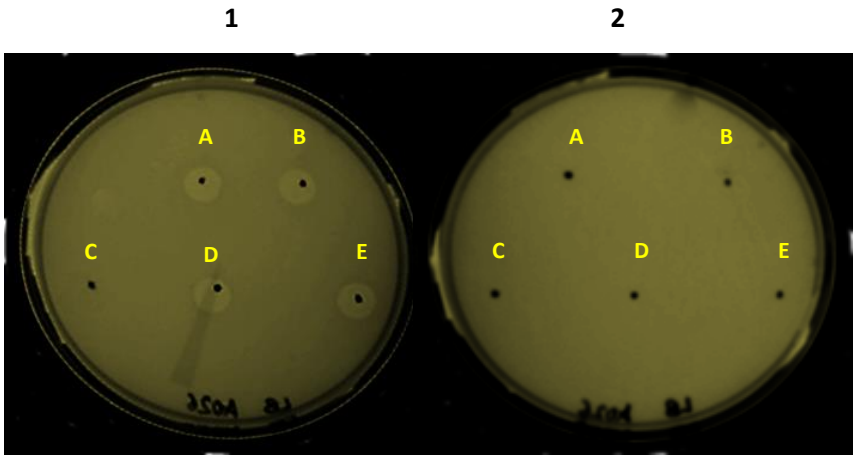


Fig. 3.3: R-pyocin-mutant strains show loss of reciprocal pyocin activity. Two representative plates of biological activity showing A026 as the indicator strain. Test strains are labelled A, B, C D and E (A = P013, B = P003, C = A026, D = A018, E = A014). In plate 1, pyocins of the wild type strains were used while in plate 2, pyocins of R-pyocin mutants of the same strains were used. There is loss of the observed pyocin activity in the mutant derivatives of the clinical isolates. The pyocin of A026 does not have activity against itself as was also observed in the other strains.

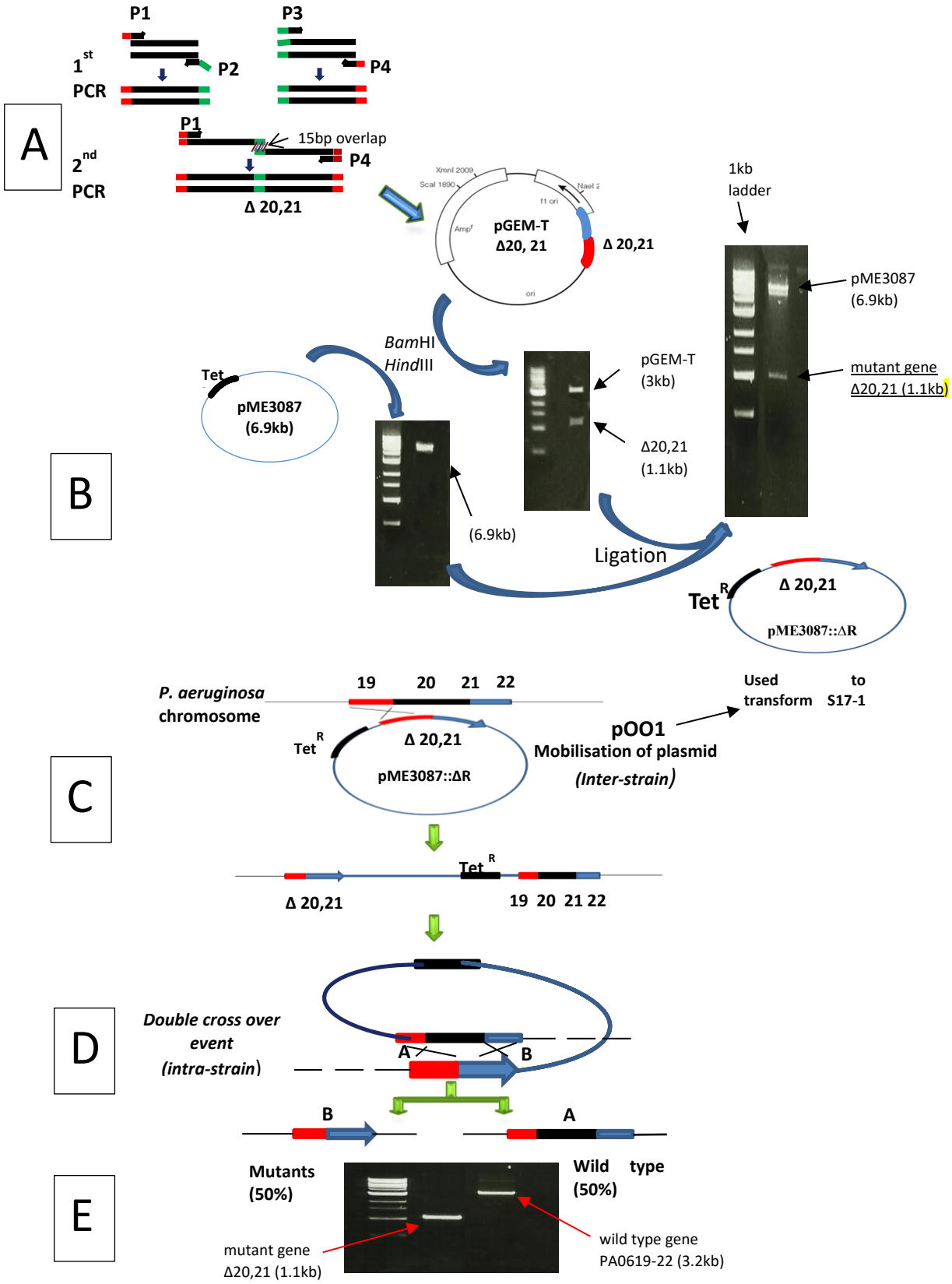


Fig. 3.4: Generation of R-pyocin deletion mutants. Mutant gene construct ΔPA0620/21 was engineered with a 2-stage PCR (A) and ligated into pME3087 to produce plasmid pOO1 (B). pOO1 was mobilised into *P. aeruginosa* by conjugation with S17-1 *E. coli* donor strain (C). Double homologous recombination (D) led to the generation of R-mutant and wild type *P. aeruginosa* strains. R-mutants were confirmed by tetracycline sensitivity and PCR.

3.2.4. R-pyocins mediate competition among strains existing in the same niche.

The concept of reciprocal cell killing in *P. aeruginosa* is as yet unreported and led to questioning the possibility of competition and strain dominance if such strain pairs co-exist in the same micro-environment. Looking closely at the pairwise activities, strain A026 showed the greatest reciprocated cell killing, acting as one member of the pair for eight other strains.

In order to translate these findings of the spot test to a more physiologically relevant interaction, pairwise interactions between two live strains were conducted using the transwell membrane plates. Further studies were conducted on one pair out of pairwise interactions, the first strain was the highly competitive A026 and the other was strain A018. The choice of A018 as the competing partner was informed by the clinical parameters of the two patients from which A026 and A018 were isolated. Both strains were isolated from adult patients with a close age range (27- 32 years), having been both diagnosed with CF as a neonate (Shanika Crusz Thesis, 2010).

Firstly, the interactions between both strains as planktonic cells were investigated. Transwell microtitre plates were used so that both strains could be grown separately in wells that allowed for cell free interaction through the 0.4 µm pores of a dividing membrane. The cell pairs included were A026/A018, A026/A018ΔR, A026ΔR/A018 and A026ΔR/A018ΔR.

Starting with comparable OD_{600} values for each member of the competing pairs, hourly samples (50 µl) of each compartment were taken and treated with LIVE/DEAD stain (BacLight®) to determine the percentage of live cells over time. Appreciable

growth inhibition and killing was noted very early – after three hours.

Figure 3.5 shows the percentage A018 live cells when A026 or A026ΔR was competing with A018 compared to when A018 was unpaired with a competitor i.e. having only sterile LB broth in the adjacent compartment of the transwell experiment. There was an appreciable decline in the percentage of A018 live cells that were paired with A026 at the onset of the third hour; this continued till the end of the experiment. The growth of the A018 cells in the unpaired sample and those paired with A026ΔR were comparable with a steady increase in the population up to the sixth hour.

The live cell counts were conducted for both top and bottom compartments of the transwell experiment. In the wells containing the pair A026ΔR/A018, the dying population was A026ΔR while in the wells with A026/A018ΔR or A026/A018, the A018 strains (A018 or A018ΔR) were dying. Survival was excellent for both members of the pair A026ΔR/A018ΔR. the experiment was repeated three times with comparable results.

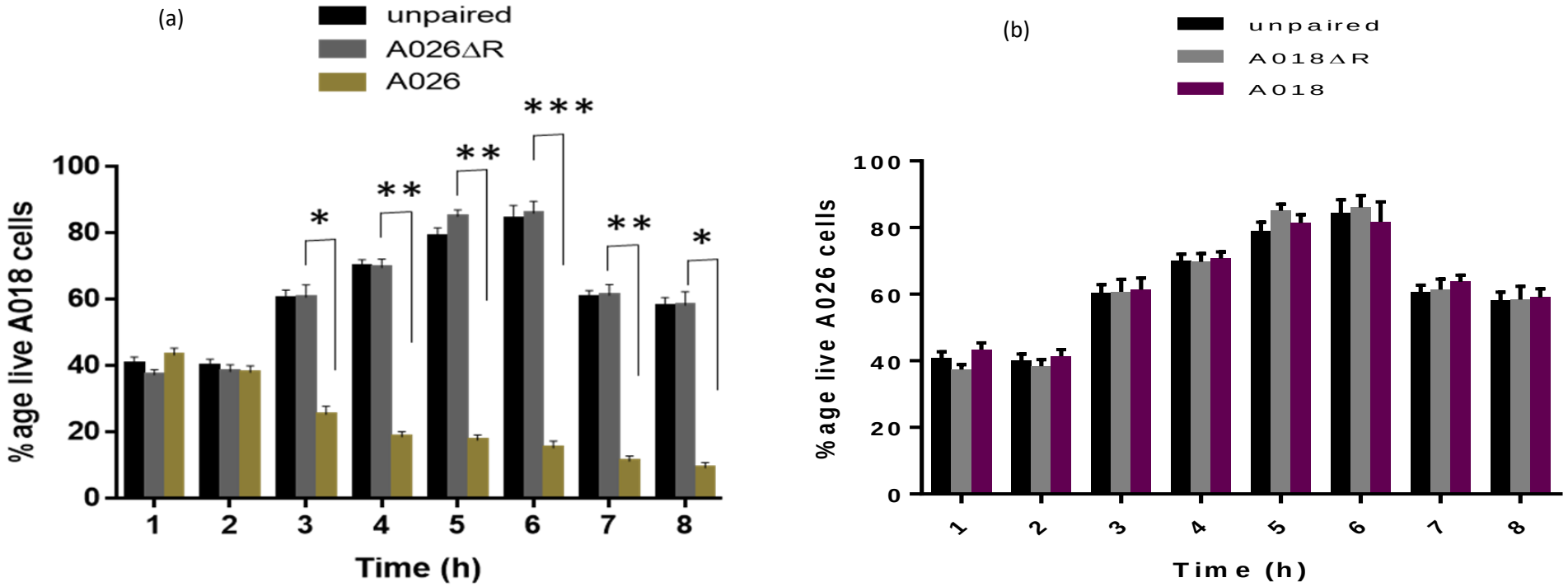


Fig. 3.5: Planktonic cell competition using transwell membrane experiment. In fig. (a), A018 strain was paired with A026 wild type, A026ΔR or plain LB (unpaired); whilst in fig. (b) A026 strain was paired with A018 wild type, A018ΔR or plain LB (unpaired). The percentage of live cells (A018 or A026 in (a) or (b) respectively) was estimated hourly by staining with BacLight® LIVE/DEAD stain and viewing with the confocal laser scanning microscope. Live cell counts were done in three fields for each reading. (Key: *p = 10^{-4}, **p = 10^{-5}, ***p = 10^{-6}).

3.2.5. Pyocins are produced from the onset of the exponential phase of growth.

Early activity (third hour) of the WT pyocins during strain competition (Fig. 3.5) led to the suspicion that R type pyocins are produced before the late exponential/stationary phases of growth. Therefore, a biological activity assay of cell free supernatants of A026 untreated isolates during all stages of the growth cycle was conducted as a spot test using the other strain A018 as the indicator strain.

It was noticed that observable pyocin activity commenced around the 4 h after commencement of growth assay (Fig. 3.6). This appeared as a mottled clearance characteristic of phage plaques, and it progressed with increased clearing of the zones as cell growth (and consequently pyocin production) increased (Fig. 3.6). The activity increased in a population dependent manner. Pyocins were produced and functional both in the mono-cultures used in the spot test and in the transwell competition experiments when the cells were present in the vicinity of a competitor.

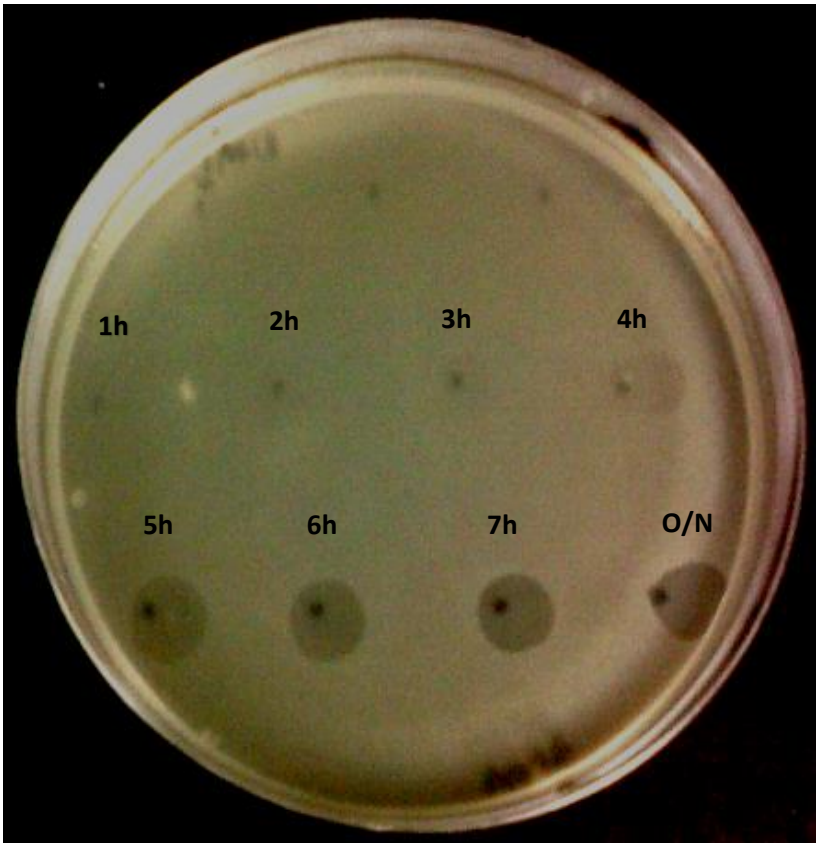


Fig. 3.6: Pyocin activity of cell-free extract prepared hourly from a mono-culture of A026 was used for spot test against indicator strain A018. Appreciable lethal activity commenced around the 4th hour and peaked in the overnight (O/N) spot.

3.2.6. Dead and dying cells from planktonic competition exist in aggregates which resemble biofilms

Estimating the percentage of live cells in the transwell experiment entailed counting individual cells under the confocal microscope. However, as the experiment progressed, it was noticeable that cell clumps were produced in the wells of the outcompeted strain. Over time, in order to count individual cells accurately it was necessary to break and disrupt these cell aggregates by making serial dilutions.

Initially, it was predicted that the cell aggregates represented the formation of small biofilm clusters as the cells became more dormant (Oliveira *et al.*, 2015) but it was noticed that the aggregates were more prominent in the weaker strain (A018). The cell aggregates were thus stained in their native state. Following staining of the cell sample with LIVE/DEAD stain, it was discovered that the clumps were made up of dead cells having taken up the red dye (Fig. 3.7). The dominant strain also showed some clumping but the aggregation was not so well formed and contained a majority of live cells (Fig. 3.7). These cell aggregates became more pronounced at later time points of the transwell competition.

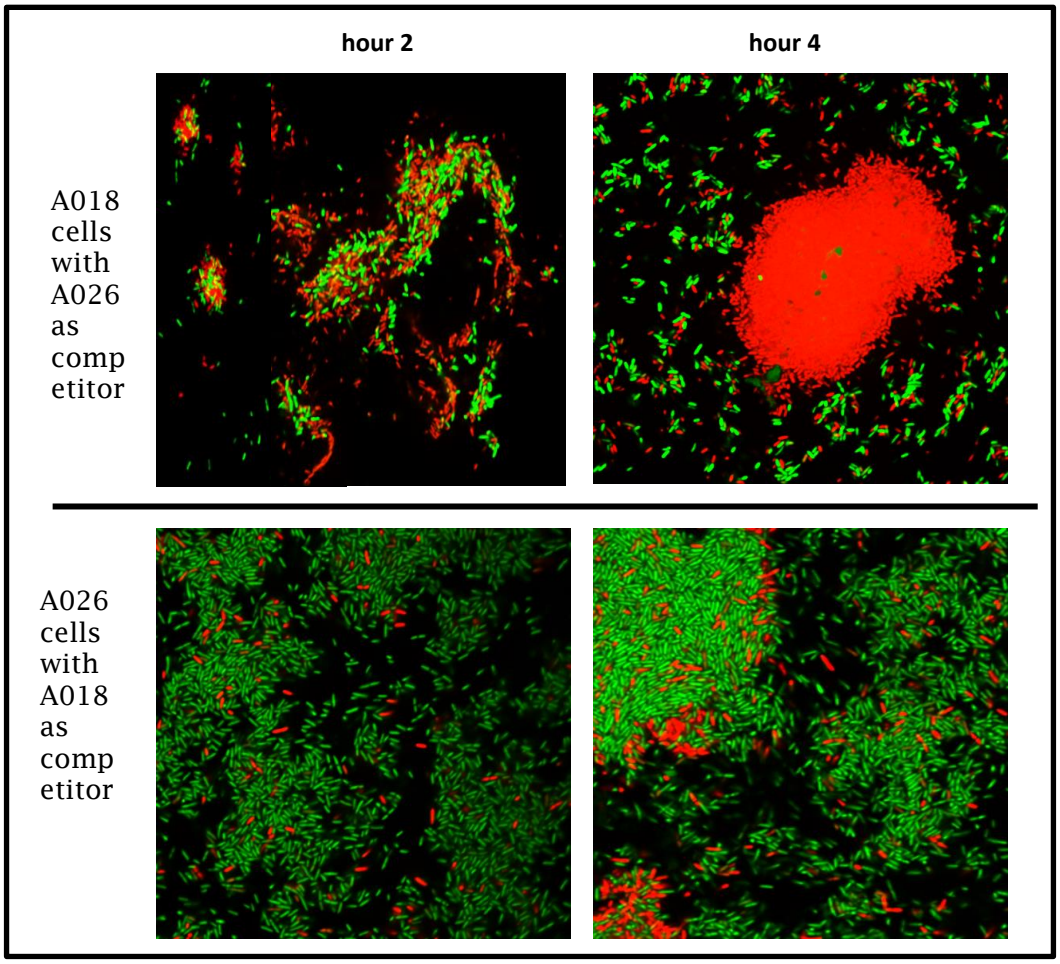


Fig. 3.7: BacLight® LIVE/DEAD staining of the clumps of cells formed over time during the transwell assay. Cell aggregates that resemble biofilms were shown to be dead cells after staining. These dead cell aggregates were greater in the weaker strain (A018) during competition (upper panel) than in A026 - the dominant strain (lower panel) and increased in size from 2 hr (left) to 4 hr (right) duration.

3.3. Discussion

More than 90% of *P. aeruginosa* strains are known to produce pyocins with different classes represented per strain (Michel-Briand & Baysse, 2002). Similarly, there was a large diversity of the distribution of pyocin subtypes in the clinical isolates in the present study (Table 3.1). While the simple dual-gene configuration of S-pyocins allows for many sub-type representations in a single strain, genetic alterations of the operon encoding the multipartite phage-like pyocins only permit a single subtype per strain. This may however be more related to the sizes of the genes/operon than the type differences since relatively modest genome modifications are probably needed to incorporate an extra S-pyocin than a bigger R/F operon. The differences that define sub-classification in the R-pyocin operon are present in the genes encoding the tail fibre and assembly/chaperoning – *prf15* and *prf16* (PA0620 and PA0621 equivalents in the PAO1 prototype strain) www.pseudomonas.com (Scholl & Williams, 2010). The differences in the sequences of these two genes enabled specific primers to be designed for use in the multiplex amplification of the R-pyocins of the isolates and this generated one amplicon per isolate (Fig. 3.1).

All the clinical strains under study had an R-pyocin type, demonstrating the importance of the presence of R-pyocins in clinical isolates. This was unlike some laboratory strains like PML14 (Holloway, 1969, Kageyama, 1975) and PAC1 (Nakayama *et al.*, 2000) which do not produce any R-pyocin subtype because they lack the whole R-pyocin locus. Over 90% of a study population of 1400 *P. aeruginosa* isolates from different habitats produced R and F pyocins whilst only approximately 70% produced the S-type pyocins (Riley, 1998). This further

enhances the importance of the R pyocins for competition especially in habitats where the micro-ecology consists of a mixed population of strains that strive for dominance particularly when space and nutrients are in limited supply.

In this study, the indispensability of the R-pyocin locus in the clinical isolates was in contrast to the S-pyocins, because some strains had none of the six sub-classes tested whilst others had one, more than one or all the tested S-subtypes. From the results of the biological activity spot test assays, the most competitive strain was A026 which demonstrated a reciprocated lethality when paired with eight other strains (A007, A014, A018, A024, A032, P003, P013 and P020). Strikingly, A026 did not have an S-type pyocin from the six sub-classes tested which diminished the impact of S-type pyocins in competition of clinical isolates.

Ghoul *et al.* noted in their study that dominant strains which are involved in chronic infections do not possess a wide diversity of pyocin types (Ghoul *et al.*, 2015). According to them, this might be due to some selective forces or to the cost of maintaining many pyocin types for isolates that persists for long periods during chronic infections. A wider diversity of pyocin types was noticed with isolates from early infections. Fewer pyocin types in isolates of prolonged infections was not due to loss of pyocin types over time but linked to the fact that competitive strains involved in chronic infections tend to produce fewer pyocins (Ghoul *et al.*, 2015, Eberl & Tummler, 2004, Romling *et al.*, 1994)

Reversal of the R-pyocin activity as demonstrated by the spot test assays using pyocins extracted from null-R-pyocin mutants confirmed the role of the R-pyocins in competition and survival. Competition as a function of the R-pyocins was first

reported with planktonic cultures of laboratory strains (PAO1, PA14 and PAK) by Heo *et al.* (Heo *et al.*, 2007). In their study, strain PAK was killed by both PAO1 and PA14 but the killing activity was not reciprocated by PAK which was attributed to the absence of the *prf16* (R-pyocin assembly) gene in PAK although this susceptible PAK strain produced lethal pyocins which killed eleven other tested strains. Kohler *et al.,* 2010 also reported that the dominance of a strain lasts until it is outcompeted by another strain having an R-pyocin whose potency is greater than that of the first strain (Kohler *et al.*, 2010). This was a finding in the isolates from one of the 61 intubated CF patients they studied. After a period of co-colonisation for ten days, one strain outcompeted the other because it had a different and more potent R-pyocin subtype (Kohler *et al.*, 2010, Koehler *et al.*, 2009).

In all these studies, there was an absence of reciprocal killing activity of the clinical isolates reported. In contrast however, this present study focused on what we choose to call a 'true' competition, when either member of the competing pair has comparable ballistic capacities for a fair duel. Planktonic cell competition using the transwell method, represented a physiologically representative assay as informed by the profile of strains that demonstrated reciprocal biological activities from spot tests.

The major factor relating the pyocin classes of A026 and its eight competing pairs was the differences in the subclasses of the R-pyocins they have. All the 8 strains had an R2-pyocin subtype compared to the R1 of A026. The emergence of a winner in the planktonic assay reveals that dominance is inevitable when two strains which are sensitive to the R-pyocins of each other co-exist in the same domain. This is buttressed by the loss of dominance in the null-R mutant of the

winning strain when exposed to the wild type of the weaker competitor.

The pyocin activities outlined in the profile (Fig. 3.2) shows that non-uniformity in the pyocin subclasses of two strains does not particularly translate to the sensitivity of either strain to the pyocins of the other. The susceptibility or resistance of a strain to a particular pyocin may involve an interplay of many factors. One of these may be related to the pyocin receptor(s) displayed by the strain. Many receptors have been identified for the pyocins e.g. the ferripyoverdine receptors FpvA1 for S2 and S4 (Elfarash *et al.*, 2012), FpvAII for S3 (Baysse *et al.*, 1999) and ferripyochelin (FptA) for S5 while the LPS outer core sugar residues are receptors for the R-pyocins (Kohler *et al.*, 2010). There appears to be a relationship between the receptors and the pyocin genes. For instance, the wild type and null-R mutant of the dominant A026 strain displayed different biological properties in the presence of the A018 wild type. Given that only the R-pyocin biosynthesis genes of A026 were mutated without tampering with the genes coding for the receptors, the exposure of these two derivatives (wild type and mutant) to the same competing strain should produce similar results if the receptors were entirely responsible for biological responses. It is therefore likely that mutations in pyocin genes induce conformational changes in the corresponding receptors. However, another possibility is that the susceptibility of A026ΔR to the R-pyocins of A018 is due to the unopposed pyocin activity of the latter in which case the defenceless A026ΔR succumbs to the offensive attack of the competitor, and is not a function of a change in the receptor status. The latter argument seems more plausible.

There appears to be some strain specific response to pyocins. The sensitivity of a strain to the R-pyocin of another does not

seem to inform a similar response to the same R-pyocin subtype from yet another strain. Therefore, although the R1-pyocin of strain A026 was effective against all eight R2-pyocin producers in this study population, only a few other R1-pyocin producing strains showed such a broad spectrum of activity. This may be a function of specific interactions between the pyocins and the receptors of the susceptible strains. Such interactions being unpredictable may therefore bring about a response in some pairwise interactions and not in others. Another possible explanation is that an interplay between all pyocin subclasses present in a strain may cause a display of an array of susceptibility and resistance patterns. The absence of S-pyocin subclasses in A026 abolished this interference, allowing some uniformity in the pairwise interactions. However, the presence of the S-pyocins in other strains might have been responsible for the mixed picture.

Understanding the responses that were due to R-pyocins was made possible by the treatment with protease which abolished the effects of protease-sensitive S-pyocins. This essentially caused all the strains to behave like A026 and display a uniform trend. With trypsin treatment, the observable trend of non-reversal of pyocin activities was uniform in the reciprocated killing. Apart from A026 as a competitor another pair (A033 and A014) which are R1 and R2- pyocin producers respectively also demonstrated this reciprocity.

The production and activity of pyocins commenced early in the growth cycle of a strain and increased with increasing cell population density. Thus, pyocin production though inducible, could be expressed in the absence of induction. In the absence of induction when pyocins are produced in monocultures, it was observed that appreciable activity using spot test, commenced around 4 h into growth curve assay. However,

during competition of planktonic cells in the transwell assay, a significant drop in the viable counts was noticed from the 3 h post inoculation. Planktonic competition mimicked an induction of pyocin production triggered by a competitor producing cell-lysing pyocins. This induction appeared to cause an earlier initiation of pyocin activity 3 h post inoculation (Fig. 3.5) compared to the first visible activity of the mono-culture at 4 h into the growth assay (Fig. 3.6). This early induction and activity of pyocin showed appreciable effects on the weaker strain causing its dead cells to clump in aggregates that resembled biofilms (Fig. 3.7).

The concentration level of the R-pyocins that triggered the clumping effect was lethal. These dead cell aggregates were earlier reported as biofilms induced at sub-inhibitory concentrations of R-pyocins (Oliveira *et al.*, 2015). The uptake of propidium iodide by these biofilm-like aggregates in the same study (Oliveira *et al.*, 2015) however revealed that these clumps are not viable biofilms since propidium iodide mainly stains damaged cells. In the present study, it was clear that these clumps were aggregates of dead cells after staining with the LIVE/DEAD stain (Fig. 3.7).

In summary, the R-pyocins appear to be a class of pyocins which are important to *P. aeruginosa,* especially strains that exist with other strains in niches where competition is central to survival. The growth advantage conferred by the R-pyocins on the strains that produce them have made them important in pathogenic and environmental niches (Heo *et al.*, 2007).

Chapter 4

The role of R-pyocins in shaping populations and biofilm interactions

4.1. Introduction

Many previous studies that have been used to investigate the pathogenesis of *P. aeruginosa* have focused on studying the free living planktonic forms of individual cells. However, in most natural and pathologic settings *P. aeruginosa* grows in multicellular biofilms. The more resilient and antibiotic resistant properties exhibited by biofilms coupled with the complexity and diversity of interactions that prevail during communal existence have led to an extensive research field studying biofilms.

The cells in biofilms are stacked up in stratified random layers and occupy a smaller surface area. This arrangement allows for highly co-ordinated behaviours while also serving as a protection for cells occurring at deeper layers of the strata. This is a well-known mechanism of resistance for antibiotics (Goldberg, 2002). Biofilm eradication has been a great challenge in clinical therapy in established chronic CF lung infections and most eradication measures have tended to target acute infections, during early colonisation stages when biofilms have not yet been established (Hansen *et al.*, 2008, Douglas *et al.*, 2009). The genetic diversity of cell members of a biofilm also ensures that some cells which have acquired more desirable resistance mechanisms for the continuity of life are sustained thus resulting in a degree of heterogeneity (Korber *et al.*, 1993).

Having established the role of R-pyocins in competition and survival of clinical isolates in free living cells, strain interactions were similarly probed in biofilm communities of *P. aeruginosa*. Various combinations of the wild type and null-R pyocin mutants (ΔR) of the competing pair – A026 and A018 – were studied as they formed biofilms and these were

compared with the biofilms formed from mono-cultures of each of these strains. Eradication of biofilms grown on plastic beads or in microfluidic wells with R-pyocins from the competing strain, showed that R-type pyocin was an effective treatment against established *P. aeruginosa* biofilms. However, the treatment of biofilms grown in a synthetic wound model with R pyocins was less effective.

4.2. Results

4.2.1. Single treatment of static biofilms grown on polystyrene beads with R-pyocins, shows significant reduction in viable biofilm populations

The spatial distribution of cells in a biofilm ensures that more cells occupy the same surface area compared to planktonic cells in a one-layer cell arrangement. Since R-pyocins have antibacterial effects against planktonic cells of susceptible strains (section 3.2.4), it is relevant to determine whether this activity extends to cells living in a biofilm. Therefore, 24 h biofilms of A018 (or A026) grown on polypropylene beads were subjected to a single treatment of R-pyocins from the competitor strain for one hour and viable cell counts were plotted before and after treatment over time.

The R-pyocins of each strain caused the reduction of viable cell counts of the competing strain by a 3-log to 5-log difference (Fig. 4.1a). However, the R-pyocins extracted from the nullR mutant of either strain did not give any appreciable difference in the viable cell count of the competitor when compared to the untreated population (Fig 4.1b). The CFU counts in the treated and the untreated populations at the same hour were compared. The analysis showed that the readings at 4 h and 5 h treatments were significant ($p < 0.05$) (Fig. 4.1a).

It was noticed that the elimination of viable cells from the biofilm population was not total, leading to an appreciable survival of some cells after the one-hour pyocin treatment in the range of 10^3 to 10^4 CFU/ml (Fig. 4.1a). Comparable results were obtained when either strain was treated with the R-pyocin of the other (Fig. 4.1a & b).

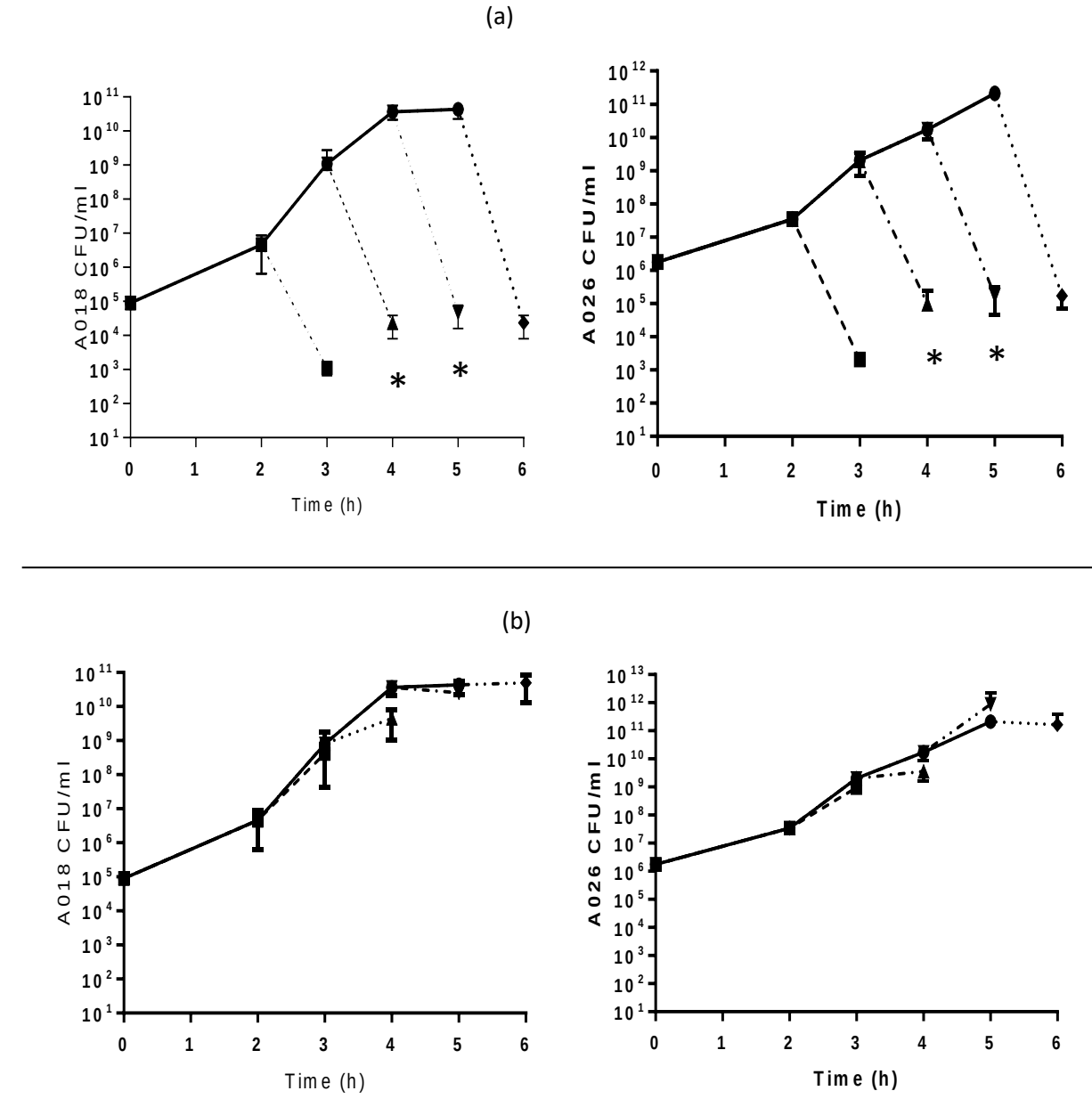


Fig. 4.1: R-pyocin treatment of static biofilms. Single treatment of static biofilm of A018 or A026 grown on polystyrene beads using R-pyocins of the competitor (A026 or A018). Hour 0 readings are the CFU/ml values of the 24 h biofilm grown on the beads. In one set of beads, growth was allowed to proceed unhindered (unbroken lines) while in the other set, three beads were harvested every hour, their biofilms were treated for one hour using purified R-pyocins and the CFU count was recorded after treatment (broken lines). Beads in (a) were treated with R-pyocins from wild type of competitor while the ones in (b) were treated with R-pyocins from the null-R mutant of the competitor. Comparing the CFU counts in the treated and the untreated populations at the same hour revealed that differences in the readings at 4 h and 5 h were significant (Key: * = $p < 0.05$).

4.2.2. R-pyocins drive competition in biofilms

As shown earlier, R-pyocins are important components in the competition of planktonic cells in strains occupying adjacent environments (section 3.2.4.) and can inhibit the growth of susceptible biofilms on a solid surface (section 4.2.1). However, whilst the bead biofilm assay gave an indication of the potential use of R-type pyocin in the treatment of *P. aeruginosa* biofilms, it does not simulate the physiological conditions found in an ecosystem where strains compete for survival. In order to investigate the effect of R-pyocins of two interacting strains which co-exist in a common ecological niche, it was necessary to co-localise both competing strains as growing biofilms. This was achieved by mixing equal cell densities (measured by comparable OD_{600} values) of both competitors, A018 and A026 that were differentially labelled with GFP or mCherry in microfluidic channels of a BioFlux® device and allowing them to form biofilms over 15 hours.

Six combinations of the labelled strains were used. When WT A026 labelled with either GFP fluorophore (A026GFP) or the mCherry fluorophore (A026m-Cherry) was incubated with WT A018 labelled with the contrasting fluorophore, the A026 strain outcompeted the A018 strain regardless of the nature of the fluorophore (Fig. 4.2a). Also, A026 dominated in pairs that involved A018ΔRm-Cherry and A026GFP or A018ΔRGFP and A026m-Cherry (Fig. 4.2b). However, when A018m-Cherry was mixed with the null-R mutant of A026 (A026ΔRGFP) or A018GFP was mixed with A026ΔRm-Cherry, A018 was able to outcompete A026ΔR (Fig. 4.2c). Thus, A018 was able to outcompete A026 only when the latter was unable to produce functional R-pyocins.

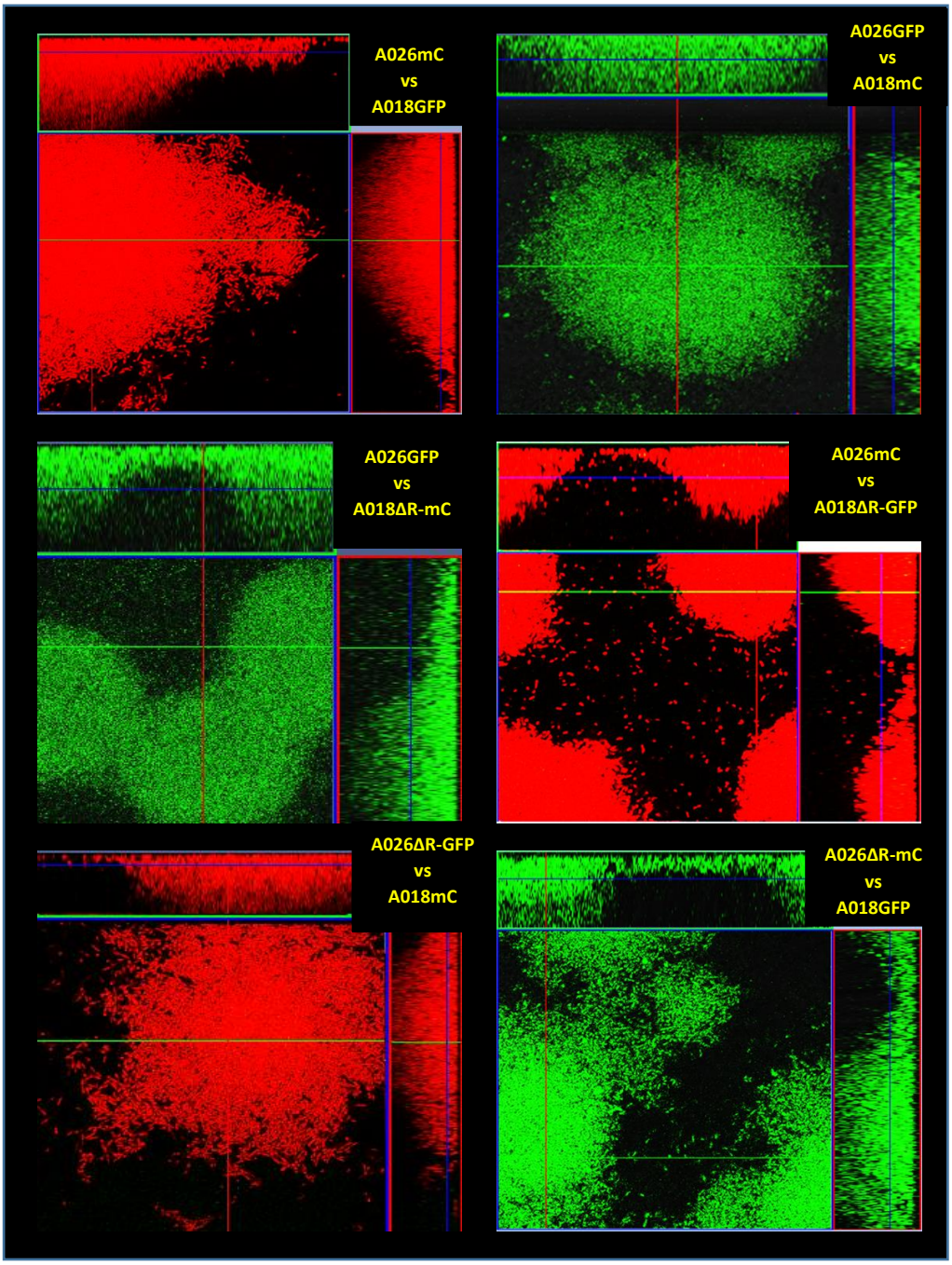


Fig. 4.2: Biofilms of competition experiments. Fifteen-hour biofilms developed in microfluidic BioFlux® device as a result of competition between A026 and A018. A026 dominated in the biofilm competition of A018 and A026 [Fig.(a)] and in the biofilm competition of A026 and A018ΔR [Fig.(b)]. However, A018 only dominated when in competition with A026ΔR [Fig. (c)]. In each of the groups (a), (b) and (c), the fluorophores – mCherry and GFP were swapped for the strain pairs.

The production of a functional R-pyocin is central to the survival of a producing strain since no null-R mutant of a strain was able to establish itself in the presence of the wild type strain of the other competing isolate. However, what happens when the two competing strains are null-R mutants? To answer this question, the null-R mutants of the two strains (A018ΔR and A026ΔR) were differentially labelled with GFP and mCherry and were also used to generate biofilms in the BioFlux® micro-channels.

Both strains were able to co-exist as biofilms but they clearly segregated from each other which enabled them to form individual pockets of biofilms which were strain specific. The split images (Fig 4.3a) at 10x lens magnification show that the two strains occupied distinct micro-niches in the biofilm and a close-up view at 63x lens objective confirmed the distribution of each strain showing minimal interaction (Fig. 4.3b).

Since the inactivity of R-pyocins in both strains could abolish competition, the ability of two strains with matching R-pyocin types to co-exist in the same niche was explored. To this end, two strains A018 and P013 which were tolerant of each other by the biological spot test assay and possessed the same R-pyocin type (R2) were mixed in the microfluidic channels to study the patterns of their biofilms.

The results showed that both strains were able to co-exist and produced biofilms with same appearance as the R-mutants of A018 and A026 (Fig. 4.3c). Although the two strains (A018 and P013) had the same S- and R-pyocin subtypes (Table 3.1), they were able to discriminate between self and non-self and thus they formed distinct micro-niches like the combination of the mutants (A018ΔR/A026ΔR).

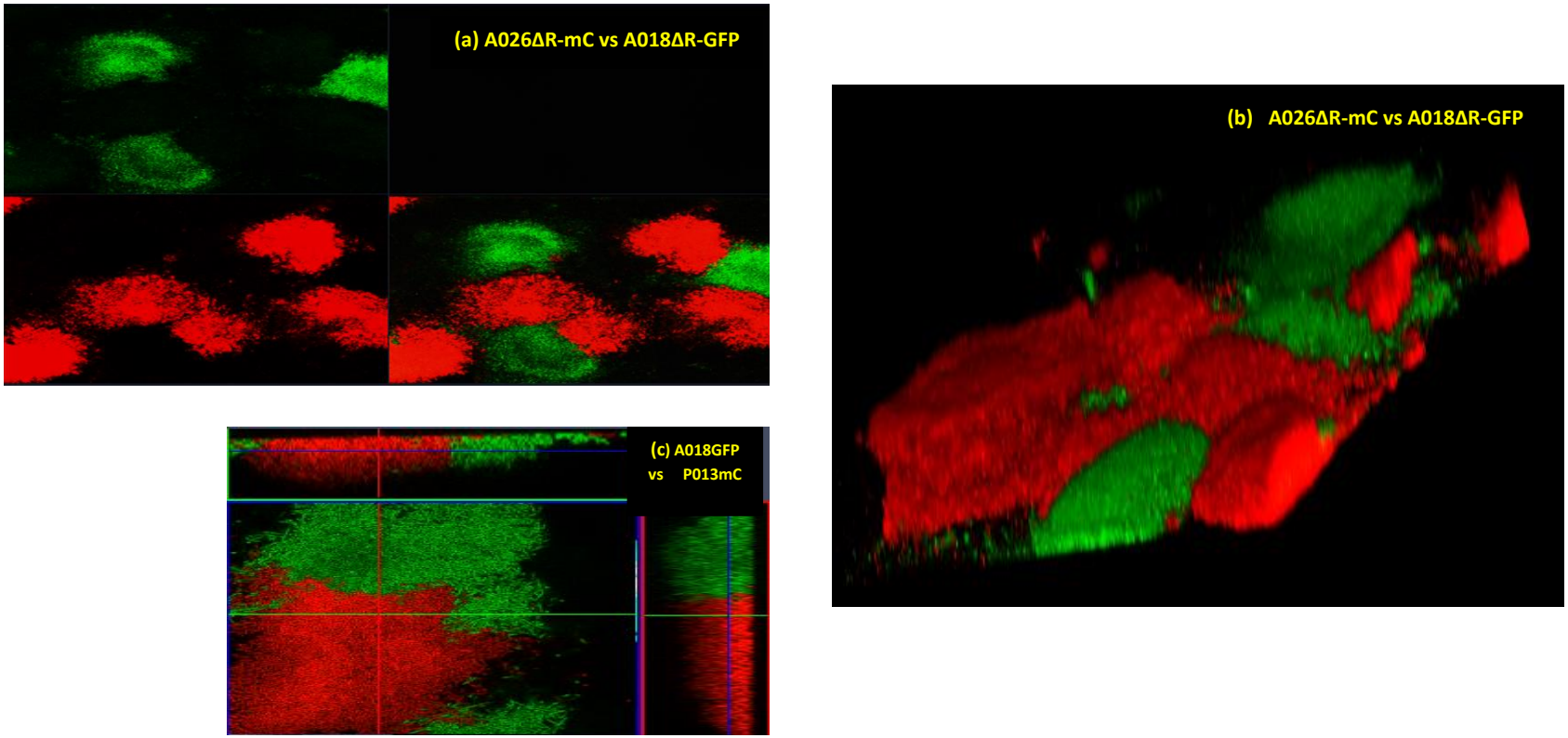


Fig. 4.3: Biofilms of mixed cultures of A018ΔR-GFP and A026ΔR-mCherry (a) & (b) and A018GFP and P013mCherry (c)**.** The BioFlux® microfluidic channels were seeded with equal concentrations (comparable OD_{600} values) and volumes of the two labelled mutants. Fifteen-hour biofilm shows the distribution of the two strains. Fig. (a) shows a split image of the fluorescence green and red channels singly (upper and lower left panel), then none and combined channels (upper and lower right panels). Fig. (a) was at 10x magnification and shows a wider distribution of the strains as they form micro-niches whilst (b) shows a closer view of the same biofilms at 63x magnification. Fig. (c) is a mixed culture of the wild type strains A018GFP and P013mCherry, they both have the same subtypes of S- and R-pyocins.

4.2.3. Strain competition enhances biofilm formation and maturation

The effect of competition on biofilms formed by a strain was studied by comparing biofilm formation in the presence or absence of a competitor. Mixed- and mono-cultures used for this assay were A026m-Cherry mixed with A018GFP and A026m-Cherry alone respectively. Biofilms were cultivated within the same time frames and cultivated at the same culture conditions throughout the experiment.

The 3D architecture of the mixed culture biofilm formed during competition between two strains had more compact packing, depth and maturation with fewer planktonic cells (Fig. 4.4a). These biofilms were essentially scattered islands which enclosed more cells in a well-structured tightly arranged architecture.

The mono-cultures of A026mCherry also produced biofilms but had more planktonic cells perhaps due to the absence of threat from a competitor (Fig. 4.4b). The biofilm groups of mono-cultures were randomly formed and were more immature showing less biomass and shallower depth than the ones formed as a result of competition.

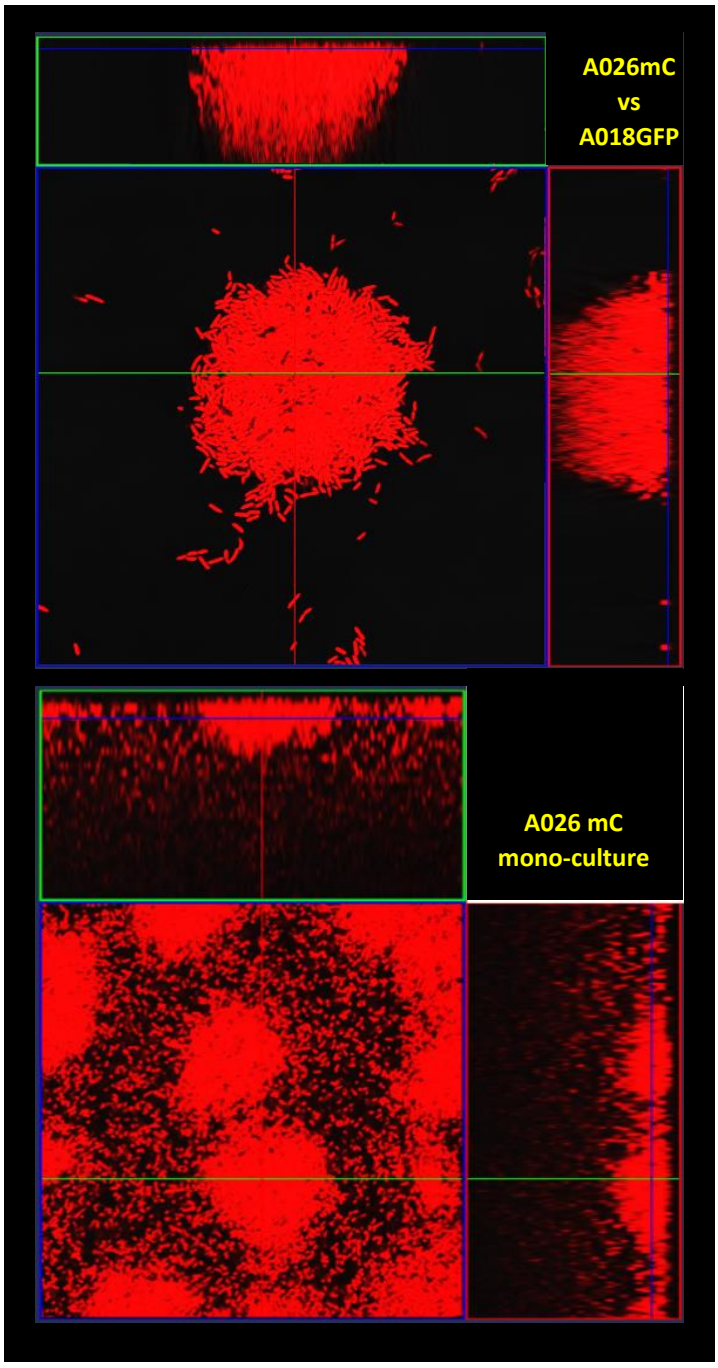


Fig. 4.4: Structural differences in biofilms formed from competition and mono-cultures. Comparing the architectural differences in the biofilms formed as a result of the survival from a competition and that of a mono-culture in the same strain. **Fig. (a)** shows A026-mCherry biofilm after out-competing A018-GFP in a mixed culture while **Fig. (b)** is the biofilm formed from a mono-culture of A026-mCherry. Biofilms were cultivated within same time frame affording no time advantage to either combination.

4.2.4. Continuous treatment in a dynamic biofilm model reveals the anti-biofilm efficacy of the R-pyocins

The R-pyocins have been shown to have good antimicrobial activity against planktonic cells using the trans-well plate experiment (section 3.2.4.) and against biofilms in the bead biofilm assays (section 4.2.1.). A total eradication of the biofilm on the beads was not however achieved. This was attributed to a short duration of pyocin treatment (one hour) and also the fixed concentration of R-pyocins used for each treatment. Therefore, in order to allow for a longer time of action of R-pyocin molecules with a dynamic exchange of spent R-pyocins for fresh doses, a microfluidic biofilm model – the BioFlux® system was employed.

Biomass killing of strain A018 was extended with increased addition of R-type pyocin of strain A026 such that after approximately 4 h, full-depth killing of the biomass was achieved with the R-pyocins of A026 (Fig 4.5). In contrast, the R-pyocins from the A026 null-R mutant (A026ΔR) showed no adverse effect on biofilm growth of strain A018 (Fig. 4.5b). There was evidence of some dying cells using the pyocins of A026ΔR, but this was attributed to factors other than pyocin addition. Cell deaths noticed during treatment with A026ΔR pyocin did not affect the full biomass thickness as was observed in the wild type. The same results were achieved when either member of the competing pair was exposed to the R-pyocins of the other.

Interestingly, biofilm architecture of cells treated with active pyocins was very different when compared with cell treated with inactive mutant pyocin or pyocin that had no effect on the cell. Biofilms treated with active pyocin showed some features

of mushroom-like/ microcolony phenotypes which could be appreciated from the base of the biomass (Fig. 4.5a, 4 h post treatment) which was absent from the inactive samples (Fig. 4.5b, 4 h post treatment).

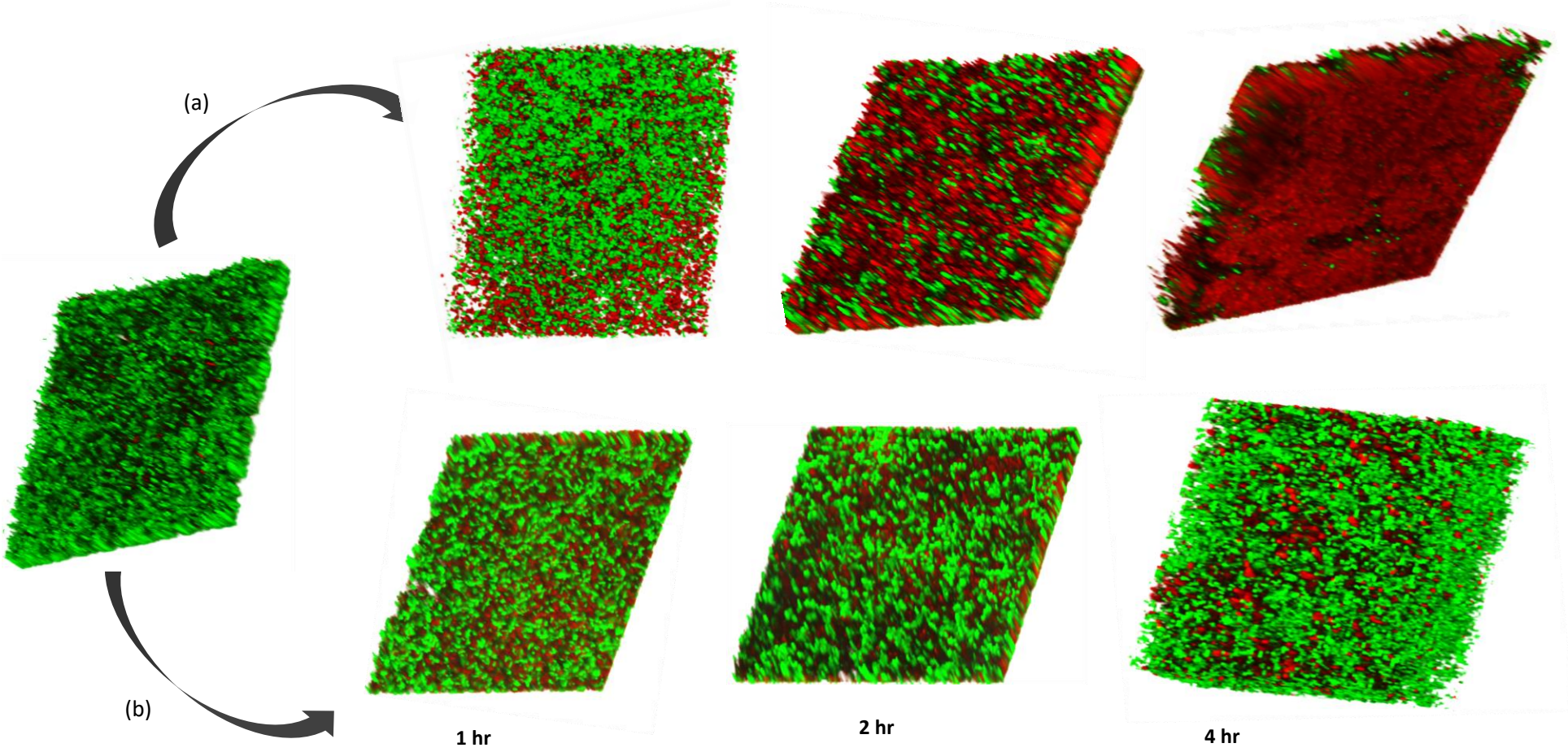


Fig. 4.5: Anti-biofilm efficacy of R-pyocins. Fifteen-hour biofilm of A018 was treated with R-pyocins extracted from A026 (a) and A026ΔR (b). In Fig. (a), appreciable portion of the biomass was killed by the 2nd hour and full-depth lethal effects on the biomass was appreciated by the 4th hour. The 4th hour biofilm also demonstrated some mushroom-like/ microcolony phenotype appreciated from the base of the biomass. All these were absent in the control experiment which utilised the R-pyocins of A026ΔR. The results obtained after treating A026 biofilm with the R-pyocins of A018 and A018ΔR were also comparable.

4.2.5. Biofilms grown in chronic wound models shows appreciable effect of R-pyocins during intermittent treatment

The effect of pyocins on biofilms grown in an *in vitro* chronic wound model was also studied. The peculiar nature of these biofilms include the typical micro- and macro-colonies which are scattered biofilm pockets that are embedded in the vital tissues – majorly collagen. The presence of these biofilms at varying depths of collagen invasion makes it possible for them to elicit systemic infections when the bacteria cells are seeded into the blood stream

The chronic wound model was designed to mimic biofilm invasion of wound collagen. Treatment of the 24 h biofilm of strain A018 grown in the model using different duration of exposure to R-pyocins of strains A026 gave some interesting results. The viable cell population (CFU/ml) harvested from the models before and after the procedure showed that the cell counts were comparable after 1 h, 2 h and 3 h post R-pyocin treatment of the collagen biofilm (Fig. 4.6).

Furthermore, the two 3 h post R-pyocin treatments produced different results. The first treatment was uninterrupted for three hours while the other was an hourly treatment using fresh R-pyocin replacements over three hours. There was an appreciable drop in the viable cell count with the intermittent treatment compared to the uninterrupted, and the latter essentially produced the same results as the other uninterrupted timed experiments. In the statistical analysis of the CFU counts, the readings of the untreated biofilm were compared to those of the treated ones at the different time points i.e. 1 h, 2 h and 3 h (Fig. 4.6).

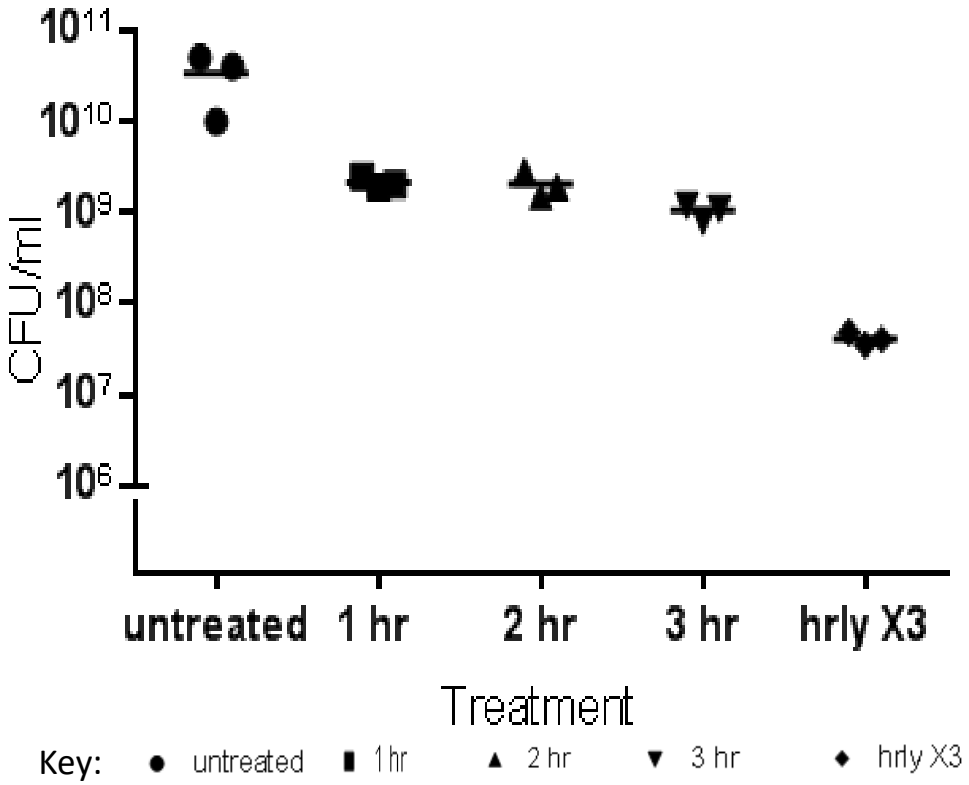


Fig. 4.6: Pyocin treatment of biofilms grown in chronic wound model (CWM). 24-hour biofilms were grown in CWM and the viable cells were counted (i.e. untreated reading). R-pyocin was used to treat the 24 h biofilm over 1 h, 2 h and 3 h periods and the viable cells were counted after treatment over the specified durations. The viable cell counts for each of these time points gave comparable cell counts after treatment. When the 3-hour treatment was broken into interrupted hourly treatments using fresh R-pyocins each time (i.e. hrly x 3), the cell count dropped appreciably compared to the untreated biofilm.

4.3. Discussion

The development of biofilms in *P. aeruginosa* is essential for its ability to sustain chronic infections (Sanchez *et al.*, 2013). Cells in biofilms are very different from their planktonic counterparts in terms of stratification, antibiotic resistance, co-ordinated behaviour and mutability. As shown in chapter 3, the R-pyocins have proven efficacy against the planktonic forms of *P. aeruginosa*. However, the clinical applicability of these findings will only be substantiated if the efficacy of R-pyocins can be reproduced against biofilms which is the preferred mode of existence of *P. aeruginosa* in virtually all chronic infections.

The antibiotic resistance of biofilms is linked among other factors to the inaccessibility of some cells to antimicrobial agents due to the spatial distribution afforded by stratification. In this study, the same factor was also predicted as a possible resistance mechanism against R-pyocins which also act as antimicrobials. Since the R-pyocins bind to lipopolysaccharide (LPS) receptors using their tail fibres, it is obvious that not all the LPS receptors of biofilm cells are exposed for binding since spatial distribution ensures that some cells exist in deeper layers. The bead biofilm treatment showed that there was an appreciable protection afforded by the inaccessibility of some cells since complete eradication of the biomass was not achieved.

Some other reasons could however explain the lack of complete activity of the R-pyocins in the bead biofilm model. The first was that probably the treatment was not allowed to proceed for long enough, with the one-hour treatment not permitting complete action. The second reason was the fact that the R-

pyocin concentration being constant meant that the molecules were used up; this was so because the mechanism of killing was the one-hit method. Both limitations were overcome in the microfluidic biofilm model which enabled a prolonged pyocin treatment and continuous renewal of the active R-pyocins. With these treatments, there was a complete full-depth biomass killing (Fig. 4.5).

This anti-biofilm activity of R-pyocins is yet unreported. Though intriguing, the mechanism is yet to be understood. It is known that R-pyocins kill susceptible cells by attaching to their LPS and causing membrane depolarisation through pore formation and subsequent efflux of intracellular ions (Michel-Briand & Baysse, 2002). Since contact is required to elicit the action of R-pyocins, the mechanism of action against inaccessible cells in the deeper layers of the biomass without sloughing off the superficial layers of the biomass is interesting. There appears to be a knock-on effect causing some chain reaction propagated down the biomass. Some explanations for this may be that the depolarisation of a cell causes a destabilisation of the membrane potential of another cell in close proximity; or that the death of a cell causes its collapse bringing intact cells underneath it to a closer contact with more R-pyocins. Although the mechanism for anti-biofilm activity is not understood at the moment, the latter scenario may not hold true since it was previously reported that cells killed by R-pyocin activity do not undergo the LPS degradation (exotoxin release) remaining as ‘ghost cells’ (Scholl & Martin, 2008).

Prolonged treatment of the biofilm with R-pyocins revealed a subtle change in the biofilm architecture as it rounded up into mushroom-like biomass by 4 h post R-pyocin treatment (Fig. 4.5a). This enhancement of biofilm maturation was induced by

the presence of active R-pyocins, presumably as a mechanism to protect the cells from the lethal effects of the pyocins. This was also noticed in the mixed-culture biofilms of paired competitors in the microfluidic experiment (Fig. 4.4). However, both experimental approaches differ. The biofilms grown on polypropylene beads encountered unopposed activity of R-pyocins because the producing strain was absent while biofilms formed in the microfluidic channels occurred as a result of competition between two live strains whose R-pyocin production depended on the population of the surviving cells. However, the enhancement of biofilm maturation in both cases suggested R-pyocin induction. The role of R-pyocins in biofilm induction and maturation has been previously reported (Oliveira *et al.*, 2015). This shows that succumbing in death or fortification to form biofilms are two outcomes which are linked to the functioning of the R-pyocins.

In the competition experiment using a mixed culture (Fig. 4.4a), R-pyocins of either strain triggered events that combined their offensive and defensive properties. Although the presence of a lethal pyocin molecule in the domain of a sensitive strain is primarily as an offensive weapon, it also sensitises the strain to the presence of an intruder, thus amplifying the need for the cells to round up in closely packed biofilms to ensure fortification (Fig. 4.4a). This defensive property induced by the presence of R-pyocins was also reported by Oliveira *et al.*, 2015 after they discovered that biofilm formation was increased in conditions when strains were mixed compared with unmixed conditions (Oliveira *et al.*, 2015). Thus, with the offensive and defensive properties of the R-pyocins, the outcome of a competition appears to be a function of the potency of the R-pyocin subtype concerned. Therefore, lower numbers of planktonic cells and the increase in biomass during

competition were respective results of the offensive and defensive tendencies exhibited by the R-pyocins (Fig. 4.4 a & b). Furthermore, although R-pyocins were also produced by the biofilm cells used in the anti-biofilm experiment (Fig. 4.5), the absence of a live competitor eventually overwhelmed the initial defense mounted by the biofilm leading to its eradication.

The R-pyocin is so important in strain interactions that the more aggressive strain, A026 was killed by the weaker strain, A018 when the former loses its ability to produce functional R-pyocins and is exposed to the wild type of A018 (Fig. 4.2.). Thus, the activity or the absence thereof, of R-pyocins when they are of different subtypes in two sensitive competing strains is a major factor in the survival of either strain.

Although, A018 possessed several classes of S-pyocins which were not present in A026, the survival of A026 in the biofilm was not a function of any of the S-pyocin sub-types, such survival was solely R-pyocin dependent. Thus, A026 retained its dominance only when its ability to express R-pyocins was preserved. The co-existence in the same ecological niche of two competing strains that will otherwise naturally out-compete each other was made possible by incapacitating the production of R-pyocins in both strains. On the other hand, two strains with same R-pyocin subtype can co-exist (A018 and P013). Thus, competition and co-operation are two phenomena which are obtainable when two strains occupy the same niche and the determination of which phenomenon predominates is solely dependent on the differences or similarities and activities of the R-pyocin subtype each possesses.

Due to the peculiar nature of biofilms found in chronic wounds, the definition of biofilms have been extended to include cell aggregates attaching to one another to form clumps of colonies

in addition to the popular definition of surface-attached typical biofilms (Percival *et al.*, 2015, Kragh *et al.*, 2016). Depending on the size of these unattached biofilms, they may be referred to as micro- or macro-colonies (Schaber *et al.*, 2007) and they exist at varying depths in the wound. The random association of these biofilm colonies with collagen was simulated by the chronic wound model (CWM).

These collagen-associated colonies were targeted in the CWM. When using the same concentration of R-pyocins at different exposure times, it became evident that the efficacy of the procedure was a function of the concentration of the R-pyocins used rather than that of the exposure time. Thus at the same concentration, the viable population recovered were comparable although the treatment times were different i.e. one, two and three hours. It might have been possible to achieve a greater killing effect using a higher concentration of the R-pyocins but instead, the R-pyocins were replenished every hour for three hours to compare the results with the uninterrupted 3-hour treatment. For the two treatments, i.e. uninterrupted and intermittent, the latter proved more efficacious, achieving the killing of a higher number of cells. Once again, the single-hit mechanism of the R-pyocins comes to play here, whereby only one R-pyocin molecule effects killing regardless of the number adsorbed to the surface of the sensitive strain (Iijima, 1978, Uratani & Hoshino, 1984) and the molecule(s) remain attached after the killing of the cell (Scholl & Martin, 2008). In the intermittent treatment, aspiration of the spent R-pyocin suspension for the introduction of a fresh one not only got rid of the spent R-pyocins but also the dead cells to which they remain attached. Essentially intermittent treatment helped to get rid of dead bacterial cells while allowing viable ones to get into a closer contact with the next

treatment of R-pyocins. Although the same duration of treatment was used for both 3-hour treatments, prolonging the time of exposure in the uninterrupted treatment did not achieve a better clearance since bound R-pyocins can neither be re-used nor re-cycled.

Chapter 5

Genomic analysis of A018 and A026 and the link between quorum sensing and R-pyocin expression.

5.1. Introduction

In previous chapters, it has been demonstrated that the R-pyocins of *Pseudomonas aeruginosa* have good efficacy against biofilms of sensitive strains, and influence strain interactions of two different populations of cells that occupy adjacent niches, both in the biofilm and planktonic states. The data presented in chapters 3 and 4 showed that one strain, A026 which produced the R1 subtype of R pyocin was able to out-compete a selection of R2 producing strains during planktonic and biofilm growth. Conversely, the strain that demonstrated dominance in another study (Kohler *et al.*, 2010), was an R2 pyocin producer having been shown to outcompete another R1 producer after a period of ten days co-habitation in the lungs of an intubated CF patient. In the present study, the reasons for strain dominance of an R1 producing strain over an R2 producer are at this stage unclear but it is unlikely that R1 subtype has greater toxicity than R2 subtypes as supported by the study done by Kohler *et al.,* 2010.

It was therefore pertinent to see whether any indications of strain dominance of A026 over A018 can be gleaned from a study of the genome of both strains. Some interesting questions that needed to be addressed included: (i) Why was A026 so competitive? Its ability to unilaterally kill a sensitive strain during strain mixing despite both strains having the propensity to kill each other was profound. (ii) Are there genetic differences in the clinical strains that contributed to their adaptation to the anatomical site of isolation and virulence compared to reference strains? (iii) What are the genetic factors that could explain the resistance of A026 to the S-pyocins which was shown by PCR that it does not produce? (iv) Is there any genetic evidence to suggest that A026 could be

a highly virulent strain? (v) Are there genetic mutations relative to a reference strains which might explain the competitiveness and survival strategies of A026?

The entire genomes of strain A026 and A018 were sequenced. *In silico* analyses of the genomes were performed with reference to known annotated strains PAO1 and LESB58, with A018 mapping closely with PAO1 and A026 with the LESB58 strain. Putative pyocin related genes were identified by analysing sequences homologous to pyocins and their immunity proteins. Other virulence genes analysed included quorum sensing regulatory gene, *lasR.*

A026 was a *lasR* mutant and displayed autolytic plaques on agar plates. The loss of this phenotype in the R-pyocin mutant of strain A026 (A026ΔR-pyo) suggested a link between R-pyocin expression and quorum sensing. Transcriptional fusion of the promoter of the first R-pyocin gene PA0615 and the bioreporter *lux CDABE* in mini-CTX *lux* plasmid was used to monitor the expression of R-pyocin in PAO1 prototype and its *lasR* mutant (ΔlasR-N). The increased R-pyocin expression in ΔlasR-N over the PAO1 wild type appeared to be RecA independent.

5.2. Results

5.2.1. Whole genome sequencing of A026 and A018 maps them as close relatives of the LES (Liverpool Epidemic Strains) and PAO1 strains respectively.

One of the major features that made strain A026 of particular interest was its ability to kill approximately one-third of all other clinical isolates used in this study and its highly competitive nature when grown adjacent to or as a mixture with other strains. Although PCR reactions showed that it did not possess any of the major six subclasses of S-pyocins currently reported, it was tolerant of the R-pyocin activities of competing strains provided its own R-pyocin was intact. These features were intriguing and prompted an understanding of the genetic make-up of both A026 and A018 using whole genome sequencing analysis.

The original mapping of both A018 and A026 against PAO1 as reference strain showed that A018 was closely related to PAO1 whereas difficulties in the mapping of A026 to the PAO1 reference strain suggested that it was distantly related to PAO1. While all single nucleotide polymorphisms (SNPs), insertions and deletions (INDELs) recorded in the A018 strain was only 65, over 22,000 of such SNPs and INDELs were discovered in A026 when compared to PAO1. Consequently, A026 was mapped against the LESB58 reference strain because it was more relevant to use a fully annotated R1- subtype pyocin producer.

The phylogenetic analysis showed that A026 is related to the LESB58 strain (Fig. 5.1). On a closer inspection, the LES sister strains (LES431 and LESB58) form an LES clade which is related to A026. The strains in the LES clade share a common internal node with A026. Thus, although the strains in the LES clade are fully annotated reference genomes, they are strains which

share a common ancestry with A026. The LES strains also appear to be descendants of a recent speciation event from a sister strain to A026. In addition, the LES clade and A026 (henceforth referred to as LES-related A026, or LES_A026) formed a nested hierarchy.

A018 and other PAO1 related strains form a clade (a PAO1 clade) with a common internal node related to the NC_022360 strain. Strain A018 could essentially be considered as a PAO1 strain because of a common root of ancestry with seven other documented PAO1 strains (Fig. 5.1) and is henceforth referred to as PA018. The ancestral relationship of PA018/PAO1 to LES_A026 is quite distant as predicted earlier from the overwhelming number of SNPs and INDELs detected in LES_A026 compared to PAO1.

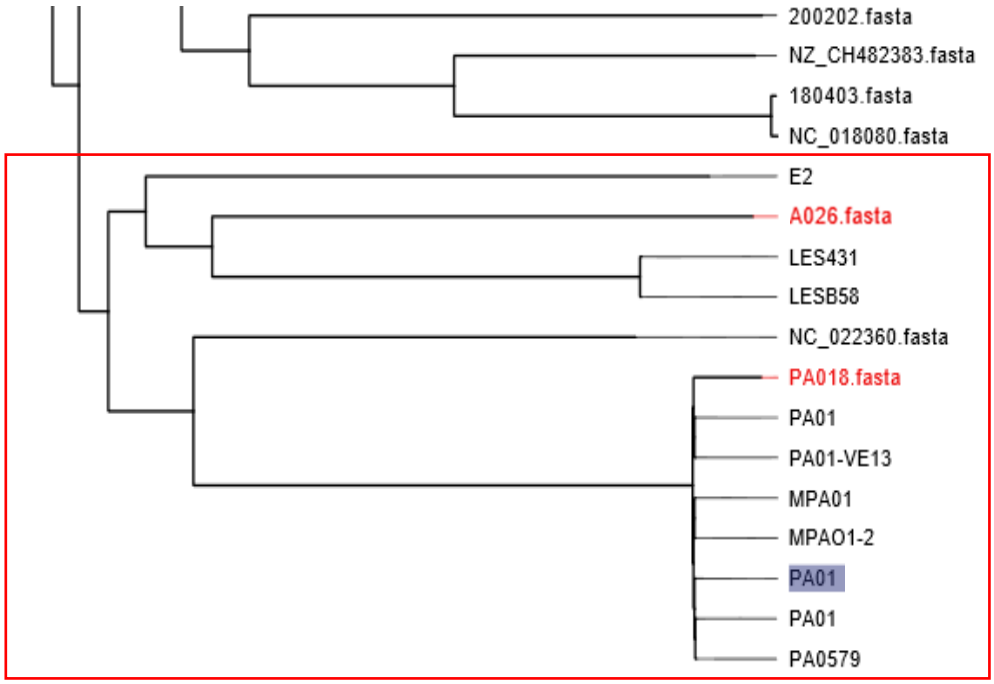


Fig. 5.1: Phylogenetic tree showing the close relatedness of PA018 with the PAO1 strain while A026 (LES-related A026) is linked in ancestry with the Liverpool Epidemic Strains LES431 and LESB58.

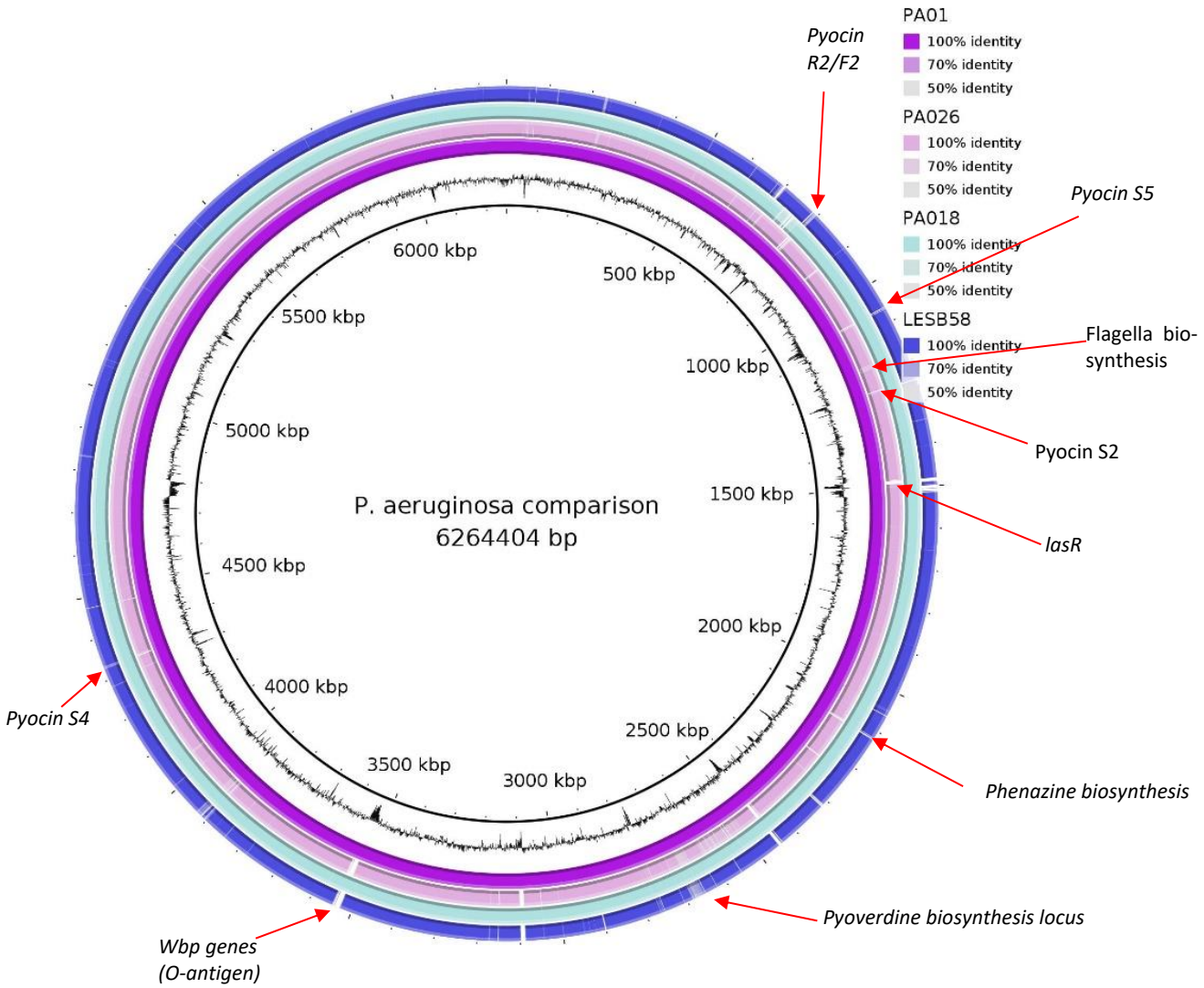


Fig. 5.2: Blast Ring Image Generator (BRIG) output image of A026 ( ), PA018 ( ) and LESB58 ( ) using PAO1 ( ) as the reference strain. Some PAO1 genes were either absent or had less than 100% identity in the other three strains and these are shown as either gaps or lighter shades respectively. All annotated gene positions (red arrows) are the locations on PAO1 reference strain while other strains are relative to it.

5.2.2. Clinical isolates, LES-related A026 and PA018 have bigger genomes than LESB58 and PAO1 reference strains due to the presence of accessory genes

The BLAST Ring Image Generator (BRIG) (Fig. 5.2) (Alikhan *et al.*, 2011) was first used for comparative genome analysis of the two clinical strains LES_A026 and PA018 and the reference strains PAO1 and LESB58. However, the image generated (Fig. 5.2) gave a broad comparison of the entire genomes using one reference genome, PAO1. In this particular analysis, the genes that were in the three genomes under comparison (LES_A026, PA018 and LESB58) but not present in PAO1 could not be analysed and it was also difficult to analyse individual genes based on inter-genomic differences in the DNA sequences. These limitations indicated that there is a need for a better tool for comparative analysis.

The tool employed was the Artemis Comparison Tool (ACT) (Carver *et al.*, 2005). ACT was used for the alignment and sequence comparison of the full genomes of the clinical isolates LES_A026 and PA018 with reference strains PAO1 and LESB58. ACT analysis revealed that the genomes of both clinical isolates were larger than the corresponding reference strains. For example, in comparison to the PAO1 genome, PA018 and LES_A026 strains contained an extra 635 kilobase pairs (kbp) and 457 kbp respectively (Table 5.1., Fig. 5.3).

In genomic comparisons, blocks of genes in different species which share the same order of sequences and are located in comparable positions in the genome are believed to be results of recent divergence in evolution and therefore show close relatedness of the isolates that have them. However, as evolutionary events become more divergent, various gene re-arrangements occur leading to localisation of genetic loci in

different relative positions or reversal of genetic order much like ‘cut and paste’ manipulations during evolution. These genetic orders are referred to as synteny and may be direct, following the same sequence or inverted, when the gene sequence is reversed.

The ACT software image (Fig. 5.3) shows appreciable regions of synteny; these are represented respectively as red coloured lines (same sequences) and blue coloured lines (inverted sequences). The width of each of the coloured lines depicts the length of the sequences over which there are regions of synteny. It is possible to optimise the images generated by adjusting the length of sequences (footprints) over which to assess synteny and therefore filter out low scoring hits. In order to exclude these low scoring hits, the score cut off was set to a footprint of 2 kilobase pairs (kbp) i.e. only genes that had homology in at least 2 kbp lengths of their sequences were included in the analysis. This generated a genome wide view for the analysis of direct and inverted synteny (Fig. 5.3).

A high degree of direct sequence order (red) was observed between PAO1 and PA018 with minimal inversions (blue) which appeared to be clustered around the third quarter (+ strand) of the A018 genome (Fig. 5.3). The comparison of PA018 and LES_026 genomes showed extensive genome re-arrangements (blue) compared to sequences with direct synteny (red) while direct and inverted synteny appeared to be equally distributed between the genomes of LESB58 and LES_A026. This further confirmed that PAO1 and PA018 showed the closest relatedness, followed by LES_A026 and LESB58 while LES_A026 and PA018 had the widest divergence of ancestry.

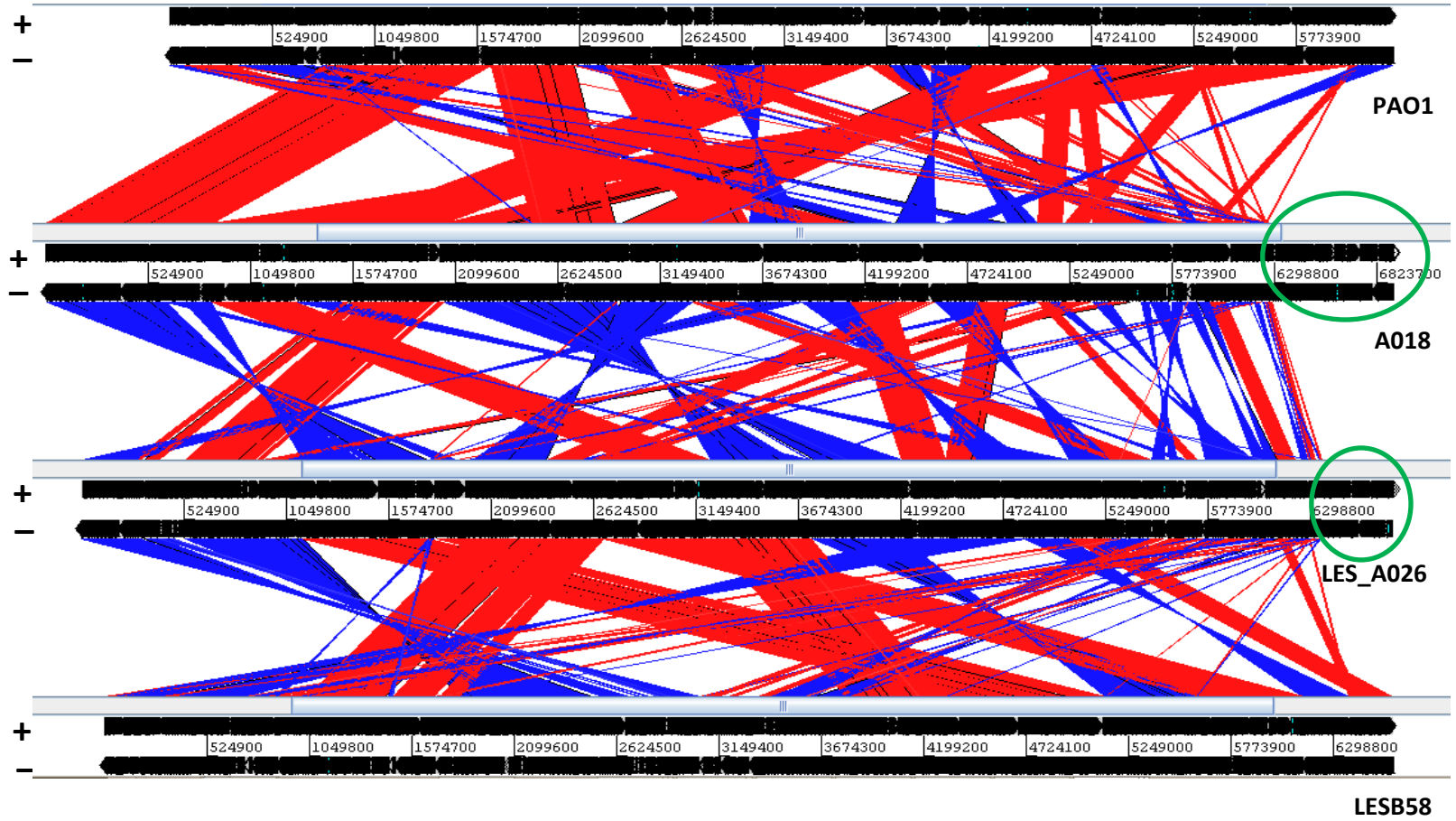


Fig. 5.3: Genome comparison of PA018, LES-related A026 and reference strains PAO1 and LESB58 in the ACT view. Forward (+) and complement (–) strands of each genome are indicated in the black genome lines. The full extent of each genome is shown and drawn to scale. Direct and inverted synteny of the coding sequences are shown in red and blue respectively; the score cut off was set to a footprint of 2 kbp in order to exclude low scoring hits while comparing the genomic arrangements. The green circles show areas of loss of synteny in both PA018 and LES-related A026 genomes towards the 3' ends of their forward strands.

On a closer inspection of the ACT images, approximate sizes of the genomes of PA018 and the LES_A026 that showed some degree of synteny (either direct or inverted) were comparable to the genome size of PAO1 i.e. ~6.3 Mbp (Fig. 5.3). Within the limits of the 2 kb homology that was used to fine tune the ACT images, there were parts of the genomes of these clinical strains that had no identity with each other or with PAO1 and LESB58 reference genomes (green circles in Fig. 5.3) and these corresponded with the extra genome sizes of approximately 457 to 635 kbp noted earlier. These genome areas existed towards the 3'-end of the forward (+) strands.

Using the DNAPlotter (Carver *et al.*, 2009), the features of the genomes of these clinical isolates were visualised and individually compared with the two reference strains (Fig. 5.4). The G+C contents of these extra parts of their genomes were particularly low ranging from 7.2% to 82.4% (in LES_A026) and 7.5% to 83.3% (in PA018) compared to their respective average values of 64.45% and 63.22%; with some overwhelming proportions falling well below these average values. These low G+C values and the wide ranges of the G+C% values spread over these parts of the genome suggested that they were accessory and not related to the *P. aeruginosa* genome which has a typical G+C average of 66.6% (Table 5.1).

Another feature that supported the presence of these foreign accessory genes was their existence in clusters (Fig. 5.4). Furthermore, it was noted that there were more transfer RNAs (tRNAs) in the clinical isolates than in the reference strains. For PA018, out of the total 91 tRNAs, 26 were present in the accessory DNA while the accessory genes of LES_A026 had 22 tRNAs out of the 85 total tRNAs found in its genome (Table 5.1). In the absence of these extra tRNAs, the number of the tRNAs found in the core genomes of these clinical isolates were

comparable to the total tRNAs of PAO1 (Table 5.1). Probing the coding sequences (CDSs) for putative transposases or integrases in the accessory genomes, two transposases apiece were found for each of the clinical strains, whereas two integrases were in the accessory genome of LES_A026 only and none in that of A018.

The BLASTn alignments of NCBI was used to probe the ancestral origin of these accessory genes in the clinical isolates. Results showed that they were at least 99% identical to the genes of species like *Clostridium difficile, Acanthamoeba castellanii* and the Enterobacteria. Some products of these accessory genes suggested an added virulence advantage e.g. some genes coded for metallo-betalactamase super family of proteins, multiple antibiotic resistance proteins, multi-drug efflux pumps and ferrous ion transport proteins. There were 44 and 42 phage-related genes respectively in LES_A026 and PA018, some of these were in clusters. Figure 5.4 shows the approximate distribution of the phage-related genes (short red lines on the outermost circle) together with the cluster arrangement of the R- and F-pyocin genes (in the R-F pyocin loci). Some other clusters were discovered within each of these genomes which are yet to be annotated while other phage-related genes were scattered throughout the genomes. Four of the genes in the accessory genome were phage-related for each of the clinical isolates (Fig. 5.4).

	PAO1	PA018	LESB58	LES_A026
Genome size (Mbp)	6.264	6.899	6.602	6.721
GC content (%)	66.56	63.22	66.30	64.45
Total no of coding sequences (CDSs)	5570	6161	5966	6069
Total tRNAs	63	91	67	85

Table 5.1: Some important differences in the genomes of clinical isolates PA018 and LES_A026 compared with the reference strains PAO1 and LESB58. The clinical strains have bigger genomes made up of more genes in the coding sequences. The number of total tRNAs was also higher but the average G+C contents were marginally lower.

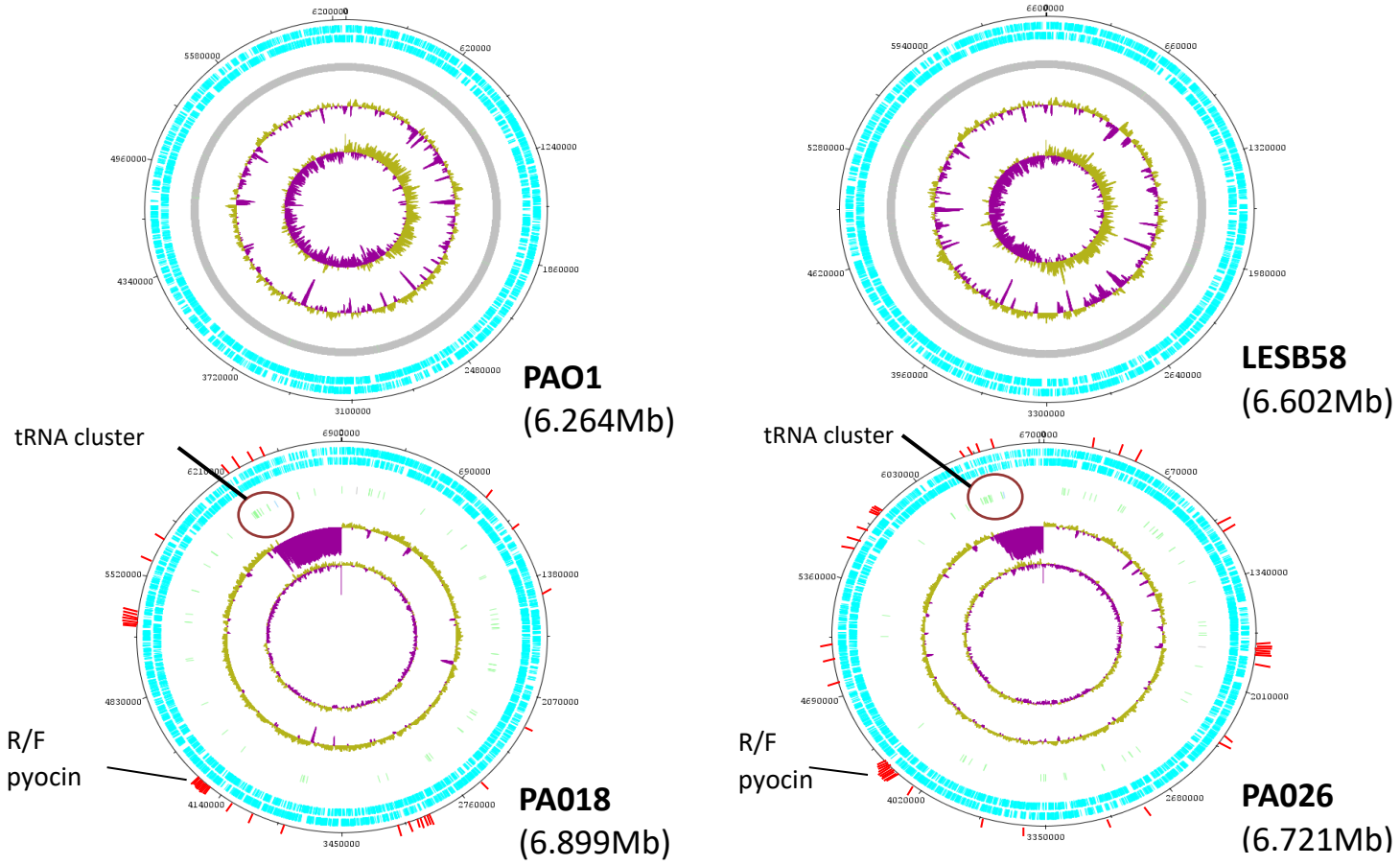


Fig. 5.4: The DNAPlotter images of LES_A026 and PA018 and the reference strains PAO1 and LESB58. From inside out, the innermost ring shows the GC skew, the next ring is the GC plot. Each GC plot of the clinical isolates shows a cluster of GC poor regions (purple) towards the 3' tail end of the forward strand. For PA018 and LES_A026, the next ring shows the distribution of tRNAs. The outermost two blue rings denote the coding sequences in both forward and reverse strands. The red spikes on the circumference show approximate locations of phage-related genes on the clinical isolates with the R/F pyocin gene cluster annotated.

5.2.3. Unique pyocin properties of LES_A026 and PA018

As LES_A026 had no S type pyocins in its genome, its resistance to any of the soluble pyocins of PA018 posed an intriguing question about the likely sources of immunity to S-type pyocins in this strain.

The Artemis® and Artemis Comparative Tool (ACT®) software packages (Carver *et al.*, 2008) were used to search for genetic determinants such as S type pyocin immunity genes which could account for the resistance. Using the Artemis software to navigate the LES_A026 genome for gene products having the property (qualifier value) of 'immunity', three hits were found: these are genes *00599, 01372* and *03011* (from the serial gene numbering of the forward strand of the LES_A026 genome). Gene *01372* is an 'orphan' pyocin S2 immunity gene present in a comparable locus as the pyocin S2 immunity gene (PA1151) of PAO1, but it does not have a cognate structural S2 gene. It bears 65.12% homology with PA1151 of PAO1 but has a closer ancestral link with colicin E2 immunity gene of *Escherichia coli* with which it has 84% homology.

Using the NCBI Sequence Alignment Tool (Clustal Omega) to assess gene alignment and phylogeny, the other two immunity genes of LES_A026 i.e. *00599* and *03011* are both closely related to the immunity gene of pyocin S3. Since the DNase mechanism of killing are shared by pyocins S1, S2, S3 and AP41, the immunity genes of these pyocins were also mapped against the *00599, 10372* and *03011* genes of the LES_A026 genome. Genes *00599* and *03011* form a clade with the S3 immunity gene and they have a common root of ancestry with the AP41 immunity gene (Fig. 5.5.). The clade formed by the S1 and S2 immunity genes also appears to be recent differentiation events from a sister group which shares the

same internal node as the 'orphan' *01372* immunity gene of LES_A026 strain (Fig. 5.5). The ancestry root of the *E. coli* colicin immunity gene E2 is distant and could not be assessed within the limit of the number of genes under analysis.

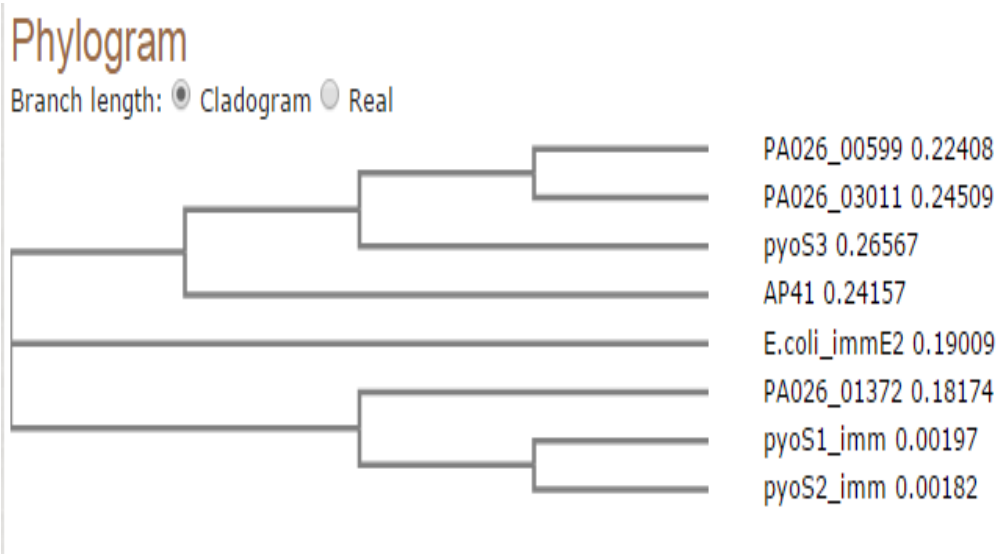


Fig. 5.5: Phylogram showing the relationship by ancestry of the immunity genes of pyocins S1, S2, S3, AP41 with the orphan immunity genes (00599, 03011, and 01372) of LES_A026 strain. All immunity sequences were compared with the immunity gene of colicin E2 (E. coli_immE2).

However, although the presence of immunity genes could explain the resistance of LES_A026 to pyocins S1 and S3, no immunity gene against the S4 pyocin was found. Since the S2 and S4 pyocins share the same ferripyoverdine receptor, FpvAI to gain access into susceptible cells (Elfarash *et al.*, 2012), an *in silico* analysis of the *fpvA1* gene encoding this receptor was done. The *fpvA1* gene of PAO1 reference strain is PA2398 (Elfarash *et al.*, 2012) and a comparative analysis of a corresponding homologous gene in the LES_A026 genome reveals that it was absent in the genome (Table 5.2, Fig. 5.6a).

Although utilised for the uptake of S2 and S4 pyocins, the FpvA1 receptor is primarily a Fe^{3+} scavenger. An alternative receptor (FpvB) has been recognised for ferripyoverdine-Fe^{3+} uptake (Ghysels *et al.*, 2004). The FpvB receptor encoded by *fpvB* (PA4168 in PAO1 genome) was present in LES_A026 (gene *05532*) and was 100% homologous to PA4168 of PAO1. The absence of *fpvA1* was also seen in LESB58 although it had identical *fpvB* gene with the PAO1 and LES_A026 genomes. Both *fpvA1* and *fpvB* are however present in the PA018 genome (Table 5.2).

Another pyocin gene present in PA018 to which LES_A026 was apparently resistant is pyocin S5. Resistance to S5 pyocin would be better analysed using pure form of the pyocin since some strains having the genes may not produce S5 pyocin under the prevailing growth conditions. The ferripyochelin receptor FptA has been recognised for the uptake of S5 pyocin (Elfarash *et al.*, 2014). The *in silico* analysis of this gene *fptA* (PA4221 in the PAO1 genome) shows that its sequence is preserved in the genomes of the clinical isolates LES_A026 (*05632*) and PA018 (*05520*) and also in LESB58 (*07061*). Although, the PA018 strain possessed an S5 pyocin gene having 100% homology with that of the PAO1 strain, it did not have the corresponding immunity gene (Table 5.2). The mode of protection against autolysis by its own S5 pyocin was not clear but suggests that other forms of immunity against the S5 pyocin exist.

The absence of a cognate immunity gene for the S2 structural gene (*41691*) of LESB58 was also pertinent but an alignment of the sequence of this gene with the pyocin S2 structural gene of PAO1 showed that the fourth domain containing the active DNase activity is grossly mal-aligned and thus presumably inactive.

The R1 pyocin sequence of LES_A026 was homologous to that of LESB58 but the F-pyocin locus present in LES_A026 was completely absent in the LESB58, thus the latter does not produce F-pyocins. By aligning the F-pyocin sequence of LES_A026 with the tail fibre sequences of F1 and F2 pyocins reported by Nakayama *et al.,* 2000, the F-type pyocin produced by LES_A026 was discovered to be the F1-type (Nakayama *et al.*, 2000).

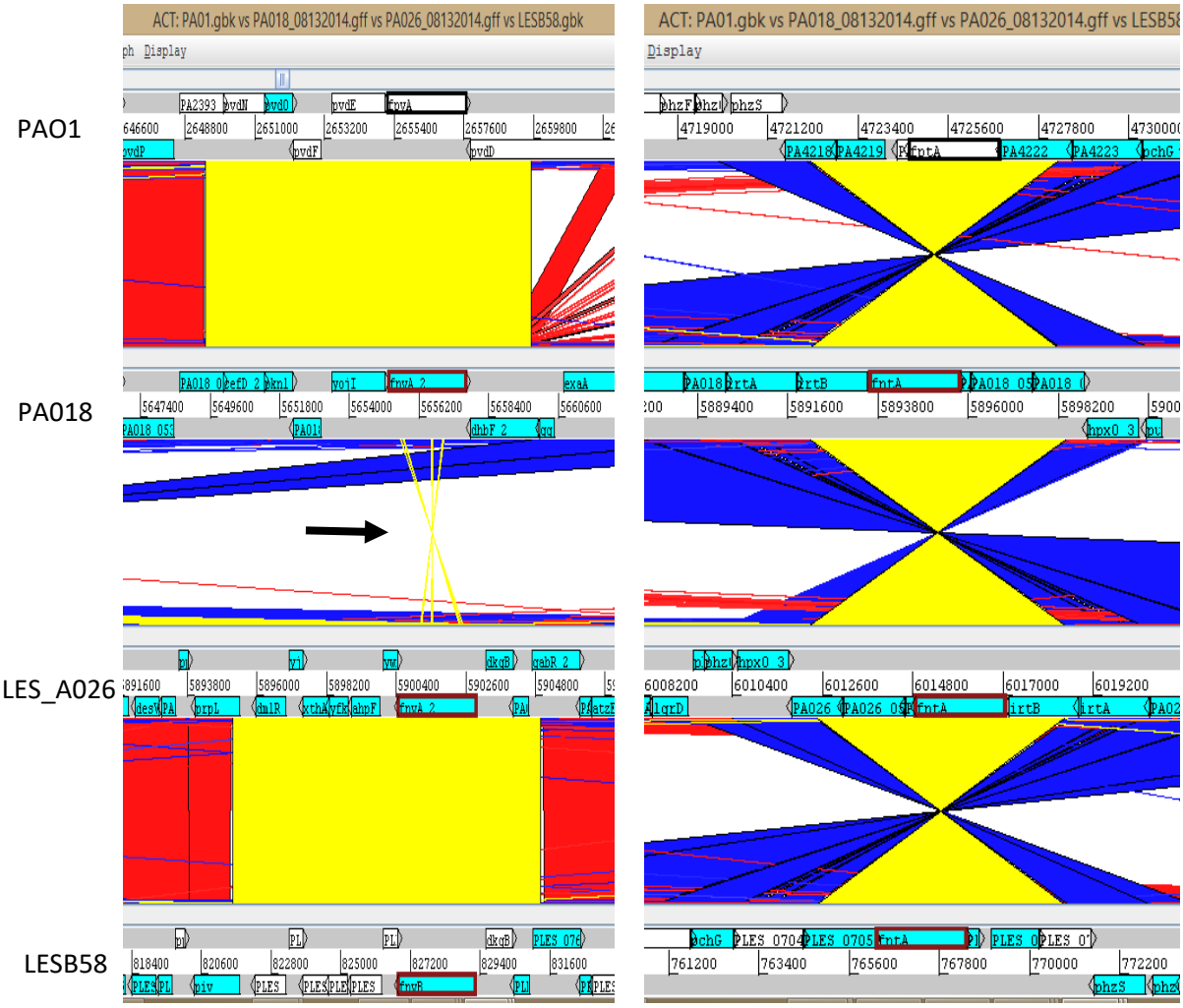


Fig. 5.6: *In silico* analysis of the genes coding for the receptors of S2/S4 and S5 pyocins (*fpvA1* and *fptA* respectively). The comparative genomics was performed by the ACT software. Yellow colour depicts the synteny of the genes under analysis, with direct synteny shown as a colour block (a) and inverted synteny as cross-overs (b). The *fpvA1* gene of PAO1 and PA018 are homologous showing direct synteny just like the versions seen in LES_A026 and LESB58 (a). However, *fpvA1* of LES_A026 or LESB58 does not have any significant relationship with *fpvA1* of PAO1 or PA018 (black arrow). The pyocin S5 receptor gene *fptA* was however preserved in all the four strains showing inverted gene order (b). Genes under analysis are shown in rectangular red borders.

PAO1 Reference strain	A018	LES_A026	LESB58
Pyocin S5 (*PA0985*)	√	X	X
Immunity S5 (*PA0984*)	X	X	X
Pyocin S2 (*PA1150*)	√	X	Variant (distorted DNase domain)
Immunity S2 (*PA1151*)	√	Variant (65.12 % homology)	X
Pyocin S4 + immunity S4 (*PA3866*)	√	X	X
Pyocin R2 (*PA0615-PA0628*)	√	Variant R1-type (*PA0620-PA0621*)	Variant R1-type (*PA0620-PA0621*)
Pyocin F2 (*PA0632-PA0648*)	√	Variant F1 type (*PA0642-PA0648*)	X
Other pyocin related immunity genes X	X	*00599, 03011* (PA026 genes)	X
Pyocin S2/S4 receptor *fpvA1* (*PA2398*)	√	X	X
Alternative ferripyoverdine receptor *fpvB* (*PA4168*)	√	√	√
Pyocin S5 receptor *fptA* (*PA4221*)	√	√	√

Table 5.2. Pyocin related genes of LES_A026, A018 and LESB58 using PAO1 as reference strain (Key: √ = gene present and 100% homologous, **X** = gene absent, any homology less than 100% are shown as variants with the corresponding percentages.

5.2.4. Other virulence properties of PA018 and LES_A026

Some of the virulence genes of LES_A026 which were investigated using the ACT and Artemis software packages include those for synthesis of the LPS, flagella, exotoxin, phenazines, pyocyanin, pyoverdine etc. (Table 5.3). While most of these were essentially homologous to the corresponding genes in the PAO1 reference strain, some including LPS and phenazines were closely related to those of LESB58 while others like pyoverdine and flagella were related to neither strains (Table 5.3). Probably the most notable genetic determinant of LES_A026 discovered was a mutation in *lasR*, one of the quorum sensing genes which regulates many genes linked to virulence (Table 5.3).

PAO1 reference strain	A018	LES_A026	LESB58
LPS O-antigen *wbp* genes (*PA3141-PA3160*)	√	Variant (100 % homology to LESB58)	Variant
Flagella biosynthesis (*PA1077-PA1105*)	√	Variant (unrelated to LESB58)	Variant
lasI (*PA1431*)	√	√	√
lasR (*PA1430*)	√	Variant (deletion of first 115 amino acids)	√
rhlI (*PA3476*)	√	√	√
rhlR(*PA3477*)	√	√	√
Exotoxin A (*PA1148*)	√	√	√
Phospholipase C (*PA0026, PA0844, PA3319*)	√	√	√
Elastase (*PA3724*)	√	√	√
Phenazine (*PA1899-PA1904*), (*PA4209-PA4216*)	√	Variant (100 % homology to LESB58)	Variant
Pyocyanin related (genes *phzS, phzM, phzH*)	√	√	√
Pyocheline siderophore (PA*4221-PA4229*)	√	√	√
Pyoverdine (PA*2385-PA2427*)	√	Variant (unrelated to LESB58)	Variant

Table 5.3. Virulence properties of A018, LES_A026 and LESB58 using PAO1 as reference strain (Key: √ = gene present and 100% homologous with PAO1 reference gene, **Variant** = any homology less than 100% or unrelated to the that of the reference strain).

5.2.5. LES_A026 strain is a *lasR* mutant with a truncated N-terminus and has the characteristic autolytic plaque phenotype on agar plates.

Comparison of the amino acid residue sequences of LasR from LES_A026 with the corresponding gene of PAO1 shows that the LES_A026 variant has a genetic deletion of 115 residues from the N terminus thus producing a truncated polypeptide missing the first 115 amino acids of the normal LasR protein. LasR from LES_A026 was 124 residues and 13.61 kDa in size compared with the normal polypeptide of 239 residues and 26.6 kDa in size. The methionine used as the first amino acid for the truncated LasR polypeptide was the 116th amino acid of the normal LasR protein (Fig. 5.7).

Phenotypically, the strain had the characteristic iridescent sheen and autolysis on agar plates associated with *lasR*$^{-}$ mutant strains (Hoffman *et al.*, 2009) (Fig. 5.8). These punched out autolytic lesions bear close resemblance with phage-like plaques.

5.2.6. Characteristic autolytic plaques seen in LES_A026 are lost in both the null-R-pyocin mutant and LasR complemented derivatives of LES_A026.

As R type pyocins are commonly referred to as tailocins with strong similarity to defective phage tails, it was pertinent to investigate a possible relationship between R type pyocin expression and the appearance of the autolytic plaques in strains with a *lasR*$^{-}$ deficient background. Thus the phenotypes of LES_A026 and its two derivatives i.e. the complemented *lasR* (LES_A026lasR^{+}) and null-R pyocin (LES_A026ΔR) derivatives were compared.

Autolysis was lost in both derivatives compared to LES_A026 wild type suggesting a relationship between LasR-dependent quorum sensing and R pyocin production. (Fig. 5.8)

```
PAO1lasR        MALVDGFLELERSSGKLEWSAILQKMASDLGFSKILFGLLPKDSQDYENAFIVGNYPAAW
PA026lasR       ------------------------------------------------------------

PAO1lasR        REHYDRAGYARVDPTVSHCTQSVLPIFWEPSIYQTRKQHEFFEEASAAGLVYGLTMPLHG
PA026lasR       ------------------------------------------------------MPLHG
                                                                       *****

PAO1lasR        ARGELGALSLSVEAENRAEANRFMESVLPTLWMLKDYALQSGAGLAFEHPVSKPVVLTSR
PA026lasR       ARGELGALSLSVEAENRAEANRFMESVLPTLWMLKDYALQSGAGLAFEHPVSKPVVLTSR
                ************************************************************

PAO1lasR        EKEVLQWCAIGKTSWEISVICNCSEANVNFHMGNIRRKFGVTSRRVAAIMAVNLGLITL
PA026lasR       EKEVLQWCAIGKTSWEISVICNCSEANVNFHMGNIRRKFGVTSRRVAAIMAVNLGLITL
                ************************************************************
```

Fig. 5.7: The LasR sequence of strain A026 shows a deletion of the first 115 amino acids from its N-terminal end when compared to the LasR sequence of PAO1. All amino acids after the truncated N terminus have the same sequence as the reference peptide.

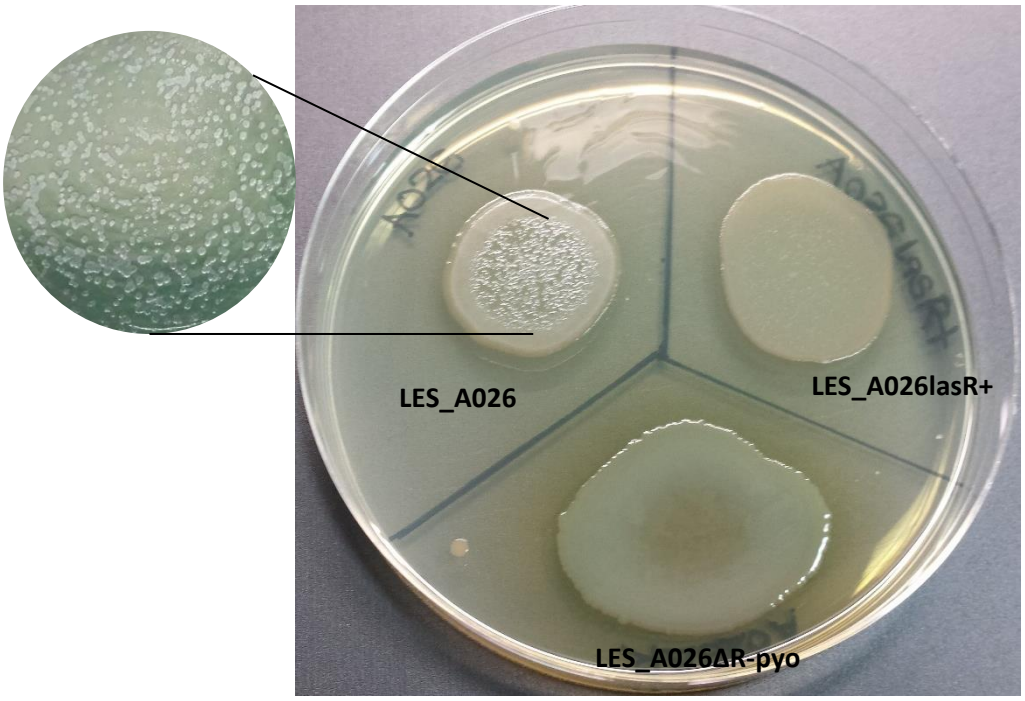


Fig. 5.8: Morphology of LES_A026 and its derivatives on agar plate. The derivatives are LES_A026 complemented with *lasR* (LES_A026lasR^{+}) and the R-pyocin defective mutant of A026 (LES_A026ΔR-pyo). The iridescent sheen and autolytic plaques which are obvious in LES_A026 are lost in these two derivatives.

5.2.7. LES_A026 (*lasR* mutant) and PAO1 Δ*lasR*-N mutants display a sustained stationary phase during growth.

To investigate the relationship between *lasR* and R pyocin production further, the Nottingham strain of PAO1 (PAO1-N) and its *lasR*⁻ defective strain (Δ*lasR*-N) were used. This allowed direct comparison between a wild type strain and its isogenic *lasR* mutant and alleviates any potential interference from the vast array of SNPs and INDELs in the LES_A026 genome.

The growth curves of PAO1-N and ΔlasR-N were plotted and compared. Results showed that the exponential growth phases of both strains were identical but the growth of ΔlasR-N plateaued at the stationary phase and did not have any lysis indicative of cell death that was prominent in the PAO1-N (Fig. 5.9).

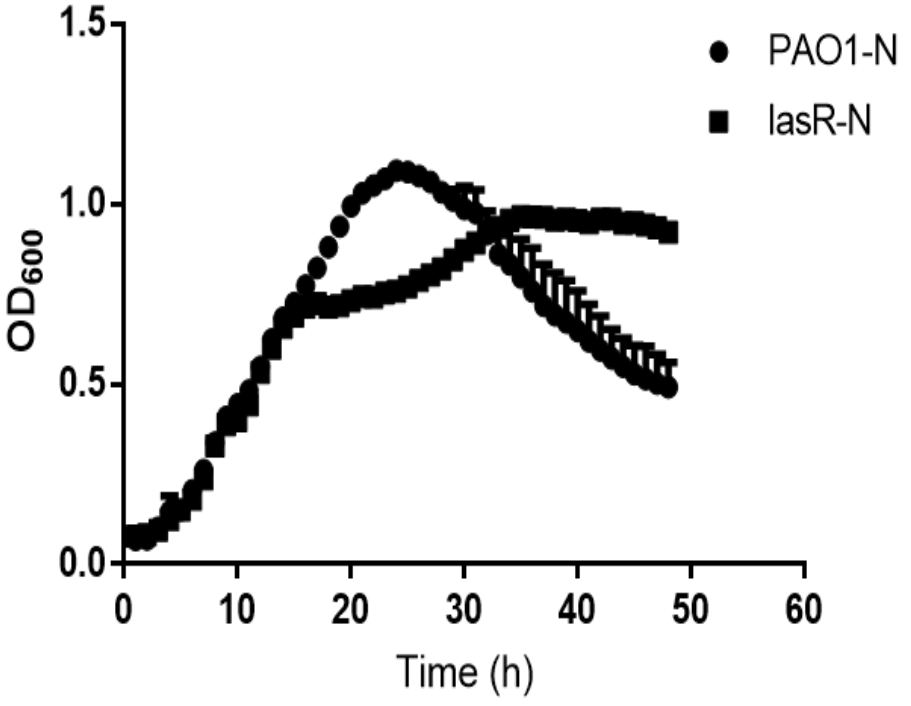


Fig. 5.9: The growth curves of PAO1 Nottingham strain (PAO1-N) and its *lasR* defective mutants (lasR-N). Growth traced the same course in the first 15 hours but thereafter, the wild type reached the highest peak of growth followed by a steep phase of decline. The *lasR* mutant however showed sustained growth until about 35 h after growth commencement, culminating in a steady plateau till the end of the experiment. This growth pattern was re-produced four times.

5.2.8. Construction of transcriptional reporters to evaluate the effects of *lasR* mutation on R-pyocin expression.

In order to evaluate the effects of the *lasR* mutation on R-pyocin expression, some transcriptional fusion constructs were made using the mini-CTX reporter system (Fig. 5.11). These constructs consisted of the promoter regions of two genes of interest (PA0615 and *recA*) fused upstream to the promoterless *luxCDABE* operon of the mini-CTX *lux* plasmid. The intergenic region upstream of the *recA* gene (133 bases) together with the first four codons in the same gene was used as the promoter sequence of *recA*. This sequence has been used before in another study (Boles & Singh, 2008) where they incorporated the intergenic region sequence and the first codon of *recA* (total of 136 bases) fused to a GFP reporter.

However, the promoter region for PA0615, the first structural gene of the R-pyocin (Chang *et al.*, 2005) has not been reported. Therefore, the sequences of the PA0615 gene, its upstream lysis gene PA0614 (Nakayama *et al.*, 2000) and the 21-base intergenic region between them were interrogated for putative promoter sequences using the BPROM software (Softberry, Inc., Mount Kisco, NY). Only one promoter was predicted in the sequence (Fig. 5.10). This promoter was used to monitor the expression of R-pyocin.

The promoter sequences of PA0615 and *recA* (PA0615p and *recAp* respectively) were amplified using primer pairs PA0615p-H3-F / PA0615p-ER1-R and N-recAp-H3-F / N-recAp-ER1-R respectively (Table 2.1) and ligated into pGEM-T Eazy vector (Fig 5.11a). Both p-mini-CTX *lux* and the recombinant pGEM-T plasmids were digested with *Hind*III and *Eco*RI in order to linearise the *lux* plasmid and release the promoter sequences from pGEM-T (Fig. 5.11b). The mobilised promoter sequences

were subsequently ligated into the mini-CTX *lux* plasmid and the new plasmids were named pOO2 (PA0615p::*lux* plasmid) and pOO3 (*recAp::lux* plasmid). The presence of the promoter sequences in the *lux* plasmid was confirmed by digestion with the same enzymes (Fig. 5.11c) and DNA sequencing.

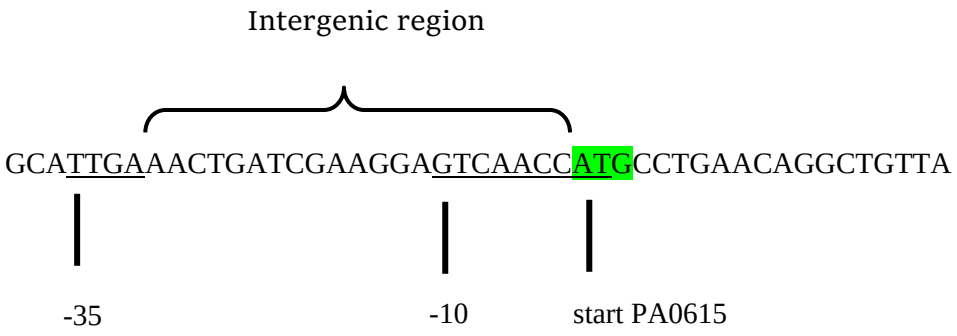


Fig. 5.10: The promoter site of gene PA0615 as predicted by the bacterial promoter finding tool, BPROM software (Softberry, Inc., Mount Kisco, NY). The predicted -35 and -10 sequences, intergenic region between PA0614 and PA0615 are shown while the start codon of PA0615 is highlighted in green.

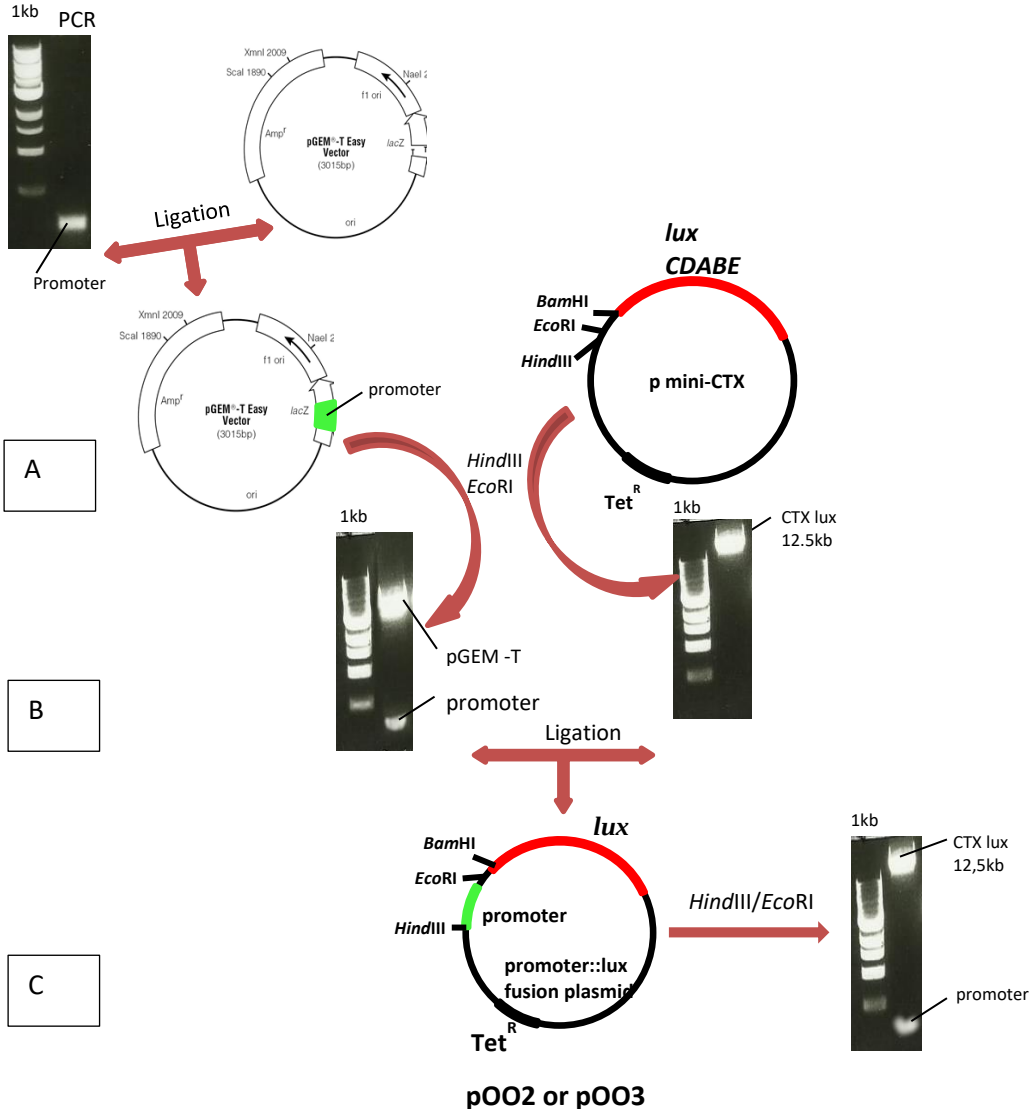


Fig. 5.11: Construction of transcriptional reporters by fusing the promoter regions of genes of interest (PA0615p and *recAp*) with the *luxCDABE* gene of mini-CTX *lux* plasmid. The promoters were amplified from the PAO1 genome and ligated into pGEM-T Easy vector (A). The promoter was subsequently mobilised from pGEM-T into p-mini-CTX *lux* by digesting the two plasmids with *Eco*RI and *Hind*III (B) and ligating the promoter with the linearised p-mini-CTX *lux*. The presence of the promoter in the plasmid was confirmed by a repeat digestion with the same enzymes (C) and DNA sequencing.

5.2.9. The *lux CDABE* bio-reporter system is suitable to study promoter response to *lasR* inactivation.

The mini-CTX-lux reporter system uses a bioluminescent reporter and is a useful tool to study gene expression at the level of transcription. Unlike the green fluorescence protein (GFP) reporter which has a long half-life (Tombolini *et al.*, 1997), the short half-life of the *lux* reporter allows it to detect very rapid changes in gene expressions thus increasing the sensitivity of the assay (Meighen, 1991). The β-galactosidase reporter (*lacZ*) is also widely used but does not allow for the direct assays and readings much like the GFP reporter which requires a long duration to fold into the fluorescent protein making it also inappropriate for real time measurements (Homburg *et al.*, 2007, Heim *et al.*, 1995). Changes in promoter activity which translates to corresponding changes in transcript levels and by extension gene expression, is signalled by equivalent intensities of light production from the luminescent protein expressed by the *lux* genes. Quantitative measurement of the light produced during the growth phase in real time can be automated for easy reading and analysis.

A mini-CTX *lux* plasmid bearing a constitutive reporter derived from the kanamycin resistance gene cassette *kan::lux* (Steve Higgins PhD thesis, 2014) was used as a control to assess whether Δ*lasR* influenced light production independent of its promoter sequences. This was done by transforming PAO1-N and ΔlasR-N strains with *kan::lux* and analysing bioluminescence response. The bioluminescence levels of both strains were plotted over time (Fig. 5.12a). The curves for both strains showed comparable areas under the curve (AUC) and light production over time (Fig. 5.12a). Furthermore, the peak values of bioluminescence attained in the two strains were also

of approximately equal values. These showed that *lasR* inactivation does not have an independent effect on the expression of *luxCDABE*. Thus the mini-CTX lux system was adjudged to be a good reporter to study different gene expressions in response to *lasR* inactivation.

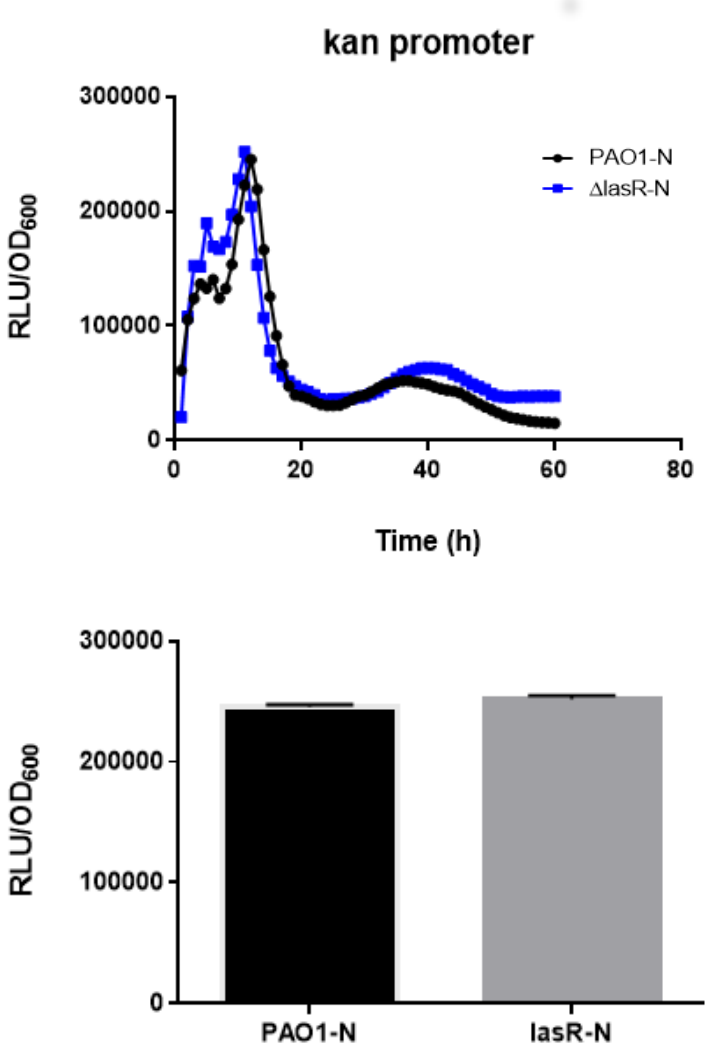


Fig. 5.12: A mini-CTX *lux* plasmid bearing a constitutive reporter derived from the kanamycin resistance gene cassette (Steve Higgins, thesis 2014) was used to transform PAO1-N and lasR-N strains by electroporation. These strains bearing the *kan::lux* fusion were used as controls in order to assess whether Δ*lasR* influenced light production independent of the promoter sequences used to study *lasR* expression. Graph (a) shows the bioluminescence (RLU/OD_{600}) over time to study the trend and compare the areas under the curves while graph (b) shows the peak value of the bioluminescence attained for each strain.

5.2.10. Mitomycin C as an inducing agent showed less cytotoxic effects than ciprofloxacin at comparable concentrations.

Two genotoxic agents, ciprofloxacin (a DNA gyrase inhibitor) and mitomycin C (MMC) (a potent DNA cross-linker) previously proven to have an effect on pyocin induction (Brazas & Hancock, 2005, Duport *et al.*, 1995) were used to assess effects on cell growth since pyocin induction involves cell lysis. Graded concentrations of 1 µg/ml, 0.5 µg/ml and 0.25 µg/ml were used for each of the genotoxins.

The aim of generating this gradient was to find a mutagenic agent (and its optimal concentration) which in addition to its documented potency as a pyocin inductor, also has a considerably reduced adverse effect on cell growth. These concentrations were added from the commencement of growth analysis and the growth curves were plotted and compared with an uninduced control that was not treated with either agent.

The results showed that ciprofloxacin prevented the growth of the cells even at the lowest concentration used (0.25 µg/ml) (Fig. 5.13). MMC treatment has minor effects on growth compared to the uninduced control with the lowest concentration (0.25 µg/ml) having a growth profile similar to that of the control (Fig. 5.13).

The concentration of MMC used for pyocin induction was thus 0.25 µg/ml. At this concentration, cell growths were optimal.

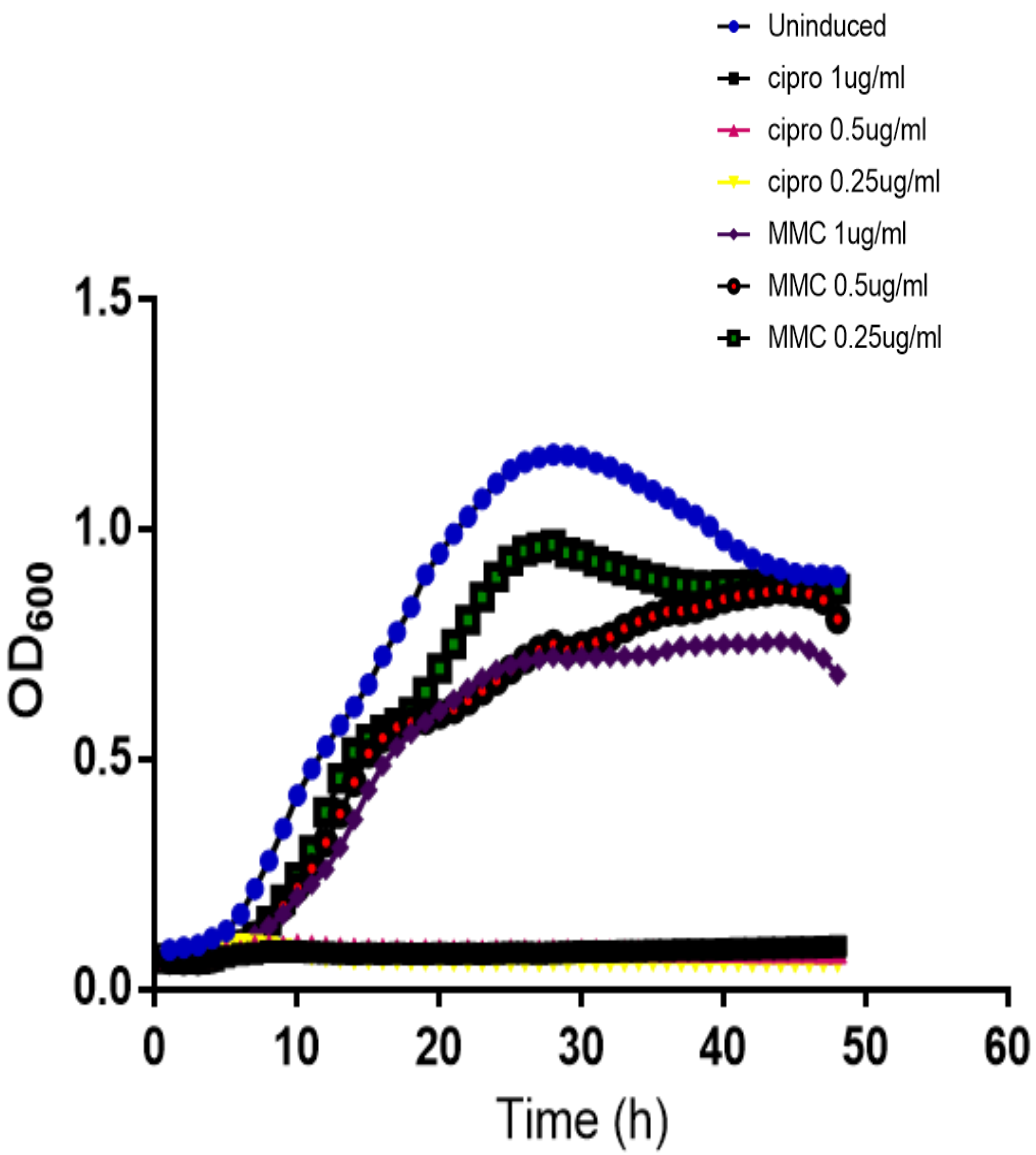


Fig. 5.13: Using genotoxic agents to assess the effect on cell growth. Genotoxic agents mitomycin C (MMC) and ciprofloxacin at different concentrations of 1 µg/ml, 0.5 µg/ml and 0.25 µg/ml were added to the growing cells and their effects on growth were measured. Ciprofloxacin was growth inhibitory at all the three concentrations used. Growth inhibition recorded from the effect of MMC reduced with decreasing concentrations. The least concentration of MMC (0.25 µg/ml) showed comparable growth with the untreated population.

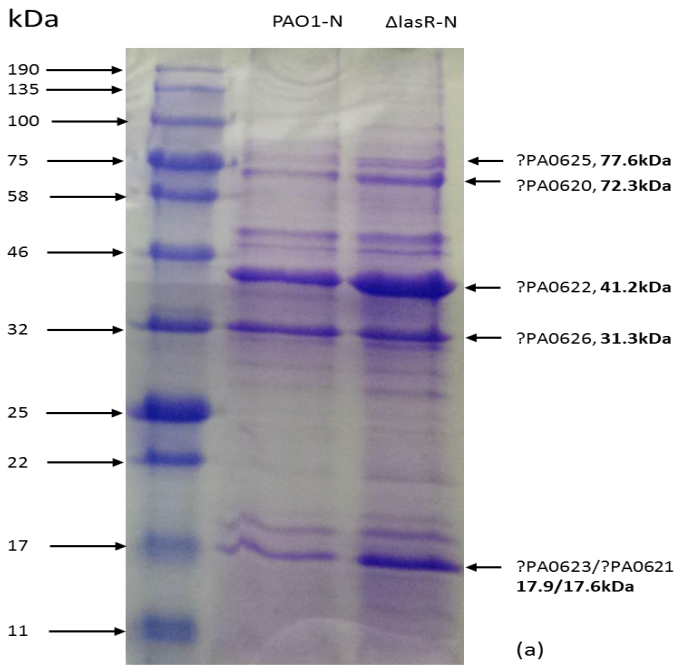
5.2.11. *lasR* mutation causes increased R-pyocin expression and activity.

R-pyocin was expressed and purified in PAO1-N and ΔlasR-N and subjected to SDS PAGE analysis and biological activity assay in order to investigate if there are difference in R-pyocin expression in the two strains.

Induction of R-pyocin expression was carried out for both strains after growing them to comparable cell populations at OD_{600}. The R-pyocins were purified and analysed on SDS PAGE. On SDS PAGE analysis, there was increased production of the R-pyocin component peptides in the ΔlasR-N compared to PAO1-N. Fig. 5.14a shows a Coomassie blue stained SDS polyacrylamide gel. Therefore, these R-pyocins were tested on a susceptible strain (A010) using a semi-quantitative spot assay.

The R-pyocins of ΔlasR-N and PAO1-N (being from the same parent strain PAO1-N) was used against the same susceptible indicator strain (A010). When used in their purified undiluted form, both R-pyocins gave a clear zone of inhibition and at first it appeared that there was no difference in their activities. However, double dilutions of the concentrated R-pyocin showed that the least dilution at which the lethal activity of the R-pyocins of PAO1-N was preserved was 32-fold while that of the lasR-N was as high as 128-fold dilutions (Fig. 5.14b).

There appears to be an increase in the R pyocin molecules produced by ΔlasR-N by at least 4-fold compared to that of PAO1-N.



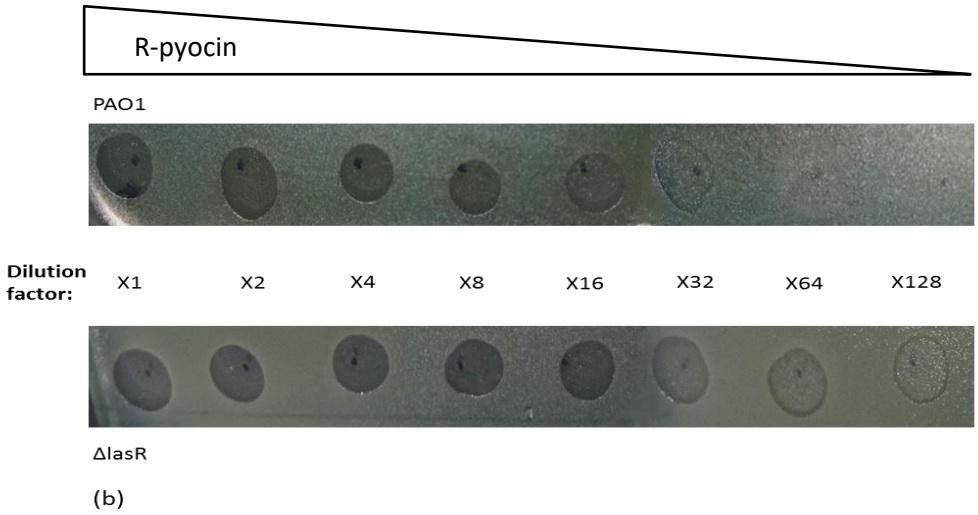


Fig. 5.14:

(a) Coomassie blue stained SDS polyacrylamide gel of purified R-pyocins of PAO1-N and ΔlasR-N. First lane is the molecular weight marker with arrows indicating the band weights.

(b) Semi-quantitative spot assay using serial double dilutions of purified R-pyocins of PAO1-N and ΔlasR-N against a susceptible indicator strain. The starting concentration was prepared from undiluted induced R-pyocin prepared from PAO1-N and lasR-N at comparable optical density values OD_{600}. Each dilution spot was 7 µl and plates were incubated at 37 °C overnight.

5.2.12. Genetic complementation of the Δ*lasR* mutant

Before any effect of the *lasR*$^{-}$ mutation on R pyocin expression was determined, it was necessary to restore *lasR* to ΔlasR-N by genetic complementation to ensure that any differences in R pyocin expression between PAO1 and PAO1Δ*lasR* were entirely due to *lasR* inactivation.

Genetic complementation of *lasR* was achieved in trans under the transcriptional control of the native promoter in pUCP18 carrier plasmid (Fig. 5.15). The entire sequence of the *lasR* gene was amplified using primers lasR-pUCP18-ER-F and lasR-XbaI-R (Table 2.1) and ligated into pGEM-T Easy vector (Fig. 5.15a). Both pUCP18 and the recombinant pGEM-T plasmids were digested with *Eco*RI and *Xba*I (Fig. 5.15b) and the mobilised *lasR* gene was subsequently ligated into the linearised pUCP18 and named pOO4. The *lasR* gene ligation was checked by digesting with the same enzymes (Fig. 5.15c) and confirmed by DNA sequencing.

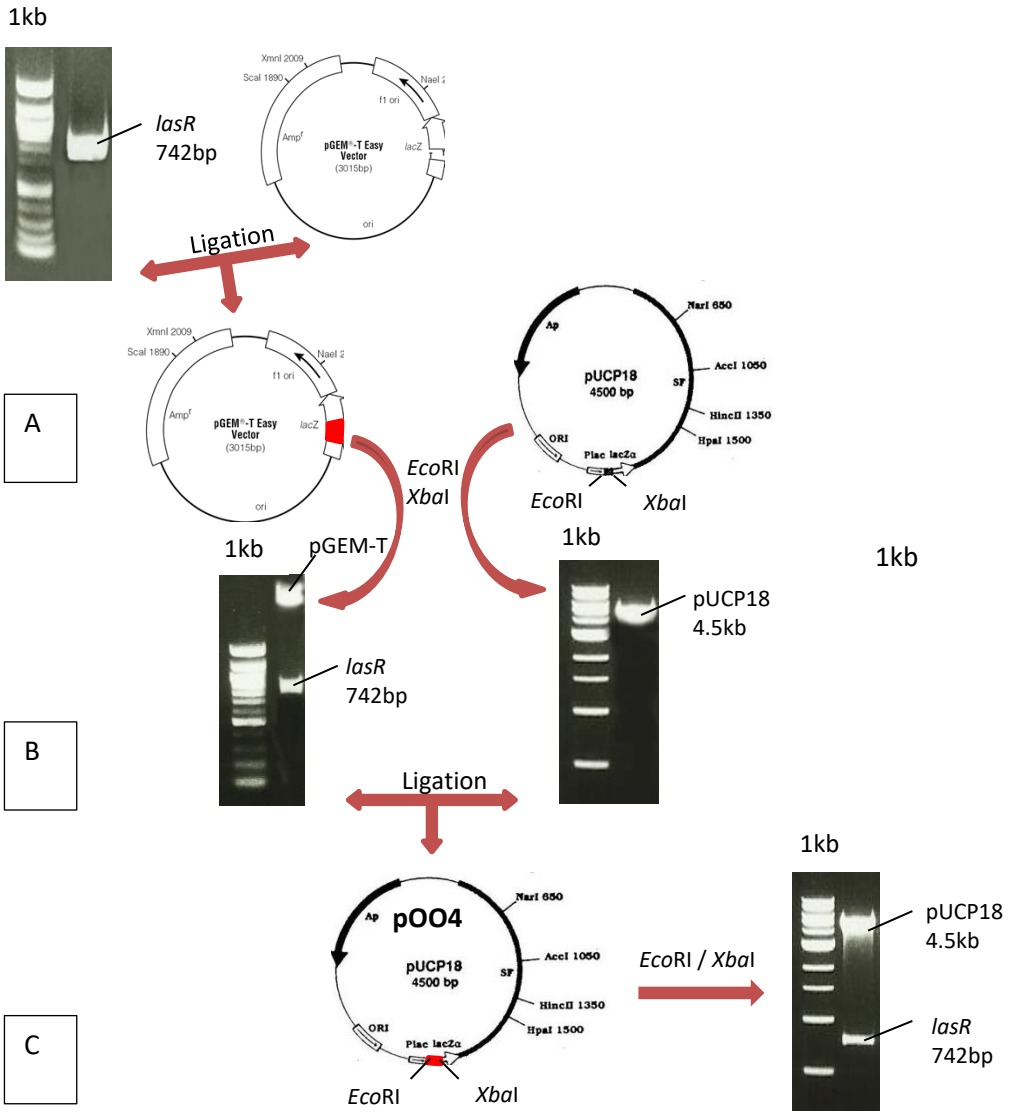


Fig. 5.15: Genetic complementation of *lasR*. The *lasR* gene was amplified by PCR and ligated into pGEM-T Easy vector (A). Thereafter, it was mobilised into the pUCP18 plasmid by digesting both plasmids with *Eco*RI and *Xba*I (B) and then ligating the *lasR* with the linearised pUCP18. The presence of *lasR* in the pUCP18 was confirmed by repeat digestion of the recombinant plasmid with the same enzymes (C) and DNA sequencing.

5.2.13. *ΔlasR* mutant strain shows increased expression of R-pyocins in real time compared to the wild type.

The SDS PAGE and spot assay only measured the end point of expression of R-pyocin in the strains and involved external induction with mitomycin C. It was important to probe the real time expression of R-pyocins in ΔlasR-N, PAO1-N and the complemented ΔlasR-N strain (*lasR*$^{+}$-N) with and without induction, to study the effect of the *lasR* mutation. The promoter of the first structural gene of R-pyocin (PA0615) was used as the transcriptional fusion reporter with the *lux* plasmid for this purpose.

The rate of transcription of the PA0615 was recorded as the relative luminescence unit (RLU) standardised by the OD_{600} nm (RLU/OD_{600}). In the uninduced state, the expression of R-pyocin recorded more than 6-fold increase in the logarithmic function of cell density (RLU/OD_{600}) in the ΔlasR-N compared to the PAO1-N strain (p values < 0.001). (Fig. 5.16a). Genetic complementation of the *lasR* gene restored the expression rate of R-pyocin in the complemented strain (lasR^{+}-N) to values comparable to that of the wild type, PAO1-N (Fig. 5.16b). Upon induction with MMC, the highest transcription levels increased slightly from 6-fold to over 7-fold in ΔlasR-N, but more significantly, the duration of expression also increased. After induction with MMC, the time taken for the transcription levels to be restored to the basal value was doubled (40 h) when compared to the uninduced assay (20 h) (Fig. 5.16a & b).

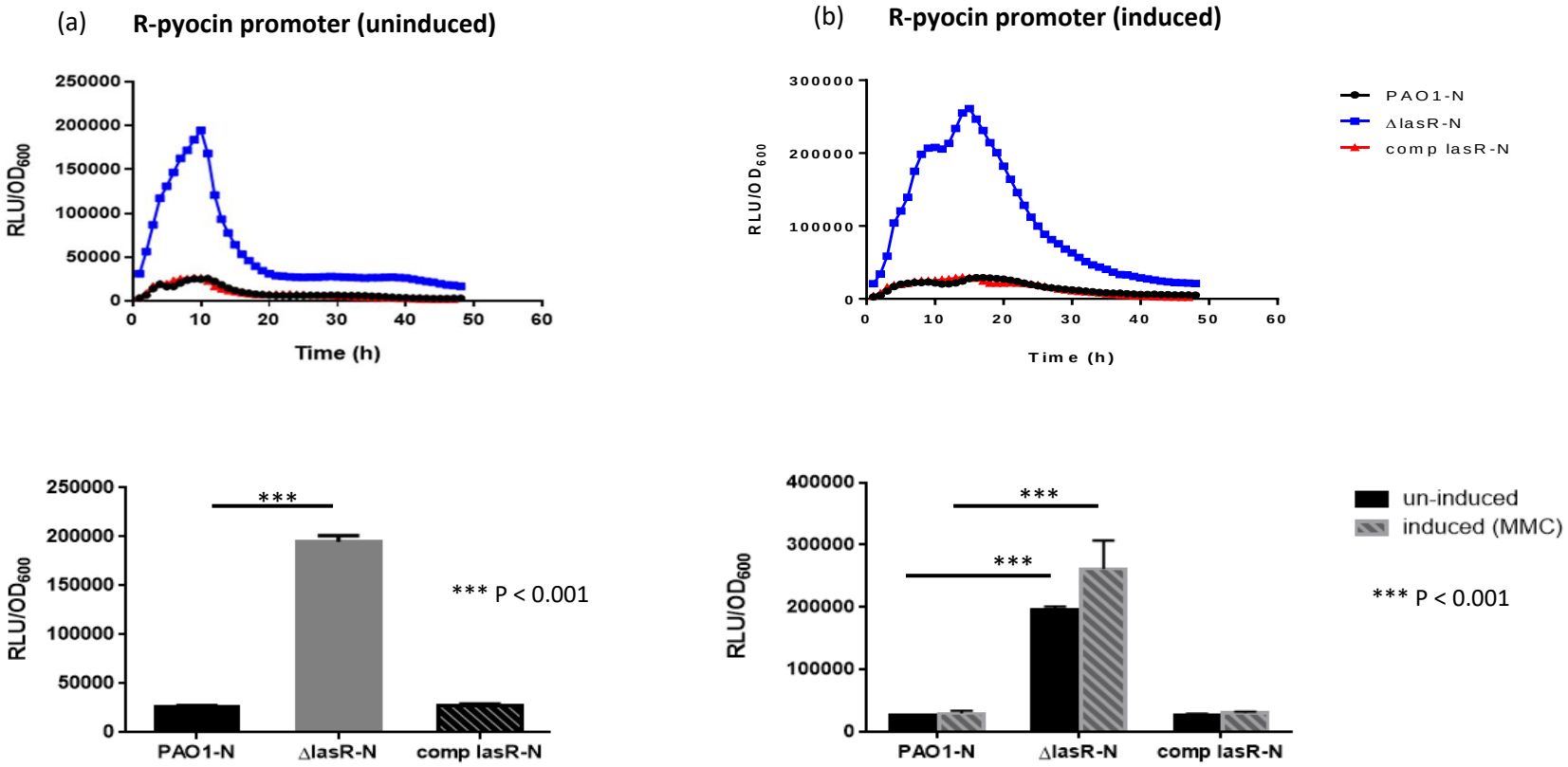


Fig. 5.16: Real time analysis of R-pyocin expression using the promoter of the first R-pyocin gene (PA0615) fused to the mini-CTX lux reporter system with or without induction with mitomycin C. The line graphs in the upper panel show bioluminescence over time. There is a relative increase in R-pyocin expression in ΔlasR-N compared to PAO1-N both in the induced and uninduced states and these were reversed in the lasR complemented strain (comp lasR-N). The bar charts in the lower panel plot the peak of bioluminescence for PAO1-N, lasR-N and the complemented lasR strain (comp lasR-N). The elevated peaks in ΔlasR-N were reverted to levels comparable to PAO1-N in the comp lasR-N strain.

5.2.14. Suppression of R-pyocin expression in PAO1-N is RecA-independent

Pyocin expression is known to be under the regulation of RecA (Michel-Briand & Baysse, 2002). Therefore, using the *P. aeruginosa* RecA promoter for *lux* expression will help to probe any relationship between RecA and R-pyocin expression in a background of intact or mutated *lasR*.

Results show that RecA expression is not correspondingly increased in ΔlasR-N. RecA expression attained comparable levels in both PAO1-N and ΔlasR-N showing that mutation in the quorum sensing gene *lasR* does not have an effect on RecA expression (Fig. 5.17).

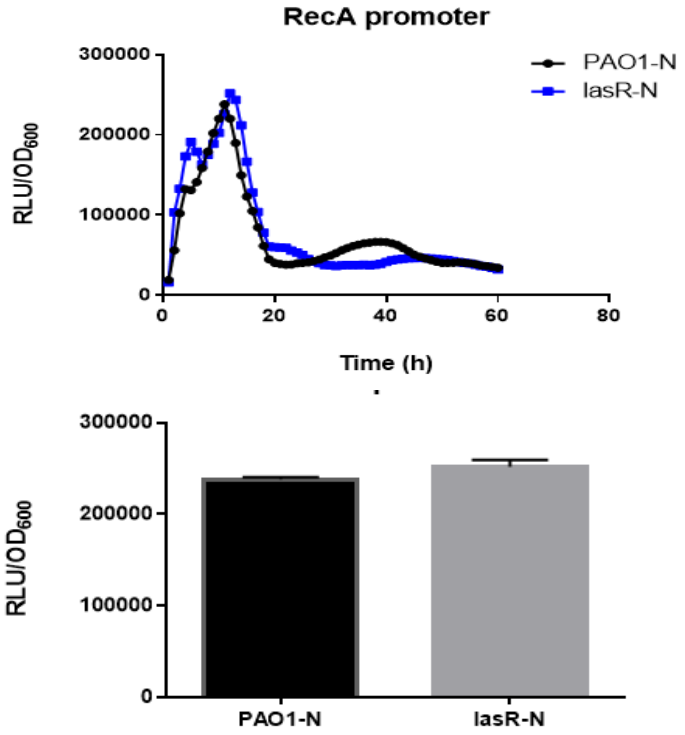


Fig. 5.17: p-mini-CTX lux bearing the promoter of RecA (*recAp:: lux*) in PAO1 and ΔlasR-N. Bioluminescence (RLU/OD_{600}) over time to compare the areas under the curves for PAO1-N and ΔlasR-N (a) and the peak values of the bioluminescence attained for each strain (b).

5.3. Discussion

The genomic study of the most competitive strain A026, revealed that it is related to the Liverpool Epidemic Strains (LES) in its ancestry. The LES have been associated with infections both in the CF and non-CF patients (Salunkhe *et al.*, 2005). It was first noted among the paediatric patients in Liverpool for its resistance to ceftazidime which was the antibiotic of choice in the mid-1990s (Cheng *et al.*, 1996) but the LES have since spread across all age groups (Panagea *et al.*, 2003) and geographical boundaries (Edenborough *et al.*, 2004, Kakinuma *et al.*, 2010, Aaron *et al.*, 2010) due to their property of high transmissibility. These strains are known to cause super-infections, taking over niches from other strains that have already established chronic infections (McCallum *et al.*, 2001, McCallum *et al.*, 2002, Mohan *et al.*, 2008). Although LES_A026 is highly competitive as a LES member, the work presented here shows that its competitiveness can be attributed (at least in part) to the high production of R pyocins.

Having compared the full sequences of the genomes of twelve reference strains (including PAO1), Ozer *et al.* concluded that approximately 11% of the genomes of *P. aeruginosa* are made up of accessory genes (Ozer *et al.*, 2014) which was in agreement with other studies (Mathee *et al.*, 2008, Spencer *et al.*, 2003). In the present study, a further genome addition of approximately 10% was present in each of the clinical isolates sequenced. The very low %GC plot of these added genes contributed to the reduction in the average %G+C of the genomes of these clinical strains compared to that of the average 66.6% of the *P. aeruginosa* genome.

Some hot spots on bacterial chromosomes which allow for site specific recombination and integration of foreign mobile

genetic elements have been identified (Williams, 2002, Reiter *et al.*, 1989). The transfer RNAs (tRNAs) have been well studied. These tRNAs have sequences which are well conserved across species and therefore offer a ready integration site using the *attB* site of the host. Reiter *et al* found that both the host DNA and the flanking regions of the integrated phage of *Haemophilus influenzae* (Hplc1) had $tRNA^{leu}$ and $tRNA^{tyr}$ which enhanced the integration of the phage into the host genome (Reiter *et al.*, 1989). In the present study, the cluster of tRNAs at the replication terminus of the genomes of clinical isolates PA018 and LES_A026 suggested that these hot spots were the portals of entry for the integration of the foreign genes.

Some of the organisms whose genes have striking homologies with the genes found in the accessory genomes of these strains include *Clostridium difficile*, (which accounted for more than 70%) and *Acanthamoeba castellanii*. These organisms which can be isolated from soil or occur as commensals in the gut are clues to the original habitats from where these *P. aeruginosa* clinical strains were acquired. This supports the notion that the accessory genome is horizontally acquired while the core genomes are vertically handed down generations (Comeau *et al.*, 2007). However, although the sources of accessory genes are external, the integration into the genome confers the property of vertical transmission.

The accessory genome also codes for additional virulence traits, thus contributing to the clinical implications they might have in infections (Sui *et al.*, 2009). Some of the specialised virulence properties encoded by the accessory genes in these clinical isolates include multi-drug efflux pumps, metallo-betalactamase super family proteins and multiple antibiotic resistance proteins. Since the accessory genes are non-universal across all strains, they are known to encode niche-

based traits which ensure the survival of the organism in its micro-environment (Kung *et al.*, 2010). One of such encoded traits identified in these clinical isolates is for ferrous iron transport which is important in the survival as a CF lung pathogen in the iron- and ferritin-rich airway secretions of CF patients (Stites *et al.*, 1998). These genes encoding virulence and patho-adaptive traits are important for survival in the host-related ecology.

In previous chapters, this study has shown that the survival of a strain during competition is a function of the R-pyocins. In addition, LES_A026, remains resistant to the R pyocin deficient PA018 (PA018ΔR) despite the presence of five S-pyocins (S1 to S5) in the latter that are not present in LES_A026. The mechanism of resistance of LES_A026, to these S-pyocins was initially unknown but the analysis of its whole genome sequence suggested that it might be related to the protection conferred by immunity proteins and lack of appropriate receptors for the different S-pyocins.

LES_A026 has at least three 'orphan' immunity genes with homology to known S type pyocin immunity genes. 'Orphan' immunity genes are genes which encode an immunity protein which is complementary to a structural toxin domain with which it does not share cognate neighbourhood; they are known to confer additional immunity on the strains that have them (Denayer *et al.*, 2007, Elfarash *et al.*, 2012, Elfarash *et al.*, 2014, Dingemans *et al.*, 2016). The three 'orphan' immunity genes present in LES_A026 are closely related to the immunity genes of pyocins S1, S2, S3 and AP41 (Fig. 5.5). These genes potentially provided the resistance to pyocins S1, S2 and S3 of strain A018. The immunity genes of pyocins S1 and S2 share more than 85% homology in their gene sequences and are known to offer a cross protection against the two pyocins in

any strain that has either of them (Michel-Briand & Baysse, 2002). The immunity gene *01372* is closely related to both S1 and S2 immunity genes and by extension, its presence in LES_A026 is likely to offer a shared protection against both S1 and S2 pyocins of PA018ΔR. The other two immunity genes *00599* and *03011* are both closely related to the immunity genes of S3 pyocin and may offer resistance to this pyocin.

Whilst the resistance of LES_A026 to pyocin S1, S2 and S3 could be explained by the presence of closely related orphan immunity genes to these pyocins, there did not appear to be any immunity gene(s) against pyocins S4 and S5 in LES_A026. Another possibility was the absence of the cell surface receptors on the outer membrane (OM) which mediate the entry of these pyocins. The absence of the common receptor – the ferripyoverdin receptor type I (FpvAI) shared by both pyocins S2 and S4 (Elfarash *et al.*, 2014) was a clue to S4 pyocin resistance. Although the high degree of sequence homology (and probably cross protection) shared by the 'orphan' gene *01372* and the immunity genes of pyocin S2 could account for resistance to S2 pyocin, a more inclusive explanation for the resistance to both pyocins S2 and S4 was the lack of the shared *fpvA1* receptor gene in LES_A026 (Fig. 5.6a).

P. aeruginosa strains have an alternative type I ferripyoverdine receptor for the uptake of extracellular Fe^{3+}, this is FpvB (Ghysels *et al.*, 2004). Double mutants in both receptor genes *fpvA1* and *fpvB* were unable to utilise type I pyoverdine (Ghysels *et al.*, 2004). In the present study, whilst PAO1 and PA018 have both genes, LES_A026 and LESB58 only have the *fpvB* receptor gene (Table 5.2, Fig. 5.6). It is known that FpvB could serve an alternative function as a type I ferripyoverdine receptor but an alternative role as a receptor for S2 or S4 pyocin is not yet reported. Therefore, the absence of *fpvA1* gene in

LES_A026 could account for resistance to both pyocins S2 and S4.

Having attributed S2 and S4 resistance to a lack of receptor, the same explanation was sought for S5 pyocin resistance. However, the comparative analysis of the genomes showed that the ferripyocheline receptor gene (*fptA*) for S5 pyocin was represented and 100% identical in all the isolates – both clinical and reference strains (Fig. 5.6b). The resistance mechanism of LES_A026 to pyocin S5 could not be explained by a lack of receptor or the presence of a related immunity gene. Interestingly, Ghoul *et al.,* 2015 has previously argued that it was possible for an organism to display resistance to a pyocin without having the immunity gene of the same pyocin (Ghoul *et al.*, 2015). In their study, the strains that used an alternative mechanism of resistance was 81% of the total study population compared to the strains that displayed resistance secondary to their immunity genes which accounted for the remaining 19% (Ghoul *et al.*, 2015).

Furthermore, strain PA018 lacks the immunity gene to its own pyocin S5 and it is not clear if the activity of this pyocin is fully preserved in the absence of the immunity gene. In the same study by Ghoul *et al.,* out of the 53 isolates that produced S-type pyocins, 35% had only the killing gene, 14% had only the immunity gene while the rest 51% had both. All the isolates that possessed the killing genes only had the DNase mode of killing (i.e. S1, S2, S3 or AP41) and although they did not produce the immunity genes, they did not show lethal activities against themselves; none had the pore-forming S5 pyocin type (Ghoul *et al.*, 2015). This is in contrast to the present study in which strain A018 has the killing gene for the pore-forming S5 pyocin without the corresponding immunity gene.

Apart from the presence of 'orphan' immunity genes to S-pyocins, LES_A026 also bears a mutation in its *lasR* gene. The LasR protein is a major transcriptional activator which regulates many virulence factors. The mutation of the LasR of LES_A026 involved the truncation of the first 115 amino acids in the N-terminus of the polypeptide (Fig. 5.7). The N-terminus of LasR has been linked to its property of multimerisation (Kiratisin *et al.*, 2002). LasR, a member of the LuxR protein family forms multimers *in vivo* in order to function as a transcriptional activator, and an intact N-terminus (not the C-terminus) is central to this function (Kiratisin *et al.*, 2002). Proper folding and translocation of a protein is also linked to intact N-terminus functions (Basharov, 2000). Thus, the *lasR* mutation of LES_A026 produces a non-functional protein.

Genetic variations in *lasR* usually leading to LasR inactivation, is a common pathoadaptive change displayed by many CF lung isolates (Schaber *et al.*, 2004, D'Argenio *et al.*, 2007, Tingpej *et al.*, 2007). Characteristic phenotypes of an iridescent sheen and autolysis on agar plates have been used for easy identification of clinical isolates with this genotype (Hoffman *et al.*, 2009). LES_A026 strain also has this phenotype (Fig. 5.8).

The increasing prevalence of *lasR* mutants in CF infections could be due to a growth advantage which they have over the wild type parent strain or other strains that do not have the mutation. A comparison of the growth pattern of the PAO1-Nottingham derivatives (ΔlasR-N and PAO1-N) showed the same growth pattern during the first 15 hours but thereafter, the population density of ΔlasR-N trailed behind that of PAO1-N. PAO1-N attained a peak by 25 h into growth followed by a steep decline indicative of cell lysis. However, ΔlasR-N maintained a steady but slower growth from 15 h up to the 35 h into the growth assay and then plateaued until the end of the

experiment (Fig. 5.6). These findings are similar to those of Heurlier *et al.* (Heurlier *et al.*, 2005). They attributed the increased cell lysis in the wild type to higher sensitivity to an alkaline environment which prevails in nutrient-rich media due to excessive production of ammonia (Hoffman *et al.*, 2010). In such an environment of high pH, biological activity is progressively lost in isolates that can produce the signal molecule N-acyl homoserine lactone while *lasR* mutants that can neither produce nor respond to these molecules enjoy a sustained growth advantage (Heurlier *et al.*, 2005).

Considering the prevalence rate of Δ*lasR* in both clinical isolates and environmental strains (Gambello & Iglewski, 1991) it is pertinent to question the added advantage that the mutation of this quorum sensing gene confers on the isolates that bear them. Virulence in terms of production of factors like exotoxins, proteases, lipases, pyocyanin, lytic enzymes etc. are positively linked to an active LasR and thus Δ*lasR* has been associated with low virulence in some animal studies (Pearson *et al.*, 2000, Rumbaugh *et al.*, 2000). However, the growth advantage of Δ*lasR* over its wild type (Fig. 5.6) may account for their ability to take over when occurring in a mixed population with their wild type *lasR* allele carriers (Smith *et al.*, 2006). Δ*lasR* strains have also demonstrated a better growth in the enriched secretions of CF lungs (Barth & Pitt, 1996) and have been shown to have increased growth compared to the wild type in the presence of amino acids like phenylalanine, isoleucine and tyrosine (D'Argenio *et al.*, 2007).

The loss of autolytic plaques in both the *lasR* complemented strain (LES_A026lasR^{+}) and the R-pyocin defective mutant (LES_A026ΔR-pyo) of LES_A026 (Fig. 5.8) suggested a link between quorum sensing and R-pyocin. Overproduction of nitric oxide (NO·) has been earlier reported to be responsible

for this Δ*lasR* phenotype (Hoffman *et al.*, 2010) but the reversal of this autolytic phenotype in LES_A026ΔR-pyo also strongly supported the role of the R-pyocins.

The increased expression of R-pyocins in ΔlasR-N compared with PAO1-N as analysed by the SDS PAGE suggested that a non-functional LasR increases R-pyocin expression (Fig. 5.14a). The non-self nature of R-pyocins previously established (Michel-Briand & Baysse, 2002) suggests that this upregulation is a defence mechanism against competitors. This increased activity against competitors was shown by serial double dilution semi-quantitative spot assay of the R-pyocins of ΔlasR-N and PAO1-N against a susceptible strain, A010 (Fig. 5.14b). The same susceptible indicator strain was used for the spot test for both strains since ΔlasR-N is essentially a derivative of PAO1-N wild type. The highest dilution of the R-pyocins of ΔlasR-N which retained lethal activities was 128-fold, compared to those of PAO1-N – 32-fold. Comparing the highest lethal dilutions of the R-pyocins of the two strains, there is a 4-fold increase in the level of biological activity for ΔlasR-N compared to its wild type. Therefore, there is a relatively higher number of functional R-pyocin molecules in ΔlasR-N.

In order to study the effect of *lasR* mutation on R-pyocin expression in real time, the promoter of the first structural gene in the R-pyocin operon (PA0615) was transcriptionally fused to the mini-CTX *lux* reporter. The choice of this promoter, having been interrogated and confirmed to possess promoter activity, was based on the reports that the upstream gene (PA0614) is a lysis gene (Nakayama *et al.*, 2000, Michel-Briand & Baysse, 2002) and that the R-pyocin operon consists of the genes PA0615 – PA0628 of the PAO1 genome (Chang *et al.*, 2005). Although the mini-CTX *lux* plasmid has a tetracycline resistance cassette for selectivity, its property of single copy

chromosomal integration into the *P. aeruginosa* genome allows the propagation of strains without the need for antibiotic selection.

Various mutagenic agents have been previously used for the induction of pyocin expression. These include ciprofloxacin (Brazas & Hancock, 2005), and MMC (Duport *et al.*, 1995). Being an inhibitor of DNA gyrase, it was not surprising to discover that ciprofloxacin inhibited cell growth even at low concentrations (Fig. 5.13). The concentration of MMC that has been used for the induction of R-pyocin expression in purification assays is 3 µg/ml (Scholl & Martin, 2008). This concentration combines the properties of R-pyocin induction and cell lysis which releases the induced R-pyocin molecules. The property of cell lysis is however less desirable in the present study due to the prolonged duration of assay. Thus the assay concentrations of 1 µg/ml, 0.5 µg/ml and 0.25 µg/ml were compared for their impacts on growth inhibition relative to an uninduced assay. The assay concentration of 0.25 µg/ml was most suitable and further reductions were not made in order not to trade off the R-pyocin induction properties of MMC.

R-pyocin transcription in ΔlasR-N induced a very high level of luminescence compared to the PAO1-N wild type without MMC induction. Thus, without an external influence, Δ*lasR* is able to attain a high peak of R-pyocin expression. Upon induction, the major effect is interestingly not only an increase in the peak of expression but a prolonged production of high levels of R-pyocins shown as a wider area under the curve (AUC) – Fig. 5.16b. The reversal of the expression rate of R-pyocin gene to the wild type levels after a re-activation of LasR function in the complemented strain supports the link between quorum sensing and R-pyocin expression. However, a direct control of

RecA regulation on R-pyocin expression could not be found. RecA transcription levels were comparable with and without LasR activity in PAO1-N and ΔlasR-N respectively.

The mechanism of induction using MMC involves increased genotoxicity and cell death. R-pyocins also cause cell death and the release of intracellular contents. Thus, their presence can mimic a genotoxic agent during competition. It follows therefore that on exposure to a competitor, Δ*lasR* senses the R-pyocins of the other strain as an inducing agent, and increases its R-pyocin expression but more significantly, the competing strain is exposed to longer periods of R-pyocin expression from the *ΔlasR* strain. Although the R-pyocins of *ΔlasR* also acts as an inducing agent for its competitor, the longer duration of R-pyocin expression from the *ΔlasR* strain affords a competitive advantage.

The LES are known to be highly competitive strains. It is suggested from the present study that an added advantage of increased R-pyocin production is bestowed during competition if such a strain is a *ΔlasR*. The exposure of a 'LES-*ΔlasR* strain' (typically LES_A026 in this study), to a competitor harnesses the combined properties being a competitive LES member and a *ΔlasR* high R-pyocin producer. This synergy can potentially improve potency in super-infections. Since more LES are becoming associated with CF chronic lung infections and the prevalence of *lasR* mutants is increasing, such a combination of *lasR* mutation in LES members is likely to be more predominant in the clinical isolates.

The role of temperate phages in improving competitive fitness in CF lung has recently been reported (Davies *et al.*, 2016). Temperate phages unlike the lytic ones are able to undergo the lysogenic phase in bacteria without inducing the immediate

lysis of the host bacterium. This property gives them the potential of getting integrated into the host genome and being propagated in the daughter cells. Competition assays set up between lysogenic and non-lysogenic variants of the PAO1 strain showed that the lysogenic variant with triple phage (PAO1φtriple) outcompeted the non-lysogenic strain (PAO1φ-) in the rat model (Davies *et al.*, 2016). This killing was attributed to the phage-mediated lysis of the non-lysogenic strain because a large number of infective virions/phages were recovered in the homogenised lung secretions (Davies *et al.*, 2016). This supported previous reports that competitive fitness of lysogens is enhanced by lysis of phage-susceptible competitors (Brown *et al.*, 2006, Burns *et al.*, 2015).

The R-pyocins have been reported previously to be related to temperate phages by showing serological cross-reactivity (Homma & Shionoya, 1967, Kageyama *et al.*, 1979). Many other properties shared by the R-pyocins and the temperate phages increase the probability of close relatedness between the two molecules. One such property was the bactericidal activity of the temperate phage PS17 which was demonstrated by isolated tails of the bacteriophage similar to that seen in the R-pyocins (Shinomiya & Shiga.S., 1979). The R-pyocins have been likened to defective prophages (Nakayama *et al.*, 2000) and the integration of their genes into *P. aeruginosa* genome ensures their propagation over generations, much like the temperate phages. Furthermore, R-pyocins also undergo a lytic phase upon induction with genotoxic agents like mitomycin C, a property they also share with the temperate phages (Fortier & Moineau, 2007). The presumed contribution of the temperate phages to the global spread of the LES was put forward by Davies *et al* (Davies *et al.*, 2016) and using the same argument,

the acquisition of *lasR* mutation by LES could generate a ‘super strain’ equipped for multiple super infections.

Chapter 6

General Discussion

Conclusions and Future directions.

The results presented in this study show that the R-pyocins of *Pseudomonas aeruginosa* are important in biological interaction and shaping strain populations during competition in both planktonic and biofilm forms of the bacteria. The distribution of the subtypes of the S- and R-type pyocins investigated was diverse in the clinical strains tested but each strain had one R-pyocin subtype but a myriad of combination of the S-pyocin subtypes was seen amongst each of the isolates (Table 3.1). The killing mechanisms of the S-pyocins differ e.g. DNase, tRNase, rRNase and pore formation, but the R-type pyocins regardless of the subtype, have a similar mechanism of action i.e. pore-formation leading to membrane depolarisation by the LPS-bound phage tail. Therefore, an isolate having S-pyocin subtypes with more than one mode of killing would be expected to be fitter than others having pyocin type(s) with a single mechanism of action. However, this study shows that increased biological activity and competition resulted from the activity of R-pyocin that was unregulated in a strain that was devoid of any S-type pyocin. The growth advantage displayed by strains due to R-pyocins are supported by other studies (Heo *et al.*, 2007, Koehler *et al.*, 2009, Kohler *et al.*, 2010).

In the competition experiments reported here, both planktonic and biofilm models were studied. Planktonic competition of strain pairs using the transwell plates showed that R-pyocins were responsible for the death of the susceptible and less competitive strain, with the dead cells aggregating into clumps that closely resembled biofilms (Fig. 3.7). In the biofilm studies, the dominant strain outcompeted the more susceptible one to establish a biofilm, a function that was linked to the activity of the R-pyocins (Fig. 4.3). Investigating the potential

therapeutic properties of the R-pyocins, this study also showed that R-pyocins are able to kill the cells of susceptible strains in established biofilms using both static and dynamic biofilm models. There was an appreciable degree of protection offered by the spatial distribution of biofilms grown on beads after R-pyocin treatment (Fig. 4.1). However, such protection seemed to be dependent on the duration of exposure and the concentration of added R-pyocins. In the treatment of microfluidic biofilms with R-pyocins, prolonged treatment and renewal of R-pyocin supply led to a full-depth biomass eradication (Fig. 4.5). The biofilm-like cell aggregation in planktonic cell cultures was previously reported as true biofilms induced by the presence of sub-inhibitory concentrations of R-pyocins from the strain in the adjacent well (Oliveira *et al.*, 2015). Apart from this link of R-pyocins to biofilms, the antimicrobial effects of R-pyocins on biofilm cells is yet unreported. However, potent activity of the S2 pyocin against biofilms of *P. aeruginosa* has previously been shown to have greater efficacy than comparable concentrations of tobramycin and aztreonam (Smith *et al.*, 2012).

Natural communities of *P. aeruginosa* exist as biofilms. The challenges of antibiotic treatment in CF lung infections is complicated by the complexity of biofilm structure and the co-ordinated organisation of the member cells. A major setback to antibiotic therapy is that development and trials are conducted by studying the mechanisms of action against bacterial planktonic phenotypes. Planktonic parameters like minimal inhibitory/bactericidal concentrations (MIC/MBC) do not necessarily translate to conditions obtainable in the biofilm state. Thus, there is minimal correlation between the response of patients during exacerbation periods and the antibiotic sensitivity profile generated (Foweraker & Govan, 2013).

Therefore, attempts to measure drug efficacy of any potential antimicrobial against *P. aeruginosa* should be conducted against stable biofilm structures. Some parameters for studying biofilm susceptibility are biofilm bactericidal concentration (BBC) which is the lowest effective antibiotic concentration that achieves 99.9% killing of an established biofilm, and biofilm inhibitory concentration (BIC) which defines the lowest concentration necessary to prevent the establishment of biofilms. However, the BBCs of antibiotics used in CF lung infections are high and may be unsafe in the body (Fernandez-Olmos *et al.*, 2012).

In this study, R-pyocin treatment was also used in the biofilms grown in chronic wound models. In contrast to the efficacy recorded in the microfluidic biofilms, the wound model unfortunately showed minimal reduction in the CFU count at different time points (Fig 4.6). This suggested that an appreciable population of the biofilm cells were located inside the collagen model following invasion since a surface inoculation of the biofilm models with *P. aeruginosa* strains was used to initiate the infection. This supported the invasive tendency of the *P. aeruginosa* wound isolates earlier reported by Rumbaugh *et al.,* 1999. In their study, *P. aeruginosa* which was used to induce a burn wound infection in a murine model spread to underlying structures and the blood stream, and was reportedly isolated from the liver and spleen within 24 h post infection (Rumbaugh *et al.*, 1999). Phage therapy has also been studied in wound infections. McVay *et al.,* 2007 reported a study of the phage therapy of burn wound infections caused by *P. aeruginosa* in a mouse model. Of the three routes of phage administration tested – intraperitoneal (i.p.), intramuscular (i.m.) and subcutaneous (s.c.), the i.p. route was the most effective as the mortality rate of only 12% compared

favourably to mortality rates of 72% and 78% for the i.m. and s.c. routes respectively (McVay *et al.*, 2007). In this example, the invasive tendency of *P. aeruginosa* in wound infections could explain the effectiveness of the i.p. route for bacterial clearance since this route offers a wider distribution of the therapeutic agent.

In contrast to the invasive nature of *P. aeruginosa* in wound infections, the chronic lung infections it causes in CF patients are usually localised to the patient's airway epithelia and seldom cross to the blood to cause systemic infections or bacteraemia even when these patients develop fever during acute exacerbations (McCarthy *et al.*, 1980, Fahy *et al.*, 1991). A case study of a CF patient with acute appendicitis who had bacteraemia from the same strain causing the chronic lung infection was attributed to swallowing of the organism from the sputum which eventually crossed the inflamed appendix to reach the blood stream (Gilchrist *et al.*, 2011). This suggests that the surface-associated biofilms of *P. aeruginosa* in CF lungs are minimally or non-invasive and may well account for the success of the inhalational regimens in some antibiotics that have been recorded in some studies (Geller *et al.*, 2007, Westerman *et al.*, 2007). In order to achieve the goal of bringing the R-pyocins in close contact with the LPS receptors for effective killing of susceptible cells, the inhalational route appears to closely mimic the R-pyocin treatment of the microfluidic biofilm model in this study.

Furthermore, this study also probed the genetic bases of resistance of a strain (A026) to five subtypes of the S-pyocin despite its inability to produce these S-pyocin subtypes or corresponding immunity genes of these pyocins. These resistance abilities were attributed to the presence of orphan immunity genes with striking homology to the immunity genes

of pyocins S1 and S3 (Fig. 5.5), and/or lack of an appropriate receptor gene (FptA) for the S2 and S4 pyocins (Fig. 5.6). The ability of orphan immunity genes to provide resistance against related pyocins in the strains that have them was earlier reported (Elfarash *et al.*, 2012, Elfarash *et al.*, 2014, Dingemans *et al.*, 2016). However, the resistance of strains to S-type pyocins has also been shown to be independent of the immunity protein as strains may display resistance to bacteriocins either in the absence of S-type immunity genes (Ghoul *et al.*, 2015) or due to prevailing environmental conditions e.g. high concentration of extracellular iron reduces sensitivity of strains to pyocin S2 due to competitive inhibition arising from sharing the same receptor (Inglis *et al.*, 2016). In addition, although the FpvB acts as an alternative receptor for the uptake of ferripyoverdine in the absence of the FpvA receptor (Ghysels *et al.*, 2004), it did not act as an alternative receptor for S2 and S4 pyocins in the present study. Thus, the FpvA receptor appears to be specific for the S2 and S4 pyocins and the mode of uptake of these pyocins may not be linked to ferripyoverdine import, otherwise, the FpvB should have served the same purpose.

The environment is a well-known source of acquisition of *P. aeruginosa* strains for CF patients especially children (Rau *et al.*, 2010, Manos *et al.*, 2013), and household drains is a well noted source yielding more than eight times of the *P. aeruginosa* isolates recovered from other household environments (Purdy-Gibson *et al.*, 2015). However, other modes of transmission include patient to patient transmission e.g. from infected patient to infection naïve ones (Fluge *et al.*, 2001, Armstrong *et al.*, 2002, O'Carroll *et al.*, 2004). Also, some strains (LES) are able to cause superinfections taking over niches from established strains without themselves

succumbing to any further strain succession (Winstanley *et al.*, 2009, Mowat *et al.*, 2011, Williams *et al.*, 2015, Winstanley *et al.*, 2016). The LESB58 strain has six prophage gene clusters, one of which is the R1 pyocin cluster and mutation in these prophage regions led to the loss of competitiveness in the LESB58 strain (Winstanley *et al.*, 2009). In the present study, the loss of the biological activities of the R-pyocin deficient mutant of A026 also linked competitiveness to the production of the phage-like R-pyocins.

Infection by LES members is typically associated with the evolution of divergent lineages which co-exist in the same patient and are easily transmissible to other patients (Williams *et al.*, 2015). The highly competitive nature of the LES which enables them to outcompete established strains to global proportions has been linked to the activities of their temperate phages (Davies *et al.*, 2016). The role of phages in infections has also been reported because they are more associated with pathogenic strains (Busby *et al.*, 2013) and increased virulence (Pallen & Wren, 2007). The findings of Davies *et al.,* 2016 therefore suggested that any factor that increases phage formation in the LES will contribute to increased competitiveness.

This study has identified a role of LasR inactivation in increasing strain competitive fitness and the strain LES_A026 had a large N-terminal deletion of LasR. The inactivation of the quorum sensing regulator, LasR is a common patho-adaptive mutation in CF lung *P. aeruginosa* isolates (Hoffman *et al.*, 2010). This strain has been attributed to increased antibiotic resistance (Cheng *et al.*, 1996), social cheating (Diggle *et al.*, 2007a, Sandoz *et al.*, 2007, Wilder *et al.*, 2011), reduced virulence (Rumbaugh *et al.*, 2009) and exaggerated inflammatory responses in the respiratory epithelial cells

(LaFayette *et al.*, 2015). Semi-quantitative analysis using serial dilutions for spot assay showed a relative increase in the number of R-pyocins released by ΔlasR-N over its PAO1-N wild type (Fig. 5.14b). Likewise, the promoter of the R-pyocin gene PA0615 that was transcriptionally fused to the CTX *lux* bioreporter also recorded an increase in the R-pyocin expression in the ΔlasR-N strain (Fig. 5.16). The inactivation of LasR in the LES_A026 strain therefore gives a better advantage for a higher level of R-pyocin expression than its competitors.

RecA control could not be linked to this increased expression of the R-pyocins (Fig. 5.17). Activated RecA is an indirect activator of pyocin gene expression. It releases the repression of PrtR on PrtN by inducing the autocleavage of PrtR. PrtN then activates the expression of the pyocin genes by recognising a consensus sequence in the P box of the pyocins upstream regulatory sequence. A P box-like sequence highly homologous to those found in pyocins AP41, S1 and S2 was reported near the R2 pyocin gene cluster (Matsui *et al.*, 1993). Recognition of and binding to this P box-like sequence is believed to trigger the expression of the genes in the R/F pyocin operon. The precise location of this P box-like sequence is in the intergenic region between genes, PA0613 and PA0614. This sequence also contains the promoter sequence of the lysis gene, PA0614.

Coordinated expression of the genes constituting an operon usually involves the activation of the promoter upstream of the first gene to transcribe a polycistronic mRNA, although some individual genes may have internal promoters for distinct regulation (Napolitano *et al.*, 2013). In the case of RecA, the location of its P box-like sequence upstream of PA0614 leads to the expression of all the genes in the R-pyocin operon, including the lysis gene PA0614 (Matsui *et al.*, 1993). LasR inactivation did not alter the expression of RecA in the present

study. However, the promoter upstream of the first transcribed R-pyocin gene PA0615 showed an increase in the expression of R-pyocins in the lasR^{-} mutant.

The rapid progression of lung disease in CF patients has been linked to LasR deficient strains (Hoffman *et al.*, 2009). The deterioration of lung functions is a leading cause of mortality in these patients (Lambiase *et al.*, 2006, Kim *et al.*, 2014b). The first signs of abnormal lung functions are usually detected when chronic inflammation and early biofilm formation have already set in (Starner & McCray, 2005). These patients have periods of exacerbation which cause significant morbidity with little improvement in lung function following treatment (Sanders *et al.*, 2010). Although most eradication measures target the pre-biofilm stage of infection, the transition from colonisation to biofilm development is ill-defined and varied, and invariably occurs over several years (Li *et al.*, 2005).

Despite many strategies that have been proposed to prevent lung inflammation and intermittent exacerbation, or to prolong remission periods, the use of antibiotics is still the main intervention in CF lung infections. Many patients are administered a prolonged course of antibiotics as treatment during acute exacerbations (Starner & McCray, 2005), and as maintenance doses for the control of bacterial populations in the airway (Hoiby *et al.*, 2011). In addition, multi-drug therapy with more than one antibiotic is common place such that at least two antibiotics with synergistic mechanisms of action are implemented (Kirkby *et al.*, 2009). Other adjuvant therapies to antibiotic intervention have been proposed. These include the detection of microbial biomarkers for early intervention to limit risks of morbidity (Rogers *et al.*, 2011) and the use of quorum sensing inhibitors or quenchers (Dong *et al.*, 2001).

Extensive and intensive therapies drive the emergence of antibiotic resistance (Oliver, 2010). This is exacerbated by the wide diversity of genotypic and phenotypic properties between different bacterial isolates from the same infection and individual having varying antibiotic sensitivity patterns for commonly used antibiotics (Darch *et al.*, 2015). Different regions or compartments of the lungs also harbour strains with markedly divergent evolution suggesting that related clones experience different selective pressures dependent on regional distribution (Jorth *et al.*, 2015, Markussen *et al.*, 2014).

In spite of the improved competitiveness of the LasR deficient clinical strain in this study and its ability to out-compete other strains, the eradication of its biofilm was possible by treating with purified R-pyocins of another competitor. The antimicrobial properties of the R-pyocins have been reported even across many other species and their spectrum of activity can be further extended due to their amenability to genetic engineering (Williams *et al.*, 2008, Ritchie *et al.*, 2011). As shown in this study simple biological spot test assay was initially used as the 'sensitivity testing' assay to identify the strain(s) that produced lethal R-pyocins to each indicator strain.

In conclusion, the R-pyocins are very important in competition and their increased expression improves competitive fitness. The anti-biofilm properties they display also makes them a potentially viable option for anti-biofilm therapy to meet the current need for non-antibiotic measures for the treatment chronic CF lung infections is being sought. They could be useful as adjuvant therapies like others that have been previously proposed. As a future work to the present study, it will be clinically relevant to compare R-pyocin efficacy with that of antibiotic regimens currently in use in CF lung therapy.

Furthermore, the assessment of the role of the orphan immunity genes in sensitivity and resistance against S-type pyocins in other susceptible strains will also be pertinent. The increased expression of R-pyocins in LasR deficient strains has added to the understanding of why such strains are becoming increasingly prevalent in clinical infections, thus reiterating the role of R-pyocins in strain interactions. A further understanding of the mechanisms that explain this link between quorum sensing and R-pyocin expression will be worthwhile to explore.

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