

Declaration

I hereby certify that this thesis incorporates original research which has not been previously submitted for a higher degree to any other university: and to the best of my knowledge and belief, it does not contain any material previously published or written by other persons except where reference has been made in the text.

Pholawat Tingpej

Date

Publications and Presentations

Tingpej P., Smith L., Rose B., Zhu H., Conibear T., Al Nassafi K., Manos J., Elkins M., Bye P., Willcox M., Bell S., Wainwright C., and Harbour C. (2007) Phenotypic characterisation of clonal and nonclonal *Pseudomonas aeruginosa* strains isolated from lungs of adults with cystic fibrosis. *Journal of Clinical Microbiology* **45**(6): 1697–1704

Smith L., Rose B., **Tingpej P.**, Zhu H., Conibear T., Manos J., Bye P., Elkins M., Willcox M., Bell S., Wainwright C., and Harbour C. (2006) Protease IV production in *Pseudomonas aeruginosa* from the lungs of adults with cystic fibrosis. *Journal of Medical Microbiology* **55**(12): 1641-1644

Tingpej P., Elkins M., Rose B., Moriarty C., Manos J., Bye P., and Harbour C. (2008) Clonal strains of *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis [Abstract]. *Infection, Genetics and Evolution* **8**(4): S10. This work was presented at *The 8th International Meeting Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases* (30th November – 2nd December 2006, Bangkok, Thailand).

Tingpej P., Fung C., Manos J., Whitchurch C., Rice S., Rose B., and Harbour C. Development of artificial sputum medium for biofilm studies of *Pseudomonas aeruginosa* isolates from people with cystic fibrosis. Presented at *The Centre for Microbes, Infection and Host Defence Showcase Symposium* (27th November 2007, Sydney, NSW, Australia).

Manos J., Curtis M., **Tingpej P.**, Arthur J., Rose B., Bye P., and Harbour C. (2007) Enhanced biofilm formation and downregulation of gene expression are features of a transmissible *Pseudomonas aeruginosa* clone from cystic fibrosis patients [Abstract]. *Pediatric Pulmonary* **42**(S30): 325 (A349). This work was presented at *The 21st Annual North American Cystic Fibrosis Conference* (3rd – 6th October 2007, Anaheim, CA, USA) by Dr. Manos.

Acknowledgements

Many individuals and organisations have been of assistance to me throughout my PhD, and I would like to express my thank to them here.

First to my supervisor, A/Prof Colin Harbour, and my associate supervisor, A/Prof Barbara Rose; I am deeply indebted to you for your guidance all through this project. To Colin, I am grateful for all of your help, your support and your encouragement. This thesis would never have become a reality without your insightful knowledge and your supervision. To Barbara, thank you for your suggestions, your comments and your questions. Your commitment to research has motivated me to finish this project.

I also would like to acknowledge the CF Microbiology Group at the University of Sydney for having me as part of the active group. My heartfelt thanks are particularly due to Dr. Helen Hu for her help in many parts of this thesis. Thank you for sharing your knowledge; I have learnt much from working with you. Thank you also to Dr. Jim Manos for his comments and suggestions over the course of the project, and to Khaled Al Nassafi for his works on PCRs. My acknowledgments should go to the previous members of the CF group, particularly Lucas Smith for his excellent works in the bioassays. I also would like to acknowledge Crystal Lee and Dr. Gratian Punch for their work on genotyping bacterial isolates. Parts of their work were incorporated into Chapter Eight of the present thesis. My thanks should go to Jenita Gunasegaram for her help with the PFGE technique when I first started.

Likewise, I wish to thank the CF clinic at the Royal Prince Alfred Hospital particularly A/Prof Peter Bye for allowing me to visit the clinic regularly. To Dr. Mark Elkins, I owe you a great debt of gratitude for all of your sincere help on statistics, manuscripts, research methodology and many parts of this thesis. My special thanks goes to Carmel Moriarty for assisting me in getting the clinical data that I needed.

Equally, I wish to express my gratitude to the Cooperative Research Centre for Eye Research and Technology at the University of New South Wales particularly Prof. Mark Willcox and Dr. Hua Zhu for allowing me to access the Centre and to use its equipment. To Hua, I am deeply grateful for your help with the TLC. Also, I would like to thank Dr. Scott Rice and his Director, Prof. Steffan Kjelleberg, at the School of Biotechnology and

Biomolecular Science, Centre for Marine Biofouling and Bio-Innovation at the University of New South Wales for providing the bacterial mutant strains used in this thesis.

My thank you and appreciation is also due to Dr. Karen Vickery and Dr. Aniko Pajkos for great assistance with the set up of the CDC Biofilm Reactor. To Aniko, I am extremely indebted to all the help that I received from you from the very first day.

My heartfelt gratitude also to Dr. Louise Cole at the School of Medical Science, Bosch Institute and Dr. Toby Knight for their great help in using confocal microscopes, and to Dr. Jane Radford at Department of Pathology for allowing me to use the fluorescence microscope.

I also would like to acknowledge A/Prof. Cynthia Whitchurch and Dr. Lynne Turnbull (formerly at Monash University, now the University of Technology, Sydney) for assisting me with the development of an artificial sputum medium. Thank you to Nathan Hare and Dr. Stuart Cordwell at the School of Molecular and Microbial Biosciences at the University of Sydney for their cooperative work on *P. aeruginosa* proteomics.

My special thank you should also go to the Department of Microbiology at the Royal Prince Alfred Hospital, particularly Michael Hoon and Barbara Thompson, for their help with the bacterial identification and antibiotic sensitivity testing.

A thankyou also to the Australian Cystic Fibrosis Trust for providing funding to the CF Microbiology Group at the University of Sydney which helped, in part, support the research performed in this thesis.

I also would like to take this opportunity to thank the Office of Educational Affairs, Royal Thai Embassy in Canberra, particularly Ms. Somchitr Wattanatassi, Ms. Anne Weir and the minister counsellor, Mr Phanu Sungkhavon for the assistance and advice throughout my time in Sydney, and to the Royal Thai Government and the Department of Preclinical Sciences at Thammasat University, Thailand for my scholarship. Thank you to A/Prof Siripen Tor-Udom for her friendly advice on all bureaucratic processes.

My life as well as this thesis would never have been completed without the help of many supportive friends and colleagues. This all started at the Department of Infectious Diseases

and Immunology. I could not possibly name everyone, but I would be remiss if I did not mention at least the following:

Rahma Tohidi-Esfahani, thank you for everything – literally everything. Dr. Veronica Yozghatlia, your friendship is deeply appreciated. Rebecca Rinaldo and Catherine Ferla, thank you for paying your careful attention whenever I needed. Vivian Gorgis, for your hugs. Jenifer Huch and Rodney Henriquez, for your laugh and all the stories. To other department members (both past and present): Carmen Lok, Kate O’Hara, Carina Fung, Michelle Curtis, Fiona Zheng, Kate Gilchrist, Dr. Jim Wildig, Dr. Robert Welschinger, Dr. Jean Zou, Dr. Nham Tran, Dr. Ivy Zhang, Dr. Belinda Herring, Dr. Allison Abendroth, A/Prof. Helen Briscoe, A/Prof. Ray Kearney, and Prof. Yvonne Cossart. Especially the latter, thank you for sharing the cooking recipes with me.

I must thank as well to all of my friends, who have supported me throughout my PhD, in Sydney: Julian Duong (you first, of course); Darren Battye; Tula Vanichakorn; Tim Rusten; Dr. Thorsten Kostulski; Dr. Aek Phakiti; John Stone; Dane Levison; Grant Mistler; Jane Cantwell; Allison Baker; Greg Wadeson; Lam Tuong Ly; Dr. Popchai Ngamskulrungrroj; and to my friends in Bangkok: Dr. Plearnpit Lickasittipun; Dr. Marinya Phongpudpun (sorry I could not be at your wedding); Trewisa Thongsawang; Napat Cheun-Im; Dr. Amara Chaikarn; Dr. Pritsana Piyabhan; Dr. Siharat Wisitthanaphorn; Dr. Napakorn Sangchay; Dr. Pornpong Luxsameesathaporn; Ben Harris.

To my beautiful housemate, Belinda Montgomery, for all of your support and putting up with me and all the mess I created.

I am deeply indebted to the Maury family. Thank you for having me as part of your family, especially Gillian and Jean Pierre for their warm love. My heartfelt thanks to Veronique, Mary, Nicolette, Doug, Claudia and Gabrielle.

To my family in Bangkok: thank you to mum and Aof for visiting me when I needed you; to my aunt for being my finance manager; and to P’X, Khun Yaa, dad and grandpa for your love and support.

My last two thanks are of course to the two most important persons in my life. First, to you, Séb, for your love and your support. Without you, I would have never finished this

thesis. Thanks for being at wherever and whenever I needed. Last, to you, Khun Yai Udom Sasanavin. Thanks for loving and thinking of me until your last breath. Sorry that I could not be there when you needed me, and thus I wish to dedicate this thesis to you.

Abstract

Cystic fibrosis (CF) is the most common inherited lethal disorder among Caucasian populations. Chronic pulmonary infections, particularly from *Pseudomonas aeruginosa*, are the major determinant of the morbidity and mortality of people with CF. It is generally accepted that people with CF acquire this pathogen independently from their surrounding environment, and that individual CF patients carry unique strains different from others. The spread of this pathogen from patient to patient is thought to be rare and occurs particularly among closely contacted cases such as CF siblings. However, over the past decade, there have been several reports of an emergence of clonal *P. aeruginosa* strains commonly found infecting a number of CF patients. One such report is from the CF paediatric clinic at the Royal Children's Hospital in Melbourne in which more than half of the patients were infected with a single strain or clone, subsequently called Australian epidemic strain 1 or AES-1. A preliminary survey showed that AES-1 had spread extensively along the Australian eastern seaboard among CF patients attending other CF centres in Melbourne, Sydney and Brisbane, including adult patients at the Royal Prince Alfred Hospital (RPAH), Sydney. Another clonal strain, subsequently called AES-2, was identified in both CF adults and children at the Prince Charles Hospital and the Royal Children's Hospital, in Brisbane. The total extent of prevalence of the AES-1 and AES-2 strains at the RPAH as well as the clinical status of patients who carried these strains was unknown. Moreover, the pathogenicity of these two clonal strains had not been investigated.

The studies presented in this thesis investigated the prevalence of these clonal strains among CF patients attending the adult CF clinic at RPAH, Sydney by using pulsed-field gel electrophoresis. Overall, 50% of 112 patients with *P. aeruginosa* were found to be infected with clonal strains. The AES-1 and AES-2 strains were identified in 38% and 5% of the patients respectively. Two new clonal strains, called Sydney-1 and Sydney-2, were also identified. Patients with clonal strains had a significant increase in their number of exacerbations and hospitalisation days, and tended to have lower pulmonary functions when compared to patients infected with non-clonal strains. By using a variety of bioassays to examine the pathogenicity of the clonal and non-clonal strains, it was found that both AES-1 and AES-2 produced more virulence factors and were more resistant to antibiotics when compared to the non-clonal strains. AES-1 and AES-2 were associated with

increased production of proteases, including elastase, alkaline protease and protease IV. Overall the results presented in this thesis suggest that there may be a link between virulence and transmissibility of this pathogen.

The studies presented in this thesis also compared the biofilm forming capacities of the AES-1 and non-clonal isolates. AES-1 was shown to have greater biofilm-forming capacity than the non-clonal strains, when they were grown on a glass surface, suggesting a possible association between clonality and biofilm formation. A model for the study of bacteria grown in conditions similar to CF sputum was also developed. *P. aeruginosa* grown in this model was found to develop into clumps which may be comparable to the biofilm structure in the CF lung. This model was shown to be beneficial for transcriptomic and proteomic studies which are underway within the research group.

AES-1 was also found to have phenotypic variations between isolates. By applying the amplified fragment length polymorphism technique, more subtypes of this clone were revealed. However, these detected subtypes did not correlate with the different phenotypes, suggesting minor mutations such as single point polymorphisms may be responsible for the phenotypic diversity within the clone.

The final part of this thesis was devoted to examining the safety of a novel CF treatment: hypertonic saline (HS) inhalation. HS was shown to increase airway mucociliary clearance, while increased osmolarity associated with the use of HS was also shown to have an inhibitory effect on the formation of biofilms. Findings in this study proved that there was no evidence of strain selection in patients who received the long-term treatment with HS. The study also demonstrated that AES-1 was significantly more persistent in the CF lung than the non-clonal strains.

The present thesis not only defines the clonal strains of *P. aeruginosa* and their implications for infected patients, but also provides a general understanding into the pathogenesis of both clonal and non-clonal strains infecting CF lungs.

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1 Chapter One: Literature Review

1.1 Cystic Fibrosis (CF)

1.1.1 An Overview and a Brief History of CF

Cystic fibrosis (CF) is the most common autosomal recessive disorder in the Caucasian population with 1 in 25 people being carriers and an incidence of approximately 1 in 2,500 live births (Gaskin, 2004; Kulczycki *et al.*, 2003; Ratjen & Doring, 2003). CF, also called mucoviscidosis, affects the entire body, causing progressive disability and early death. The most common presentations of the disease involve pulmonary infection as well as gastrointestinal and nutritional abnormalities. Other symptoms, such as meconium ileus and failure to thrive, appear in infancy and childhood.

CF is caused by a mutation in a gene called the cystic fibrosis transmembrane conductance regulator (*CFTR*). A defect in this gene results in a myriad of clinical problems, but the most troublesome symptom is chronic pulmonary infection, particularly from a bacterial pathogen called *Pseudomonas aeruginosa* (*P. aeruginosa*). Approximately 80 to 95% of CF individuals succumb to respiratory failure caused by chronic bacterial infection (Lyczak *et al.*, 2002).

Prior to 1938, CF was not recognised as a single disease but as a collection of diverse syndromes. Early descriptions of CF were mainly about defects of the alimentary system, such as meconium ileus, difficulty in feeding and failure to gain weight. Children born with CF almost uniformly died in their first year of life.

In 1938, Anderson published a detailed study describing the similarity of pathological characteristics among a group of young children who died from intestinal obstruction or from respiratory complications (Andersen, 1938). It was the first observation that led to an understanding of CF as a single disease with diverse effects. Anderson also proposed that CF was an hereditary disease, but it was not until 1989 that the gene responsible for CF was discovered (Kerem *et al.*, 1989; Riordan *et al.*, 1989; Rommens *et al.*, 1989). Since then, over 1,000 mutations in the *CFTR* gene have been identified, being responsible for a wide range of CF clinical severity (Kiesewetter *et al.*, 1993), and there have been substantial advances in basic and clinical research leading to new diagnostic tests and new

treatments. To date, the median survival age of people with CF exceeds 30 years (**Figure 1.1**). In the future, developments relating to gene therapy and treatments directed at solving the basic defect of CF will hopefully afford longer life expectancy and better quality of life.

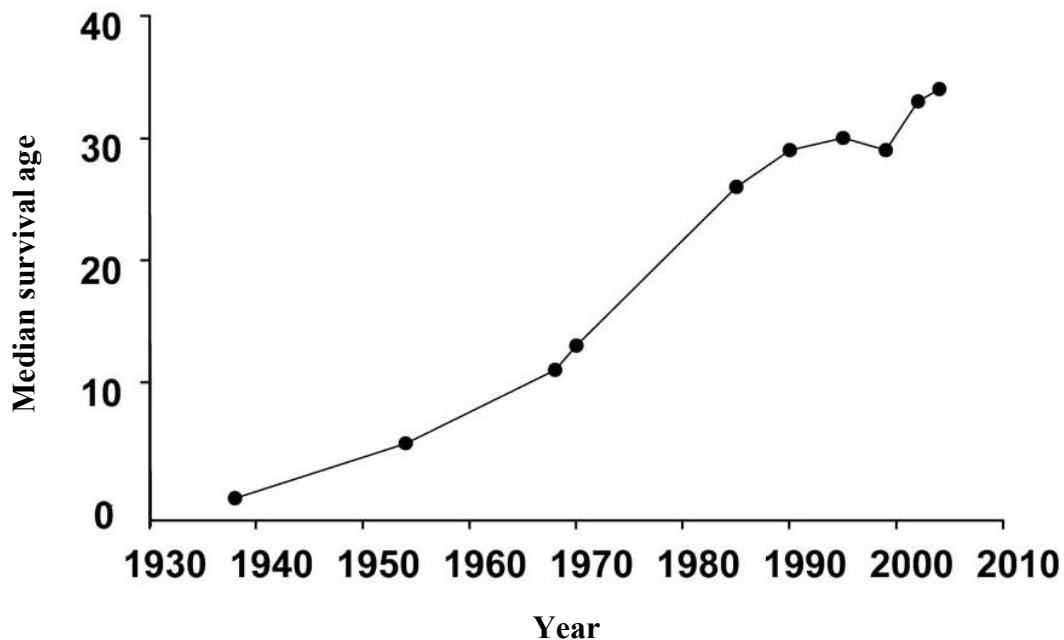


Figure 1.1 Median survival age for people with CF at various times since the first description of CF (Davis, 2006)

1.1.2 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

1.1.2.1 *CFTR* Gene

The *CFTR* gene is located on the long arm of chromosome 7 (Knowlton *et al.*, 1985). It is 250,000 base pairs long and encodes a protein of 1,480 amino acids, the CFTR protein. The most common mutation, $\Delta F508$, is a deletion (Δ) of three nucleotides that results in a loss of the amino acid phenylalanine (F) at the 508th position on the protein and creates a protein that does not fold normally (Welsh *et al.*, 1993). This defective protein does not transfer to the Golgi network, a requirement for membrane expression, but is retained in the endoplasmic reticulum and is eventually degraded intracellularly (Gilbert *et al.*, 1998; Ward *et al.*, 1995).

1.1.2.2 Structure of the CFTR

Structurally, the CFTR protein is classified as an ATP-binding cassette transporter (ABC transporter) (Rowe *et al.*, 2005). It contains two ATP-hydrolysis domains (also termed nucleotide-binding domains) and two membrane-spanning domains, each of which contains six membrane-spanning alpha helices (**Figure 1.2**). Activation of the CFTR protein relies on phosphorylation via cyclic AMP-dependent protein kinase which occurs at the Regulatory domain or R domain. The nucleotide-binding domains are responsible for the binding and hydrolysis of ATP and provide the energy necessary for channel activity. The carboxyl terminal which consists of threonine, arginine and leucine (TRL) is anchored to the cytoskeleton in close approximation to a number of important proteins (Short *et al.*, 1998).

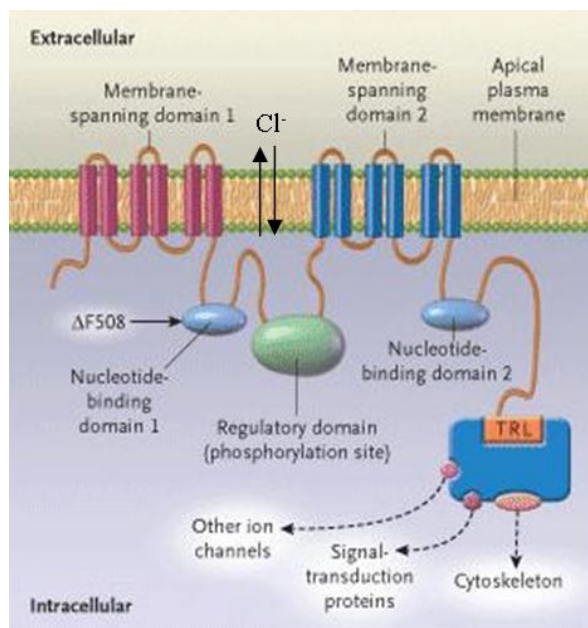


Figure 1.2 Proposed structure of CFTR protein (adapted from Rowe *et al.*, 2005). The CFTR functions as a chloride (Cl⁻) channel.

1.1.2.3 Function of the CFTR

The biological function of the CFTR was revealed by the observation that the sweat of CF individuals contains abnormally high electrolyte levels. Normally, the sweat glands release the isotonic secretion and while this secretion travels to the skin surface the CFTR proteins, situated within luminal plasma membranes of epithelial cells lining the ducts, act to reabsorb sodium chloride (NaCl). The sweat ducts of CF people however are impermeable to chloride (Cl⁻), resulting in increasing luminal negative charges and decreasing sodium (Na⁺) flux (Quinton & Bijman, 1983; Schulz, 1969). Thus, the NaCl remains abnormally high in the secretion. This observation revealed the primary role of the CFTR protein as the Cl⁻ ion channel (Quinton, 1983). More recent research has revealed that the CFTR protein is multifunctional: it down-regulates transepithelial Na⁺ transport via epithelial Na⁺ channel (ENaC) (Ismailov *et al.*, 1996); it regulates calcium-activated Cl⁻ channels and potassium channels (Kunzelmann & Schreiber, 1999). It also interacts with several molecules, including Aquaporin 3 (water channels in airway epithelial cells) (Schreiber *et al.*, 1999), NBC-1 (Na⁺/HCO₃⁻ cotransporter) and NKCC-1 (Na⁺/K⁺/2Cl⁻ cotransporter) (Shumaker *et al.*, 1999; Shumaker & Soleimani, 1999). Ultimately, impaired CFTR function leads to an imbalance of electrolyte transportation in many vital organs.

1.1.2.4 Mutation of the CFTR

There have been more than 1,000 described mutations in the *CFTR* gene associated with the disease. These mutations can be classified into six classes (**Figure 1.3**). The $\Delta F508$ is categorised as a class II defect. The CFTR protein is expressed with tissue specificity; it has been shown that CFTR resides on the apical membrane of exocrine epithelial cells, including in the sweat gland, pancreatic ducts, bile ducts, intestine, male genital ducts, and respiratory tract (Cohn *et al.*, 1991; Crawford *et al.*, 1991; Marino *et al.*, 1991; Trezise & Buchwald, 1991). The mutation of CFTR thus affects those organs accordingly.

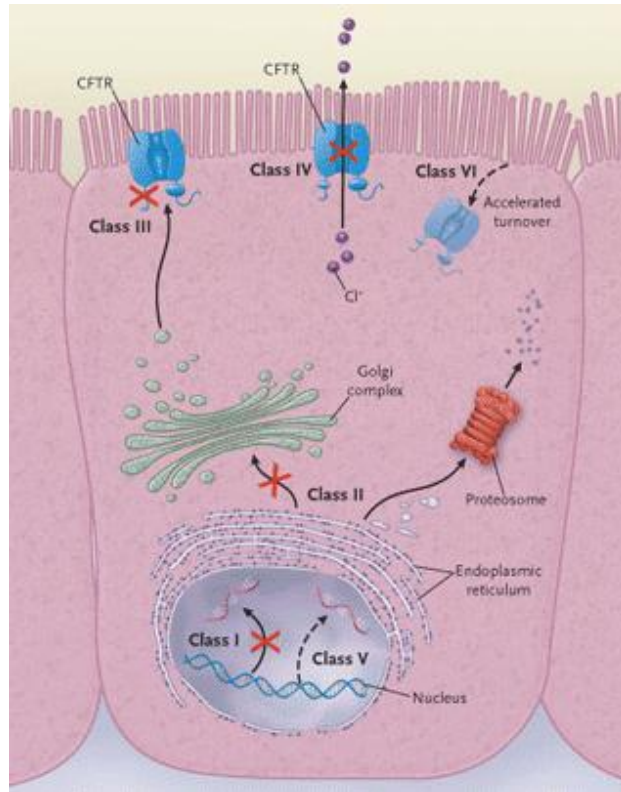


Figure 1.3 Categories of CFTR mutations (Rowe *et al.*, 2005).

CFTR mutation can be categorised into six classes as follows:

Class I, the absence of CFTR synthesis;

Class II, defect of CFTR protein maturation causing premature degradation;

Class III, disordered regulation, such as diminished ATP binding;

Class IV, defective chloride conductance or channel gating;

Class V, a reduced number of CFTR transcripts; and

Class VI, accelerated turnover of the CFTR proteins from the cell surfaces.

1.1.3 Clinical Manifestations of CF

The symptoms of CF differ from person to person, depending on the severity of the disease. In general, the basic pathology of CF is due to abnormal CFTR function, causing electrochemical alterations leading to dehydration and formation of mucous plugging exocrine ducts. **Table 1.1** summarises pathology and the symptoms of the affected organs.

Table 1.1 Pathology and related symptoms manifested in people with CF

Organ	Pathology	Symptoms	References
Sweat gland	Decrease NaCl reabsorption via the sweat ducts	Salty-tasting skin	(Quinton & Bijman, 1983)
		Pseudo-Bartter's syndrome (salt wasting with metabolic alkalosis)	(Devlin <i>et al.</i> , 1989)
Pancreas	Retention of digestive proenzymes in the pancreatic ducts, causing premature digestion of the pancreatic tissue	Pancreatic insufficiency	(Kopelman <i>et al.</i> , 1985)
		Malnutrition and failure to thrive	(Pencharz & Durie, 2000)
	Destruction of insulin-producing pancreatic islets	CF-related diabetes mellitus	(Lanng <i>et al.</i> , 1994)
Liver	Intraluminal concretions in the bile ducts	Biliary cirrhosis	(O'Brien <i>et al.</i> , 1992)
Intestine	Blockage of digestive enzymes	Malabsorption and malnutrition	(Wilschanski & Durie, 1998)
	Thickened faeces	Intestinal obstruction	(Wilschanski & Durie, 1998)
Male genital system	Congenital absence of vas deferens	Infertile	(Dodge, 1995)
Female genital system	Thickened cervical mucous	Fertility difficulties	(Gilljam <i>et al.</i> , 2000)
Lung	Thickened mucous causing obstruction of lower airways	Chronic pulmonary infection	(Lyczak <i>et al.</i> , 2002)

Symptoms usually appear within the first year after birth. The salty-tasting skin, which is caused by abnormal salt homeostasis of the sweat glands and is a common characteristic of CF people, led to the development of a sweat test. First developed in 1953 (Di Sant' Agnese *et al.*, 1953), the sweat test, which measures the amount of Na⁺ and Cl⁻ in sweat, remains the gold-standard method for CF diagnosis.

While people with CF experience a wide range of symptoms, the most important health problem leading to morbidity and mortality is chronic lung infection. Respiratory failure from the chronic bacterial respiratory infection is the leading cause of death in CF, and the bacterium, *P. aeruginosa*, is the most common causative pathogen (Lyczak *et al.*, 2002). Even though accumulated studies provide a great understanding of the genetic and molecular mechanisms leading to CF, the link between physiological properties of CF lungs and the chronic infection remains elusive. The following sections will discuss the current knowledge of the pathophysiology of CF lungs and the roles played by microorganisms.

1.2 Pathogenesis of Pulmonary Disease in CF

1.2.1 Normal Defences of the Lung

In a healthy individual, airways below the first bronchial division (or lower airways) are sterile, despite continuous exposure to airborne microbes. This sterile condition is a result of effective defence mechanisms of the lungs which involve (i) a mechanical clearance, (ii) secreted antimicrobial mediators and (iii) an innate immune system (**Figure 1.4**).

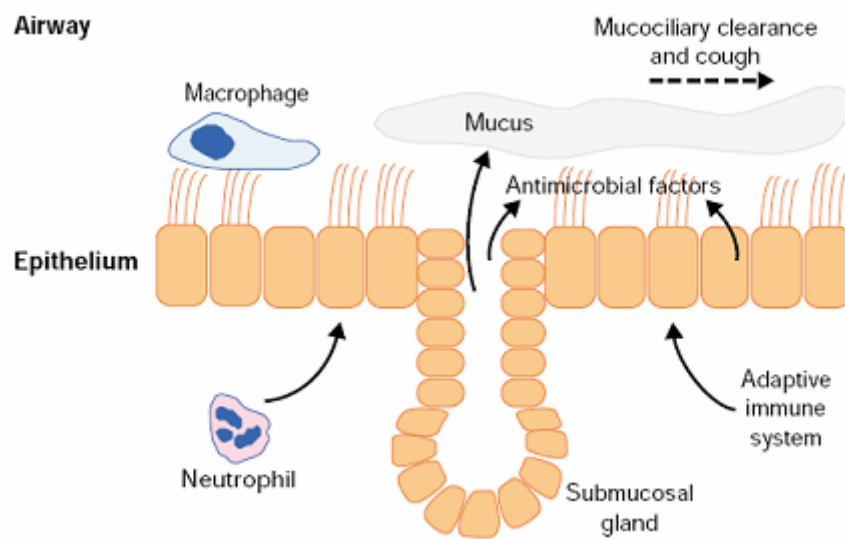


Figure 1.4 The normal defences of human airways (Travis *et al.*, 2001)

1.2.1.1 Mechanical Clearance

The mechanical clearance of any foreign body or pathogen within airways involves simple reflexes such as coughing and sneezing, but the underlying strategy of these reflexes – the mucociliary clearance (MCC) – is more complex and not yet completely understood.

MCC functions to trap and expel particles inhaled into an airway (Robinson & Bye, 2002). MCC requires the coordinated regulation of ciliary beat and a volume of airway surface liquid (ASL) (Robinson & Bye, 2002; Tarran *et al.*, 2006). The ASL is normally around 20 – 25 μm deep and is composed of water, ions and macromolecules generated locally by surface epithelial cells and/or submucosal glands (Tarran *et al.*, 2006). It is partitioned into

two compartments: a periciliary liquid layer (PCL) through which cilia beat freely (Paradiso *et al.*, 2001); and an overlying mucus layer (Guggino, 2001) (**Figure 1.5**). The ASL acts in coordination with ciliated epithelium driving trapped foreign particles toward the pharynx where it is eventually expectorated from the airways

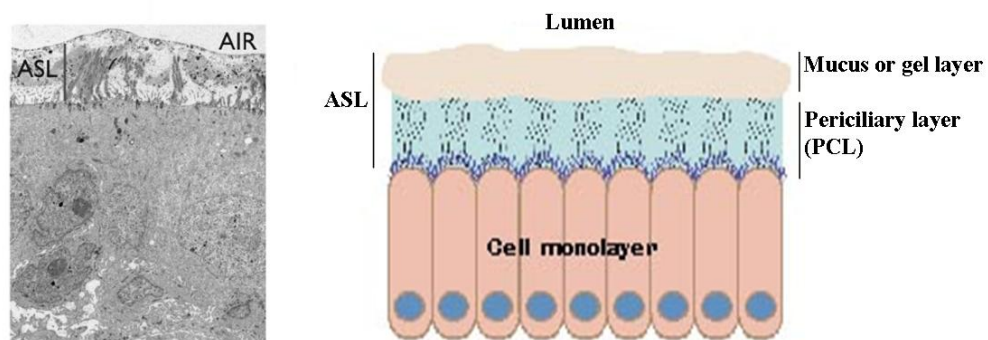


Figure 1.5 (Left) Transmission electron micrograph of human airway epithelia (Durairaj *et al.*, 2006). (Right) Schematic model depicting compartments within ASL (Paradiso *et al.*, 2001).

1.2.1.2 Antimicrobial Mediators

ASL also contains a rich diversity of antimicrobial proteins and peptides, including lysozyme, lactoferrin, human β -defensin 1 and 2, human neutrophil peptides (HNPs) and cathelicidin (Travis *et al.*, 2001). These factors provide a first line of defence against inhaled microbes. The contribution of an individual factor depends on its function or ability to eliminate microbes. For example, cathelicidin has been shown to act against *P. aeruginosa* and *Staphylococcus aureus* (Travis *et al.*, 2000) while lactoferrin works synergistically with lysozyme killing many types of microbes (Singh *et al.*, 2000b).

1.2.1.3 Innate Immune System

Certain microbes, for example *Burkholderia* species, have been shown to be resistant to the antimicrobial mediators (Sahly *et al.*, 2003). In such cases, the innate immune system may play a major role in pathogen elimination. Components of the airway innate immune system include neutrophils and alveolar macrophages. If either neutrophils or macrophages

are overwhelmed by microbes or foreign particles, they initiate an inflammatory response by releasing chemotaxins, recruiting more inflammatory cells which eventually leads to chronic inflammation if the microbes or particles are persistent or cells cannot eliminate them (Baeza-Squiban *et al.*, 1999; Hart & Winstanley, 2002; Travis *et al.*, 2001).

1.2.2 Pathophysiology of Airway Disease in CF

Although the genetic defect causing CF was discovered in 1989, the mechanisms by which CFTR mutations cause airway diseases remain uncertain. Several hypotheses have been proposed for the mechanism that links the CFTR dysfunction to the clinical disease, including suggestions of abnormal composition and functions of ASL (Matsui *et al.*, 1998; Smith *et al.*, 1996), defective airway submucosal gland secretion (Jayaraman *et al.*, 2001; Verkman *et al.*, 2003), defective ingestion and clearing of pathogens by epithelial cells (Pier *et al.*, 1997), abnormal ASL pH which leads to a decrease in the MCC (Coakley *et al.*, 2003), and increased availability of bacterial receptors (Saiman & Prince, 1993). Of these, two models relate directly to alteration in ASL: the “high-salt hypothesis” and the “low-volume hypothesis”, and the general consensus is that these are the most likely explanations of airway disease in CF.

1.2.2.1 The Low-Volume Hypothesis

This model, proposed by Boucher and colleagues, emphasises the roles of CFTR as both a Cl⁻ channel and a regulator of ENaC (Quinton, 1983; Stutts *et al.*, 1995) (see also Section 1.1.2.3). These roles are crucial in an optimisation of ASL volume which is essential for the efficiency of the MCC (Boucher, 1999). In this model, the absence of CFTR results in increased ENaC activity, leading to hyper-absorption of Na⁺ through airway epithelia (**Figure 1.6**). As a result of increased Na⁺ absorption, the mucosal surface then has a more negative charge. Subsequently, Cl⁻ molecules follow Na⁺ by permeating the epithelia through non-CFTR Cl⁻ channels (**Figure 1.6**). The secretion of Cl⁻ through CFTR itself is also defective, and thus the overall result is an increase in the absorption of both Na⁺ and Cl⁻. Accordingly, water is reabsorbed, leading to the dehydration of ASL (Matsui *et al.*, 1998).

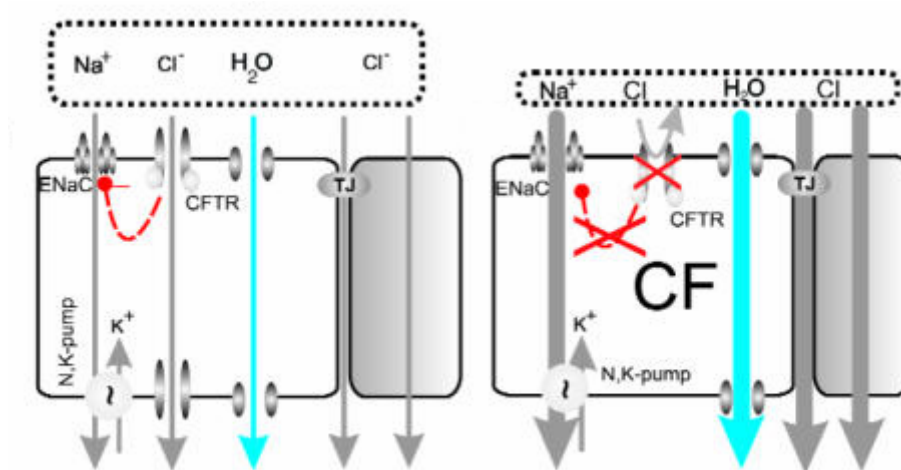


Figure 1.6 Schematic diagram of the low-volume hypothesis illustrating normal ASL and respiratory epithelia (left), and CF respiratory epithelia (right) (Wine, 1999).

This hypothesis has been supported by evidence from an *ex vivo* airway epithelial cell culture study showing that normal airway epithelia regulate the volume of ASL by setting the height of the PCL to approximately the height of the extended cilium ($\sim 7 \mu\text{m}$), but this PCL regulation fails in CF airway cultures. PCL height is significantly reduced ($\sim 3 \mu\text{m}$), and cilia are collapsed onto cell surfaces (**Figure 1.7**) (Matsui *et al.*, 1998).

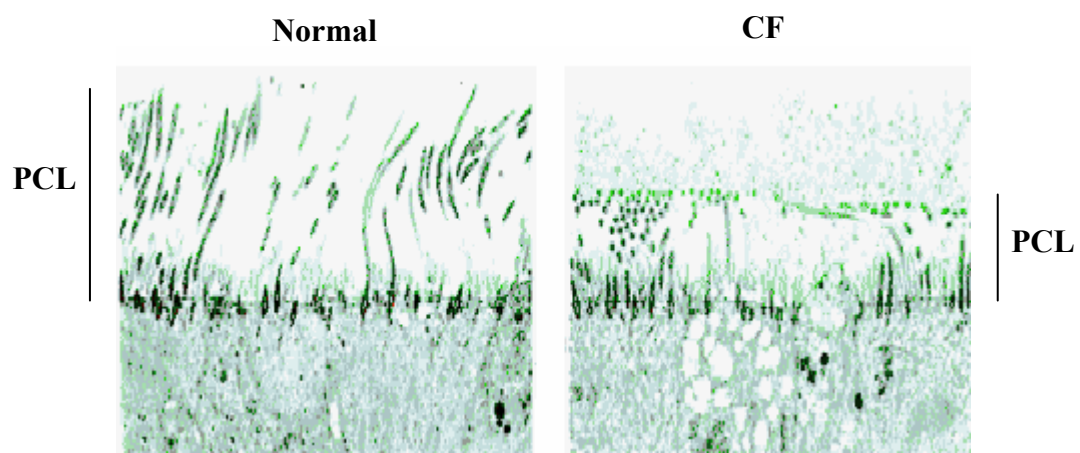


Figure 1.7 ASL volume depletion observed in primary human respiratory epithelia cultures from normal and CF subjects (Matsui *et al.*, 1998).

Importantly, PCL volume depletion is associated with significantly reduced mucus transport rates in CF compared to normal airways, and this causes mucus plugging in CF

airways (Matsui *et al.*, 1998; Tarran *et al.*, 2001). Consequently, any inhaled particle or pathogen is entrapped in this mucus, but the CF lungs fail to clear it, triggering release of inflammatory chemokines leading, eventually, to chronic inflammation.

1.2.2.2 The High-Salt Hypothesis

In contrast, Welsh and colleagues have postulated that ASL in CF is relatively hypertonic when compared with normal ASL (Zabner *et al.*, 1998). This hypothesis suggests that the absence of CFTR leads to a decreased reabsorption of Cl^- through the airway epithelia. Consequently, the transepithelial potential difference becomes hyperpolarised (increasing negative charge in the lumen), and thus Na^+ molecules remain in the lumen in order to neutralise the charge. As a result, the level of NaCl increases in the ASL (**Figure 1.8**)

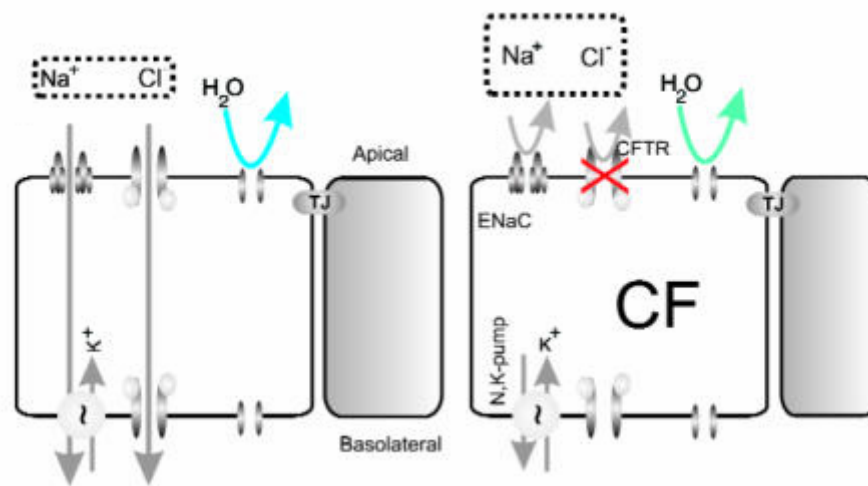


Figure 1.8 Schematic diagram of the high-salt hypothesis illustrating normal ASL and respiratory epithelia (left), and CF respiratory epithelia (right) (Wine, 1999).

Smith *et al.* demonstrated that an elevated level of NaCl in the ASL inactivated salt-sensitive antimicrobial mediators such as β -defensin 1 and 2 (Smith *et al.*, 1996). These two mediators require a low NaCl concentration for their efficient bactericidal activity (Bals *et al.*, 1998; Goldman *et al.*, 1997), and thus a high NaCl concentration as proposed in this model fails the bacterial killing activity. This bactericidal activity was also restored by lowering the salt concentration. This hypothesis concluded that the increasing salt level in the ASL (caused by the defective CFTR) resulted in the malfunction of airway defence mechanism. This condition is prone to a predisposal of bacterial infection, which may lead to chronic lung infection (Smith *et al.*, 1996).

In summary, it is important to understand the pathophysiology of CFTR as this has therapeutic implications. While the two hypotheses explain the roles of CFTR in the CF lungs in a completely opposite way, there is general agreement that the pathogenesis of CF lung disease reflects a defect in the defence system of airway surfaces against infection. The next section describes the microbiology of lung infection in CF focusing on mechanisms of infection of bacteria commonly found causing the CF lung infection.

1.3 Microbiology of the Lungs of CF Individuals

The pathophysiology of the CF lung as described in Sections 1.2, whereby the host defence functions are inhibited, promotes a suitable environment for microbial growth. The prevalence of bacterial respiratory pathogens by age is shown in **Figure 1.9**. The most common pathogens are *S. aureus*, *P. aeruginosa*, *H. influenzae* and the *B. cepacia* complex.

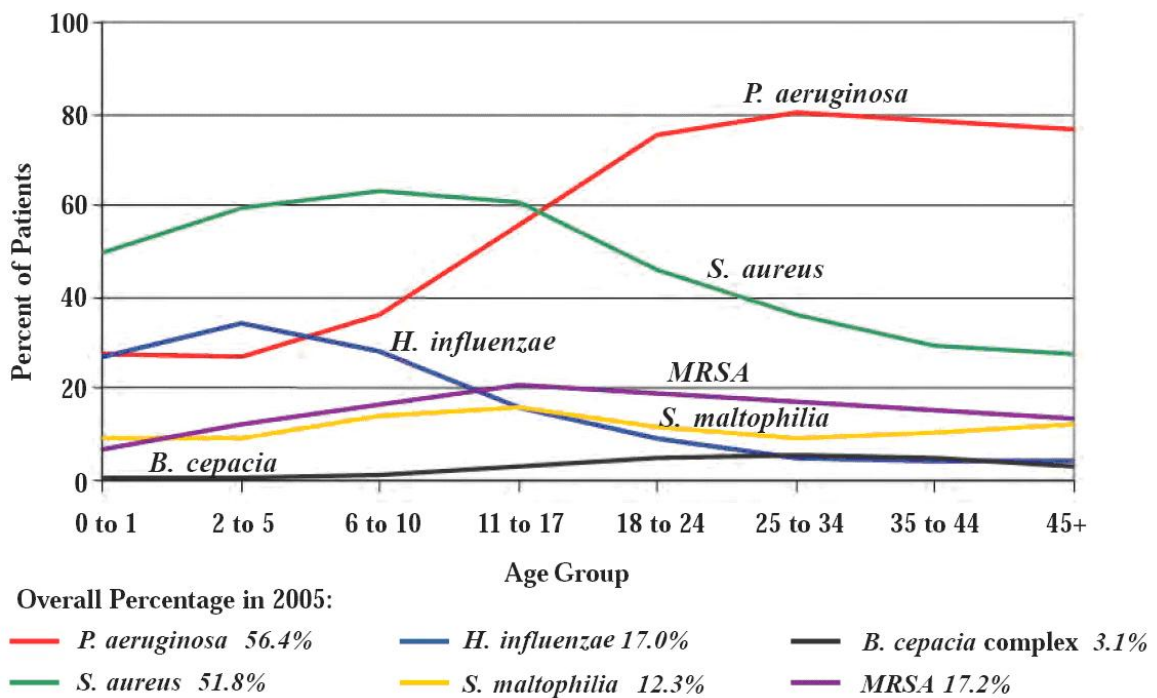


Figure 1.9 Prevalence of bacterial respiratory infections by age group among CF individuals (Cystic Fibrosis Foundation, 2005)

The microbiology of CF lungs has been studied since the earliest descriptions of this disease (Davis, 2006). The first study (in 1946 by Di Sant’Agnese and Andersen) was done in post-mortem specimens from the lungs of 14 CF infants; twelve of which yielded *S.*

aureus while *H. influenzae* and *P. aeruginosa* were each isolated from a single sample (Davis, 2006; Govan & Deretic, 1996). Today it is recognised, as illustrated in **Figure 1.9**, that *S. aureus* is predominant from infancy to mid-childhood (0 – 10 years old). The prevalence of *H. influenzae* and *P. aeruginosa* is also common in these young age groups, although that of *H. influenzae* decreases as patients age. Antibiotic therapy particular for *S. aureus* and *H. influenzae* obviously contributes to the decline of these pathogens. In contrast, by adulthood, the prevalence of infection by *P. aeruginosa* rises to 80% in the CF population, which is thought mainly due to the increased antibiotic resistance characteristics of this pathogen (Bradley *et al.*, 2007).

Reports of infection by *B. cepacia* complex and MRSA are relatively recent. Both *B. cepacia* and MRSA emerged as multiresistant pathogens among the CF population during the early 1980s (Boxerbaum *et al.*, 1988; Thomassen *et al.*, 1985b). Although the overall prevalence of *B. cepacia* complex infection among CF patients is comparatively low (3.1%) (**Figure 1.9**), the clinical implication is significant because *B. cepacia*-colonised patients may develop a rapid and fatal clinical deterioration known as ‘cepacia syndrome’ (Isles *et al.*, 1984) (see Section 1.3.3).

Co-infections within the Lungs of CF Individuals

Pathogens residing in the airways of CF subjects rarely exist as only single species, and co-infections involving different species of bacteria, fungi or viruses are quite common (Harrison, 2007; Hoiby, 1974). Moore *et al.* reported that 53% of patients were colonised by one organism while the remainder (47%) harboured two or more species (Moore *et al.*, 2005). Burns *et al.* recovered bacterial pathogens from CF sputum samples ranging from 0 – 10 species (average 2.9 species per sample) (Burns *et al.*, 1998). Moreover, even within a single species, and in particular *P. aeruginosa*, it was observed that there was an average of 2.38 phenotypically distinct isolates recovered from CF sputum samples (range from 1 – 6), highlighting the intra-species diversity (Burns *et al.*, 1998). This observation was supported by studies by Smith *et al.*, which genotyped *P. aeruginosa* isolates taken from a single CF patient over a period of 8 years and showed the coexistence of multiple genetic-variants and ultimately the selection of a dominant clone over the studied period (Smith *et al.*, 2006).

New approaches to studying the diversity of the bacterial community within CF lungs by using techniques such as 16S Ribosomal RNA identification or Terminal Restriction

Fragment length polymorphism (T-RFLP) profiling, have improved upon the conventional approach using agar-based cultivation, and the findings have widened the general understanding of CF lung infections (Rogers *et al.*, 2003; Rogers *et al.*, 2004).

The T-RFLP technique is based on ribosomal gene fragment profiles generated by a specific restriction endonuclease digestion followed by gel electrophoresis (Marsh, 1999). The band profiles are then assigned to bacterial species by comparison of band size with the existing database. By using T-RFLP to analyse 71 sputa from 34 CF adults, a single sputum sample was shown to have an average bacterial 'species richness' (the number of separate species present in a community) of 13.3 species (or an average of 13.4 species per patient) (Rogers *et al.*, 2004). All species assignment detected by T-RFLP are shown in **Table 1.2**. Many of these species have not been previously detected in the CF sputum samples. This is, however, not surprising since they are mainly anaerobic species and conventional bacterial detection from clinical samples has usually been done in an aerobic condition. Nevertheless, the presence of these anaerobes is consistent with studies that found that the CF lung has anaerobic regions (Worlitzsch *et al.*, 2002). It is also noteworthy that in T-RFLP, nucleic acids are directly extracted from samples (which are sputum in this case) and there is a possibility that oropharyngeal contamination could contribute to the diversity of the species (Rogers *et al.*, 2006). Most importantly, the clinical impact of the colonisation of these species within the CF hosts, and the degree of interaction among those species, are still unknown.

Table 1.2 Detection frequencies of bacterial species assigned according to T-RFLP profiles (Rogers *et al.*, 2004)

Bacterial species assignment	% of patients
<i>Pseudomonas aeruginosa</i>	88
<i>Porphyromonas endodontalis</i> , <i>Prevotella denticola</i> , <i>P. melaninogenica</i> , <i>P. nigrescences</i> , <i>P. veroralis</i> , <i>P. intermedia</i>	71
<i>Craurococcus roseus</i>	62
<i>Prevotella loescheii</i> , <i>P. salivae</i> , <i>P. buccae</i> , <i>P. oris</i> , <i>Porphyromonas gingivalis</i>	57
<i>Rhizobium loti</i> , <i>Ochrobactrum anthropi</i> , <i>Peptostreptococcus anaerobius</i>	44
<i>Ralstonia pickettii</i> , <i>Oligella urethralis</i>	35
<i>Selenomonas sputigena</i> , <i>Streptococcus intermedius</i>	32
<i>Paracoccus halodenitrificans</i>	26
<i>Ralstonia gilardii</i>	21
<i>Staphylococcus aureus</i> , <i>S. epidermidis</i> , <i>S. cohnii</i> , <i>S. hominis</i>	18
<i>Comamonas testosteroni</i>	18
<i>Acinetobacter johnsonii</i>	15
<i>Burkholderia cepacia</i> complex	15
<i>Abiotrophia paraadiacens</i>	15
<i>Salmonella enterica</i> serovar typhimurium, <i>Bifidobacterium pseudocatenulatum</i> , <i>P. mirabilis</i>	15
<i>Actinomyces viscosus</i> , <i>A. naeslundii</i> , <i>Xanthomonas campestris</i> , <i>X. hyacinthi</i> , <i>Morganella morgani</i> , <i>Neisseria</i> species, <i>Pseudomonas aureofaciens</i> , <i>Legionella pneumophila</i>	12
<i>Bilophila wadsworthia</i>	9
<i>Mycobacterium tuberculosis</i>	9
<i>Burkholderia gladioli</i>	9
<i>Stenotrophomonas maltophilia</i> , <i>Fusobacterium gonidiaformans</i>	9
<i>Haemophilus paraphrophilus</i> , <i>H. influenzae</i> , <i>Actinobacillus actinomycetemcomitans</i>	9
<i>Veillonella parvula</i>	9
<i>Treponema pallidum</i>	9
<i>Bacteroides gracilis</i>	6
<i>Aeromonas caviae</i> , <i>Abiotrophia defectiva</i>	6
<i>Flavobacterium indologenes</i>	6
<i>Staphylococcus sciuri</i>	6
<i>Mycobacterium chelonae</i>	6
<i>Eubacterium saburreum</i>	6
<i>Halomonas variabilis</i>	6
<i>Streptococcus pneumoniae</i> , <i>S. salivarius</i> , <i>S. pyogenes</i> , <i>S. macedonicus</i> ,	6
<i>Peptococcus</i> -like species oral clone JM048	6
<i>Desulfovibrio desulfuricans</i> , <i>Tistrella mobilis</i>	3
<i>Wolinella succinogenes</i> , <i>Campylobacter rectus</i> , <i>Bacteroides forsythus</i>	3
<i>Acinetobacter junii</i>	3
<i>Pandoraea pulmonicola</i>	3
<i>Lactobacillus murinus</i>	3
<i>Rhizobium radiobacter</i>	3
<i>Methylobacterium radiotolerans</i>	3
<i>Mycobacterium flavescens</i>	3
<i>Bordetella hinzii</i>	3
<i>Pseudomonas huttiensis</i>	3
<i>Capnocytophaga sputigena</i>	3
<i>Streptococcus anginosus</i> , <i>Veillonella atypica</i> , <i>V. ratti</i>	3
<i>Alcaligenes xylosoxidans</i>	3

Microbial Ecological Interactions within the Lungs of CF Individuals

Co-infections simultaneously lead to ecological interactions between species (inter-species) and even within species (intra-species) (West *et al.*, 2006). The interactions can be both synergistic and antagonistic (Harrison, 2007). For example, oropharyngeal microflora may contribute to the up-regulation of *P. aeruginosa* genes linked with pathogenesis (Duan *et al.*, 2003), suggesting the synergistic relationship. On the other hand, quantitative microbial studies by Moore *et al.* revealed that there was a significantly higher bacterial density among patients who had only Gram-negative infection compared to those who had a mixed Gram-negative/Gram-positive infection, suggesting the antagonistic interactions may play roles in this kind of co-infection (Moore *et al.*, 2005). Indeed, it has been shown that *P. aeruginosa* is capable of killing the cells of *S. aureus* and other Gram-positive bacteria especially in the CF lung environment (Mashburn *et al.*, 2005; Palmer *et al.*, 2005). It has been proposed that the possible ecological interactions between CF microbes can be categorised into four groups as shown in **Table 1.3** (Moore *et al.*, 2005).

Table 1.3 Possible interactions between microbes within the lungs of CF patients (Moore *et al.*, 2005)

Ecological Interaction	Effect on pathogen	Effect of co-coloniser
Antagonism	Reduction in cell density Increase in cell density	Increase in cell density Reduction in cell density
Neutral	No effect	No effect
Symbiosis	Presence of co-coloniser essential for growth and proliferation	Presence of pathogen essential for growth and proliferation
Synergism	Presence of co-coloniser, although not essential, promotes growth of pathogen, more so than effect of pathogen growing alone	Presence of pathogen, although not essential, promotes growth of co-coloniser, more so than effect of co-coloniser growing alone

The diversity of pathogens, the complexity of microbial ecology and the sophisticated interactions among microbes dwelling in the CF lungs are beyond simple explanations. More details regarding the four most studied bacterial species – *S. aureus*, *H. influenzae*, *P. aeruginosa* and *B. cepacia* complex – are summarised hereafter. Research data of *P. aeruginosa* infections, particularly CF lung infections, are reviewed in section 1.4.

1.3.1 *Staphylococcus aureus*

In the 1940s, *S. aureus* was recognised as the first pathogen to cause chronic lung infections in children with CF (Andersen, 1949). In that pre-antibiotic era, *S. aureus* was the most common pulmonary pathogen isolated and was responsible for the mortality of most CF patients, as shown in autopsy studies (Andersen, 1949; Zuelzer & Newton, 1950). Today, *S. aureus* is still the most common organism found in 50% of CF patients who are under 10 years old (**Figure 1.9**), and the infection can be found in infants with CF as young as 3 months old (Armstrong *et al.*, 1997).

S. aureus possesses a variety of virulence factors. Teichoic acid (a component of the cell wall) and fibronectin-binding proteins were shown to be associated with the binding of *S. aureus* to the respiratory epithelial cells in the CF lungs (Aly & Levit, 1987; Mongodin *et al.*, 2002). Once bound, *S. aureus* secretes virulence factors such as catalase, coagulase, hemolysin and several exotoxins which all contribute to host tissue damage (Cohen, 1986). Moreover, capsules which coat the bacterial cells and virulence factors (namely leucocidins) facilitate an immune evasion, contributing to its persistence within the hosts (Foster, 2005).

The discovery of penicillin by Alexander Fleming in 1928 and its introduction into clinical practice in the 1940s did initially ease the staphylococcal infection problem, and the subsequent synthesis of β -lactam antibiotics such as oxacillin, dicloxacillin has played a major role in controlling *S. aureus* infection in CF patients (Szaff & Hoiby, 1982). However, soon after the introduction of these antibiotics, resistant strains of *S. aureus* were reported (Barber & Rozwadowska-Dowzenko, 1948; Kirby, 1944). *S. aureus* firstly developed resistance to β -lactam antibiotics by acquiring β -lactamases. Subsequently, resistance to β -lactamase resistant antibiotics, such as methicillin and flucloxacillin, was identified in *S. aureus* in 1960 (Jevons, 1961), leading to the emergence of methicillin resistant *S. aureus* (MRSA) which now circulate among CF population with prevalence

ranging from 0% to 23% (Burns *et al.*, 1998; Thomas *et al.*, 1998). The MRSA have acquired *mecA*, a gene encoding a penicillin binding protein 2a (PBP-2A), which binds β -lactams with much lower affinity than the native PBPs (Berger-Bachi, 1994).

Data suggest that *S. aureus* is no longer a common cause of significant morbidity or mortality in CF (Conway & Denton, 2006). A recent clinical trial showed that there was no significant difference between pulmonary symptoms shown by CF patients colonised with *S. aureus* compared to non-colonised patients (Stutman *et al.*, 2002). However, lung damage caused by *S. aureus* may predispose to *P. aeruginosa* infection (Stutman *et al.*, 2002), leading to a worse prognosis in patients infected with both pathogens compared to patients infected with *S. aureus* alone (Marks, 1990). Early treatment and eradication of *S. aureus* infection are therefore recommended.

Similarly, MRSA infection has been shown to have no significant deleterious effect to the lungs of CF patients (Boxerbaum *et al.*, 1988; Conway & Denton, 2006; Miall *et al.*, 2001; Solis *et al.*, 2003; Thomas *et al.*, 1998). Despite that, patients with MRSA are often stigmatised and are limited in their social contact, causing psychological damage (Mackenzie & Edwards, 1997). A study of the risk of transmission of MRSA among CF and non-CF patients at the Royal Prince Alfred Hospital (RPAH) in Sydney by Givney and colleagues demonstrated that acquisition of MRSA occurred during hospital admission periods but not from social contacts outside the hospital, indicating that the problem was mainly associated with the nosocomial transmission but not with the socialisation outside the hospital (Givney *et al.*, 1997). On this basis, most CF centres including the CF centre at the RPAH currently suggest isolating patients with MRSA from the rest of the clinic population (Givney *et al.*, 1997; Thomas *et al.*, 1998).

Persistence of the *S. aureus* infection is also associated with the small colony phenotype named ‘small colony variant (SCV)’ (Proctor *et al.*, 2006). In contrast to the normal *S. aureus* phenotypes, SCVs are ten times smaller, grow slower, are able to synthesise organic compounds (auxotrophy) such as haemin or thymidine, and express distinct biochemical characteristics (**Figure 1.10**) (Proctor *et al.*, 2006). *S. aureus* SCVs survive inside host cells without releasing virulence factors to lyse the host cells, hiding from the host immune response (Vesga *et al.*, 1996; von Eiff *et al.*, 1997). Their slow growth and ability to survive intracellularly contribute to their extreme resistance to anti-staphylococcal drugs (Chuard *et al.*, 1997). Their tiny size and unusual biochemical

phenotype make them difficult to isolate in clinical specimens (Proctor & Peters, 1998). Prevalence of *S. aureus* SCVs in the patients with CF ranges from 17% to 49% (Besier *et al.*, 2007; Kahl *et al.*, 1998).

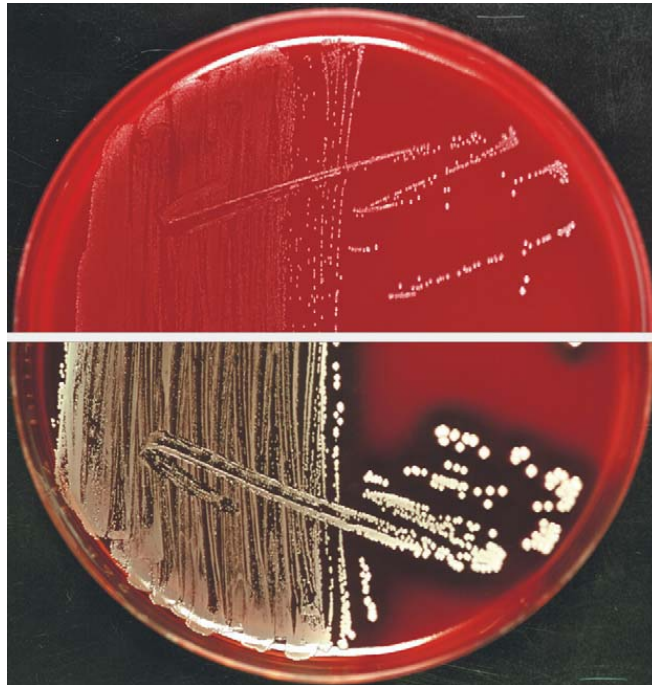


Figure 1.10 *S. aureus* colonies on Columbia horse blood agar (48-hour cultivation) exhibiting the SCV phenotype (upper part) and the normal phenotype (lower part) (Proctor *et al.*, 2006).

The adverse clinical impact of *S. aureus* SCVs infection has been reported in clinical conditions such as chronic osteomyelitis or foreign-body related infection (Proctor *et al.*, 2006). Despite their slow growth rate, *S. aureus* SCVs have higher expression of surface adhesins than the normal phenotype and thus have increased virulence (Vaudaux *et al.*, 2002). However, in various animal models, *S. aureus* SCVs express different virulence compared to the normal phenotype; *S. aureus* SCVs were more virulent in a murine model but less virulent in a *Caenorhabditis elegans* model (Jonsson *et al.*, 2003; Sifri *et al.*, 2006). The clinical significance of *S. aureus* SCVs in the lungs of people with CF has been recently investigated, and it was shown that CF individuals with *S. aureus* SCVs were significantly older and had lower lung function and body weight (Besier *et al.*, 2007). Patients with *S. aureus* SCVs were also significantly more co-colonised with *P. aeruginosa* (80% in patients with SCVs compared to 53% in patients with the normal phenotype *S. aureus*), which could contribute to the patients' poorer clinical status (Besier *et al.*, 2007).

The startling result from this study was the association between *S. aureus* SCVs and the advanced age of the patients since *S. aureus* is usually found during infancy and is often replaced in adulthood by *P. aeruginosa* (Gilligan, 1991). This observation is supported by a recent data which has suggested that the presence of *P. aeruginosa* in the CF airways selected for *S. aureus* SCVs with enhanced antibiotic resistance (**Figure 1.11**) (Hoffman *et al.*, 2006; O'Connell, 2007). A *P. aeruginosa* exoproduct called 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) was found to be responsible for this complex interspecies interaction in the CF milieu (Hoffman *et al.*, 2006). This finding emphasises the complexity of microbial interactions within the CF lungs.

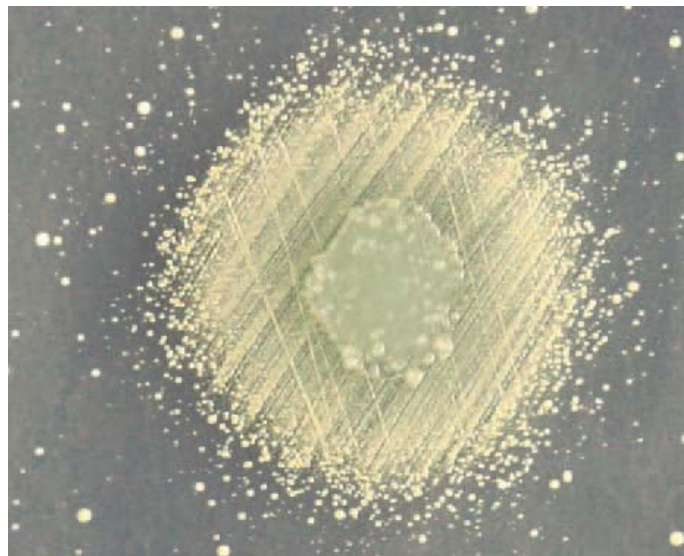


Figure 1.11 The illustration shows a colony of *P. aeruginosa* (confluent colony in the centre) secreting HQNO which protects *S. aureus* (haze surrounding the *P. aeruginosa* colony) from the antibiotic (tobramycin) included in the medium (Hoffman *et al.*, 2006).

1.3.2 Haemophilus influenzae

H. influenzae is a small (1 x 0.3 µm), pleomorphic, Gram-negative coccobacillus. It is a nonmotile, non-spore forming, fastidious, facultative anaerobe. Some strains of *H. influenzae* possess a polysaccharide capsule which can be serotyped into 6 different types (a-f) based on their biochemical structure. The most virulent strain is *H. influenzae* type b (Hib), which accounts for more than 95% of *H. influenzae* invasive diseases, including bacteraemia, meningitis, cellulitis, epiglottitis, septic arthritis, and pneumonia (Fleischmann *et al.*, 1995).

In the CF, however, the common *H. influenzae* isolated from lower respiratory tract infections is nonencapsulated and hence called “Nontypeable *H. influenzae* (NTHi)” (Foxwell *et al.*, 1998). The NTHi is part of the normal flora in the human upper respiratory tract, colonising the nasopharynx in up to 80% of individuals (Vitovski *et al.*, 2002). Nevertheless, they can cause a wide range of diseases, including otitis media, conjunctivitis, sinusitis, bronchitis, and pneumonia (Foxwell *et al.*, 1998). Particularly, in patients with underlying pulmonary conditions such as CF, the NTHi can colonise the lower respiratory tract and can exacerbate the disease (Foxwell *et al.*, 1998).

The prevalence of NTHi isolated from lower airways of CF individuals is highest during infancy period. Rosenfeld *et al.* showed that 38% infants with CF at one year of age were colonised by the NTHi in their lower airways (Rosenfeld *et al.*, 2001). This figure reduces to 24% in the infants at the age of three (Rosenfeld *et al.*, 2001) and to only 10% in CF patients over 15 years of age (Pressler *et al.*, 1984). However, this low figure may reflect difficulties in isolating this fastidious microorganism. Indeed, Bilton *et al.* demonstrated that NTHi strains were found in up to 30% of clinically stable CF adults (Bilton *et al.*, 1995).

While the complete pathogenic roles of NTHi in CF are still unclear, it is hypothesised that NTHi causes inflammation and damage to the airways and acts as a gateway organism for other colonisers such as *P. aeruginosa* (Smith, 1997). Recently, it has been found that NTHi is capable of forming biofilms both *in vitro* and in CF lower airways (**Figure 1.12**) (Starner *et al.*, 2006). The NTHi biofilms, which displayed a decreased antibiotic susceptibility, were found on the apical surface of airway epithelia (Starner *et al.*, 2006). Moreover, the airway epithelial cells that were adherent to the NTHi biofilms exhibited inflammatory and host defence responses by increased chemokine and cytokine secretion, supporting the role of NTHi in causing airway damage (Starner *et al.*, 2006).

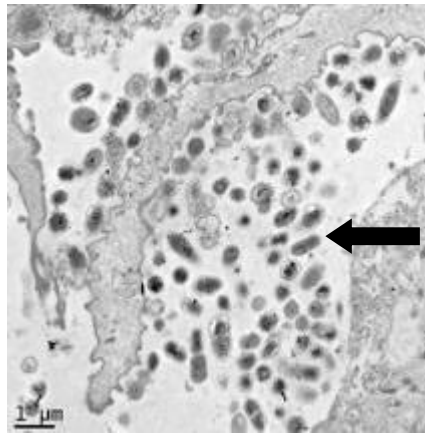


Figure 1.12 Evidence of NTHi biofilm formation in CF. Transmission electron microscopy reveals the NTHi adherent (arrow) to surfaces of airway epithelial cells (Starner *et al.*, 2006).

In summary, although NTHi strains form part of the normal flora, increasing evidence of a pathogenic role of NTHi in CF individuals suggests that NTHi strains may be partly responsible for disease progression. A European consensus therefore recommends considering a treatment of early or asymptomatic colonisation with NTHi (Doring & Hoiby, 2004).

1.3.3 *Burkholderia cepacia* complex

Burkholderia cepacia complex (Bcc) is a group of Gram-negative bacilli composed of at least ten species. A term “genomovar” is commonly used with the Bcc to denote species which are phenotypically indistinguishable but have sufficiently genetic distinctness (Vandamme *et al.*, 1997). To date, ten genomovars have been identified and each genomovar has been given formal scientific names as shown in **Table 1.4**.

The Bcc is ubiquitous in nature and can be found in soil, water, plants and animals. The first member of this group, *B. cepacia* (genomovar I), was discovered in 1950 as a causative agent of bacterial soft rot on onion bulbs; hence the name “cepia” which in latin means onion. Other members of the Bcc were subsequently found throughout the environment (Coenye & Vandamme, 2003).

Table 1.4 Genomovars and species within the *B. cepacia* complex (Coenye *et al.*, 2001; Coenye *et al.*, 2003)

Genomovars	Species
I	<i>B. cepacia</i>
II	<i>B. multivorans</i>
III	<i>B. cenocepacia</i>
IV	<i>B. stabilis</i>
V	<i>B. vietnamiensis</i>
VI	<i>B. dolosa</i>
VII	<i>B. ambifaria</i>
VIII	<i>B. anthina</i>
IX	<i>B. pyrrocinia</i>
X	<i>B. ubonensis</i>

The genome of Bcc has a multireplicon structure, which serves to ensure functionality if any copy of a gene is knocked out (Lessie *et al.*, 1996). It also contains an array of plasmid insertion sequences, which have abilities to promote genomic rearrangements and to increase the expression of neighbour genes (Gaffney & Lessie, 1987). Such complexity of the Bcc genome contributes to its capacity to utilise a variety of organic compounds as nutritional sources, which is beneficial to the natural environment (**Figure 1.13**) (Mahenthalingam *et al.*, 2005). Because it can use and degrade various complex carbon compounds including chlorinated aromatic substrates commonly found in pesticides and herbicides, the Bcc has been widely used as bioremediation agents to degrade chemicals contamination in soils.

The Bcc is an opportunistic human pathogen and does not normally infect healthy individuals but only those that are immunocompromised such as people with CF. The first report of a Bcc isolation from CF airways was from a paediatric CF clinic from Toronto, Canada in 1984 (Isles *et al.*, 1984). In that report, Isles *et al.* observed an increased prevalence of the Bcc infection from 10% to 18% over a ten-year period along with an association of those who were infected with an increased morbidity and mortality (Isles *et al.*, 1984). They also described a syndrome, subsequently called “cepacia syndrome”, which was characterised by high fever, bacteremia, and severe progressive respiratory failure leading to a very high fatality rate that occurred in approximately 20% of colonised patients (Isles *et al.*, 1984; Thomassen *et al.*, 1985b).

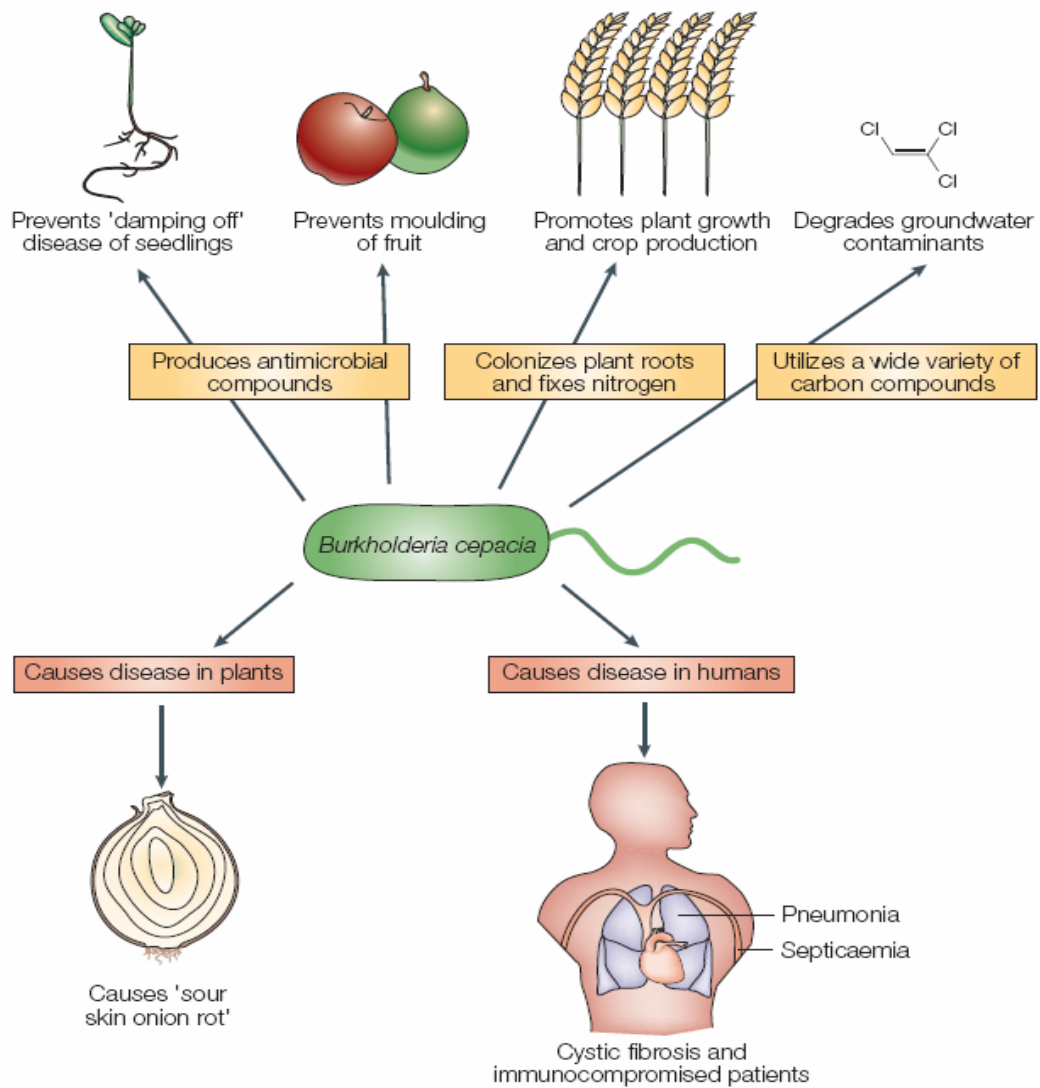


Figure 1.13 Beneficial and detrimental effects of the *Burkholderia cepacia* complex (Mahenthiralingam *et al.*, 2005). Although the Bcc was originally recognized as a pathogen of onions and is now known to be an important opportunistic human pathogen, it also has positive environmental effects and has been widely used as a biopesticide in the protection of crop against fungal diseases and as a bioremediation agent.

Among all Bcc genomovars, *B. cenocepacia* is the most common species isolated from CF airways, and the second most common is *B. multivorans*. Recently a newly characterised member of the Bcc, *B. dolosa* (genomovar VI), has been recovered from airways of CF individuals and has been associated with the rapid decline in lung function and a significantly increased short-term risk of death (Kalish *et al.*, 2006).

Although prevalence levels of the Bcc infection are relatively low (10% or fewer) compared to the prevalence of *P. aeruginosa* colonisation, it has generated considerable anxiety among CF communities due to three major concerns:

- (i) cepacia syndrome,
- (ii) multiple-drug-resistant characteristic, and
- (iii) person-to-person transmission.

Cepacia Syndrome

The cepacia syndrome is more commonly associated with *B. cenocepacia* than other Bcc species. As previously mentioned, the cepacia syndrome occurs in about 20% of patients with Bcc infection. It is important to note that majority of people with Bcc remain stable for many years and the infection may be transient (Govan *et al.*, 1996). Although several studies have shown the association of poor clinical consequences with the Bcc infection (Corey & Farewell, 1996; Muhdi *et al.*, 1996), it is difficult to predict outcomes which are variable in patients colonised by different strains (Ledson *et al.*, 2002). Indeed, a study from the adult CF clinic at the Royal Prince Alfred Hospital, Sydney showed that the Bcc infection had a significant adverse effect on survival, but this outcome was variable within subgroups of the Bcc (Soni *et al.*, 2002).

Multidrug Resistance of the Bcc

All species in the Bcc are innately resistant to many antibiotics and even panresistant in some cases. It has been shown that some Bcc used penicillin as a sole carbon source and could potentially grow in the presence of the penicillin G (Beckman & Lessie, 1979). This high resistant characteristic derives from multiple mechanisms including (i) a specific efflux pump, (ii) reduced permeability of a cell membrane, (iii) enzymatic inactivation such as production of β -lactamase, and (iv) an alteration of cell target such as penicillin-binding proteins (Mahenthiralingam *et al.*, 2005). As a result, a conventional antibiotic susceptibility test, which suggests only a single choice of antibiotic to use, can no longer apply for Bcc treatment. Multiple combination bactericidal antibiotic testing (MCBT), a

recently developed method which combines antibiotics that together have *in vitro* bactericidal activity, has become available for use (Aaron *et al.*, 2000). Results from the MCBT studies showed that triple antibiotic combinations are more likely than single antibiotics to be bactericidal against Bcc isolates (Aaron *et al.*, 2000).

Transmission of the Bcc between People with CF

The first evidence that suggested the possibility of Bcc transmission was from a case of a CF patient who became infected with a Bcc strain after having close contact with another CF patient who had had a Bcc airway infection for more than 18 months following attendance at a CF summer camp (LiPuma *et al.*, 1990). Molecular investigations using Ribotyping subsequently showed that the Bcc strains from the two patients were identical suggesting transmission occurred from one to another (LiPuma *et al.*, 1990). Govan *et al.* confirmed the transmissibility of the Bcc by reporting evidence based on the use of RFLP-PFGE and a detailed epidemiological investigation (Govan *et al.*, 1993). Their study showed that an increased incidence of the Bcc infection in CF clinics in Edinburgh and Manchester in the United Kingdom between 1986 and 1992 was primarily due to an emergence of a transmissible *B. cenocepacia* strain ET12, and the epidemiological evidence indicated that social contact such as regular attendance at a CF fitness class, social kissing and more intimate contact facilitated the spread of the ET12 strain within and between clinics (Govan *et al.*, 1993). Twelve of 54 patients who acquired the ET12 strain developed rapid deterioration and died within a short period (Govan *et al.*, 1993). Subsequently, the ET12 strain was shown to be associated with a fourfold increase risk of mortality (Ledson *et al.*, 2002). In addition to the study by Govan *et al.*, several studies from the United States and Canada demonstrated transmission associated with close contacts in social settings (Mahenthiralingam *et al.*, 1994a; Pegues *et al.*, 1994; Smith *et al.*, 1993). These results have led to the disbanding of CF summer camps worldwide; the US Cystic Fibrosis Foundation discontinued sponsorship and support of CF summer camps (Saiman & Siegel, 2004). Strict segregation policies were also introduced into many CF centres leading to a fall in incidence of Bcc cross infection while ongoing transmission of epidemic clones were documented in clinics that did not implement segregation (Chen *et al.*, 2001; Paul *et al.*, 1998). In 2004, the UK Cystic Fibrosis Trust recommended that patients with Bcc infection should avoid close contact with other patients and should not attend meetings where there are other people with CF (The UK Cystic Fibrosis Trust Infection Control Group, 2004).

Although the stringent infection control policies have helped to limit cross infection, they have not eliminated the risk of sporadic acquisition of the Bcc from natural environment sources. A more recent report identified a highly transmissible *B. cenocepacia* strain in soil samples from a field in the USA, suggesting that there may be environmental reservoirs of this pathogen (LiPuma *et al.*, 2002). However, the risk of acquisition from the environment has not been fully documented.

In summary, the Bcc can be transmitted from one CF patient to another. While the segregation of colonised from non-colonised patients generates a controversial attitude within the CF communities, the acquisition of this pathogen from cross infection, which leads to increasing mortality, cannot be ignored.

1.4 *Pseudomonas aeruginosa* Pathogenesis in CF

1.4.1 General Microbiology

P. aeruginosa is a Gram-negative bacillus belonging to the genus *Pseudomonas* and the family *Pseudomonadaceae*. Members of the genus *Pseudomonas* are common inhabitants of soil, marshes, coastal marine habitats, and both fresh and salt water. *P. aeruginosa* also colonises the surfaces of both plants and animals, causing both plant diseases (Walker *et al.*, 2004) and human diseases.

P. aeruginosa has the largest genome among all known bacteria, and its 6.3 million base pairs (Mbp) genome contains 5,570 predicted genes (Stover *et al.*, 2000), contributing to its extraordinary versatility, adaptability and virulence. *P. aeruginosa* has very simple nutritional requirements. It can even grow in distilled water (Favero *et al.*, 1971). Like other members of the pseudomonads, *P. aeruginosa* possesses metabolic versatility and is able to grow by utilising a large variety of substrates and at temperatures as high as 42°C. Despite being categorised as an obligate aerobic bacterium, *P. aeruginosa* can grow in anaerobic conditions by using nitrogenous oxides as respiratory electron acceptors (Filiatrault *et al.*, 2005; Ye *et al.*, 1995) as well as by metabolising arginine (Vander Wauven *et al.*, 1984) and pyruvate (Eschbach *et al.*, 2004). Collectively, these reasons explain why *P. aeruginosa* is a successful coloniser and ubiquitous.

P. aeruginosa is the perfect example of an opportunistic pathogen. The bacterium hardly ever infects healthy hosts. Nevertheless, it can colonise any tissue with compromised defences such as burns or exposed wounds. It causes urinary tract infections, respiratory system infections, skin and soft tissue infections, bone and joint infections, bacteraemia and a variety of systemic infections particularly in people with compromised immune systems including cancer and AIDS patients, burn sufferers, and patients treated with immunosuppressive agents as well as people with CF.

Apart from people with CF who acquire *P. aeruginosa* from natural environments, infections by this bacterium generally occur in hospital settings. Patients with indwelling catheters, such as an endotracheal tube, nasogastric tube or urinary tubes, are prone to *P. aeruginosa* colonisation. Indeed, *P. aeruginosa* was the most common pathogen isolated from hospitalised patients (Cardo *et al.*, 2004). The ability to grow in various hospital niches including sinks, drains, ventilators or even in antiseptic or disinfectant solutions together with its large variety of cell associated and secreted virulence factors and its antimicrobial resistance render *P. aeruginosa* a feared nosocomial pathogen.

1.4.2 Virulence Factors of *P. aeruginosa*

The capacity of *P. aeruginosa* to cause such a wide range of infections is due to the fact that it possesses a large array of virulence factors. These virulence factors can be classified into (i) cell-associated virulence factors and (ii) secreted or extracellular virulence factors.

1.4.2.1 Cell-Associated Virulence Factors

1.4.2.1.1 Lipopolysaccharide

Lipopolysaccharide (LPS) is a major component of the outer membrane of *P. aeruginosa* and other gram negative bacteria. It consists of a hydrophobic domain, Lipid A, which embeds into the phospholipid bilayer of the outer membrane, and a hydrophilic tail composed of core polysaccharide and O-specific polysaccharide, projecting from the surface. The O-specific polysaccharide or O-side chain is the immunodominant antigen (Goldberg & Pier, 1996), and because of its variation in structure, the O-side chain was historically used in serologic typing schemes such as the Fisher serotyping scheme or International Antigenic Typing System (IATS) scheme for epidemiologic studies of *P. aeruginosa* before the availability of molecular typing techniques (Fomsgaard *et al.*, 1988).

Typically, the LPS of *P. aeruginosa* isolated from environmental sources or from patients with acute infection expresses many long O-side chains (also called smooth LPS) (Fomsgaard *et al.*, 1988). In acute infection such as septicaemia, the smooth LPS is a prominent pathogenesis factor because of its toxic nature and its capability to resist complement-mediated killing in serum (Cryz *et al.*, 1984; Pier, 2007). It has been shown that complement molecules such as C3 or C5b-9 preferentially attach to an external end of the long-chain LPS molecules (Joiner *et al.*, 1986). As a result, the long LPS molecules sterically hinder the insertion of C5b-9 into the bacterial cell wall where it would otherwise kill the bacteria (Joiner *et al.*, 1984), thus the smooth LPS functions as a barrier against host immune response.

In contrast, the LPS of *P. aeruginosa* recovered from the lungs of chronically infected CF patients has few, short or no O-side chains (also called rough LPS) (Fomsgaard *et al.*, 1988; Hancock *et al.*, 1983), and thus the rough LPS *P. aeruginosa* is very sensitive to normal human serum (Hancock *et al.*, 1983), which could explain why people with CF rarely have *P. aeruginosa* septicaemia despite having high bacterial loads in the lungs. In addition, this suggests that the survival of *P. aeruginosa* in a CF lung environment does not rely on the LPS barrier but evades host immunity by different strategies. Indeed, it was found that clearance of bacteria in respiratory lumens depends mainly on the innate host defence mechanism involving shedding epithelial cells that have ingested bacteria (desquamation) (Pier *et al.*, 1996a; Pier *et al.*, 1996b; Pier *et al.*, 1997), whereas the complement activation system plays a minor role (Pier & Ames, 1984). The CFTR on the epithelial cells itself acts as a bacteria receptor by binding to the O-side chains and mediates the ingestion or internalisation of *P. aeruginosa* into the epithelial cell (Pier *et al.*, 1996b). A defective CFTR cell such as a CF cell is therefore unable to uptake *P. aeruginosa*; conversely, the rough LPS feature of *P. aeruginosa* allows these bacteria to evade the ingestion. This is supported by the histological evidence of CF airways demonstrating that *P. aeruginosa* is confined generally in airway lumens while extraluminal presence is rare (Baltimore *et al.*, 1989). The loss of O-side chains also makes *P. aeruginosa* non-typable by serotyping techniques (Hancock *et al.*, 1983).

In addition to the adaptive mechanism by modifying the O-side chain, *P. aeruginosa* recovered from chronic lung infections in CF was also found to synthesise a specific structure of Lipid A (Ernst *et al.*, 1999). This unique form of Lipid A containing palmitate

and aminoarabinose was associated with enhanced resistance to cationic antimicrobial peptides such as aminoglycosides and induced interleukin 8 (IL-8) production (Ernst *et al.*, 1999; Ernst & Miller, 2000; Pier, 2000), suggesting bacterial adaptability to survive in the CF lung environment and its roles in the pathogenesis of CF.

1.4.2.1.2 Flagellum

A bacterial flagellum, a filamentous structure projecting from the cell membrane, is a complex bacterial organelle consisting of three sub-assemblies: (i) a basal body embedded in the cell surface, (ii) a filament and (iii) a hook which connects the filament and the basal body (Shapiro, 1995). The flagellum not only enables bacteria to move and thus respond to environmental signals (chemotaxis) but also facilitates the acquisition of nutrients (Shapiro, 1995). Structures and genetic regulation of the bacterial flagella are surprisingly well conserved among diverse strains or even bacterial species (Macnab, 1992).

In nature, *P. aeruginosa* possess a monotrichous flagellum (a single polar flagellum) which confers motility and chemotaxis (Drake & Montie, 1988). In respiratory tracts, the flagellum of *P. aeruginosa* can also function as an adhesin to receptors of host cells which is essential for an initial phase of infection (Feldman *et al.*, 1998). FliD, the flagellar cap protein encoded by *fliD*, has been demonstrated to adhere to mucin on the respiratory tract surface (Arora *et al.*, 1998), whereas flagellin, the major flagellar protein encoded by *fliC*, has been shown to bind to the respiratory epithelial cell glycolipids receptors such as GM1 and asialoGM1 (Feldman *et al.*, 1998) and to Muc1 mucin on the epithelial cell surface (Lillehoj *et al.*, 2002). However, the flagella are highly immunogenic (Mahenthiralingam *et al.*, 1994b), and the binding of the flagella to the host cells can strongly initiate inflammatory responses especially IL-8 expression which is a potent chemokine recruiting neutrophils and macrophages (DiMango *et al.*, 1995). In the normal host, the presence of neutrophils and macrophages coupled with the intact mucociliary clearance can readily eradicate any inhaled *P. aeruginosa*.

Intriguingly, in the CF host, after the initial colonisation in the airway, *P. aeruginosa* rapidly adapt to the host by repression of *fliC* (Wolfgang *et al.*, 2004); the flagellin and the flagellar filaments have been reported to be undetectable within 2 hours after an exposure to the CF muco-purulent respiratory liquid (Wolfgang *et al.*, 2004). By not expressing the flagella, the host immune responses cannot detect the presence of the bacteria

(Mahenthiralingam *et al.*, 1994b). This particular feature of *P. aeruginosa* was demonstrated in isolates from chronically infected CF lungs which were mostly non-motile.

It has been reported that the flagella are also important in an initial step of in the formation of complex colonial structures called biofilms (O'Toole & Kolter, 1998a) (see Section 1.4.5).

1.4.2.1.3 Pili

Like the flagella, pili also play important roles in the bacteria-host cell interactions including the attachment of *P. aeruginosa* cells to host surfaces. *P. aeruginosa* pili can bind to the asialoGM1 receptors of the respiratory cells (Saiman & Prince, 1993), and the numbers of the asialoGM1 receptors has been shown to be increased in the CFTR defective cells, suggesting that CF respiratory surfaces are essentially susceptible to *P. aeruginosa* binding (Saiman & Prince, 1993). As with the flagellum, *P. aeruginosa* promptly ‘turn off’ the expression of pili after binding to the host cells (Hallet, 2001; Henderson *et al.*, 1999) as the pili acts as the bacterial ligands in the initial stages of nonopsonic phagocytosis (Kelly *et al.*, 1989).

The pili in *P. aeruginosa* are type IV pili (Mattick, 2002) and, as well as having a role in the attachment to host epithelial cells, type IV pili are also required for a translocation of bacterial cells along solid surfaces which is termed “twitching motility” (Mattick, 2002). The term twitching motility, derived from the observation of the jerking characteristic movement of the cells, was first used to describe flagella-independent surface motility (Mattick, 2002). Twitching motility allows *P. aeruginosa* cells to move or glide toward environment and nutritional signals (Merz *et al.*, 2000) and facilitates rapid colonisation of cells on surfaces as well as biofilm formation (O'Toole & Kolter, 1998a) (see Section 1.4.4). In *P. aeruginosa*, almost 40 genes including genes controlling type IV pili formation and genes regulating the complex chemosensory pathway (Chp system encoded by *chp* genes cassette) are responsible for twitching motility (Alm & Mattick, 1997). In addition, *pilA*, a gene encoding a transcription of pilin which is a major structure of pili, is regulated by the alternative sigma factor (σ or RpoN) (Ishimoto & Lory, 1989).

1.4.2.2 Secreted Virulence Factors

1.4.2.2.1 Pyocyanin

P. aeruginosa produces a blue, chloroform-soluble pigment called pyocyanin, causing a blue-pus wound, a typical characteristic of *P. aeruginosa* wound infection which has been recognised for more than a century (Jordan, 1899). Indeed, its former name, *Bacillus pyocyaneus*, simply means a blue-pigment producing bacillus (Jordan, 1899), and even the name “*aeruginosa*” translated from the Latin word also means “copper rust” describing the blue-green pigment seen in laboratory cultures (Young, 1947). Pyocyanin has been shown as an important factor playing various roles in pathogenesis including: (i) an ability to cause transferrin to release iron which is a crucial requirement for growth (Cox, 1986); (ii) acting as an antibiotic against a wide variety of competing microorganisms by inducing the production of toxic oxygen compounds such as superoxide (O_2^-) or hydrogen peroxide (H_2O_2) (Hassan & Fridovich, 1980); (iii) inducing an apoptosis of neutrophils as shown in both *in vitro* (Usher *et al.*, 2002) and *in vivo* (Allen *et al.*, 2005); (iv) disrupting a host protease-antiprotease regulatory system which controls the balance of protease levels in the lungs (Britigan *et al.*, 1999); (v) inhibiting catalase activity in respiratory epithelial cells which is essential in preventing toxicity from oxygen radical products (O'Malley *et al.*, 2003); and (vi) stopping ciliary beating by decreasing intracellular cAMP and ATP of the respiratory epithelial cells (Kanthakumar *et al.*, 1993; Wilson *et al.*, 1988). Lau *et al.* demonstrated that *P. aeruginosa* pyocyanin-mutant strains had substantially less pathogenicity compared to the wild-type strains as tested in mice models (Lau *et al.*, 2004). Nonetheless, the mutant strains were still able to cause local inflammation (Lau *et al.*, 2004), highlighting the importance of other virulence factors.

1.4.2.2.2 Siderophores

Iron is a key component of basic metabolism and thus a vital element for most organisms including *P. aeruginosa*. In humans, free-form iron is scarce as it is strongly bound to iron-binding proteins such as transferrin, lactoferrin and hemoglobin (Aisen & Listowsky, 1980). In order to grow with in the human hosts, *P. aeruginosa* excretes siderophores which are bacterial products mediating bacterial iron uptake systems (Crosa, 1997). Siderophores compete for iron by converting the transferrin-bound iron into a soluble form which bacteria can utilise (Sriyosachati & Cox, 1986). Proteases such as elastase and alkaline protease secreted by *P. aeruginosa* also mediate siderophores uptake iron by

proteolytic cleavage of iron from host transferrin (Doring *et al.*, 1988). *P. aeruginosa* produces two types of siderophores, pyoverdinin and pyochelin. Pyoverdinin, which is a yellow-green fluorescent pigment, formerly known as fluorescein, was shown to be more important for iron acquisition (Ankenbauer *et al.*, 1985; Cox & Adams, 1985). However, both are essential for virulence and infectivity (Cox, 1982; Meyer *et al.*, 1996).

1.4.2.2.3 Proteases

Proteases, also known as proteinases, by definition are a group of hydrolase enzymes which catalyse the reaction of hydrolysis of protein molecules at various bonds. Proteases are involved in digesting long protein chains into short fragments, detaching amino acids from the protein chain, or attacking internal peptide bonds of a protein. To date, four types of proteases have been known to be secreted by *P. aeruginosa*; (i) LasB elastase, (ii) LasA elastase or staphylolysin, (iii) alkaline protease and (iv) protease IV. These proteases are associated with virulence by enhancing the ability of *P. aeruginosa* to invade tissues.

(i) LasB Elastase

Among the numerous extracellular virulence factors produced by *P. aeruginosa*, elastase is considered to be a major contributor in its pathogenesis. Originally purified and described in 1965 (Morihara *et al.*, 1965), elastase was named because of its ability to degrade elastin. However, the proteolytic activity of elastase exceeds its elastolytic activity (Galloway, 1991). In fact, elastase is capable of degrading several biological molecules including laminin, fibrin and collagen in human hosts (Heck *et al.*, 1986) and surfactant proteins A and D in the respiratory tract (Mariencheck *et al.*, 2003). Elastase has also been shown to destroy epithelial cell junctions (Azghani *et al.*, 1993) and to disrupt intact respiratory epithelia and damage corneal tissue (Twining *et al.*, 1993). It can also cleave immunoglobulin (such as IgG and IgA), complement components, interferon- γ , interleukin and tumour necrosis factor (Doring *et al.*, 1985; Heck *et al.*, 1990; Schultz & Miller, 1974). On the basis of these studies, elastase is clearly an important factor in tissue invasion.

Elastase is encoded by the *lasB* gene (Schad *et al.*, 1987). The production of active elastase involves three steps, including (i) synthesis of prepro-elastase which occurs in the bacterial cytosol, (ii) cleavage to form pro-elastase during transport across the cytoplasmic membrane, and (iii) formation of two disulfide bonds to become mature (or active) elastase

when it is secreted extracellularly (Braun *et al.*, 2001). The secretion of elastase is via the type II secretion system (Braun *et al.*, 1996) and is controlled by a signalling system called “quorum sensing” (Gambello & Iglewski, 1991). (see Section 1.4.3 for the type II secretion and Section 1.4.4 for quorum sensing)

(ii) LasA Elastase or Staphylolysin

LasA elastase was discovered as a result of a study of a LasB-mutant strain in which the strain still produced and exhibited the elastolytic activity (Ohman *et al.*, 1980). Later, the *lasA* gene was discovered and was found to be responsible for the production of another form of elastase (Goldberg & Ohman, 1987; Schad *et al.*, 1987). Like the *lasB* gene, the transcription of *lasA* is also controlled by the *lasR* gene of the quorum sensing system (Toder *et al.*, 1991).

LasA acts directly upon elastin and enhances the elastolytic activity of LasB elastase as well as human neutrophil elastase, leading to tissue destruction (Peters & Galloway, 1990). Kessler *et al.* showed that LasA also had staphylolytic activity, resulting from cleavages within the pentaglycine cross-links in the peptidoglycan of *S. aureus* cells (Kessler *et al.*, 1993). It has also been shown that LasA can inhibit the growth of *S. aureus* cells *in vitro* (Mansito *et al.*, 1987). This favours LasA as a useful agent in enzyme-based treatment of *S. aureus* infections particularly keratitis, where, in a mouse model, the LasA itself causes little or no damage to the cornea (Barequet *et al.*, 2004; Preston *et al.*, 1997). It has been proposed that secreted LasA or staphylolysin may contribute to an observation in the CF lung that *S. aureus* is often displaced by the colonisation of *P. aeruginosa* (Machan *et al.*, 1991; Palmer *et al.*, 2005)

(iii) Alkaline Protease

Encoded by the *aprA* gene, alkaline protease is another virulence protein important in the pathogenesis of *P. aeruginosa*. It has been shown that alkaline protease is required for the establishment of corneal infections (Howe & Iglewski, 1984). It can suppress host immunity by inhibiting the production of interferon- γ from T cells (Horvat & Parmely, 1988). It can also inhibit neutrophil function, particularly chemotaxis, and is responsible for an evasion of this pathogen from the phagocytic defence system (Kharazmi *et al.*, 1984). Alkaline protease appears to act in concert with both LasB and LasA elastase causing notable destruction of tissues such as airways of CF patients (Suter, 1994). Similar

to LasB and LasA elastase, transcription of alkaline protease is under the influence of the *lasR* gene (Gambello *et al.*, 1993).

(iv) Protease IV

Protease IV was first described in 1992 (Toder & Gambello, 1992), and its pathogenic role was further explained in a study of corneal infections caused by *P. aeruginosa* (O'Callaghan *et al.*, 1996). Encoded by the *prpL* gene, protease IV is a 26 kDa lysine-specific endoprotease (Engel *et al.*, 1998a). Several studies have demonstrated the roles of protease IV in corneal infections, including the destruction of ocular fibrinogen and elastin (Caballero *et al.*, 2004; Engel *et al.*, 1998a; Engel *et al.*, 1998b; O'Callaghan *et al.*, 1996). However, its role in the pathogenesis of the airways is still elusive with to date only one published study of protease IV and its effect in the lung. The study showed that protease IV degrades surfactant proteins, decreasing both the host defence and the biophysical properties of pulmonary surfactant, and thus it may contribute to acute lung injury associated with *P. aeruginosa* (Malloy *et al.*, 2005). However, its role in chronic lung infections such as CF lungs needs to be investigated further.

In summary, proteases are crucial virulence factors of *P. aeruginosa*. These proteases work together leading to significant damage of host tissues. The elastolytic activity of these enzymes plays a major role in *P. aeruginosa* pathogenesis because a number of organs or tissues are composed of elastin (e.g. lung tissue, vascular tissue and ocular tissue) and need elastic properties for their physiologic functions. In CF, it has been suggested that the roles of these proteases (except protease IV) are important in the establishment of *P. aeruginosa* infection (Doring *et al.*, 1983; Doring *et al.*, 1985; Suter, 1994). Strains that do not produce proteases were shown to have significantly reduced virulence (Cowell *et al.*, 2003). Any treatment targeting these proteases could be a potential therapeutic option (Kipnis *et al.*, 2006).

1.4.2.2.4 Chitinase

Chitin is one of the most abundant polysaccharide polymers found in nature. Numerous bacterial species from the genera including *Bacillus*, *Vibrio*, *Enterobacter*, *Serratia*, *Aeromonas* and *Pseudomonas* have been reported to secrete chitinolytic enzymes or chitinase (Yu *et al.*, 1991). These enzymes are able to break down polymeric chitin into oligosaccharides, which can be used as energy sources (Bassler *et al.*, 1991). Chitinase

produced by *P. aeruginosa* has been investigated for many years as a virulence factor associated with plant diseases (Collinge *et al.*, 1993); however, its role in clinical pathogenesis has been rarely explored. The first report of *P. aeruginosa* chitinase involvement in a clinical setting arose from the development of a whole-cell *P. aeruginosa* vaccine (Cripps *et al.*, 1994). By using western blotting to analyse the whole-cell extracts, chitinase was found to be one of the detected antigens (Cripps *et al.*, 1994). It was later characterised as a 58 kDa enzyme encoded by *chiC* (Thompson *et al.*, 2001) and was found to be regulated by the quorum sensing system (Winson *et al.*, 1995). Folders *et al.* reported that ChiC chitinase was produced by clinical isolates of *P. aeruginosa* (Folders *et al.*, 2001). Where chitin is applied as a wound-healing agent (Cho *et al.*, 1999), bacterial chitinase may be released upon the presence of chitin and may contribute to infections of wounds. However, the role of its pathogenesis in the CF lung has not yet been identified.

1.4.2.2.5 Exotoxin A

Exotoxin A (ETA) is another important factor involved in the virulence of *P. aeruginosa*. The production of this toxin was found in both environmental and clinical strains of *P. aeruginosa* (Bjorn *et al.*, 1977). Once inside the host cell, ETA can cause cytotoxicity by inhibiting protein synthesis via ADP-ribosylation of elongation factor-2 at the protein translation level (**Figure 1.14**) (Iglewski *et al.*, 1977). It was suggested that ETA enters into the cell via a system of endocytosis via the interpolar receptor (Bourke *et al.*, 1994). However, the responsible receptor is yet to be identified. ETA causes tissue damage, including liver cell necrosis, renal necrosis and pulmonary haemorrhage and is also responsible for bacterial invasion and suppression of immune responses in the CF lung (Pitt, 1986).

1.4.2.2.6 Exoenzymes

P. aeruginosa produces four exoenzymes: exoenzyme S (ExoS), exoenzyme T (ExoT), exoenzyme U (ExoU) and exoenzyme Y (ExoY) which are encoded by *exoS*, *exoT*, *exoU* and *exoY* genes respectively (Aktories & Barbieri, 2005). The production of exoenzymes is induced by contact between bacterial cells and eukaryote cells and in an environment that is low in Ca²⁺ (Hornef *et al.*, 2000). Following cell contact, *P. aeruginosa* delivers these exoenzymes into host cells via a complex protein secretion system called the type III secretion system (Galan & Collmer, 1999). Different *P. aeruginosa* isolates express these

four cytotoxins differently. Feltman *et al.* investigated the presence of these exoenzymes genes among environmental isolates and isolates from various clinical specimens including the sputum from CF and found that all studied isolates contained *exoT* while the presence of *exoS*, *exoU* and *exoY* appear to be mutually exclusive (Feltman *et al.*, 2001). Interestingly CF isolates harboured the *exoS* gene more frequently and the *exoU* gene less frequently compared to isolates from the other sites of infection (Feltman *et al.*, 2001).

As shown in **Figure 1.14**, ExoS and ExoT disrupt the actin cytoskeleton through Rho GTPase-activating protein (GAP) activity and ADP-ribosylation (Goehring *et al.*, 1999; Krall *et al.*, 2000; Krall *et al.*, 2002). The ExoS ADP-ribosylates the host protein ERM which is the protein that has roles in cell shape, microvilli, motility, cell adhesion and phagocytosis (Maresso *et al.*, 2004). The ExoT ADP-ribosylates Crk proteins, resulting in an inactivation of Rac1 which is essential in cell migration (wound healing) and phagocytosis (Sun & Barbieri, 2003). Both ADP-ribosylation and inactivation of Rho GTPase by ExoS and ExoT cause an alteration in the cytoskeleton of host cells and ultimately cell apoptosis (Kaufman *et al.*, 2000).

ExoU is a lipase (or phospholipase) which disrupts membrane function in host cells (**Figure 1.14**) (Dacheux *et al.*, 2000). It is a potent necrotic toxin and has been shown to be over 100-fold more cytotoxic than ExoS (Lee *et al.*, 2005). *In vitro* studies showed that ExoU mediated killing of a variety of mammalian cells, including macrophages, epithelial cells and fibroblasts (Coburn & Frank, 1999; Finck-Barbancon *et al.*, 1997).

ExoY is an adenylate cyclase that elevates intracellular cyclic AMP (cAMP) to supra-physiological levels (over 500-fold above normal level), which indirectly disrupts the actin cytoskeleton (**Figure 1.14**) (Yahr *et al.*, 1998).

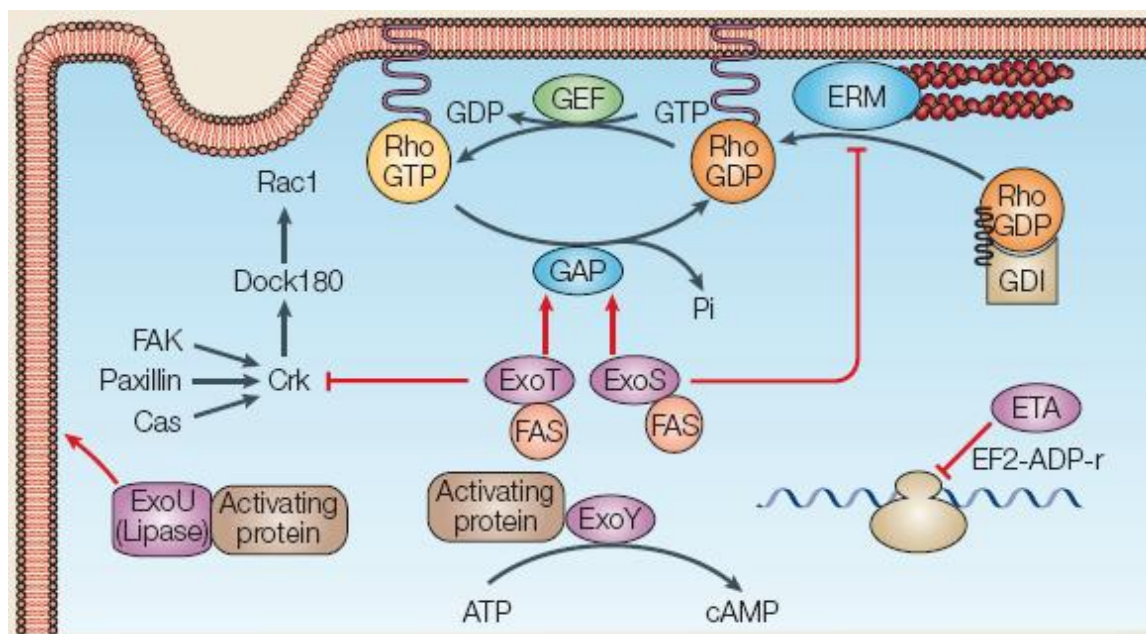


Figure 1.14 Schematic diagram showing the mechanisms of action of exotoxin A and exoenzymes inside the host cell (Aktories & Barbieri, 2005).

The functions of these type III secreted exoenzymes have been investigated in both acute and chronic infections. In acute infections, such as burn wounds, acute pneumonia or corneal infection, the exoenzymes were clearly shown to be an important virulence mechanism (Holder *et al.*, 2001; Kudoh *et al.*, 1994; Lee *et al.*, 2003). In chronic infections, however, the roles of these exoenzymes remain controversial. The presence of antibodies against exoenzymes in sera of adult CF patients indicated that the type III cytotoxins are expressed during chronic infection of the CF lung (Moss *et al.*, 2001). Nicas *et al.* also demonstrated that *P. aeruginosa* strains producing ExoS caused greater degree of lung damage than ExoS deficient mutant strains in a rat chronic lung infection model (Nicas *et al.*, 1985). In contrast, more recent research compared the secretion of these exoenzymes from genotypically identical *P. aeruginosa* strains from the same CF patient isolated at the initial onset of infection and ten years later and found that initial strains secreted higher levels of exoenzymes than strains isolated later after the development of chronic infection (Lee *et al.*, 2005).

1.4.2.2.7 Rhamnolipid

Rhamnolipid is a surfactant secreted by bacteria including *P. aeruginosa*. While its roles in clinical settings are yet to be studied, its industrial use as an alternative potential surfactant compound (biosurfactant) have been widely recognised (Fiechter, 1992). In *P. aeruginosa*,

rhamnolipids are synthesised through a catalysing reaction of rhamnose by a specific rhamnosyltransferase (Burger *et al.*, 1963). This rhamnosyltransferase is encoded by the *rhlAB* genes, which were found to be regulated by the quorum sensing system (RhII/RhlR) (Ochsner & Reiser, 1995). (also see Section 1.4.4) The clinical importance of the rhamnolipids has been revealed recently and they have been shown to have roles in development and maintenance of biofilms (a cluster of bacterial cells encased by exopolysaccharide matrix) (Davey *et al.*, 2003). (also see Section 1.4.5) More recently, *ex vivo* studies of airway epithelia showed that the rhamnolipids produced by *P. aeruginosa* were able to modulate and break the tight junctions between respiratory epithelial cells (Zulianello *et al.*, 2006).

1.4.2.2.8 Other Virulence Factors

In addition to all of the mentioned, *P. aeruginosa* secretes other virulence factors which contribute to its pathogenicity (Wilson & Dowling, 1998), but they are less well understood. These factors are summarised in **Table 1.5**. Moreover, *P. aeruginosa* has been shown to possess the quorum sensing system, to exist in biofilm, and to express mucoid phenotype especially the strains from CF lungs. All of these features are directly associated with its virulence. The next sections review data regarding the properties of the quorum sensing, biofilm formation and mucoidy.

Table 1.5 Virulence factors of *P. aeruginosa* (adapted from Wilson & Dowling, 1998)

Virulence factor	Biological action
Phospholipase C	Haemolysis; tissue damage; destroy surfactant
Histamine	Impair epithelial integrity
Leukocidin	Cytotoxic to neutrophils and lymphocytes

1.4.3 Exoprotein Secretion Systems

In addition to a wide range of virulence factors, *P. aeruginosa* possesses secretion systems facilitating transportation of those secreted virulence factors out of the cell (Bitter, 2003). To date, six different pathways have been identified in *P. aeruginosa*. However, three of these systems – type I, type II and type III secretion systems – are well understood and involved in the secretion of major virulence factors.

Type I Secretion System

The type I secretion system, also known as an ATP-binding cassette (ABC pathway) or a general export pathway (GEP), is a one step transportation system (**Figure 1.15**) (Andersen *et al.*, 2000). This simple system, which comprises three protein subunits: the ABC protein, membrane fusion protein (MFP) and outer membrane protein (OMP), transports molecules of various sizes ranging from 20 – 100 kDa (Filloux *et al.*, 1998). Alkaline protease is secreted through this pathway.

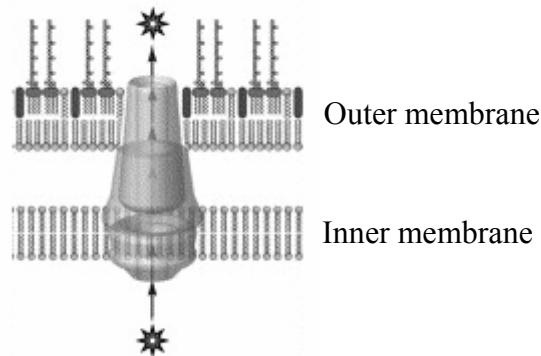


Figure 1.15 Type I secretion system (adapted from Filloux *et al.*, 1998).

Type II Secretion System

The type II secretion system, also known as a general secretory pathway (GSP), is a two-step protein transportation process. The type II system initially uptakes proteins through the inner membrane via the Sec system (**Figure 1.16**) and then modifies the proteins within the periplasmic space prior to secreting out of the cells via a multimeric complex structure (**Figure 1.16**) (Filloux *et al.*, 1998). Virulence factors including LasB and LasA elastase, protease IV, exotoxin A and phospholipase C, are known to be secreted via this pathway (Kipnis *et al.*, 2006).

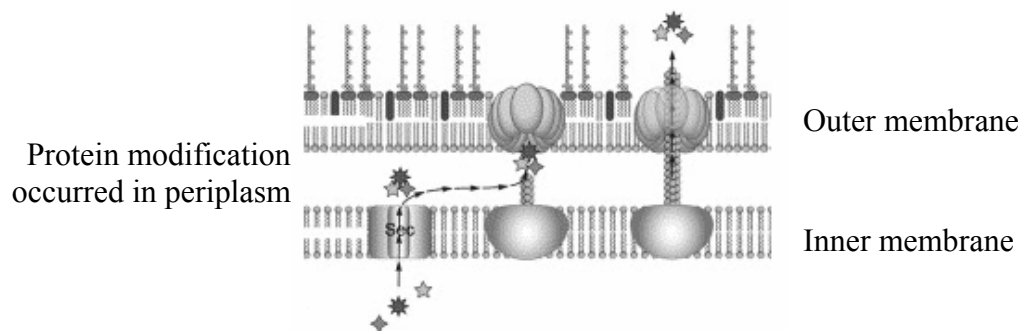


Figure 1.16 Type II secretion system (adapted from Filloux *et al.*, 1998).

Type III Secretion System (TTSS)

Made up of more than 20 proteins, the TTSS is the most complex of all known protein secretion systems (Hueck, 1998). Its structure is evolutionarily related to the bacterial flagellar basal body (Galan & Collmer, 1999). By contact with host cells, this system acts like a needle and delivers (or injects) effector proteins, including ExoS, ExoT, ExoU and ExoY, across host cellular membranes into their cytosol (Cornelis & Van Gijsegem, 2000; Cornelis, 2006). At the tip of the TTSS needle is a protein complex containing a low-calcium-response (LcrV) protein subunit, which functions at the low Ca^{2+} concentration (**Figure 1.17**) (Cornelis, 2006). The protein LcrV is a translocator – a TTSS protein that actively assembles a transmembrane pore in the host cell membrane (**Figure 1.17**). As described in Section 1.4.2.2.6, the exoproteins of *P. aeruginosa* secreted through this type of secretory system can influence host cells, especially the cytoskeleton functions.

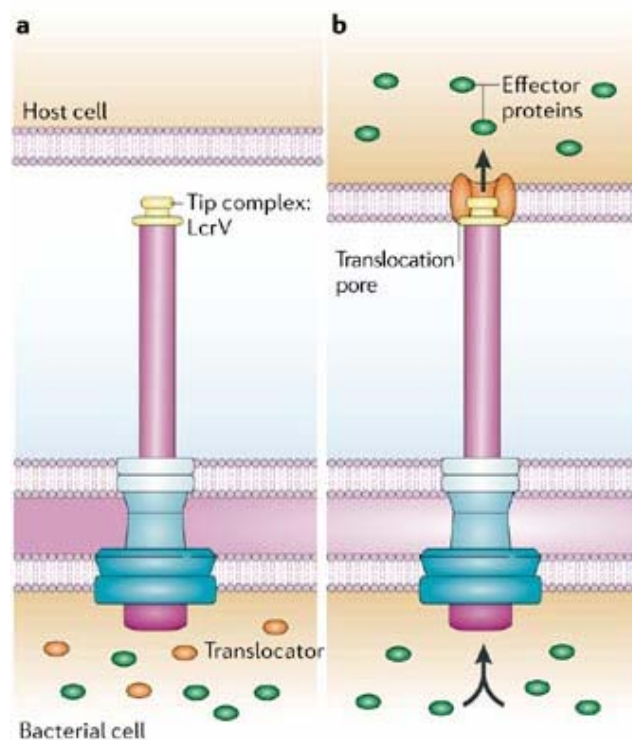


Figure 1.17 Type III secretion system. (a) LcrV forms the complex at the tip of the TTSS needle. (b) Once contact with the host cell membrane is made, the tip complex assists with the assembly of translocation pore. Adapted from Cornelis (2006).

1.4.4 Quorum Sensing System

Quorum sensing (QS) is a term that has been given to the regulation process which bacteria use to communicate with one another in a cell density-dependent manner. Generally, bacteria sense and respond to their population density via self-produced small diffusible molecules, and by responding to these specific chemical signals, bacterial cells can regulate a variety of physiological functions or even function as a group.

It has been known for more than 25 years that bacteria can communicate by releasing and responding to certain signalling molecules. This phenomenon, now known as QS, was first described in the bioluminescent bacterium *Vibrio fischeri* (Nealson *et al.*, 1970). In nature, *V. fischeri* lives in symbiosis with its marine eukaryotic hosts – the squid *Eurprymna scolopes* (Ruby, 1996). The squid hosts provide *V. fischeri* with a nutrient-rich environment inside the body cavity while *V. fischeri* produces light (bioluminescence) that the host uses as an antipredation strategy (Ruby, 1996). Light emission is triggered by the transcription of luciferase gene, which occurs when the *V. fischeri* cell-population density

is sufficient to produce a threshold accumulation of the signal substance, called an autoinducer (Nealson *et al.*, 1970). The autoinducer structure was later determined as 3-oxo-hexanoyl-homoserine lactone (3-oxo-C6-HSL or OHHL) (a molecule belonging to a group of chemical compounds called acyl-homoserine lactones or AHL).

The QS in *V. fischeri* is regulated by *luxI/luxR* genes. The biosynthesis of the autoinducer OHHL is controlled by the gene *luxI* via LuxI protein, an autoinducer synthase enzyme. The OHHL then binds the LuxR protein, and the LuxR-OHHL complex activates the transcription of the *luxCDABE* gene encoding luciferase enzymes responsible for the bioluminescent process (**Figure 1.18**).

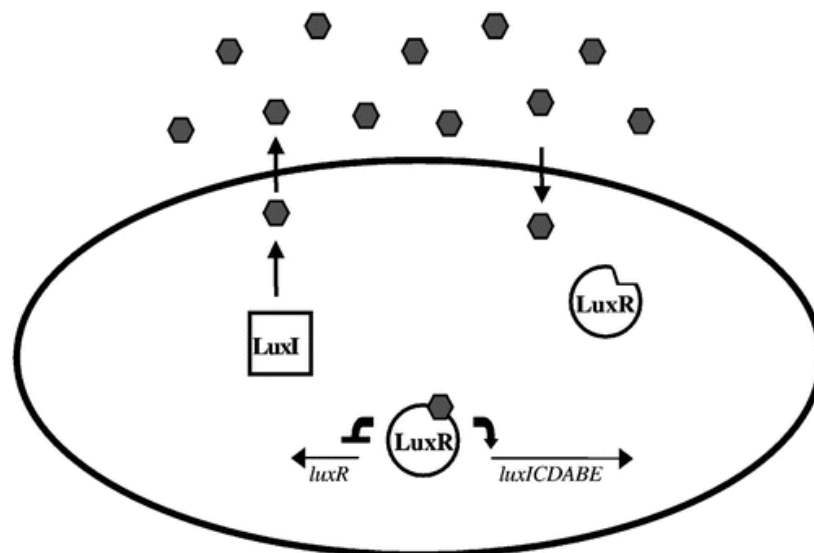


Figure 1.18 The *V. fischeri* LuxI/LuxR quorum sensing circuit (Schauder & Bassler, 2001). The oval represents a bacterial cell, and the hexagons represent the OHHL.

To date, QS resembling that first observed in *V. fischeri* have now been discovered in over 25 species of Gram-negative bacteria including *Agrobacterium tumefaciens*, *Chromobacterium violaceum*, *Erwinia caratovora*, the *B. cepacia* complex, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Aeromonas hydrophila* and *P. aeruginosa* (Camara *et al.*, 2002; de Kievit & Iglewski, 2000; Miller & Bassler, 2001).

Quorum Sensing System in P. aeruginosa

The QS in *P. aeruginosa* consists of two main pairs of LuxI/LuxR homologues termed LasI/LasR and RhII/RhIR. Similar to the LuxI, both LasI and RhII are the autoinducer

synthase enzymes, and the resulting complexes of both LasR-autoinducer and RhIR-autoinducer activate a transcription of various target genes including genes controlling virulence production and formation of biofilms (**Figure 1.19**) (Latifi *et al.*, 1996).

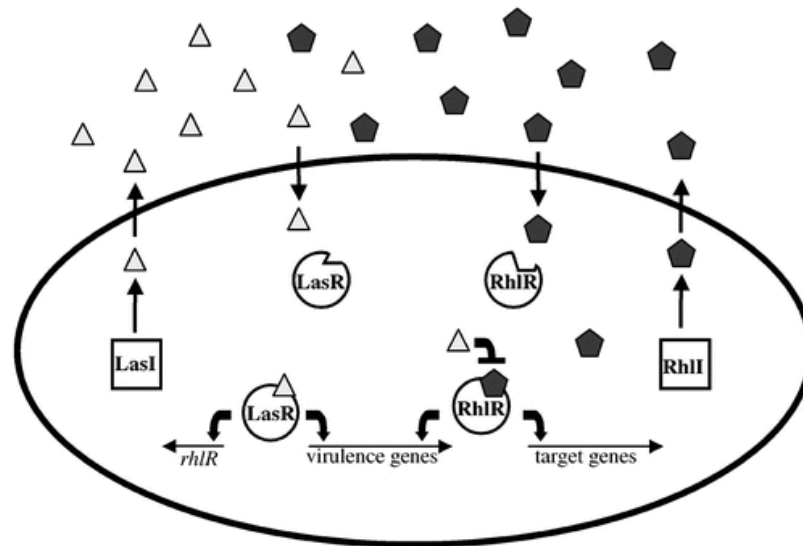


Figure 1.19 The *P. aeruginosa* LasI/LasR and RhII/RhIR quorum sensing systems (Schauder & Bassler, 2001). The oval represents a bacterial cell; the triangles represent the OdDHL, and the pentagons represent the BHL.

The LasI/LasR system was first discovered from a study of an elastase (*lasB*) expression regulation system in *P. aeruginosa* (Gambello & Iglewski, 1991). The expression of the *lasB* was found to be activated by the protein LasR which was also controlled by an open reading frame at the distal end of the *lasR* gene (Gambello & Iglewski, 1991). This open reading frame was found to have high similarity to the *luxI* gene of *V. fischeri* and its product also had a homologous structure to the autoinducer synthase enzyme (LuxI protein) (Passador *et al.*, 1993). On the basis of the similarities in structure and location of the genes, this open reading frame was renamed *lasI* (Passador *et al.*, 1993). The autoinducer synthesised by the LasI was subsequently identified as a homoserine lactone cognate called N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL or OdDHL) (Pearson *et al.*, 1994). The LasR-OdDHL complex was later found to control the expression of various virulence genes including *lasB*, *lasA*, *aprA* and *toxA* (encoding ETA) (Gambello *et al.*, 1993; Passador *et al.*, 1993).

The discovery of a second QS (RhlI/RhlR) has revealed the complexity of this system. By studying an elastase mutant and a mutant strain with multiple defects, Latifi *et al.* and Brint *et al.* found that despite adding the exogenous autoinducer OdDHL, only the elastase production was restored while alkaline protease, hemolysin and pyocyanin were still defective (Brint & Ohman, 1995; Latifi *et al.*, 1995). In addition, the gene *rhlR*, which was previously reported to control the production of rhamnolipid (Ochsner & Reiser, 1995), was found to play an important role in regulating the synthesis of those exoproducts. RhlR was subsequently identified to have a very similar structure with LasR and function in a similar fashion to the LasR and LuxR. The gene responsible for the production of autoinducer that binds to RhlR was then discovered and it has a high homology to the *lasI*; hence it was renamed as *rhlI*. The structure of this autoinducer was later identified as N-butyrylhomoserine lactone (C4-HSL or BHL) (Pearson *et al.*, 1995). On the basis of these data, it has been concluded that RhlI/RhlR is an additional QS system in *P. aeruginosa*.

The third QS system called the Pseudomonas Quinolone Signal (PQS) has been reported recently (Pesci *et al.*, 1999) and involves a compound, 2-heptyl-3-hydroxy-4-quinolone, which is not of the homoserine lactone class but a quinolone (Pesci *et al.*, 1999). PQS has been shown to be another component involved in the control and expression of *lasB* and *lasA* as well as acting as another regulation link between the first two QS circuits.

Figure 1.20 demonstrates the gene regulation of the QS system and the virulence factors expression in *P. aeruginosa*. Overall, LasI protein produces the signalling molecule OdDHL. At a concentration above the threshold level, OdDHL molecules bind to LasR. The activated LasR-OdDHL compounds then attach to promoter elements and stimulate transcription of many virulence genes. The LasR-OdDHL molecules also have a positive feedback effect by activating *lasI* expression as well as inducing the expression of *rhlR*, which encodes the transcriptional activator protein, RhlR. Subsequently, the RhlR binds to the BHL molecules, and the activated RhlR-BHL complexes then induce the expression of target genes including *lasB*, *aprA*, *rhlAB* and *rpoS* (encoding the stationary phase sigma factor). The OdDHL also interferes with binding of the BHL to RhlR. Presumably, this action ensures that the two systems initiate their cascades sequentially and in the appropriate order (Pesci *et al.*, 1997). The PQS is an additional regulatory link between the Las and Rhl circuits. LasR is required for the synthesis of PQS which in turn induces transcription of *rhlI*. The PQS partially controls the expression of the elastase genes *lasB* and *lasA* in conjunction with the Las and Rhl systems. In addition, at the top of the

hierarchy, Vfr protein was found to regulate the transcription of *lasR*, making it another crucial component of *P. aeruginosa* virulence (Albus *et al.*, 1997). Moreover, GacA was shown to control the transcription of both *lasR* and *rhIR* positively (Reimmann *et al.*, 1997), and the recently discovered RsaL was shown to be a negative regulator at the Las level (Rampioni *et al.*, 2006).

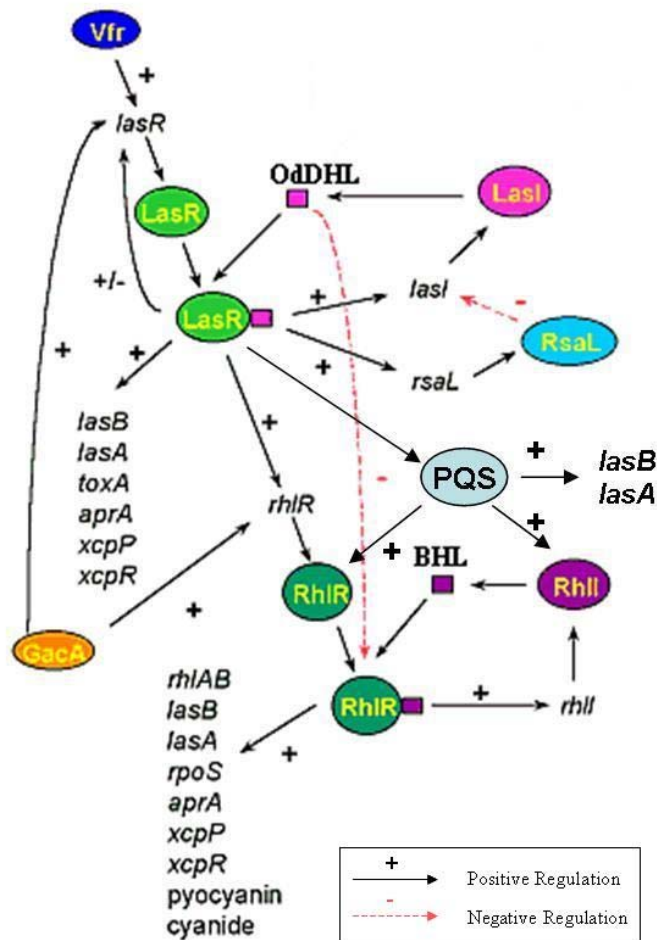


Figure 1.20 Schematic diagram of the QS system and the virulence factors regulation modified from (Latifi *et al.*, 1995).

Microarray analysis data has shown that more than 600 genes of a *P. aeruginosa* QS-mutant strain were significantly differentially expressed in response to the exogenous autoinducers (Schuster *et al.*, 2003; Wagner *et al.*, 2003). These genes were involved in the production of virulence factors, the development of biofilms, cellular responses to the environment and antibiotic resistance. The AHL molecules themselves also have biological

functions responsible for pathogenesis of this bacterium, including stimulating interleukin-8 production (DiMango *et al.*, 1995) and suppressing production of interleukin-12 and tumour necrosis factor (Telford *et al.*, 1998). The *las* and/or *rhl* mutants were shown to have markedly reduced virulence in various animal models (Tan *et al.*, 1999; Tang *et al.*, 1996; Wu *et al.*, 2001).

Given the significance of the QS in the pathogenesis of *P. aeruginosa* and other bacteria, strategies designed to interfere with these signalling systems are likely to have potential as biological controls (Hartman & Wise, 1998). Compounds such as furanones, which are isolated from marine algae *Delisea pulchra*, have been shown to inhibit the QS and thus enhance bacterial clearance from the leaves of this alga (Wu *et al.*, 2004). This field of research has future potential in combating *P. aeruginosa* infections especially in people with CF.

1.4.5 Biofilm Formation

Generally, bacterial studies are conducted by conventional methods in which bacteria are grown in liquid or broth culture before experiments are carried out. Bacteria grown by such methods are individually free-floating and/or free-swimming (planktonic) in a fluid environment. In nature and in most infectious diseases, however, bacteria, including *P. aeruginosa*, live in a “community” attached to surfaces, and this mode of existence is called “biofilms”. Biofilms, by definition, are a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Costerton *et al.*, 1999). Bacteria growing on biofilms have been increasingly recognised as an important cause of human diseases.

Bacteria thrive in the biofilm mode of growth in order to survive in a hostile environment (Donlan & Costerton, 2002). It has been suggested that an attachment to surfaces not only provides some degree of stability in the growth environment to the biofilms but may also affect physiological functions of the adherent cells (O'Toole & Kolter, 1998a). Moreover, biofilm formation offers protection from various environmental challenges ranging from heavy metal toxicity (Teitzel & Parsek, 2003) to host immune response (Leid *et al.*, 2002) and antimicrobial agents (Stewart & Costerton, 2001).

Biofilm Development

The visual characteristics of biofilms growing in diverse environments are markedly similar, indicating structural specialisation may contribute to this survival strategy (Hall-Stoodley *et al.*, 2004). *In situ* studies of biofilms revealed highly hydrated structures, composed of 73 to 98% extracellular materials and space, and bacteria growing as microcolonies interspersed with voids or water channels (Lawrence *et al.*, 1991). By analysis of gene and protein expression and microscopic images of *P. aeruginosa* biofilm during a developmental process, Sauer and colleagues proposed that the maturation of biofilms of *P. aeruginosa* involves five different stages, as depicted in **Figure 1.21** (Sauer *et al.*, 2002).

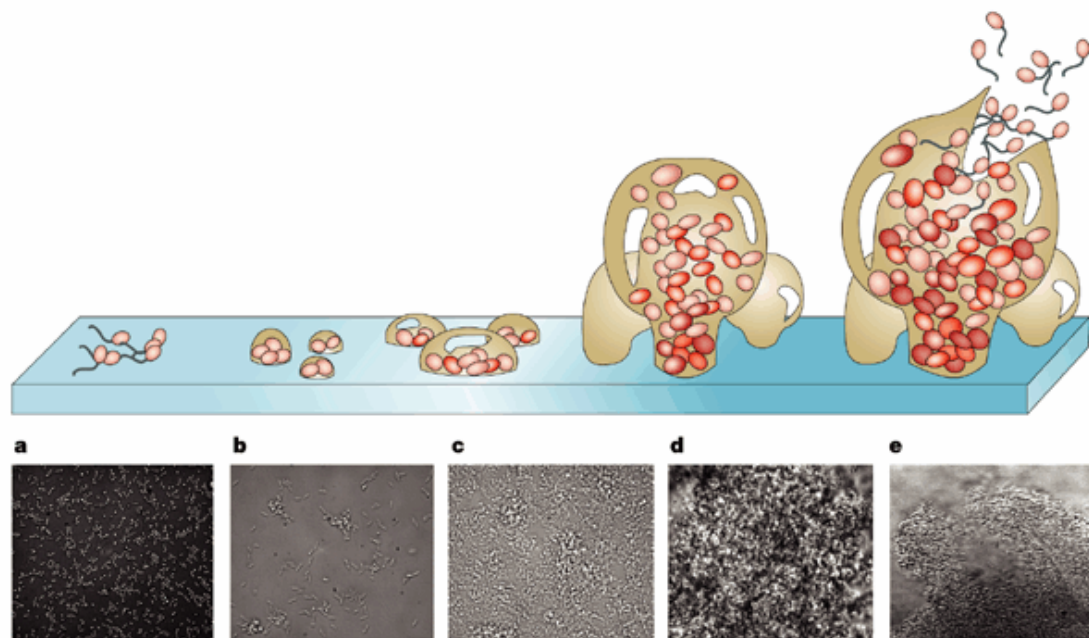


Figure 1.21 Schematic diagram showing five stages of biofilm development. Each stage is paired with an image of a developing *P. aeruginosa* biofilm (Sauer *et al.*, 2002).

(i) Reversible attachment (**Figure 1.21 a**)

This is an initial event in biofilm development; planktonic bacteria are attached to substratum at cell pole using flagella. *P. aeruginosa* non-motile mutants showed a significant decrease in an attachment efficacy compared to flagellated strains (O'Toole & Kolter, 1998a). In this stage, *P. aeruginosa* is transiently fixed to the substratum and is able to detach freely (Sauer *et al.*, 2002). It has been shown that this attachment phase is independent of the QS signal (de Kievit *et al.*, 2001).

(ii) Irreversible attachment (**Figure 1.21 b**)

Bacterial cells reorientate to the longitudinal cell axis, and the swimming motility ceases possibly due to the loss of flagella (Sauer *et al.*, 2002). *P. aeruginosa* cells then aggregate and form microcolonies. Type IV pili, which are responsible for the twitching motility (surface-associated motility), have been shown to have an important role in the aggregation of cells and the microcolony formation (O'Toole & Kolter, 1998a). There is evidence that after attachment, genes, including *algC*, *algD* and *algU*, are activated leading to the synthesis of extracellular matrix, which in *P. aeruginosa* is composed of alginate (also see Section 1.4.8) (Davies & Geesey, 1995). Activation of *lasB* was also shown to occur within 24 hours of the attachment (Sauer *et al.*, 2002).

(iii) Maturation-1 (**Figure 1.21 c**)

At this stage, approximately 540 genes were shown to be upregulated (Sauer *et al.*, 2002). Some of these genes were controlled by the QS system, indicating that additional regulatory systems might have roles in this phase (Sauer *et al.*, 2002). Proteins involved in anaerobic processes have been suggested to contribute during this maturation period (Hassett, 1996; Hassett *et al.*, 2002), implying that there is an oxygen limitation at least in some part of biofilms.

(iv) Maturation-2 (**Figure 1.21 d**)

Biofilms reach the maximum thickness during this stage of development. The biofilms in this phase have a mushroom-shaped multicellular structure, although the formation of such a structure might depend on nutrient sources (Klausen *et al.*, 2003a). Evidence indicates that the complex architecture of the mature biofilm is actively maintained (Davey *et al.*, 2003). Rhamnolipids, produced by cells within the biofilms, have been proposed as a maintenance factor for the open spaces or channels surrounding microcolonies (Davey *et al.*, 2003). Furthermore, it has been shown that the bacterial population within the biofilm during this stage exhibits different physiological activities (Xu *et al.*, 1998). This may correlate to the oxygen distribution within the mature biofilms which was shown to be depleted 30-fold at the centre of microcolonies (DeBeer *et al.*, 1994).

(v) Detachment (**Figure 1.21 e**)

As the biofilms develop, single cells or groups of cells can disperse out and may proceed to form new biofilms. The detachment can be caused by external perturbations, such as

shearing forces from flowing fluid (Stoodley *et al.*, 2002). However, Sauer *et al.* suggested that this could be an active process that the biofilm is programmed to do, allowing colonisation of new niches (Sauer *et al.*, 2002). It was observed that bacteria within microcolonies actively swim away from the interior part of cell clusters (Sauer *et al.*, 2002). It has been suggested that enzymes degrading the exopolysaccharide matrix might also play roles in the dispersion (Boyd & Chakrabarty, 1994).

***P. aeruginosa* Biofilms in the CF Lungs**

P. aeruginosa forms biofilms both in the natural environment and in diseases. Several studies have shown that this pathogen exists in the biofilm mode of growth in the lungs of CF patients (Koch & Hoiby, 1993; Lam *et al.*, 1980; Potts *et al.*, 1995; Singh *et al.*, 2000a). Direct examination by scanning electron microscope of CF sputum has confirmed the presence of biofilm-like structures of *P. aeruginosa* enclosed in exopolysaccharide matrix (**Figure 1.22**) (Singh *et al.*, 2000a). In addition, studies by Singh *et al.* detected the QS signals in CF sputum, corresponding with data from other studies of the roles of QS in the formation of biofilms (Davies *et al.*, 1998). Recently, it has been shown that mucin within the CF lungs can interact and promote *P. aeruginosa* to form the biofilms (Landry *et al.*, 2006).

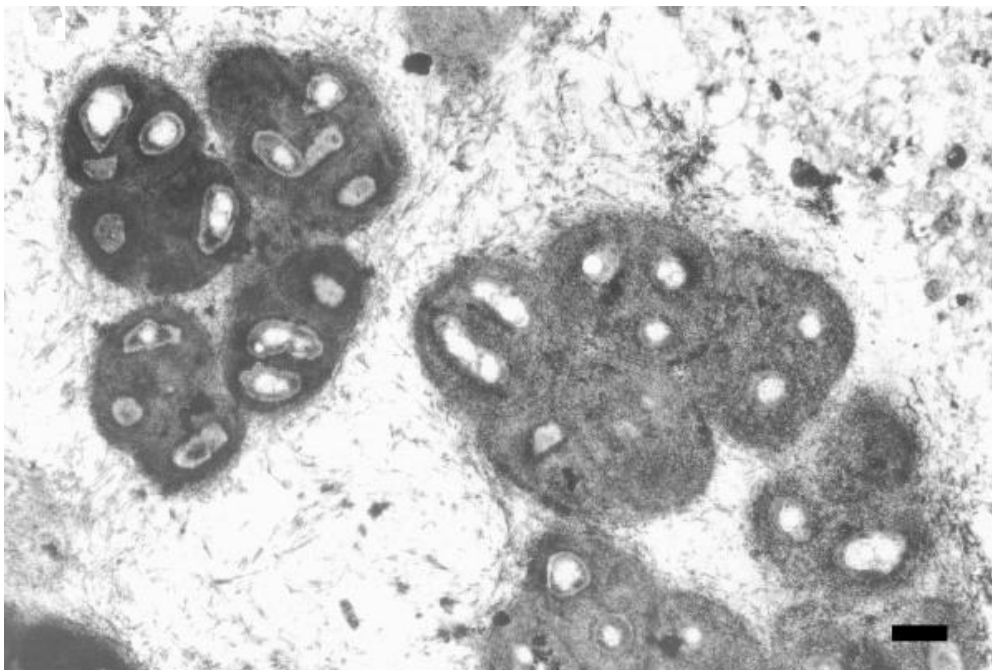


Figure 1.22 Scanning electron microscope image of *P. aeruginosa* biofilms in CF sputum. Bacterial cells (white rods) are surrounded by the exopolysaccharide matrix (dark). Scale bar, 1 μm . (Singh *et al.*, 2000a)

Bacteria grown in the biofilm mode have been shown to have increased resistance to antimicrobial agents compared to planktonic bacteria (up to 1,000-fold more resistant) (Davies, 2003). The failure of an agent to penetrate into the core of biofilms has been suggested as one of mechanisms responsible for this phenomenon, and the biofilm exopolysaccharide matrix might act as a barrier, reducing the amount of antimicrobial agent interacting with the cells inside (Campanac *et al.*, 2002; Davies, 2003). The exopolysaccharide matrix encasing the *P. aeruginosa* biofilms has also been shown to be able to protect the biofilms from the host immune response (Meluleni *et al.*, 1995). Ultimately, the formation of biofilms is an important reason for the persistence of *P. aeruginosa* in the CF lungs (see also Section 1.4.8).

1.4.6 Acquisition of *P. aeruginosa* in CF

Because *P. aeruginosa* lives in the water and soil, it can be found on vegetables and living plants as well as in water drains or wet surfaces. Individuals may contract the organism from such sources. Thus it is generally accepted that people with CF acquire this pathogen from their surrounding environment. Indeed, a study from Stuttgart, Germany detected *P. aeruginosa* in 73 of 102 (72%) households of people with CF – most frequently in the drains of showers, kitchen basins and bathrooms (Regnath *et al.*, 2004). However, this study did not demonstrate any association between contaminated households and patients' bacteriological status. In the natural environment, Green *et al* found that 24% of soil samples contained various strains of *P. aeruginosa*, and some strains were of a similar type to those isolated from clinical samples (Green *et al.*, 1974). Sprinkler watering was suggested as a contributory factor in spreading this pathogen (Green *et al.*, 1974). Other environmental sources where evidence suggests that people with CF may encounter *P. aeruginosa* include fresh water lakes (van Asperen *et al.*, 1995), domestic pets such as dogs (Petersen *et al.*, 2002) and hot tubs or hydrotherapy pools (Gregory & Schaffner, 1987; Moore *et al.*, 2002). Swimming pools are generally safe provided the recommended programme of maintenance is carried out; however, reports of *P. aeruginosa* contamination was not unheard of (Barben *et al.*, 2005). In fact, it was persistent contamination of *P. aeruginosa* that forced the City of Sydney to close the 50-metre pool at Cook and Phillip Park; although, there were no reported cases of illnesses associated with this incidence (Munro, 2007).

Food and drinking water can also be a source of *P. aeruginosa*. Several studies have reported *P. aeruginosa* contamination in food or even distilled water from hospitals (Favero *et al.*, 1971; Hardalo & Edberg, 1997; Kominos *et al.*, 1972; Shooter *et al.*, 1971; Wright *et al.*, 1976). One study recovered *P. aeruginosa* from 82% of tomatoes (Kominos *et al.*, 1972). As for drinking water, it is generally safe to drink tap water. One study showed that none of the running water specimens from water taps were infected (Raj, 2005). However, water in large dispensers (coolers) was shown to be contaminated in up to 25% of samples (Baumgartner & Grand, 2006). Commercial bottled water was also reported as a source of *P. aeruginosa* contamination (Wilkinson & Kerr, 1998).

Other possible sources of *P. aeruginosa* include dental equipment (Jensen *et al.*, 1997; O'Donnell *et al.*, 2005): it was found in 3% of water samples from dental equipment (Jensen *et al.*, 1997). Nevertheless the risk of acquisition through this route is considered low.

In conclusion, it is likely that people with CF acquire *P. aeruginosa* from the environment. However, the risk of acquiring this organism differs in various settings. The risk of acquisition from other individuals or other CF patients will be reviewed in Section 1.4.11.

1.4.7 Adhesion of *P. aeruginosa*

After entering the lungs, bacteria must adhere to hosts in order to establish a successful colonisation and resist innate host defences. In healthy individuals, bacteria entering the lung are removed by the mucociliary clearance (MCC) as described in Section 1.2.1. In CF lungs, however, the MCC is impaired, and it has been shown that *P. aeruginosa* can readily adhere to CF mucin (Nelson *et al.*, 1990). This bacterium-mucin adherence was also found to be species-specific as only *P. aeruginosa* (but neither *E. coli* or *K. aerogenes*) showed this interaction (Vishwanath & Ramphal, 1984). The flagellar cap protein, FliD, was shown to be responsible for the adherence to mucin on the CF respiratory tract surface (Arora *et al.*, 1998) (Section 1.4.2.1.2). In addition to the mucin attachment, *P. aeruginosa* also possesses other cell components aiding the adherent process. These components include (i) LPS, in which the O-side chain binds to the CFTR (Pier *et al.*, 1997) (Section 1.4.2.1.1); (ii) flagellin (encoded by *fliC*) which binds to epithelial cell receptors such as GM1 and asialoGM1 (Feldman *et al.*, 1998) (Section 1.4.2.1.2); and (iii) pili which also adhere to the asialoGM1 receptors (Saiman & Prince,

1993) (Section 1.4.2.1.3). The adherence between *P. aeruginosa* and host mucosal surface or host cells is important in bacterial colonisation and subsequent infection.

1.4.8 Early or Transient Infection with *P. aeruginosa*

With the recent introduction of a bronchoalveolar lavage (BAL) technique which assists in obtaining specimens for culture from the lower airway in young children with CF, who usually cannot expectorate sputum, acquisition and colonisation of *P. aeruginosa* were found to occur as early as in the first year of life (Armstrong *et al.*, 1996; Armstrong *et al.*, 1997; Rosenfeld *et al.*, 2001). Longitudinal studies following the status of *P. aeruginosa* infection in young CF children also found that infected children had some intermittent periods in which bacteria were undetectable (Burns *et al.*, 2001), suggesting either that CF hosts could eradicate the organisms or that some *P. aeruginosa* strains established a transient rather than chronic infection. Moreover, genotypic studies demonstrated that such strains from distinct infecting events in the same CF subjects were not a single specific clone; infection developed and was subsequently cleared, and the next infection was caused by a new strain (Burns *et al.*, 2001). These transient-infection strains also had non-mucoid and antibiotic susceptible phenotypes, which were distinct from *P. aeruginosa* isolated from chronic infected CF subjects (Burns *et al.*, 2001). This transient or early infection period is thus the optimal time for successful eradication of *P. aeruginosa* which then may preserve lung function and may delay onset of the chronic infection (Frederiksen *et al.*, 1997; Ratjen *et al.*, 2001; Valerius *et al.*, 1991). Unfortunately, this stage of early infection is temporary and, without treatment, the pathogen often persists and eventually becomes a chronic infection.

1.4.9 Chronic *P. aeruginosa* Infection

Once *P. aeruginosa* establishes a chronic infection in the lungs, the same bacterial lineage persists continuously for years and cannot be eradicated by any known therapy. The most characteristic feature of chronic *P. aeruginosa* infection in the CF lungs is the conversion from non-mucoid to mucoid phenotype and the formation of biofilms (Govan & Deretic, 1996). The mucoid character is a result of production of mucoid exopolysaccharide called “alginate” (Govan & Deretic, 1996), and it has been shown that the level of alginate production was clinically correlated to poor prognosis in people with CF (Pedersen *et al.*, 1992). Recently, it was shown that thick mucous plaques within the CF airways, which provided an anaerobic niche, induced the alginate production in which *P. aeruginosa* cells

were embedded (**Figure 1.23**) (Worlitzsch *et al.*, 2002). Motile *P. aeruginosa* (especially strains from the environment) can penetrate into the thickened mucus plaques and evade host neutrophils and macrophages (**Figure 1.23 d-f**), which appear unable to penetrate the thickened mucus. *P. aeruginosa* is also able to adapt well to mucus hypoxia – it can grow in hypoxic environments, using the nitrate within ASL as a terminal electron acceptor (Worlitzsch *et al.*, 2002). Recently, it has been demonstrated that *P. aeruginosa* also employed microaerobic respiration in concert with anaerobic nitrate respiration in chronically infected CF lungs (Alvarez-Ortega & Harwood, 2007). Importantly, the bacteria exhibit adaptations by producing alginate and forming biofilms (Hassett, 1996; Worlitzsch *et al.*, 2002). Together, the alginate production and the formation of biofilms within the anaerobic milieu lead to resistance to host defence mechanisms and to antibiotics, and ultimately contribute to the chronic persistence of *P. aeruginosa* within the CF lungs (Hassett *et al.*, 2002; Worlitzsch *et al.*, 2002; Yoon *et al.*, 2002).

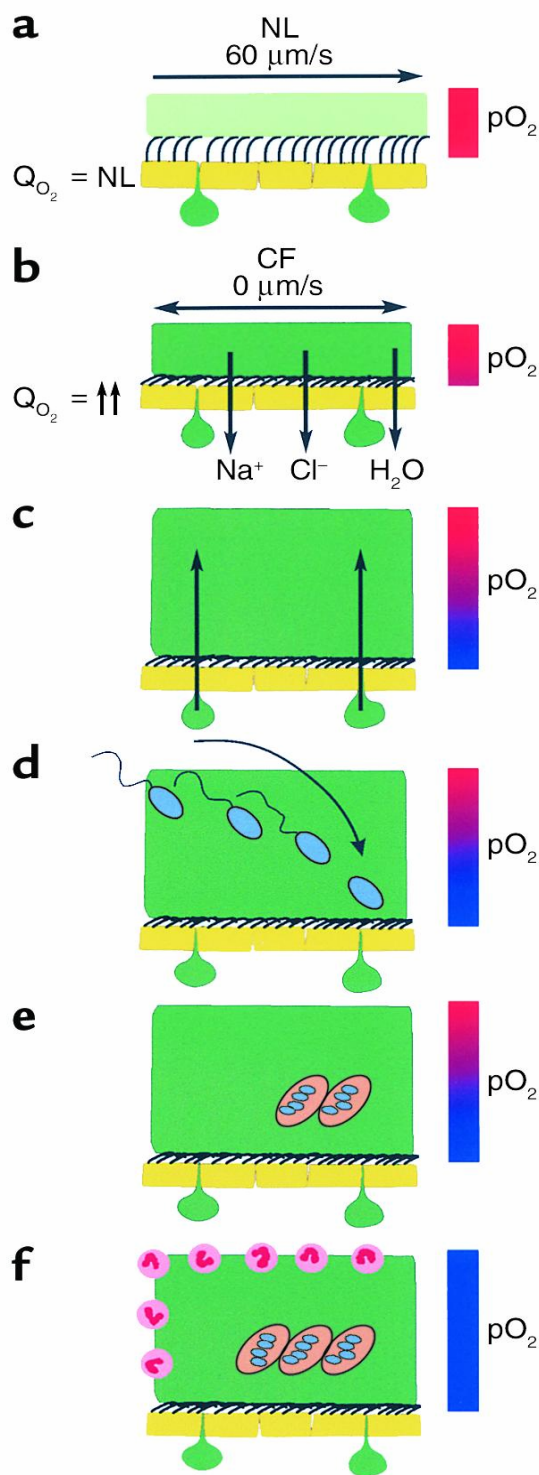


Figure 1.23 Schematic model describing the series of steps that are thought to possibly lead to persistent chronic *P. aeruginosa* infection in airways of CF patients. **(a)** On normal airway epithelia, a thin mucus layer resides atop the PCL. A normal rate of epithelial O_2 consumption (Q_{O_2}) produces no O_2 gradients within this thin ASL (denoted by red bar). NL means normal level. **(b–f)** CF airway epithelia. **(b)** PCL depletion, mucus adherent to epithelial surfaces, and increased Q_{O_2} . **(c)** Persistent mucus hypersecretion from mucus secretory glands with time increases the height of luminal mucus masses. The raised CF epithelial Q_{O_2} generates steep hypoxic gradients (blue colour in bar) in thickened mucus masses. **(d)** *P. aeruginosa* deposited on mucus surfaces penetrate into hypoxic zones within the mucus masses. **(e)** *P. aeruginosa* adapts to hypoxic niches with increased alginate formation and the creation of biofilms. **(f)** Biofilms (alginate embedded cells) resist host defences (i.e. neutrophils), setting the stage for chronic infection. The presence of increased bacterial density renders the now mucopurulent mass hypoxic (blue bar). Adapted from Worlitzsch *et al.*

(2002).

1.4.9.1 Mucoïd Conversion of *P. aeruginosa* Isolated from Chronic Lung Infection in People with CF

P. aeruginosa isolated from environments (wild type) or from patients with non-CF diseases or even from early CF lung infection typically appears to be flat or non-mucoïd (**Figure 1.24**). In contrast, most strains (nearly 85 – 90%) recovered from chronically infected CF lungs exhibit the mucoïd morphology (**Figure 1.24**) (Govan & Deretic, 1996).

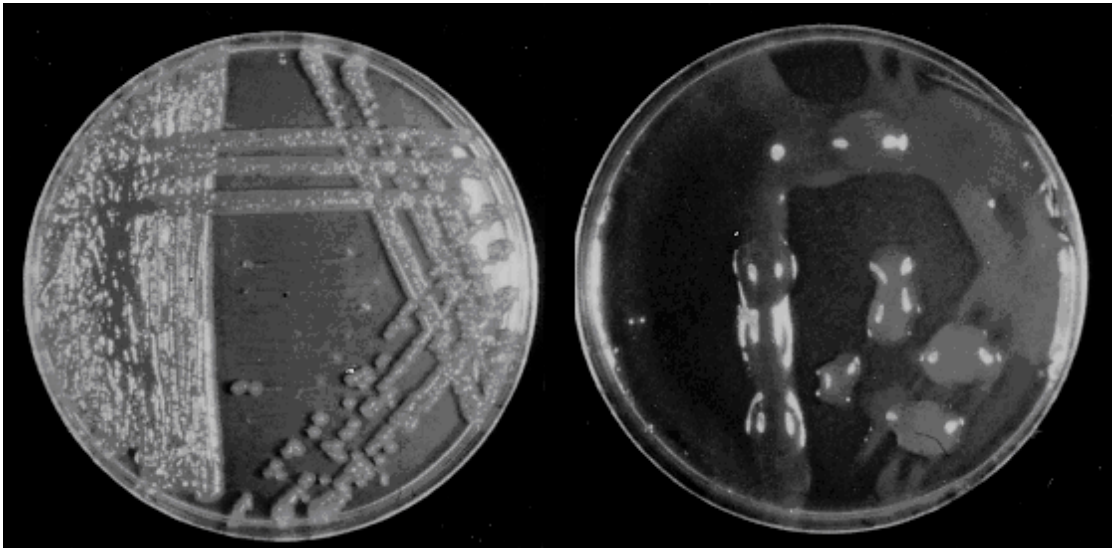


Figure 1.24 Phenotypic difference between non-mucoïd or flat colony (left) and mucoïd colony (right). (Govan & Deretic, 1996)

It is believed that the mucoïd strain of *P. aeruginosa* is transformed from the non-mucoïd strain that entered into the lungs at an early stage because it was shown by a molecular genotyping technique that the mucoïd isolates were genotypically similar to the initially-acquired isolates (Romling *et al.*, 1994). However, there is no clearly defined interval between the non-mucoïd colonisation and transition to mucoïd forms.

The genetic regulation of mucoïd or alginate production has been intensively investigated over the past decades. The mucoïd production is controlled by transcriptional regulation of the GDP-mannose dehydrogenase (an alginate biosynthetic enzyme), encoded by *algD* (Chitnis & Ohman, 1993). This *algD* gene is under the control of a complex regulatory mechanism, and one major regulator is thought to be the *algU mucABCD* cluster (Martin *et al.*, 1993). AlgU, also known as sigma factor 22 (σ^{22}), positively regulates the *algD* gene while *mucA*, *mucB*, *mucC* and *mucD* act as negative regulators of AlgU (Boucher *et al.*, 1997; Deretic *et al.*, 1993).

As shown in **Figure 1.23**, the alginate production is related to the growth of bacteria in an anaerobic condition. Hassett discovered mucoid strains reverted to non-mucoid after being grown in culturing medium under aerobic conditions (Hassett, 1996). This, however, did not happen when the bacteria were grown anaerobically (Hassett, 1996). It was postulated that an inner membrane-bound oxygen sensor may be responsible for this process (Taylor *et al.*, 1999). Other factors that may induce *P. aeruginosa* to produce alginate are limitations in nutrients, phosphate or iron (Boyce & Miller, 1982; Terry *et al.*, 1992). Interestingly, Mathee *et al.* demonstrated that hydrogen peroxide produced by polymorphonuclear leukocytes (PMNs) within the CF airways can induce a mutation in *mucA* resulting in conversion to the mucoid phenotype of *P. aeruginosa* (Mathee *et al.*, 1999). In addition, it has been shown that high osmolarity can induce alginate synthesis through an *algD* transcription (Berry *et al.*, 1989).

Pathogenic Roles of Alginate

Pedersen *et al.* clearly showed that the clinical course of CF patients was significantly worsened as alginate-producing *P. aeruginosa* emerge in the airways (Pedersen *et al.*, 1992). It was found that both direct and opsonin-mediated phagocytosis was inhibited due to the alginate capsule surrounding bacterial colonies (Oliver & Weir, 1985). Alginate may also have an immunomodulation role – it can suppress lymphocyte functions (Mai *et al.*, 1993). Importantly, alginate is known to be responsible for the biofilm growth of *P. aeruginosa*, which is highly resistant to both host immune defences and exogenous antibiotics (Davies, 2003).

1.4.10 Transmission of *P. aeruginosa* between People with CF

While person-to-person transmission of the Bcc has been well documented (Govan *et al.*, 1993; LiPuma *et al.*, 1990), as described in Section 1.3.3, it was thought until recently that people with CF acquired *P. aeruginosa* from their natural environment (as previously described in Section 1.4.5). It was generally accepted that people with CF harbour individual unrelated strains of *P. aeruginosa* which are uniquely different from those of other infected CF patients. Cross infection of *P. aeruginosa* among CF individuals was considered uncommon (Govan & Deretic, 1996; Govan, 2000; Pollack, 1995). Sharing of the same strain of *P. aeruginosa* between patients had been reported only from CF siblings or in cases of close contact (Grothues *et al.*, 1988; Kelly *et al.*, 1982). More recently

however several studies have reported the transmissibility of *P. aeruginosa* among unrelated CF patients. The first study of this kind dates back to 1980 in Denmark when Hoiby and Rosendal reported one strain infecting 22% of the patients in their CF centre (Hoiby & Rosendal, 1980). This strain also appeared to colonise patients' airways and replaced another strain of *P. aeruginosa* after treatment. Following further extensive epidemiological investigations, this reported strain was subsequently isolated from sinks, soap, baths, tables, brushes and air in the same CF centre suggesting that environmental contamination possibly caused the cross-infection rather than transmission from person to person (Zimakoff *et al.*, 1983).

1.4.10.1 Transmission between CF Individuals Who Are Siblings

Studies by Kelly *et al.* provided more convincing evidence that *P. aeruginosa* could be transmitted between people with CF; they divided patients into three groups according to their degree of contact and found that each of six pairs of CF siblings (the close-contact group) shared the same strain of *P. aeruginosa*. In contrast, the low-contact and medium-contact groups showed no evidence of strain sharing among the patients (Kelly *et al.*, 1982). A similar study using serotyping and antibiotic susceptibility patterns revealed that 20 of 24 sibling pairs had at least one identical serotype in common, and of the four pairs that had no shared serotype, two did not live together (Thomassen *et al.*, 1985a). Speert and Campbell also demonstrated that three of four sibling pairs shared the same serotype although this occurred transiently and rarely among unrelated CF patients (Speert & Campbell, 1987). By using both genotyping and pyocin typing, Grothues *et al.* analysed 22 CF siblings from eight families and 72 unrelated CF patients and demonstrated that CF siblings harboured either identical or closely related strains while all unrelated CF patients were colonised with different strains (Grothues *et al.*, 1988).

Overall, accumulating evidence has shown that CF individuals who are siblings are likely to share common strain(s), however, whether this is due to the person-to-person cross infection or the acquisition from the same environment source still remains unknown.

1.4.10.2 Transmission of *P. aeruginosa* in Recreational CF Camps

Following the report of transmission of Bcc occurring among patients attending a CF camp (LiPuma *et al.*, 1990), several investigations of the possibility of *P. aeruginosa* cross

infection have been conducted in CF camps. In these settings, people with CF spend a certain period of time (usually about 1-2 weeks) all together in the same environment, staying in the same hotels and participating in the same daytime activities. Using a serotyping technique, Speert *et al.* found that there was a low risk of acquiring *P. aeruginosa* in CF children who were free of this organism from other children with CF; however, whether or not *P. aeruginosa* strains were spread among CF children who were already colonised was inconclusive (Speert *et al.*, 1982). Applying both serotyping and genotyping methods, Hoogkamp-Korstanje and colleagues discovered that the incidence of becoming colonised with *P. aeruginosa* in previously negative children was about 7.7%. However, after two months of follow up, the prevalence of permanent colonisation was only 1.9%, which was not substantially different from the risk of becoming colonised and infected in the community (Hoogkamp-Korstanje *et al.*, 1995). They therefore concluded that the risk of cross-infection among the CF campers was outweighed by the benefits of the camp to participants. Two similar epidemiology studies from an 8-day camp in Ohio, USA (Thomassen *et al.*, 1985a) and from a 3-week camp in Israel (Greenberg *et al.*, 2004) also reported that none of the *P. aeruginosa*-negative patients became colonised with *P. aeruginosa* during the camp attendance. In contrast to those studies, a 100%-cross-colonisation rate was reported among five CF patients who were free of *P. aeruginosa* prior to attending a one-week CF camp in Spain (Ojeniyi *et al.*, 2000). These five patients subsequently developed a chronic colonisation lasting six months after the initial isolation, and all of their *P. aeruginosa* isolates appeared to be the same genotype. This same strain was also found in two previously infected patients who also attended the same camp (Ojeniyi *et al.*, 2000). In addition to the findings by Ojeniyi and coworkers, a study from Norway examined *P. aeruginosa* isolates from 60 patients and found a large cluster of the same strain shared by 27 patients who were more likely to have attended the summer camp but not hospitals (Fluge *et al.*, 2001). A prospective study conducted in four different CF camps in The Netherlands also recently reported that 21 of 80 participant children may possibly acquire *P. aeruginosa* during camp attendance (Brimicombe *et al.*, 2008), adding more evidence supporting the risk of *P. aeruginosa* transmission in this setting.

Summer camps for people with CF (especially children) have existed for decades. The clear benefits of attending CF camps include improved pulmonary functions, physical endurance and nutritional status as well as the provision of psychological support (Blau *et al.*, 2002). Nonetheless, the actual risk-benefit of such camps still needs to be carefully evaluated.

1.4.10.3 Transmission of *P. aeruginosa* within Clinical Settings

Several researchers from different CF centres around the world have investigated the transmissibility of *P. aeruginosa* in the hospital environment. Not all studies have revealed evidence of *P. aeruginosa* cross infection in CF centres. Early studies by a Danish CF group using serotyping and bacteriophage typing indicated that cross infection occurred (Hoiby & Rosendal, 1980; Zimakoff *et al.*, 1983) while Speert and colleagues using the same techniques failed to detect the cross colonisation of *P. aeruginosa* (Speert & Campbell, 1987). However, the serotyping technique has been subsequently shown to be an unsuitable technique for typing *P. aeruginosa* strains isolated from CF patients, and this was due to polyagglutinated and nonagglutinated strains which were observed among the great majority of *P. aeruginosa* isolates (Hancock *et al.*, 1983).

The DNA-based genomic typing techniques, such as random amplification of polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) of macrorestriction DNA using pulsed-field gel electrophoresis (PFGE) analysis, were introduced in the 1990s as tools for epidemiology investigations. Since then, there have been several studies applying these molecular methods to investigate the possibility of *P. aeruginosa* transmission. One of the most significant studies was a report by Cheng and colleagues from the CF clinic at the Alder Hey Children's hospital at Liverpool in the United Kingdom (Cheng *et al.*, 1996). They found a very high proportion (70.6%) of the patients harboured a ceftazidime-resistant clone of *P. aeruginosa* whereas a previous surveillance in the same clinic (Dalzell *et al.*, 1991) only detected a prevalence of 14%. The investigators were alerted to the possibility of person-to-person transmission especially because some patients infected with the ceftazidime-resistant strain had never received this agent for treatment. Using PFGE, it was shown that 85% of patients who harboured the β -lactam-resistant *P. aeruginosa* were infected with an identical strain. Further investigations failed to detect this strain in the ward or hospital environment. They concluded that the selection of an antibiotic resistant strain and the following person-to-person transmission were most likely responsible for this outbreak (Cheng *et al.*, 1996); the epidemic strain was later called Alder Hey strain (strain AH) or Liverpool strain. This study has raised concern over the possibility of person-to-person *P. aeruginosa* transmission particularly when the strain is multiresistant.

Studies by Jones *et al.* also reported similar findings at the adult CF centre at the Wythenshawe Hospital in Manchester, United Kingdom (Jones *et al.*, 2001). In this

prospective surveillance of 154 CF adults, 22 (14%) harboured the same strain of multidrug-resistant *P. aeruginosa* which was genomically distinct from the Alder Hey epidemic strain (**Figure 1.25**). Moreover this epidemic strain, subsequently called Manchester strain, was not isolated from inpatient or outpatient environments or from non-CF patients who were infected by *P. aeruginosa*, suggesting no common hospital environmental sources were implicated in this outbreak. Furthermore, of 24 patients who were coinfecting with *P. aeruginosa* and the Bcc and had been segregated from the main group of patients for 8 years, none had this epidemic strain. This suggested that patient segregation, designed primarily to prevent Bcc cross infection, may have prevented the acquisition of *P. aeruginosa* epidemic strain via person-to-person route. This finding of the possibility of the person-to-person transmission of *P. aeruginosa* highlighted the importance of microbiological surveillance as well as the significance of segregation.

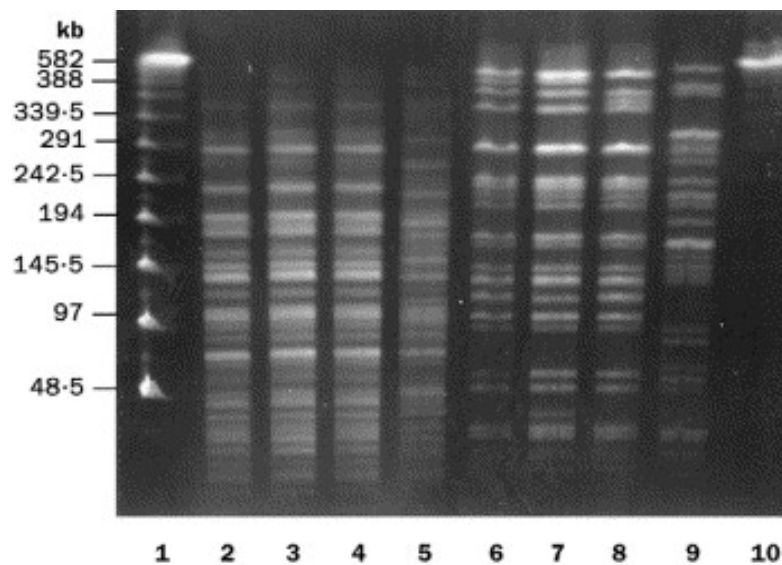


Figure 1.25 PFGE-generated DNA banding pattern comparing the Liverpool epidemic strain and the Manchester epidemic strain (Jones *et al.*, 2001).

Lanes 1 and 10 are molecular weight marker; lanes 2 – 4 are Manchester strain (digested by *Xba*I); lane 5 is Liverpool strain (digested by *Xba*I); lanes 6 – 8 are Manchester strain (digested by *Spe*I); and lane 9 is Liverpool strain (digested by *Spe*I).

Other studies have also reported the *P. aeruginosa* cross infection in both adults and children with CF (Denton *et al.*, 2002; Edenborough *et al.*, 2004; McCallum *et al.*, 2001). Due to the accumulating reports of *P. aeruginosa* transmissibility, a nationwide survey was

conducted to determine the extent of the problem among individuals with CF in England and Wales (Scott & Pitt, 2004). A total of 1,250 *P. aeruginosa* isolates from 31 CF centres throughout England and Wales were genotyped using PFGE. Findings showed that 93 isolates from 15 centres clustered with the Alder Hey or Liverpool epidemic strain and 11 isolates from three centres clustered with the Manchester epidemic strain. Novel epidemic strains were also identified. The survey confirmed the incidence of cross infection within and between CF centres.

Whilst several studies have provided evidence to support the view that *P. aeruginosa* is transmitted person to person, many investigators have failed to detect transmission of *P. aeruginosa* among CF patients. Studies by Speert and colleagues demonstrated that only prolonged close contact led to strain sharing (Speert *et al.*, 2002). They evaluated at least three isolates (first, midpoint and most recent isolates during the course of infection) from each of 174 CF individuals and found a total of 157 distinct types of *P. aeruginosa*, using the genotypic techniques (both PFGE and RAPD). Of these, 123 types were unique to a single patient whereas the remaining 34 types were shared by two or more patients. Of the 34 types, seven were unique to a single sibling pair; three were shared by one or more sibling pairs and with other unrelated patients with CF; and 24 were shared by unrelated CF individuals. Two dominant strains were recovered from 21 patients and 18 patients respectively. With a team of physicians, nurses, social workers and dieticians, the extensive epidemiologic investigations (including patients' domicile location, any contact between patients, social interactions of patients both within and outside the hospital, and date of acquisition) were applied to the patients who were infected with the same strain. The findings showed that besides siblings, only two patients, who had the same strain, had strong evidence of social contact both in and out of the hospital. The findings also demonstrated that the majority of patients who had the two dominant strains were infected transiently. Moreover, there was no obvious geographic clustering to support patient-to-patient spread outside the hospital or to support acquisition of the two dominant types from the common place. From these data, the investigators concluded that the risk of acquiring *P. aeruginosa* from other patients was very small and that prolonged close social contact was necessary for patient-to-patient transmission.

Similarly, surveillance studies from Germany and from France also reported the low incidence of cross infection among CF patients (Boukadida *et al.*, 1993; Spencker *et al.*, 2000). Mahenthalingam *et al.* found only one instance of strain sharing among 385

sequential isolates from 20 CF patients (Mahenthiralingam *et al.*, 1996). Another investigation performed in Sao Paulo in Brazil also showed that the cross colonisation was rare among the patients attending the outpatient clinic (da Silva Filho *et al.*, 2001).

Recently, Jelsbak *et al.* used the single nucleotide polymorphism typing technique to study *P. aeruginosa* isolates from 22 patients over a period of more than 30 years follow up, and found that there were two common strains, called strain “r” and strain “b”, transmitted between patients (Jelsbak *et al.*, 2007), adding more evidence supporting the emergence of *P. aeruginosa* transmissible strains.

1.4.10.4 Clonal *P. aeruginosa* Transmission in Australia

In Australia, the presence of so called epidemic or clonal strains of *P. aeruginosa* has been reported from CF centres along the Australian east coast. In 2002, the CF clinic at the Royal Children’s Hospital in Melbourne first detected a clonal strain of *P. aeruginosa* among children with CF in 2002 (Armstrong *et al.*, 2002). Alerted by the unexpected deaths from severe lung disease of five unrelated cohort children under the age of five, Armstrong and colleagues initiated a cross-sectional survey of 326 children attending their clinic. Of 118 children who harboured *P. aeruginosa*, 65 (55%) children shared an indistinguishable or closely related strain. Twelve *P. aeruginosa* isolates from environmental sources including inpatient wards, physiotherapy room and pulmonary function laboratory were genotypically distinct and none were related to the epidemic strain. Common source of infection outside the hospital was also unlikely since patients were unrelated and lived in geographically diverse areas. This *P. aeruginosa* strain (or the “Melbourne epidemic strain” as named by the authors) appeared to be resistant to commonly used antibiotics more than other strains. The study also showed that children infected by the Melbourne clonal strain spent more days in hospital due to respiratory exacerbations compared to children with unrelated strains. Findings from this study have raised awareness of the possibility of *P. aeruginosa* cross infection across Australia, and the authors urged other CF clinics to consider molecular epidemiological surveillance.

Later in 2002, molecular screening of *P. aeruginosa* isolates from patients attending the adult CF clinic at the Royal Prince Alfred Hospital (RPAH) in Sydney also revealed the emergence of a dominant strain of *P. aeruginosa* among adult patients (Anthony *et al.*, 2002). By using PFGE to type 50 *P. aeruginosa* isolates from 18 patients, Anthony *et al.*

found that 10 (56%) patients were infected by an identical or closely related strain (Anthony *et al.*, 2002). Following the collaboration between the CF centres at RCH and at RPAH, the dominant strains from both clinics were exchanged and subsequently identified using PFGE which revealed that both strains were indistinguishable (Armstrong *et al.*, 2003). Subsequently, eight (five adults, three paediatric patients) of 100 patients at the Prince Charles Hospital in Brisbane and at Royal Children's Hospital in Brisbane were also found to harbour this dominant strain (Armstrong *et al.*, 2003). This strain was then called Pulsotype-I or Australian epidemic strain-1 (AES-1). Further studies carried out at Brisbane CF clinics, i.e. the Royal Children's Hospital and the Prince Charles Hospital, identified another dominant strain (O'Carroll *et al.*, 2004). *P. aeruginosa* isolates from 50 children and 50 adults with CF were analysed using PFGE; 39 patients (25 children, 14 adults) shared a common strain called Pulsotype II (or AES-2). Interestingly, eight of the 14 adult patients had received their paediatric care at the Royal Children's Hospital, suggesting the transfer of the strain from the childrens' clinic to the adult clinic. However this could not explain the colonisation in the remaining six adults who had never attended the paediatric clinic at the Royal Children's Hospital. The transmission of the common strains was shown to spread within and possibly between these two clinics. Yet again, acquisition from common environmental sources still remained one of the possible explanations.

Table 1.6 summarises all published investigations (from 1980 – present) of *P. aeruginosa* transmission. The data tend to support the view that there is a strain variation in transmission potential of *P. aeruginosa*, and that some strains are more easily transmissible from patient to patient. Previous evidence also showed that the incidence of acquisition and cross infection of *P. aeruginosa* could be reduced by patient segregation. While the routes of transmission are not fully understood, infected respiratory secretions are likely to be the primary source of contamination, which may contaminate the health care environment and serve as a potential reservoir for *P. aeruginosa* or spread directly from person to another person via air.

Table 1.6 Summary of published data from 1980 – present investigating the transmission of *P. aeruginosa* among people with CF

Year	Location and Country	Technique	Samples and Population	Results	Authors
1980	Copenhagen, DNK	Serotype, Phage type	45 pt. (484 isolates)	10 pt. shared same strain	Hoiby & Rosendal
1982	British Columbia, CAN	Serotype	18 pt.	No cross infection	Speert <i>et al.</i>
1982	Dublin, IRL	Serotype, Pyocin type	116 pt. (3 groups)	No cross infection except in sibling group	Kelly <i>et al.</i>
1983	Copenhagen, DNK	Serotype, Phage type	115 env. isolates	sink, soap, baths and air had same strain	Zimakoff <i>et al.</i>
1985	Ohio, USA	Antibiogram, Serotype	47 pt. (CF campers)	No cross infection	Thomassen <i>et al.</i>
1987	British Columbia, CAN	Serotype	91 pt.	No cross infection, Negative env. culture	Speert & Campbell
1988	Hannover, GER	FIGE	72 pt. and 22 siblings.	No cross infection except in siblings	Grouthes <i>et al.</i>
1993	Brussels, BEL	PFGE, Antibiogram	31 pt. (166 isolates)	Clonal strains exist, 28% infected	Struelens <i>et al.</i>
1993	Paris, FRA	PFGE	21 pt. (187 isolates)	Low risk of cross infection	Boukadida <i>et al.</i>
1995	Utrecht, NLD	Phage type, RAPD	18 pt. (91 isolates)	Low risk of cross infection	Hoogkamp-Korstanje <i>et al.</i>
1996	Liverpool, GBR	PFGE	65 pt. (92 isolates)	55 pt. shared same strain (Liverpool strain)	Cheng <i>et al.</i>
2000	Leipzig, GER	PFGE	30 pt. (469 isolates)	Low risk of cross infection	Spencer <i>et al.</i>
2000	Copenhagen, DNK	PFGE	22 pt. (CF campers)	100% cross infection to previous-PA-negative pt.	Ojeniyi <i>et al.</i>
2001	Liverpool, GBR	PFGE	4 cases reported	Superinfection by Liverpool strain	McCallum <i>et al.</i>
2001	Manchester, GBR	PFGE	154 CF pt. and 52 non-CF pt.	22 pt. shared same strain (Manchester strain)	Jones <i>et al.</i>
2001	Sao Paulo, BRA	RAPD	86 pt. and env. isolates	No cross infection, Negative env. culture	da Silva Filho <i>et al.</i>
2001	Oslo, NOR	PFGE	60 pt.	27 pt. shared same strain	Fluge <i>et al.</i>
2002	Melbourne, AUS	RAPD, PFGE	118 pt.	65 pt. shared same strain (AES-1)	Armstrong <i>et al.</i>
2002	Sydney, AUS	PFEG	18 pt. (50 isolates)	10 pt. shared same strain (AES-1)	Anthony <i>et al.</i>
2002	Liverpool, GBR	PFGE	1 case report	Cross infection to non-CF parent	McCallum <i>et al.</i>
2002	British Columbia, CAN	RAPD, PFGE	174 pt.	Cross infection only in close contact cases	Speert <i>et al.</i>
2002	Leeds, GBR	PFGE	6 children	4 children shared same strain	Denton <i>et al.</i>
2003	Mel, Syd and Bris, AUS	PFGE	Reports from 3 centres	A dominant strain over 5 centres (AES-1)	Armstrong <i>et al.</i>
2004	Brisbane, AUS	PFGE	100 pt. (163 isolates)	39 pt. had AES-2, 8 pt. had AES-1	O'Carroll <i>et al.</i>
2004	GBR	PFGE	849 pt.	6 clones identified among 233 pt.	Scott & Pitt
2004	Beer Sheva, ISR	Antibiogram, PFGE	19 pt. and env. isolates	No cross infection, Negative env. culture	Greenberg <i>et al.</i>
2004	Sheffield, GBR	PFGE	43 pt.	17 pt. had clonal strains	Edenborough <i>et al.</i>
2007	Copenhagen, DNK	SNP genotype	22 pt. (150 isolates)	2 clonal strains found in 7 pt.	Jelsbak <i>et al.</i>
2008	The Hague, NLD	AFLP	80 pt. (CF campers)	Possible transmission of PA in 21 pt.	Brimicombe <i>et al.</i>

FIGE, field inversion gel electrophoresis. PFGE, pulsed-field gel electrophoresis. RAPD, random amplification of polymorphic DNA. AP-PCR, arbitrary primers-PCR. SNP, single nucleotide polymorphism. AFLP, amplified fragment length polymorphism. pt., patients. env., environment. PA, *P. aeruginosa*.

1.5 Update in Treatment of CF

Advances in knowledge of the pathophysiology of the CF airways (as described in Section 1.2.2) as well as a clearer understanding of the microbiology of this disease (as described in Section 1.3) have contributed greatly to the improvement of options for treatment of people with CF, allowing many CF patients to live into adulthood. Although to date there is no cure for this condition, CF researchers have recently discovered promising compounds (i.e. Moli1901 – peptide derived from *Streptomyces cinnamoneum*, Curcumin – a compound found in turmeric, and even gentamicin) that can correct the common abnormality of the CFTR protein ($\Delta F508$) and partially restore the functions of the CFTR (Egan *et al.*, 2004; Wilschanski *et al.*, 2003; Zeitlin, 2004; Zeitlin *et al.*, 2004). These compounds are currently only in either phase I or phase II trials (**Figure 1.26**) and their clinical use for CF treatment may be some way into the future.

Perhaps the most important aspect of the CF therapy is to provide supportive treatments according to the symptoms. It has been established since 1964 that three therapeutic facets are essential for people with CF: relief of airway obstruction, treatment of the lung infection and nutritional repletion (Matthews *et al.*, 1964).

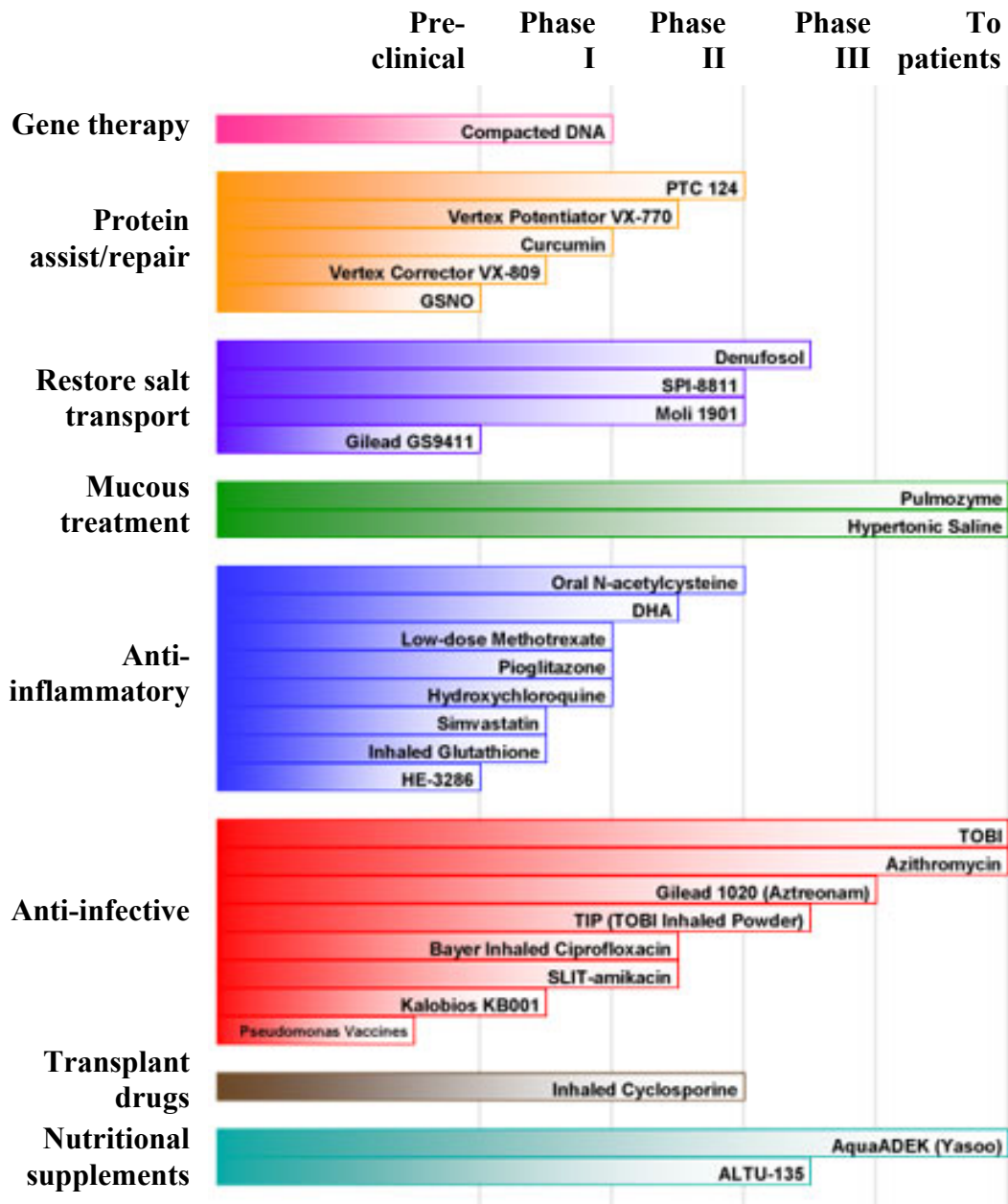


Figure 1.26 Current status of the development of potential drugs and agents for CF therapies (from the Cystic Fibrosis Foundation <http://www.cff.org/treatments/Pipeline/> accessed 28th February 2008. Data are snapshot of those potential CF therapies that are currently in development as of January 2008.)

Hypertonic Saline Inhalation

Clearance of secretions relieving airway obstruction has a prominent role in CF therapy. An inhalation of hypertonic saline has been reported to have a therapeutic benefit in CF. Robinson *et al.* studied its efficacy in ten CF adults and found that a single administration of the hypertonic saline significantly improved the mucociliary clearance (Robinson *et al.*, 1996). However, studies by Suri and coworkers in 48 children over 12-week period showed that twice-daily hypertonic saline inhalation had little effect on pulmonary function (increase mean FEV₁ by only 3%) compared to other proven therapies such as recombinant human deoxyribonuclease (rhDNase) (Suri *et al.*, 2001). Nonetheless, it is noteworthy from the studies by Suri *et al.* that there were some children who had greater improved lung function from hypertonic saline than rhDNase and that treatment with nebulised hypertonic saline was five times cheaper than the rhDNase (Suri *et al.*, 2001). A recent double-blind, parallel-group trial conducted by the CF group at RPAH and the University of Sydney testing the effect of hypertonic saline therapy in 164 patients over 48 weeks, found therapeutic benefit of this treatment for people with CF (Elkins *et al.*, 2006). Results from this large multicentre trial showed that patients treated with hypertonic saline (7% saline solution) twice daily had significant improvement of lung function and had (treated with normal saline twice a day for 48 weeks) (Elkins *et al.*, 2006). Similar findings by Donaldson and colleagues also showed that administration of hypertonic saline four times daily over a 2-week period improved lung function and mucociliary clearance (Donaldson *et al.*, 2006). These landmark studies by both Elkins *et al.* and Donaldson *et al.* have contributed greatly to current therapeutic regimens for people with CF (Accurso, 2007).

1.6 Research Aims and Objectives

Background and Scope

Clinical studies have demonstrated the importance of *P. aeruginosa* infection as a major determinant of morbidity and mortality in people with CF. Generally, CF individuals acquire *P. aeruginosa* early in life and, by their teenage years, up to 80% of the CF population is infected by this pathogen. Initial isolates of *P. aeruginosa* from young CF children usually display a non-mucoid characteristic associated with an environmental phenotype. Therefore, it is assumed that the environment is the major source for *P. aeruginosa* infection and that transmission from person to person is rare, occurring mainly in close-contact cases such as CF siblings. However, a number of recent clinical and epidemiological studies, as described in Section 1.4.9, have suggested that acquisition of *P. aeruginosa* by cross infection may be more common than previously thought. Over the past decade clonal strains of *P. aeruginosa* have emerged as a life-threatening cause of infection in patients with CF from many countries, including Australia. Following the identification of the clonal strain AES-1 from the CF paediatric clinic at the Royal Children's Hospital in Melbourne (Armstrong *et al.*, 2002), the CF group at the University of Sydney conducted a preliminary epidemiology survey at the adult CF clinic at the Royal Prince Alfred Hospital (RPAH) in Sydney and found that 16 of 32 CF adults were infected with this strain. This preliminary data highlighted the need for a more comprehensive analysis to determine the prevalence of clonal strains in the RPAH clinic.

In the first report of the detection of AES-1, Armstrong *et al.* suggested that AES-1 might be responsible for the unexpected deaths of five CF children under five years of age. This report also showed that children infected with this strain had increased hospitalisation rates (Armstrong *et al.*, 2002). According to the current literature, however, it was possible that this association would be less likely to occur in the adult CF population because it has been shown that the production of virulence factors by *P. aeruginosa* decreases over long-term colonisation within the CF airways (Smith *et al.*, 2006). In order to address this issue, it was therefore important to investigate the clinical implications of infection of the clonal strains, AES-1 and AES-2, in adults with CF.

While there have been a number of reports of the *P. aeruginosa* clonal strains emerging worldwide, there are few studies in the literature that investigate factors that facilitate transmission. One recent study showed that LES displayed enhanced virulence, including

high expression of virulence factors and increased antimicrobial resistance (Salunkhe *et al.*, 2005a) however bacterial factors that may be associated with the transmissibility of both AES-1 and AES-2 have not yet been fully investigated. Thus, an important part of this thesis was devoted to the investigation of the association between the clonal strains and the production of virulence factors. Ultimately, results from this study should provide insight into the physiological properties of the transmissible strains, and such knowledge then used to develop more effective control strategies to address the clinical problems presented by transmissible strains.

One important property of *P. aeruginosa* pathogenesis is the formation of biofilms. There is a growing body of evidence highlighting the significance of biofilms in clinical settings, including infection in the lungs of people with CF. As described in Section 1.4.5, *P. aeruginosa* biofilms have been shown to be associated with increased antibiotic resistance as well as infectivity in CF hosts. Recent preliminary research conducted by the CF group at the University of Sydney described the characteristics of biofilm structures among different strains of *P. aeruginosa* grown in a Flow Chamber System (Whitnall, 2003). In this thesis, the formation of biofilms by representative isolates of the clonal strain AES-1 was investigated and then compared with *P. aeruginosa* laboratory strain PAO1 and non-clonal strains isolated from adults with CF: these studies employed a biofilm model developed by the Centres for Disease Control and Prevention, USA and called the CDC Biofilm Reactor and image-analysis software for analysis.

While biofilms grown on abiotic surfaces such as those grown in the Flow Chamber system or the CDC Biofilm Reactor provide insight into their structure, they may not accurately represent what is actually happening in the lungs of CF patients. Moreover, there is compelling evidence demonstrating that *P. aeruginosa* can form biofilm clusters surrounded by a densely staining matrix in sputum from a CF airway without any surface supporting the growth (Singh *et al.*, 2000a). These clusters are thus very likely to be most like the natural biofilms that occur in CF airways. Understanding the pathophysiology of this cluster biofilm may provide a crucial clue to intervening in the formation of biofilm in the CF lung. To do so, it is essential to have a model which could support bacterial growth in the biofilm mode while, at the same time, mimicking the CF airway milieu. This present research set out to develop a growth medium that is akin to the CF lung environment for generating growth of *P. aeruginosa* in the biofilm mode.

An early study from the CF group at the University of Sydney showed that different isolates from the same clonal strain, AES-1 clone displayed distinct phenotypes based on namely mucoidy (Anthony *et al.*, 2002). It was shown that there was some mutation at the level of the gene controlling the mucoidy production (Anthony *et al.*, 2002). This suggested that despite being classified as the same strain, members of the AES-1 clone have important differences in both phenotype and genotype. Generally, bacterial isolates are categorised as the same strain based on the DNA banding patterns generated by a rare restriction enzyme followed by the PFGE/RFLP as suggested by Tenover and colleagues (Tenover *et al.*, 1995). However, it has been suggested that this technique may not have the highest discriminatory power, even though to date it is still the gold standard method used for molecular typing. Another molecular technique, i.e. AFLP, has been shown to have a superior discriminating power, and this technique was used to determine whether or not further differences within strains could be detected by using AFLP to analyse isolates from the AES-1 clone as compared to PFGE/RFLP.

The final part of this thesis addressed safety issues surrounding a novel treatment for the CF patients – hypertonic saline inhalation. As described in Section 1.5, this treatment was recently developed by the CF group at RPAH and the University of Sydney, led by Prof. Bye (Elkins *et al.*, 2006). This clinical trial established the safety of the hypertonic saline inhalation with regard to bronchospasm, tolerability and bacterial density (Elkins *et al.*, 2006). However, the possibility that this treatment selects for particular strains of *P. aeruginosa*, notably clonal strains, has not yet been examined. This part of the present thesis investigated whether or not there was any change in the genotypes of *P. aeruginosa* isolated from the participants at the beginning and the end of the trial.

Specific Research Aims

The aims of the research performed during this thesis were to:

- (i) establish the genotypic profile of *P. aeruginosa* isolated from the CF patients attending an adult CF clinic at RPAH;
- (ii) evaluate clinical outcomes of patients infected by clonal strains compared to those infected with non-clonal strains of *P. aeruginosa*;
- (iii) compare and contrast the production of virulence factors in clonal and non-clonal strains, including total proteases, elastase, staphylolysin, protease IV, chitinase, rhamnolipid and exoenzyme S and the production of QS molecules;
- (iv) evaluate the biofilm structures of selected clonal and non-clonal *P. aeruginosa* isolates using the CDC Biofilm Reactor;
- (v) develop a novel model to study biofilm formation in conditions that mimic the CF lung environment and to compare the biofilm formation of selected clonal and non-clonal strains grown in this model;
- (vi) analyse the major *P. aeruginosa* clonal strain, AES-1, by using the AFLP technique; and
- (vii) determine the effects of the treatment with hypertonic saline inhalation on the possibility of genotypic changes of *P. aeruginosa* isolates recovered from the participants at the beginning and end of the trial.

2 Chapter Two: Material and Methods

2.1 Introduction

Research conducted during this thesis consists of six different but related studies. The study population for each part is different and is therefore described in the relevant chapter. In this chapter, general experimental procedures, which were used throughout the project, are explained whereas the more specific protocols are described in the relevant sections. Methods of processing the sputum samples from patients with CF in order to obtain *P. aeruginosa* isolates are described hereafter. The PFGE technique – the main technique used for genotyping strains of *P. aeruginosa* in Chapter Three and Chapter Eight – is also described in this chapter along with its interpretation methods. Details of preparation methods and ingredients of broths, buffers and solutions are described in Appendix 11.1 and 11.2.

2.2 General Experimental Procedures

2.2.1 Sputum Specimen Processing

2.2.1.1 Sputum Storage and Transportation

Specimens obtained from CF patients were stored at 4°C and processed within 48 hours. If sputum specimens were collected from patients outside RPAH, they were couriered to the CF group at the University of Sydney within 24 hours of production and were kept on ice in an esky box until arrival. Shipment packages and transportation were done in accordance with IATA (The International Air Transport Association) packing and shipping regulations.

2.2.1.2 Sputum Processing

Samples were weighed and were then homogenised with a liquefying agent, Sputasol[®] (0.1% dithiothreitol; Oxoid) at 1:1 w/v unless the sample weighed less than 5 g in which case it was made up to 1 mL with Sputasol[®]. The homogenising process followed the manufacturer's instruction: 1-minute vortexing, 5-minute resting and then 3-minute vortexing.

Serial 10-fold dilutions of the homogenised samples were then made with sterilised PBS to the dilution of 10^{-3} , 10^{-5} , 10^{-6} and 10^{-7} . These dilutions were then plated out (100 μ L from each dilution) onto *Pseudomonas* selective agar (cetrimide fucidin cephaloridine or CFC agar; Oxoid) and onto mannitol salt agar (MSA; Oxoid) to detect *S. aureus*. These inoculated agar plates were incubated at 37°C for 48 hours.

2.2.2 Identification of *P. aeruginosa*

After 48 hours incubation, all colonies with distinct phenotypes which grew on the inoculated plates (CFC) were subcultured onto Columbia horse blood agars (HBA; Oxoid). All *P. aeruginosa* isolates were identified by colony morphology, growth on CFC agar, a positive oxidase test, growth at 42°C, and resistance to C390 [9-chloro-9-(4-diethylaminophenyl)-10-Phenylacridan; Dutec Diagnostics] when grown on Mueller-Hinton agar (Oxoid). *P. aeruginosa* isolates were also evaluated for the level of mucoid production as – (non mucoid), + (low), ++ (medium) and +++ (high).

2.2.3 Storage of *P. aeruginosa*

2.2.3.1 Storage in Protect[®] Bacterial Preserver Beads

P. aeruginosa subcultured on HBA was preserved either in Protect[®] preserver beads (Technical Service Consultants Ltd.) or in 15% (v/v) Glycerol. Protect[®] preservation procedures followed the manufacturer's instruction. Briefly, approximately 5 colonies of bacteria was inoculated into a Protect[®] vial, and then mixed by spinning for 1 minute. The mixture was left standing for at least 30 seconds followed by decanting the cryopreservative fluid, leaving bacteria to attach with preserver beads. Vials were then stored at -70°C for further analysis.

2.2.3.2 Storage in 15% Glycerol

Purified *P. aeruginosa* culture was transferred into a sterilised 15% (v/v) Glycerol (see Appendix) vial using a sterile loop. It was then mixed and stored at -70°C for further analysis.

2.2.4 Resuscitation of *P. aeruginosa* from Cryopreservation

2.2.4.1 Recovery from Protect[®]

P. aeruginosa isolates were recovered from Protect[®] by placing a single bead from the vial onto a Columbia HBA plate (using a sterile hook) and then rolling that bead thoroughly over a portion of the plate. It was then incubated at 37°C for 48 hours.

2.2.4.2 Isolate Recovery from Glycerol

P. aeruginosa isolates were recovered from 15% (v/v) Glycerol by using a sterile inoculating loop to transfer a small amount of Glycerol-bacteria mixture onto a portion of a HBA plate. It was then streaked and incubated overnight at 37°C for 48 hours.

2.2.5 Cultivation of *P. aeruginosa*

2.2.5.1 Cultivation on Agar Plates

Cultivation of *P. aeruginosa* and other bacteria in this thesis was done on agar plates including HBA, CFC, MSA and Nutrient agar (Oxiod). Flame-sterilised inoculating loops were used to streak plates as shown in **Figure 2.1**.

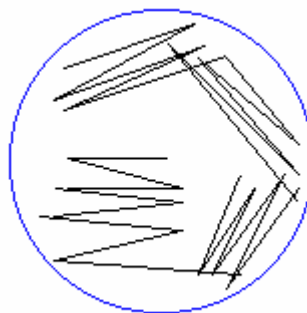


Figure 2.1 Plate streaking method. Sterile inoculating loops were used to spread the bacteria out.

2.2.5.2 Cultivation in Broth

Bacterial broth cultivation was done by inoculating up to three colonies from a pure culture of bacteria grown on agar plates into sterilised broth (10 mL) in sterile test tubes or in McCartney bottles. Inoculated broth was mixed briefly and then incubated at 37°C or otherwise as indicated in an Orbital shaking incubator (BioLab) at 250 rpm. Broth used in this thesis included Nutrient broth (Oxoid), Lauria-Bertani broth (LB; Oxoid), Triple Soy broth (TSB; Oxoid), and Miller Hinton broth (MHB; BD Company).

2.3 Pulsed-Field Gel Electrophoresis (PFGE)

Developed in 1984 by Schwartz and Cantor (Schwartz & Cantor, 1984), PFGE is considered the gold standard for molecular techniques in epidemiological studies of pathogenic organisms. Unlike other molecular methods such as RAPD in which strain differentiation is based on length polymorphisms of some parts of DNA randomly amplified, the PFGE method pertains to the entire bacterial genome. Using a rare cutting restriction enzyme, large DNA segments (macrorestriction) are created from the whole bacterial genome. Typically, DNA molecules larger than 20 kb migrate through a standard gel at the same rate (Helling *et al.*, 1974). Thus the theory behind the PFGE technique is simply to separate large DNA fragments (up to 2,000 kb). It is also necessary to preserve and protect the relatively fragile large DNA fragment from DNA shear forces (Anand, 1986). PFGE involves several steps as follows:

2.3.1 Bacteria Cultivation

P. aeruginosa were grown in Nutrient broth overnight as described in Section 2.2.5.2.

2.3.2 Cell Extraction and Cell Washing

Two millilitres of an overnight broth culture was transferred to a 2-mL microtube, and the tube was centrifuged at 4,500 rpm for 15 minutes. Supernatant was then decanted, and a pellet of *P. aeruginosa* cells was subject to a washing process. Briefly, cell pellets were re-suspended in 1.5 mL of PIV Buffer (see Appendix) and were then centrifuged at 4,500 rpm for 15 minutes. Supernatant was again decanted, and the pellets were kept. This washing

step with PIV was repeated three times. At the completion, cells were resuspended in 400 μL of PIV Buffer and incubated at 37°C.

2.3.3 DNA Plug Formation

To avoid damaging by shearing forces during extraction of large DNA molecules, whole bacterial cells are embedded and lysed in an agarose plug (Anand, 1986; Schwartz & Cantor, 1984), which involved an agarose solution preparation and a cell-embedded gel plug formation.

To prepare the agarose solution, pulsed-field electrophoresis grade agarose (Pulsed Field Certified™ Agarose, Bio-Rad) was used. Briefly, 0.2 g agarose was dissolved in 10 mL of 50mM ESP Proteolysis Buffer (see Appendix). This solution was kept molten at 50°C in a water bath prior to use.

To cast the cell-embedded plug, 200 μL of the warm cell suspension from the previous step was mixed with an equal volume of the melted agarose and swiftly transferred into a preformed plug mold (Perspex Plug Mold, Bio-Rad; the mold was cleaned thoroughly by using 70% (v/v) ethanol followed by sterile dH₂O rinsing and was assembled when dry.) *P. aeruginosa* cell-agarose mixture was allowed to solidify at room temperature (15 – 20 minutes).

2.3.4 Cell Lysis

When the agarose set, the plug mold was disassembled and each agarose plug was removed using a clean spatula. Each plug was submerged into 1 mL of EC Lysis Buffer (in a 2-mL microtube) followed by an incubation at 37°C in a shaking water bath overnight. EC Lysis Buffer contains lysozyme (see Appendix) diffusing into the gel plug and lysing bacterial cells *in situ*. To minimise any possible contamination particularly by DNAase, this process was done cautiously on a clean bench. Microtubes were wrapped with clean parafilm prior to the incubation.

2.3.5 Proteinase K Treatment

After the overnight incubation, the EC Lysis Buffer was replaced with 1 mL of ESP Proteolysis Solution containing proteinase K (Astral Scientific), followed by an overnight

incubation at 50°C in a shaking water bath. The proteinase K inactivates nucleases that might otherwise degrade the DNA.

2.3.6 Plug Cutting

In order to fit the plug (10 mm long x 5 mm wide x 1.5 mm thick) into a single well of an electrophoresis gel, each plug was cut into three smaller segments (approximately 3 mm long). To do so, the plug was removed from the microtube and placed on a sterile glass slide and cut using a flame-sterile surgical blade. A single glass slide was used for each plug and the blade was thoroughly flamed before cutting another plug in order to avoid carrying over of DNA between samples. The three segments were re-immersed into the ESP-Proteinase K Solution and incubated for a further 10 minutes.

2.3.7 Inactivation of Proteinase K and Plug Washing

Following the 10-minute incubation, the ESP-Proteinase K Solution was removed. The plugs were then washed with 1 ml of TE Buffer (see Appendix) by gently pipetting up and down several times, and TE was then discarded. In order to inactivate the proteinase K activity, the plugs were re-suspended in 1 mL of TE containing 0.175 mg of phenylmethylsulfonyl fluoride (PMSF) and incubated at the room temperature for 30 minutes. This step was repeated once. After that, the TE-PMSF Solution was removed, and the plugs were suspended into 1 mL of TE Buffer and incubated for 30 minutes at the room temperature. After which, the buffer was gently pipetted up and down before removal. This washing step with the TE Buffer was repeated four times. After the last wash, the agarose plug segments were stored in 1 mL of TE Buffer at 4°C until required.

2.3.8 Endonuclease Digestion

Since the whole chromosome of bacteria (6.3 Mbp for *P. aeruginosa*) is subjected to an endonuclease digestion, it is important that the restriction enzyme used in PFGE recognises long stretches of bases. Restriction enzymes that recognise frequent sites would cut many times in a chromosome and, as a result, it would be difficult to interpret the resulting pattern. For the construction of DNA fragmented polymorphisms that create high discriminatory power and are still easy to interpret, a total of approximate 15 – 40 DNA bands is optimal. *SpeI*, which is an infrequently cutting restriction enzyme, has been previously shown to be the most appropriate choice for *P. aeruginosa* (Grundmann *et al.*,

1995). A diagram below shows the recognition site of *SpeI*. The process for this step is as follows.



A single agarose plug segment was removed from storage in the TE Buffer and transferred to an 1.5-mL microtube containing 1 mL of sterile dH₂O. This was then left standing on ice for 20 minutes. The step was repeated once, and the dH₂O was finally decanted and replaced with 250 μ L of NE Buffer-2 Solution (New England Biolabs) containing BSA (New England Biolabs). The plug was then left to stand on ice for 30 minutes, allowing it to equilibrate in the NE Buffer-2 Solution. After which, the buffer was removed and replaced with the *SpeI* solution (see Appendix) and then incubated at 37°C overnight in a shaking water bath. It was important to ensure that the DNA plug was totally submerged under the enzyme solution.

2.3.9 Cessation of Restriction Enzyme Reaction

Prior to the electrophoresis, the restriction enzyme reaction was halted with the addition of 1 mL of ice-cold TE Buffer and was then left standing on ice for at least 30 minutes.

2.3.10 Agarose Preparation

Pulsed-field electrophoresis grade agarose (Pulsed Field Certified™ Agarose, Bio-Rad) was used as its average pore size in an agarose matrix is relatively large (100 - 200 nm). To prepare the gel, 2.4 g of Pulsed Field Certified™ Agarose were mixed in 200 mL of 0.5x TBE Buffer (final concentration, 1.2%). The mixture was dissolved by heating in an autoclave using a free-steam mode set at 100°C for 10 minutes. Because the free steaming generally reduces the amount of air bubbles which otherwise might interfere in a casting process, this method was preferred to the use of a microwave oven for heating. The molten mixture was kept in a water bath set at 50°C. Approximately 5 mL of the aliquot of molten agarose were separated in a clean McCartney bottle and kept molten at 50°C. The remaining mixture was poured into a pre-assembled clean gel casting mold with a ten-lane well comb (Bio-Rad). (The gel casting stand and the comb were cleaned using 70% (v/v)

ethanol followed by sterile dH₂O and left dry prior to assembly.) The gel was allowed to set at room temperature.

Once the gel solidified and the restriction enzyme reaction completely ceased, each DNA plug from the previous step was inserted into a single well. When all plugs were loaded, the aliquot of molten agarose was pipetted over each plug until the wells were full, ensuring that there were no gaps or air bubbles between the plug and the gel. This was allowed a further 10 minutes to solidify.

2.3.11 Electrophoresis

The CHEF-DR II electrophoresis apparatus (Contour-Clamped Homogeneous Electrophoresis Field; Bio-Rad) was used to separate large DNA fragments generated by the macrorestriction. The CHEF-DR II system administers its electric field as “pulses” which allows DNA molecules trapped in agarose-matrix pores to re-align themselves before further migration (Chu *et al.*, 1986). The CHEF-DR II unit delivers the pulsed current from a hexagonal array of electrodes which creates two fields – one moving “upper right” to “lower left” (direction A, **Figure 2.2**), and the other from “upper left” to “lower right” (direction B, **Figure 2.2**) – which intersect at 120° angles. As a result, large DNA molecules travel in direction A for a short time and eventually get caught in the agarose matrix, but then as the electric channel is changed to direction B, molecules re-align and move in the B direction. As a result, the net movement is essentially in the forward direction (Chu *et al.*, 1986).

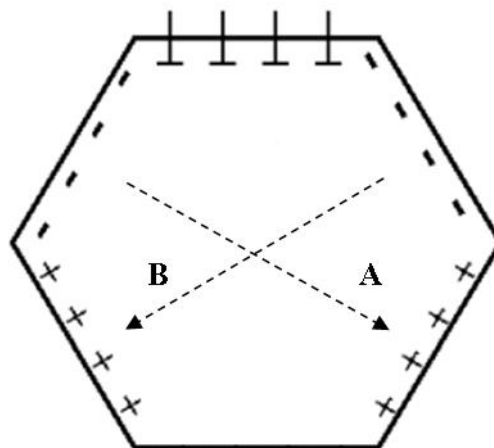


Figure 2.2 CHEF-DR II electric field and current directions (arrows) generated by its electrodes.

Prior to the electrophoresis, the CHEF-DR II apparatus was cleaned with 70% (v/v) ethanol followed by a wipe down with dH₂O. Two litres of dH₂O was then poured into the tank and pumped through for at least 10 minutes in order to clean the entire system. After which, the water was drained completely, and 2 L of cold 0.5x TBE Buffer was filled into the tank and again pumped through the system. A cooling module was then set to keep the temperature of circulating buffer at 14°C. This was done 20 minutes before the gel was loaded into the system so that the buffer was at 14°C throughout. After which, the gel was placed in the middle of the CHEF-DR II electrophoresis tank.

An electrophoresis field was set at 6 V/cm (or 200 Voltage). Pulse times were approximated using an equation suggested by the manufacturer:

$$\text{Pulse time (seconds)} = \text{size of DNA fragments to separate (kb)} / 10$$

For *P. aeruginosa*, DNA fragments sized ranging from 5 – 600 kb were expected from the macrorestriction. Electrophoresis conditions were thus set into two different ramping “Blocks” in order to separate DNA molecules with different sizes. In “Block 1”, the pulse times was set to linearly increase from 0.5 second (initial switch time) to 25 seconds (final switch time) for 20 hours in order to separate DNA fragments around 5 – 250 kb. In “Block 2”, immediately following the Block 1, the pulse times was set to linearly increase from 30 seconds (initial switch time) to 60 seconds (final switch time) for 4 hours in order to separate DNA fragments around 300 – 600 kb. **Table 2.1** summarises settings used for electrophoresis. The total duration of electrophoresis was 24 hours.

Table 2.1 Electrophoresis settings for PFGE

Setting	Block 1	Block 2
Initial switch time (seconds)	0.5	30
Final switch time (seconds)	25	60
Voltage (V/cm)	6	6
Running time (hours)	20	4

2.3.12 Staining DNA Restriction Pattern and Visualisation

Once the electrophoresis was finished, the gel was removed from the tank and stained with 0.5 mg/ml Ethidium Bromide in approximately 1 L of 0.5x TBE for 20 – 30 minutes and

followed by de-staining in dH₂O for 30 minutes – 1 hour. After which, the gel was visualised under a UV Transilluminator (Novex Australia Pty. Ltd.), and images were captured using an Olympus C-3040 Zoom digital camera (Olympus) incorporated with a DigiDoc-It Imaging System version 1.1.25 (UVP Inc.). All images were stored in TIFF format.

2.3.13 DNA Banding Pattern Analyses

2.3.13.1 Analysis Using Criteria Proposed by Tenover *et al.*

Tenover *et al.* established guidelines for analysing DNA fragment patterns generated by PFGE in 1995 (Tenover *et al.*, 1995), and they have been widely used and accepted since then. It was proposed that any genetic event, including gain or loss of restriction site and insertion or deletion of DNA fragments, created a DNA banding pattern distinct from the original, and that the number of band differences from the original DNA pattern varied depending on the type of genetic event. For example, as shown in **Figure 2.3 Lane B**, any point mutation creating a new restriction site results in loss of one fragment present in the original pattern and, concomitantly, gaining two new smaller fragments. In a case of an insertion in which the insertion part does not have a restriction site, the new DNA banding pattern should gain a new fragment of a larger size to the fragment that is inserted (**Figure 2.3 Lane D**). From this basis, criteria for interpreting the DNA banding patterns generated by PFGE were created, as shown in **Table 2.2**.

Table 2.2 Criteria for interpreting PFGE patterns, developed by Tenover and colleagues (Tenover *et al.*, 1995)

Category	Number of genetic differences	Typical number of DNA banding fragment differences
Indistinguishable	0	0
Closely related	1	2 - 3
Possibly related	2	4 - 6
Different	≥3	≥7

According to these criteria, the relatedness of strains of bacteria was categorised into four levels: indistinguishable, closely related, possibly related and unrelated (Tenover *et al.*, 1995). While indistinguishable and unrelated are obvious, the closely related and possibly related are problematic. A single genetic event (such as a spontaneous mutation) creates a strain categorised as closely related and may occur spontaneously over time (Sader *et al.*, 1993). In contrast, isolates categorised as possibly related may have the same genetic lineage as that of the original strain but are less likely to be responsible for the outbreak or transmission of the original isolate (Tenover *et al.*, 1995). In this thesis, a cut-off at three bands or fewer differences was used as a standard point to differentiate bacterial strains.

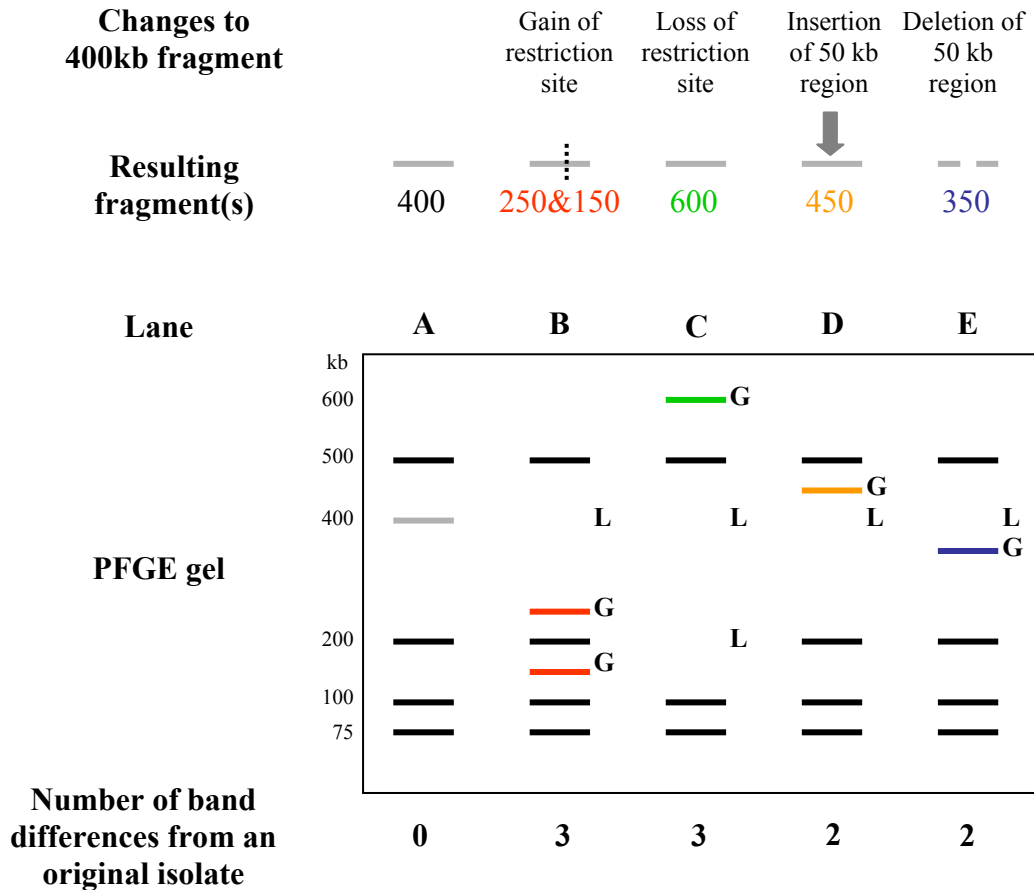


Figure 2.3 Schematic diagram illustrating the changes in the DNA banding patterns of an isolate as a result of various genetic events (Tenover *et al.*, 1995).

Lane A represents an original isolate;

Lane B represents a pattern of an isolate with a gain of a restriction site;

Lane C represents a pattern of an isolate with a loss of a restriction site;

Lane D represents a pattern of an isolate with an insertion of DNA from an existing fragment; and

Lane E represents a pattern of an isolate with a deletion of DNA from an existing fragment.

G indicates fragments present after a genetic event but absent from the original pattern; **L** indicates fragments present in the original pattern and missing after a genetic event.

2.3.13.2 Analysis using GelCompar II™

An analysis of the DNA banding patterns was done using cluster-aiding software, GelCompar II™ (Applied Maths, Belgium). The DNA banding patterns generated by the PFGE were converted to distance between isolates according to the Dice coefficient of similarity (Dice, 1945). Dendrograms were constructed using the unweighted pair group method with arithmetic means (UPGMA).

3 Chapter Three: Prevalence of *P. aeruginosa* Clonal Strains in Adults with Cystic Fibrosis and Their Clinical Impact

3.1 Background

Clonal strains of *P. aeruginosa* are an emerging threat to CF communities in several countries including Australia (Cheng *et al.*, 1996; Fluge *et al.*, 2001; Jones *et al.*, 2001). In order to design rational control strategies for these strains, it is important to know the prevalence and clinical impact, if any, of these strains. In Australia, two clonal strains, AES-1 and AES-2, have been found among CF individuals attending CF centres along the eastern coastline (Armstrong *et al.*, 2002; O'Carroll *et al.*, 2004). These clones show evidence of an adverse impact on clinical status, particularly the AES-1 clone among CF children (Armstrong *et al.*, 2002; Nixon *et al.*, 2001). Following a preliminary study from the adult CF clinic at RPAH Sydney which showed that 16 of 32 patients were infected with AES-1 (Armstrong *et al.*, 2003), it was recognised that there was a need for a more comprehensive analysis to determine the prevalence of clonal strains and the clinical impact of different strains, including the AES-1 and AES-2 clonal strains, on CF adults. Studies in this chapter were designed to:

- establish the genotype of *P. aeruginosa* strains isolated from CF patients attending the adult CF clinic at RPAH;
- evaluate clinical outcomes of patients infected by different strains comparing the relative impact of clonal and non-clonal strains of *P. aeruginosa*.

The genotyping of strains was performed by using PFGE/RFLP which is the “gold-standard” molecular typing technique. A laboratory standard *P. aeruginosa* strain, PAO1, which has been genome sequenced (Stover *et al.*, 2000), was used as an inter-gel reference. Its PFGE/RFLP DNA-banding pattern was also validated by a comparison with a banding result generated by an *in silico* study.

3.2 Material and Methods

3.2.1 Study Population

All sputum samples for this study were obtained from patients with CF who attended the adult CF clinic at the RPAH during December 2000 to November 2004. At the time of study, the RPAH CF clinic had 189 patients in total, and of those, 28 patients had undergone lung transplantation; 11 had not produced sputum; and three were non-compliant. Investigations reported in this study were carried out on sputum specimens from 112 patients who were chronically infected with *P. aeruginosa* for a minimum of three years. All participants gave informed consent for this study. This study was approved by the Sydney South West Area Health Service Ethics Committee (Approval X002-0320) and the University of Sydney Human Research Ethics Committee (Project Reference Number 6999).

3.2.2 Sample Processing

Sample processing was described in Section 2.2.1. *P. aeruginosa* isolates were resuscitated from cryopreservation [either Protect[®] (Technical Service Consultants Limited) or 15% Glycerol] when tested (See Section 2.2.4). Cultivation steps were restricted to a maximum of two subcultures prior to testing in order to minimise the spontaneous genetic alteration.

3.2.3 Genotyping of *P. aeruginosa*

3.2.3.1 Macrorestriction and PFGE

P. aeruginosa isolates were genotyped using pulsed-field gel electrophoresis (PFGE) following macrorestriction by the *SpeI* endonuclease as previously described in Section 2.3. DNA macrorestriction analyses and clonal strain identification were performed using GelCompar II[®] and the criteria established by Tenover and colleagues (Tenover *et al.*, 1995) (see Section 2.3.13). A clone is defined here as one showing the same PFGE banding pattern (or three bands or fewer difference) in three or more patients.

3.2.3.2 *In Silico* Genotyping

An *in silico* technique was developed for simulating experiments, including PFGE/RFLP, on computers (Bikandi *et al.*, 2004). The following website: <http://insilico.ehu.es/> was used according to procedures described by San Millán and coworkers (San Millan *et al.*, 2005). By selecting “Restriction digest and PFGE” (**Figure 3.1** Upper), the programme provided a choice of microorganisms to be studied (which included *P. aeruginosa*). As this programme is based on the published complete genome sequences of microorganisms, only PAO1 is available to represent *P. aeruginosa*. The programme also provides a vast variety of restriction enzymes to choose from, and here the “*SpeI*” restriction enzyme was selected (**Figure 3.1** Lower).

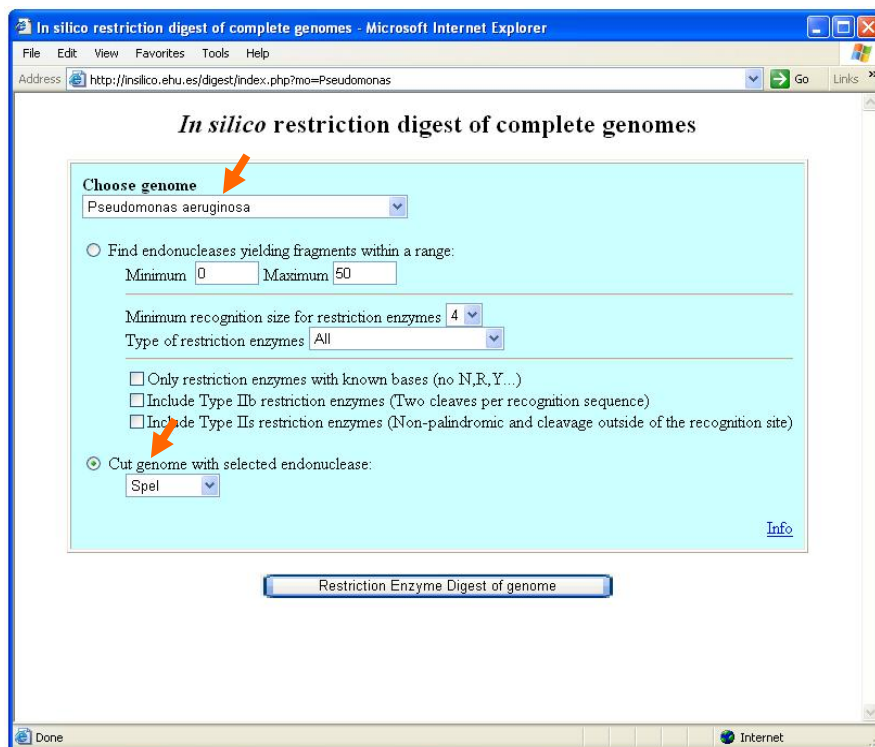
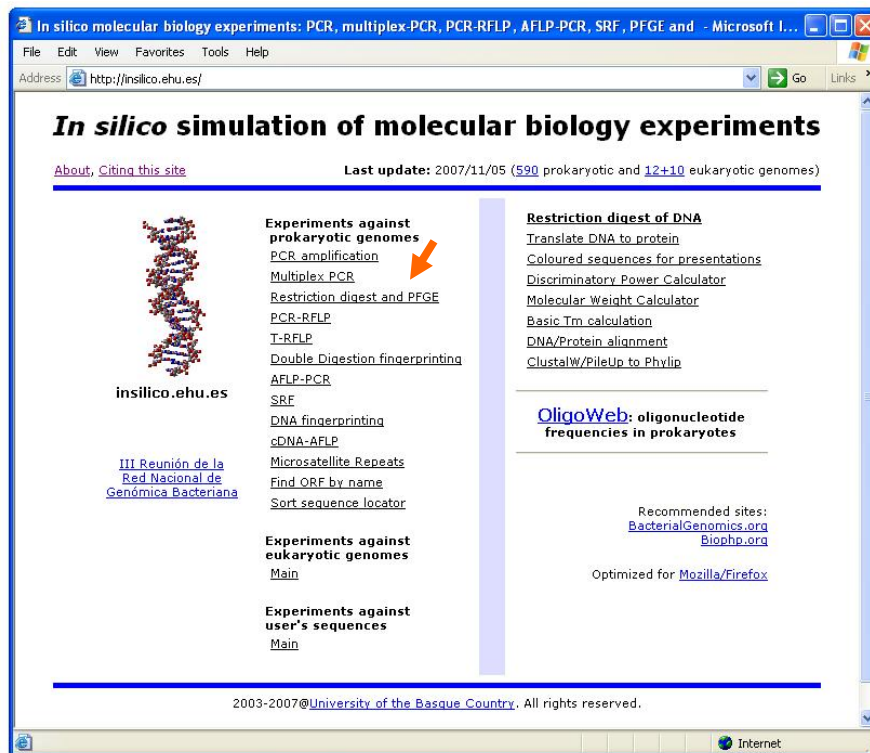


Figure 3.1 Illustration of *in silico* webpage from <http://insilico.ehu.es/>. Upper figure presents the home page of this site; “Restriction digest and PFGE” (red arrow) was selected. Lower figure shows the page of restriction digest of complete genomes step. *P. aeruginosa* and *SpeI* were selected as indicated by red arrows.

3.2.4 Patient Demographic and Clinical Outcome Evaluation

This study was a clinical audit, and clinical data were obtained by medical record review. This was approved by the Ethics Committee, and each patient provided informed consent. Patient demographic data including patient's age, gender and CF genotypes were obtained from the Cystic Fibrosis National Data Registry. Pulmonary function, including per cent predicted of forced expiratory volume in 1 second (%FEV₁) and per cent predicted of forced vital capacity (%FVC), and body mass index (BMI) were measured on the day of sputum collection. Respiratory exacerbations defined according to criteria established by Fuchs and colleagues (Fuchs *et al.*, 1994) and hospital-admission days were documented from hospital records over the twelve months from collection date.

3.2.5 Statistical Analyses

Clinical data from patients with non-clonal strains were first compared to those from patients with all clonal strains combined. Non-clonal strains were compared with each clonal strain only if statistical significance was observed. All comparisons were made using the unpaired *t*-test, or Wilcoxon's rank sum test where data were not normally distributed

3.3 Results

3.3.1 Characteristics of the Study Population

The mean age of the 112 patients was 26 years, and the male-to-female ratio 65:47. The mean BMI, %FEV₁ and %FVC were 21, 55 and 73 respectively. These characteristics are consistent with those of the entire clinic.

3.3.2 DNA Fingerprints Generated by PFGE/RFLP

DNA fingerprints from *P. aeruginosa* isolates are illustrated in **Figure 3.2**. The *SpeI* restriction enzyme generated approximately 18 to 25 bands in each lane. Lane 2, 9 and 10 display an identical banding pattern which was previously identified as AES-1. Lane 3 is also identical to Lane 4, which was previously identified as AES-2. Each of Lanes 5 – 8 shows a unique banding pattern.

3.3.3 Comparison of PAO1 Banding Pattern between PFGE/RFLP and *In Silico* Experiment

Advances in computer technique have created a simulation model to allow users to test or perform various genotyping methods on a computer (“*in silico*”). These methods include PFGE/RFLP. The *in silico* PFGE/RFLP with the *SpeI* digestion shows a total of 37 cleaves which are sorted according to the length of the sequence (**Figure 3.3**). As shown, both *in silico* and the actual PFGE/RFLP create almost identical patterns. Only the bands below 43 kb showed some discrepancies between the two experiments (**Figure 3.3**). This result from the *in silico* experiment is consistent with and supports the result from the actual PFGE/RFLP.

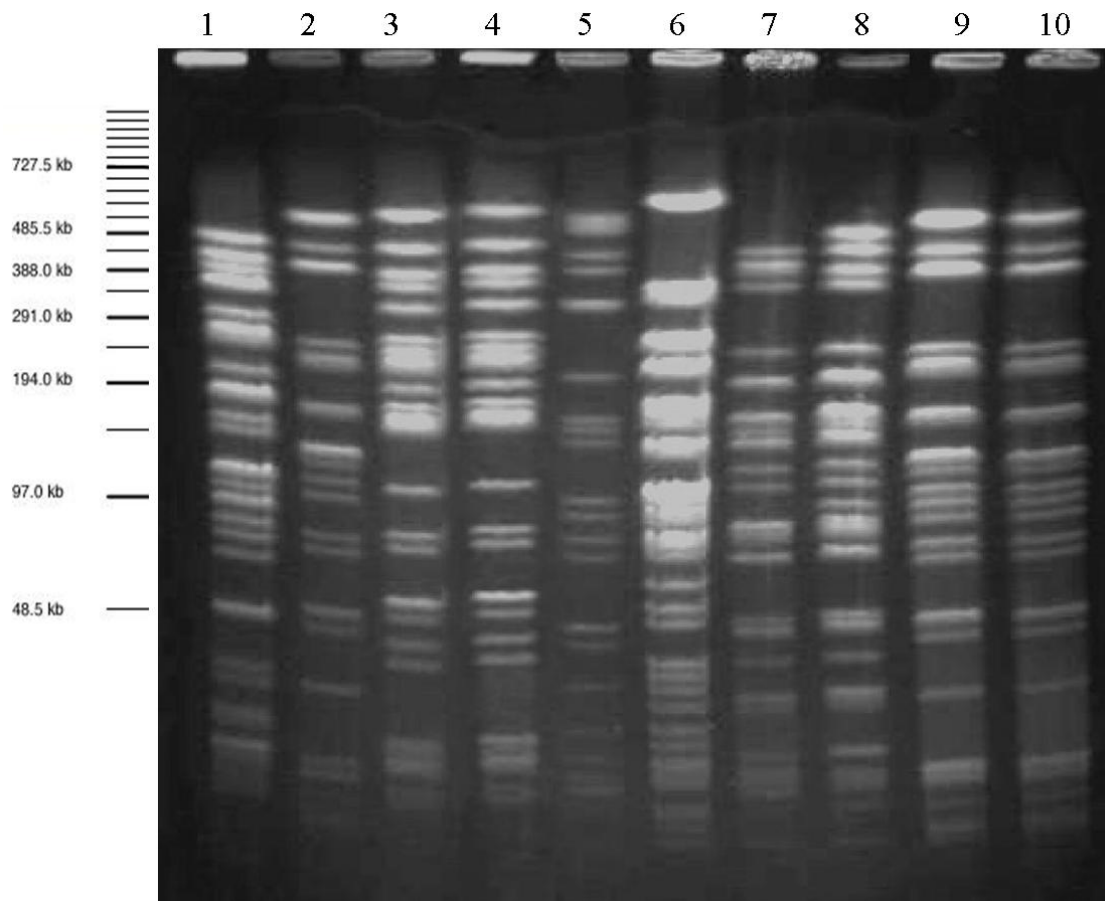


Figure 3.2 DNA banding pattern of *P. aeruginosa* strain PAO1 (Lane 1) and strains recovered from CF patients (Lane 2 – 10). Lambda ladder (New England BioLabs) is used as a molecular weight marker.

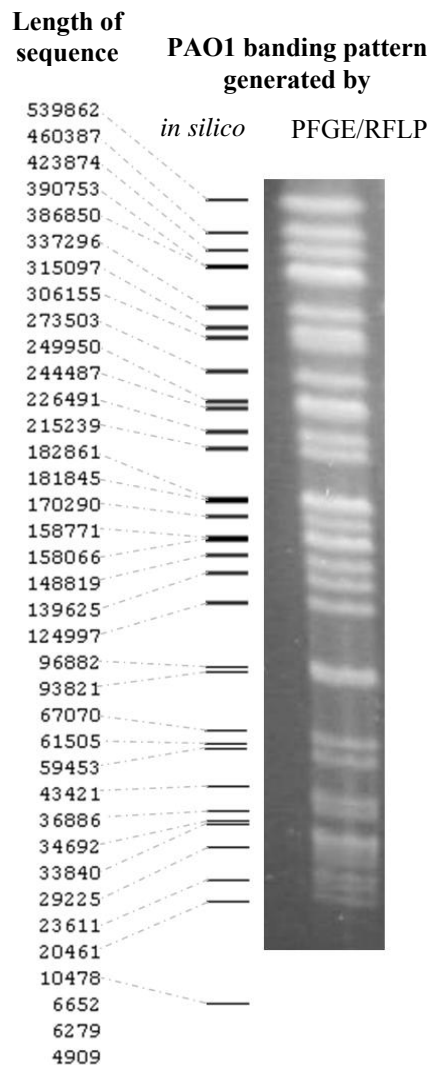


Figure 3.3 Comparison of DNA banding patterns generated by *in silico* and an actual PFGE/RFLP experiment with indication of the length of sequence of each band.

3.3.4 Prevalence of *P. aeruginosa* Clonal Strains

A total of 258 *P. aeruginosa* isolates from 112 patients was genotyped. The mean number of *P. aeruginosa* isolates tested per sputum sample was two (range one to six). **Figure 3.4** shows a dendrogram of 112 *P. aeruginosa* isolates (one isolate per patient). Isolates from 43 patients (38%) branching from Node A are indistinguishable or closely related strains according to the criteria by Tenover *et al.* (isolates have three bands or fewer difference) (Tenover *et al.*, 1995). DNA banding patterns of these isolates also show a similarity higher than 80% as calculated using Dice Coefficient. The DNA fingerprints of these isolates are similar to that of the AES-1 strain, indicating that these 43 patients harboured AES-1. Isolates from six patients (5%) also have similar banding patterns both to themselves and to the AES-2 strain. Two new clonal strains were also identified; one designated Sydney-1 (S-1) infected six patients (5%) and the other, Sydney-2 (S-2), infected three patients (3%). One patient with AES-1 was co-infected with S-1 and another had S-2. Fifty-six patients (50%) harboured only non-clonal strains.

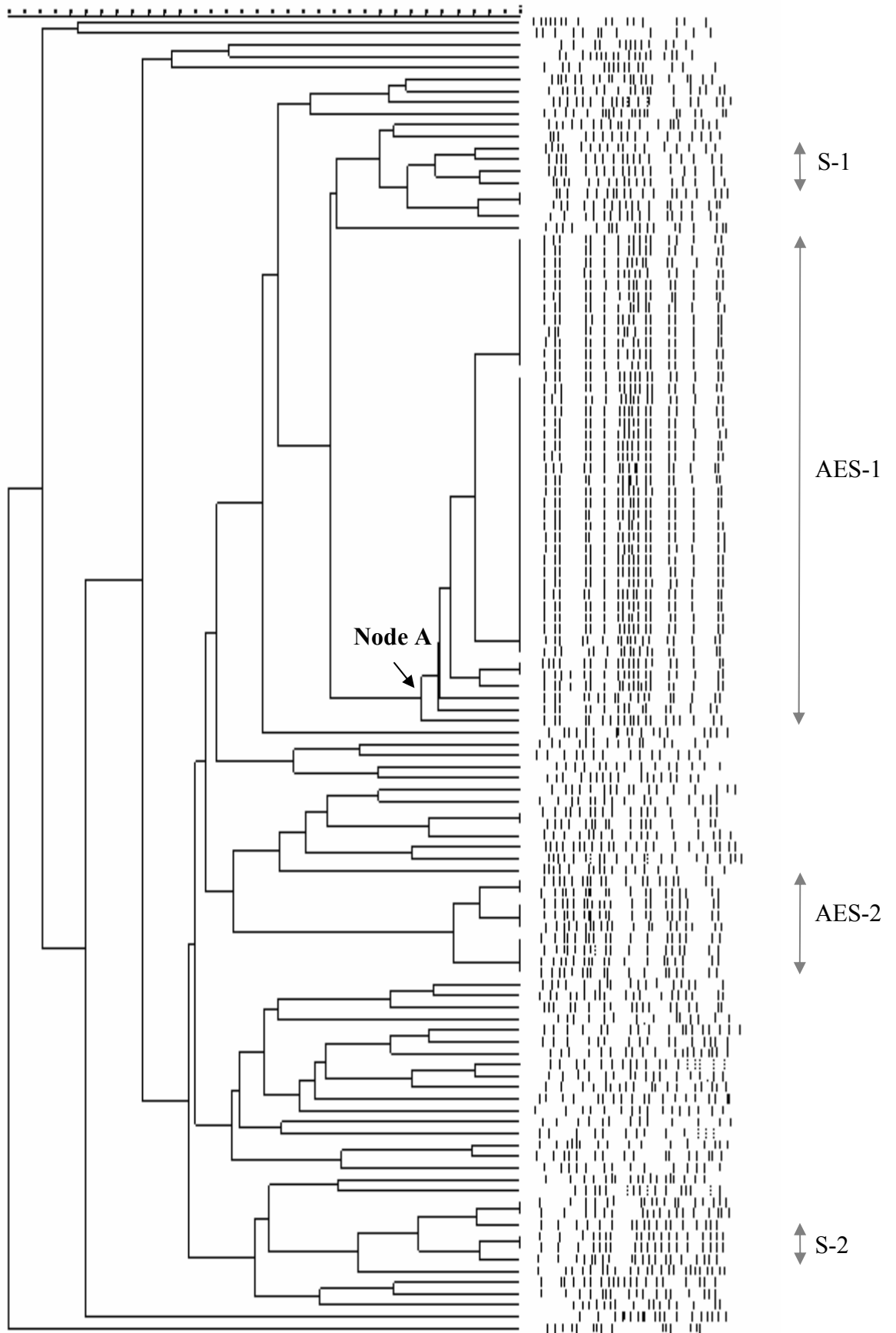


Figure 3.4 Dendrogram showing the relatedness of *P. aeruginosa* isolates from 112 patients.

3.3.5 Relationship between *P. aeruginosa* Strains and Characteristics of Patients

Overall, there was no association between patient's age and an infection by any clonal strain. The male and female ratios were also similar between the clonal and non-clonal groups (**Table 3.1**). Of 112 CF patients, 88 (79%) patients had a Δ F508 homozygous genotype. The proportion of CF patients who had a Δ F508 homozygous genotype was higher in the clonal group, especially in patients who were infected with AES-1 (**Table 3.1**), but this relationship was not statistically significant ($p = 0.106$ when comparing between non-clonal and clonal groups, or $p = 0.087$ when comparing between non-clonal and AES-1 group).

Table 3.1 Characteristics of CF patients infected by clonal and non-clonal strains

	Non-clonal (n = 56)	Clonal (n = 56)	AES-1 (n = 43)	AES-2 (n = 6)	S-1 (n = 5) ^a	S-2 (n = 2) ^b
Age (range)	28 (18-51)	25 (18-40)	24 (18-36)	30 (18-40)	25 (18-33)	31 (30-32)
Males (%)	34 (61%)	33 (59%)	24 (56%)	4 (67%)	3 (60%)	2 (100%)
CF genotype						
Δ F508 Homozygous (%)	24 (43%)	32 (57%)	27 (63%)	3 (50%)	2 (40%)	0
Δ F508 Heterozygous (%)	14 (25%)	10 (18%)	10 (23%)	0	1 (20%)	0
Other genotypes (%)	6 (11%)	3 (5%)	0	1 (17%)	0	1 (50%)
No data (%)	12 (21%)	11 (20%)	6 (14%)	2 (33%)	2 (40%)	1 (50%)

^a excluding one patient co-infected with AES-1.

^b excluding one patient co-infected with AES-1.

3.3.6 Clinical Outcomes of Patients Infected by Clonal and Non-Clonal Strains

Six patients with inadequate follow-up or whose medical records could not be retrieved were excluded. Eight patients (one with AES-1 and seven with non-clonal strains) were found to be co-infected with Bcc. Of those, one patient died, four had no record of pulmonary functions, and three had average BMI, %FEV₁ and %FVC of 20, 51% and 82%, respectively. After excluding the above 14 patients, clinical outcomes of 98 patients

who were infected with either clonal or non-clonal strains, were compared (**Table 3.2**). Comparisons of combined data from patients with any clonal strain (n = 52) with those with only non-clonal strains (n = 46) showed that patients with clonal strains had a greater number of exacerbations and a higher median number of hospital days (**Table 3.2**). These associations remained significant when data from AES-1 and AES-2 and S-1 were analysed separately (**Table 3.2**). While patients from all groups had the same BMI (mean BMI = 21), patients with clonal strains tended to have lower %FEV₁ and %FVC when compared with those with non-clonal strains, but this relationship was not statistically significant (**Figure 3.5**). Two patients with AES-1 who were co-infected with other clonal strains (S-1 and S-2) were grouped in the AES-1 group for convenience in a statistical analysis. The one with AES-1 and S-1 had 29% of FEV₁ and 47% of FVC with three exacerbation episodes within the twelve-month period, whereas the other with AES-1 and S-2 had 110% of FEV₁ and 112% of FVC without any exacerbation episode.

Table 3.2 Clinical characteristics of patients infected with clonal and non-clonal strains

	Non-clonal (n = 46)	Clonal (n = 52)	AES-1 (n = 39)	AES-2 (n = 6)	S-1 (n = 5) ^a	S-2 (n = 2) ^b	<i>P</i> values				
							<i>P</i> ₁	<i>P</i> ₂	<i>P</i> ₃	<i>P</i> ₄	
Mean BMI (SD)	21 (3)	21 (3)	21 (3)	21 (3)	21 (2)	21 (1)	0.250				
Mean %FEV ₁ (SD)	59 (24)	52 (23)	53 (24)	55 (25)	56 (15)	31 (2)	0.190				
Mean %FVC (SD)	75 (23)	71 (20)	69 (22)	77 (20)	76 (13)	61 (7)	0.316				
Median exacerbation episodes (range)	0 (0-3)	1 (0-7)	1 (0-7)	1 (0-2)	1 (0-3)	0	0.005	0.009	0.156	0.027	
Median hospitalisation days (range)	0 (0-36)	12 (0-95)	11 (0-95)	23 (0-29)	18 (0-43)	0	0.003	0.008	0.033	0.021	

*P*₁: all clonal versus non-clonal; *P*₂: AES-1 versus non-clonal; *P*₃: AES-2 versus non-clonal; *P*₄: S-1 versus non-clonal

^a excluding one patient co-infected with AES-1.

^b excluding one patient co-infected with AES-1. Clinical outcomes data of the patients with S-2 were not statistically comparable due to a small number of patients (n = 2).

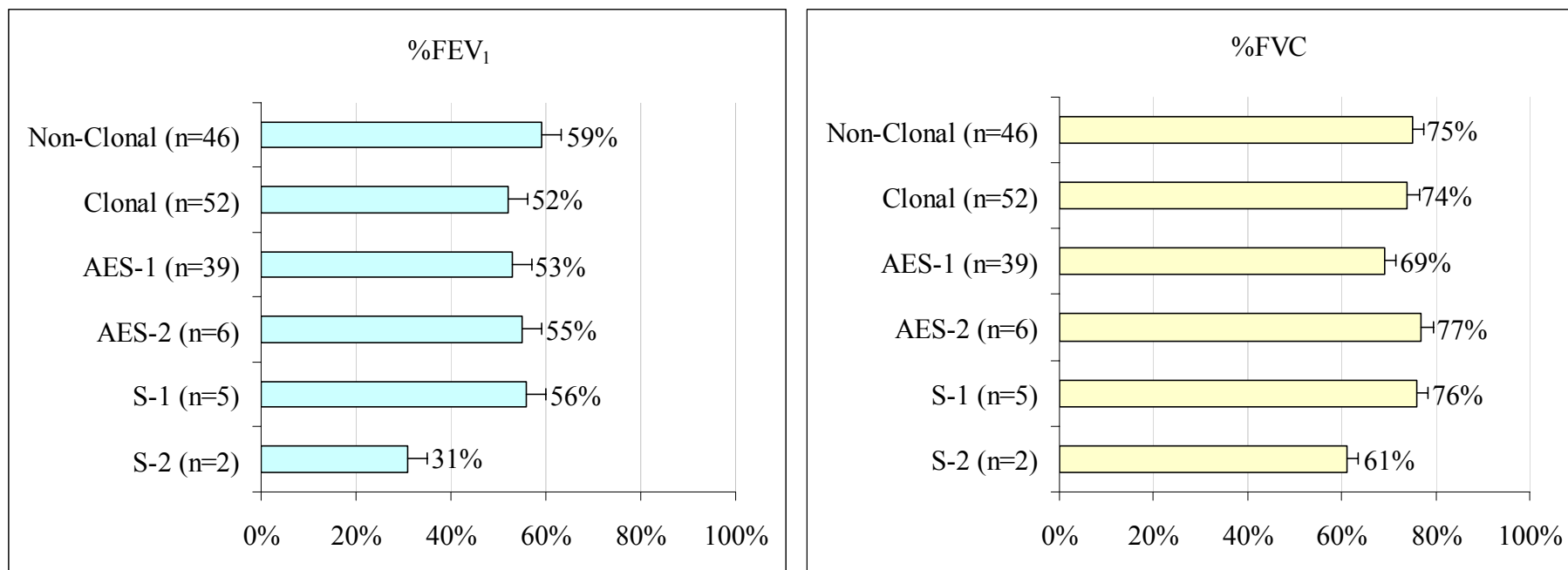


Figure 3.5 Comparison of pulmonary functions (mean %FEV₁ and mean %FVC) between patients infected with either clonal or non-clonal strains. Patients with clonal strains tend to have lower %FEV₁ compared to patients with non-clonal strains. Patients with S-2 have the lowest pulmonary functions, but this data could not be compared statistically due to a small sample size.

3.4 Discussion

By employing PFGE following macrorestriction, which remains the gold standard epidemiology technique, this study has shown that approximately half of the patients at the adult CF clinic at RPAH, who carried *P. aeruginosa*, are infected with a clonal strain. Findings have also provided evidence that the identified clonal strains were associated with negative clinical implications. This study has also integrated the experiments done by the real PFGE/RFLP and the *in silico* model. Results from this study are important for patient management at the clinic.

The major clone, AES-1, affecting 38% of the patients, was first linked with an adverse clinical course in the paediatric CF clinic at the Royal Children's Hospital in Melbourne (Armstrong *et al.*, 2002). The prevalence of the AES-1 strain reported in this study is in line with the previous reports from the Royal Children's Hospital, Melbourne (55%) (Armstrong *et al.*, 2002) and from the preliminary survey in Sydney (50%) (Armstrong *et al.*, 2003). AES-1 has been associated with the death of five unrelated cohort children and with the increased hospitalisation of CF children who carried this strain (Armstrong *et al.*, 2002; Nixon *et al.*, 2001). This study provides further evidence that this association also extends to adults chronically infected with *P. aeruginosa*; patients with AES-1 had significantly more frequent pulmonary exacerbation episodes and were more likely to have been hospitalised within the preceding 12 months than other patients.

The present study also identified AES-2 among six patients (5%). This is the first report of the AES-2 strain outside Brisbane, and it supports the early findings of the spread of clonal strains among CF clinics (O'Carroll *et al.*, 2004). AES-1, in particular, has been identified in patients from five CF clinics in Melbourne, Sydney and Brisbane (Armstrong *et al.*, 2003). This result confirms that *P. aeruginosa* cross-infection may be more extensive than previously believed. Patients with AES-2 also had significantly more hospitalised days within 12 months and tended to have lower %FEV₁.

Interestingly, the relative prevalence of the AES-1 and AES-2 strains at the RPAH clinic is the opposite of that at the Prince Charles Hospital and The Royal Children's Hospital in Brisbane. This result shows that AES-1 infected 38% and AES-2 infected 5% of patients at the RPAH whereas the study from the clinics in Brisbane found that AES-1 and AES-2 infected 8% and 38% of patients, respectively.

Two emerging clonal strains, S-1 and S-2, were also detected. The association between patients carrying one of these clonal strains and a poorer clinical impact was also observed. Patients with S-2 had the lowest %FEV₁ and %FVC, but this data cannot be confirmed statistically due to the small sample size (n = 2).

Overall, there was no significant correlation between patient's age, gender and CF genotype and an infection by a clonal strain. This finding is consistent with the previous report of AES-1 (Armstrong *et al.*, 2002). However, in the study that first reported AES-2, O'Carroll *et al.* found that patients with AES-2 were significantly younger when compared to patients with non-clonal strains (O'Carroll *et al.*, 2004).

In general, the view that people with CF were infected by unique lineages of *P. aeruginosa* acquired from the environment has been challenged since the first compelling evidence of the Liverpool epidemic strain (LES) (Cheng *et al.*, 1996). This study again confirms the possibility of transmission between patients. However, it is not known from the present study if transmission occurs directly from one patient to another or indirectly via a common source/environment such as respiratory equipment or a CF centre itself.

A recent environmental survey at the Liverpool adult CF clinic detected the LES in the air samples taken inside the ward corridor and the outpatient clinic and the patients' rooms (up to three hours after departure), and no other *P. aeruginosa* strains apart from LES were isolated from these air samples. This suggests that aerosol dissemination may contribute to the transmission of LES (Panagea *et al.*, 2005). Moreover, the LES was isolated from patients' hands, clothes and bed linen and external surfaces of spirometry (Panagea *et al.*, 2005). However, this contamination happened only transiently (less than two hours) and no persistent environment reservoirs were found (Panagea *et al.*, 2005). Research from the Manchester centre has also isolated the clonal strain (the Manchester epidemic strain or MES) from room air when colonised patients performed spirometry (Jones *et al.*, 2003), suggesting this may be the most important factor in patient-to-patient spread.

As for AES-1, in the first report by Armstrong and colleagues, environment samples were also tested but none had the AES-1 strain (Armstrong *et al.*, 2002). A preliminary survey of respiratory equipment and environment at the RPAH also failed to detect the presence of this strain (Whitnall, 2003). These findings suggest that direct patient-to-patient

transmission plays a role in the spread of AES-1, but a definite conclusion requires a larger prospective study in both patients and environment.

Factors such as a resistance to antibiotics may contribute to the spread of the clonal strains. Antibiotic selective pressure has been proposed as the cause of the emergence of the LES (ceftazidime-resistant strain) (Cheng *et al.*, 1996). This may be an additional reason to account for the domination of the clonal strains identified in this study.

The clinical implications of clonal strains of *P. aeruginosa* identified in this study are concordant with the previous reports by Armstrong (Armstrong *et al.*, 2002) and O'Carroll (O'Carroll *et al.*, 2004) and with those of the LES and MES (Al-Aloul *et al.*, 2004; Jones *et al.*, 2002). However, other clonal strains such as Midlands-1 were reported to be not associated with more severe outcomes (Chambers *et al.*, 2005), indicating that there are some variations of virulence among individual clonal strains.

Decisions to reorganise CF clinics to address the challenge of clonal strains must be evidence-based. This cross-sectional study reveals the extensive presence of clonal strains within the clinic and adds weight to the growing body of evidence linking clonal strains with poorer clinical outcomes and increased treatment requirement. Recently, there has developed a clear consensus documenting the potential threat of the person-to-person transmission of *P. aeruginosa*, which appears to be strain dependent (Doring & Hoiby, 2004; Kerem *et al.*, 2005). Further investigations to define risk factors for the spread of the clonal *P. aeruginosa* strains are therefore needed for the development of policies that limit spread and minimise the social implications associated with segregation.

In the next chapter, phenotypic factors, including antibiotic susceptibility profiles and production of virulence factors, were investigated in representative isolates. Comparison of these characteristics between clonal and non-clonal strains was expected to lead to a better understanding of the pathogenesis of those strains.

4 Chapter Four: Characterisation of Virulence Factor Production and Antibiotic Resistance in Clonal and Non-Clonal *P. aeruginosa* Strains

4.1 Background

In the previous chapter, two major clonal strains, AES-1 and AES-2, were found infecting a large proportion of the patients (43%) attending the CF clinic at the RPAH. Patients infected by these strains had a significant increase in the number of exacerbation episodes and hospitalisation days, and tended to have a lower pulmonary function than patients who harboured their own unique (non-clonal) strains. It was hypothesised that this observation could be explained by the fact that the clonal strains were more virulent than non-clonal strains.

As discussed in Section 1.4, the pathogenesis of *P. aeruginosa* infection depends on cell-associated and secreted virulence factors such as proteases (LasB elastase, staphylolysin, alkaline protease and protease IV) and toxin (i.e ExoS). Proteases (apart from protease IV) are known to cause tissue destruction and disruption of intact respiratory epithelia as well as contributing to an evasion of host immunity (Doring *et al.*, 1985; Heck *et al.*, 1986; Horvat & Parmely, 1988; Mariencheck *et al.*, 2003). The ExoS also contributes to the pathogenesis in CF lungs through an alteration of the cytoskeleton of host cells, resulting in cell apoptosis (Goehring *et al.*, 1999). Other virulence factors including chitinase and rhamnolipids are also thought to be important in pathogenesis, but their exact roles in the CF lungs are not yet fully understood.

It is now known that many *P. aeruginosa* virulence factors are regulated by a cell-to-cell communication or a QS (quorum sensing) system. This QS system relies mainly on two AHL-regulated circuits: (i) *las*, consisting of the transcriptional activator LasR and the AHL synthase LasI that directs the synthesis of OdDHL; and (ii) *rhl*, consisting of RhlR and RhlI that directs the synthesis of BHL (see more details in Section 1.4.4). It has been suggested that QS may play an important role in pathogenesis; strains with a mutation in the QS genes have been shown to have reduced virulence in several animal models (Imamura *et al.*, 2005; Pearson *et al.*, 2000; Wu *et al.*, 2001).

The properties contributing to the infectivity of *P. aeruginosa* clonal strains AES-1 and AES-2 have not been defined. The fact that these strains were found to be associated with inferior clinical outcomes suggests that they may have an increased production of virulence factors, and this may promote tissue destruction and, ultimately, facilitate transmission between patients. The success of AES-1 and AES-2 as dominant clones within CF centres along the Australian eastern seaboard may also be due to their resistance to antibiotics. Therefore, the experimental studies reported in this chapter were designed to:

- compare and contrast the production of virulence factors of *P. aeruginosa* isolates from clonal strains and from non-clonal strains; and
- investigate antibiotic susceptibility patterns of isolates from clonal and non-clonal groups.

4.2 Material and Methods

4.2.1 Study Population and Bacterial Strains and Culture Conditions

Investigations were carried out on 43 *P. aeruginosa* isolates from the sputum samples of 43 patients attending the adult CF clinic at the RPAH. These patients were randomly selected from the study population (112 patients) described in Chapter Three. All isolates were identified as *P. aeruginosa* according to methods shown in Section 2.2.2, and were genotyped using PFGE/RFLP as described in the previous chapter. Genotypic details of each isolate and the infected patients' data are shown in **Table 4.1**. Overall, the male to female ratio was 21:22. The mean age was 27 years (range 18 – 58). The total 43 isolates were comprised of 14 AES-1, 5 AES-2 and 24 non-clonal isolates. The study was approved by the Institutional Ethics Committee (Approval X02-0320) and the University of Sydney Human Research Ethics Committee and subjects provided informed consent for the work.

P. aeruginosa PAO1, a well-characterised wound-derived strain, was included as a control. *S. aureus* strain ATCC12600 was used for the staphylolysin assay, and *Chromobacterium violaceum* (*C. violaceum*) strain CV026 and *Agrobacterium tumefaciens* (*A. tumefaciens*) strain A136 were used for AHL assays.

All tested isolates were recovered from the Protect[®] Bacterial Preserver Beads or 15% Glycerol storages according to methods described in Section 2.2.4. In order to reduce genetic and/or phenotypic changes, a restriction of sub-cultivation to a maximum of two generations was applied to all isolates prior to testing. Culture conditions of bacteria used in this study are summarised in **Table 4.2** according to testing assays. Colony morphology (i.e. size, shape and colour) and mucoid production of each isolate were recorded when bacteria were grown on a Columbia HBA (Oxoid) at 37°C for 48 hours.

Table 4.1 *P. aeruginosa* isolates in this study with its genotype and clinical data

Isolate	Gender	Age	FEV₁ % predicted	Genotype
C1	F	20	62	AES-1
C2	F	24	44	AES-1
C3	M	22	28	AES-1
C4	F	31	29	AES-1
C5	F	24	68	AES-1
C6	M	20	33	AES-1
C7	M	19	51	AES-1
C8	M	25	22	AES-1
C9	M	22	89	AES-1
C10	F	20	15	AES-1
C11	F	22	40	AES-1
C12	F	29	40	AES-1
C13	M	32	63	AES-1
C14	F	22	72	AES-1
C15	F	18	35	AES-2
C16	F	28	40	AES-2
C17	M	29	100	AES-2
C18	M	26	65	AES-2
C19	M	37	76	AES-2
U1	M	20	68	Non-clonal
U2	M	21	89	Non-clonal
U3	F	28	87	Non-clonal
U4	F	29	58	Non-clonal
U5	F	21	35	Non-clonal
U6	M	19	98	Non-clonal
U7	M	48	33	Non-clonal
U8	F	35	38	Non-clonal
U9	M	34	37	Non-clonal
U10	F	36	69	Non-clonal
U11	M	33	51	Non-clonal
U12	M	26	56	Non-clonal
U13	M	19	58	Non-clonal
U14	F	20	75	Non-clonal
U15	F	58	30	Non-clonal
U16	M	22	59	Non-clonal
U17	M	26	71	Non-clonal
U18	F	38	47	Non-clonal
U19	M	31	70	Non-clonal
U20	F	19	58	Non-clonal
U21	F	30	58	Non-clonal
U22	F	34	55	Non-clonal
U23	M	32	23	Non-clonal
U24	F	25	32	Non-clonal

Table 4.2 Bacterial strains and culture conditions

Bioassay	Tested Strains	Reporter Strains	Culture Medium	Culture Condition	Reference
Proteases, Chitinase, Rhamnolipids	43 <i>P. aeruginosa</i> isolates and PAO1		TSB	10 mL in McCartney bottles, 37°C, 250 rpm, overnight or until OD ₆₀₀ above 1.2	Zhu <i>et al.</i> (2006)
Staphylolysin	43 <i>P. aeruginosa</i> isolates and PAO1		TSB	10 mL in McCartney bottles, 37°C, 250 rpm, overnight or until OD ₆₀₀ above 1.0	Zhu <i>et al.</i> (2006)
		<i>S. aureus</i> ATCC 12600	LB	20 mL in 50-mL sterile Falcon™ tube, 37°C, 250 rpm, overnight	Kessler <i>et al.</i> (1993)
Exoenzyme S	43 <i>P. aeruginosa</i> isolates and PAO1		LB with 1 mM EGTA	10 mL in McCartney bottles, 37°C, 250 rpm, overnight or until OD ₆₀₀ above 1.2	Jain <i>et al.</i> (2004)
AHLs	43 <i>P. aeruginosa</i> isolates and PAO1		AB medium supplemented with 0.2% glucose	10 mL in McCartney bottles, 37°C, 250 rpm, overnight or until OD ₆₀₀ above 1.2	Geisenberger <i>et al.</i> (2000)
		<i>C. violaceum</i> CV026	TSB	10 mL in McCartney bottles wrapped with aluminium foil, 30°C, overnight	Zhu <i>et al.</i> (2002)
		<i>A. tumefaciens</i> A136	minimal A ⁺ medium	10 mL in McCartney bottles wrapped with aluminium foil, 30°C, overnight	Zhu <i>et al.</i> (2002)

4.2.2 Sample Preparation

Following broth cultivation, the optical density at 600 nm (or at 660 nm for the staphylolysin assay), of each isolate tested was measured and was then subjected to a supernatant extraction. The cultures were transferred into sterile 15-mL Falcon™ tubes and were centrifuged at 5,000 rpm at 4°C for 15 minutes using a Beckman JA-14 rotor in a Beckman J2-21M/E High Speed Centrifuge. After which, supernatant was decanted and filtered through a 0.22-µm Millex™ Syringe Driven unit (Millipore). The filtered supernatant was either used immediately or stored at 4°C until required but for no longer than two weeks.

4.2.3 Bioassays for Virulence Factors

4.2.3.1 Total Proteases

Total protease activity was determined by using Hide azure powder (Sigma-Aldrich) as substrate, as described previously (Woods *et al.*, 1986). Briefly, 0.5 mL of *P. aeruginosa* culture supernatant was mixed with 1.5 mL of Hide azure powder substrate solution (see Appendix) and was then incubated in a shaking water bath set at 100 rpm and at 37°C for 3 hours. The precipitation was removed by centrifugation at 3,000 rpm for 10 minutes, and the optical density of each supernatant read at 595 nm using a Beckman DU®640 spectrophotometre (Beckman). The protease activity of each sample was normalised to the densities (OD₆₀₀) of the cultures grown. Experiments were carried out in triplicate on two separate occasions.

4.2.3.2 LasB Elastase

LasB elastase was measured by using Elastin-Congo red (ECR; Sigma-Aldrich) as substrate (Schad *et al.*, 1987). ECR solution (10 mg/mL) was made up by adding ECR into Tris-HCl-CaCl₂ buffer (0.1 M Tris HCl, 1 mM CaCl₂) and was mixed continuously using a magnetic stirrer until it was aliquoted into 24-well plates. Bacterial culture supernatant (0.25 mL) and 20 mM L-cysteine solution (10 µL) were then mixed with the aliquot (0.75 mL) of ECR solution. The mixture was placed in an Orbital shaking incubator (Biolab) set at 37°C, 100 rpm for 18 hours. Following incubation, the reaction was stopped with the addition of 0.1 mL 0.12 M EDTA (see Appendix). The mixture from each well was then

transferred to a sterile 1.5-mL microtube and was centrifuged at 15,000 rpm for 10 minutes using a Sigma 1K15 centrifuge (Sigma). The absorbance of supernatants was then measured at 495 nm using a Multiscan MCC (Titertek). A mixture of sterile (un-inoculated) TSB and ECR solution was included as a negative control. All isolates were tested in triplicate on two separate occasions.

4.2.3.3 Staphylolysin (or LasA Elastase)

S. aureus was used as substrate in this assay (Kessler *et al.*, 1993). Briefly, *S. aureus* cells were collected by centrifugation of an overnight culture of *S. aureus* ATCC 12600 (**Table 4.2**) at 5,000 rpm, 4°C for 15 minutes using a Beckman J2-21M/E High Speed Centrifuge (JA14 rotor). Cells were washed once with PBS and were then resuspended in 20 mL of 0.02 M Tris HCl pH 8.5. The cell solution was then boiled for 15 minutes. Once cooled to room temperature, it was diluted to an OD₆₆₀ of 2.0. One millimetre of *P. aeruginosa* culture supernatant was then mixed with 1-mL OD₆₆₀-adjusted *S. aureus* cell suspension. The absorbance of this mixture was read at 595 nm and was then incubated at 25°C in a water bath for 20 hours. The absorbance was then read again at 595 nm. The staphylolysin activity was determined as the rate of decrease in the absorbance caused by cell lysis. All isolates were tested in triplicate and two separate occasions.

4.2.3.4 Zymography

Zymography is based on the use of sodium dodecyl sulfate (SDS)-polyacrylamide gels co-polymerised with a protein substrate, which in this study was gelatin. All four types of *P. aeruginosa* proteases have the ability to re-nature after removal of SDS. Their proteolytic activities on a co-polymerised substrate could be analysed with this method by staining gels (zymograms) with Coomassie Blue. The sites of proteolysis can be seen as translucent bands on a Coomassie-Blue background (Frederiks & Mook, 2004). In this study, 7.5% SDS-polyacrylamide gels containing 0.1% w/v gelatin from porcine skin (Sigma-Aldrich) were used. Methods of making 7.5% SDS-polyacrylamide gels are described in the Appendix.

Twenty-one microlitres of unconcentrated supernatants were denatured in 7 µl of 4x SDS sample buffer (Appendix), and each denatured supernatant was then loaded into a single well of the gel. An electrophoresis was conducted in a Mini-Protean™ II electrophoresis

tank (Bio-Rad) at 100 Volts using a Model-200 power supply (Bio-Rad) for 1.5 – 2 hours, and was performed in a cold room (approximately 4°C). Precision-Plus Protein All Blue Standard (Bio-Rad) was used as a molecular weight marker.

Following electrophoresis, gels were carefully removed and were then washed in a 200-mL 2.5% (v/v) Triton X-100 solution (Sigma-Aldrich) for 1 hour in order to remove SDS and to re-nature the proteins followed by thoroughly rinsed in dH₂O. Gels were then incubated overnight at 37°C in 200-mL Tris-Gelatin reaction buffer (Appendix) to induce protease activities. Proteases, if present, would now react and lyse the gelatin at where they migrated to, according to their molecular weight.

After allowing proteases to digest the gelatin, gels or zymograms were stained in 0.25% (w/v) Coomassie Blue for 2 hours with gentle shaking at room temperature, and were de-stained for 3 hours in a de-staining solution (Appendix). The area of digestion appeared as clear bands against a darkly stained background where the substrate has been degraded by the protease (**Figure 4.1**). Zymograms were imaged using an Olympus C-3040 Zoom digital camera (Olympus). All samples were tested in triplicate on two separate occasions.

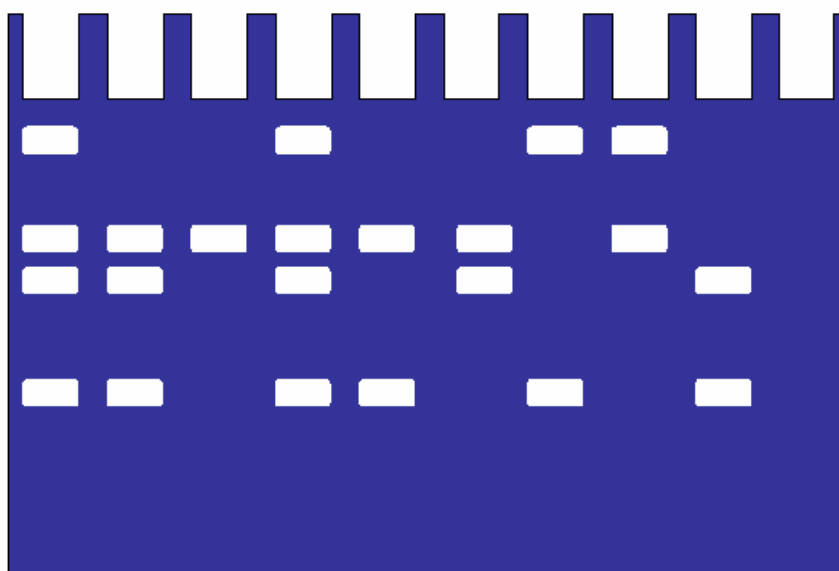


Figure 4.1 Illustration of a zymogram. Clear bands indicate proteolytic activities of corresponding proteases according to their molecular weight.

4.2.3.5 Chitinase

Chitinase activity was determined using Carboxymethyl-Chitin-Remazol Brilliant Violet (CM-Chitin-RBV) as substrate [method adapted from the manufacturer (Loewe Biochemica)]. Briefly, in a 1.5-mL microtube, 560 μ L of bacterial supernatant was added to 200 μ L CM-Chitin-RBV (Loewe Biochemica) with 40 μ L 1 M sodium phosphate buffer (Appendix). The mixture was incubated for 18 hours at 40°C. The reaction was then stopped with the addition of 200 μ L 2 M HCl and was left on ice for 15 minutes. Adding the 2 M HCl solution also resulted in a precipitation of non-degraded CM-Chitin-RBV. Similar to assays for total proteases and LasB elastase, chitinase present in supernatants cleaved RBV from CM-Chitin. RBV, which was soluble in aqueous, was separated from non-degraded and precipitated CM-Chitin-RBV by a centrifugation at 4°C for 15 minutes at 14,000 rpm using a Sigma 1K15 centrifuge (Sigma). The absorbance of supernatants containing liberated RBV was then measured at 550 nm. Sterile (un-inoculated) TSB was used as a negative control. All isolates were tested in triplicate on two separate occasions.

4.2.3.6 Rhamnolipids

The presence or absence of rhamnolipids was assessed by using a minimal supplement plate containing cetyltrimethylammonium bromide (CTAB) assay (Siegmund & Wagner, 1991). This agar plate test was based on an anionic property of the rhamnolipids. The rhamnolipids (anion) when paired with CTAB (cation) formed insoluble ion pairs. The agar plates also contained a basic dye (methylene blue) to enhance visibility of these insoluble compounds. Two microlitres of each *P. aeruginosa* culture (**Table 4.2**) were spotted on the detecting plates and allowed to dry prior to incubation at 37°C for 24 hours. After which, plates were incubated at 25°C for further 48 hours. *P. aeruginosa* that produced the rhamnolipids appeared as a blue halo-surrounded colony. Methods of making the rhamnolipids detection plates are described in the Appendix.

4.2.3.7 Exoenzyme S (ExoS)

Production of ExoS was detected using Western blot. Briefly, supernatants of overnight cultures (as described in **Table 4.2**) were boiled for 5 minutes prior to 12% (w/v) non-reducing SDS-PAGE. The electrophoresis was conducted in a Mini-Protean™ II electrophoresis tank (Bio-Rad) at 100 Volts using a Model-200 power supply (Bio-Rad) for 2 hours. Separated protein was electrophoretically blotted at 30 Volts overnight onto a

Tris-Glycine buffer equilibrated polyvinylidene difluoride (PVDF) membrane (Immuno-Blot™, Bio-Rad) using the mini-blotting system (Hoefer Scientific) This blotting step was performed in a cold room (approximately 4°C). Prior to an antibody conjugation, blots were blocked in 3% (w/v) BSA (Sigma-Aldrich) in PBS for 1 hour.

An optimisation of a primary antibody was conducted. PAO1, which was previously shown to produce ExoS but not ExoU (Hauser *et al.*, 2002), was used as a positive control. Chicken polyclonal ExoS antibody (Abcam) was diluted in dH₂O to dilutions at 1:2,500 (recommended dilution by the manufacturer), 1:5,000, 1:8,000 and 1:10,000. Blots of PAO1 were incubated with 5 mL of each antibody dilution overnight and were developed in anti-chicken antibody alkaline phosphatase conjugate (1:1000) (Sigma-Aldrich) using BCIP/NBT solution (Sigma-Aldrich). Dilution of the primary antibody at 1:10,000 gave a clear and visible band, and thus was chosen for subsequent testing.

Blots of all isolates were performed as described above. Precision-Plus Protein All Blue Standard (Bio-Rad) was used as a molecular weight marker. Blotted PVDF membranes were incubated in 10 mL 1:10,000 ExoS antibody (Abcam) at room temperature overnight on the rotating platform (Ratex). After this, the PVDF membranes were washed three times with PBS for 1, 5 and 10 minutes, respectively. Then, blots were immersed in AP buffer (Appendix) for 15 minutes followed by incubation in the anti-chicken antibody conjugated with alkaline phosphatase (1:1000) (Sigma-Aldrich) for 90 minutes. They were again washed three times with PBS for 1, 5 and 10 minutes, respectively, and were immersed in AP buffer for 15 minutes with repeated immersion in AP buffer for another 10 minutes. Blots were then developed using BCIP/NBT solution (Sigma-Aldrich). Tests were carried out in duplicate on two different occasions.

4.2.4 AHLs Production

Production of AHLs was detected by using a thin-layer chromatography (TLC). Briefly, tested *P. aeruginosa* isolates were grown in AB media supplemented with 0.2% glucose as described in **Table 4.2**. Approximately 10 mL of overnight culture supernatants were subjected for an AHL extraction. In 50-mL glass test tubes, the culture supernatants were extracted by thoroughly mixing with equal volumes of HPLC-graded ethyl acetate (Sigma-Aldrich). The mixtures were then allowed to separate into two layers, and AHL molecules were dissolved in the top layer of ethyl acetate. Liquid from this layer was then removed

into a sterile McCartney bottle and allowed to evaporate and dry in a fume hood (Plastic Constructions). The residue of AHLs precipitant was then re-dissolved in 100 – 200 μ L of ethyl acetate (Sigma-Aldrich) and was preserved in a glass vial (Samco) and stored at - 20°C until tested.

Prior to the chromatography, 200 mL of methanol/dH₂O (60:40 v/v) were poured into a TLC tank (Cole-Parmer), the tank was closed with a glass lid and allowed to acclimatise for 30 minutes. Four microlitres of each sample were spotted on a pre-warmed C₁₈ reversed-phase TLC plate (Whatman) as illustrated in **Figure 4.2**. Once all spots were dry, the plates were transferred into the TLC tank. The chromatograms were developed as the solvent moved (**Figure 4.2**) and the AHLs migrated along with the solvent. The chromatograms were stopped once the solvent front moved to within 2 cm of the top of the plates (approximately 3 – 4 hours), and were left to dry in a fume hood (approximately 30 – 60 minutes).

C. violaceum CV026 and *A. tumefaciens* A136 were used as AHLs detecting strains. *C. violaceum* generally used hexanoyl-homoserine lactone (C6-HSL or HHL) to produce purple pigment (violacein). The strain CV026 was a mutant strain that could not produce HHL but could still produce violacein in respond to external (or administered) AHLs with short side-chains (i.e. BHL and HHL) (McClellan *et al.*, 1997; Winson *et al.*, 1995). Similarly, *A. tumefaciens* strain A136 had no autoinducer synthase but contained TraR and a TraR-regulated *traI-lacZ* plasmid. TraR recognised all AHLs, including OdDHL, N-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL or ODHL), OHHL and HHL but not BHL, and subsequently activated *traI-lacZ* to produce β -galactosidase which reacted with 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) and produced blue/green pigment (Zhu *et al.*, 1998). To prepare the cultures of reporter strains, bacteria were grown overnight as described in **Table 4.2**.

The overnight cultures of *C. violaceum* CV026 and *A. tumefaciens* A136 were mixed with either 100 mL of 1.5% melted TSB agar or 100 mL of 1.5% melted minimal A⁺ medium agar containing X-Gal (40 μ g/mL) (maintained at approximately 45°C in a water bath), respectively, and were immediately poured on to the dry TLC plates. Once solidified, the plates were covered with aluminium foil (to protect light) and incubated at 30°C for 48 hours. Images of the chromatograms were obtained by using a scanner (Canon). Purified BHL and OdDHL used as positive controls were kindly supplied by Dr. Kumar

(Department of Chemistry, University of New South Wales). All samples were tested three times.

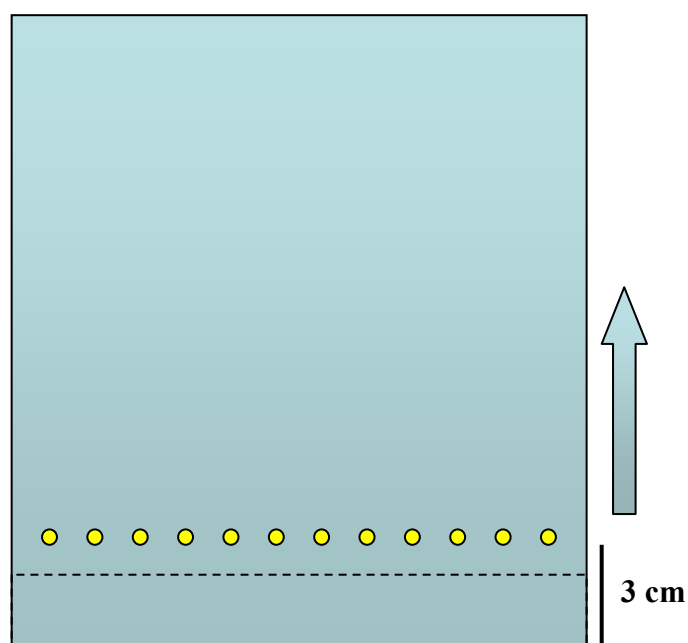


Figure 4.2 C_{18} reverse-phase TLC plate. Samples were spotted at 3 cm above the bottom edge (maximum of 12 spots per one 20 x 20 cm plate). Plates were then placed in a tank containing solvent (60:40 methanol/dH₂O). Dash line indicates the level of solvent in the tank. Arrow indicates the direction of solvent migration.

4.2.5 Antibiotic Susceptibility Test

All isolates were tested for antibiotic susceptibility using the VITEK[®] Automated system (BioMérieux). All tests were performed by the Department of Microbiology at RPAH.

4.2.6 Statistical Analyses

Because of the small sample size of AES-2, data of both AES-1 and AES-2 groups were combined, and comparison analyses were performed between the clonal (AES-1 and AES-2) group and the non-clonal group. Pearson's Chi Square test was used to analyse the secretion of the virulence factors and AHL molecules between clonal and non-clonal groups. *P* values of equal or less than 0.05 were considered significant. Relative Risk and

95% confidence intervals were used to determine the strength of the associations. The levels of virulence factor production were analysed using standard *t*-test for normally distributed data and using Mann-Whitney *U* test for nonparametric data. Relationships between protease, elastase activity and patient age were calculated using Pearson's correlation coefficient (*r*).

4.3 Results

4.3.1 Characteristics of Colony Morphology and Mucoid Production

The colony morphology of each isolate grown on a Columbia HBA is shown in **Table 4.3**. Mucoid production was found in 41 (95%) isolates. Of these, 9 (21%) showed + mucoidy, 25 (58%) ++ mucoidy, and seven (16%) +++ mucoidy (**Table 4.3**). A greater proportion of non-clonal isolates produced +++ mucoidy than clonal isolates (7/24 versus 0/19) but proportions of isolates with mucoidy at the + and ++ level were similar between clonal and non-clonal groups. Overall, there was no relationship between mucoidy and genotype, and there were no specific colonial morphologies found within AES-1, AES-2 and non-clonal groups.

Table 4.3 Colony morphology of all isolates

Isolate	Shape	Margin	Size	Elevation	Mucoidy	Colour	Hemolysis
AES-1							
C1	Circular	entire	1-2 mm	raised	++	grey/white	no
C2	Circular	entire	2-4 mm	raised	++	shiny creamy	no
C3	Circular	entire	1-2 mm	raised	++	greeny grey	partial
C4	Circular	entire	1 mm	raised	+	shiny creamy white	complete
C5	Circular	entire	2-3 mm	raised	++	dark grey	partial
C6	Circular	entire	1 mm	raised	++	white	no
C7	Circular	entire	2-3 mm	raised	+	grey/white	no
C8	Circular	entire	1-2 mm	raised	++	grey	no
C9	Circular	entire	4-5 mm	flat	-	grey	no
C10	Circular	entire	1-2 mm	raised	++	creamy grey	partial
C11	Circular	entire	3-4 mm	raised	++	shiny creamy	no
C12	Circular	entire	3-4 mm	convex	++	creamy grey	no
C13	Circular	entire	2-4 mm	raised	++	grey/white	no
C14	Circular	entire	2 mm	raised	+	dark grey	partial
AES-2							
C15	Circular	entire	1-2 mm	raised	++	creamy grey	partial
C16	Circular	entire	1 mm	raised	++	grey	no
C17	Circular	entire	1 mm	raised	+	grey with metallic sheen	partial
C18	Circular	irregular	3-4 mm	raised	++	brownish grey	no
C19	Circular	entire	1-2 mm	raised	++	greenish brown	partial

(continued next page)

Table 4.3 continued

Isolate	Shape	Margin	Size	Elevation	Mucoidy	Colour	Hemolysis
Non-Clonal							
U1	Circular	entire	1.5-2 mm	convex	+++	grey	no
U2	Circular	entire	2 mm	raised	++	greeny grey	partial
U3	Circular	entire	<1-1 mm	raised	++	grey	no
U4	Circular	entire	1-2 mm	raised	++	grey with metallic sheen	no
U5	Circular	entire	1 mm	raised	++	grey with metallic sheen	partial
U6	Circular	entire	1 mm	convex	+++	grey	partial
U7	Circular	entire	3-5 mm	convex	+++	shiny clear	complete
U8	Circular	entire	1-2 mm	flat	-	creamy	no
U9	Circular	entire	1-2 mm	raised	+++	shiny creamy	no
U10	Circular	entire	1-2 mm	raised	++	grey	complete
U11	Circular	entire	1-2 mm	raised	+++	shiny grey	no
U12	Circular	entire	<1-1 mm	raised	++	shiny grey	no
U13	Circular	undulate	1-2 mm	raised	+	shiny grey	partial
U14	Circular	entire	3-5 mm	convex	+++	greeny grey	complete
U15	Circular	entire	1 mm	raised	+	grey	no
U16	Circular	entire	2-3 mm	raised	++	greeny grey	no
U17	Circular	entire	1 mm	raised	+	greeny grey	partial
U18	Circular	entire	1 mm	convex	+++	creamy grey	no
U19	Circular	undulate	1-3 mm	raised	++	grey with metallic sheen	no
U20	Circular	irregular	1-2 mm	raised	++	dark green	no
U21	Circular	entire	0.5 mm	raised	+	creamy	no
U22	Circular	entire	1-2 mm	raised	++	shiny creamy	no
U23	Circular	entire	1-2 mm	raised	+	shiny white/clear	no
U24	Circular	irregular	2-4 mm	raised	++	grey white	partial
PAO1	Circular	irregular	4-5 mm	flat	-	greeny brown	partial

4.3.2 Comparison of Total Proteases, Elastase, Staphylolysin Production between Clonal and Non-Clonal Strains

Proteases showed the ability to degrade Hide azure powder and the level of production was directly measured from the colour (blue) intensity of the assay's supernatant (**Figure 4.3**). Overall, 37 (86%) of the 43 isolates produced the proteases. Of these, clonal isolates (14 AES-1 and 5 AES-2) were significantly more likely than non-clonal isolates to produce the proteases (all 19 clonal isolates versus 18 of the 24 non-clonal isolates; $p = 0.019$). As shown in **Table 4.4**, levels of the total proteases among all isolates were variable (and not normally distributed), and the median level of total protease production of clonal isolates (160 mU) was higher than non-clonal isolates (78 mU) (**Figure 4.4**), but this was not statistically significant [$p = 0.152$ (Mann-Whitney U test)].

Like the total proteases, LasB elastase activity was measured from the colour (red) intensity of soluble dye in the assay using ECR as the substrate (**Figure 4.3**). Of the 43 tested isolates, 32 (74%) isolates exhibited elastase activity detected by this assay. Again, clonal isolates [17 (89%) of the 19] were more likely to produce the LasB elastase than non-clonal isolates [15 (63%) of the 24] ($p = 0.044$). Levels of the elastase activity ranged from 3 to 367 mU (**Table 4.4**). The median level of LasB elastase production of clonal isolates (42 mU) was greater than non-clonal isolates (9 mU) (**Figure 4.4**), but this was not statistically significant [$p = 0.120$ (Mann-Whitney U test)].

Staphylolysin or LasA elastase was quantified by measuring the differences in density of *S. aureus* cells that were lysed after a 20-hour incubation. Overall, 40 (94%) of the 43 isolates produced staphylolysin. There was no significant difference in the number of productive isolates between clonal [16/19 (84%)] and non-clonal [24/24 (100%)] groups ($p = 0.079$). Activities of this enzyme were variable among isolates, and in some cases were very low (**Table 4.4**). The median level of production of clonal isolates (2 mU) was lower than non-clonal isolates (4.5 mU) (**Figure 4.4**), but this was not statistically significant [$p = 0.055$ (Mann-Whitney U test)].

As expected, there was a significant correlation between total proteases and elastase activities ($r = 0.847$, $p < 0.001$) (**Figure 4.5**). However, this correlation did not occur between the total proteases and the activity of staphylolysin (**Figure 4.5**).

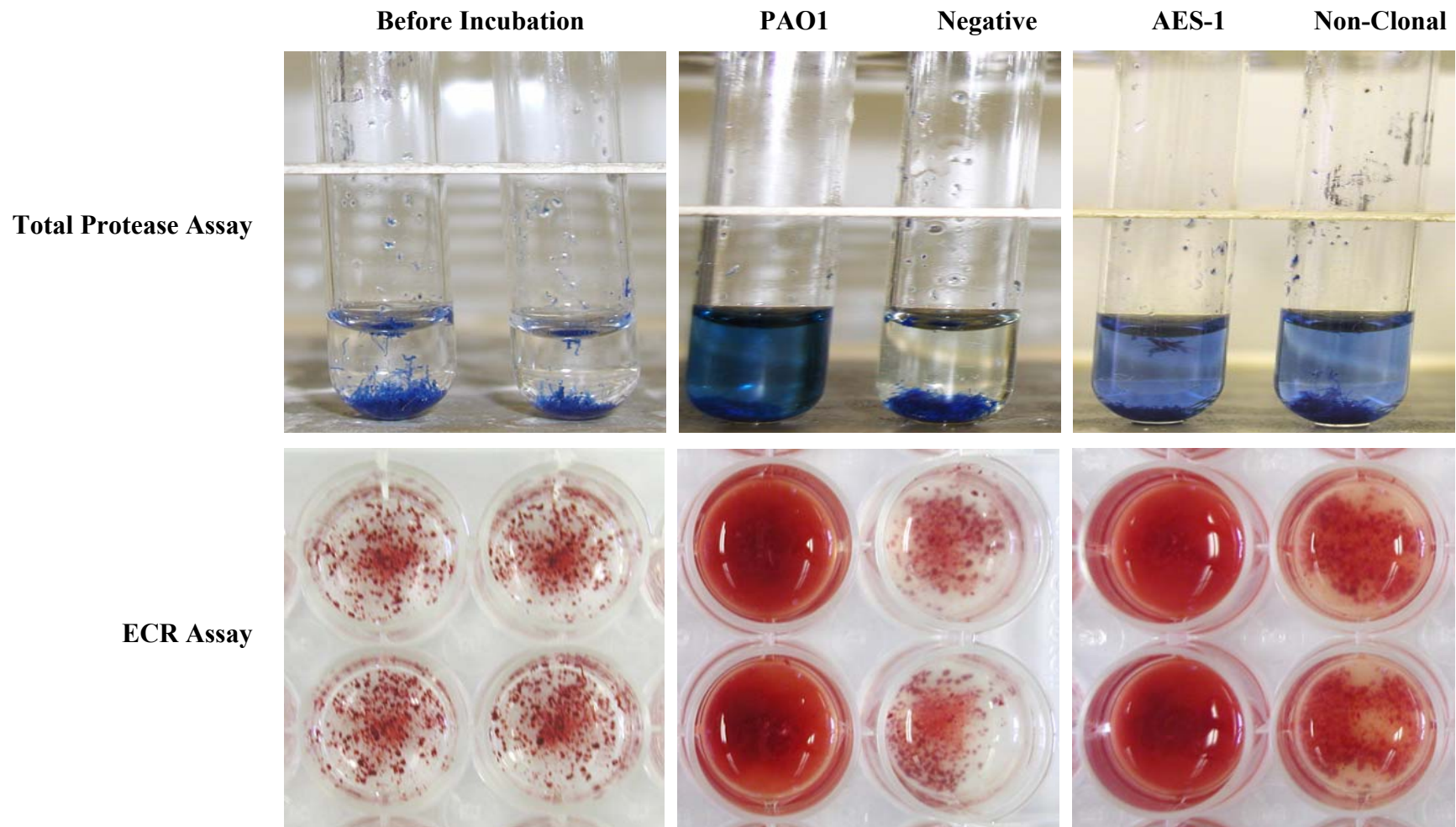


Figure 4.3 Illustration of total protease assay (upper panel) and ECR elastase assay (lower panel).

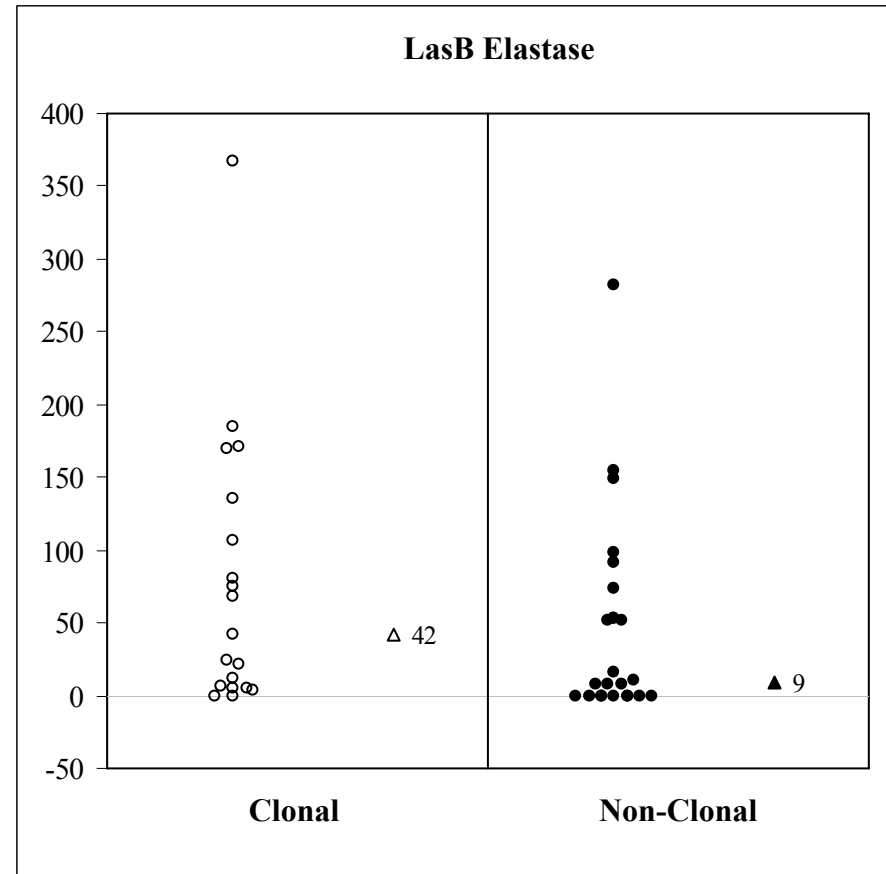
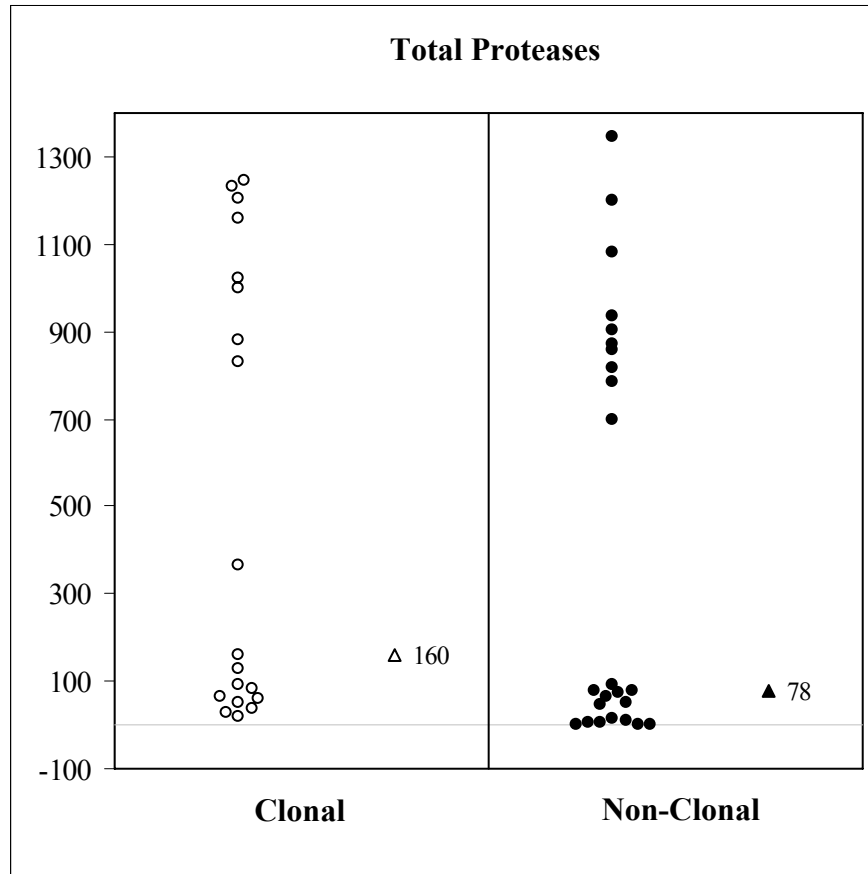
Table 4.4 Virulence factors of all tested isolates

Isolate ID	Age	Mucoidy	Protease Activities			7.5% Zymogram			Chitinase Activity	Rhamnolipids	ExoS (Western Blot)
			Total Protease	LasB Elastase	LasA Elastase	LasB/LasA Elastase	Alkaline Protease	Protease IV			
AES-1											
C1	20	++	60 ± 14	0 ± 0	88 ± 0	-	-	-	0 ± 0	+	+
C2	24	++	35 ± 4	0 ± 0	1 ± 1	-	-	-	0 ± 0	+	+
C3	22	++	1244 ± 188	367 ± 168	18 ± 6	+	+	+	38 ± 12	+	+
C4	31	+	28 ± 17	5 ± 6	2 ± 3	-	+	+	40 ± 8	+	+
C5	24	++	51 ± 24	5 ± 2	2 ± 1	+	+	+	15 ± 7	+	+
C6	20	++	66 ± 18	12 ± 10	2 ± 0	+	+	+	18 ± 4	-	+
C7	19	+	1024 ± 103	75 ± 53	0 ± 0	+	+	+	16 ± 8	+	-
C8	25	++	830 ± 115	42 ± 12	0 ± 0	+	+	+	37 ± 28	+	+
C9	22	-	1232 ± 50	81 ± 61	16 ± 0	+	+	+	60 ± 8	+	-
C10	20	++	1205 ± 70	171 ± 121	51 ± 13	+	+	+	108 ± 16	+	+
C11	22	++	17 ± 5	22 ± 17	1 ± 0	+	+	+	6 ± 5	-	+
C12	29	++	129 ± 19	107 ± 98	1 ± 1	+	+	+	76 ± 22	-	+
C13	32	++	1157 ± 46	135 ± 17	3 ± 0	+	-	+	48 ± 6	+	+
C14	22	+	160 ± 16	24 ± 14	5 ± 3	+	+	+	59 ± 25	+	+
AES-2											
C15	18	++	997 ± 92	170 ± 51	3 ± 4	+	+	+	89 ± 14	+	+
C16	28	++	92 ± 12	3 ± 0	2 ± 0	-	-	+	89 ± 35	+	-
C17	29	+	878 ± 35	184 ± 35	6 ± 0	+	+	-	58 ± 8	+	+
C18	26	++	81 ± 33	6 ± 7	0 ± 0	-	-	+	0 ± 0	+	+
C19	37	++	366 ± 76	68 ± 0	3 ± 1	+	+	+	50 ± 26	+	+

(continued next page)

Table 4.4 continued

Isolate ID	Age	Mucoidy	Protease Activities			7.5% Zymogram			Chitinase Activity	Rhamnolipids	ExoS (Western Blot)
			Total Protease	LasB Elastase	LasA Elastase	LasB/LasA Elastase	Alkaline Protease	Protease IV			
Non-Clonal											
U1	20	+++	1198 ± 84	282 ± 57	81 ± 0	+	-	+	77 ± 30	+	+
U2	21	++	699 ± 259	73 ± 23	88 ± 2	+	+	+	35 ± 11	+	+
U3	28	++	857 ± 47	53 ± 21	9 ± 3	+	+	-	51 ± 3	-	-
U4	29	++	0 ± 0	0 ± 0	2 ± 0	-	-	-	17 ± 14	+	-
U5	21	++	1346 ± 74	91 ± 5	31 ± 0	+	+	+	66 ± 4	+	+
U6	19	+++	784 ± 78	149 ± 137	91 ± 100	+	-	+	40 ± 7	+	-
U7	48	+++	901 ± 351	53 ± 59	2 ± 3	+	+	+	168 ± 41	+	+
U8	35	-	74 ± 77	0 ± 0	2 ± 1	-	-	-	0 ± 0	-	+
U9	34	+++	1082 ± 26	98 ± 29	69 ± 1	+	-	-	44 ± 2	+	+
U10	36	++	66 ± 9	0 ± 0	1 ± 0	-	-	-	22 ± 16	+	+
U11	33	+++	0 ± 0	0 ± 0	2 ± 1	-	+	-	53 ± 4	+	+
U12	26	++	78 ± 16	7 ± 9	3 ± 0	-	-	-	0 ± 0	+	-
U13	19	+	0 ± 0	0 ± 0	5 ± 3	-	-	-	25 ± 8	+	+
U14	20	+++	935 ± 30	155 ± 31	4 ± 1	+	+	+	87 ± 27	+	+
U15	58	+	49 ± 18	8 ± 22	2 ± 1	-	-	-	48 ± 39	-	+
U16	22	++	90 ± 70	10 ± 14	2 ± 0	-	-	-	59 ± 14	+	+
U17	26	+	818 ± 34	52 ± 74	29 ± 0	+	-	-	61 ± 9	+	+
U18	38	+++	47 ± 52	7 ± 10	7 ± 5	-	-	-	0 ± 0	-	-
U19	31	++	13 ± 1	0 ± 0	3 ± 0	-	-	-	0 ± 0	+	-
U20	19	++	0 ± 0	16 ± 13	2 ± 1	-	-	-	71 ± 40	+	-
U21	30	+	873 ± 100	51 ± 25	61 ± 1	+	+	-	39 ± 30	-	+
U22	34	++	0 ± 0	0 ± 0	5 ± 1	-	-	-	0 ± 0	+	+
U23	32	+	0 ± 0	0 ± 0	9 ± 12	-	-	-	0 ± 0	+	+
U24	25	++	77 ± 9	0 ± 0	3 ± 3	-	+	-	116 ± 42	+	+
PAO1		-	957 ± 91	277 ± 20	87 ± 5	+	+	+	106 ± 12	+	+



(Figure 4.4 explanation in the next page)

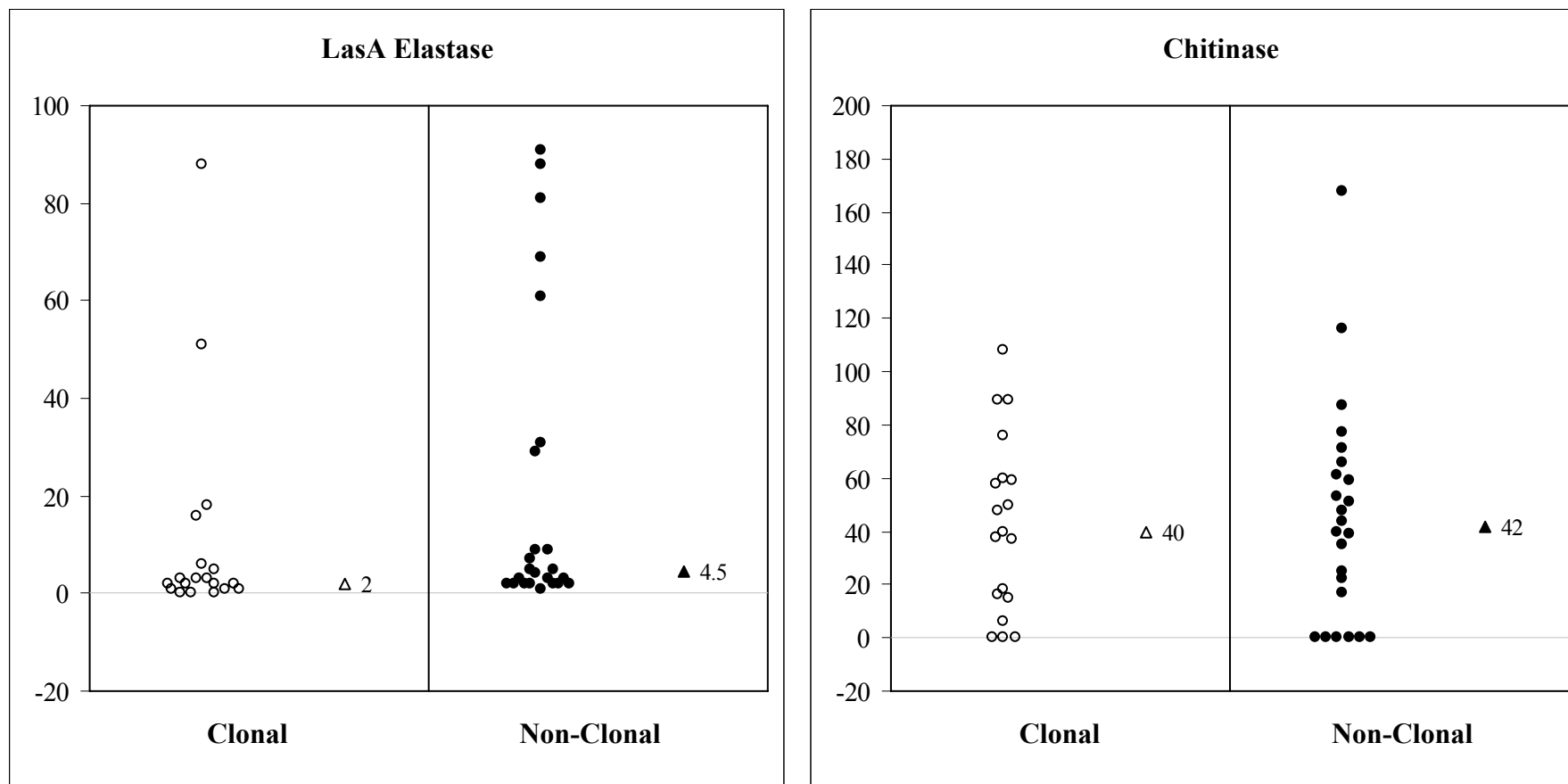


Figure 4.4 Comparison of levels of activities of total proteases, LasB elastase, LasA elastase and chitinase between clonal and non-clonal isolates. Y-axis indicates levels of activities (mU) according to each chart. Each circle represents an individual isolate; blank circles represent clonal isolates while filled (black) circles represent non-clonal. Blank triangles and filled triangles represent median levels of clonal group and non-clonal group, respectively. Median levels of each data were also indicated as number next to triangles.

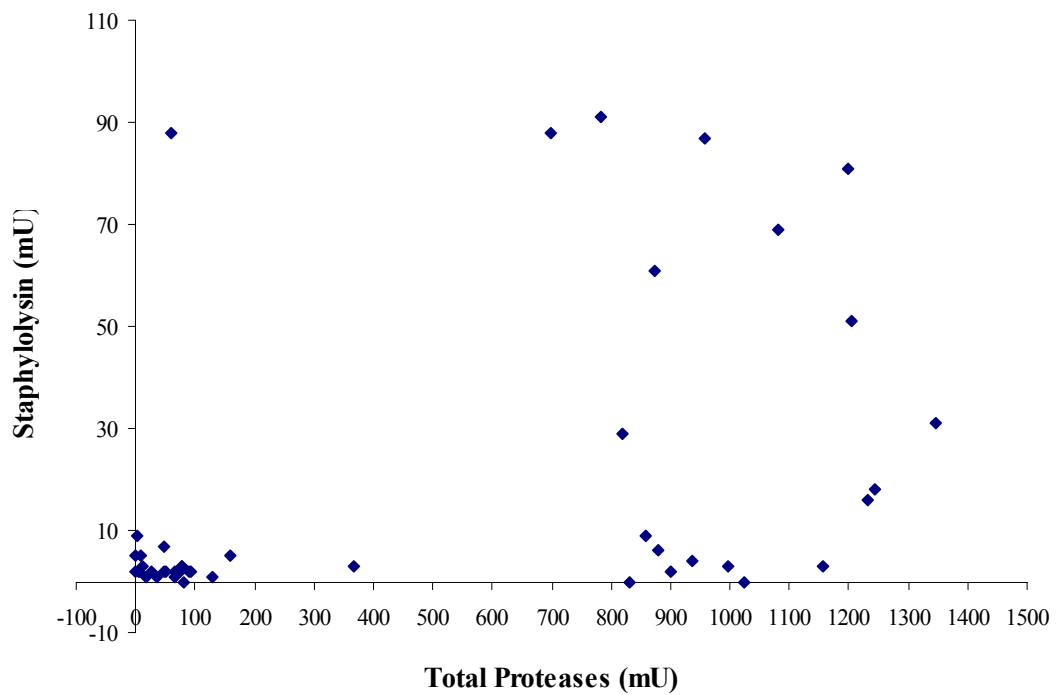
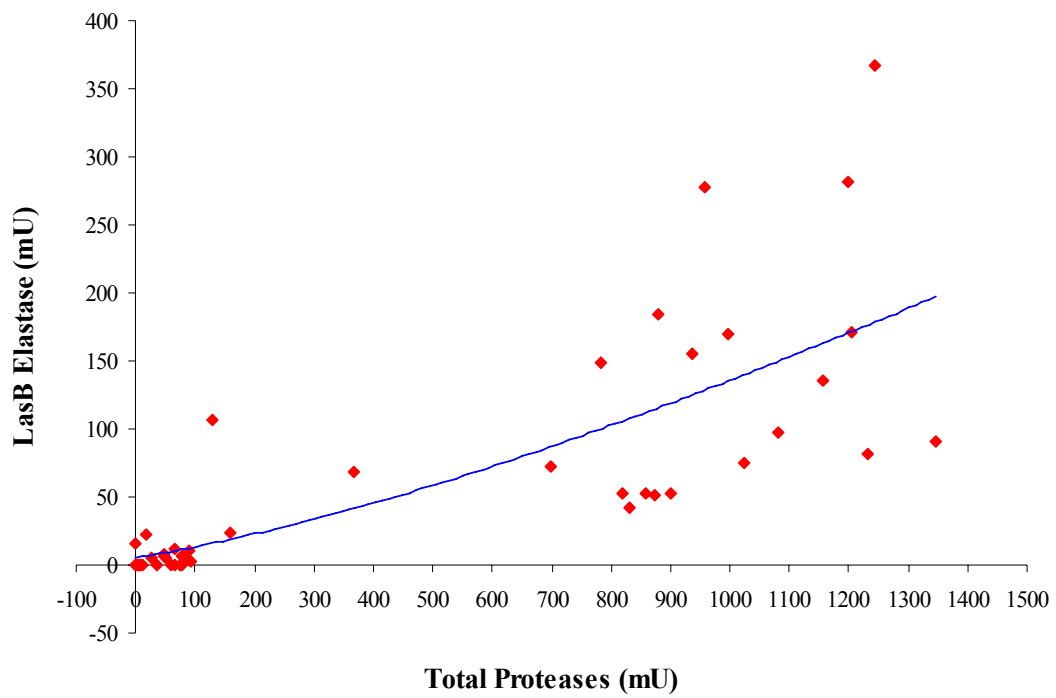


Figure 4.5 Correlation of the level of total protease production with LasB elastase (upper) and with LasA elastase (lower). The significant relationship was found only between total protease and LasB elastase activities ($r = 0.847$, $p < 0.001$).

4.3.3 Zymographic Profiles of Clonal and Non-Clonal Strains

Zymography was conducted using 7.5% SDS-PAGE incorporating gelatin as substrate. A protease profile produced by zymograms contained up to four bands for each sample. **Figure 4.6** shows a zymogram with bands detected at molecular masses of approximately 350 kDa, 120 kDa, 98 kDa and 56 kDa. LasB elastase and LasA elastase were represented by bands at 120 kDa and 98 kDa, but in many cases, these two bands were very close to each other making it difficult to accurately differentiate (**Figure 4.6**). Thus, the presence of these bands was interpreted as a positive of a combined LasB elastase and LasA elastase. Alkaline protease was represented by the band at 51 kDa. Although the protease IV enzyme has a molecular mass of 26 kDa, it aggregates under mild SDS denaturing conditions, and is resolved at a molecular weight of 350 kDa (Caballero *et al.*, 2001) (**Figure 4.6**).

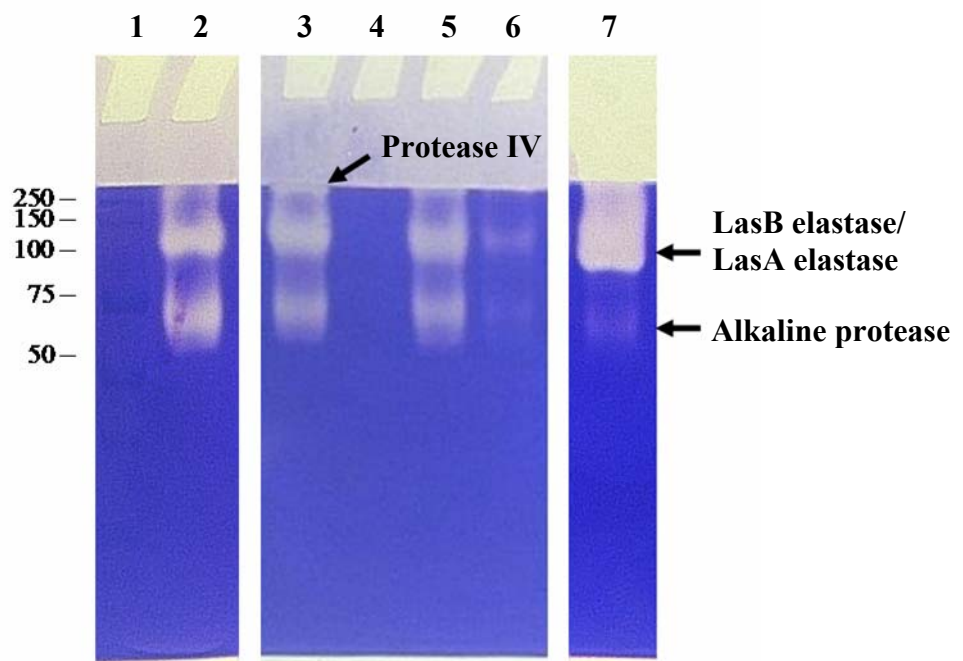


Figure 4.6 Zymographic patterns of proteases. As seen in lane 7 (PAO1), bands of LasB elastase and LasA elastase join together and are difficult to be separately identified. Lane 1 is molecular weight marker. Lanes 2 – 7 are U3, C3, C2, C15, C17 and PAO1, respectively.

Overall, 24 (56%) of the 43 isolates produced LasB elastase/LasA elastase and 22 (51%) of 43 alkaline protease (**Table 4.4**). The amount of protease secreted by individual isolates, which was indirectly indicated by band intensity, was variable. All 24 isolates that produced elastase on zymography also showed elastase activity in the ECR assay (**Table 4.4**). However, 8 isolates that had negative zymographic LasB elastase/LasA elastase displayed low levels of activity using the ECR assay (**Table 4.4**). Similar to the results of the ECR assay, the isolates producing LasB elastase/LasA elastase detected by zymography occurred significantly more frequently in the clonal group [14/19 (74%)] than in the non-clonal group [10/24 (42%)] ($p = 0.036$). There were similar findings in the production of alkaline protease and of protease IV. Fourteen (74%) of the 19 clonal isolates showed a band representing the alkaline protease compared to only 8 (33%) of the 24 non-clonal isolates ($p = 0.009$); 16 (84%) clonal isolates showed a band representing the protease IV while this was found in only 6 (25%) of the non-clonal isolates ($p < 0.001$).

4.3.4 Production of Chitinase and Rhamnolipids

Thirty-four (79%) of the 43 isolates showed chitinase activity, but there was no relationship between the clonality and the production of this virulence factor with 16 (84%) of the 19 clonal isolates producing chitinase versus 18 (75%) of the 24 non-clonal isolates producing ($p = 0.461$) (**Table 4.4**). The mean levels of activity were also not significantly different between the two groups (42 mU for clonal group and 45 mU for non-clonal; $p = 0.831$) (**Figure 4.4**). Similar results were also found in the production of rhamnolipids. The rhamnolipids were produced by 16 (84%) clonal isolates and 19 (79%) non-clonal isolates ($p = 0.673$) (**Table 4.4**).

4.3.5 Production of Exoenzyme S

Western blot was conducted to detect the production of ExoS. The concentration of a primary antibody was optimised, and the concentration at 1:10,000 gave a single strongly visible band at 36 kDa corresponding to the ExoS molecular weight (Hauser *et al.*, 2002). Overall, the ExoS production was found in 33 (77%) of the 43 isolates (**Table 4.4**). The intensity of the bands was variable among isolates (**Figure 4.7**). There was no significant relationship between bacterial strains and the production of ExoS [16/19 (84%) clonal isolates versus 17/24 (71%) non-clonal isolates; $p = 0.302$].

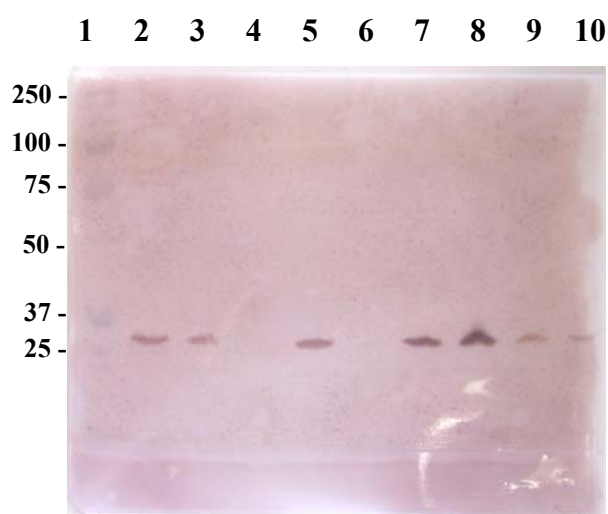


Figure 4.7 ExoS Western blotting. Lane 1 is molecular weight marker. Lanes 2 – 10 are PAO1, C6, C7, C8, C9, C10, C11, C12 and C13, respectively.

4.3.6 Production of AHLs and Comparison between Clonal and Non-clonal Strains

When results from the two AHL biosensors were combined, the number of spots varied from none to five. Thirty-two (74%) of the 43 isolates produced spots corresponding to one or more AHLs (**Table 4.5**). Using the reporter strain *C. violaceum* CV026, two spots, most likely representing BHL and HHL (Geisenberger *et al.*, 2000; Zhu *et al.*, 2002) were noted. Nineteen (44%) of all isolates were found to produce both BHL and HHL spots while 12 (28%) isolates showed only a single spot corresponding to HHL but not BHL (**Table 4.5**). There was no isolate that produced only BHL but not HHL. Using the *A. tumefaciens* A136 reporter strain, up to 4 spots were detected, most likely corresponding to OddHL, ODHL, OHHL and HHL (Zhu *et al.*, 2002) (**Figure 4.8**). At least one of these AHL spots was detected in 16 (37%) isolates. Of these, OddHL was detected in 8 (19%) isolates, ODHL in 11 (26%) isolates and OHHL in 9 (21%) isolates. Eleven (26%) isolates did not show any AHL spots at all using these two reporter strains. Overall, there was no relationship between the production of AHLs and the clonality of *P. aeruginosa*, apart from that of OHHL. Clonal strains were more likely to secrete OHHL than the non-clonal strains ($p = 0.030$) Results of individual isolates are shown in **Table 4.5** and data comparing clonal and non-clonal groups are shown in **Table 4.6**.

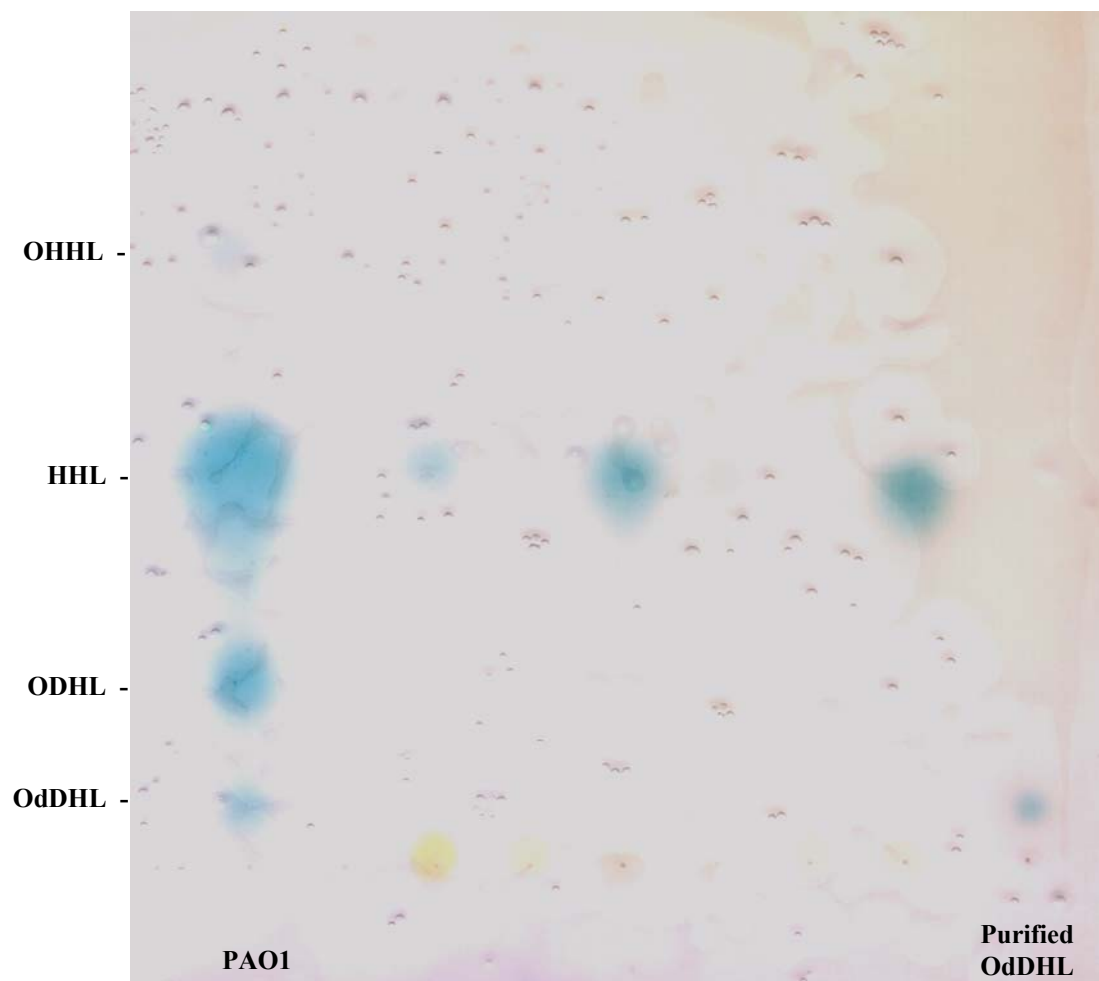


Figure 4.8 Illustration of TLC overlaid by *A. tumefaciens* A136. Spots of four different types of AHLs (OddDHL, ODHL, HHL and OHHL) are indicated.

Table 4.5 Production of AHLs of all tested isolates

Isolate	BHL	HHL	OHHL	ODHL	OdDHL
AES-1					
C1	+	+	+	-	-
C2	-	-	-	-	-
C3	+	+	+	-	-
C4	+	+	-	-	-
C5	-	-	-	-	-
C6	-	+	+	-	-
C7	-	+	-	-	-
C8	-	+	+	-	-
C9	+	+	-	-	-
C10	+	+	-	-	-
C11	-	-	-	-	-
C12	-	+	-	-	-
C13	-	+	-	-	-
C14	-	+	-	-	-
AES-2					
C15	-	+	+	+	+
C16	+	+	-	-	-
C17	+	+	+	+	+
C18	+	+	+	+	+
C19	+	+	-	-	-
Non-Clonal					
U1	+	+	-	+	+
U2	-	+	-	+	+
U3	+	+	-	-	-
U4	-	-	-	-	-
U5	+	+	-	+	+
U6	-	+	-	-	-
U7	-	+	-	-	-
U8	-	-	-	-	-
U9	+	+	-	+	-
U10	-	-	-	-	-
U11	-	-	-	-	-
U12	-	+	-	-	-
U13	-	-	-	-	-
U14	+	+	-	+	-
U15	+	+	-	-	-
U16	+	+	+	+	+
U17	+	+	-	-	-
U18	-	+	-	+	+
U19	-	-	-	-	-
U20	+	+	-	-	-
U21	+	+	+	-	-
U22	-	-	-	-	-
U23	-	-	-	+	-
U24	-	-	-	-	-
PAO1	+	+	+	+	+

Table 4.6 Summary of virulence factors and AHLs production comparing between clonal isolates and non-clonal isolates

	Total isolates (n=43)	Clonal (n=19)	Non-clonal (n=24)		Statistics Clonal vs Non-clonal
Total proteases					
Presence	37 (86%)	19 (100%)	18 (75%)	$p = 0.019$	RR = 1.3 (95% CI = 1.1-1.7)
production level ^a	92	160	78	$p = 0.152$	
ECR Elastase					
Presence	32 (74%)	17 (89%)	15 (63%)	$p = 0.044$	RR = 1.4 (95% CI = 1.0-2.0)
production level ^a	22	42	9	$p = 0.120$	
Zymography					
LasB/LasA elastase	24 (56%)	14 (74%)	10 (42%)	$p = 0.036$	RR = 1.8 (95% CI = 1.0-3.0)
Alkaline protease	22 (51%)	14 (74%)	8 (33%)	$p = 0.009$	RR = 2.2 (95% CI = 1.2-4.1)
Protease IV	22 (51%)	16 (84%)	6 (25%)	$p < 0.001$	RR = 3.4 (95% CI = 1.6-6.9)
Staphylolysin					
Presence	40 (94%)	16 (84%)	24 (100%)	$p = 0.079$	
production level ^a	3	2	4.5	$p = 0.055$	
Chitinase					
Presence	34 (79%)	16 (84%)	18 (75%)	$p = 0.461$	
production level ^b	44	42	45	$p = 0.831$	
Rhamnolipids	35 (81%)	16 (84%)	19 (79%)	$p = 0.673$	
ExoS production	33 (77%)	16 (84%)	17 (71%)	$p = 0.302$	
AHL detection					
BHL	19 (44%)	9 (47%)	10 (42%)	$p = 0.708$	RR = 2.2 (95% CI = 1.2-3.9)
HHL	31 (72%)	16 (84%)	15 (63%)	$p = 0.115$	
OHHL	9 (21%)	7 (37%)	2 (8%)	$p = 0.030$	
ODHL	11 (26%)	3 (16%)	8 (33%)	$p = 0.190$	
OdDHL	8 (19%)	3 (16%)	5 (21%)	$p = 0.673$	
any AHLs	32 (74%)	16 (84%)	16 (67%)	$p = 0.190$	

^a Median values of production level. ^b Mean values of production level.

Relative risk (RR) and 95% Confident intervals (CI) are shown when comparisons between clonal and non-clonal strains are significant.

4.3.7 Relationships between AHLs Production and Virulence Factors

AHLs, as part of the QS system, are known to have roles in the regulation of virulence factor production. Isolates with positive results for one or both of BHL and HHL were significantly more likely to produce total proteases ($p = 0.004$), LasB elastase ($p = 0.006$), and protease IV ($p = 0.007$) than BHL/HHL-negative isolates. The average levels of the total proteases activity (623 mU) and LasB elastase activity (82 mU) among BHL/HHL-positive isolates were also significantly higher than those of negative isolates (28 mU for total proteases and 2 mU for LasB elastase) (both $p < 0.001$). When interpreting the BHL production alone, this relationship was found only in the LasB elastase production [18 (95%) of the 19 BHL-positive isolates versus 14 (58%) of the 24 BHL-negative isolates; $p = 0.012$]. Interestingly, there was no relationship between BHL/HHL and other virulence factors, or relationship between the production of any long-chain AHLs (OHHL, ODHL and OdDHL) and all tested virulence determinants.

4.3.8 Association between Virulence Factor Production and Patient's Age

It has been reported previously that *P. aeruginosa* adapts for long-term survival in the CF lungs by down-regulating the production of several virulence factors (Smith *et al.*, 2006). The association between duration of infection (indirectly determined by patient's age) and the levels of virulence factor production was thus investigated, and it was found that there was a significant trend for decreasing LasB elastase activity with increasing patient age ($r = -0.34$, $p = 0.028$) (**Figure 4.9**). This association was found only in the production of LasB elastase but not other virulence factors. Interestingly, this trend was not significant statistically when clonal and non-clonal groups were analysed separately. However, LasB elastase levels tended to reduce with increasing patient age in the non-clonal ($r = -0.39$, $p = 0.07$) while this was not found in the clonal group ($r = -0.08$, $p = 0.75$) (**Figure 4.9**).

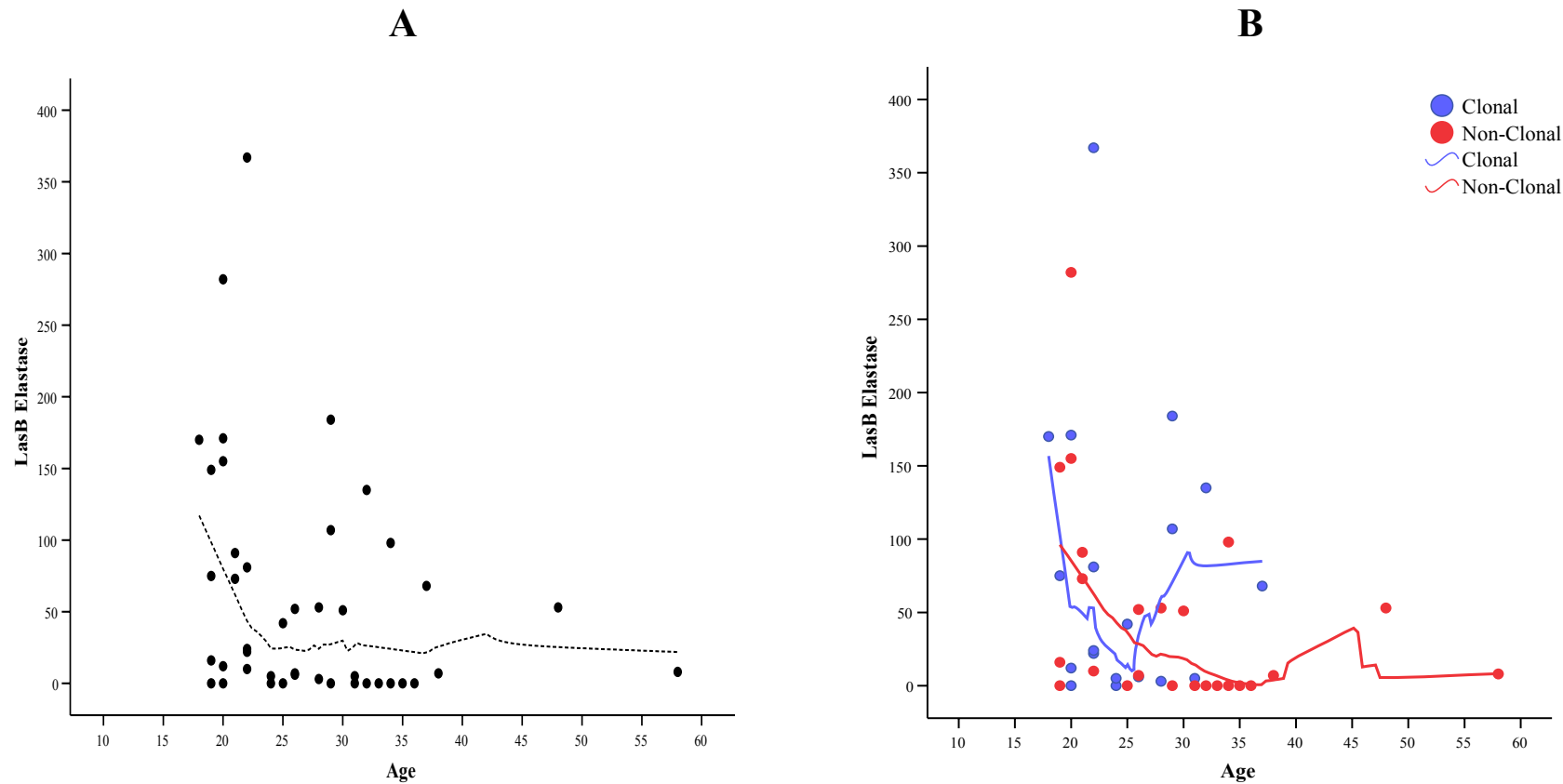


Figure 4.9 Correlation between patient's age and the level of LasB elastase production. **(A)** Among all tested isolates, there was a significant trend of decreasing LasB elastase level with increasing patient's age ($r = -0.34$, $p = 0.028$). Black dots represent individual isolates, and black dash line is a trend line. **(B)** This trend was not significant statistically when clonal and non-clonal group were separately analysed. However, the trend of the LasB elastase production in the non-clonal group (red line) was likely to reduce with increased patient's age compared with the clonal group (blue line). Blue dots represent individual clonal isolates; red dots represent individual non-clonal isolates.

4.3.9 Comparison of Antibiotic Susceptibilities between Clonal and Non-Clonal Isolates

Results showed that the percentages of clonal isolates that were resistant to ticarcillin-clavulanate, amikacin and gentamicin were significantly higher than in the non-clonal group ($p = 0.05$, 0.05 and 0.008 , respectively). To explore the possibility that these antibiotics were responsible for the domination of any particular clonal strains, further analysis was performed by separately comparing AES-1 to non-clonal and AES-2 to non-clonal. As shown in **Figure 4.10**, the AES-1 isolates were still significantly more likely to be resistant to ticarcillin-clavulanate, amikacin and gentamicin. The AES-1 strain was also 100% resistant to ceftriaxone, but this was not significant statistically when compared to the non-clonal strains ($p = 0.07$). However, AES-2 did not show these significant data possibly due to the small sample size. Antibiotic susceptibility patterns of all tested isolates are shown in the Appendix 11.3.

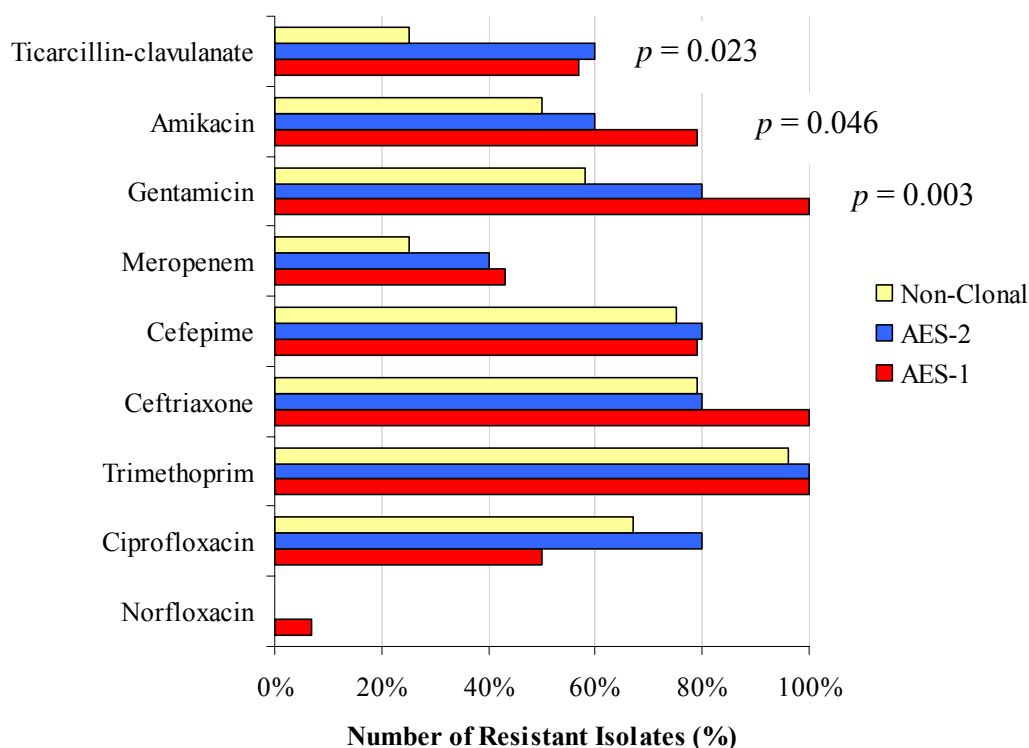


Figure 4.10 Percentages of antibiotic resistant isolates of AES-1, AES-2 and non-clonal group. P values, representing comparison between AES-1 and the non-clonal strains, are shown when there is a significant comparison.

4.4 Discussion

4.4.1 Production of Virulence Factors by Clonal and Non-Clonal Isolates

The major aim of this study was to gain insight into the properties that distinguish *P. aeruginosa* clonal strains from non-clonal strains. Findings showed that the clonal strains were more likely than the non-clonal strains to possess protease activities. The results not only indicate that the clonal strains are more virulent, but also suggest that the increased virulence as such or the capacity of the clonal strains to retain virulence over time may contribute to the transmissibility. In addition these findings could help explain why the clonal strains are associated with a negative impact on infected patients as described in the previous chapter.

The high percentage of the clonal isolates secreting proteases is of particular interest as significant differences between clonal and non-clonal strains were found in the production of LasB elastase (as detected by the ECR assay), LasB elastase/LasA elastase (as detected by zymography), alkaline protease and protease IV. These findings are consistent with recent reports of LES (or the Liverpool epidemic strain) (Salunkhe *et al.*, 2005a) and an epidemic ocular *P. aeruginosa* strain (Lomholt *et al.*, 2001). LES has been shown to have increased LasB elastase, LasA elastase and pyocyanin activities (Salunkhe *et al.*, 2005a). It therefore seems possible that genetically distinct clonal strains have evolved common mechanisms, such as producing proteases, to facilitate infectivity and transmissibility, which in turn have damaging effects on host tissues.

All tested isolates from this study were obtained from CF patients with chronic *P. aeruginosa* infection (infected for at least three years), and were shown to possess genes encoding the virulence factors including *lasA*, *lasB*, *aprA*, *rhlAB* and *exoS* (personal communication, Al Nassafi, PhD student, CF group, University of Sydney) (see Appendix 11.4). The fact that some of the isolates (mainly non-clonal) did not exhibit these virulence factors is consistent with the general assumption that *P. aeruginosa* persists in the CF airways for prolonged periods by adapting via down-regulating the production of virulence factors (Burke *et al.*, 1991; Nguyen & Singh, 2006; Yahr & Greenberg, 2004). Particularly, in the case of LasB elastase in the present study, the production levels were found to be significantly reduced

when the patient's age increased. Interestingly, this figure was changed when data were analysed separately between clonal and non-clonal groups. It appeared that while the non-clonal isolates from older patients tended to have lower LasB elastase activities ($p = 0.07$), the trend in the clonal group was more or less opposite (**Figure 4.9**). Indeed, an isolate from the oldest patient from the clonal group (37 years old) showed a considerably high level of LasB elastase (68 mU) (**Table 4.4**). These results were interpreted with awareness that patient's age may not perfectly represent the duration of infection, but it was the most practical indicating parameter in this cross sectional study. Overall, based on these findings, it has been shown here that unlike isolates from the non-clonal group, clonal isolates retain their virulence by resisting the down-regulation process and may employ other modes of adaptation for long-term colonisation in CF lungs.

ExoS is a toxin secreted by the type III secretion system (or TTSS) (Yahr *et al.*, 1996). In this study, there was no evidence of any association between the clonality of the *P. aeruginosa* strains and the ExoS production. Detection of this exoprotein was performed in isolates grown in culture medium with low-calcium conditions to induce maximum type III protein secretion (Jain *et al.*, 2004). EGTA (ethylene glycol tetraacetic acid) is a calcium-chelating agent, and the culture condition used here (LB with 1 mM EGTA) has been used previously (Jain *et al.*, 2004). While it is not surprising that all tested isolates had the *exoS* gene but not *exoU* (see Appendix 11.4) as a similar figure has been reported previously (Feltman *et al.*, 2001), the finding that the majority of isolates (77%) showed ExoS on Western blots are higher than expected. Loss of production of ExoS and other TTSS proteins over prolonged infection period has been widely reported (Jain *et al.*, 2004; Lee *et al.*, 2005; Yahr & Greenberg, 2004). In one instance, the secretion of ExoS was found in only 4% of isolates from chronically infected adults with CF (Jain *et al.*, 2004), which is almost 20 times lower than findings in this study (isolates were grown in similar culture conditions in both studies).

4.4.2 Quorum Sensing Molecules Produced by Clonal and Non-Clonal Isolates

QS is believed to have significant roles in establishment of infection (de Kievit & Iglewski, 2000; Wu *et al.*, 2001). Findings from this study that less than half of all isolates produce

either BHL [44% (19/43)] or OddHL [19% (8/43)] are therefore surprising considering that all isolates tested in this study were grown in the culture conditions reported to promote the production of AHLs (Geisenberger *et al.*, 2000). All but one (isolate U4) of the isolates have been shown to have all *lasI*, *lasR*, *rhlI* and *rhlR* genes (Appendix 11.4), and there was no evidence of major genetic changes with the amplified regions of these QS genes to account for these findings. However, minor mutations, if occurring, could be responsible for the low prevalence of BHL and OddHL producing isolates. Indeed, a similar report of the LES isolates showed that some LES carried mutations in *lasR* resulting in a QS-deficient phenotype (Salunkhe *et al.*, 2005a). Nevertheless, the QS-mutant LES isolates still exhibited a virulent nature even more than PAO1 in a *Drosophila melanogaster* (fruit fly) killing assay (Salunkhe *et al.*, 2005a). In addition to the report of LES, Schaber *et al.* showed that QS-deficient *P. aeruginosa* strains were still capable of causing infections of various sites including wound, urinary tract and lower respiratory tract (Schaber *et al.*, 2004). Similarly, Zhu *et al.* also detected a *P. aeruginosa* isolate with defect in *lasI* and *lasR* associated with contact lens induced acute red eyes (Zhu *et al.*, 2002; Zhu *et al.*, 2004). Overall findings suggest that even when lacking QS production, *P. aeruginosa* cannot be considered avirulent.

One explanation of the low prevalence of BHL and OddHL positive isolates may be from the technique used. In this study, the TLC technique with two AHLs biosensors was used to detect the AHL molecules which were extracted from culture supernatants with ethyl acetate. It is possible that some of the AHLs were lost during this extraction process. In fact, Erickson *et al.* demonstrated that up to 20% of AHL samples production may not be recoverable after extraction (Erickson *et al.*, 2002). This technical problem was unforeseen at the time the study was conducted and unresolved when the study was completed. However, as suggested by Erickson *et al.*, the amount of AHLs lost should not substantially alter the detection process (Erickson *et al.*, 2002).

Smith *et al.* recently reported that *P. aeruginosa* underwent adaptations to the CF lung environment by developing mutations in several genes including *lasR* (Smith *et al.*, 2006). The isolates of *P. aeruginosa* in their study were collected from a single CF subject at different time points over an eight-year period (Smith *et al.*, 2006). The authors suggested that the mutation of *lasR* was important for a positive-selection process, rendering the mutant a better chance to survive and to persist as a dominant strain in the CF lungs (Smith *et al.*, 2006).

Indeed, the OdDHL molecule itself has been shown to have immunomodulatory effects (Telford *et al.*, 1998). It may be possible that the *P. aeruginosa* OdDHL-deficient strain can effectively evade host immunes and thus chronically persists in the CF lungs. Overall, this data is consistent with the findings of the low prevalence of OdDHL secreting isolates in this study, and might explain the number of OdDHL negative in all tested AES-1 isolates (**Table 4.5**).

The discovery of detectable levels of other AHLs (i.e. HHL, OHHL and ODHL) is also interesting. Similar findings were shown from other CF strains of *P. aeruginosa* (Geisenberger *et al.*, 2000) and even in sputum samples from CF subjects (Erickson *et al.*, 2002). Overall, these results suggest that *P. aeruginosa* produces different and various autoinducer molecules in the CF lung environment. However, the significance of these molecules in the pathogenesis of *P. aeruginosa* is yet to be defined. When results of these AHLs (HHL, OHHL and ODHL) are included, the prevalence figure of AHL-producing isolates rose; 72% (31/43) and 37% (16/43) of all isolates had positive detections for short-chain AHLs (BHL or HHL) and long-chain AHLs (OdDHL, OHHL or ODHL) respectively. It may be possible that these AHLs, besides BHL and OdDHL, have some roles in the regulation of virulence factors. This is supported by the findings of the significant associations between protease production and HHL-positive isolates in this study. However, similar associations were not found in OHHL/ODHL/OdDHL-positive isolates. The finding that OHHL secretions were detected more frequently in the clonal strains suggests that this molecule may play an important role in pathogenesis, but these figures need to be interpreted cautiously because the total number of OHHL-positive isolates is small.

It is also possible that *P. aeruginosa* uses other QS signalling system such as Pseudomonas quinolone signal or PQS. PQS, in particular, has been shown to regulate the production of LasB elastase and rhamnolipids (Pesci *et al.*, 1999), suggesting that this signal may have a role in the virulence regulation in AHL-deficient strains. The detection of the PQS molecules in CF sputum samples indicated the importance of this signal *in vivo* (Collier *et al.*, 2002). However, the exact prevalence of PQS-producing *P. aeruginosa* strains particularly from CF patients with chronic lung infection is still unknown and was not investigated in this thesis.

4.4.3 High Prevalence of Antibiotic Resistance in Clonal Isolates

Findings in this study showed that the AES-1 isolates were more resistant to ticarcillin-clavulanate, amikacin and gentamicin, when compared to non-clonal strains, similar to the report from the CF centre at Royal Children's Hospital in Melbourne (Armstrong *et al.*, 2002). The percentage of AES-2 strains that were resistant to these three antibiotics was also higher than the non-clonal group; although, these results were not statistically significant (possibly due to the small sample size). Nevertheless, the finding is consistent with the previous report of this clone from CF centres in Brisbane (O'Carroll *et al.*, 2004). It should be noted that amikacin and, in particular, gentamicin are rarely prescribed for the patients at the CF clinic at the RPAH. The fact that the AES-1 strain is more resistant to these aminoglycosides suggests that this strain may have an intrinsic resistant nature. Indeed, multidrug active efflux systems including the MexAB-OprM and MexXY efflux pumps, which contribute to the natural resistance to aminoglycosides (Aires *et al.*, 1999), have been shown to be up-regulated in the LES strain (Salunkhe *et al.*, 2005a). However, the expression of these efflux pumps in AES-1 has not yet been studied.

4.4.4 Clinical Significance of This Study

This study has provided several important clinical observations. First, there was no specific morphology (**Table 4.3**) or specific antibiogram (see Appendix 11.3) among the clonal isolates. To date, the only way to distinguish clonal isolates from non-clonal is to perform genotyping using techniques such as PFGE/RFLP. Second, the clonal strains usually produced more proteases than the non-clonal strains. However, when the levels of protease activities in strains were compared with clinical outcomes in corresponding patients, there was no significant evidence supporting this link ($r = 0.106$, $p = 0.497$ when total protease activities were correlated with patient's FEV₁). One explanation for the lack of an association here may be that only one isolate from each patient was tested. The protease activity of this isolate may not have been representative of that of other phenotypes that may have been present in the CF lungs. Also, *P. aeruginosa* strains with QS deficiency are still capable of causing different infections including CF lungs. This finding has an important implication since the QS molecules have received vast attention as a potential therapeutic target (Smith & Iglewski, 2003a; Williams, 2002). Compounds such as furanones (Hentzer *et al.*, 2002) and AHL-

lactonase (Dong *et al.*, 2001) have been shown to have an inhibitory effect on the QS signals, but the data presented here suggest that they may not be applicable and effective for all kinds of *P. aeruginosa* infections. Lastly, this study has examined the possible roles of protease IV, chitinase and rhamnolipids in the CF pathogenesis. Protease IV, in particular, was expressed more frequently in the clonal strains, suggesting that it might have some role in infectivity/transmissibility. The high proportions of isolates that produced rhamnolipids and chitinase among all tested isolates suggest that they may play a role in chronic CF lung infection. However, further investigations are needed to explore the mechanisms of actions of these virulence factors.

In summary, this study has addressed the issue of clonal strains, particularly AES-1 and AES-2, which have become an emerging threat to the CF centre at the RPAH, if not nation-wide. This study also extends the general understanding of the pathogenesis of this bacterium in the CF lung infections. AES-1 and AES-2 were shown in the previous chapter to be associated with poorer clinical outcomes. The findings from studies in this chapter demonstrate that these two strains were not only hypervirulent but also highly antibiotic resistant. These could be potential factors contributing to their infectivity and transmissibility. Identifying these factors is crucial for the development of new strategies to combat the threat of clonal strains as well as *P. aeruginosa* in general.

5 Chapter Five: Production of Biofilms in *P. aeruginosa* Clonal and Non-Clonal Strains

5.1 Background

The previous two chapters showed that the 38% of CF patients at the adult CF clinic at RPAH were infected with *P. aeruginosa* clonal strain AES-1 and that AES-1 isolates were more likely to express virulence factors than the non-clonal isolates, providing one explanation for the observation of poorer clinical outcomes among patients infected with this strain. In addition to the virulence factors, several studies have reported the significance of biofilms in clinical settings. Compelling evidence has shown that *P. aeruginosa* exists in the biofilm mode of growth within the lungs of people with CF, contributing to its persistence and leading to the stage of chronic lung infection (Singh *et al.*, 2000a). Bacteria grown in this mode of growth are known to be more resistant to antimicrobial agents. A descriptive study conducted by Whitnall from the CF group at the University of Sydney showed that different *P. aeruginosa* isolates had different capacities to form biofilms (Whitnall, 2003). It is hypothesised that strains with greater biofilm-forming capability may become more persistent in the CF airways, and this may explain the high proportion of the AES-1 strain and, perhaps, the poorer clinical consequences. To test this hypothesis, a quantitative study that measured the biofilm forming capacity of both clonal strains, in particular AES-1, and non-clonal strains, was conducted. Recently, biofilm researchers from the Biofilm Laboratory at Centres for Disease Control and Prevention (USA) developed a novel model system to study bacterial biofilms, called “CDC biofilm reactor” (Donlan *et al.*, 2004), and the reliability and reproducibility of this system has been demonstrated (Goeres *et al.*, 2005). By employing this novel technique incorporating image analysis software, the aims of studies in this chapter were to:

- grow *P. aeruginosa* biofilms using the CDC biofilm reactor; and
- quantitatively analyse the biofilms produced by representatives of the AES-1 and non-clonal strains and control strain PAO1 using the ImageJ software (National Institute of Health [NIH], USA).

5.2 Material and Methods

5.2.1 Study Population

Twelve clinical isolates of *P. aeruginosa* (four isolates of AES-1, and eight non-clonal strains) from the study population described in the previous chapter and two laboratory strains – PAO1 and PAO $\Delta pilA/\DeltafliM$ – were included in the studies. The twelve clinical isolates were chosen from patients who had matching profiles (age and gender) (**Table 5.1**). The strain PAO1 was used as a positive control while PAO $\Delta pilA/\DeltafliM$, which is a mutant strain defective in the type IV pili (PilA, type IV pili precursor) and flagella (FliM, flagellar motor switching protein) (Klausen *et al.*, 2003b), was used as a negative control. PAO $\Delta pilA/\DeltafliM$ was kindly donated by Dr. Rice, School of Biotechnology and Biomolecular Sciences, University of New South Wales.

5.2.2 Bacterial Growth and Conditions

All isolates were grown overnight at 37°C on Columbia HBA plates (Oxoid) as described in Sections 2.2.5.1 and then transferred to 10 mL of LB broth and incubated at 37°C overnight as described in Section 2.2.5.2.

Table 5.1 *P. aeruginosa* strains used in this study

Isolate	Strain	Patient profile		Reference
		Gender	Age	
C3	AES-1	M	22	
C4	AES-1	F	31	
C5	AES-1	F	24	
C12	AES-1	F	29	
U4	Non-clonal	F	29	
U7	Non-clonal	M	48	
U8	Non-clonal	F	35	
U12	Non-clonal	M	26	
U16	Non-clonal	M	22	
U18	Non-clonal	F	38	
U22	Non-clonal	F	34	
U23	Non-clonal	M	32	
PAO1	wild type ATCC 15962			Holloway <i>et al.</i> (1979)
PAO $\Delta pilA/\DeltafliM$	type IV pili and flagellar mutant			Klausen <i>et al.</i> (2003)

5.2.3 Normalisation of Bacterial Inoculum

It was imperative that the inoculum size (an amount of bacterial cells to be inoculated into the CDC biofilm reactor) of all experiments was as consistent as possible. In order to achieve this, the bacterial culture density from the previous step was measured at OD₆₀₀ using a Beckman DU[®] 640 spectrophotometer (Beckman). All cultivation solutions were diluted with pre-warmed (approximately 37°C) LB broth to the OD₆₀₀ of 0.5. This ensured that a standard inoculum with similar bacterial numbers was used throughout the study.

5.2.4 Description of the CDC Biofilm Reactor

The CDC Biofilm Reactor (BioSurface Technologies Corporation, USA) consisted of a one-litre glass vessel with an effluent spout attached with a Tygon[®] tube and closed by a clamp (**Figure 5.1**). A polyethylene top plate supported eight independent and removable

polypropylene rods, a medium-inlet port and a gas-exchange port. Each rod held three removable coupons (biofilm growth surfaces) for a total of 24 sampling opportunities (coupons were held by a small screw as shown in **Figure 5.1**). A coupon was a disk, 1.27 cm in diameter and 0.3 cm thick, made of a material such as polycarbonate or borosilicate glass (**Figure 5.1**). The glass vessel was submerged in a five-litre water tank containing water maintained at 35°C using a heating element. The water tank was then placed on a digitally controlled magnetic stir plate (Super-Nova™, BioSurface Technologies Corporation) to provide constant rotation of the baffled stir bar. Rotation of the baffle provided constant mixing and consistent shear to the coupon surface.

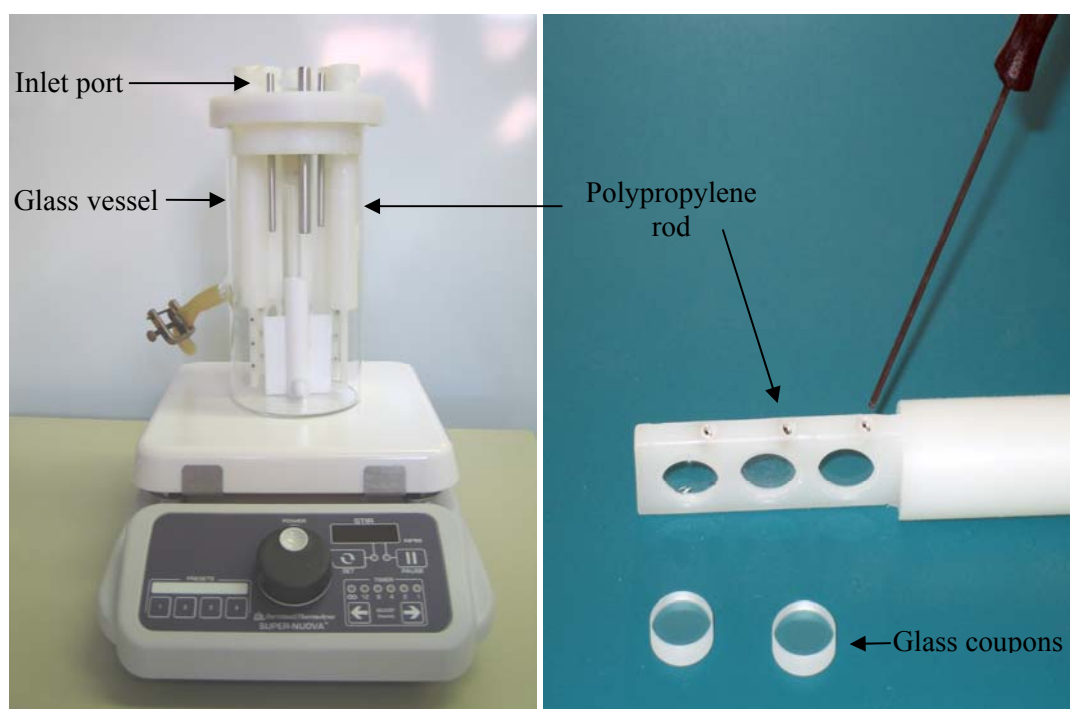


Figure 5.1 CDC Biofilm Reactor apparatus. (Left) Glass vessel (coupon-holding rods were removed to expose the baffled stirrer) placed on the digital controlled magnetic stirring platform. (Right) Polypropylene coupon-holding rod and glass coupons. The rod has three holding spaces where coupons can be fitted and tightened by a small screw.

5.2.5 Biofilm Experiment Set Up

Prior to assembly, all parts of the CDC biofilm reactor were thoroughly cleaned with 70% ethanol followed by sterile dH₂O and left to dry. Once assembled, the reactor was sterilised at 121°C for 15 minutes. The sterile LB (pre-warmed at approximately 35°C) was then filtered

into the sterile vessel via the medium-inlet port through a 0.22- μm MillexTM Syringe Driven unit (Millipore). This process was done in a class II biological safety cabinet (Westinghouse Pty. Ltd.).

Five millilitres of the bacterial solution normalised to the OD₆₀₀ of 0.5 (Section 5.2.3) were inoculated into the reactor via the medium-inlet port. All inlet ports were then sealed with sterile aluminium foil. The vessel top was also covered with two layers of aluminium foil. The biofilm reactor was then placed into the water tank and then on the magnetic stir platform as described in the previous section. The bacterial mixture was left without stirring for one hour in order to allow cells to attach to the coupon surfaces. After which, the magnetic stir plate was set at 120 rpm, and the cultivation was conducted for a total of 72 hours.

5.2.6 Removal of Coupons

Six coupons (from two opposite rods) were removed at set time points ($t = 24, 48$ and 72 hours after incubation) in a sterile manner. Briefly, the biofilm reactor vessel was taken from the water tank, dried and wiped down with 70% ethanol and placed in the class II biological safety cabinet. Two rods were gently removed, and the coupons held in each rod were then unscrewed and were immediately collected in a container containing sterile LB for further analyses. The top of the reactor vessel was then sealed with sterile tape at the positions where the rods were taken out, and the vessel was then replaced in the water tank.

5.2.7 Biofilm Visualisation

5.2.7.1 Biofilm Preparation and Staining

Biofilms grown on the coupons were subjected for imaging. Each coupon was washed in 0.9% saline three times in order to remove unattached (planktonic) cells and cell debris.

The green-fluorescent Syto-9[®] stain, which was packaged as part of the LIVE/DEAD[®] BacLightTM Bacterial Viability Kits (Molecular Probes Inc.), was used to stain the bacterial biofilms. To do so, the Syto-9 staining solution was prepared according to the manufacturer's instruction (900 μL of Syto-9 in 3-mL 0.9% saline), and the solution stored in a light-

protection container. The coupons were then stained in this solution for ten minutes at room temperature. After which, each coupon was rinsed with 0.9% saline to remove excess dye, placed on a clean glass slide and covered with a glass coverslip prior to the microscopic visualisation. The Syto-9 dye has excitation/emission wavelengths at about 480/500 nm and stains both live and dead bacteria.

5.2.7.2 Visualisation using Fluorescent Microscopy

The fluorescent microscopic visualisation was carried out on an Olympus Provis AX70 microscope (Olympus Co. Ltd.) with the UV illumination setting at a green-fluorescent wavelength. All removed coupons were microscopically examined, and biofilms were randomly chosen. Biofilms grown on the edge of the coupons were avoided. At least ten biofilms were captured using an image capturing software, Studio Lite[®] (Olympus Co. Ltd.). All images were saved in a TIFF file for further analysis.

5.2.7.3 Visualisation using Confocal Laser Scanning Microscopy (CLSM)

Prior to the CLSM process, the coupons were washed three times with 0.9% saline as previously described. After which, biofilms grown on coupons were fixed with a 4% (w/v) paraformaldehyde solution for ten minutes (Harrison *et al.*, 2006). Following the fixation step, the biofilms were stained with Syto-9 as previously described.

Biofilms from eight *P. aeruginosa* isolates (a PAO1 strain, two AES-1 isolates and five non-clonal isolates) were examined using a FV-1000 Confocal system on a IX-81 inverted microscope (Olympus Co. Ltd.) which incorporated an image capturing software, Fluoview version 1.5 (Olympus Co. Ltd.). A Laser at the green-fluorescent-protein wavelength (approximately 470 nm) was used, and at least three randomly selected biofilms of each isolate were scanned on both x-y and x-z (cross section) panels. All images were saved into a TIFF file for further analysis.

5.2.7.4 Image Analyses

Images of biofilms were analysed using the ImageJ (version 1.24) software (NIH, USA). This software allowed the area of selected subjects to be measured. Biofilm thickness was determined by measuring the x-z section of the cross-sectional images using the Fluoview software (Olympus Co. Ltd.).

5.2.8 Experiment Validation

To reduce the subjectivity in choosing the biofilms for analysis, all experiments apart from that of PAO1 were double blindly conducted. All isolates grown on the HBA (Oxoid) were cultivated directly from the glycerol storage, and the sub-culturing process was limited to only one time.

At the end of each experiment, 100 mL of the culture medium in the biofilm vessel was tested for contamination by plating on HBA (Oxoid). Results from any experiment found to be contaminated were excluded, and the experiment of that isolate was repeated.

5.2.9 Biofilm Cleaning Method

When a single experiment was completed, all parts of the CDC biofilm reactor were decontaminated by soaking in detergent (Pyronex[®]; DiverseyLever Australia) overnight. After which, they were sonicated for 30 minutes using a Soniclean sonicator (Soniclean Pty. Ltd.) and were gently scrubbed (particularly the glass coupons which were scrubbed using a sponge to avoid scrubbing marks). All equipments were then rinsed thoroughly with dH₂O and left to dry. Prior to performing experiments, all equipments were cleaned thoroughly again and sterilised as described in section 5.2.5.

5.2.10 Statistics Analyses

Statistical analysis was performed using SPSS[®] version 14.0 (SPSS Inc.). Comparisons between areas of biofilms of the AES-1 and non-clonal strains were made using the unpaired *t*-test. *P* values of less than 0.05 were considered significance.

5.3 Results

5.3.1 Polycarbonate Coupons are Autofluorescent

The first biofilm experiment was conducted using the polycarbonate coupons. All coupons sampled at 24 hours were completely green in every image, and the structure of biofilms could not be seen. A clean polycarbonate coupon (not from the biofilm experiment) clearly exhibited the green autofluorescence under the fluorescent microscope when excited by UV. This result led to this material being discarded and a change to borosilicate glass coupons for the biofilm experiments.

5.3.2 Biofilm Development

5.3.2.1 Biofilm Development of PAO1

Coupons sampled at 24 hours after incubation showed multiple cell clusters or microcolonies (green cells on black background; **Figure 5.2 A**). Single cells (not in a cluster) were also seen scattered throughout the coupon surfaces. Under the microscope, tiny movement of these cells could also be observed. This was likely to be the twitching motility. Overall, these results suggested that at 24 hours, some bacterial cells were undergoing early maturation (such as cells in small clusters) whereas some were attaching either reversibly or irreversibly.

At 48 hours, larger cell clusters were seen over the coupon surfaces (**Figure 5.2 B**). The numbers of individual cells attached to the surface had decreased (**Figure 5.2 B**). The data suggested that biofilms might undergo the maturation stage of the biofilm development at this time point.

Biofilms observed at the 72-hour time point appeared to be somewhat larger than the 48-hour biofilms. Under the fluorescent microscope, the shape of each biofilm did not appear to be the mushroom-like structure described previously (Klausen *et al.*, 2003b) (**Figure 5.2 C**), however the CLSM revealed a ball-like structure (**Figure 5.2 D and E**).

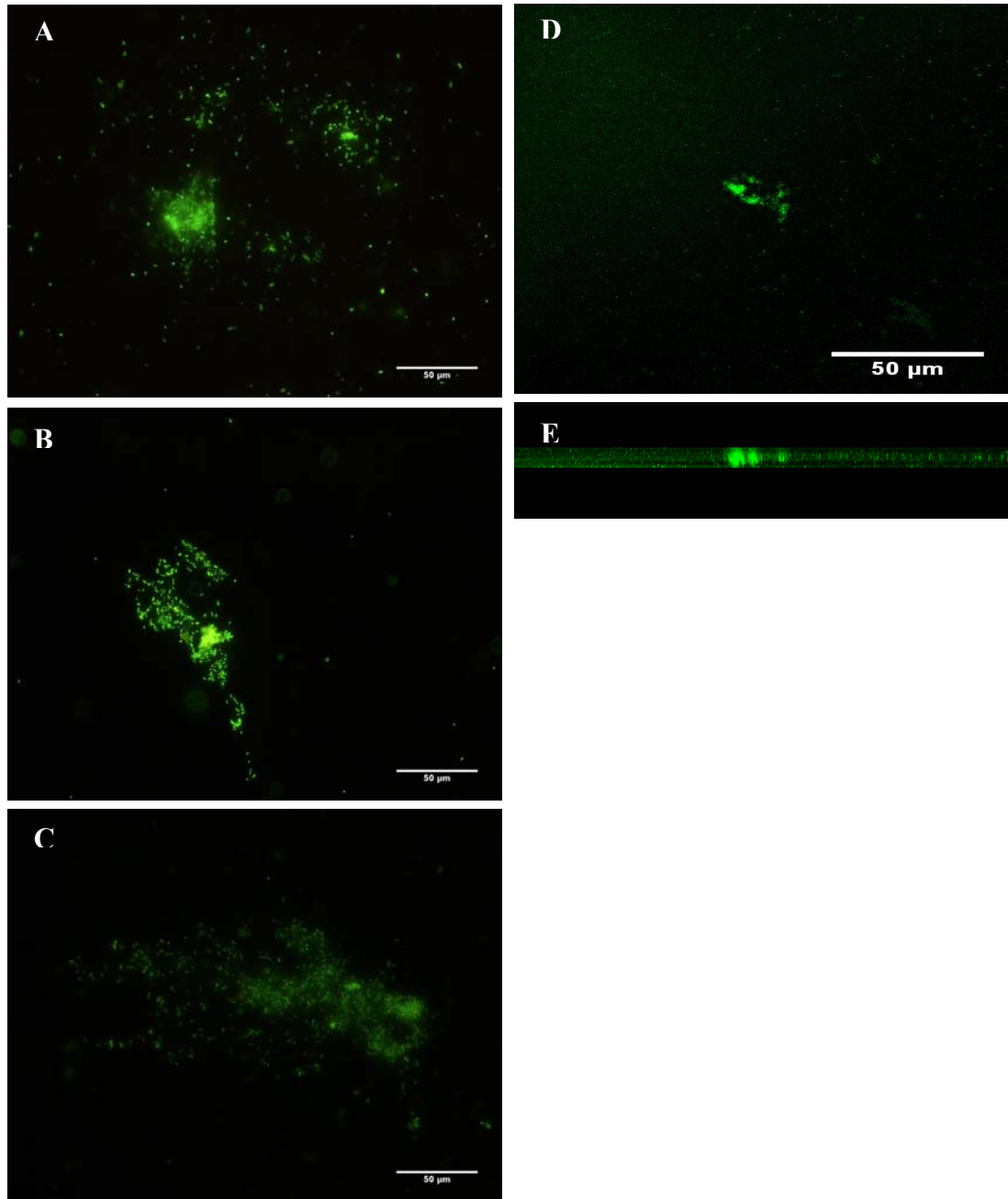


Figure 5.2 Biofilm development of PAO1. Images (A), (B) and (C) were captured by a fluorescent microscopy at 24, 48 and 72 hours, respectively. Image (D) shows a biofilm at 72 hours captured by a confocal microscopy. Image (E) shows the x-z cross-sectional view of the biofilms depicted in the image (D). Scale bars, 50 μm .

5.3.2.2 Biofilm Development of the AES-1 Isolates

Biofilms of four AES-1 isolates are illustrated in **Figure 5.3**. Generally, biofilms became larger with longer culture in the biofilm reactor. There was no specific shape of the biofilm clusters among these four isolates.

At 24 hours of incubation, cells were found aggregating in large and dense clusters spreading over the coupon surfaces. Single planktonic cells were also found attached to the surface and scattered, but the number of these cells seemed to be fewer than those of PAO1 at the same time point (24 hours). Findings were consistent with either irreversible attachment or maturation phases of the biofilm development.

Biofilms at the 48-hour time point showed strongly bright fluorescent staining spots at the centre, indicating a denser (or more compact) structure (**Figure 5.3** Panel **A**, **B** and **D**). This suggested that cell aggregates progressively grew and formed structured biofilms. However, this dense structure was not found in the biofilms of isolate C5 (**Figure 5.3** Panel **C**) at this time point. Interestingly, structures of a filamentous shape were often observed in the biofilms of isolate C4 (**Figure 5.3** Panel **B**). Higher magnification images, as shown in **Figure 5.4**, revealed that these structures were a continuously unidirectional arrangement of multiple elongated cells. This is likely to be evidence of cell multiplication.

At 72 hours, biofilms were enlarged compared to those at 24 and 48 hours. Biofilms of isolate C5 (**Figure 5.3** Panel **C**) showed a darker or less-stained centre. This feature was also found in different biofilms of this isolate, as illustrated in **Figure 5.5**, and was likely to be consistent with the dispersion stage. However, this feature was not observed in other AES-1 isolates.

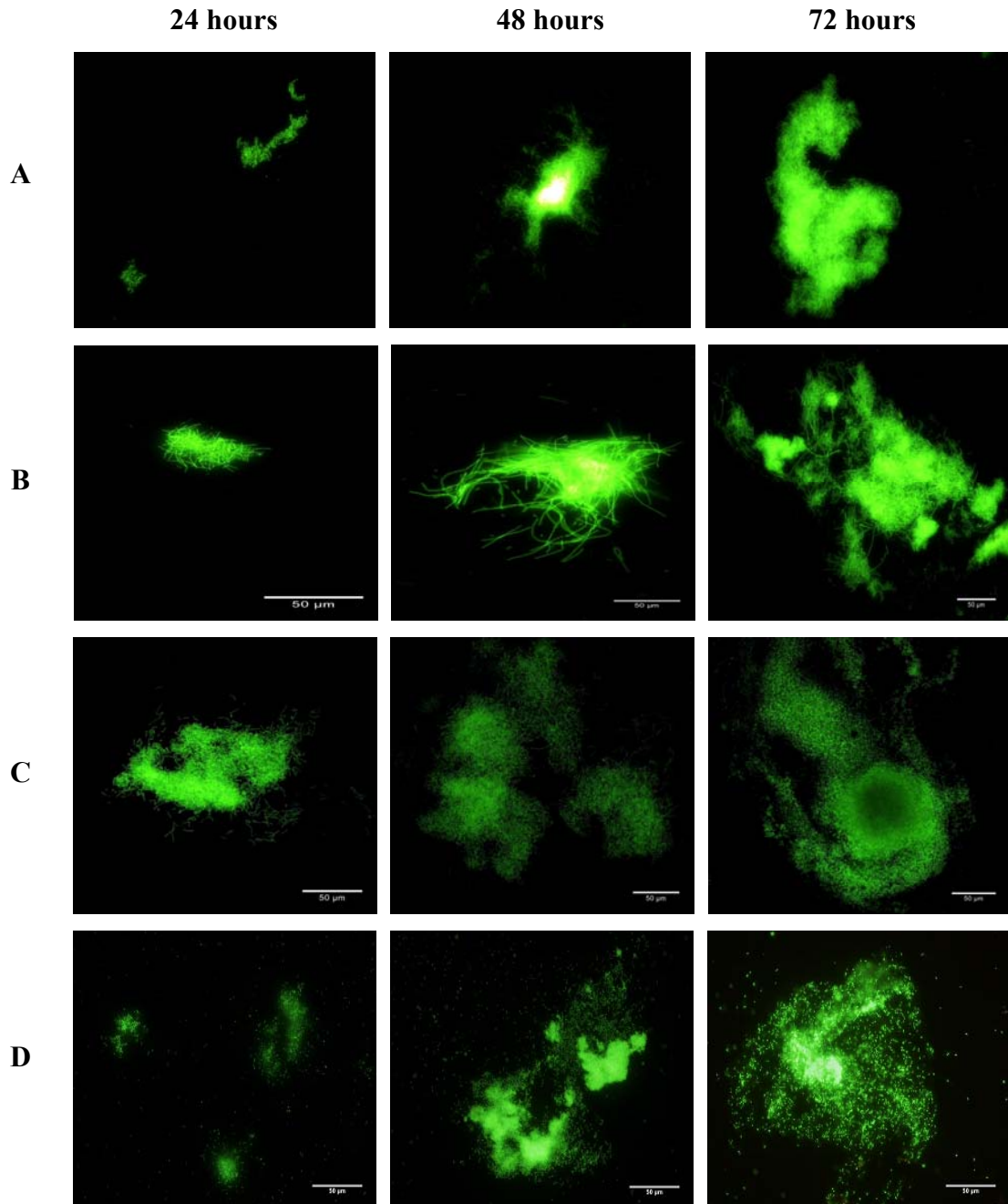


Figure 5.3 Biofilm development at 24, 48 and 72 hours of AES-1 isolates: (A) isolate C3; (B) isolate C4; (C) isolate C5; and (D) isolate C12. All images were captured by a fluorescent microscope. Bars, 50 µm.

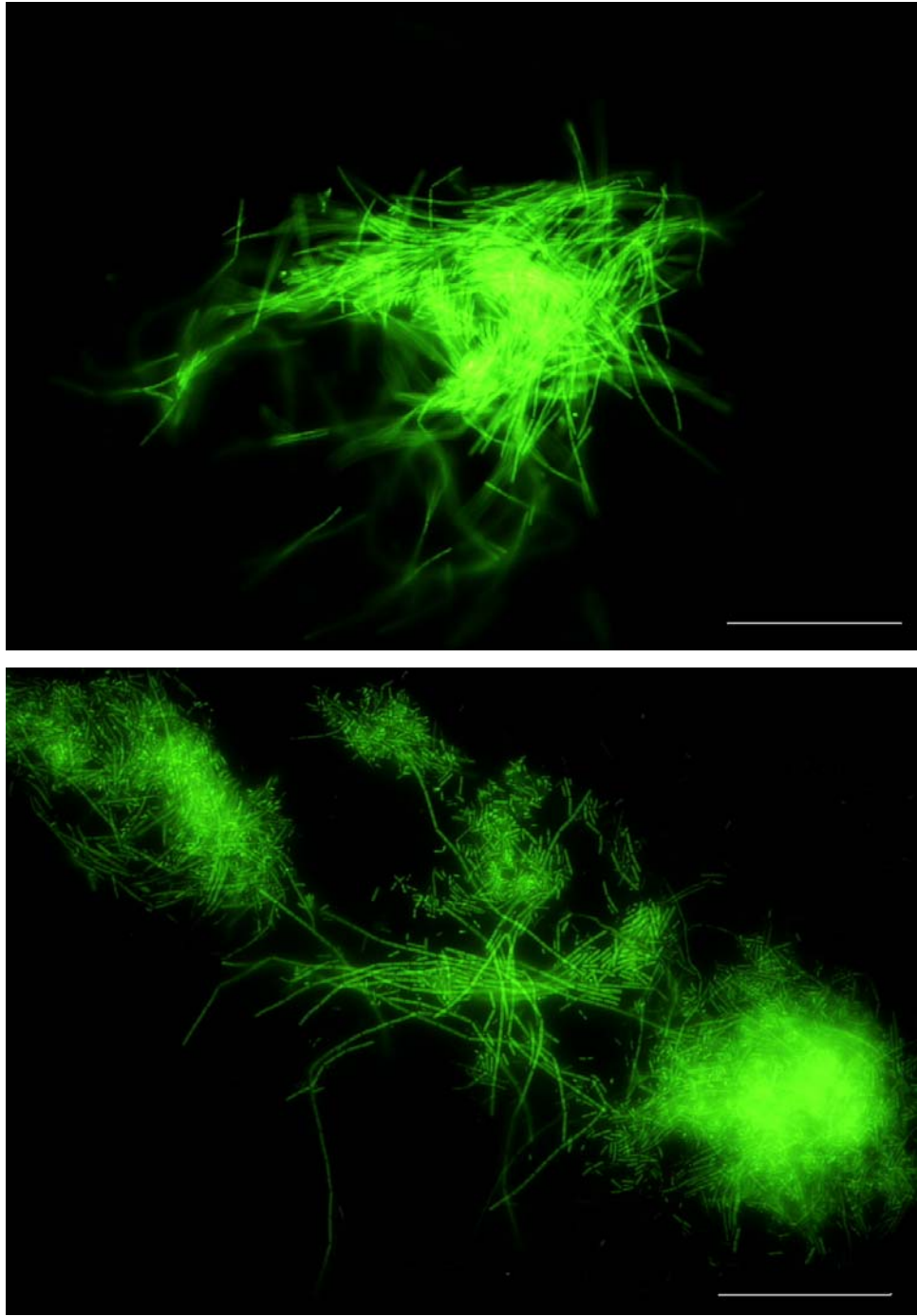


Figure 5.4 Examples of the filamentous structures found in biofilms of isolate C4. Bars, 50 μm .

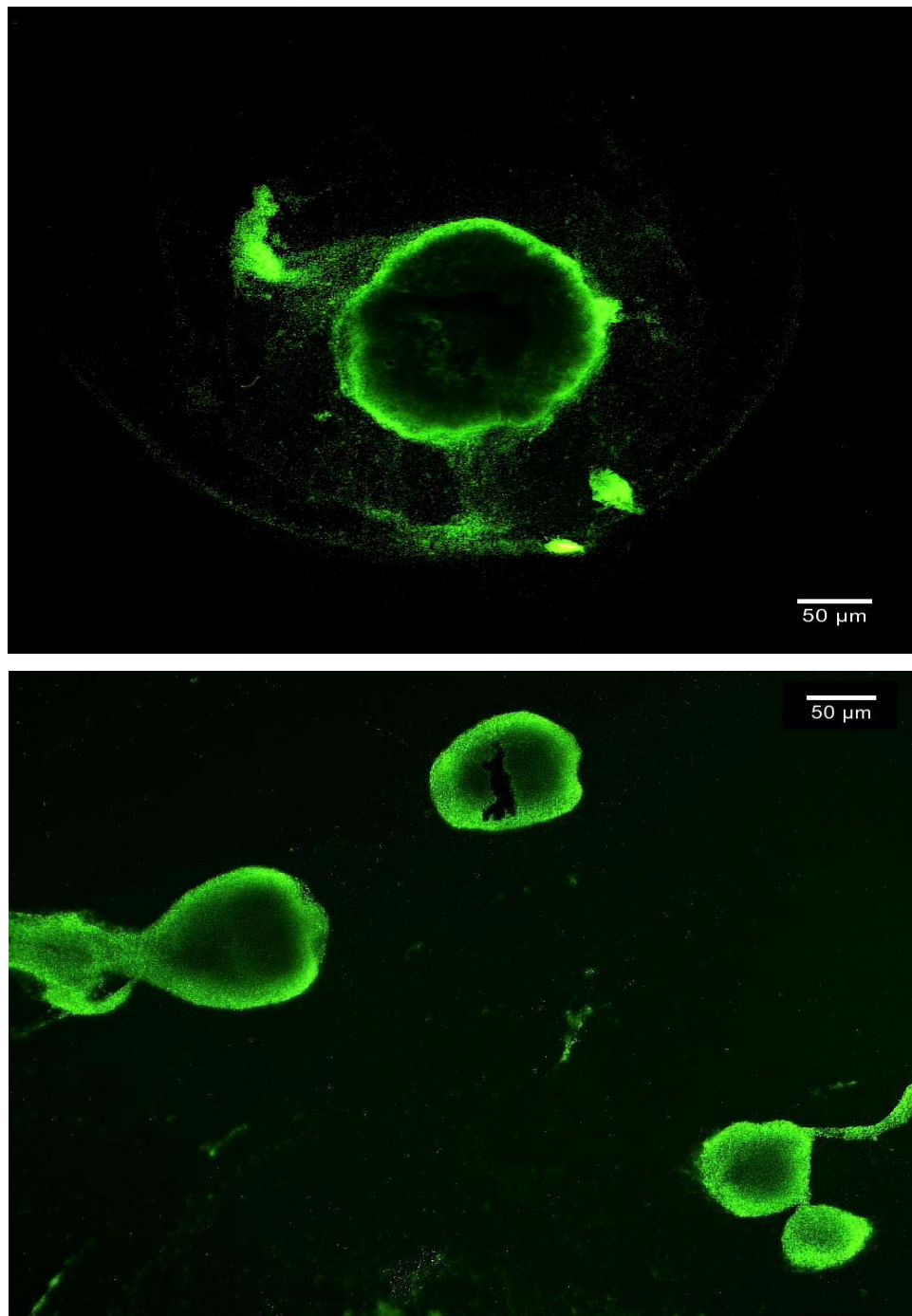


Figure 5.5 Hollow centres found the biofilms of isolate C5 at 72 hours. This feature is likely to be consistent with the dispersion stage of the biofilm development. Bars, 50 μm .

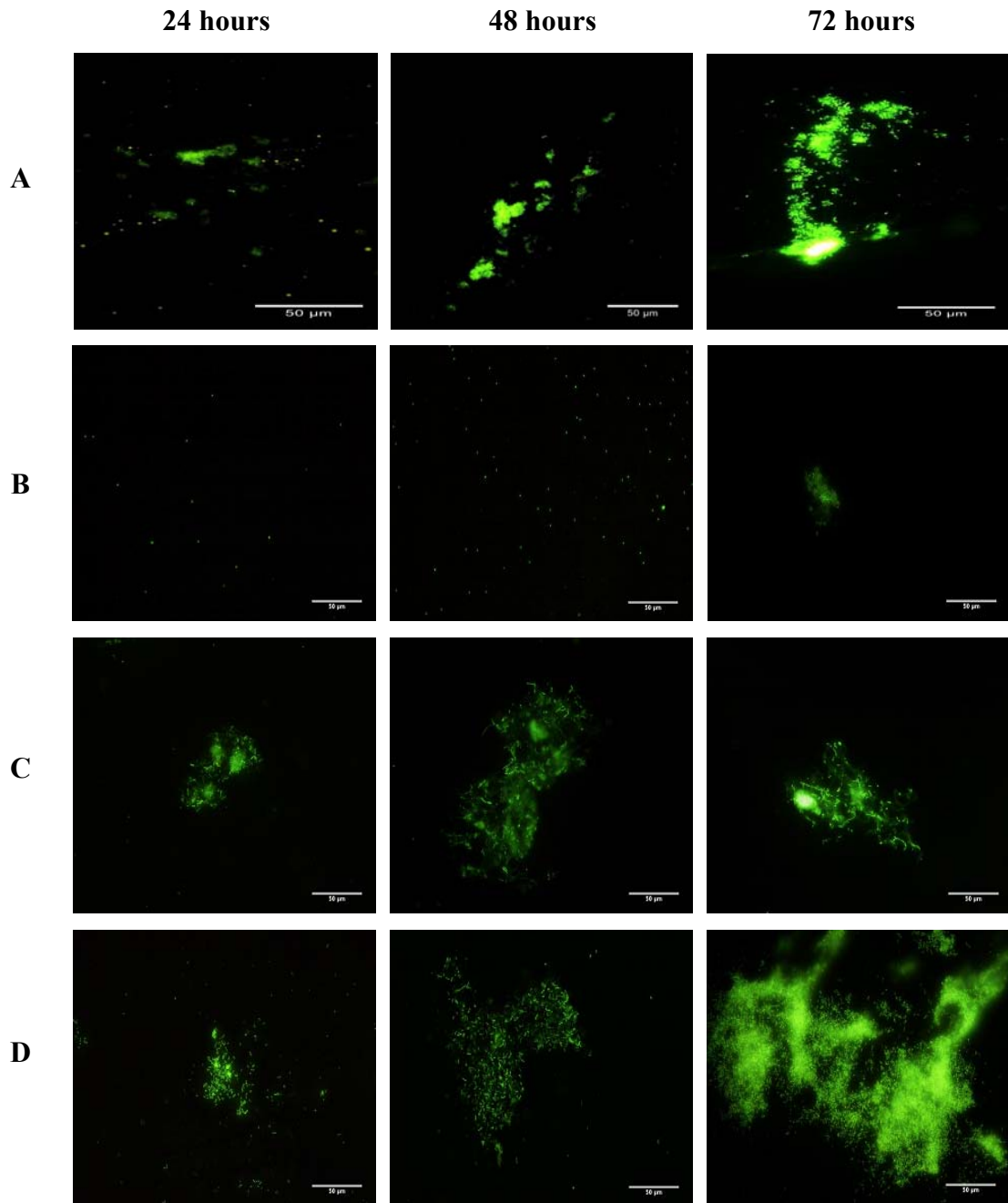
5.3.2.3 Biofilm Development of the Non-Clonal Isolates

Eight non-clonal isolates were grown for the biofilm formation study. All isolates seemed to grow differently and there were no uniform structures of the biofilms of these isolates. In addition, biofilms became larger over time as with AES-1 strains. Two strains, isolate U7 and isolate U18, did not form any biofilm over the coupon surfaces at 24 hours (**Figure 5.6 Panel B and F**). Even at 48 hours, the isolate U7 did not form biofilms (**Figure 5.6 Panel F**).

Apart from isolates U7 and U18, small cell aggregates were observed over the coupon surfaces from the cultures of the other six non-clonal isolates, at 24 hours. Single cells attaching to the surfaces were also found dispersed, although the number of these cells seemed to be varied among strains. Isolates U7 and U18 in particular seemed to have the fewest number compared to other isolates.

At 48 hours, larger and denser cell clusters were found especially in isolates U4, U16 and U22 (**Figure 5.6 Panel A, E and G**). These compact clusters could be seen throughout the surfaces, suggesting that the biofilms were likely to be in the maturation phase. The cell clusters of isolates U8, U12 and U23, however, seemed to have a loose and flat structure; cells were found aggregating in a monolayer (**Figure 5.6 Panel C, D and H**). These isolates could be in the irreversible attachment or early maturation stages.

At 72 hours, the number and the size of cell clusters were increased compared to the 24-hour and 48-hour biofilms. Isolates U7 and U18 also showed the structures of cell aggregation (**Figure 5.6 Panel B and F**), but only two cell clusters were found on each coupon. The cell clusters of the isolate TH-2B appeared to be decreasing in size but became structured biofilms (**Figure 5.6 Panel H**), although the monolayer clusters could still be seen.



(Continued next page)

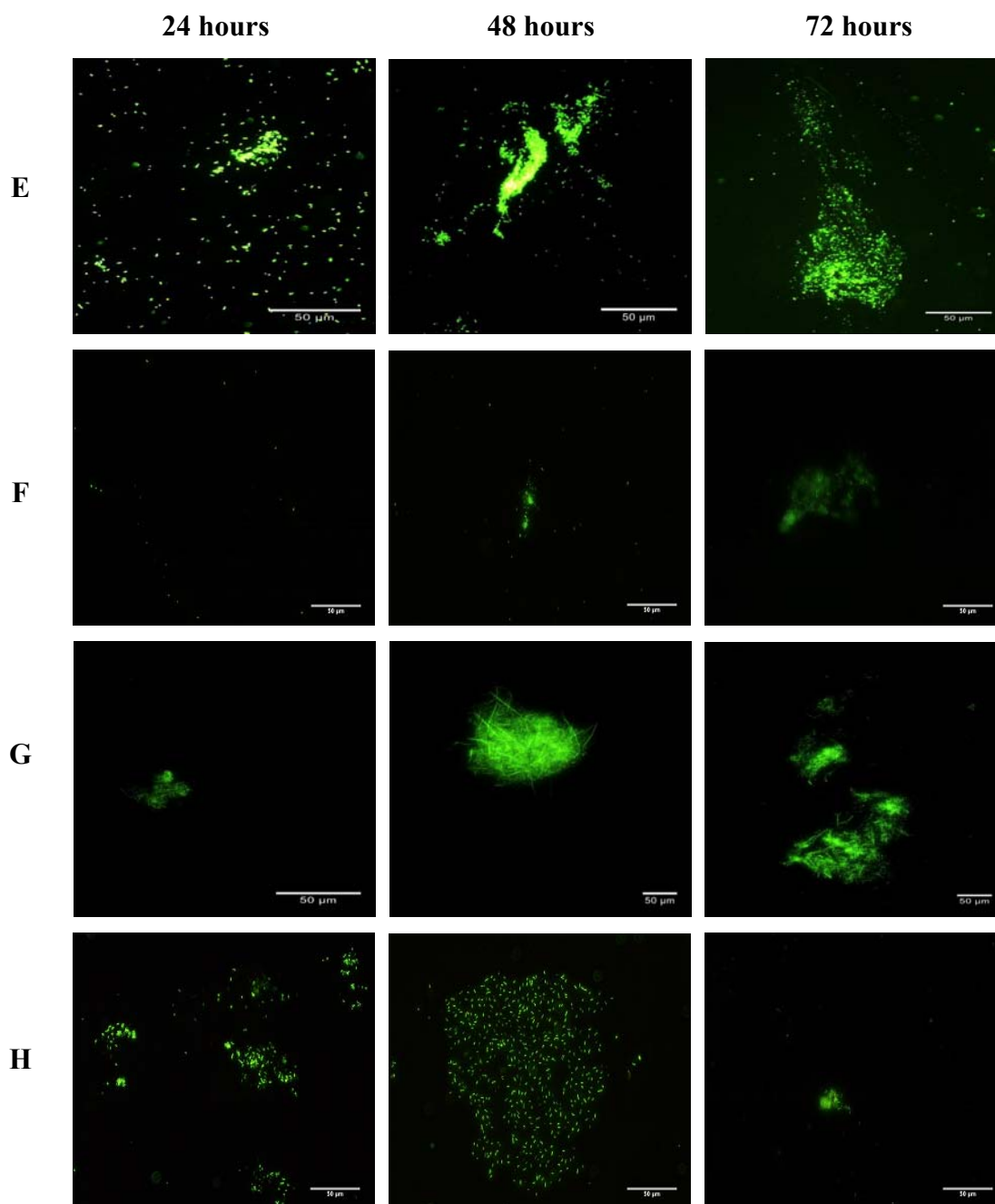


Figure 5.6 Biofilm development at 24, 48 and 72 hours of non-clonal isolates: (A) isolate U4; (B) isolate U7; (C) isolate U8; (D) isolate U12; (E) isolate U16; (F) isolate U18; (G) isolate U22; (H) isolate U23. All images were captured by a fluorescent microscope. Bars, 50 μm .

5.3.2.4 Biofilm Development of PAO $\Delta pilA/\Delta fliM$

As shown in **Figure 5.7**, there was no formation of biofilm by the strain PAO $\Delta pilA/\Delta fliM$ at any time point. Few single cells attaching to the coupon surfaces could be seen under the fluorescent microscope, but there was no progression of those attached cells into cell aggregations.

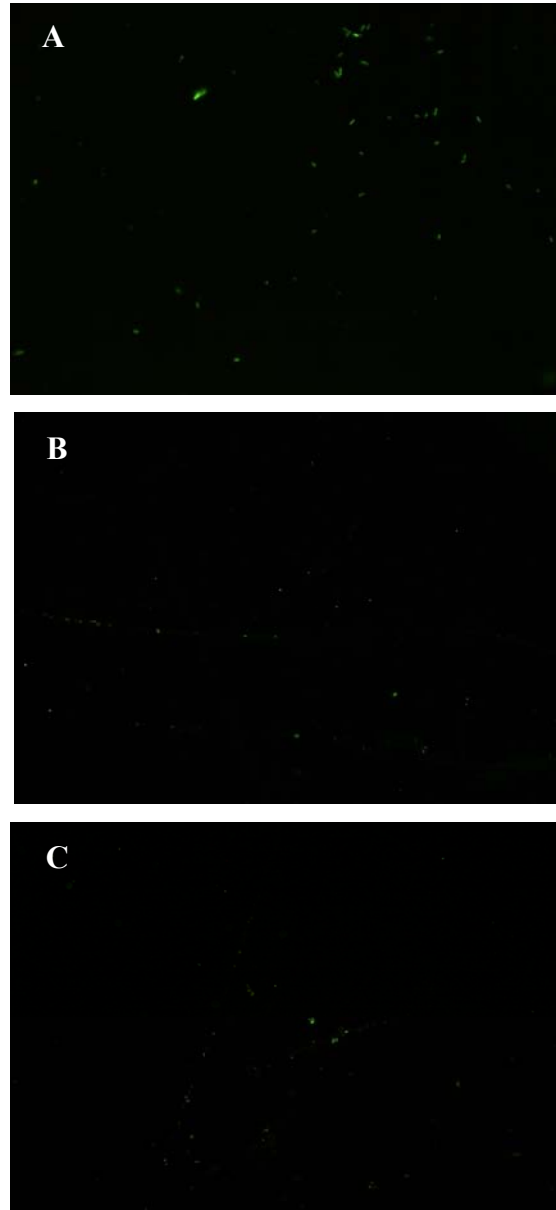


Figure 5.7 Biofilm development by the strain PAO $\Delta pilA/\Delta fliM$. Images were captured at 24, 48 and 72 hours as shown in (A), (B) and (C), respectively.

5.3.3 Quantitation of Biofilm Structures

5.3.3.1 Application of ImageJ Software in Biofilm Quantitative Analysis

ImageJ is an image processing programme developed by NIH in the USA and is freely available. Scale (pixels) of certain distance was set according to a slide micrometre (Brunel Microscopes Ltd.). For example, a scale of 380 pixels was equal to 100 μm (**Figure 5.8**). Each image was converted into a binary image (**Figure 5.9**) using the adjust threshold command to obtain an optimum black and white image, so that the least possible information from the image was lost. This allowed the software to detect a boundary of each biofilm (yellow line at the edge between black and white areas; **Figure 5.9 B**). By applying this binary threshold, it allowed the measurement of area as accurately and less subjectively as possible. The programme then calculated the area according to the set scale.

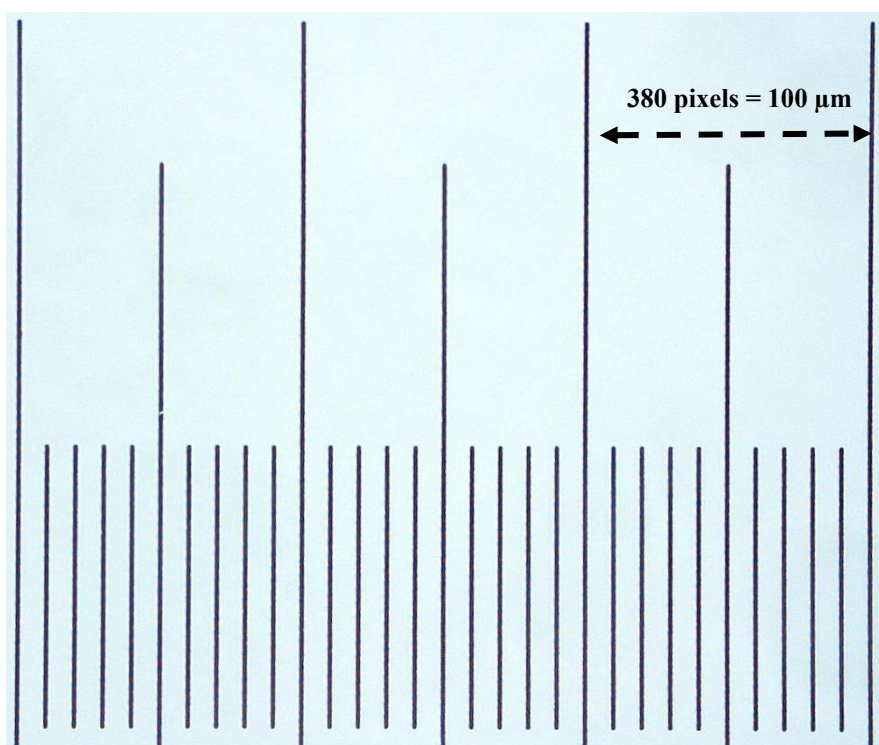


Figure 5.8 A slide micrometre. A scale of 380 pixels was equal to 100 μm . This image was captured using an Olympus Previs AX70 microscope with a 60x objective lens.

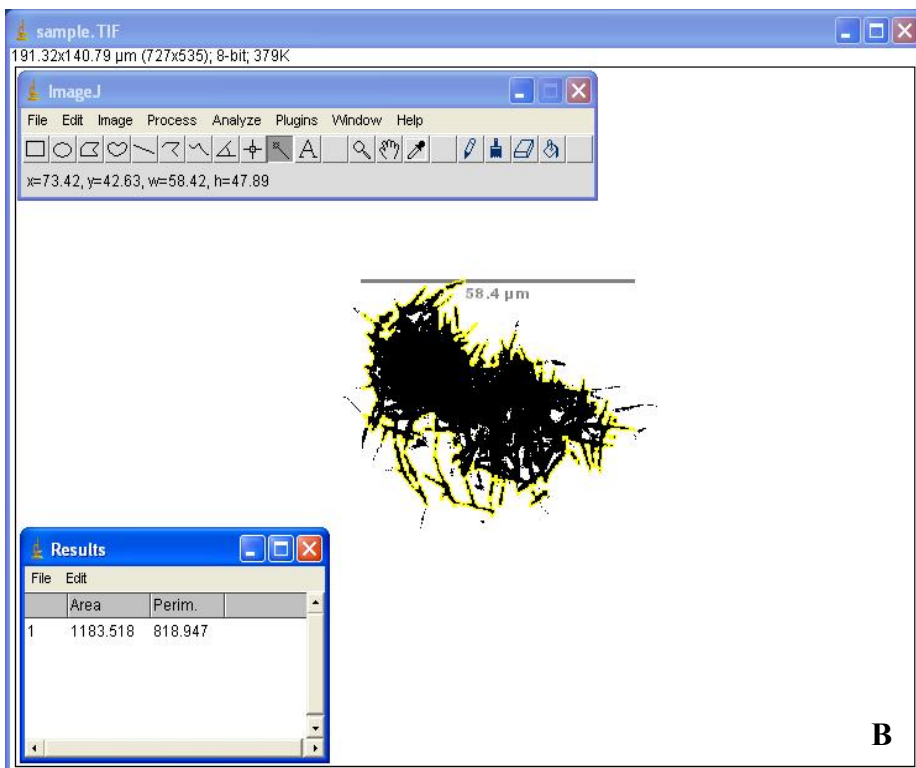
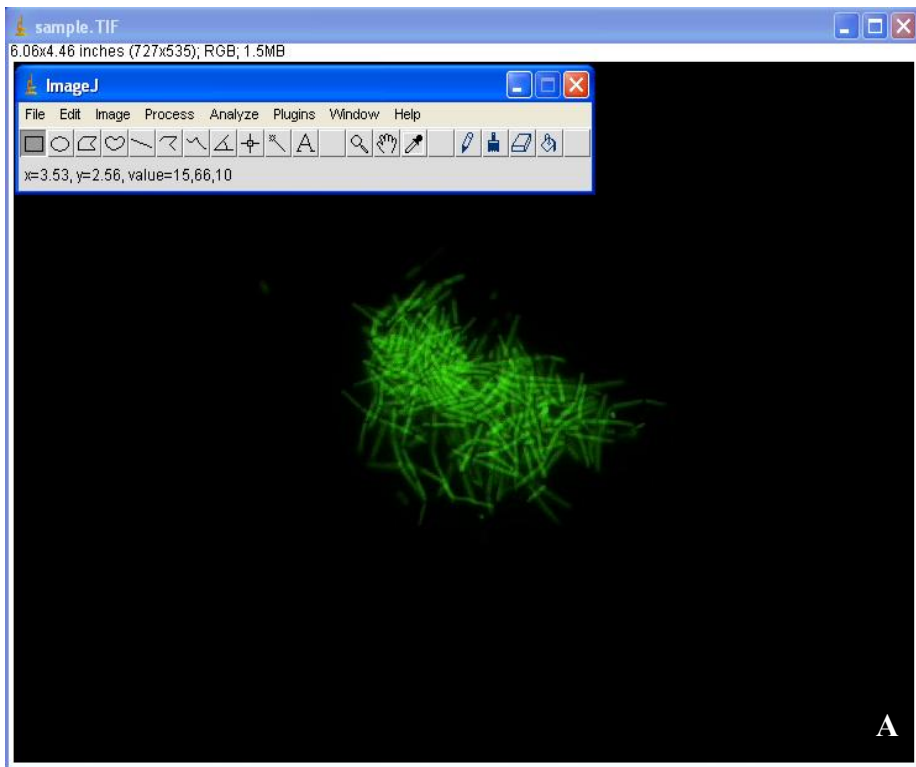


Figure 5.9 ImageJ programme. A fluorescent image (A) was converted to a binary (black and white) image (B) so that the boundary (yellow line) of the subjected could be detected and an area was calculated (lower-left small box).

5.3.3.2 Areas of Biofilms

The average areas (μm^2) of ten randomly selected biofilms formed by each isolate at 24, 48 and 72 hours are shown in **Figure 5.10** and in **Table 5.2**. At all time points, the four AES-1 isolates showed biofilms with a larger area compared to the non-clonal isolates and PAO1. Isolate C5 (AES-1) showed the greatest capacity for biofilm formation; an average biofilm area of this isolate measured at 72 hours was about 75,000 μm^2 . In contrast, biofilms of the non-clonal isolates were generally smaller. By combining the biofilm areas of all tested isolates, a comparison between average biofilm areas of a group of the AES-1 isolates and a group of the non-clonal isolates showed that there was a strong evidence of a significant difference in biofilm areas between two groups ($p = 0.001$ at 24 hours and at 48 hours; $p = 0.007$ at 72 hours).

By comparing the areas of biofilms at different time points of each individual isolate, the data suggested that the AES-1 isolates were able to form mature biofilms more promptly than the non-clonal isolates. As shown in **Table 5.2**, biofilms of all four AES-1 isolates had grown significantly from the 24-hour time point to the 48 hours while only three of eight non-clonal isolates (U8, U12 and U16) showed this similar growth. Comparison of the average areas of biofilms at 48 hours with those at 72 hours showed that two of four AES-1 isolates (C5 and C12) and three of eight non-clonal isolates (U4, U12 and U16) had made significant increases in the size of biofilms ($p = 0.005$, 0.015 and 0.008, respectively).

Table 5.2 Average biofilm areas of tested isolates at 24, 48 and 72-hour time point

Strain	Isolate	Average biofilm area (μm^2) and statistic values				
		24 hr	P_1	48 hr	P_2	72 hr
	PAO1	1178	< 0.001	1726	0.381	1791
AES-1	C3	2365	0.011	16768	0.534	26848
	C4	3230	0.044	25774	0.746	22770
	C5	4754	< 0.001	19727	0.032	74534
	C12	1274	0.006	3199	0.015	9045
	U4	107	0.205	164	0.005	810
Non-Clonal	U7	0	<i>n/a</i>	0	<i>n/a</i>	148
	U8	899	0.035	1805	0.118	1084
	U12	373	0.031	1667	0.015	7627
	U16	519	0.042	1456	0.008	5093
	U18	0	<i>n/a</i>	48	0.141	311
	U22	544	0.471	1154	0.089	5551
	U23	641	0.556	826	0.210	375

P_1 and P_2 represent P values of t-tests between the average biofilm areas at 24-hour and 48-hour time points, and between 48-hour and 72-hour time points, respectively.

n/a means not applicable.

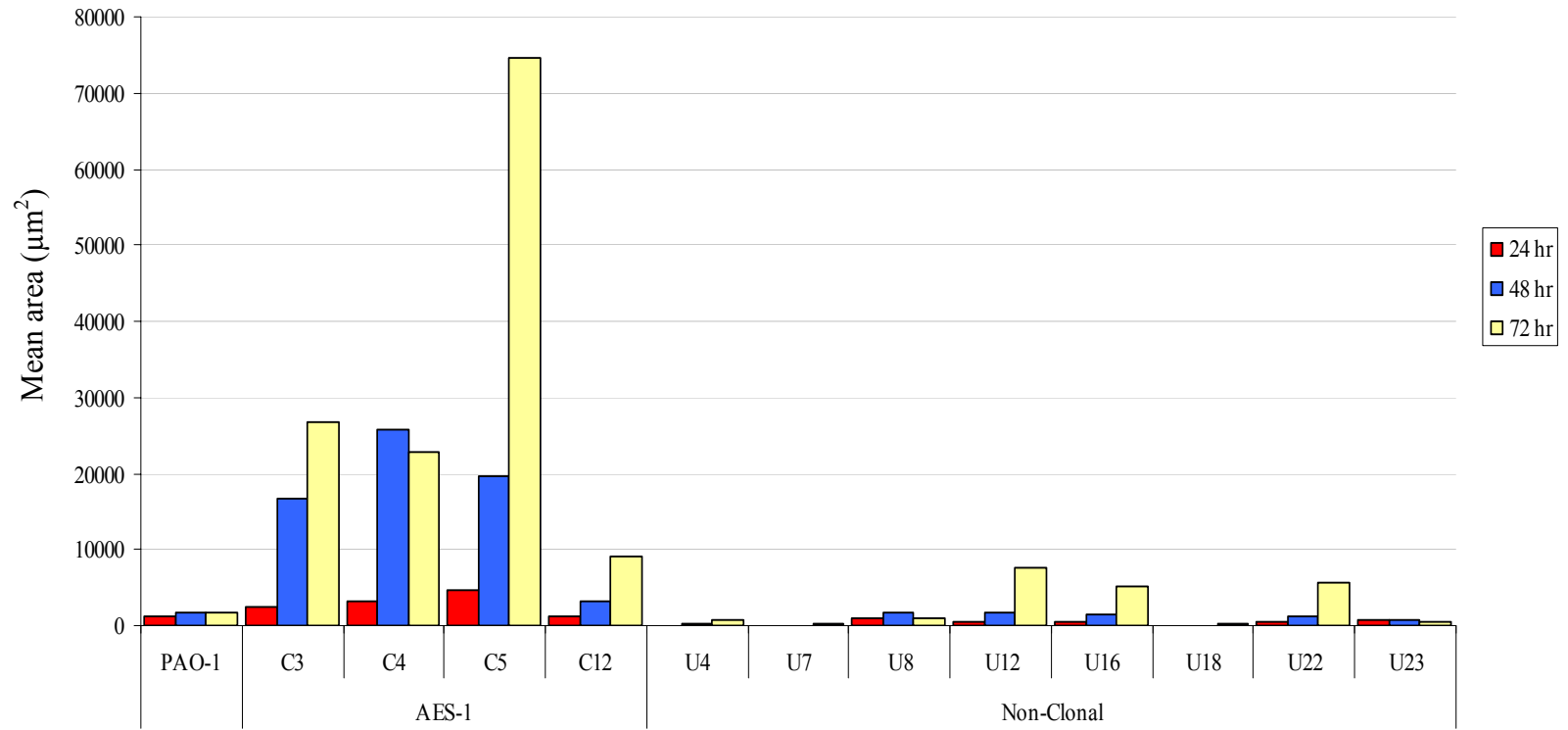


Figure 5.10 Average area of biofilms of *P. aeruginosa* isolates at 24, 48 and 72 hours.

5.3.3.3 Biofilm Thickness

The thickness of biofilms was measured using CLSM. Two AES-1 (isolates C5 and C12), five non-clonal (isolates U7, U8, U12, U18 and U23) and PAO1 were examined. Examples of the paraformaldehyde-fixed biofilms of isolate C12 (AES-1) and isolate U12 (non-clonal) are illustrated in **Figure 5.11**.

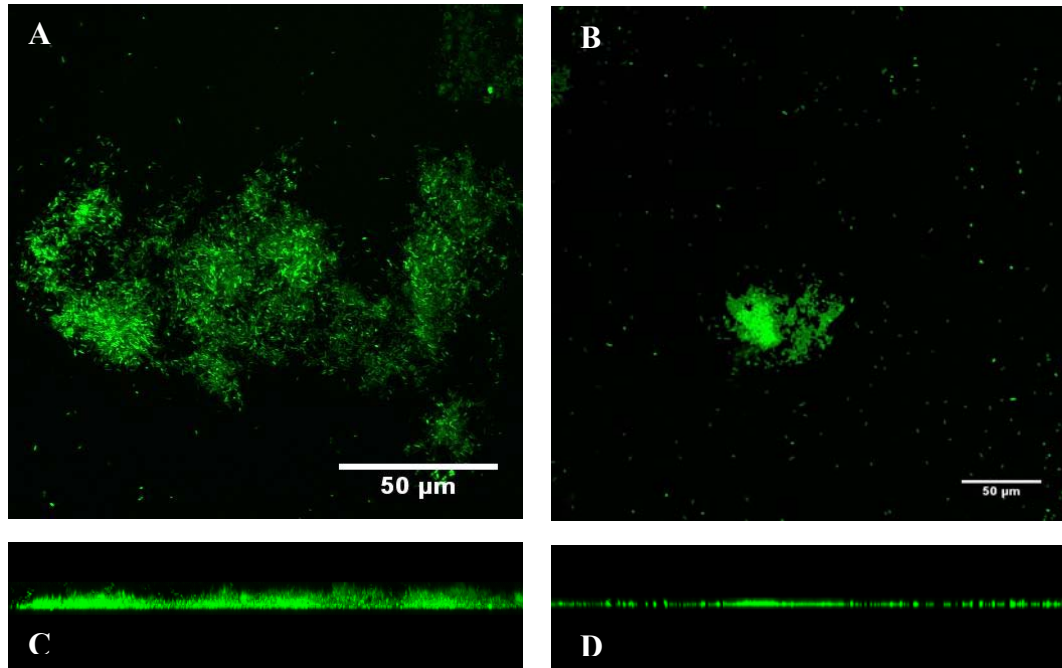


Figure 5.11 CLSM images of biofilms. Isolate C12 (AES-1) at x-y plane (**A**) and its cross-sectional (x-z) image (**C**). Isolate U12 (non-clonal) at x-y plane (**B**) and its cross-sectional (x-z) image (**D**).

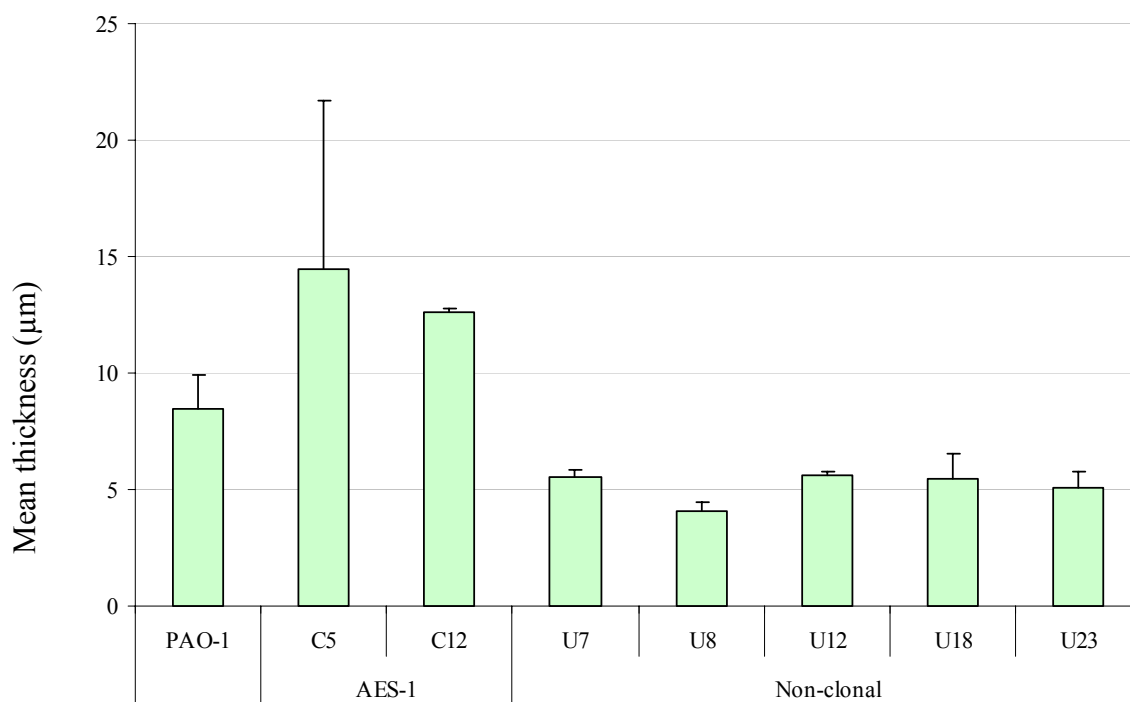


Figure 5.12 Average thicknesses of biofilms at 72 hours

Figure 5.12 shows average thickness of the biofilms as measured by CLSM. Both isolates of AES-1 tended to have a thicker (greater than two-fold) biofilm mass compared to the non-clonal isolates, suggesting a greater capacity in the biofilm formation of these strains. The average thickness of biofilms of PAO1 grown in this model is also consistent with a previous report (approximately 10 µm) (Heydorn *et al.*, 2002).

5.4 Discussion

5.4.1 Biofilm Formation Study Using the CDC Biofilm Reactor

There are a variety of *in vitro* model systems developed for growing and examining biofilms. A descriptive study of the biofilm structures by Whitnall in 2003 was conducted using a Flow Cell Chamber system, and the experiment involved cultivation of bacteria labelled with a green fluorescent protein (GFP) (Whitnall, 2003). To grow the GFP-tagged *P. aeruginosa*, an addition of antibiotics (i.e. carbenicillin and kanamycin) was required at different concentrations depending on the strains. These additional antibiotics can interfere with the biofilm formation and, with the different concentrations used, can also contribute to the variation of biofilms that were formed by different *P. aeruginosa* strains (Spoering & Lewis, 2001).

In contrast to the Flow Cell Chamber system, bacterial biofilms grown on coupons in the CDC Biofilm Reactor can be taken out without disturbing the remaining growing biofilms. The biofilms can also be stained and examined microscopically.

The CDC Biofilm Reactor was developed in 2004 by researchers at the Biofilm Laboratory at the Centre for Disease Control and Prevention in USA. It was first used to study the biofilm formation of *Streptococcus pneumoniae* (Donlan *et al.*, 2004). The reliability and reproducibility of the system has been demonstrated (Goeres *et al.*, 2005). This is the first study to apply this model system to study *P. aeruginosa* biofilms.

In the original study by Donlan *et al.*, the coupons used were made of germanium and the biofilms grown on these coupons were captured using an attenuated total reflectance-Fourier transform infrared (ATR-FTIR) laser spectrometer (Donlan *et al.*, 2004). In this experiment, the polypropylene coupons were initially used for the biofilm cultivation according to advice from the manufacturer that it had no autofluorescent property. However, it appeared that this material showed a strong autofluorescence under an excitement by the green fluorescent light. In fact, this observation has been previously reported (Piruska *et al.*, 2005). The polypropylene coupons were thus substituted with borosilicate glass coupons.

To visualise the biofilms, instead of using GFP-expressing *P. aeruginosa*, bacterial cells were stained with Syto-9, which is a freely diffusible green fluorescent dye that intercalates intracellular nucleic acid. This dye was first used to assist a quantification of bacterial cells (*Salmonella typhimurium*) through a flow cytometry (Lebaron *et al.*, 1998). It was shown here that this dye can also be applied to stain cells within the biofilm population. The recently available TRITC-ConA (Molecular Probes) has been shown to stain exopolysaccharide matrix of *P. aeruginosa* biofilms, which will greatly assist our understanding of biofilm structure in the future (Harrison *et al.*, 2006).

5.4.2 Formation of Biofilms by AES-1 and Non-Clonal Isolates

The major goal of this study was to compare biofilms formed by different strains of *P. aeruginosa* quantitatively. This goal was achieved by applying the image processing and analysis software to the biofilm images, and the results showed that the AES-1 isolates had a greater biofilm-forming capacity compared to the non-clonal isolates.

Initial Attachment

As described in Section 1.4.5, it has been proposed that biofilm formation proceeds through five stages. Biofilms formed by all four AES-1 isolates were likely to be in the attachment and/or early maturation (maturation-1) phases within 24 hours whereas the two non-clonal isolates (isolates U7 and U18) did not appear to be in this developmental phase. The attachment phase is an initial step of the biofilm development, and involves the expression of flagella and type IV pili (O'Toole & Kolter, 1998a). Both isolates U7 and U18 might not express these motility factors hence the non-attachment. In contrast to the flagella and type IV pili, it is likely that QS has no role in this process (Beatson *et al.*, 2002; Kirisits & Parsek, 2006). Consistent with this suggestion, either the attachment of cells or the existence of small cell clusters was observed in isolates whose QS signals could not be detected. (The production of the QS signals of tested isolates is shown in **Table 5.3.**)

Table 5.3 The QS signals and rhamnolipid production by tested isolates. This result was from the studies of Chapter Four.

Strain	Isolate	QS signals	Mucoidy
		BHL/HHL/OHHL/ODHL/OdDHL	
AES-1	PAO1	+/+/+/+/+	-
	C3	+/+/+/-/-	+
	C4	+/+/-/-/-	+
	C5	-/-/-/-/-	+
	C12	-/+/-/-/-	+
Non-Clonal	U4	-/-/-/-/-	+
	U7	-/+/-/-/-	+
	U8	-/-/-/-/-	-
	U12	-/+/-/-/-	+
	U16	+/+/+/+/+	+
	U18	-/+/-/+/+	+
	U22	-/-/-/-/-	+
	U23	-/-/-/+/+	+

Maturation

Following attachment, cell clusters or microcolonies develop into mature biofilms. PAO1, all four AES-1 isolates, and three out of eight non-clonal isolates (U8, U12 and U16)

showed this significant progression as their biofilms became significantly larger at 48 hours. Although the three non-clonal isolates showed this substantial growth, the average areas of biofilms at 48 hours of the non-clonal isolates were significantly smaller than those of the AES-1 isolates. Of the remaining five non-clonal isolates that had non-progressive growth, one isolate (isolate U7) did not form a biofilm at all. As shown in **Table 5.3**, it is noteworthy that these isolates apart from PAO1 and isolate U16 did not produce *las*-regulated QS signals (i.e. OdDHL) – intercellular signalling molecules which have been previously reported as a major requirement for the formation of biofilms (Davies *et al.*, 1998). Nonetheless, findings from this study are consistent with a report by Molin and colleagues that biofilms formed by the *lasI* mutant are structurally indistinguishable from the wild-type (Heydorn *et al.*, 2002). These results suggest that the QS system may not be entirely necessary for *P. aeruginosa* biofilm formation. Recently, Smith *et al.* applied a whole-genome analysis to *P. aeruginosa* isolates recovered from chronic CF lungs with an 8-year duration course and demonstrated that during the course of infection, the environment in the CF lungs selects for mutations in the QS regulatory gene, *lasR* (Smith *et al.*, 2006). Again, this finding suggests that at least in the CF lungs, QS may play little or no role in *P. aeruginosa* biofilm maintenance.

Dispersion

As biofilm formation progresses, cells within the biofilms may be released into the surrounding liquid, either from erosion created by fluid shear or by bacteria actively swimming away (also known as ‘seeding dispersal’) (Purevdorj-Gage *et al.*, 2005). It has been reported that seeding dispersal was associated with cell death, creating voids (or hollows) within microcolonies which simultaneously resulted in the dispersal of a viable subpopulation (Webb *et al.*, 2003). Findings from the present study that isolate C5 (AES-1) formed a ‘hollow’-like structure are consistent with this seeding dispersal process. The fact that the hollowing biofilms were found in only this isolate, but not the remaining tested isolates, is possibly due to its dimension. This observation is consistent with the report that a microcolony diameter of approximately 80 μm was required for the seeding dispersal process in PAO1 (Purevdorj-Gage *et al.*, 2005); mature biofilms of isolate C5 had a diameter of more than 150 μm (**Figure 5.13**). Interestingly, the wild-type PAO1, which was reported to exhibit the seeding dispersal, did not appear to display this hollowing structure within the time-frame of this experiment. This discrepancy is possibly due to the experimental course; Webb *et al.* observed this structure in PAO1 after twelve days while

Purevdorj-Gage *et al.* found it within five days of their experiments (Purevdorj-Gage *et al.*, 2005; Webb *et al.*, 2003).

Other factors that may play roles in this dispersal stage include the QS system (Purevdorj-Gage *et al.*, 2005), rhamnolipids [Schooling *et al.* suggested that rhamnolipids promoted the dispersal (Schooling *et al.*, 2004) whilst Purevdorj-Gage *et al.* suggested the opposite (Purevdorj-Gage *et al.*, 2005)], nutritional status [either starvation (Gjermansen *et al.*, 2005) or carbon nourishment (Sauer *et al.*, 2004)], and the mucoid/non-mucoid morphotype (Purevdorj-Gage *et al.*, 2005). In the latter instance, the mucoid CF strain has been previously linked with the non-seeding-dispersal characteristic (Purevdorj-Gage *et al.*, 2005). This contradicts the findings of hollowing biofilms formed by isolate 19-334 which is a mucoid isolate (**Table 5.3**). Recent studies also showed that the seeding dispersal did occur in mucoid CF strain biofilms (Kirov *et al.*, 2005; Kirov *et al.*, 2007), supporting the result from the present study. Importantly, it has been suggested that the seeding dispersal may contribute to the transmission of bacteria (Kirov *et al.*, 2005; Stoodley *et al.*, 2005). This may be associated with the transmission mechanism of the AES-1 isolate.

Cross-sectional measurement of 72-hour biofilms using CLSM revealed that the AES-1 biofilms were two-fold thicker than the non-clonal strains. Thicker biofilms have been linked with an increase of resistance to antimicrobial agents (Cochran *et al.*, 2000; Landry *et al.*, 2006). This may contribute to the resistant nature of the AES-1 strain.

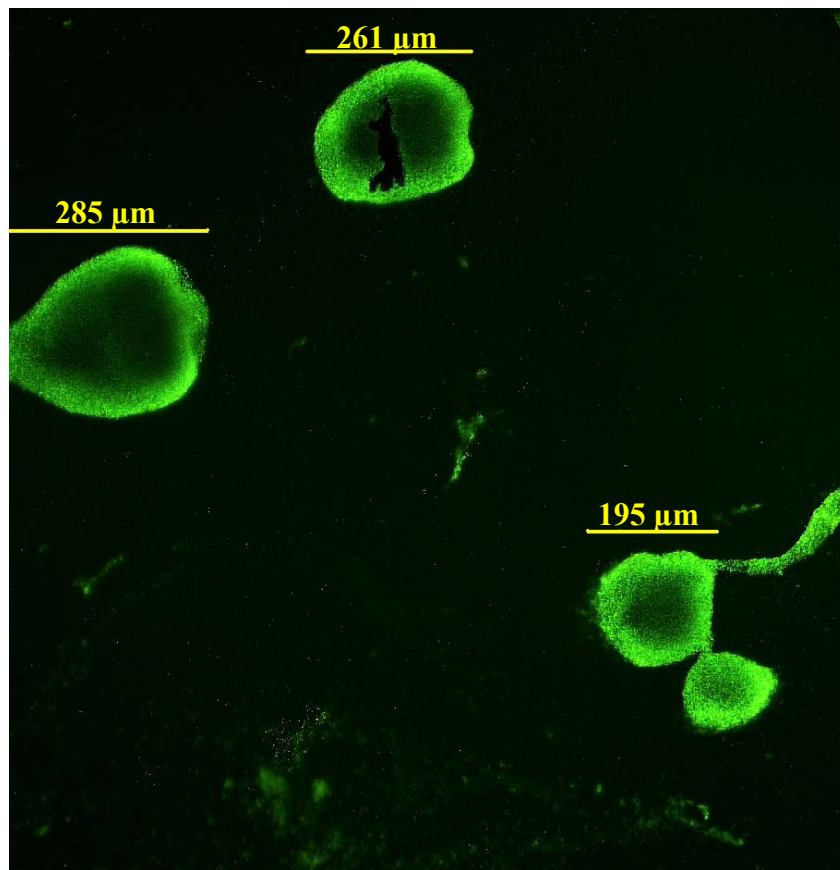


Figure 5.13 Diametres of biofilms formed by isolate C5 at 72 hours.

In summary, it has been widely accepted that the biofilm mode of growth contributes greatly to the survival of bacteria in both natural and clinical settings. Particularly in *P. aeruginosa*, the formation of biofilms allows this pathogen to persist within the lungs of people with CF, causing chronic infections. It is obvious from this study that AES-1 has a better capacity for biofilm formation when compared to the non-clonal strains. The biofilms produced by the AES-1 isolates formed more quickly and were significantly larger at all time points and tended to be thicker as measured by CLSM when compared to those of the non-clonal strains. Overall, this data suggest that the clonal strain AES-1 has more potential to be persistent and perhaps out grow the non-clonal strains. Moreover, the observation of the seeding dispersal in one clonal isolate may be related to its transmissibility. These results have large implications for the pathogenesis of this pathogen in the CF lungs.

6 Chapter Six: Development of an Artificial Sputum Medium for Biofilm Studies

6.1 Background

Almost all biofilm studies reported in the literature, including experiments from the previous chapter, have been performed using a model in which bacterial biofilms grew on solid abiotic surfaces (i.e. glass slides in a flow-chamber model or borosilicate glass coupons in the CDC Biofilm Reactor) (Christensen *et al.*, 1999; Sternberg *et al.*, 1999). While these studies have advanced our understanding of bacterial biofilms, the key question still remains – can these findings be extrapolated to the nature of *P. aeruginosa* in clinical settings, such as in the lungs of people with CF?

The literature suggests that the development of *P. aeruginosa* biofilms in the CF airways may not be associated with surface growth (Baltimore *et al.*, 1989; Simel *et al.*, 1984; Singh *et al.*, 2000a; Worlitzsch *et al.*, 2002). An immunolocalisation study demonstrated that in freshly excised CF airways, *P. aeruginosa* grows as ‘clumps’ or ‘macrocolonies’ in the intraluminal space rather than an epithelial surface compartments (**Figure 6.1**) (Worlitzsch *et al.*, 2002). In addition, TEM examination of CF sputum revealed that bacteria were clustered together, encased in a densely stained matrix without attachment to surfaces (**Figure 6.1**) (Singh *et al.*, 2000a). By staining with a Gram stain, a similar structure of the bacterial clusters was also observed in sputum samples from CF patients (**Figure 6.1**).

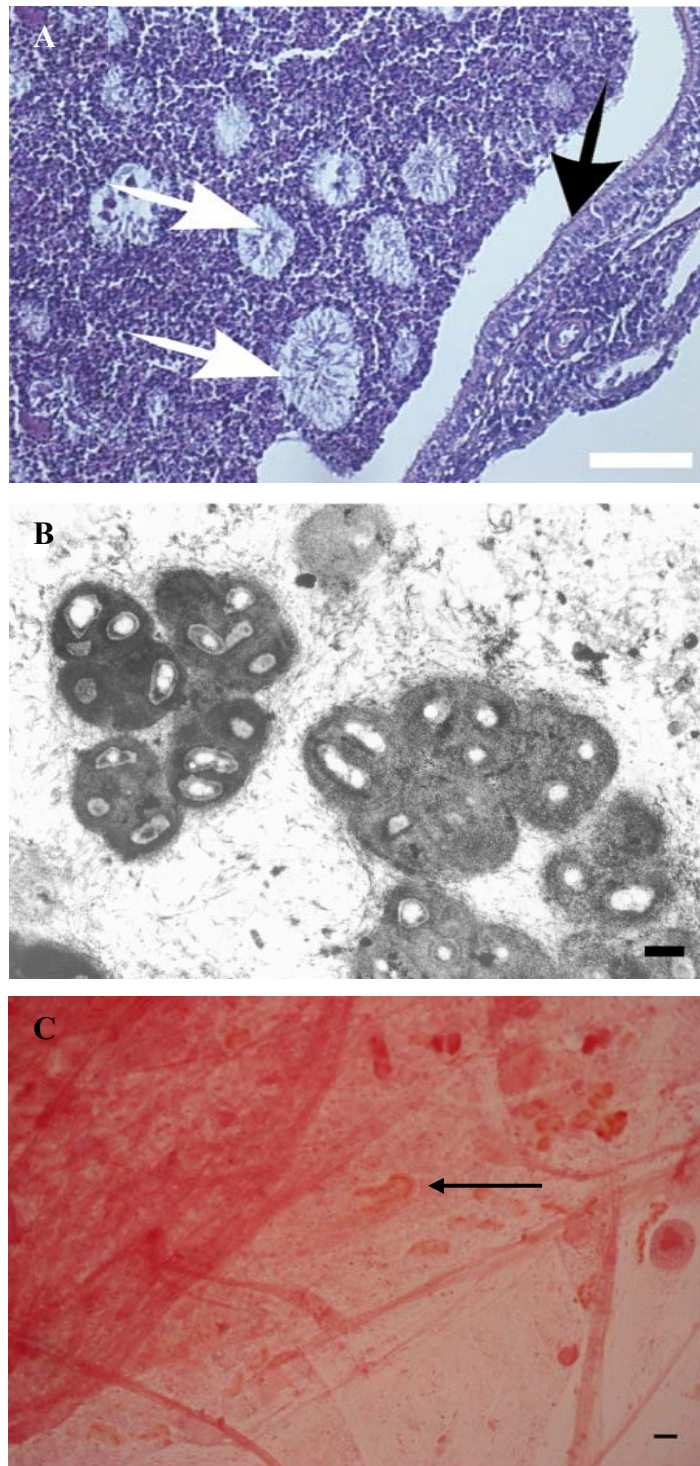


Figure 6.1 Characteristics of bacterial clusters in the CF airways: (A) from freshly excised CF airways. Note the absence of *P. aeruginosa* on epithelial surface (black arrow) and presence of *P. aeruginosa* ‘macrocolonies’ within intraluminal material (white arrows) (Worlitzsch *et al.*, 2002); (B) TEM of CF sputum. Note the bacterial cells surrounded by dense matrix (Singh *et al.*, 2000a); (C) Gram stain of CF sputum. Note the cluster of bacteria surrounded by a matrix (black arrow) (author’s preliminary work).

It is very likely that these bacterial clusters that are present in the CF airways are a form of *P. aeruginosa* biofilms in which bacteria adhere to each other and probably to sputum components but not to the solid surface. In order to understand the nature of such unique biofilms, it is important to have an *in vitro* model to grow biofilms in an environment similar to the CF lungs (while a direct isolation of living biofilms from the CF lung or the sputum is still impossible). Thus, studies in this chapter aimed to:

- develop a study model of growth medium that closely resembled sputum from CF airways and simultaneously supported the growth of bacteria in the biofilm mode.

Recently, Sriramulu and colleagues developed such a model, called an artificial sputum medium (ASM), containing chemical components resembling CF sputum and demonstrated that *P. aeruginosa* grown in this model displayed a biofilm-like structure (Sriramulu *et al.*, 2005). The present study will elaborate upon the practicality of this model for a large-scale study.

6.2 Material and Methods

6.2.1 Bacterial Strains and Growth Condition

The following strains were used in this study: control strain, PAO1; strain PAO-JP2, a mutant derivative of PAO1 with double deletions of two major QS components – *lasI* and *rhlI* ($\Delta lasI/\Delta rhlI$) (Pearson *et al.*, 1997); and AES-1R, a representative isolate of the clonal strain, AES-1. The mutant strain PAO-JP2 has been shown to have no biofilm forming capacity (de Kievit *et al.*, 2001). This strain was a gift by Dr. Zhu from the Cooperative Research Centre for Eye Research and Technology (CRCERT) at University of New South Wales. AES-1R is an original strain that was isolated from a *P. aeruginosa* infected CF child, who attended the CF clinic at the Royal Children's Hospital, Melbourne and subsequently suffered from a fulminant pneumonia as previously reported (Armstrong *et al.*, 2002). This strain was a gift by Dr. Armstrong from Department of Paediatrics at Monash University.

6.2.2 Artificial Sputum Medium

The components of an artificial sputum medium (ASM) proposed by Sriramulu *et al.* are shown in **Table 6.1**.

Table 6.1 Components of an artificial sputum medium developed by Sriramulu *et al.* (2005)

Component	Content per 1 litre of ASM
NaCl	5 g
KCl	2.2 g
DTPA (diethylene triamine pentaacetic acid)	5.9 mg
Amino acids	5 g
Mucin	5 g
DNA	4 g
Egg yolk emulsion	5 ml

In this study, some chemical components and their concentrations were modified to make the medium more similar to CF sputum.

Salts

Analyses of salt compositions of sputa from CF patients have shown that the average concentration of Na⁺ was 91.6 mmol/L or approximately 5 g/L, and the average concentration of K⁺ was 30.6 mmol/L or approximately 2.2 g/L (Kilbourn, 1978). Calcium (Ca²⁺) was also present in the CF sputum at the average concentration of 2 mmol/L (Kilbourn, 1984). As per Sriramulu *et al.*, Na⁺ and K⁺ were added at according concentrations, and Ca²⁺ (0.22 g/L) was included as an extra ASM ingredient.

Iron

In CF sputa, iron was present mainly in transferrin or ferritin-bound iron (Stites *et al.*, 1998). Concentrations of transferrin and ferritin were 1.09 µg/mg and 242 ng/mg, respectively (Stites *et al.*, 1999) while concentration of free-form iron was only 0.03 mmol/L (Kilbourn, 1984). *P. aeruginosa* is able to use iron for growth by producing siderophores (i.e. pyocyanin) to scavenge it from iron-bound proteins (Wolz *et al.*, 1994).

Ideally, the iron component in the ASM should be in the protein-binding form such as “iron-saturated ferritin”. However, this product was not commercially available at the time. Therefore, DTPA, an iron chelating agent, was substituted and used at the concentration according to the recipe by Sriramulu *et al.*

DNA

An amount of DNA was present in CF sputum, presumably originating from disrupted leukocytes, macrophages or airway epithelial cells (Yeager, 1971). Picot *et al.* reported that the average DNA content in sputum was approximately 1.39 g/L (Picot *et al.*, 1978). The DNA content in the original recipe from Sriramulu *et al.* was at 4 g/L, but in a recent study using a similar medium, a concentration of DNA at 1.39 g/L was used (Alkawash *et al.*, 2006). In this study, DNA at 1.39 g/L was chosen as an ingredient of the ASM.

Mucin

Concentration of mucin in the CF sputum was about 10 g/L (Yeager, 1971). The ASM recipe of Sriramulu *et al.* was adapted from a work done by Ghani and Soothill (1997) in which the concentration of mucin at 5 g/L was used (as it was the maximum concentration that allowed the culture medium to flow through their biofilm model) (Ghani & Soothill, 1997). In this study, the mucin concentration was adjusted to 10 g/L, and the mucin derived from porcine stomachs (Sigma-Aldrich) was used because it was reported to resemble human tracheobronchial mucin in its carbohydrate, amino acids and sulfate ester compositions (Scawen & Allen, 1977).

Proteins

Studies consistently showed high amounts of amino acids in CF sputum (Barth & Pitt, 1996; Thomas *et al.*, 2000). In the present model, casamino acids were used to substitute the addition of 20 individual amino acids. Albumin was also reportedly present in the CF sputum at a concentration of around 1% (w/v) (Yeager, 1971), and was thus added in this model at this level.

Lecithin

Lecithin has been proposed as a component of CF sputum. Although its actual amount has not yet been defined, an estimated concentration at 5 mL/L was used in studies by Govan (Govan, 1975) and by Sriramulu *et al.* (Sriramulu *et al.*, 2005). In this study, egg yolk emulsion (phosphatidylcholine) was used as a source of lecithin.

pH

The range of pH in the CF sputum was 6.5 to 7.1 (Kwart *et al.*, 1963). In this ASM, pH was monitored and, if necessary, adjusted to 7 using Tris-base (Amresco[®]).

Viscoelasticity

Certain types of mucins (MUC5AC and MUC5B) that are produced by CF airway epithelia constitute CF sputum (Rubin, 2007), and have been reported as “gel-forming mucins” (Hovenberg *et al.*, 1996), contributing to the viscoelasticity of the sputum. In this model, purified bacteriological agar (Oxoid) was added to the final concentration of 0.1% in order to increase the viscoelasticity of the ASM.

Collectively, the ingredients of the ASM in this study are summarised and shown below (Table 6.2).

Table 6.2 Summary of the ASM ingredients and final concentrations in this study

Component (Supplier)	Content per 1 litre of ASM
NaCl (Biolab)	5 g
KCl (Univar)	2.2 g
CaCl ₂ (AnalaR [®])	0.22 g
DTPA (Sigma-Aldrich)	5.9 mg
DNA from Salmon Sperm (Sigma-Aldrich)	1.39 g
Mucin from Porcine Stomach (Sigma-Aldrich)	10 g
Casamino Acids (Difco)	5 g
Albumin from Bovine Serum (BSA; Sigma-Aldrich)	10 g
Lecithin from Egg Yolk Emulsion (Oxoid)	5 ml

ASM Preparation, Sterilisation and Culturing Condition

Separated components were made up at high concentration (stock solutions) and sterilised accordingly. Methods of making the stock solutions are explained in the Appendix.

Salt solution was sterilised by autoclaving at 121°C for 15 minutes. Casamino acids solution and BSA solution were filter sterilised using Millex[®] syringe driven filter units with 0.22-µm pore size (Millipore), instead of autoclaving in order to avoid denaturing proteins. Sterility tests of DTPA, DNA, mucin and egg yolk emulsion were performed by

inoculating each component on a Columbia HBA (Oxoid). DNA, DTPA and egg yolk emulsion showed no growth of any contamination while mucin was contaminated with *Bacillus subtilis* and *Streptococci spp.* (organisms were identified by Department of Microbiology at the RPAH). By avoiding the autoclave which would denature the mucin, sterilisation methods including UV-irradiation (placed under UV light overnight), dry-heat sterilisation (140°C for 2 hours), filter sterilization (both through 0.22- and 0.45- μ m filters) and microwave (putting in microwave at medium heat for 1 minute) were applied. None of these methods was able to successfully eliminate the contamination, because *B. subtilis* existed in a spore form. Ultimately, antibiotics including tetracycline, penicillin and ampicillin were included in the final recipe of ASM. These antibiotics were shown to have no growth inhibitory effect on any of the tested *P. aeruginosa* strains; although it has been previously shown that tetracycline at subinhibitory concentrations can reduce the production of virulence factors including protease and elastase (LeVatte *et al.*, 1990).

To make 100-mL ASM, 10 mL of 10x salt solution, 20 mL of 5x BSA solution, 10 mL of 10x casamino acids solution, 10 μ L of DTPA solution, and 0.5 mL of egg yolk emulsion were mixed with 1 g mucin and 0.139 g DNA. The mixture was made up to 85 mL with dH₂O and was stirred vigorously for 15 – 20 minutes followed by mashing in a sterile glass tissue grinder in order to homogenise all undissolved ingredients. Tetracycline, penicillin and ampicillin were added to the final concentrations of 16 μ g/mL, 1 μ g/mL and 1 μ g/mL, respectively. The mixture was then made up to 90 mL with dH₂O and was then aliquoted into sterile McCartney bottles or 24-well plates in equal portions before adding agar solution for the final concentration of 0.1% (v/v).

Bacterial solution was diluted with PBS to an optical density equivalent to a 0.5 MacFarland Standard (BioMerieux) and was then added to the ASM – 50 μ L for the 10-mL ASM in each McCartney bottle or 5 μ L for the 1-mL ASM in a single well of 24-well plates. Cultures were then incubated at 37°C for 72 hours, and results were observed every 24 hours.

6.3 Results

6.3.1 Biofilm Formation within the ASM

After 48 hours of culture in the McCartney bottles containing 10 mL of the ASM, both PAO1 and AES-1R isolates displayed several ball-like structures (clumps or macrocolonies) within the cultivation matrix which did not attach to the glass wall. These clumping structures were likely to be clusters of bacteria or biofilms. Pellicles were also observed attached to the glass surface at an interface between the medium and air (**Figure 6.3**). Both isolates also had a layer of green pigment diffusing at the top of the ASM surface. After 72 hours, the clumping structures were more prominent, and the pellicles were noticeably thicker (**Figure 6.2**). In some cases, these clumps appeared to be a single large biomass (**Figure 6.3**). The green pigmentation also tended to be darker than at 48 hours, indicating that the amount of pigment was increasing. There was no discrepancy in growth characteristics between PAO1 and AES-1R. In contrast, the ASM cultivation of PAO-JP2 (a *lasI* and *rhlI* mutant) appeared to grow homogeneously. There was no formation of such clumping structures or pellicles and no production of green pigmentation. However, a clear layer of liquid was observed at the top of the culturing medium (**Figure 6.4**).

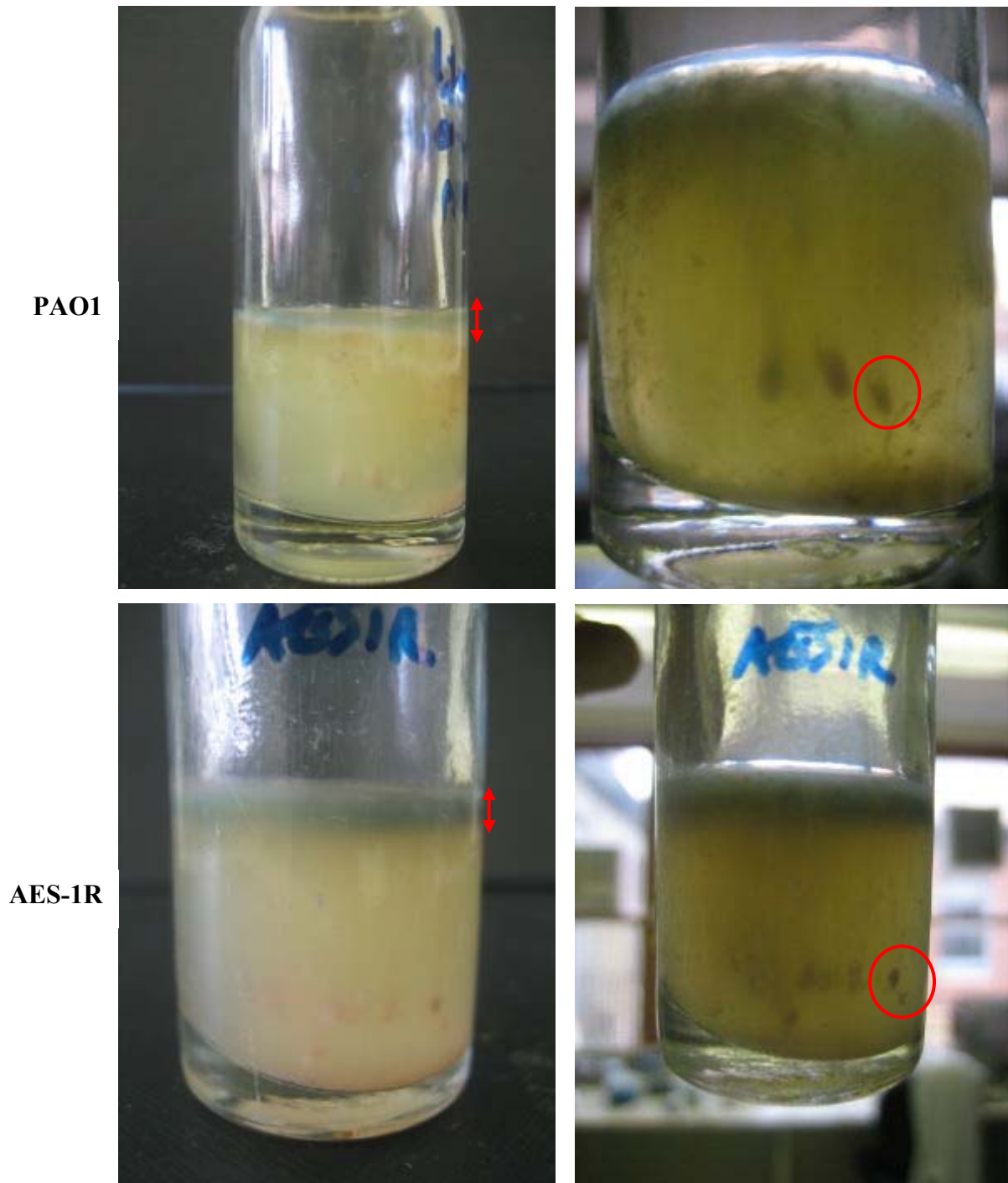


Figure 6.2 Clumping structures (examples in red circles) of PAO1 (top images) and AES-1R cultivations (bottom images) suspended in the ASM without surface attachment. Notice a green pigment layer and pellicles atop medium surface or at an air-medium interface (red arrows). Images on left and right hand sides were taken from same sample but with different background.

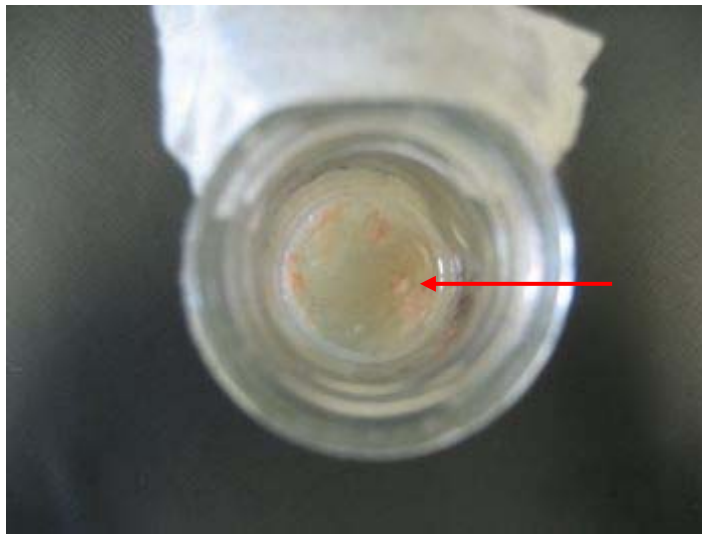
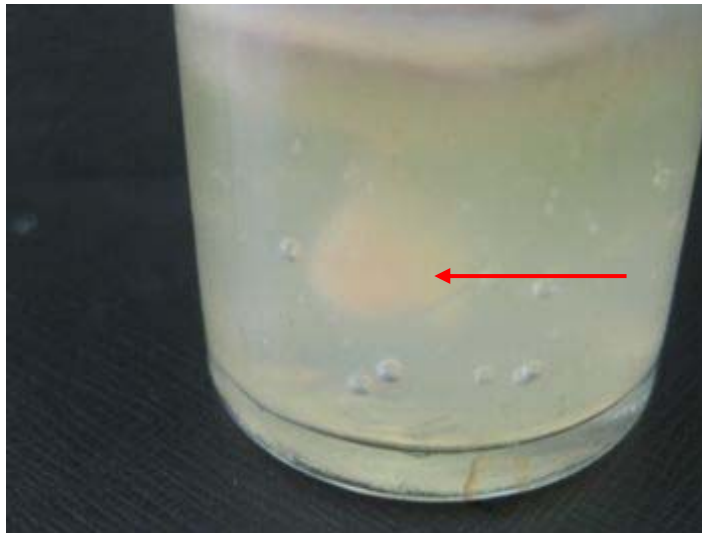
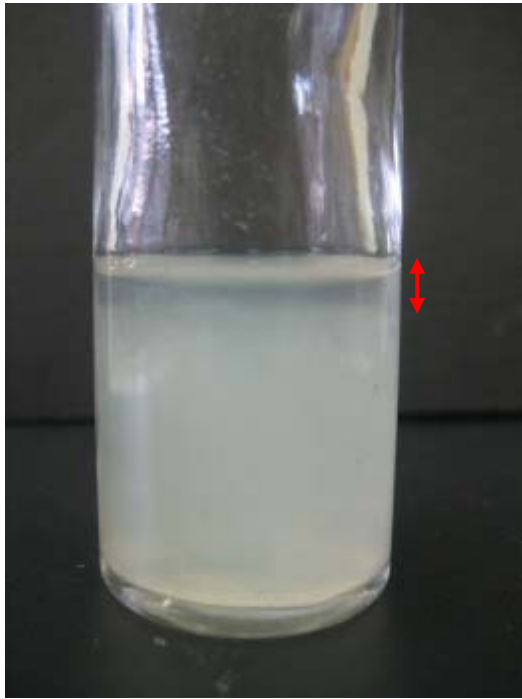


Figure 6.3 A single biomass of the AES-1R isolate in the ASM cultivation at 72 hours (top). Surface pellicles can be seen at the air-medium interface (middle image; a bird-eye view of the culturing bottle) or attaching to the glass wall (bottom).

PAO-JP2



Un-Inoculated ASM

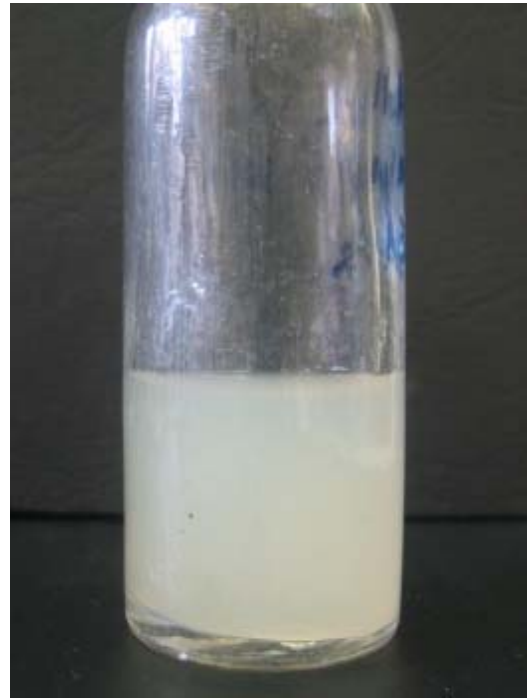


Figure 6.4 Cultivation of PAO-JP2 ($\Delta lasI/\Delta rhlI$) in the ASM at 72 hours (left). Note the clear liquid layer on top of the medium (red arrow). An un-inoculated ASM is also illustrated here (right).

6.3.2 Staining of Bacterial Clumps

Bacterial clumps of both PAO1 and AES-1R isolates were removed from the culture medium by pipetting using a blunt end pipette tip. Each sample was mixed with approximately equal amounts of the Syto-9 staining solution (as described in Section 5.2.7.1) and was then visualised under a fluorescent microscope (Olympus Provis AX70). An un-inoculated ASM and a PAO-JP2 culture were also included for the visualisation. Green-fluorescent stained cells were found as a major component of the clumping structures of both PAO1 and AES-1R, and a relatively small number of individual cells was also observed outside the clumps (**Figure 6.5**). Samples from the culture of PAO-JP2 showed a considerable amount of individual cells in the medium matrix, and no clumping structure was observed microscopically. The un-inoculated ASM appeared to be stained slightly (**Figure 6.5**).

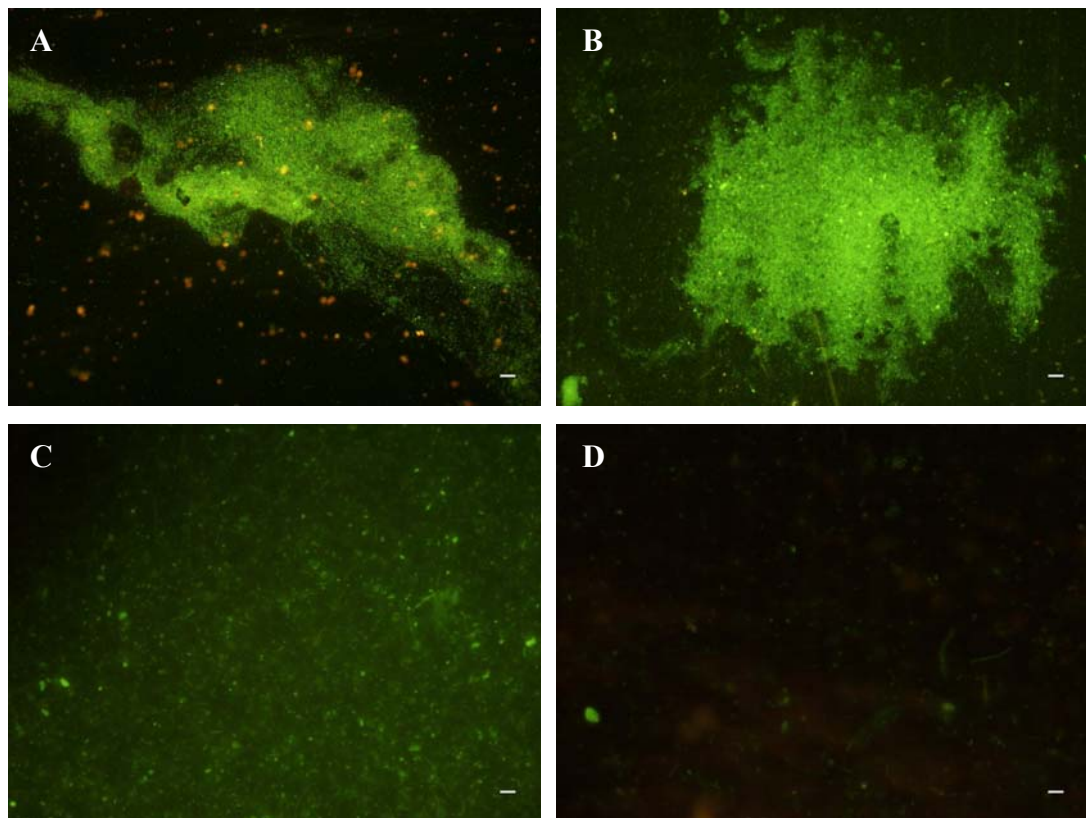


Figure 6.5 Illustration of microscopic images of ASM-grown bacteria: PAO1 (**A**), AES-1R (**B**), PAO-JP2 (**C**) and un-inoculated ASM (**D**). Bars, 50 μm .

6.3.3 Comparison of Bacterial Growth in the ASM with Non-Autoclaved Mucin versus Autoclaved Mucin

In the beginning of this study, a problem of contamination was experienced which was subsequently shown to have originated from the porcine stomach mucins. The only method which was found to completely eliminate the contamination was autoclaving at 121°C for 15 minutes. This would result in denaturing the mucins and might be expected to have some effect on the growth of bacteria/biofilms. To test the effect of denatured mucins, PAO1 and AES-1R were grown in the ASM containing autoclaved mucins and in the one containing non-autoclaved mucins. As shown in **Figure 6.6**, PAO1 and AES-1R formed clumping structures or macrocolonies in the non-autoclaved-mucin ASM while in the autoclaved-mucin ASM, both strains grew homogeneously.

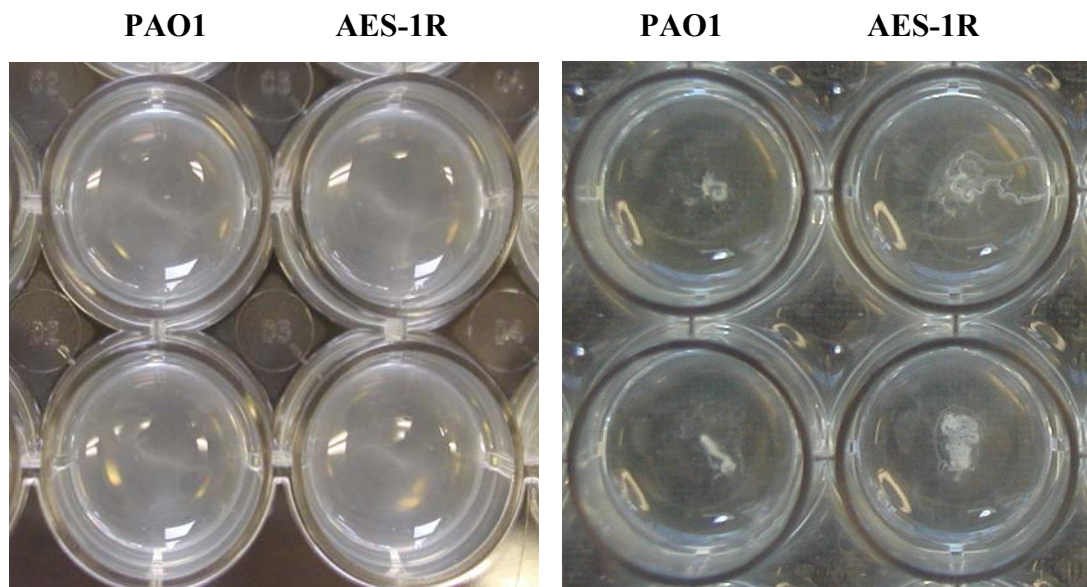


Figure 6.6 Comparison of bacterial growth in the ASM containing autoclaved mucin (left) and non-autoclaved mucin (right). Bacterial clumps of both PAO1 and AES-1R were observed in the non-autoclaved mucin ASM.

6.4 Discussion

6.4.1 Significance of the ASM Model

Several biofilm-study models have been developed for experimental use, and most of them focus on cells and/or biofilms attached to a solid surface. While the nature of biofilms grown in this setting can represent those grown on medical catheters or in surgical instruments (Pajkos, 2004), it may not mirror the biofilms that are found in the CF lungs. Indeed, several studies have demonstrated that *P. aeruginosa* grows in the CF airways without attachment to any surface (which in the CF airways is the epithelium) (Singh *et al.*, 2000a; Worlitzsch *et al.*, 2002). Worlitzsch and colleagues showed that 94.5% of *P. aeruginosa* resided in a cluster form at 5 – 17 μm distant and the rest (5.5%) resided 2 – 5 μm distant from the epithelial cell surface; none of which attached to the airway (Worlitzsch *et al.*, 2002). This special form of biofilm may have different properties both physiologically and genetically from the surface grown biofilms. For the validity of this study, it was important to have the model that most closely resembled the sputum from the CF lungs which also supported biofilm growth adequately.

Recently, two studies have initiated an exploration of biofilms grown in realistic milieu (Palmer *et al.*, 2005; Sriramulu *et al.*, 2005). Palmer and colleagues grew *P. aeruginosa* in a morpholinopropanesulfonic acid (MOPS)-buffered medium mixed with CF-sputum powder (lyophilised sputa from CF subjects) and found that bacteria showed an up-regulation of genes involved in amino acids transportation and degradation and genes for a synthesis of PQS (Pseudomonas quinolone signal) (Palmer *et al.*, 2005). The authors did not state any observation of special growth characteristics such as the biofilm mode of growth in their report. In the ASM model developed by Sriramulu *et al.*, *P. aeruginosa* was shown to grow readily in a cluster where cells adhered to each other (Sriramulu *et al.*, 2005). Similar structures were also observed in this study in which the model was adapted to resemble the CF sputum closely. Success in consistently obtaining the bacterial clumps in all cultures in a larger container (10-mL McCartney bottles) has important implications for a future large-scale study.

6.4.2 Bacterial Growth and Biofilm Formation in the ASM

It is likely that the bacterial clumps grown in the ASM are biofilms even though they do not entirely fit into the conventional biofilm definition – “a structured community of bacterial cells enclosed in a self-produced polymeric matrix ‘and’ adherent to an inert or living surface (Costerton *et al.*, 1999)” (see also Section 1.4.5). This was supported by the fact that the clumping structures were observed in both PAO1 and AES-1R but not in PAO-JP2, a strain that is unable to form biofilms. Microscopic studies also showed that the clumps were composed of bacterial cells attaching to each other (**Figure 6.5 A and B**), while in the culture of PAO-JP2, cells were dispersing within the medium matrix (**Figure 6.5 C**). This clumping structure was also similar to the ‘macrocolonies’ of bacteria observed in the freshly excised CF airways (**Figure 6.1 A**) (Worlitzsch *et al.*, 2002).

It was not surprising that the formation of pellicles at the interfaces either between air and medium or between medium and glass surface was also observed. These pellicles have previously been shown to be a dense mat of bacteria (in other word ‘biofilms’) under an electron microscope (Cole *et al.*, 1989). As expected, there were no pellicles found in the cultivation of PAO-JP2.

Ultimately, the existence of biofilms in both clumps in the ASM (or macrocolonies in the CF sputa) and air/medium interface pellicles leads to a re-consideration of the definition of biofilms. Indeed, Stoodley and Costerton recently proposed a new biofilm definition as “a matrix-enclosed bacterial population adherent to each other ‘and/or’ to surfaces or interfaces” (Hall-Stoodley *et al.*, 2004).

Components of the ASM that contribute to the biofilm formation

It has been shown previously that certain chemicals, such as DNA, amino acids or lecithin, are important in the formation of *P. aeruginosa* biofilms (Sahu & Lynn, 1978; Thomas *et al.*, 2000; Whitchurch *et al.*, 2002). Extracellular DNA, in particular, has been shown to function as an intercellular connector or a stabiliser of the biofilms (Whitchurch *et al.*, 2002). Sriramulu *et al.* also tested the effect of each individual ASM component and found that all components, including DNA, amino acids, lecithin, salt, iron (at low level) and mucins, contributed to the growth and the clump formation of *P. aeruginosa* (Sriramulu *et al.*, 2005). In the absence of amino acids, the clumping structures became ‘loose’ and were easily disrupted by pipetting whereas the absence of mucin not only caused no biofilm formation but also reduced the overall growth of *P. aeruginosa* (Sriramulu *et al.*, 2005). In

this study, it has been further shown that mucins that aided the biofilm formation have to be in their natural form. Denatured mucins caused *P. aeruginosa* to grow planktonically. Interaction between mucins and *P. aeruginosa* has been previously reported: the bacteria were found to adhere to mucin from human tracheobronchial airways (Ramphal & Pyle, 1983; Vishwanath & Ramphal, 1984); to bovine conjunctival mucin (Aristoteli *et al.*, 2003); and to porcine gastric mucin (Aristoteli & Willcox, 2001). This observation is also consistent with a recent work which showed that mucin can interact with *P. aeruginosa* and have a profound effect on the development of biofilms (Landry *et al.*, 2006). Bacteria grown on mucin displayed clumping structures (which were called ‘microcolonies’ in their report) (Landry *et al.*, 2006), similar to those found in this study. The observations by Landry *et al.* and those from this study suggest that the development of biofilms in the ASM may be initiated by an adhesion of bacterial cells to mucins, and this bacterium-mucin interaction may play a role in the initial stage of the CF airway infections.

Bacterial pigment production in the ASM

The iron level in the ASM is expected to be low as a result of an iron chelating agent, DTPA. It is not surprising to find that both PAO1 and AES-1R produced abundant levels of green pigments such as pyocyanin, as these pigments act as iron scavengers (Cox, 1986). As expected, PAO-JP2 did not produce this pigment because its production is controlled by the QS system (de Kievit & Iglewski, 2000). Pyocyanin is also a potent virulence factor (see Section 1.4.2.2.1). The high production level of this pigment may reflect damages that could occur within the CF lungs.

In summary, a model which closely mimicked chemical constituents of sputum from CF airways was successfully developed. *P. aeruginosa*, when grown in this model, clustered together and formed structures resembling biofilms. This biofilm formation may be promoted by the unique composition of the CF sputum. In addition, other unique conditions of the CF lungs have been reported to be possible contributors to the development of biofilms and the subsequent persistent infections. These conditions include (i) an ASL (airway surface liquid) hyperabsorption (Matsui *et al.*, 2006); (ii) an increased accumulation of human neutrophils (Walker *et al.*, 2005); and (iii) an anaerobic condition of mucus in the CF airways (Yoon *et al.*, 2002).

It is also important to note that PAO1 is a burn wound isolate. The fact that this strain readily grew with similar characteristics to the CF lung strain (AES-1R) suggested that the

growth condition may be more influential than the characteristic background of bacteria. Indeed, a transcriptomic study by Fung in our group using this model showed that there were no genes significantly differentially expressed between AES-1R and PAO1 when grown in the ASM (Fung, 2007).

Finally, the efficacy of the ASM has been shown by this study and in the study by Fung (Fung, 2007). The ASM described in this study has been used for growing bacteria for a proteomic investigation conducted by N. Hare (PhD student) and Dr. Cordwell from School of Molecular and Microbial Biosciences at the University of Sydney in collaboration with the CF Microbiology group at the University of Sydney.

7 Chapter Seven: Amplified Fragment Length Polymorphism (AFLP) Analysis of *P. aeruginosa* AES-1 Isolates

7.1 Background

Studies reported in Chapter Four and Five have shown that AES-1 displayed a more virulent phenotype when compared with non-clonal isolates. It was noted however that there was considerably variation of phenotypes among the AES-1 isolates. For example, isolate C2 had a low total protease activity and did not show any protease at all in the zymogram gel, while isolate C3 displayed a completely opposite phenotype (refer to **Table 4.4**). Moreover, the colonial morphologies and/or antibiograms of each isolate were also different from other isolates belonging to the same clone. The research in this chapter was designed to determine whether there is a genetic basis for the observed phenotype variation which is not detectable by PFGE/RFLP.

PFGE/RFLP is a whole-genome DNA fingerprinting technique which compares DNA-fragment patterns generated by restriction with an infrequent-cutting restriction endonuclease. Because of the use of this rare-cutting enzyme, it generally generates a maximum of approximately 30 – 40 DNA bands, and thus may not detect minor genetic distinctions or DNA micro-heterogeneity. A technique called ‘amplified fragment length polymorphism’ (AFLP), which is another whole-genome typing method, has been reported to be highly sensitive for detecting polymorphisms in DNA (Vos *et al.*, 1995). In one example, it has been shown to have higher discriminatory power than PFGE/RFLP (Speijer *et al.*, 1999). By applying this technique to AES-1 isolates, it may be possible to discover ‘sub-strains’ of the AES-1 genotype, which may explain the phenotypic variations within this clone. The aim of this study was therefore to:

- analyse the *P. aeruginosa* strain AES-1 by using the AFLP technique.

The AFLP technique is based on the selective PCR amplification of restriction fragments from endonuclease digestions of a whole genomic DNA (Vos *et al.*, 1995). The procedure of this technique is divided into three steps (**Figure 7.1**):

- (i) Digestion of the whole cellular DNA with two endonucleases and ligation of restriction specific 'adaptors' to all restriction fragments.
- (ii) Selective amplification of subset of restriction fragments with primers that have corresponding adaptor and restriction site specific sequences.
- (iii) Electrophoretic separation of amplicons on a gel and visualisation of the band pattern. [This step can be done using a automate DNA sequencer (Goulding *et al.*, 2000)].

AFLP primers consist of sequences corresponding to adaptor and restriction sites and an additional selective base. PCR amplification is achieved by using the adaptor and restriction site as target for primer annealing. Selective amplification is achieved by the use of primers with selective extension base. Only those restriction fragments in which nucleotide flanking the restriction site match the primer selective extension base are amplified.

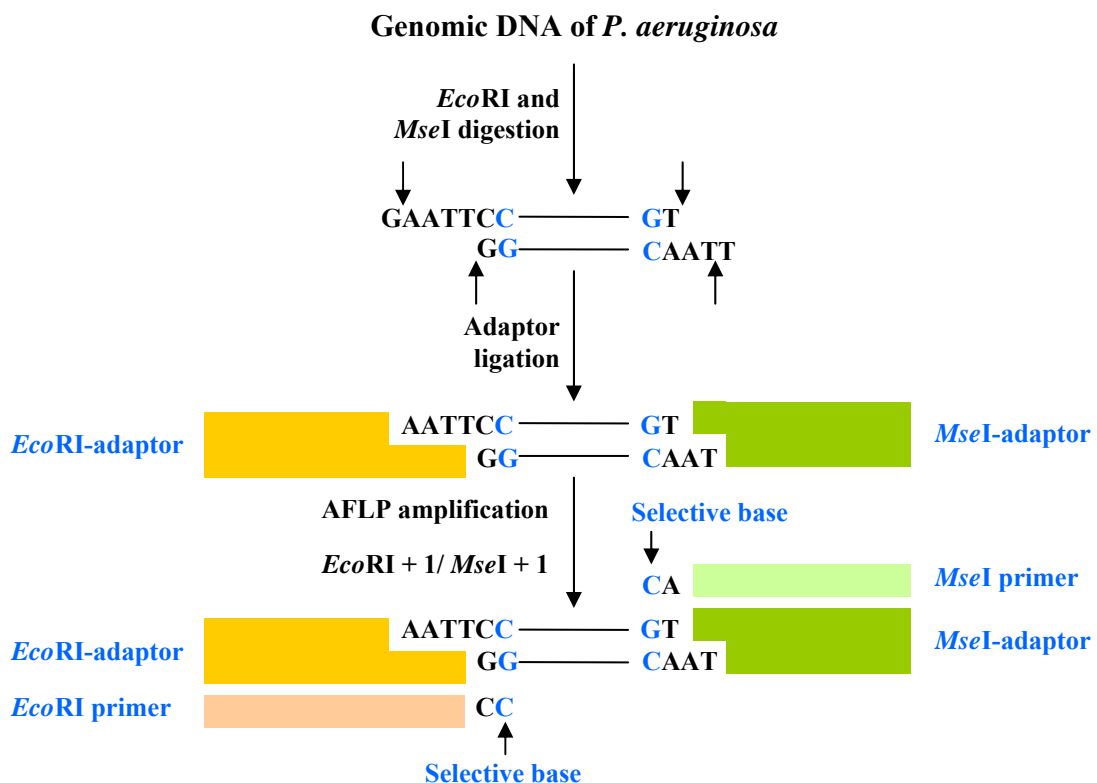


Figure 7.1 Schematic representation of AFLP principle and procedure.

7.2 Material and Methods

7.2.1 Study Population and Culturing Conditions

Seven representatives of the 14 AES-1 isolates from Chapter Four were chosen on the basis of their phenotypes in order to get the most diverse study population. These isolates are shown in **Table 7.1**. PAO1 and AES-1R were also included in this study. All isolates were recovered from Glycerol storage on a Columbia HBA (Oxoid) at 37°C for 48 hours and were sub-cultured in TSB and incubated overnight at 37°C with shaking at 250 rpm (see Section 2.2.4 and 2.2.5).

Table 7.1 Study population and phenotypic characteristics (refer to **Table 4.3** and **Table 4.4** for more details)

Isolate	Colony morphology		Virulence factors			Antibiotic Sensitivity		
	Size	Mucoidy	Protease Level	Rhamnolipids	ExoS	Tim	Mem	Cip
C2	medium	++	low	+	+	S	S	R
C3	small	++	high	+	+	R	R	R
C4	small	+	low	+	+	S	S	S
C5	medium	++	low	+	+	S	S	S
C6	small	++	low	–	+	S	S	S
C9	large	–	high	+	–	R	S	R
C12	medium	++	medium	–	+	S	S	R
AES-1R	small	–	high	+	+	R	R	S
PAO1	medium	–	high	+	+	S	S	S

Tim, ticarcillin-clavulonate; Mem, meropenem; Cip, ciprofloxacin.

S, susceptible; R, resistant.

7.2.2 DNA Extraction

Chromosomal DNA was prepared from an overnight culture from the previous step. One millilitre of each culture was transferred to 1.5 mL microtubes, and cell pellets were collected by centrifugation at 14,000 rpm for 1 minute using Hermle Z200 centrifuge (D&A Laboratory Sciences). The pellet was resuspended in 1 mL of TNE buffer

(Appendix). The tubes were then again re-centrifuged at 14,000 rpm for 1 minute. After which, supernatant was discarded and the cell pellet was resuspended in 135 μ L TNE buffer, and a further 135 μ L of TNE buffer containing 2% (v/v) Triton X-100 (Sigma-Aldrich) was then added. Then, 30 μ L of (5 mg/mL) freshly prepared lysozyme (Sigma-Aldrich) was added to the mixture followed by 30-minute incubation at 37°C in a water bath. After which, 15 μ L of (20 mg/mL) proteinase K solution (Sigma-Aldrich) was added to each tube. The tubes were thoroughly mixed and incubated in a 65°C water bath for 2 hours.

After incubation, the DNA was extracted using the phenol/chloroform/isoamyl alcohol method (Sambrook *et al.*, 1989). Briefly, an equal volume of 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (Sigma-Aldrich) was added to each tube and mixed thoroughly. The tubes were then centrifuged at 14,000 rpm at room temperature for 15 minutes. The aqueous phase (top layer) containing DNA was then carefully transferred to a new 1.5-mL microtube. The extraction was done again using the phenol/chloroform/isoamyl alcohol. The final extraction was then carried out in the aqueous phase using just chloroform.

To precipitate the DNA, a 1/10th volume of 3 M sodium acetate (Sigma-Aldrich) and 2 volumes of 100% (v/v) molecular grade ethanol (Sigma-Aldrich) were added to each tube. The mixtures were thoroughly mixed and left at -80°C overnight.

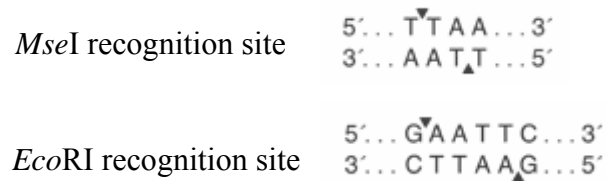
After the precipitation process, each tube was centrifuged at 14,000 rpm for 45 minutes at 4°C to pellet the DNA. The supernatant was discarded and 150 μ L of 70% (v/v) ethanol was added to each tube to wash the DNA pellet. Once ethanol was evaporated and the pellet dried, the pellet was resuspended in 100 μ L dH₂O and stored at -20°C for future use.

Quality and quantity of the extracted DNA was determined by measuring absorbance at 260 nm and 280 nm using a Beckman DU[®]640 spectrophotometer (Beckman) reading at absorbance of 260 nm and 280 nm. Purity of DNA was determined by the ratio OD₂₆₀ / OD₂₈₀ and samples with OD₂₆₀ / OD₂₈₀ value lower than 1.8 were excluded from further analysis.

7.2.3 AFLP

7.2.3.1 Digestion of DNA and Ligation of Adaptors

In this step, DNA was digested with two restriction enzymes – one that cut frequently (*MseI*; New England Biolabs) and one that cut less frequently (*EcoRI*; New England Biolabs). *MseI* and *EcoRI* have a recognition site as shown below.



DNA was digested and restriction fragments were simultaneously ligated to adaptors. All primers and adaptors used in this study were kindly given by Prof. Reeves at School of Molecular and Microbial Biosciences, University of Sydney. The sequences of oligonucleotide primers and adaptor are shown in **Table 7.2**.

Table 7.2 Sequences of primers and adaptors used in this study

Primer	Description	Sequence (5' to 3')
924	<i>Eco</i> RI adaptor	ACTGCGTACC ^N (3' amino group)
925	<i>Eco</i> RI adaptor	^P AATTGGTACGCAGTCAGTGAGGTTTCATTACCATCC (5' phosphorylated)
930	<i>Mse</i> I adaptor	TACTCAGGACTCN (3' amino group)
931	<i>Mse</i> I adaptor	CCTGATTGCTACAACGATGAGTCCTGAG
926	<i>Eco</i> RI+0	GGATGGTAATGAACCTCACT
932	<i>Mse</i> I+0	CCTGATTGCTACAACGATGAGTCCTGAG
1753	<i>Mse</i> I+C (6-FAM TM labelled)	GATGAGTCCTGAGTAAC ^a

^a The underlined nucleotide is the selective nucleotide for the primer.

Adaptors for the *EcoRI*-restriction site were prepared by combining 20 μL of both 924 and 925 *EcoRI* adaptors with 6 μL 10x One-phor-all buffer (OPA; Applied Biosystems) and 74 μL dH_2O . The mixture was then placed in boiling water for 2 minutes and allowed to cool gradually to room temperature. Once cooled, it was then diluted 1:10 to a final concentration of 5 $\text{pMol}/\mu\text{L}$. Similarly, adaptors for the *MseI*-recognition site were prepared by mixing 20 μL of both 930 and 931 *MseI* adaptors with 6 μL of OPA buffer and 74 μL of dH_2O , and the mixture was boiled and cooled gradually to room temperature. The *MseI*-adaptor solution was left undiluted with the final concentration at 50 $\text{pMol}/\mu\text{L}$. Both *EcoRI*-adaptor solution and *MseI*-adaptor solution were store at -20°C until used.

For DNA digestion and adaptor ligation, 0.1 μg of DNA was mixed with 0.5 μL of (10 $\text{U}/\mu\text{L}$) *EcoRI*, 0.5 μL of (10 $\text{U}/\mu\text{L}$) *MseI*, 10 μL OPA buffer, 1 μL of T4 ligase (ABI) 1 μL of (5 $\text{pMol}/\mu\text{L}$) *EcoRI*-adaptor solution, 1 μL of (50 $\text{pMol}/\mu\text{L}$) *MseI*-adaptor solution, 1 μL of 10 mM ATP (ABI) and 5 μL of (10 mg/mL) BSA (New England Biolabs). This mixture was then made up to 50 μL using dH_2O . The final mixture was incubated overnight at 37°C . After that, the digestion and ligation processes were terminated by placing on a heating block setting at 94°C for 2 minutes.

7.2.3.2 Amplification

7.2.3.2.1 Pre-amplification

This step was done using two primers without selective nucleotide: primer 926 (*EcoRI*+0) and primer 932 (*MseI*+0). Briefly, 1 μL of the DNA product from the digestion and ligation step was mixed with 2 μL 10x PCR buffer II (ABI), 1.6 μL 25 mM MgCl_2 (ABI) 1 μL 4 mM dNTP (ABI), 0.4 μL of 10 mg/mL BSA (New England Biolabs), 1 μL of primer 926 *EcoRI*+0 (6 pMol), 1 μL of primer 932 *MseI*+0 (6 pMol), 0.1 μL of 5 $\text{U}/\mu\text{L}$ AmpliTaq Gold[®] (Applied Biosystems) and 11.9 μL dH_2O . This mixture was then overlaid with sterile liquid paraffin prior to the amplification. Pre-amplification was performed in a PC-960 Air Cooled Thermal Cycler (Corbett Research). PCR running conditions consisted of an initial denaturation at 94°C for 10 minutes to activate AmpliTaq Gold[®], and then 20 cycles of amplification. These consisted of 15 seconds at 94°C , annealing at 56°C for 30 seconds, and extension at 72°C for 2 minutes with additional 5 minutes for the last cycle. DNA products from this pre-amplification step were used as a template for the selective amplification step.

7.2.3.2.2 Selective Amplification

Selective amplification was achieved by the use of primers complementary to each of the two adaptor sequences, except for the presence of one additional base at the 3' end. In this study, primers 926 [*EcoRI*+0 (no specific additional base)] and 1753 [*MseI*+C (selective C complementary base)] were used. The primer 1753 *MseI*+C was labelled with 6-carboxyfluorescein (6-FAMTM; ABI) (Hu, 2005), in order to facilitate the visualisation process. The mixture for the selective amplification step consisted of 1 μ L of 10-times diluted DNA product from the pre-amplification step, 2 μ L 10x PCR buffer II (ABI) 1.6 μ L 25 mM MgCl₂ (ABI), 1 μ L 4 mM dNTP (ABI), 0.4 μ L of 10 mg/mL BSA (New England Biolabs), 1 μ L of (6 pMol/ μ L) primer 926 *EcoRI*+0, 1 μ L of 6-FAMTM-labelled primer 1753 *MseI*+C (6 pMol/ μ L), 0.1 μ L of (5 U/ μ L) AmpliTaq Gold[®] (Applied Biosystems) and 11.9 μ L dH₂O. The amplification was done in a PC-960 Air Cooled Thermal Cycler (Corbett Research). The running conditions consisted of an initial denaturation at 94°C for 10 minutes to activate AmpliTaq Gold[®] followed by 10 cycles of amplification. These consisted of 15 seconds at 94°C, annealing which started at a temperature of 66°C for 30 seconds and reduced 1°C in every cycle until 56°C in the tenth cycle, followed by annealing at 56°C for 30 seconds for further 20 cycles, and extension at 72°C for 1 minutes with additional 15 minutes for the last cycle. DNA product from this step was subjected to visualisation.

7.2.3.3 Visualisation of Fluorescent AFLP

Amplified DNA was sent to the Sydney University Prince Alfred Molecular Analysis Centre (SUPAMAC) for analysis using an ABI 373 DNA Sequencer equipped with GeneScan[®] Analysis software (Applied Biosystems). Results were displayed as an electropherogram.

7.2.3.4 Dendrogram Construction

An electropherogram of each sample was analysed. A single AFLP amplicon displayed as a “peak” in an electropherogram, and its presence or absence was designated as “1” and “0” in an Excel[®] spreadsheet (Microsoft Office 2003). Similarity among all tested isolates was calculated using Dice coefficient (S_D). The formula of S_D is shown below:

$$S_D = \frac{2 n_{AB}}{n_A + n_B}$$

where n_{AB} is the number of peaks found in both A and B, n_A is a total number of peaks found in A, and n_B is a total number of peaks found in B (Dice, 1945). S_D values toward 1 indicated increasing similarity between isolates. These coefficients were transformed into distances, and a dendrogram was constructed using the DendroUPGMA programme (<http://genomes.urv.es/UPGMA/>).

7.3 Results

7.3.1 Relatedness of AES-1 Isolates Analysed by AFLP

AFLP electropherograms of PAO1 and AES-1R are shown in **Figure 7.2**. PAO1 displayed a total of 28 peaks whereas AES-1R displayed 30 peaks. There were 21 peaks in common between PAO1 and AES-1R. The S_D between these two isolates was 0.724. AFLP patterns of all AES-1 isolates are shown in **Figure 7.3**, and the presence/absence of AFLP amplicons transformed into binary data (“1” or “0”) are shown in **Table 7.3**. Generally, all tested AES-1 isolates displayed almost identical AFLP patterns. However, some micro-heterogeneity was found among isolates. Isolates C2 and C12 displayed an extra AFLP amplicon when compared to the others (**Figure 7.3**), while isolate C3 lost one amplicon (**Figure 7.3**). Thus, overall AES-1 isolates could be divided into three subgroups according to these AFLP patterns: (i) subgroup-A consisting of AES-1R and isolates C4, C5, C6 and C9, which displayed 30 AFLP peaks; (ii) subgroup-B consisting of isolates C2 and C12, which displayed 31 AFLP peaks; and (iii) subgroup-C consisting of isolate C3, which displayed 29 AFLP peaks (**Figure 7.3**, **Table 7.3**). The S_D values among all isolates are shown in **Table 7.4**. Isolates from subgroup-B were more similar to subgroup-A ($S_D = 0.984$) than isolate C3 ($S_D = 0.983$). The similarity between isolates C3 and subgroup-B isolates was 0.967. An UPGMA dendrogram based on Dice coefficient is shown in **Figure 7.4**.

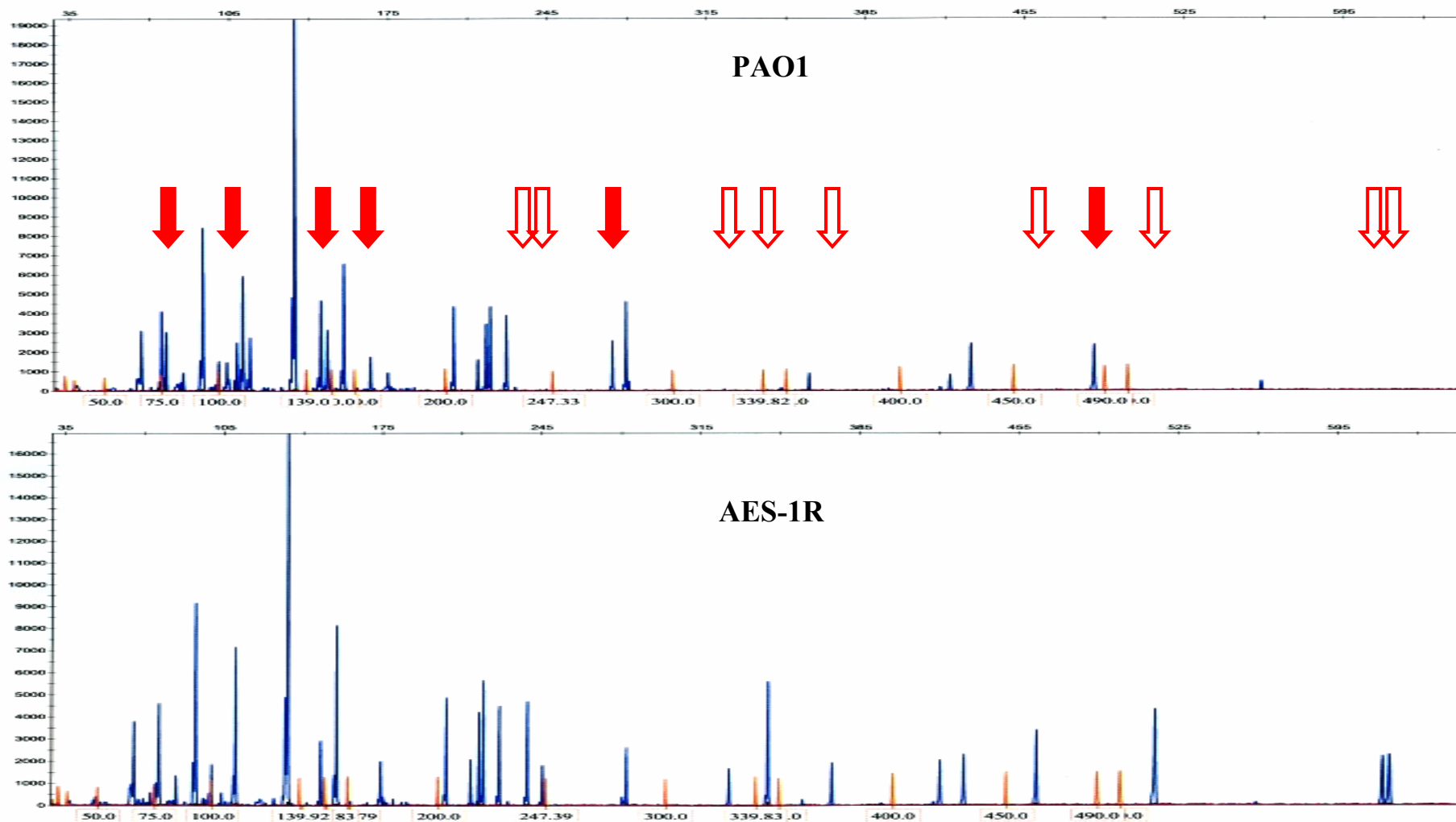


Figure 7.2 AFLP electropherograms of PAO1 (upper) and AES-1R (lower). Blue peaks indicate presence of AFLP fragments, orange peaks are molecular weight marker. Filled red arrows indicate peaks present in PAO1 but not in AES-1R, and empty red arrows indicate peaks present in AES-1R but not in PAO1.

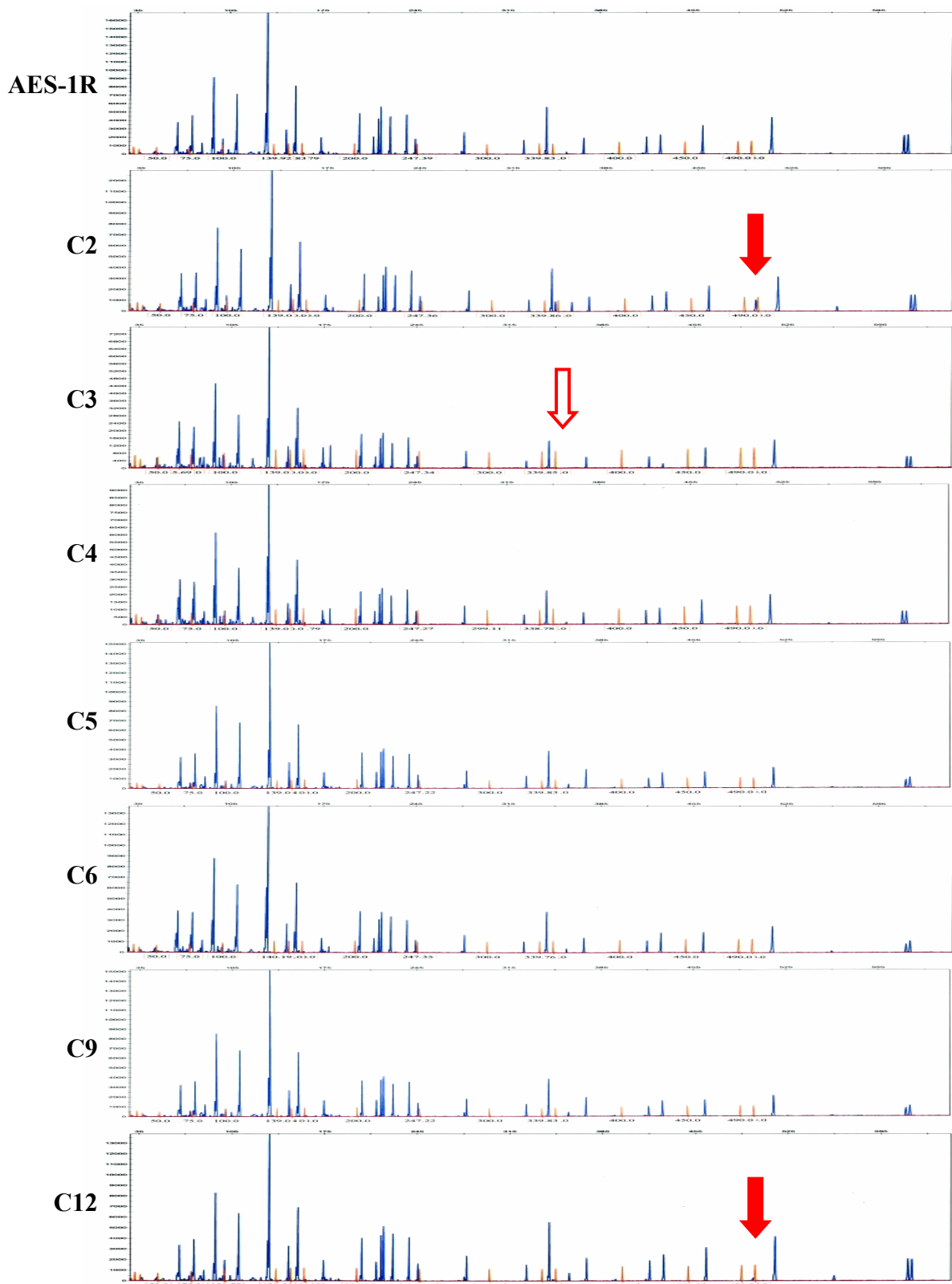


Figure 7.3 AFLP comparison among AES-1 isolates. Filled red arrows indicate peaks that are present in indicated isolates (isolate C2 and C12) but not in others. An empty red arrow indicates a peak that is present in other AES-1 isolates but not in an indicated isolate (isolate C3).

Table 7.3 Binary data of AFLP fragments.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	38	39
PAO1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	0	1	1	0	1	0	0	0	0
AES-1R	1	1	0	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1
C2	1	1	0	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	
C3	1	1	0	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	0	0	1	1	1	
C4	1	1	0	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	
C5	1	1	0	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1
C6	1	1	0	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1
C9	1	1	0	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1
C12	1	1	0	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1

“1” represents presence of the fragment, and “0” represents absence. Grey boxes indicate AFLP fragments different from other AES-1 isolates.

Table 7.4 Similarity matrix computed with Dice coefficient

	PAO1	AES-1R	C2	C3	C4	C5	C6	C9	C12
PAO1	1	0.724	0.712	0.702	0.724	0.724	0.724	0.724	0.712
AES-1R		1	0.984	0.983	1	1	1	1	0.984
C2			1	0.967	0.984	0.984	0.984	0.984	1
C3				1	0.983	0.983	0.983	0.983	0.967
C4					1	1	1	1	0.984
C5						1	1	1	0.984
C6							1	1	0.984
C9								1	0.984
C12									1

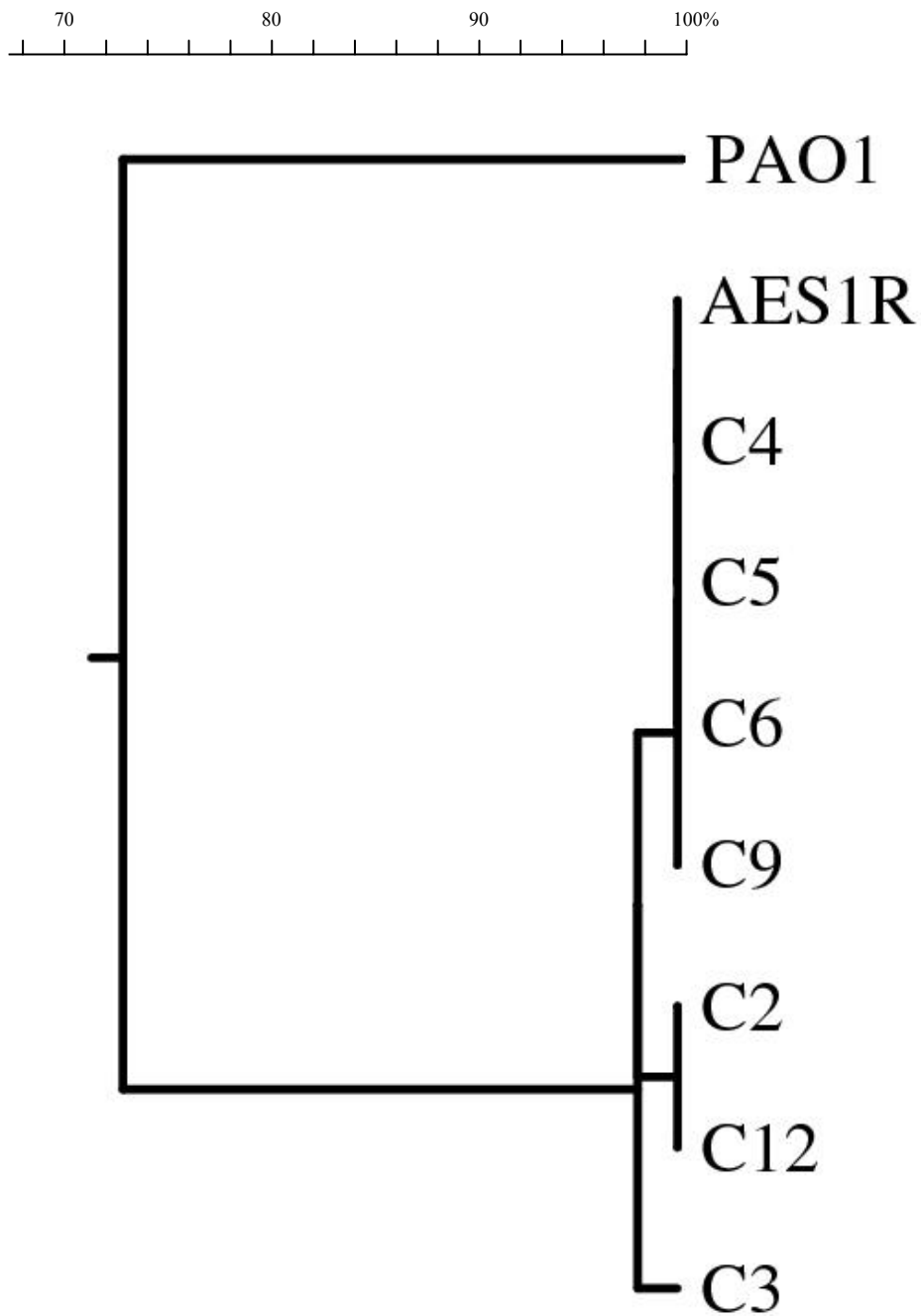


Figure 7.4 Dendrogram of PAO1, AES-1R and seven AES-1 isolates based on AFLP patterns.

7.3.2 Comparison between AFLP and PFGE/RFLP Analyses

As shown in **Figure 7.5**, all AES-1 isolates including AES-1R in this study had identical DNA banding patterns generated by PFGE/RFLP, and thus comparison among these isolates generated the S_D values of 1 or a straight-line dendrogram. The AFLP method, however, further revealed some micro-heterogeneity and defined these PFGE-identical isolates into three subgroups.

7.3.3 Correlation between AFLP Patterns and Phenotypes

Overall, there were no correlations between AFLP patterns and phenotypic characteristics. As shown in **Table 7.5**, isolates within the same subgroup displayed different phenotypes (i.e. isolates C6 and C9) while isolates from the different subgroups had very similar phenotypes (isolate C5 and isolate C2).

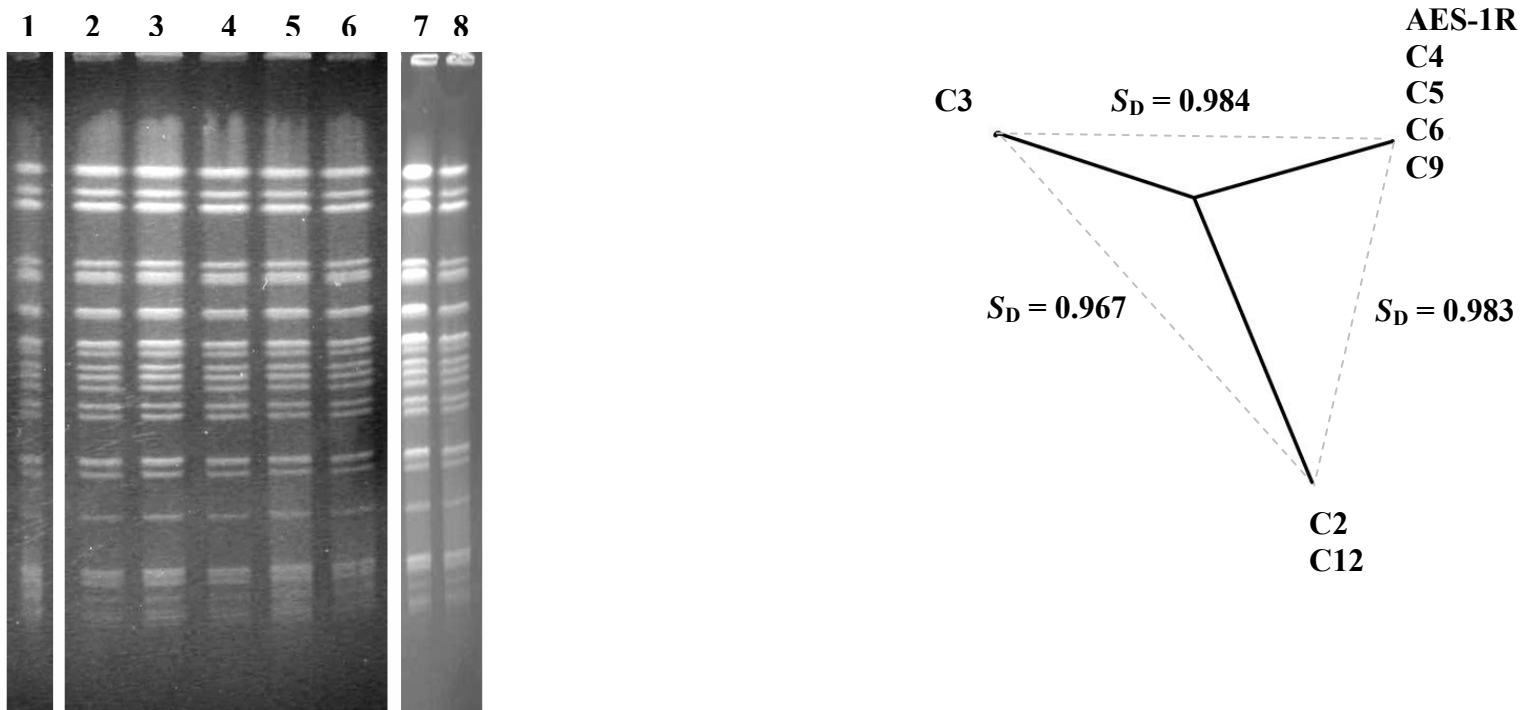


Figure 7.5 Comparison between PFGE/RFLP and AFLP. (Left) PFGE banding patterns of AES-1R, isolates C2, C3, C4, C5, C6, C9 and C12 (lanes 1 – 8, respectively). (Right) Dendrogram based on AFLP patterns with indicating S_D values between subgroups.

Table 7.5 Phenotypic characteristics of AES-1 isolates as grouped by AFLP patterns

AFLP subgroup	Isolate	Colony morphology		Virulence factors			Antibiotic susceptibility		
		Size	Mucoidy	Protease Level	Rhamnolipids	ExoS	Tim	Mem	Cip
A	AES-1R	small	–	high	+	+	R	R	S
	C4	small	+	low	+	+	S	S	S
	C5	medium	++	low	+	+	S	S	S
	C6	small	++	low	–	+	S	S	S
	C9	large	–	high	+	–	R	S	R
B	C2	medium	++	low	+	+	S	S	R
	C12	medium	++	medium	–	+	S	S	R
C	C3	small	++	high	+	+	R	R	R

Tim, ticarcillin-clavulonate; Mem, meropenem; Cip, ciprofloxacin.

S, susceptible, R, resistant.

7.4 Discussion

7.4.1 Identification of AES-1 Substrains Using AFLP

Despite being classified by PFGE/RFLP as the same genotype, AES-1 strains displayed different phenotypes. The aim of this study was, therefore, to further analyse these isolates by using another molecular typing technique. Findings showed that using AFLP, AES-1 isolates can be further divided into three substrains, according to the AFLP patterns.

Phenotypic studies reported in Chapter Four showed that the majority of clonal strains displayed hypervirulent phenotypes by producing high levels of virulence factors such as proteases. The results, however, also revealed that among the tested AES-1 clonal isolates (n = 14), there were some that produced proteases at very low levels or did not produce any proteases in some cases. These isolates also displayed various colony morphotypes (i.e. colony size or the level of mucoid production) and antibiotic susceptibility patterns. These observations are concordant with an early report from the CF Microbiology group at the University of Sydney that showed differences in the mucoid production among the AES-1 isolates (Anthony *et al.*, 2002). Altogether, these findings have emphasised the variations of phenotype that occur even among isolates of the same clone as defined by PFGE/RFLP.

In this study, the AFLP technique was used to further examine eight isolates belonging to the AES-1 clone: seven AES-1 strains recovered from adults with CF who attended the CF clinic at the RPAH (Chapter Four) and AES-1R, a strain originally recovered from an infected CF child who attended the CF clinic at Royal Children's Hospital, Melbourne at the time of the first epidemic spread of this clone (Armstrong *et al.*, 2002). Results have shown that four AES-1 isolates from adult patients (isolates C4, C5, C6 and C9) had an identical AFLP pattern to the AES-1R, confirming the similarity of this clone between Melbourne and Sydney. Two isolates (C2 and C12) have one amplicon extra while another isolate (C3) has one amplicon fewer when compared to the AFLP pattern of the AES-1R group, suggesting that these three isolates originate from the AES-1R group. Scott and Pitt also applied the AFLP method to analyse genetically similar strains and found that isolates belonging to the LES clone (Liverpool epidemic strain) had a higher level of DNA micro-heterogeneity when compared to

other clones that have spread in England and Wales (Scott & Pitt, 2004). They suggested that the number of years (age) since the emergence of LES may be responsible for its high level of micro-heterogeneity compared to another clone, the Midlands 1 (**Figure 7.6**) (Scott & Pitt, 2004). In contrast to the LES strain, the overall differences among the AES-1 isolates are very low (the S_D values = 0.983 and 0.984), which, according to the above suggestion, means that this clone is relatively recent. However, the sample size in the present study was small and, thus, might not represent the whole AES-1 population.

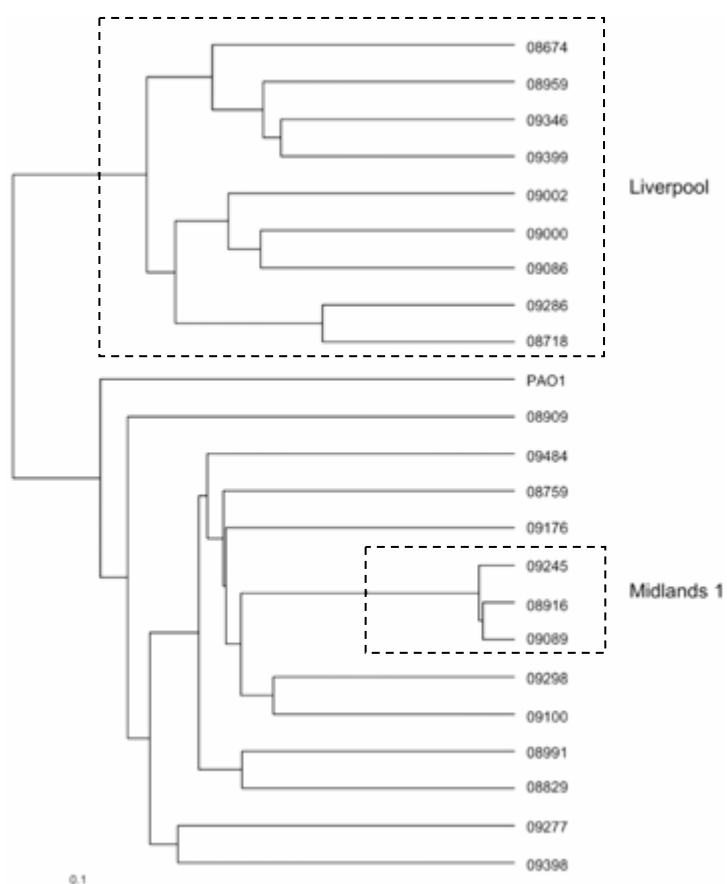


Figure 7.6 Micro-heterogeneity as revealed by AFLP analysis of the two most common *P. aeruginosa* clones infecting people with CF in England and Wales (Scott & Pitt, 2004).

7.4.2 Comparison between PFGE and AFLP

Findings from the present study support other work showing that AFLP has greater discriminatory power than PFGE/RFLP (Hu *et al.*, 2002; Melles *et al.*, 2007; Neeleman *et al.*, 2004; Ross & Heuzenroeder, 2005). Because of the use of the rare-cutting restriction endonuclease, the PFGE/RFLP procedure generates a small number of large DNA fragments and therefore can detect mutational changes only in a number of the restriction sites. Large-scale insertions and/or deletions of DNA are thought to be more responsible for the variability of macrorestriction fingerprints than single-point mutations. Indeed, Römling *et al.* demonstrated that changes in fragment pattern of *P. aeruginosa* are caused in 92% of cases by insertions and/or deletions while in only 8% by point mutation at the *SpeI* recognition sites (Romling *et al.*, 1997). In contrast, AFLP uses two restriction enzymes and generates several small DNA fragments and has, therefore, more power to detect mutations at the restriction sites as well as insertions/deletions of smaller DNA fragments when compared to PFGE/RFLP. Moreover, the number of DNA fragments can be increased by the use of different endonucleases or primers, which allows AFLP to be able to discriminate even very closely related strains. To date, there have been few published studies that applied AFLP to genotype *P. aeruginosa*, however this study has demonstrated the feasibility of using this technique to analyse genetically closely related strains.

7.4.3 No Correlation between AFLP Patterns and Phenotypes

Even though three subgroups of AES-1 have been identified by AFLP, isolates from each subgroup displayed variations of phenotypes. For example, AES-1R and isolate C9 are non-mucoid while isolate C5 and C6 from the same AFLP group are mucoid. This suggests that genetic changes responsible for the phenotypic polymorphisms maybe only very minor ones that even AFLP cannot detect. Indeed, Anthony *et al.* demonstrated that mutations (mainly single point mutations) in the *mucA* gene were responsible for the variation of the mucoid production of this clone (Anthony *et al.*, 2002). Similarly, Hocquet *et al.* reported the intraclonal variation of drug resistant profiles; *P. aeruginosa* isolates of the same clone displayed isolate-to-isolate variations (up to 64-fold) in the MICs of β -lactams (Hocquet *et al.*, 2003). Again, single-base mutations in the *mexR* gene, leading to increased expression of the MexAB-OprM efflux system, contributed to these variations (Hocquet *et al.*, 2003).

Intraclonal variations have also been shown in studies by Salunkhe *et al.* (Salunkhe *et al.*, 2005b). Two identical *P. aeruginosa* strains, TB and 892, which exhibited identical *SpeI*-macrorestriction patterns and with DNA sequences 100% identical in more than 100 kb of randomly selected loci of the core genome, responded differently when exposed to the stressor hydrogen peroxide (Salunkhe *et al.*, 2005b). 24% and 17% of the 5,900 ORFs were significantly differentially expressed in *P. aeruginosa* strain TB and strain 892, respectively (Salunkhe *et al.*, 2005b). 729 genes showed isolate-specific responses while 501 genes were similarly regulated in the two isolates (Salunkhe *et al.*, 2005b). This again has confirmed that even genetically very closely related *P. aeruginosa* isolates present an isolate-specific gene expression profile distinct from members of the same clone (Salunkhe *et al.*, 2005b).

It has become clear that there is intraclonal diversity of *P. aeruginosa* phenotypes. Wehmhoner *et al.* used a two-dimensional gels electrophoresis to compare differences of protein expression of inter- and intraclonal *P. aeruginosa* isolates (Wehmhoner *et al.*, 2003). The proteome analysis revealed that the cellular-extract components were almost identical among tested isolates, whereas the secretomes were distinctive both between and within clones (Wehmhoner *et al.*, 2003). This study has demonstrated that the proteomic approach, in particular the secretomes, is not only a sensitive method to detect *P. aeruginosa* strain variation but may provide more clinically relevant data of pathogens than the genotyping methods.

In summary, the present study has successfully applied the AFLP method to analyse *SpeI*-macrorestriction identical *P. aeruginosa* isolates that displayed variations of phenotypes. Findings have shown that the tested AES-1 isolates can be further divided into substrains according to the AFLP patterns. However, there are no relationships between each subgroup and particular phenotypes – isolates from the same substrain still displayed different phenotypes. Overall, the results have suggested that despite having high discriminatory power, AFLP may not have advantages over the PFGE/RFLP in terms of the correlation between genotypes and phenotypes. Findings also give some insight into the mechanisms underlying the variation of phenotypes of *P. aeruginosa*.

8 Chapter Eight: Effects of Inhaled Hypertonic Saline on the Genotype of *P. aeruginosa* Infecting the Lungs of Adults with Cystic Fibrosis

8.1 Background

Despite steady improvements in clinical management of people with CF, chronic lung infections, particularly with *P. aeruginosa*, remain the leading cause of morbidity and mortality (Koch & Hoiby, 1993). The vicious cycle of airway obstruction and infection is believed to be initiated by an impairment of mucociliary clearance (MCC) as a result of abnormal ASL and airway-electrolyte imbalance (excessive Na⁺ absorption) (Boucher, 2004) (see Section 1.2.2). Therefore, any intervention that can restore functions of MCC should be beneficial.

The CF centre at the RPAH and the CF Microbiology group at the University of Sydney found that inhalation of hypertonic saline (HS) significantly improved MCC and lung function (Elkins *et al.*, 2006; Robinson *et al.*, 1996; Robinson *et al.*, 1997). By measuring the MCC using a radioaerosol technique, it has been shown that a single inhalation of a 7% solution of HS markedly increased MCC clearance, not solely due to increased coughing (Robinson *et al.*, 1996; Robinson *et al.*, 1997). Evidence of the long-term efficacy of the HS treatment has also been provided. Elkins *et al.* conducted a double-blind, parallel-group, randomised trial over a 48-week period in 164 patients with stable CF, and found that participants who received 7% HS twice daily had significantly higher lung function and fewer exacerbation episodes when compared to the control (0.9% NaCl) group. The trial also established the safety of the use of the HS inhalation with regard to bronchospasm, tolerability and bacterial density, demonstrating that neither *S. aureus* nor *P. aeruginosa* increased in density following treatment (Elkins *et al.*, 2006).

Accumulated evidence suggests that certain strains of *P. aeruginosa* can be transmitted between patients. Results from early chapters in this thesis as well as from studies by Armstrong *et al.* (Armstrong *et al.*, 2003; Armstrong *et al.*, 2002) and O'Carroll *et al.*

(O'Carroll *et al.*, 2004) have shown that two clonal strains – AES-1 and AES-2 – have spread extensively within and between CF clinics along the Australian eastern seaboard. The fact that patients infected with the AES-1 or AES-2 strains had poorer clinical outcomes (Chapter Three) and that these strains were more virulent (Chapter Four), has caused great concern in the CF community. The possibility that CF participants in the above HS trial might carry these two strains was very likely but was unexplored.

In vitro studies have shown that increased osmolarity of culture conditions which induces hyperosmotic stress can contribute to an inhibition of biofilm formation (Anderson *et al.*, 2006; Bazire *et al.*, 2005; O'Toole & Kolter, 1998b; Stanley, 1983). Bazire *et al.* demonstrated that hyperosmotic shock significantly inhibits the production of QS signals (Bazire *et al.*, 2005). The OdDHL concentration was 7.5-fold lower in osmotically shocked cultures when compared to un-stressed cultures of *P. aeruginosa*. Moreover, the BHL signal was totally inhibited in the culture condition with an addition of 0.5 M NaCl (Bazire *et al.*, 2005). Recently, Anderson *et al.* found that hyperosmotic shock down-regulated several genes including the pilus-related genes, leading to biofilm inhibition (Anderson *et al.*, 2006). As shown in Chapter Five, AES-1 isolates had a greater biofilm forming capacity than non-clonal isolates, and thus it was speculated that AES-1 might have an increased ability to tolerate the hyperosmolarity. Preliminary results from a study done by Dr. Hu at the CF group at the University of Sydney have also supported this speculation. 48-hour old biofilms of AES-1 isolates were shown to be more stable than those of non-clonal isolates when they were grown in hyperosmotic conditions. This observation has led to the question of whether or not long-term therapy with HS could alter the *P. aeruginosa* population in CF lungs by promoting the selection for particular strains, such as the AES-1 clone, that are more resistant to a high osmolarity environment. Although the total density of *P. aeruginosa* was unchanged during the trial period (Elkins *et al.*, 2006), the long-term use of the HS inhalation could still contribute to a selection of such clones, notably AES-1. This safety issue had not been addressed, and thus research reported in this chapter aimed to:

- determine whether or not treatment with 7% HS inhalation preferentially selected for certain strains of *P. aeruginosa*.

P. aeruginosa isolates recovered from sputum samples of CF participants, who enrolled in the long-term inhaled HS trial conducted by Elkins *et al.* (2006), were examined in this study. The PFGE/RFLP technique was applied to test all isolates.

8.2 Material and Methods

8.2.1 Study Population

Fifty-four patients from those that participated in the long-term inhaled HS trial carried out between September 2000 and December 2003 (Elkins *et al.*, 2006), were randomly selected for this study. In that trial, participants were randomly assigned to inhale either 7% HS or 0.9% NaCl (control) twice daily for 48 weeks, and patients' sputa were collected for quantitative microbiology and other laboratory tests at baseline, at the conclusion of the trial, and at various time points in between (Elkins *et al.*, 2006). The long-term inhaled HS trial involved several CF centres in Australia. Infection control policies differed between centres participating in the trial, but at that time no centre segregated patients by *P. aeruginosa* infection or by strain of *P. aeruginosa*, and there were no changes to infection control procedures during the study period. The characteristics of the 54 patients were representative of the full cohort of 164 participants in the original trial. The proportion on active treatment was 54% and the proportion of males was 54%, versus 51% and 56% in the full cohort, respectively. The mean age was 23 years (range 11 to 48 years) versus 19 (6 to 48) years in the full cohort. The mean lung function was 73 ± 22 % predicted at baseline and was 74 ± 21 % predicted in the full cohort. These investigations arising from the trial were approved by the Institutional Ethics Committee (approval X02-0320).

8.2.2 Sputum processing and Bacterial Culture Conditions

Sputum processing and *P. aeruginosa* identification procedures were described in Section 2.2.1 and 2.2.2. Isolates representing each colony morphology were preserved in cryopreservative vials according to methods described in Section 2.2.3 and were resuscitated prior to the test (Section 2.2.4). In this study, *P. aeruginosa* isolates of all distinctive colony morphologies from each sample were tested. Bacteria were grown on a Columbian HBA

(Oxoid) and were subcultured into 10-ml Nutrient broth (Oxoid) and incubated at 37°C in an Orbital shaking incubator (Biolab) set at 250 rpm overnight. The culturing process of all isolates was performed with a restriction of sub-cultivation to a maximum of two generations in order to reduce spontaneous genetic changes.

8.2.3 Genotyping of *P. aeruginosa* using PFGE/RFLP

Methods of genotyping using PFGE/RFLP and interpretation criteria were described in Section 2.3.

8.2.4 Statistical Analyses

Comparisons between characteristics of the HS and control groups and the isolates were made using the unpaired *t*-test. Acquisition and loss of *P. aeruginosa* genotypes in the HS and control groups were compared using the chi-square test or, in cases in which subgroups were small, Fisher's exact test.

8.3 Results

8.3.1 Microbiological characteristics

Of the 54 patients, 47 (87%) patients had *P. aeruginosa* isolated in their sputa at both baseline and end of the trial. Six patients (11%) did not have *P. aeruginosa* at the baseline but were found to carry this pathogen in their end-of-trial sputum samples. One patient with *P. aeruginosa* at baseline did not have it at the end. In total, 367 *P. aeruginosa* isolates were investigated. Of which, 158 (93 from the HS group and 65 from the control group) were from baseline sputa, and 209 (116 from the HS group and 93 from the control group) were from end-of-trial samples.

Overall, there was no evidence that the long-term HS inhalation contributed to increased phenotype diversity. The number of *P. aeruginosa* phenotypes per sample at baseline ranged from zero to eight (mean = 4) in the HS group and from zero to six (mean = 3) in the control

group. The corresponding figures at the end of trial were from one to nine (mean = 4) and from zero to six (mean = 4) in the HS group and the control group, respectively.

8.3.2 Effect of Long-term HS Inhalation on *P. aeruginosa* Genotype Population

Overall, 57 different strains including AES-1 and AES-2 were identified (**Table 8.1**). AES-1 was found in 142 isolates from 22 (41%) of the 54 patients, and 11 isolates from two patients (4%) were identified as AES-2. There was no evidence of the emergence of new clonal strains.

In the 47 patients from whom *P. aeruginosa* was isolated in both beginning- and end-of-trial samples, it was found that at the baseline, 35 patients had a single *P. aeruginosa* strain, 11 patients had two strains and two had three strains (**Table 8.1**) while at the end of the trial, 41 patients had a single strain, 10 had two strains, and one had three strains (**Table 8.1**). Among these, four patients (patients 34, 35, 49 and 50) from the control group acquired an additional strain in their end-of-trial sputa compared to six patients (patients 12, 13, 22, 24, 25 and 26) in the HS group (three of them gained one strain but lost one baseline strain) (**Table 8.1**). Overall, the long-term usage of HS inhalation did not significantly increase the acquisition of new strains ($p = 0.658$). This relationship was still not significant when six patients who did not have *P. aeruginosa* at baseline but acquired a strain by the end of the trial were included (three from each group) ($p = 0.808$).

There was no evidence to suggest that long-term use of HS selected for or against clonal strains. The proportions of patients infected with AES-1 in the HS and control groups at baseline were 45% (13/29) and 36% (9/25), respectively, and these figures were unchanged at the end of the trial (**Table 8.1**). The acquisition of a clonal strain was found in only one patient (patient 12) in the HS group who had AES-1 at baseline but both AES-1 and AES-2 at trial completion. This subject did not deteriorate acutely during the study, but over the subsequent four years has shown deterioration in lung function: FEV₁ has declined from 42.2% to 26.8% of predicted (annual average was -3.4%) and FVC has declined from 70.8% to 54.3% (annual average was -3.7%) (**Figure 8.1**). The annual deterioration in FEV₁ in this patient was twice higher than the average rate of the CF clinic at RPAH (-1.7%) and other reported data (Corey

et al., 1997; Que *et al.*, 2006). Interestingly, there was no loss of a clonal strain over the 48-week trial period in any patient.

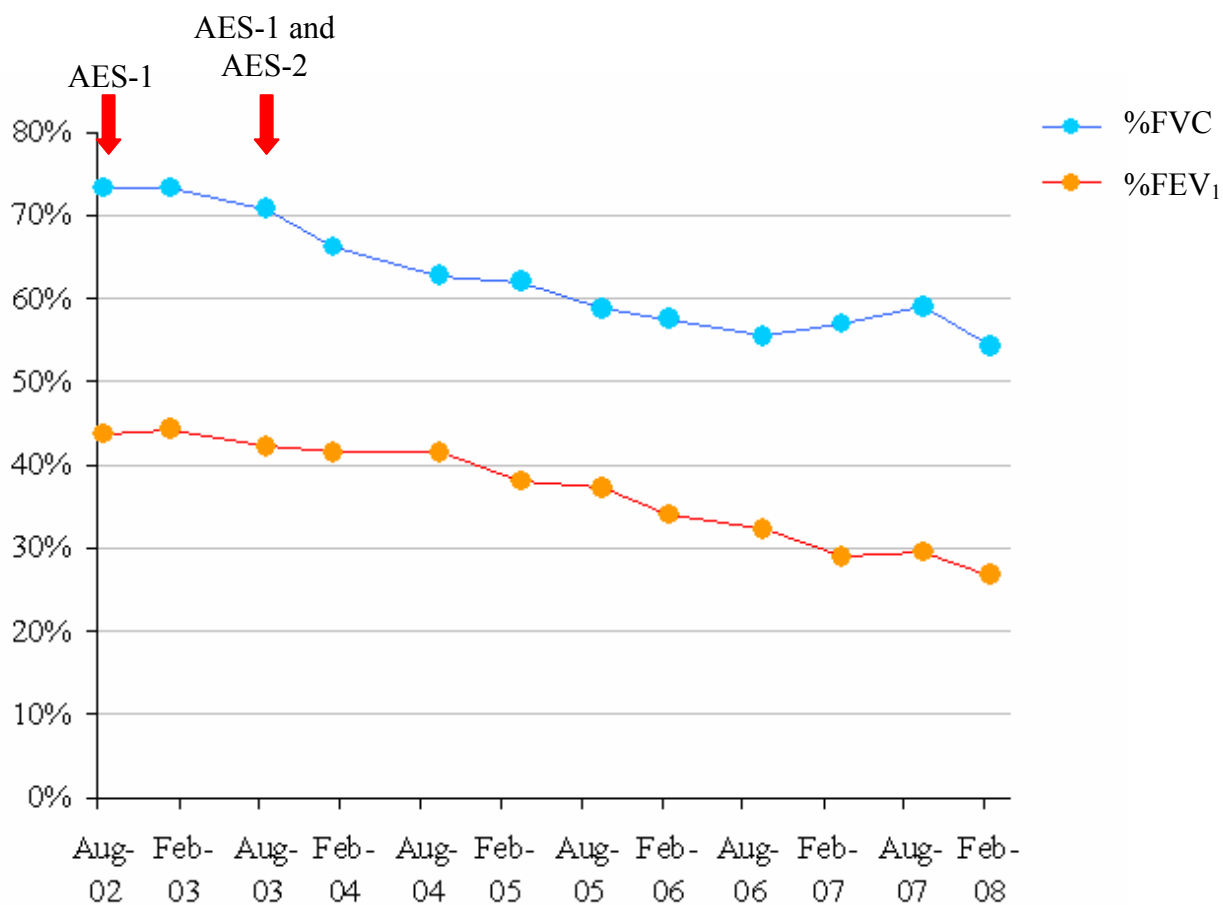


Figure 8.1 Pulmonary function of patient 12 follow up over five and a half years since the patient participated in the trial. Arrows indicate the time when sputum samples were collected for PFGE/RFLP.

Five patients (patients 10 and 11 from the HS and patients 36, 37 and 38 from the control group), who were infected with both AES-1 and a non-clonal strain at baseline, did not have a non-clonal strain but only AES-1 in their sample at the end of the trial. This was evidence of this clone's capability in replacing other strains. However, this was not due to the use of long-term inhaled HS ($p = 0.653$). Interestingly, the strain replacement was also observed in two

non-clonal strains: the strain XVII in patient 23 replaced the strain XVIII, and the strain XLVI in patient 48 replaced the strain XLVII.

Comparison between baseline and end-of-trial *P. aeruginosa* populations within each patient showed that AES-1 was significantly more persistent than non-clonal strains ($p < 0.001$). AES-1 isolates recovered from the baseline samples of 22 patients were all [22/22 (100%)] present in the end-of-trial samples of the same patients, whereas only 28 (70%) of the 40 non-clonal strains from baseline samples were still present. However, this persistence and/or disappearance of the strains was not associated with the long-term HS treatment (**Table 8.2**). Of the lost 12 non-clonal strains, six strains were from each group.

Table 8.1 Relationships between genotype and phenotype of *P. aeruginosa* isolates and the long-term treatment with HS

Group	Patient ID	Age	Gender	Strains (Number of phenotypes) ^a		Strain Population ^b
				Baseline	End	
HS	1	20	F	AES-1 (4)	AES-1 (6)	Same
	2	24	F	AES-1 (2)	AES-1 (1)	Same
	3	19	F	AES-1 (4)	AES-1 (5)	Same
	4	24	M	AES-1 (4)	AES-1 (2)	Same
	5	23	M	AES-1 (5)	AES-1 (1)	Same
	6	19	M	AES-1 (8)	AES-1 (8)	Same
	7	17	M	AES-1 (2)	AES-1 (3)	Same
	8	23	M	AES-1 (1)	AES-1 (6)	Same
	9	13	F	AES-1 (2)	AES-1 (2)	Same
	10	30	F	AES-1 (3), I (1)	AES-1 (3)	Loss
	11	15	M	AES-1 (3), II (1)	AES-1 (3)	Loss
	12	25	F	AES-1 (2), III (1)	AES-1 (3), III (1), IV (2)	Gain
	13	29	F	AES-1 (2)	AES-1 (1), AES-2 (1)	Gain
	14	18	F	AES-2 (6)	AES-2 (4)	Same
	15	21	M	V (5)	V (4)	Same
	16	19	M	VI (5)	VI (9)	Same
	17	11	M	VII (6)	VII (9)	Same
	18	21	F	VIII (4)	VIII (7)	Same
	19	16	F	IX (1)	IX (2)	Same
	20	24	M	X (4), XI (1)	X (5), XI (1)	Same
	21	18	F	XII (3), XIII (1), XIV (1)	XII (1), XIII (1), XIV (4)	Same
	22	28	F	XV (4)	XV (4), XVI (1)	Gain
	23	13	F	XVII (2), XVIII (1)	XVII (2)	Loss
	24	21	M	XIX (1), XX (1)	XIX (1), XXI (1)	Loss/Gain
	25	48	M	XXII (1)	XXIII (2)	Loss/Gain
	26	29	M	XXIV (1)	XXV (1)	Loss/Gain
	27	13	M	nil	XXVI (1)	Gain
	28	44	F	nil	XXVII (2)	Gain
	29	20	M	nil	XXVIII (3)	Gain

(continued next page)

Table 8.1 (continued)

Group	Patient ID	Age	Gender	Strains (Number of phenotypes) ^a		Strain Population ^b
				Baseline	End	
Control	30	24	F	AES-1 (3)	AES-1 (5)	Same
	31	22	F	AES-1 (2)	AES-1 (5)	Same
	32	16	M	AES-1 (5)	AES-1 (6)	Same
	33	21	M	AES-1 (6)	AES-1 (4)	Same
	34	21	M	AES-1 (3)	AES-1 (3), XXIX (1)	Gain
	35	20	F	AES-1 (3)	AES-1 (2), XXX (2)	Gain
	36	32	M	AES-1 (3), XXXI (1)	AES-1 (3)	Loss
	37	13	F	AES-1 (1), XXXII (1)	AES-1 (4)	Loss
	38	28	M	AES-1 (1), XXXIII (1), XXXIV (1)	AES-1 (2)	Loss
	39	22	M	XXXV (1)	XXXV (1)	Same
	40	30	M	XXXVI (4)	XXXVI (4)	Same
	41	28	F	XXXVII (3)	XXXVII (5)	Same
	42	42	M	XXXVIII (3)	XXXVIII (6)	Same
	43	14	M	XXXIX (3)	XXXIX (3)	Same
	44	11	M	XL (2)	XL (5)	Same
	45	42	M	XLI (2)	XLI (5)	Same
	46	32	M	XLII (1), XLIII (1)	XLII (2), XLIII (4)	Same
	47	20	F	XLIV (2), XLV (1)	XLIV (1), XLV (2)	Same
	48	13	F	XLVI (3), XLVII (1)	XLVI (5)	Loss
	49	30	F	XLVIII (2)	XLVIII (4), XLIX (1)	Gain
	50	24	M	L (4)	L (3), LI (1)	Gain
	51	21	F	LII (1)	nil	Loss
	52	21	F	nil	LIII (2)	Gain
	53	43	M	nil	LIV (1)	Gain
54	14	F	nil	LV (1)	Gain	

^a Roman numerals are used to designate non-clonal strains of *P. aeruginosa*. Clonal strains are indicated as shown (i.e. AES-1 and AES-2). Numbers in brackets indicate number of distinctive colonial morphotypes that are displayed by a respective strain.

^b Indication of the change of *P. aeruginosa* genotypes over the study period. Loss/Gain means that one baseline strain was lost but patients gained another strain at the end of the trial.

Table 8.2 Number of patients with *P. aeruginosa* strain changes in their sputum samples between the beginning and the end of the trial

Changes of strain population	Number of patients (%)		Statistics
	HS (n = 29)	Control (n = 25)	
Same strain(s)	17 (59%)	13 (52%)	$p = 0.625$
Gain of strain(s)	6 (21%)	7 (28%)	$p = 0.531$
Loss of strain(s)	3 (10%)	5 (20%)	$p = 0.449$
Loss/Gain of strain(s)	3 (10%)	0	$p = 0.240$

8.4 Discussion

8.4.1 Safety of the Long-Term Inhaled HS Treatment

Since inhaled HS has become a recognised therapy for people with CF (Flume *et al.*, 2007; Wark *et al.*, 2005), the safety aspects of this treatment are of prime importance. Even though it has been shown in the long-term inhaled HS trial that there were no changes of *P. aeruginosa* and *S. aureus* bacterial density over the study period (Elkins *et al.*, 2006), the possibility that HS therapy could alter the composition of *P. aeruginosa* populations was untested. A study in this chapter was carried out on *P. aeruginosa* isolates from participants of that trial, and provided evidence that HS inhalation over a 48-week period did not predispose to the selection of particular strains of *P. aeruginosa* during infection.

Major concern centred on the hypervirulent AES-1 clone. This is because there has been some evidence that HS inhalation might advantage the infectivity of this clone (Anderson *et al.*, 2006; Bazire *et al.*, 2005; Stanley, 1983). It has been reported that increased osmotic pressure disrupted biofilm formation (Anderson *et al.*, 2006), but AES-1 was less susceptible to this effect when compared to non-clonal isolates (Dr. Hu's unpublished data). It was thus speculated that the long-term usage of HS inhalation could promote AES-1 selection. However, reassuringly, this study did not show evidence to support this speculation. Nevertheless, the observation of acquisition of AES-2 in patient 13 from the HS group has suggested that there might be some level of strain selection and has emphasised the importance of genotype monitoring. The fact that this patient's clinical condition has worsened is consistent with the growing evidence of the virulent nature of clonal strains. It would be interesting to closely observe both clinical outcomes and the *P. aeruginosa* population in this patient.

The long-term treatment with HS did not have significant effects on the *P. aeruginosa* genotype population within individual subjects. As shown in **Table 8.2**, the proportions of patients stably infected with similar genotypes as well as gained and/or lost strain(s) are similar between the two groups. Overall, the findings support the safety of long-term treatment

with HS by providing evidence that it did not promote a selection of particular strains or an alteration of *P. aeruginosa* population structure.

8.4.2 Persistence of *P. aeruginosa* within the CF Lungs

While there have been several genotyping studies of *P. aeruginosa* population within the CF subjects, there are only two studies that genotyped all isolates with differences in colony morphologies (Spencer *et al.*, 2000; Van Daele *et al.*, 2006). This study not only tested all *P. aeruginosa* isolates exhibiting different colonial morphotypes but also followed up these populations over the 48-week period, and thus provided a greater insight into *P. aeruginosa* pathogenesis within the CF lungs. Overall findings reveal the complexity of CF *P. aeruginosa* infections.

One of the interesting findings is the fact that the AES-1 clone was found to be persistent in 100% of cases over the study period. All patients with AES-1 at baseline retained this strain in their sputum at the end of trial. In contrast, of 31 patients who had non-clonal strains at baseline (including patients co-infected with both AES-1 and non-clonal strains), 11 patients (37%) lost at least one of their non-clonal strains by the end of the trial. Thus it is clear that AES-1 is more likely to persist in the CF lungs than the non-clonal strains, which could be one of the factors contributing to its high prevalence.

Strain replacement by AES-1 was also observed in this study. Five patients who were infected with both AES-1 and non-clonal strains at the baseline, were found to carry only AES-1 at the end. This phenomenon has been previously reported to occur with LES (McCallum *et al.*, 2001). However, it is important to note that this phenomenon is not exclusive to the clonal strains. Two non-clonal strains were found to replace two other non-clonal strains in the respective patients.

This study also demonstrates that the majority of CF patients with *P. aeruginosa* are chronically infected with a single strain. Of the 48 patients who had *P. aeruginosa* at the baseline, 35 patients (73%) were infected with one strain, 11 patients (23%) with two strains, and two patients (4%) with three strains. This result is consistent with previous studies (Grothues *et al.*, 1988; Spencer *et al.*, 2000; Van Daele *et al.*, 2006). In line with the studies

from Chapter Four and other previous reports (da Silva Filho *et al.*, 2001; Van Daele *et al.*, 2006), it is shown here again that there was no relationship between genotype and colony morphology.

In summary, this study investigated whether the population of *P. aeruginosa* infecting the CF lung might be affected by long-term treatment with HS. By applying PFGE/RFLP to all isolates displaying different morphology, it has been shown that the HS treatment did not change genotypic patterns of *P. aeruginosa*. The lack of detection of increased AES-1 prevalence arising from the trial (as was speculated) provides reassuring safety data about the use of HS. This study also contributes to the general understanding of the pathophysiology of *P. aeruginosa* in the CF lungs.

9 Chapter Nine: General Discussion and Future Research

Chronic *P. aeruginosa* infection occurs in up to 80% of CF patients and is the major determinant of the mortality of people with CF. In order to control and to ultimately cure this infection, it is important to understand the pathogenesis of this bacterium in people with CF. More recently, evidence has emerged to demonstrate that some *P. aeruginosa* strains can be transmitted between CF patients and that some of these strains have a negative impact on patient health. The research reported in this thesis focussed on Australian epidemic strains and investigated the basis of the pathogenicity of these strains and their clinical impact.

Because there is no direct proof that CF patients can acquire *P. aeruginosa* directly from other patients, the existence of patient-to-patient transmission of this pathogen remains controversial. Consequently, the most appropriate means to define the transmission is to apply epidemiological surveillance to bacterial populations among people with CF. *P. aeruginosa* is ubiquitous in the environment, and thus it was believed that CF individuals acquired unique *P. aeruginosa* strains independently from environmental sources (Mahenthalingam *et al.*, 1996; Romling *et al.*, 1994). Research in the past using both molecular and non-molecular (phenotyping) typing techniques demonstrated that CF patients carried *P. aeruginosa* strain(s) uniquely different from other patients (Mahenthalingam *et al.*, 1996; Speert *et al.*, 1982; Speert & Campbell, 1987; Thomassen *et al.*, 1985a). Transmission of *P. aeruginosa* from patient to patient, which was indicated by sharing of similar bacterial strains between patients, was thought to be rare and found only among CF siblings (Grothues *et al.*, 1988; Kelly *et al.*, 1982). However, since 1995, many studies using PFGE/RFLP, the current gold standard for bacterial typing, have reported cases of *P. aeruginosa* cross-infection (Cheng *et al.*, 1996; Jones *et al.*, 2001; Ojeniyi *et al.*, 2000). One such report was from the discovery of a clone, later called AES-1, among CF children attending the Royal Children's Hospital in Melbourne, and it was proposed that this strain was associated with the unexpected deaths of five infected children (Armstrong *et al.*, 2002). Subsequently, this strain was found in other CF patients attending CF centres along the Australian eastern seaboard, including at the RPAH in Sydney (Armstrong *et al.*, 2003). However, the prevalence of this clone and its clinical impact on adult CF patients were unknown at the commencement of this thesis in 2004. Research in this thesis

thus began with a study designed to establish the prevalence of this *P. aeruginosa* strain among infected adults with CF at the RPAH.

In Chapter Three, by applying PFGE/RFLP to *P. aeruginosa* isolates recovered from patients' sputum samples, it was shown that almost half of the CF population was infected by clonal strains. AES-1 was found to infect 38% (43/112) of adult CF patients, and 5% (6/112) of patients were found to be infected by AES-2, a more recently reported clonal strain first isolated in Queensland (O'Carroll *et al.*, 2004). Moreover, two small clones that have not yet been previously reported (called S-1 and S-2) were also found in 5% (6/112) and 2% (3/112) of patients, respectively.

Further research then investigated the clinical consequences of transmissible *P. aeruginosa* infections in patients attending the CF clinic at RPAH, Sydney. Patients with CF who were infected with the clonal strains were shown to have significantly increased numbers of exacerbations and hospitalisation days compared with those patients infected with non-clonal strains, and this was consistent with the previous reports from Armstrong *et al.* (2002) and O'Carroll *et al.* (2004). They also tended to have poorer pulmonary function when compared to patients with sporadic *P. aeruginosa*. The association between transmissible *P. aeruginosa* strains and adverse clinical outcomes has been previously found with the Liverpool epidemic strain (LES) and the Manchester epidemic strain (Al-Aloul *et al.*, 2004; Jones *et al.*, 2002). LES, in particular, has been associated with severe deterioration in lung function and body mass index (Al-Aloul *et al.*, 2004).

Nevertheless, it should be noted that not all reported transmissible strains have been linked with adverse consequences. The Midlands-1 clonal strain, the second most common genotype infecting CF patients in England and Wales (Scott & Pitt, 2004), was not shown to be associated with poor clinical impacts (Chambers *et al.*, 2005). Moreover, two transmissible *P. aeruginosa* clones at the Danish CF centre in Copenhagen have recently been shown to be completely non-virulent in a *C. elegans* killing assay (Jelsbak *et al.*, 2007) although the clinical outcomes of patients who were infected by those clones were not reported in that study. Overall, this emphasises the need for assessments of the clinical implications of individual clonal strains.

Findings in Chapter Three of this thesis also demonstrated that there was no association between patient demographic factors and the clonal strain infections. Gender, age and CF genotype did not favour infections of any particular clone. This is consistent with previous reports by Armstrong *et al.* (2002) and O'Carroll *et al.* (2004).

The discovery of the clonal strains (especially the high prevalence of AES-1 among patients at the CF clinic at the RPAH) and their negative clinical impact on CF patients at RPAH led to an investigation of the virulence factors that might contribute to the transmissibility and pathogenesis of these strains. Chapter Four and Chapter Five conducted in this thesis aimed to characterise bacterial phenotypic attributes associated with the clonality. Studies in Chapter Four showed that the clonal strains exhibited enhanced virulence. AES-1 and AES-2 were significantly more likely to produce proteases including LasB elastase, alkaline protease and protease IV when compared with non-clonal isolates. A similar observation has been previously reported in the LES clone (Salunkhe *et al.*, 2005a). These findings have led to the proposition that such virulence factors are likely to have roles in both the transmissibility and pathogenesis of these highly infectious clones.

Smith *et al.* demonstrated that virulence factors of *P. aeruginosa* that are required for the initiation of acute infections are lost due to mutations during long-term colonisation within the CF airways (Smith *et al.*, 2006). In this study, it was found that the production level of LasB elastase was reduced in isolates recovered from older patients (as reported in Chapter Four). However, this figure was significant only when data from both clonal and non-clonal isolates were combined. In contrast, when results from the two groups were analysed separately, clonal strains seemed to display a different trend to the non-clonal strains (no decreasing correlation between LasB levels and ages in the clonal group) (**Figure 4.9**). Similar findings were also observed in the production of ExoS (an exoprotein secreted by the type III secretion system). Previous studies have shown that *P. aeruginosa* lost the production of ExoS and other type III secreted proteins over a long period of CF lung infections (Jain *et al.*, 2004; Lee *et al.*, 2005; Yahr & Greenberg, 2004). Studies in this thesis demonstrated that the clonal strains had higher prevalence of ExoS-secreting isolates than the non-clonal strains (84% and 71% respectively), but that this relationship was not statistically significant. It is thus possible that during the adaptation of non-clonal strains to the CF airways their virulence-factor expression is lost or reduced, but, in contrast, the clonal strains are more resistant to change and retain virulence

factors during chronic lung infection for longer leading to the properties of enhanced virulence and transmissibility associated with the clonal strains.

Studies in Chapter Four further investigated the production of cell-to-cell signal molecules or QS (quorum sensing), which are now known to control genes including those encoding virulence factors. There are two major QS circuits in *P. aeruginosa*, *las* and *rhl*, which are regulated by acyl-homoserine lactones (AHLs) called OdDHL and BHL, respectively. QS is required for *P. aeruginosa* virulence in several animal models (Pearson *et al.*, 2000; Rumbaugh *et al.*, 1999; Tang *et al.*, 1996) and is thought to have a crucial role in CF lung infections (Smith & Iglewski, 2003b). However, using a thin-layer chromatography (TLC) technique incorporating AHL-reporters (*A. tumefaciens* A136 and *C. violaceum* CV026), this study showed that only 19% and 44% of 43 clonal and non-clonal representatives produced OdDHL and BHL, respectively. Most strikingly, none of the 14 AES-1 isolates tested secreted the OdDHL molecule. Recently, it has been suggested that QS may create a metabolic burden and have a negative impact on *P. aeruginosa*'s fitness (Heurlier *et al.*, 2006). The finding of the high prevalence of QS-deficient isolates indicates that such mutant isolates may have an advantage over the wild-type. Indeed, previous studies have demonstrated that in alkaline-induced stress conditions, *lasR* mutants are more resistant to cell lysis and death than the wild-type (Heurlier *et al.*, 2005). This finding is also consistent with previous reports of the QS-deficient strains that have been isolated from a variety of different infections including wounds (Schaber *et al.*, 2004), urinary tract (Schaber *et al.*, 2004), cornea (Zhu *et al.*, 2004) and endotracheal intubation (Denervaud *et al.*, 2004). Even in the LES strain, three of the six LES isolate tested were shown to be a *lasR* mutant (Salunkhe *et al.*, 2005a). It has been proposed that *P. aeruginosa* isolates from chronic lung infections stop producing the QS signal molecules to avoid host immune responses because molecules such as OdDHL have been shown to have a strong immunomodulatory effect (Telford *et al.*, 1998). However, this adaptative response was found in both the clonal and non-clonal groups with no significant difference between the two groups. It would be interesting in the future to study the prevalence of QS-producing isolates within a *P. aeruginosa* population recovered from acute or early CF infections and to compare them with the figures in this report obtained from chronically-infecting isolates.

By using TLC to detect the QS signals, it was revealed that besides OdDHL and BHL, *P. aeruginosa* produced other AHL molecules including HHL, OHHL and ODHL. While the exact roles of these signals are still unknown, the findings showed that there is an association between HHL-producing isolates and the production of LasB elastase and protease IV, suggesting that this molecule may also be partly responsible for the pathogenicity of *P. aeruginosa*. The finding that the clonal strains were more likely to produce OHHL than the non-clonal strains implies this molecule may have some role in the transmissibility of the clonal strains.

Studies in Chapter Four also revealed that the AES-1 clonal strains were more resistant to ticarcillin-clavulanate, amikacin and gentamicin than the non-clonal strains. Moreover, 100% of AES-1 isolates were shown to be ceftriaxone resistance, but this figure was not statistically significant when compared to the non-clonal strains. While ticarcillin-clavulanate and ceftriaxone are commonly used, amikacin and gentamicin are rarely prescribed for patients at the CF clinic at RPAH. The finding that AES-1 is more resistant to these aminoglycosides suggests that this strain may intrinsically possess antibiotic resistance mechanisms. This is consistent with the antibiotic profile of the isolate AES-1R, which is an original strain isolated from an infected CF child during the *P. aeruginosa* outbreak (1991 – 1995) at the Royal Children’s Hospital in Melbourne (Armstrong *et al.*, 2002), that is resistant to all antipseudomonas agents except ciprofloxacin. Overall, it is possible that this resistant characteristic provides an advantage via selective pressure and may contribute to the high prevalence of this clone.

It has been shown that in *Vibrio cholerae*, the capacity to form biofilms contributes to its infectivity and transmissibility (Faruque *et al.*, 2006). *P. aeruginosa* is known to form biofilms in a variety of ecological niches including the CF airways (Prince, 2002). The role of *P. aeruginosa* biofilms in contributing to the persistence of various infections is well described (Costerton *et al.*, 1999), but its function relating to cross-infection is not fully understood. Research in Chapter Five compared the biofilm formation of AES-1 and non-clonal representatives by using a recently developed biofilm study model (called a CDC Biofilm Reactor) (Donlan *et al.*, 2004) to cultivate *P. aeruginosa* and by applying an image analysis programme to evaluate images of grown biofilms. It was found that the clonal strain AES-1 had an enhanced biofilm forming capacity. The biofilms produced by the AES-1 isolates

formed more quickly and were significantly larger when compared to the non-clonal strains, suggesting that this clone is a more persistent form of infections, once colonised. Moreover, it was observed that within 72 hours of an experimental course, one isolate from the AES-1 group displayed a biofilm structure corresponding to a dispersion stage of biofilm (**Figure 5.5**). Recent research has suggested that biofilm dispersal may contribute to transmission (Kirov *et al.*, 2005). This finding can be added as extra evidence linking transmission and the biofilm formation of *P. aeruginosa*. It may be possible that bacteria grow and accumulate within biofilms, creating a sufficient number of *P. aeruginosa* cells for an infective dose which is ready to disperse when the biofilms reach a certain size (Hall-Stoodley & Stoodley, 2005), and this phenomenon may be enhanced in the clonal strain AES-1.

Thus far, factors that may contribute to the patient-to-patient transmission of *P. aeruginosa* have been investigated in both infected human hosts (i.e. patient's demographic) and the pathogen, i.e. phenotypes including antibiotic susceptibility. The present thesis has defined the likely determinants that may be associated with the transmissibility of the clonal strains. These include a high production level of virulence factors (proteases), increased antibiotic resistance and an enhanced biofilm forming capacity.

One possible explanation for the relationship between bacterial virulence and transmissibility is that the virulence factors, which causes damage in airway tissues induce physical reactions in hosts, such as sneezing or coughing, facilitating the spread of the clonal strains (Lipsitch & Moxon, 1997). However, a recent report of two transmissible clones in Copenhagen, Denmark did not find this link between virulence and transmissibility (Jelsbak *et al.*, 2007).

It is clear that there may be other external factors facilitating this cross-infection (such as sharing respiratory equipment between patients). Environment surveys conducted in response to the spread of the LES and the Manchester epidemic strains have found the two clonal strains in air samples taken from rooms of infected patients, ward corridors and their outpatient clinic, suggesting that airborne dissemination is likely to be a potential mode of transmission (Jones *et al.*, 2003; Panagea *et al.*, 2005). Moreover, it is possible that patients acquire the clonal strains from common environmental reservoirs. However, the current thesis was not intended to investigate this possibility or designed to define the mode of transmission.

Nevertheless, the findings from this thesis have led to the implementation of infection control measures within the CF clinic at RPAH.

Currently, the CF group at the Royal Children's Hospital in Brisbane and at the Department of Paediatrics and Child Health at the University of Queensland (led by A/Prof. Wainwright) in conjunction with the CF group at the University of Sydney and RPAH has been conducting a large-scale research project designed to identify the determinant of *P. aeruginosa* acquisition and transmission among infants or young children with CF by defining genotype (using PFGE/RFLP) and phenotype (using specific bioassays) of *P. aeruginosa* isolated from broncho-alveolar-lavage (BAL) specimens and from home environmental samples. The study is ongoing and will hopefully lead to a clearer understanding of the bacterial factors important in both transmission and persistence.

Bacterial phenotypic characterisation of the clonal and non-clonal strains in Chapter Four and Chapter Five was mainly conducted in isolates that were grown in laboratory culture conditions. This may or may not represent the physiological condition of the CF lung, and thus it was decided that there was a need for a cultivation model that more closely resembled or simulated the CF environment. Studies in Chapter Six successfully developed such a model, by growing cultures in an artificial sputum medium (ASM), which contains chemical components at similar concentration to sputum from a CF airway. Interestingly, *P. aeruginosa* grown in this model (PAO1 and AES-1R) showed a clumping structure or a macrocolony in the medium matrix but did not attach to the surface wall of culture tubes (**Figure 6.2**). This macrocolony is likely to represent characteristics of biofilms grown in the CF airway since *P. aeruginosa* has been previously shown to organise in clusters embedded in mucous secretion in airway lumens and not adhering to the epithelial tissue (Bjarnsholt *et al.*, 2007; Worlitzsch *et al.*, 2002).

PAO1 and AES-1R grown in this model were also genomically compared to those planktonically grown in a laboratory medium (LB broth) as part of an honours project performed in the CF group at University of Sydney (Fung, 2007). Several genes were found to be significantly differentially expressed when comparing the ASM-grown to the planktonically-grown bacteria. One of the up-regulated genes was *narI*, which encodes the membrane-bound nitrate reductase γ chain (Fung, 2007). This membrane-bound nitrate

reductase was shown to be important in anaerobic growth (Palmer *et al.*, 2007). This finding suggests that the ASM model provided an anaerobic condition for the growth of bacteria, consistent with the condition naturally occurring in the CF lungs (Worlitzsch *et al.*, 2002; Yoon *et al.*, 2002).

Overall, the ASM offers a new approach for biofilm studies. Bacteria grew in the biofilm mode without the need to attach to any surface, which was not consistent with the biofilm developmental stage (reversible and irreversible attachment stages; **Figure 1.21**) as shown in the *in vitro* model. In contrast, the ASM-grown biofilm is likely to conform with the model of biofilms in CF airways as suggested by Worlitzsch *et al.* (**Figure 1.23**) (Worlitzsch *et al.*, 2002). Although, this model helps describe the process of infection from bacterial acquisition to biofilm formation, it does not explain the dispersal process since it is assumed that there has to be such a stage in the transmissible strain of *P. aeruginosa*. In the future, it would be interesting to apply this ASM model to long-term cultivation and observe any changed characteristics of grown biofilms and bacterial cells. Currently, the ASM model has been used to grow PAO1 and AES-1R as part of a proteomic comparison of both excreted and cell bound proteins (a study conducted by N. Hare and Dr. Cordwell at the School of Molecular and Microbial Biosciences at the University of Sydney). This study is underway.

The study in Chapter Seven arose from the observation in Chapter Four and Chapter Five that there was some degree of phenotypic variations among AES-1 isolates. For example, the majority of the AES-1 strains produced LasB elastase while a few isolates did not. This intraclonal variation has been previously reported in the AES-1 isolates which displayed various morphotypes especially mucoidy (Anthony *et al.*, 2002). The PFGE/RFLP technique was unable to detect this micro-heterogeneity and thus classified those isolates as identical. Thus in experiments reported in Chapter Seven, another molecular typing method was used to analyse those AES-1 isolates. The method selected, AFLP (amplified fragment length polymorphism), was used because it has been previously reported to have a superior discriminatory power to PFGE/RFLP (Speijer *et al.*, 1999). Findings showed that the eight identical AES-1 isolates tested (AES-1R and seven AES-1 isolates from chronically infected adults with CF) were further divided into three different substrains. However, the differences among each group were very small – only one AFLP peak difference between each AFLP

pattern (**Figure 7.3**). More importantly, there was still no correlation between AFLP subtypes and phenotypes.

In PFGE/RFLP, a rare-cutting endonuclease is used and a small number of large DNA bands (macrorestriction) are generated. It has been shown that the differences between these macrorestriction fingerprints are mostly caused by large-scale insertions and/or deletions of DNA (Romling *et al.*, 1997). In contrast, AFLP uses two restriction enzymes – one frequent cutter (*MseI*) and one less frequent cutter (*EcoRI*) – and generates several small DNA fragments. The finding of the phenotypic diversity of the AES-1 clone indicates that this intraclonal variation results from small-scale mutations (such as single point mutations).

Overall, this finding suggests that the phenotypic variations among isolates from the same clones are likely to be caused by single point mutations or single nucleotide polymorphisms (SNPs), while the variations between different clones/strains are likely to be caused by DNA insertions/deletions. Genotyping techniques that can detect SNPs such as SNP genotyping or whole-genome genotyping are now available (Morales *et al.*, 2004; Steemers *et al.*, 2006). It would be interesting to apply these techniques to *P. aeruginosa* population, particularly the AES-1 clone.

The final part of this thesis addresses the safety issue of a treatment for CF patients with hypertonic saline (HS) inhalation. HS therapy was developed by the CF group at RPAH and the University of Sydney (Robinson *et al.*, 1996; Robinson *et al.*, 1997), and long-term use in people with CF has been recently shown to be effective in a large-scale randomised controlled trial (Elkins *et al.*, 2006).

It has been shown, however, that increased osmolarity of growth conditions can contribute to an inhibition of biofilm formation (Anderson *et al.*, 2006), and it was shown that the biofilms of AES-1 isolates were more stable in the hyperosmotic condition compared to biofilms of non-clonal isolates (a study done by Dr. Hu at the CF Microbiology group at the University of Sydney). This created a concern that the HS therapy might facilitate the selection and hence spread of *P. aeruginosa* transmissible clones.

Reassuringly, the study in this chapter did not show evidence of the selection of the clonal strains. The long-term treatment with HS did not have a significant effect on *P. aeruginosa* strain diversity within individual CF subjects.

This study, which followed up *P. aeruginosa* strain diversity over a 48-week period, also demonstrated the dynamism of the population of this pathogen within individual patients. One interesting observation was the finding that AES-1 was more persistent in the lung than non-clonal strains. Moreover, AES-1 showed the ability to replace other non-clonal strains colonising the same patients. However, this phenomenon was also observed in two non-clonal strains that replaced two other non-clonal strains in the respective patients.

Recently, Nguyen and Singh proposed two alternative models explaining the dynamics of *P. aeruginosa* infection in CF lungs (**Figure 9.1**) (Nguyen & Singh, 2006). The first model suggests that the population of *P. aeruginosa* within CF lungs involves a dominant strain which predominates and precludes other strains or other subclones. The second model suggests that instead of competing among different clones/strains, the pathogens co-evolve and eventually co-exist as a diverse community. The prevailing evidence from the study in Chapter Eight tends to support the first model, especially in patients infected with AES-1. However, some patients who were infected with non-clonal strains demonstrated an infection pattern similar to the second model. It is important to note that this observation was obtained from the genotyping studies at two time-points and only 48 weeks apart, and was conducted in chronically infected CF adults. It would be more interesting if this genotype population study were conducted in patients with early infection (such as paediatric patients) and followed up over a long period of time.

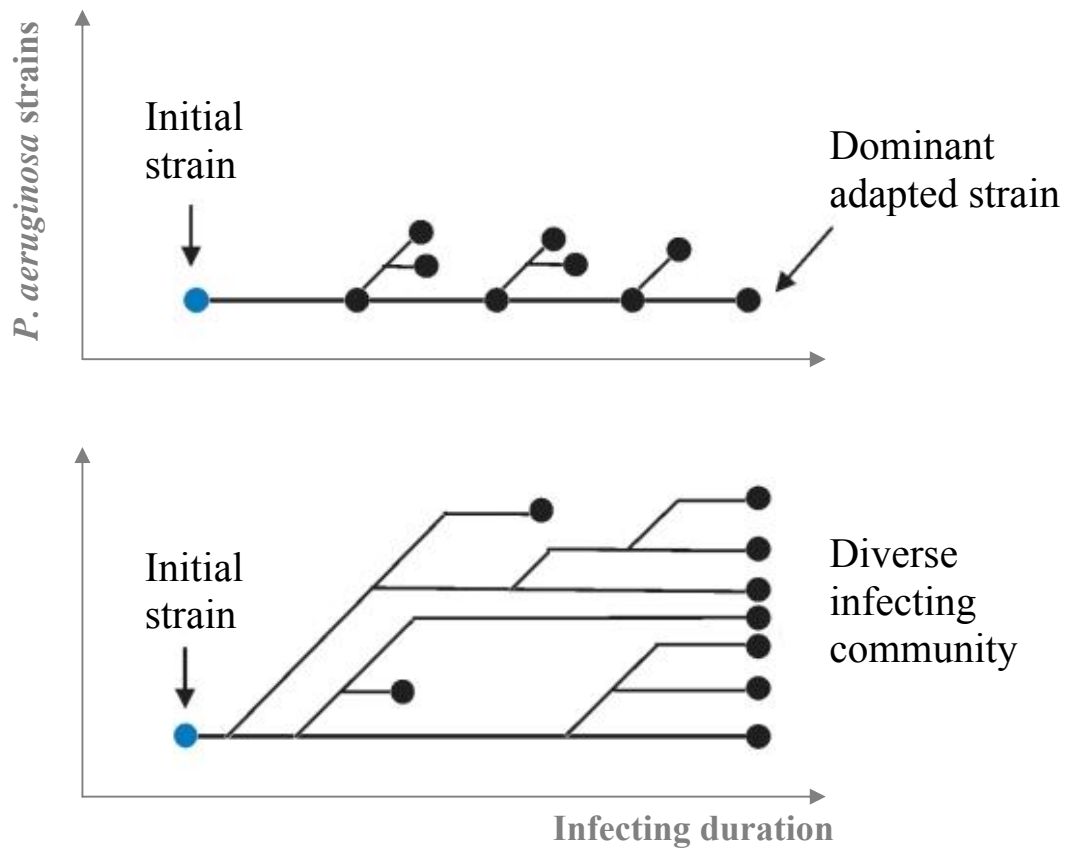


Figure 9.1 Hypothetical diagrams of two alternative models representing bacterial adaptation in chronic CF lung infections. The blue dot represents the initial infecting strain, and the black dots represent genetic variants descendent from the initial strain. Adaptation could produce a dominant strain (upper) or a diverse community of infecting bacteria (lower). (Nguyen & Singh, 2006)

Conclusions

This thesis has provided a clear picture of the strain diversity of *P. aeruginosa* within the CF population. It has also demonstrated that prevalence of a transmissible clone of this pathogen in an adult CF clinic. The emergence of clonal strains has presented a major challenge to those involved in the care of people with CF (including specialists, researchers, care-takers and even patients) requiring urgent actions to deal with and/or control their spread. In the light of this concern, the present thesis has provided a greater understanding of the pathogenesis of these clonal strains and has highlighted the connection between virulence and the negative impact on the health of those patients chronically infected with clonal strains. The hypervirulent nature of AES-1 in particular, has been revealed. This clone is linked with an increase in virulence factor production, high resistance to antibiotics, high biofilm forming capacity, and greater persistence within CF lungs. Finally, it is hoped that this thesis will assist in leading the way towards a strategy for combating *P. aeruginosa* infections not only in people with cystic fibrosis, but also in the wider community.

10 Chapter Ten: References

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11 Chapter Eleven: Appendix

11.1 Reagents and Chemical Solutions

AP buffer:

12.1 g Tris-HCl (Sigma-Aldrich), 5.8 g NaCl (Biolab), and 4.76 g MgCl₂ (BDH) dissolved in 1 L dH₂O. Stored at room temperature.

10% APS:

2 g APS (Astral Scientific) dissolved in 20 mL dH₂O. Dispensed into aliquots of 1 mL in 1.5-mL microtubes and stored at 4°C for a maximum of 2 weeks.

1% B1 (Thiamine hydrochloride) solution:

1 g B1 (Sigma-Aldrich) dissolved in 100 mL dH₂O. Stored at 4°C in an aluminium wrapped container.

5% Brij 58 solution:

5 g Brij 58 (Polyoxyethylene 20 cetyl ether; Sigma-Aldrich) dissolved in 100 mL dH₂O. Autoclaved for 20 minutes at 121°C and stored at room temperature.

3% Bovine serum albumin (BSA) solution:

3 g BSA powder (Sigma-Aldrich) dissolved in 100 mL dH₂O. Filter sterilised [through 0.22- μ m MillexTM Syringe Driven unit (Millipore)] and stored at 4°C.

5% BSA solution:

5 g BSA powder (Sigma-Aldrich) dissolved in 100 mL dH₂O. Filter sterilised and stored at 4°C.

0.25% (w/v) Coomassie Blue solution:

0.5 g Coomassie Blue (Hopkin & Williams) dissolved in 90 mL dH₂O, 90 mL Methanol (Ajax Chemicals Ltd.), and 20 mL Glacial Acetic Acid (Ajax Chemicals Ltd.). Filtered through Whatman No.1 filter papers and stored at room temperature.

De-staining solution:

200 mL Absolute ethanol (Univar), 300 mL Glacial Acetic Acid (Ajax Chemicals Ltd.) and 1.5 L dH₂O was mixed thoroughly. Stored at room temperature.

EC Lysis buffer:

0.6 mL 1M Tris pH 7.6, 20 mL 5M NaCl, 20 mL 0.5M EDTA pH 8, 0.2 g Sodium deoxycholate (Sigma-Aldrich), 0.5 g Sodium laurylsarcosine (Sigma-Aldrich), and 10 mL 5% Brij 58 solution dissolved and made up to 100 mL with dH₂O. Autoclaved for 20 minutes at 121°C and stored at room temperature.

0.12M EDTA solution:

3.9 g EDTA (Sigma-Aldrich) dissolved in 100 mL dH₂O. Autoclaved for 20 minutes at 121°C and stored at 4°C.

0.5M EDTA solution pH 8:

186.1 g EDTA·2H₂O (Sigma-Aldrich) added to 800 mL dH₂O and mixed thoroughly. pH adjusted to 8 with NaOH pellets (Pronalys). Volume adjusted to 1 L with dH₂O. Autoclaved for 20 minutes at 121°C and stored at 4°C.

10mM EGTA:

3.8 g EGTA (Sigma-Aldrich) dissolved in 1 L dH₂O. Autoclaved for 20 minutes at 121°C and stored at room temperature

ESP Proteinase K solution:

1 g Sodium laurylsarcosine (Sigma-Aldrich) dissolved in 95 mL 0.5M EDTA solution pH 8. Autoclaved for 20 minutes at 121°C and stored at 4°C. A final concentration of 1 mg/ml of Proteinase K (Sigma-Aldrich) was added prior to the use.

ESP Proteolysis buffer:

1 g Sodium laurylsarcosine (Sigma-Aldrich), 10 mL 0.5M EDTA solution pH 8 dissolved and adjusted to total volume of 100 mL with dH₂O. Autoclaved for 20 minutes at 121°C and stored at 4°C.

10 mg/mL Ethidium bromide (stock solution):

1 g Ethidium bromide (Sigma-Aldrich) dissolved in 100 mL dH₂O. Stored at 4°C in an aluminium foil wrapped container.

Ethidium bromide staining solution:

75 µL of 10 mg/mL Ethidium bromide was added to 500 mL TBE buffer. Stored at room temperature in a sealed, light protection container.

1% Gelatin:

0.2 g Gelatin from porcine skin (Sigma-Aldrich) added to 20 mL dH₂O. The solution was heated in a microwave oven until dissolved. Stored at 4°C. Re-dissolved by heating in a microwave prior to the use.

15% (v/v) Glycerol for bacterial storage:

15 mL Glycerol (Sigma-Aldrich) mixed and dissolved with dH₂O. The volume was adjusted to 100 mL with dH₂O. Dispensed into aliquots of 1 mL in cryopreservative tubes (Nunc). Autoclaved for 20 minutes at 121°C and stored at 4°C.

0.2% Glucose:

20 g Glucose (Amresco) dissolved in 1 L dH₂O. Filter sterilised and stored at 4°C.

40% Glucose:

400 g Glucose (Amresco) dissolved in 1 L dH₂O. Filter sterilised and stored at 4°C.

2M HCl solution:

16.7 mL of 12M HCl (stock solution; Unilab) diluted to final volume of 100 mL with dH₂O. Stored at 4°C.

Hide azure powder substrate solution:

100 mg Hide azure powder (Sigma-Aldrich) mixed with 20 mL Total protease buffer (Final concentration, 5 mg/mL). Stored at 4°C.

20mM L-cysteine solution:

0.024 g L-cysteine (Carbiochem Behring) dissolved in 10 mL Tris-HCl-CaCl₂ buffer. Filter sterilised and stored at 4°C.

1M MgSO₄·7H₂O:

24.65 g MgSO₄·7H₂O (Biolab) dissolved in 100 mL dH₂O. Autoclaved for 20 minutes at 121°C and stored at room temperature.

0.9% NaCl solution:

9 g NaCl (Biolab) dissolved in 1L dH₂O. Autoclaved for 20 minutes at 121°C and stored at room temperature.

1M NaCl solution:

58.44 g NaCl (Biolab) dissolved in 1L dH₂O. Autoclaved for 20 minutes at 121°C and stored at room temperature.

5M NaCl solution:

292.2 g NaCl (Biolab) dissolved in 1L dH₂O. Autoclaved for 20 minutes at 121°C and stored at room temperature.

1x NE Buffer 2 solution:

5 µL BSA (0.1 mg/mL) (New England Biolabs), 500 µL NE Buffer 2 (New England Biolabs) mixed in 4,500 µL dH₂O. Immediately used.

4% (w/v) Paraformaldehyde:

4 g Paraformaldehyde (Sigma-Aldrich) mixed with 100 mL PBS. The mixture was stirred and heated (using a heating, magnetic stirring platform) until completely dissolved. Once dissolved, allowed to cool and stored at 4°C. Paraformaldehyde is toxic; all steps were performed in a fume hood.

PBS (Phosphate buffer solution):

40 g NaCl (Biolab), 5.7 g Na₂HPO₄ (Univar), 1 g KCl (Univar) and 1 g KH₂HPO₄ (Univar) dissolved in 5 L dH₂O. Autoclaved for 20 minutes at 121°C and stored at room temperature.

PIV buffer:

10 mL 1M Tris pH 7.6 combined with 200 mL 5M NaCl and 790 mL dH₂O. Autoclaved for 20 minutes at 121°C and stored at room temperature.

10 mM PMSF (Phenylmethyl sulfonyl fluoride):

17.5 mg PMSF (Sigma-Aldrich) dissolved in 10 mL Isopropanol (Unilab). Dispensed into aliquots of 0.5 mL and stored at -20°C. PMSF is toxic; all steps were performed in a fume hood.

4% Polyacrylamide stacking gel:

3.17 mL dH₂O, 1.25 mL 0.5M Tris pH 6.8, 0.5 mL 40% Acrylamide/bisacrylamide (Sigma-Aldrich), 50 µL 10% SDS, 5 µL TEMED (Sigma-Aldrich), and 25 µL 10% APS were mixed together and immediately poured into clean gel cassettes (Bio-Rad) on top of solidified resolving gels. A ten-well plastic combs (Bio-Rad) was used to create the sample loading wells.

7.5% Polyacrylamide resolving gel containing gelatine:

4.5 mL dH₂O, 1 mL 1% Gelatin, 2.5 mL 1.5M Tris pH 8.8, 1.88 mL 40% Acrylamide/bisacrylamide (Sigma-Aldrich), 100 µL 10% SDS, 5 µL TEMED (Sigma-Aldrich), and 50 µL 10% APS were mixed together and immediately poured into clean gel cassettes (Bio-Rad). 2 mL of saturated butanol (Sigma-Aldrich) was poured on top of the gels to ensure the interface between the resolving and stacking gels was smooth. Gels were allowed to polymerise for 40 minutes. Once set, remaining butanol was decanted and gels were rinsed thoroughly with dH₂O. Remaining dH₂O was removed using Whatman No. 42 filter papers.

12% Polyacrylamide gel:

4.45 mL dH₂O, 2.5 mL 1.5M Tris pH 8.8, 3 mL 40% Acrylamide/bisacrylamide (Sigma-Aldrich), 100µL 10% SDS, 5 µL TEMED (Sigma-Aldrich), and 50 µL 10% APS were mixed together and immediately poured into clean gel cassettes (Bio-Rad). 2 mL of saturated butanol (Sigma-Aldrich) was poured on top of the gels to ensure the interface between the resolving and stacking gels was smooth. Gels were allowed to polymerise for 40 minutes. Once set, remaining butanol was decanted and gels were rinsed thoroughly with dH₂O. Remaining dH₂O was removed using Whatman No. 42 filter papers.

20 mg/mL Proteinase K solution:

100 mg Proteinase K (Sigma-Aldrich) dissolved in 5 mL dH₂O. Dispensed into aliquots of 0.5 mL and stored at -20°C.

10% SDS (Sodium dodecyl sulphate):

10 g SDS (Bio-Rad) dissolved in 100 mL dH₂O. Stored at room temperature.

10x SDS running buffer:

30 g Trizma[®] base (Sigma-Aldrich), 144 g Glycine (Sigma-Aldrich), and 10 g SDS (Bio-Rad) dissolved in 950 mL dH₂O. pH adjusted to 8 with 12M HCl (Unilab). Adjusted volume to 1 L with dH₂O. Stored at 4°C.

4x SDS sample buffer:

0.2 g SDS (Bio-Rad), 0.394 g Tris-HCl (Sigma-Aldrich), 4 mL Glycerol (Sigma-Aldrich), 0.08 g Bromophenol Blue (Sigma-Aldrich) dissolved in 10 mL dH₂O. Dispensed into aliquots of 1 mL in 2-mL microtubes and stored at 4°C.

3M Sodium acetate pH 5.2:

40.83 g Sodium acetate (CH₃COONa·3H₂O) (Sigma-Aldrich) dissolved in 80 mL dH₂O. pH adjusted to 5.2 with Glacial Acetic Acid (Ajax Chemical Ltd.). Adjusted the volume to 100 mL with dH₂O. Dispensed into aliquots and autoclaved for 20 minutes at 121°C. Stored at room temperature.

1M Sodium phosphate buffer:

70.98 g Na₂HPO₄ (Univar) dissolved in 500 ml dH₂O. 78.005 g NaH₂PO₄ (Univar) dissolved in 500 mL dH₂O. Mixed 80.95 mL Na₂HPO₄ solution with 19.05 mL NaH₂PO₄ solution. Autoclaved for 20 minutes at 121°C and stored at room temperature.

***SpeI* solution:**

20 µL *SpeI* (New England Biolabs) mixed in 2,480 µL 1x NE Buffer 2 solution. Immediately used.

Sputasol[®] (0.1% dithiothreitol) solution:

1 vial Sputasol[®] (7.5 mL) mixed with 92.5 mL dH₂O. Stored at 4°C for up to 3 months.

Syto-9[®] staining solution:

900 µL Syto-9[®] mixed with 3 mL 0.9% NaCl. Store at -20°C in an aluminium foil wrapped container.

0.5x TBE (Tris Borate Electrophoresis) buffer:

200 mL 5x TBE mixed with 1,800 mL dH₂O.

5x TBE buffer:

54 g Trizma[®] base (Sigma-Aldrich), 27.5 g Boric acid (Sigma-Aldrich), and 20 mL 0.5M EDTA pH 8 dissolved in 800 mL dH₂O. Adjusted the volume to 1 L with dH₂O. Autoclaved for 20 minutes at 121°C and stored at 4°C.

TE buffer:

5 mL 1M Tris pH 7.6, 1 mL 0.5M EDTA pH 8 mixed with 494 mL dH₂O. Autoclaved for 20 minutes at 121°C and stored at 4°C.

10x TNE buffer:

12.1 g Trizma[®] (Sigma-Aldrich), 3.7 g EDTA (Sigma-Aldrich), and 116.8 g NaCl (Biolab) dissolved in 800 mL dH₂O. pH adjusted to 7.4 with 12M HCl (Unilab). Volume adjusted to 1 L with dH₂O. Autoclaved for 20 minutes at 121°C and stored at 4°C.

1x TNE buffer:

10 mL 10x TNE buffer mixed with 90 mL dH₂O. Autoclaved for 20 minutes at 121°C and stored at 4°C.

Total protease buffer:

10 mL 1M Tris-HCl pH 8, 0.011 g CaCl₂ anhydrous (BDH), and 5.84 g NaCl (Biolabs) dissolved in 80 mL dH₂O. Volume adjusted to 100 mL with dH₂O. Autoclaved for 20 minutes at 121°C and stored at 4°C.

0.5M Tris pH 6.8:

60.55 g Trizma[®] base (Sigma-Aldrich) dissolved in 800 mL dH₂O. pH adjusted to 6.8 with 12M HCl (Unilab). Adjusted volume to 1 L with dH₂O. Autoclaved for 20 minutes at 121°C and stored at 4°C.

1M Tris pH 7.6:

121.1 g Trizma[®] base (Sigma-Aldrich) dissolved in 800 mL dH₂O. pH adjusted to 7.6 with 12M HCl (Unilab). Adjusted volume to 1 L with dH₂O. Autoclaved for 20 minutes at 121°C and stored at 4°C.

1.5M Tris pH 8.8:

181.65 g Trizma[®] base (Sigma-Aldrich) dissolved in 800 mL dH₂O. pH adjusted to 8.8 with 12M HCl (Unilab). Adjusted volume to 1 L with dH₂O. Autoclaved for 20 minutes at 121°C and stored at 4°C.

Tris-Gelatin reaction buffer:

6.057 g Trizma[®] base (Sigma-Aldrich), 1 g CaCl₂ anhydrous (BDH), 0.1363 g ZnCl₂ (Sigma-Aldrich), and 8.776 g NaCl (Biolab) dissolved in 950 mL dH₂O. pH adjusted to 8.0 with 12M HCl (Unilab). Adjusted volume to 1 L with dH₂O. Stored at 4°C.

0.02M Tris-HCl pH 8.5:

3.15 g Tris-HCl (Sigma-Aldrich) dissolved in 800 mL dH₂O. pH adjusted to 8.5 with NaOH (Pronalys). Autoclaved for 20 minutes at 121°C and stored at 4°C.

0.1M Tris-HCl pH 8:

15.759 g Tris-HCl (Sigma-Aldrich) dissolved in 800 mL dH₂O. pH adjusted to 8 with NaOH (Pronalys). Autoclaved for 20 minutes at 121°C and stored at 4°C.

1M Tris-HCl pH 8:

157.59 g Tris-HCl (Sigma-Aldrich) dissolved in 1 L dH₂O. pH adjusted to 8 with NaOH (Pronalys). Autoclaved for 20 minutes at 121°C and stored at 4°C.

Tris-HCl-CaCl₂ buffer:

3.152 g Tris-HCl (Sigma-Aldrich) and 0.0222 g CaCl₂ anhydrous (BDH) dissolved in 150 mL dH₂O. pH adjusted to 8 with 12M HCl (Unilab). Adjusted volume to 200 mL with dH₂O. Stored at 4°C.

2.5% (v/v) Triton X-100 solution:

5 mL Triton X-100 (Sigma-Aldrich) mixed and dissolved in 195 mL dH₂O. Immediately used.

20 mg/mL X-Gal:

20 mg X-Gal (Sigma-Aldrich) dissolved in 10 mL Dimethylformamide (Sigma-Aldrich). Dispensed into aliquots of 1 mL and stored at 4°C in an aluminium wrapped microtubes.

11.2 Cultivation Media

A+ minimal medium:

10.5 g K₂HPO₄ (Univar), 4.5 g KH₂PO₄ (Univar), and 1 g (NH₄)₂SO₄ (Univar), C₆H₅O₇Na₃·2H₂O (Sigma-Aldrich) dissolved in 1 L dH₂O. Autoclaved for 15 minutes at 121°C. Once cooled, added 1 mL 1M MgSO₄·7H₂O, 0.5 mL 1% B1, 10 mL 40% Glucose, 0.5 mL of 100 mg/mL Streptomycin stock solution, and 0.5 mL of 9 mg/mL Tetracycline stock solution. Dispensed into aliquots of 10 mL in sterile McCartney bottles wrapped with an aluminium foil and stored at 4°C for up to 2 weeks. For solid media, added 15 g Ultra pure agar (Sigma-Aldrich) per one litre prior to sterilisation.

AB minimal medium (supplemented with 0.2% Glucose):**- 20x AB salts solution:**

20 g NH₄Cl (Univar), 6 g MgSO₄·7H₂O (Biolab), 3 g KCl (Univar), 0.2g CaCl₂ (Univar), 50 mg FeSO₄·7H₂O (Sigma-Aldrich) dissolved in 1 L dH₂O. Autoclaved for 20 minutes at 121°C and stored at room temperature.

- 20x AB buffer:

60 g K₂HPO₄ and 23 g NaH₂PO₄ dissolved in 800 mL dH₂O. pH adjusted to 7 using 12M HCl (Unilab). Volume adjusted to 1 L with dH₂O. Autoclaved for 20 minutes at 121°C and stored at room temperature.

- AB minimal medium (supplemented with 0.2% Glucose):
50 mL 20x AB salts solution, 50 mL 20x AB buffer mixed with 900 mL 0.2% Glucose.
Dispensed into aliquots of 10 mL in sterile McCartney bottles and stored at 4°C.

CFC (Cetrimide Fucidin Cephaloridine):

Ready-to-use CFC agar plates were purchased from Oxoid. Stored at 4°C until expiry date.

Columbia HBA (Horse Blood Agar):

Ready-to-use Columbia HBA plates were purchased from Oxoid. Stored at 4°C until expiry date.

LB:

25 g LB powder (Oxoid) dissolved in 1 L dH₂O. Dispensed into aliquots and autoclaved for 15 minutes at 121°C. For solid agar, added 15 g Ultra pure agar (Sigma-Aldrich) per one litre prior to sterilisation. Stored at 4°C.

LB with 1mM EGTA:

100 mL 10mM EGTA mixed with 900 mL LB broth. Autoclaved for 15 minutes at 121°C. Stored at 4°C.

MH (Mueller Hinton):

Ready-to-use MH plates were purchased from Oxoid. Stored at 4°C until expiry date.

MSA (Mannitol Salt Agar):

Ready-to-use MSA plates were purchased from Oxoid. Stored at 4°C until expiry date.

Nutrient agar:

Ready-to-use Nutrient agar plates were purchased from Oxoid. Stored at 4°C until expiry date.

Nutrient broth:

13 g Nutrient-broth powder (Oxoid) dissolved in 1 L dH₂O. Dispensed into aliquots and autoclaved for 15 minutes at 121°C. Stored at 4°C.

Rhamnolipids detecting agar:

3 g Na₂HPO₄ (Univar), 1.5 g KH₂PO₄ (Univar), 0.25 g NaCl (Biolab), 0.25 g Tyrosine (Carbiochem Behring), 1 g Glucose (Sigma-Aldrich), 2.5 µL 1% Methylene Blue (Ajax Chemical Ltd.), 0.1 g Cetyltrimethyl ammonium bromide (Sigma-Aldrich), 0.246 g MgSO₄·7H₂O (Biolab), and 7.5 g Ultra pure agar dissolved in 500 mL dH₂O. Autoclaved for 20 minutes at 121°C. Once cooled, aliquoted approximately 25 mL into each sterile plate and allowed to set at room temperature. Immediately used or stored at 4°C for up to one week.

TSB (Tryptic Soy Broth):

30 g TSB powder (Oxoid) dissolved in 1 L dH₂O. Dispensed into aliquots and autoclaved for 15 minutes at 121°C. For solid agar, added 15 g Ultra pure agar (Sigma-Aldrich) per one litre prior to sterilisation. Stored at 4°C.

11.3 Antibiotic Susceptibility Patterns of 43 *P. aeruginosa* Isolates

Isolate	Beta-lactam								Quinolones		Aminoglycosides		Macrolide		Other classes									
	Pen	Amp	Cex	Ctx	Cfp	Oxa	Tim	Mem	Cip	Nor	Gen	Amk	Ery	Tet	Rif	Fus	Chl	Tri	Nit	Van	Met	Nov	Pmx	
AES-1																								
C1	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
C2	R	R	R	R	RS	R	S	S	R	S	R	RS	R	R	R	R	R	R	R	R	R	R	R	S
C3	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S
C4	R	R	R	R	RS	R	S	S	S	S	R	RS	R	R	R	R	R	R	R	R	R	R	R	S
C5	R	R	R	R	RS	R	S	S	S	S	R	RS	R	R	R	R	R	R	R	R	R	R	R	S
C6	R	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S
C7	R	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S
C8	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S
C9	R	R	R	R	R	R	R	S	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S
C10	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S
C11	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S
C12	R	R	R	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S
C13	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S
C14	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S
AES-2																								
C15	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S
C16	R	R	R	R	R	R	R	S	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S
C17	R	R	R	R	RS	R	S	S	S	S	R	RS	R	R	R	R	R	R	R	R	R	R	R	S
C18	R	R	R	R	R	R	R	R	R	S	RS	RS	R	R	R	R	R	R	R	R	R	R	R	S
C19	R	R	R	RS	R	R	S	S	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S

(continued next page)

Appendix 11.3 continued

Isolate	Beta-lactam								Quinolones		Aminoglycosides		Macrolide		Other classes								
	Pen	Amp	Cex	Ctx	Cfp	Oxa	Tim	Mem	Cip	Nor	Gen	Amk	Ery	Tet	Rif	Fus	Chl	Tri	Nit	Van	Met	Nov	Pmx
Non-clonal																							
U1	R	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	R	R	R	R	R	R	S
U2	R	R	R	R	S	R	S	S	S	S	RS	S	R	R	R	R	R	R	R	R	R	R	S
U3	R	R	R	RS	S	R	S	S	R	S	R	RS	R	R	R	R	R	R	R	R	R	R	S
U4	R	R	R	R	R	R	S	R	R	S	RS	RS	R	R	R	R	R	R	R	R	R	R	S
U5	R	R	R	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R	R	R	R	R	S
U6	R	R	R	R	S	R	S	R	S	S	S	S	R	R	R	R	R	R	R	R	R	R	S
U7	R	R	R	RS	S	R	S	S	R	S	RS	RS	R	R	R	R	R	R	R	R	R	R	S
U8	R	R	R	R	R	R	S	S	R	S	RS	RS	R	R	R	R	R	R	R	R	R	R	S
U9	R	R	R	R	R	R	S	S	S	S	R	RS	R	R	R	R	R	R	R	R	R	R	S
U10	R	R	R	RS	R	R	S	S	S	S	RS	RS	R	R	R	R	R	R	R	R	R	R	S
U11	R	R	R	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R	R	R	R	R	S
U12	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R	R	R	S
U13	R	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	S	R	R	R	R	R	S
U14	R	R	R	RS	R	R	S	S	S	S	R	R	R	R	R	R	R	R	R	R	R	R	S
U15	R	R	R	R	R	R	R	S	R	S	RS	S	R	R	R	R	R	R	R	R	R	R	S
U16	R	R	R	R	R	R	R	S	R	S	R	R	R	R	R	R	R	R	R	R	R	R	S
U17	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	S
U18	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	S
U19	R	R	R	R	RS	R	S	S	R	S	RS	RS	R	R	R	R	R	R	R	R	R	R	S
U20	R	R	R	R	RS	R	S	S	R	S	R	R	R	R	R	R	R	R	R	R	R	R	S
U21	R	R	R	RS	R	R	S	S	R	S	RS	S	R	R	R	R	R	R	R	R	R	R	S
U22	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	S
U23	R	R	R	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R	R	R	R	R	S
U24	R	R	R	R	R	R	S	S	R	S	S	S	R	R	R	R	R	R	R	R	R	R	S

Appendix 11.3 Abbreviations

- Beta-lactam

Pen, penicillin; Amp, ampicillin; Cex; cephalixin; Ctx; Ceftriaxone; Cfp; cefepime; Oxa; oxacillin; Tim; ticarcillin-clavulanate; Mem, meropenem.

- Quinolones

Cip, ciprofloxacin; Nor, norfloxacin.

- Aminoglycosides

Gen, gentamicin; Amk, amikacin.

- Macrolide

Ery, erythromycin; Tet, tetracycline.

- Miscellaneous

Rif, rifampicin; Fus, fusidic acid; Chl, chloramphenicol; Tri, trimethoprim; Nit, nitrofurantoin;

Van, vancomycin; Met, metronidazole; Nov, novobiocin; Pmx, polymyxin.

S; susceptible; R, resistant; RS, intermediate.

11.4 PCR results of genes encoding virulence factors, QS and TTSS

Isolate ID	<i>vfr</i>	<i>lasI</i>	<i>lasR</i>	<i>lasB</i>	<i>rhlI</i>	<i>rhlR</i>	<i>rhlAB</i>	<i>lasA</i>	<i>algR</i>	<i>aprA</i>	<i>exoU</i>	<i>exoS</i>
AES-1												
C1	+	+	+	+	+	+	+	+	+	+	-	+
C2	+	+	+	+	+	+	+	+	+	+	-	+
C3	+	+	+	+	+	+	+	+	+	+	-	+
C4	+	+	+	+	+	+	+	+	+	+	-	+
C5	+	+	+	+	+	+	+	+	+	+	-	+
C6	+	+	+	+	+	+	+	+	+	+	-	+
C7	+	+	+	+	+	+	+	+	+	+	-	+
C8	+	+	+	+	+	+	+	+	+	+	-	+
C9	+	+	+	+	+	+	+	+	+	+	-	+
C10	+	+	+	+	+	+	+	+	+	+	-	+
C11	+	+	+	+	+	+	+	+	+	+	-	+
C12	+	+	+	+	+	+	+	+	+	+	-	+
C13	+	+	+	+	+	+	+	+	+	+	-	+
C14	+	+	+	+	+	+	+	+	+	+	-	+
AES-2												
C15	+	+	+	+	+	+	+	+	+	+	-	+
C16	+	+	+	+	+	+	+	+	+	+	-	+
C17	+	+	+	+	+	+	+	+	+	+	-	+
C18	+	+	+	+	+	+	+	+	+	+	-	+
C19	+	+	+	+	+	+	+	+	+	+	-	+

(continued next page)

Appendix 11.4 continued

Isolate ID	<i>vfr</i>	<i>lasI</i>	<i>lasR</i>	<i>lasB</i>	<i>rhlI</i>	<i>rhlR</i>	<i>rhlAB</i>	<i>lasA</i>	<i>algR</i>	<i>aprA</i>	<i>exoU</i>	<i>exoS</i>
Non-Clonal												
U1	+	+	+	+	+	+	+	+	+	+	-	+
U2	+	+	+	+	+	+	+	+	+	+	-	+
U3	+	+	+	+	+	+	+	+	+	+	-	+
U4	+	-	-	+	+	+	+	+	+	+	-	+
U5	+	+	+	+	+	+	+	+	+	+	-	+
U6	+	+	+	+	+	+	+	+	+	+	-	+
U7	+	+	+	+	+	+	+	+	+	+	-	+
U8	+	+	+	+	+	+	+	+	+	+	-	+
U9	+	+	+	+	+	+	+	+	+	+	-	+
U10	+	+	+	+	+	+	+	+	+	+	-	+
U11	+	+	+	+	+	+	+	+	+	+	-	+
U12	+	+	+	+	+	+	+	+	+	+	-	+
U13	+	+	+	+	+	+	+	+	+	+	-	+
U14	+	+	+	+	+	+	+	+	+	+	-	+
U15	+	+	+	+	+	+	+	+	+	+	-	+
U16	+	+	+	+	+	+	+	+	+	+	-	+
U17	+	+	+	+	+	+	+	+	+	+	-	+
U18	+	+	+	+	+	+	+	+	+	+	-	+
U19	+	+	+	+	+	+	+	+	+	+	-	+
U20	+	+	+	+	+	+	+	+	+	+	-	+
U21	+	+	+	+	+	+	+	+	+	+	-	+
U22	+	+	+	+	+	+	+	+	+	+	-	+
U23	+	+	+	+	+	+	+	+	+	+	-	+
U24	+	+	+	+	+	+	+	+	+	+	-	+
PAO1	+	+	+	+	+	+	+	+	+	+	-	+