Phenotypic adaptation of *Pseudomonas aeruginosa* to the Cystic Fibrosis lung environment

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Declaration of Originality

I, Natasha A. E. Wierre-Gore, declare that all the work contained in this thesis is my own except where indicated and all other work is appropriately referenced.

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ABSTRACT

Pseudomonas aeruginosa is one of the major pathogens of the Cystic Fibrosis (CF) lung, infecting up to 50% of CF patients. The CF lung is a complex and heterogeneous environment in which *P. aeruginosa* establishes long term chronic infections through extensive adaptation after an initial acute infection stage.

The phenotypic adaptation of *P. aeruginosa* was investigated through two different sets of clinical isolates. The first was a previously available set of 154 longitudinally collected isolates from 18 patients. The second consisted of multiple isolates collected from 7 early and 17 chronically infected patient samples. These 593 isolates were specifically collected for this study. Multi-locus variable number tandem repeat analysis (MLVA) genotyping showed that all isolates from individual patients typed were clonally related.

Through the measurement of multiple phenotypes linked to virulence (motility, proteases, pyocyanin, pyoverdine, hydrogen cyanide, biofilm formation and speed of growth) and regulatory quorum sensing molecules, the evolution of the phenotypes between stages of infection was first assessed. The measurements for all phenotypes were significantly ($p<0.05$) lower in chronic stage isolates than early stage isolates. Results showed statistically significant ($p<0.01$) positive moderate correlations between QS regulated phenotypic and the QS autoinducer molecules.

The hypothesis that within-patient diversity is greater than between-patient diversity was tested. The results showed that between-patient diversity was in fact greater than within-patient diversity for all phenotypes for both early and chronic stages of infection. However, significant ($p<0.05$) within-patient diversity in almost all patients was observed for one phenotype or more, and within-patient diversity was higher in chronic stage infection samples than in early stage samples.

This study contributes to the body of work demonstrating the degree and complexity of adaptation of *P. aeruginosa* and brings further insight into the extent of diversity within and between patients.

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ABBREVIATIONS

3-oxo-C12-AHL: N-3-oxododecanoyl-L-homoserine lactone

ABC: ABC-binding cassette

AMP: Antimicrobial peptide

APR: Alkaline protease

ARHAI: Antimicrobial resistance and healthcare associated infections

ASL: Airway surface liquid

ATP: Adenosine triphosphate

Bp: Base pair

BPI: Bactericidal permeability increasing protein

C4-HSL: N-butanoyl-homoserine lactone

CBA: Columbia agar

CF: Cystic fibrosis

CFRD: Cystic fibrosis related diabetes

CFTR: Cystic Fibrosis Transmembrane Conductance Regulator

CIO: Cyanide insensitive oxidase

Cl: Chloride

DIOS: Distal intestinal obstructive syndrome

DNA: Deoxyribonucleic acid

DUOX: Dual oxidase

ENaC: Epithelial sodium channel

FEV_1: Forced expiratory volume in the 1^{st} second

Gm: Gentamicin

H_2O_2: Hydrogen peroxide

HCl: Hydrochloric acid

HCN: Hydrogen cyanide

HCO_3: Bicarbonate

$HOCl^-$: Hypochlorous acid

IL-8: Interleukin-8

IQS: Integrating quorum sensing system

LB: Lysogeny broth

LES: Liverpool epidemic strain

LPO: Lactoperoxidase

LPS: Lipopolysaccharide

MCC: Mucociliary clearance

MI: Meconium ileus

MLVA: Multi-locus variable number tandem repeat analysis

MPO: Myeloperoxidase

MS: Mass spectrometry

NAD: Nicotinamide adenine dinucleotide

NADH: Nicotinamide adenine dinucleotide + Hydrogen

NET: Neutrophil extracellular trap

NMR: Nuclear magnetic resonance

O_2^-: superoxide

$OSCN^-$: Hypothiocyanite

P. aeruginosa: Pseudomonas aeruginosa

PAMP: Pathogen associated molecular pattern

PCA: Principal Component Analysis

PCL: Periciliary layer

PCR: Polymerase chain reaction

PHE: Public Health England

Phox: Phagocytic oxidase

PMN: Polymorphonuclear neutrophil

PQS: Pseudomonas quinolone signal

PSA: *Pseudomonas* selective agar

RBH: Royal Brompton Hospital

ROS: Reactive oxygen species

SCFM: Synthetic CF Medium

SCV: Small colony variant

SP-A: Surfactant protein A

SP-D: Surfactant protein D

TLR: Toll-like receptor

UBC: University of British Columbia

VNTR: Variable Number Tandem Repeat

WT: Wild type

Chapter 1: Introduction

1.1 Cystic Fibrosis

1.1.1 Overview of Cystic Fibrosis and the CFTR mutations

Cystic fibrosis is one of the most common recessive genetic diseases, observed in 1 in 2500 births (Zvereff et al., 2014). The disease affects approximately 48 000 people across Europe, and over 10 000 people in the United Kingdom (Zolin A, 2019, Cystic Fibrosis Trust, 2019). Globally, at least 80 000 are affected by the disease, with at least 30 000 in the United States alone (Cystic Fibrosis Trust, 2019).

This monogenic autosomal disease is caused by a mutation in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene on chromosome 7 (Riordan et al., 1989). The CFTR is a unique ATP-binding cassette (ABC) transporter that functions as a channel for chloride (Cl^-) and bicarbonate (HCO_3^-) anions (Hyde et al., 1990, Ballard et al., 1999, Poulsen et al., 1994, Anderson et al., 1991, Curran et al., 2018, Quinton, 1983, Quinton, 2008).

Just over 2000 mutations of the CFTR gene have been identified to date (CFTR1, 2011). These mutations are grouped in six different classes depending on the effect of the mutation on the CFTR protein and functional consequence (Figure 1-1) (Fernandez Fernandez et al., 2018). Class I mutations cause premature termination signals resulting in an unstable mRNA and no functional CFTR protein (Hamosh et al., 1991, Hentze and Kulozik, 1999). Class II mutations cause the CFTR to not be trafficked to the correct location. The CFTR protein is not glycosylated and is misfolded, which leads to its destruction (Cheng et al., 1990, Amaral, 2004). These class I and II mutations lead to a lack of CFTR at the cell surface. Class III mutations lead to a CFTR protein that does reach the cell surface but possesses a gating defect, meaning the channel does not open properly due a problem binding to ATP and lack of conformational change (Rowntree and Harris, 2003). Class IV mutants display correct localisation and presence of CFTR protein on the cell membrane but these channels have decreased conductance and reduced anion current (Sheppard et al., 1993). Class V mutants are similar to Class I mutants but milder, with alternative splicing occurring which causes a

severely reduced amount of the full length mRNA (Beck et al., 1999, Highsmith et al., 1994). This in turn leads to a smaller number of functional CFTR proteins at the cell surface. Finally, class IV mutations cause CFTR to be unstable at the cell surface, with its half-life reduced considerably (Haardt et al., 1999).

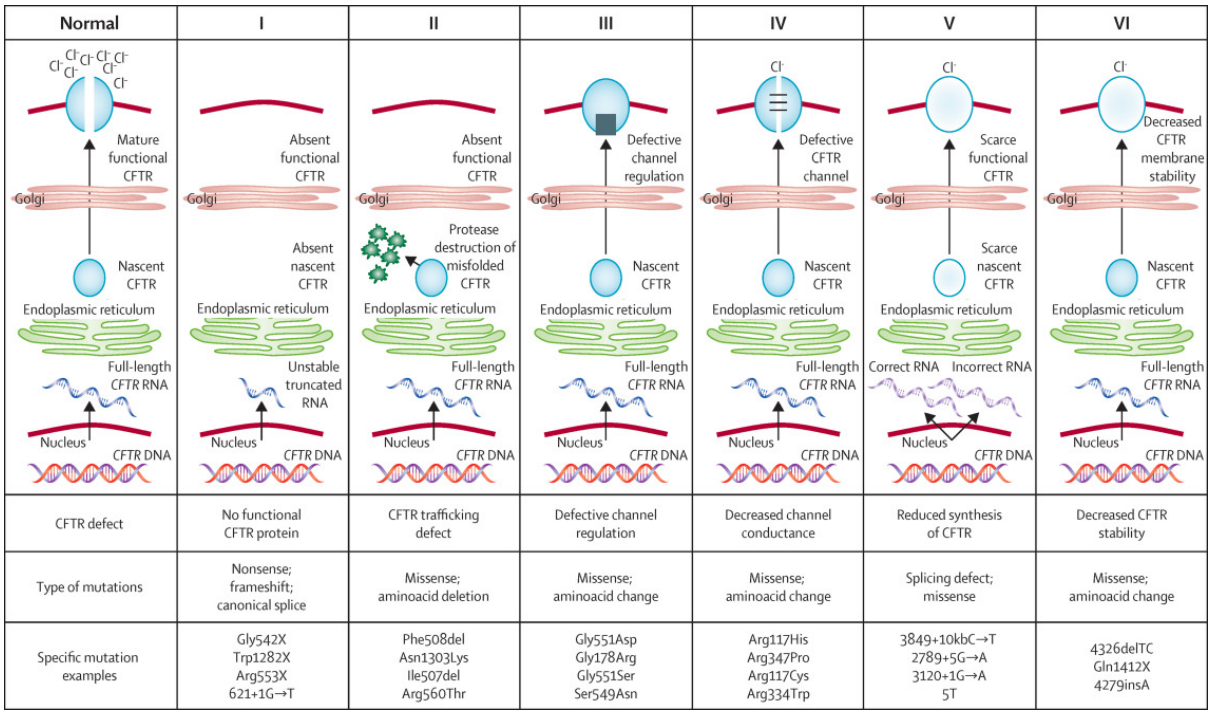


Figure 1-1: Classification of CFTR mutations and resulting phenotypes. Image taken from Elborn, 2016 (Elborn, 2016).

The multitude of CF mutations lead to various disease phenotypes (Kiesewetter et al., 1993, Kerem et al., 1990). The first three classes of mutations lead to more severe disease phenotypes than class IV, V and VI mutations (Marson et al., 2016, De Boeck and Amaral, 2016). Having an allele with the class IV mutation was found to lead to a milder phenotype regarding several clinical outcomes (Koch et al., 2001). As all CF patients have two alleles of the gene, many patients will be heterozygous, thus the final CFTR phenotypes may be unclear (Kerem and Kerem, 1996). Also some mutations can be included in several classes as they can lead to multiple sorts of defect, this being the case of the most common mutation Phe508del (F508del) (Farinha et al., 2013, Amaral, 2015). The resulting phenotypes can thus be varied, being a mix of different class mutations (Hamosh et al., 1992, Amaral et al., 2001).

Although there are a multitude of mutations altering the CFTR gene, the Phe508del (F508del) is the most common, present in at least one allele for 89.8% of CF patients in the UK (2019). This mutation was discovered simultaneously to the CFTR gene itself in 1989 (Riordan et al., 1989). Additionally to Phe508del, only 11 other mutations (G551D, R117H, G542X, 621+1G>T, N1303K, 1717-1G>A, 1898+1G>A, P76L, D1152H, 3659delC and 3272-26A>G) were detected in at least 1% of patients in the UK CF population (2019). However the frequency of these relatively common mutations varies from country to country (De Boeck et al., 2014, 2019).

Understanding the CFTR phenotypes linked to the different mutations has led to research for the development of therapies restoring or increasing CFTR functionality (Amaral, 2015). Compounds are being developed today that are correctors for mutations from class II, and potentiators for class III and IV for mutations mainly, but can be used in some cases simultaneously for additional functionality restoration (Van Goor et al., 2014, Middleton et al., 2019a, Solomon et al., 2015, Rowe et al., 2014). These new therapies are crucial as even a small percent increase in functional CFTR can lessen the severity of symptoms (Ramalho et al., 2002, Boyle et al., 2014).

1.1.2 Pathophysiology of the disease

The defect in the CFTR leads to multi organ symptoms for patients, which makes CF a life limiting disease**.** The median age of death in 2018 in the UK is 32 years old (Cystic Fibrosis Trust, 2019). However, the median predicted survival age for people born with CF has increased over the last decades and is today of 47.3 years according to the UK CF Trust (Cystic Fibrosis Trust, 2019).

The CFTR is found in the apical membrane of epithelial cells and when unfunctional results in altered secretions impacting multiple organs (Ballard et al., 1999, Anderson et al., 1992). The defect in ion transport leads to the hyper absorption of water and dehydration of the epithelial surface, which in turn leads to increased mucus viscosity, leading to the name mucoviscidosis used in certain countries (Boucher, 2007b, Quinton, 2008, Ehre et al., 2014). The dysfunctional CFTR thus affects the digestive system, the pancreas, the liver and the reproductive system causing digestive problems, potential diabetes and male infertility (Boom et al., 2007, Strong

et al., 1994, Stuhrmann and Dork, 2000). The disease was first described as a pancreatic defect (Andersen, 1938, Di Sant Agnese, 1956). However, the main life-shortening symptoms relate to the airways (O'Sullivan and Freedman, 2009).

Up to 85% of CF patients also suffer from digestive problems, mainly due to the lack of digestive enzymes being able to travel through the thick mucus (Cystic Fibrosis Trust, 2019). In the pancreas the thick mucus builds up and blocks the ducts leading to early onset exocrine insufficiency; eventually it also damages the beta cells producing insulin, leading to the development of CF related diabetes (CFRD). Almost 50% of adult CF patients suffer from CFRD, which is different from common type 1 and 2 diabetes (Moran et al., 2009, 2019, Gottlieb et al., 2012). CFRD can lead to complications also found in non CF diabetes (Kayani et al., 2018). Another digestive complication is meconium ileus (MI), which is the obstruction of the ileum of the small intestine, which can rupture in the most serious cases if not operated upon (De Lisle and Borowitz, 2013). While MI occurs in new-borns or infants, obstructions can also occur in older patients, which is referred to as distal intestinal obstructive syndrome (DIOS) (Houwen et al., 2010). Liver complications are also observed in 5-15% of CF patients, especially during childhood (De Lisle and Borowitz, 2013).

Although the lack of CFTR function does affect the previously mentioned organs, the effect on the lungs is the main cause of clinical deterioration and mortality for CF patients. In fact the cause of death was linked to respiratory symptoms in 73.1% of patients deceased between 2016 and 2018 in the UK (Cystic Fibrosis Trust, 2019).

The lungs are lined with airway surface liquid (ASL) and its hydration is ensured by ion channels, including the CFTR and the epithelial sodium channels (ENaC). While the CFTR transports chloride anions out of the cell, the ENaC absorbs Na^+ across the epithelial membrane. The balance between CFTR and ENaC activity is essential for homeostasis of the ASL. The exact relationship between the two channels remains unclear and controversial (Collawn et al., 2012, Berdiev et al., 2009). While several studies have found an inhibitory effect of CFTR on ENaC, others dispute this (Hobbs et al., 2013, Itani et al., 2011, König et al., 2002, Donaldson et al., 2002). Nonetheless in CF patients, absorption of Na^+ by the ENaC in turn leads to absorption of water by osmotic gradient from the periciliary layer (PCL) of the ASL (Matsui et al., 1998, Birket et al., 2014). Thus in the absence of a functional CFTR in the lung the ionic balance in the ASL of the lung is disturbed causing its dehydration and the presence of a viscous mucus layer (Figure 1-2) (Matsui et al., 1998, Williams and Davies,

2012).

The mucus layer consists of mucin glycoproteins, cellular debris, DNA, and proteins (Thornton and Sheehan, 2004). The mucins are produced by epithelial goblet cells and are encoded by 5 MUC genes in humans (Thornton et al., 2008, Voynow and Rubin, 2009). These mucins are densely glycosylated which can represent up to 80% of their mass and thus participate to mucus viscosity (Thornton et al., 2008). In the CF lung, entanglement of mucins is increased as a result of higher concentrations due to dehydration (Morrison et al., 2019, Henderson et al., 2014). The mucus can also adhere to the epithelial cell surfaces and leads to airway plugging and inhibition of mucociliary clearance (MCC) (Matsui et al., 1998, Hoegger et al., 2014).

Cilia are found in the PCL in which these can beat freely thanks to its low viscosity. The beating cilia in turn move the top mucus layer towards the upper airway, which is the mechanism of mucociliary clearance. This MCC is the primary defence mechanism for the lungs and is hindered in CF patients (Knowles and Boucher, 2002). Indeed, in CF the PCL of the ASL is dehydrated leading to the compression of the cilia, which become stuck in the mucus layer, rendering them immobile (Birket et al., 2014, Matsui et al., 1998).

As well as Cl^- the secretion of HCO_3^- by the CFTR also plays a role in ASL viscosity. Bicarbonate is an alkalinising molecule, its absence therefore leads to acidification of the airways (Song et al., 2006, Tate et al., 2002). A study showed a direct link between the CFTR and acidification as the pH level was proportionate to the amount of functional CFTR channels in porcine airway epithelia (Shah et al., 2016). The acidification of the CF ASL was found to alter viscosity in piglet and human cells by influencing mucin structure through electrostatic interaction (Tang et al., 2016). This effect was reversible and was found to be linked to the ASL pH. The viscosity could be reduced by increasing the pH with hypertonic saline (Tang et al., 2016). Bicarbonate was confirmed to play a role in the expansion of mucins after extracellular secretion in at least two studies (Chen et al., 2010, Cooper et al., 2013). Before being excreted, mucins are highly condensed in granules intracellularly (Verdugo et al., 1987). Thus the lack of HCO_3 in CF ASL was found to prevent expansion of the mucins leaving them highly compacted which causes the mucus to be two or three times denser than in healthy controls (Gustafsson et al., 2012).

This impacts the ability of cilia to beat and results in compromised mucociliary clearance leading to further mucus accumulation, a favourable environment for bacterial infection, and

the lack of physical removal of the bacteria (Boucher, 2007a). The overall increased viscosity and pH acidification of the ASL results in the CF lungs becoming prone to bacterial infection (Quinton, 2008, Pezzulo et al., 2012). The lack of CFTR function also leads to impaired function of the innate immune system, increasing the difficulty to fight bacterial infection. CF is therefore considered to be a mucosal immunodeficiency illness (Cohen and Prince, 2012). The effect of CF impairment of the immune system will be further discussed below.

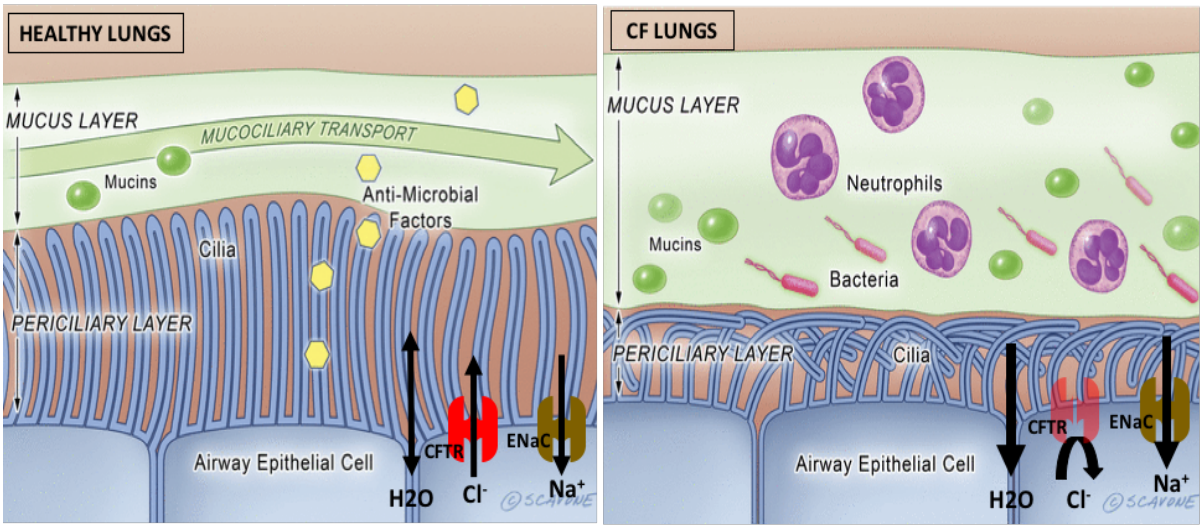


Figure 1-2: ASL changes between the healthy lung and CF lung. In the healthy lung the CFTR and the ENaC ensure the maintenance of the ASL hydration homeostasis with CFTR secreting Cl^- while the ENaC absorbs Na^+ across the epithelial cells. In the CF lung the absence of functional CFTR, chloride anions remain in the epithelial cells while sodium is still absorbed by the ENaC resulting in the re-intake of H2O from the PCL and causing dehydration of the ASL. This dehydration affects MCC and makes the airways prone to infection. Image taken from Stanton, 2017 and modified (Stanton, 2017).

1.1.3 Susceptibility to infection and major pathogens

The CFTR defect makes CF patients prone to airway infections. For example, a study found that mucins can be used as a source or carbon for the CF lung bacteria. Indeed some anaerobic bacteria are able to degrade the mucins, thus making more nutrients available for other bacterial species (Flynn et al., 2016).

In fact 34.1% of under 16 year old CF patients and 57.8% of adults suffer from acute or persistent respiratory infections in the UK (2019). In paediatric patients (<16 years old) the most common bacterial infections include *Haemophilus influenza* for 25.6% of children and

intermittent *Staphylococcus aureus* for 25.8% (2019). Nevertheless, over time *P. aeruginosa* becomes the predominant species, chronically infecting 41.4% of CF adults (>16 years old), and over 50% of 28 to 43 year olds in the UK (Figure 1-3) (Hoiby, 1982, Ramsey et al., 1991, 2019). Chronic *P. aeruginosa* infections are the main cause of inflammation due to the sustained innate immune response, which over time damages the lungs and in some cases leads to respiratory failure (Williams and Davies, 2012).

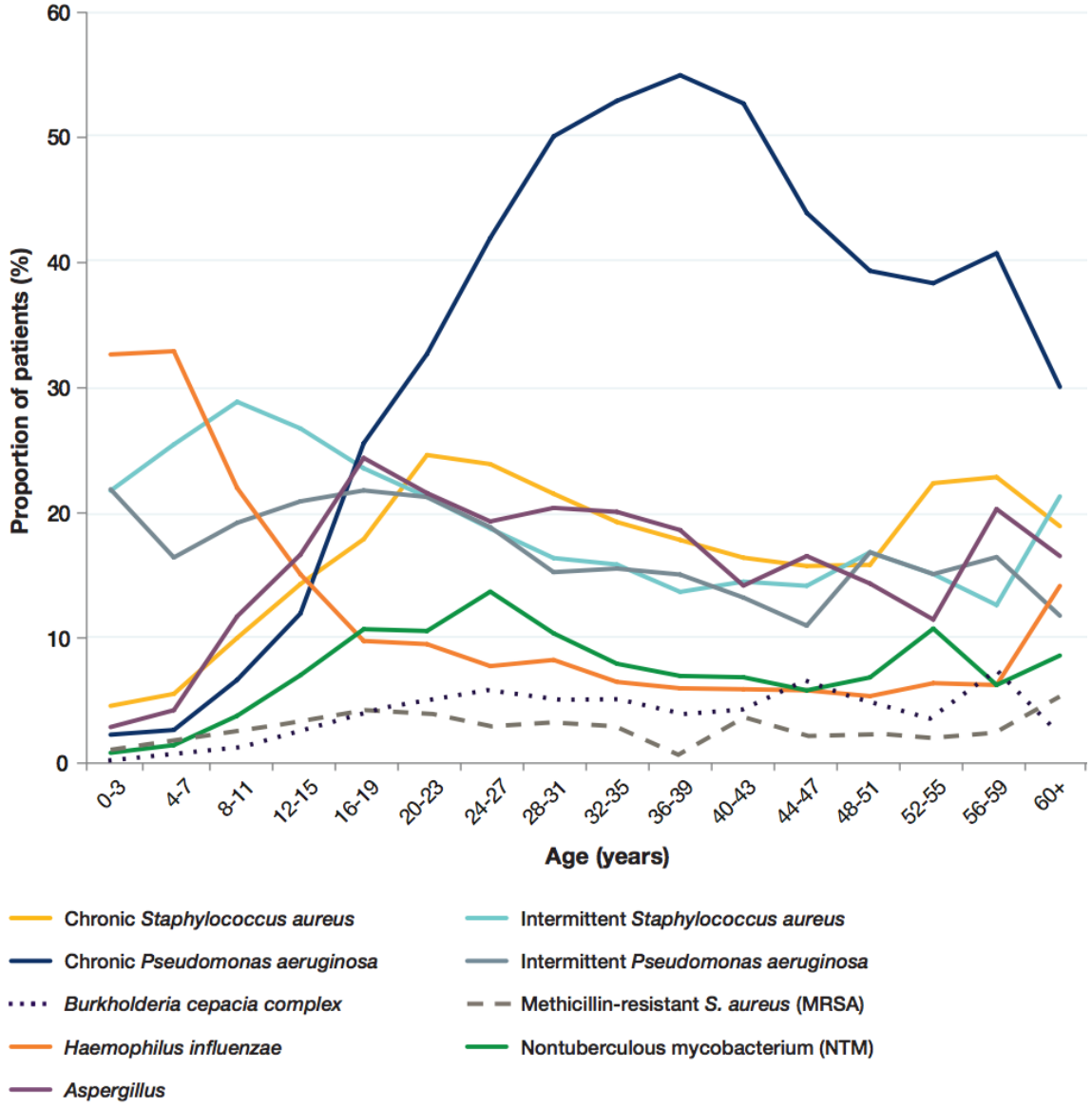


Figure 1-3: Infection rate of common CF pathogens in different age groups. Data from 9,632 patients from whom bacteria were cultured during the year. Image taken from the UK Cystic Fibrosis registry 2018 Annual data report.

1.1.4 Changing landscape of CF therapeutics: Modulators

The CF lung environment, which makes patients more prone to infection, is caused by the abnormal function of the CFTR protein stemming from mutations in the *cftr* gene. Until recently the only cystic fibrosis lung treatments available focussed on slowing the decrease in lung function with mucus clearance, the prevention and treatment of infection, and the resultant inflammation.

In the last decade there has been a leap in therapeutic approaches and care for patients with new drugs helping to tackle the causes of the disease itself, that is a dysfunctional CFTR. These newly available drugs directly target the functional defects in CFTR. They are called modulators and two types of them currently exist with several variations (Van Goor et al., 2008). These modulators aim to restore some activity in the defective CFTR protein and numbers on the cell surface. These modulators allow for a more personalised therapeutic approach to the disease, depending on patients' type of mutation.

The first type of modulators developed were potentiators and are beneficial when the CFTR protein is correctly located on the cell surface, as is the case in class III (gating) or IV (conductance) mutations (Van Goor et al., 2009). These potentiators improve the function of the channel by increasing the amount of time the channel is open, allowing flow of chloride ions (Eckford et al., 2012). Potentiators thus benefit patients with the relatively common mutation G551D (Class III gating mutation,~4% patients) or others such as R117H (Class IV conductance mutation, ~2% patients) (Ramsey et al., 2011, Accurso et al., 2010, De Boeck et al., 2014, Moss et al., 2015). The first potentiator clinically approved in 2012 was Ivacaftor (VX-770 or Kalydeco for Vertex) and in recent years several studies have unveiled other potential potentiators (Lopes-Pacheco, 2020).

The second type of modulators studied more recently are correctors. These correctors help the CFTR fold in the right configuration and traffic correctly to the cell surface, leading to increased density and activity of CFTR protein (Ren et al., 2013). These correctors aim to benefit patients possessing class II mutations, such as the most common F508del. Initially the use of correctors in monotherapies showed limitations, nevertheless in combination with the potentiator Ivacaftor, these were found to have moderate benefits on lung function with many

studies finding an increase in FEV1 (Clancy et al., 2012, Wainwright et al., 2015, Southern et al., 2018, Boyle et al., 2014, Wu et al., 2019, Fajac and De Boeck, 2017). The first combination therapy, Orkambi (combination of Lumacaftor and Ivacaftor) from Vertex, was approved in 2015 in the US and EU for F508del homozygous patients (Fajac and De Boeck, 2017). In 2018 another combination therapy, Symkevi or Symdeko (Vertex Pharmaceuticals), was approved by the FDA and EMA, using Ivacaftor alongside a new corrector called Tezacaftor (Taylor-Cousar et al., 2017, Lopes-Pacheco, 2020). A new triple combination therapy has been approved for patients as Trikafta in the US and Kaftrio in the UK, using a combination of Elexacaftor, Tezacaftor, and Ivacaftor and is beneficial to both homozygous and heterozygous F508del patients (Lopes-Pacheco, 2020, Middleton et al., 2019b, Heijerman et al., 2019, Ridley and Condren, 2020). This new triple combination therapy now enables 85-90 % of CF patients to benefit from these new therapies (Cuevas-Ocaña et al., 2020, Ridley and Condren, 2020).

The biggest effects of these modulators are the rapid improvement in FEV1 and long term sustained higher lung function over time with slower rate of decline (Gramegna et al., 2020, Volkova et al., 2020, Ridley and Condren, 2020). The new triple combinations recently available have shown FEV1 increase of over 10% in several clinical trials (Heijerman et al., 2019, Middleton et al., 2019b). Many studies have demonstrated that sustained lung function is enabled through reduced pulmonary exacerbations, which limit long term decline of lung function and increase the overall quality of life (Gramegna et al., 2020, Ridley and Condren, 2020, Wainwright et al., 2015). The partial restoration of CFTR function has also been found to lead to ASL rehydration and in turn increase in mucociliary clearance (Keown et al., 2020, Rogers et al., 2020). This enables the primary defence mechanism against pathogens to be restored. The impact of these modulators on the lung microbiota is now being studied as effects of the drugs on mucociliary clearance and ASL rehydration could be expected to lead to better physiological clearance of the bacteria (Rogers et al., 2020). The limited data currently available indicates that modulators could lead to a decrease in inflammatory markers and overall inflammation, although restoration of mucociliary clearance could also play a direct role in the decrease in inflammation (Favia et al., 2020, Hisert et al., 2020, Keown et al., 2020, Ronan et al., 2018).

In addition to indirectly helping reduce infections through restoring a healthier lung environment, some studies have found that modulators could directly have an impact on the bacteria (Reznikov et al., 2014, Schneider et al., 2016). Although individually unable to kill

bacteria, some of these modulators seem to enhance already known antibiotics (Schneider et al., 2016, Cho et al., 2019).

Finally, while the long term effect of modulators on *P. aeruginosa* chronic infections remains unclear, studies suggest that the use of Ivacaftor may help reduce the burden of *P. aeruginosa* infections and protect patients against acquiring new infections (Hisert et al., 2017, Rowe et al., 2014, Bernarde et al., 2015, Volkova et al., 2020, Singh et al., 2019, Ronan et al., 2018). All clinical findings about the effect of modulators on *P. aeruginosa* are based on patients receiving Ivacaftor or Lumacaftor/Ivacaftor (Orkambi) but the benefit of the new triple combination therapies against *P. aeruginosa* chronic infections is currently unknown.

1.2 *Pseudomonas aeruginosa* in the context of the CF lung

1.2.1 *Pseudomonas aeruginosa* as a pathogen - overview

P. aeruginosa is a Gram negative, ubiquitous, opportunistic pathogenic bacterium. Its large 6,3 million base pair (bp) genome has enabled it to be present in a wide range of environments and infect many hosts such as plants, insects and mammals (Mahajan-Miklos et al., 2000, Stover et al., 2000). The pathogen can quickly evolve to specific niches through the integration of genomic segments or by discarding these, thus easily moving from environmental reservoirs to human infections (Mathee et al., 2008). In humans it rarely causes infections in healthy individuals. *P. aeruginosa* is opportunistic and causes a range of nosocomial infections, urinary tract infections, infections in immunocompromised patients or burn wound infections, but is predominantly known as an infecting agent of the CF lungs (Bodey et al., 1983, Lyczak et al., 2000, Bitsori et al., 2012).

1.2.2 *P. aeruginosa* in CF

At the time of initial infection *P. aeruginosa* can usually be eradicated by the aggressive use of antibiotics. Apparent clearance may be followed by a so-called 'intermittent' infection stage but, in most cases, the infection eventually becomes chronic as bacteria start adapting to the CF lung environment (Pressler et al., 2011). Defining the different stages of infection has not been straightforward; in the 1990s different countries had several definitions (Kerem et al.,

1990, Ballmann et al., 1998). The current most common definition is known as the Leeds criteria describing four groups of patients (Lee et al., 2003): never infected; free of *P. aeruginosa* (no positive cultures in the previous year but previously infected); intermittently infected (50% or fewer of *P. aeruginosa* cultures positive over the previous 12 months); chronic infection (50% or more of samples are positive to *P. aeruginosa*). The accuracy of these definitions will depend on the frequency of sampling and the quality of the samples (e.g. young children may not reliably expectorate sputum). The UK Cystic Fibrosis Trust recommends airway sampling at least every 2 months for children and every 3 months for adult patients. In 2006, Proesmans et al. validated the Leeds criteria and were able to differentiate chronic from intermittent stages with anti-*Pseudomonas aeruginosa* antibody levels, although this was less useful in adults than in children (Proesmans et al., 2006).

1.2.3 Host response to *P. aeruginosa* infection

The infection of the lungs leads to complex bacteria-host interactions that have yet to be fully understood (Hiemstra et al., 2015). The primary innate immune response is triggered by the presence of bacteria through the recognition of Pathogen Associated Molecular Patterns (PAMPs), known to include LPS and flagellin (Ramphal et al., 2008, Sonawane et al., 2006). Several toll-like receptors (TLRs) recognise the *P. aeruginosa* PAMPs and trigger the chain of reactions activating the immune response (Figure 1-4) (MacRedmond et al., 2005, McIsaac et al., 2012, Raoust et al., 2009, Zhang et al., 2005).

The inflammatory response is largely driven by the influx of polymorphonuclear neutrophils (PMNs), moving from the systemic circulation into the airways (Khan et al., 1995, Bals et al., 1999). PMNs are recruited to the lung by cytokines produced by the epithelial cells and alveolar macrophages in response to the presence of bacteria sensed by the TLRs (Bonfield et al., 1995, McIsaac et al., 2012).

The recruited neutrophils (Brinkmann et al., 2004) produce molecules including reactive oxygen and nitrogen species, hypochlorous acid ($HOCl^-$) produced by myeloperoxidase (MPO), and antimicrobial peptides (AMPs), which are known to have bactericidal properties (Moskwa et al., 2007, Winterbourn et al., 2016).

The dysfunctional CFTR directly affects host immune cells such as neutrophils and macrophages. Indeed, even though a debated subject, CF neutrophils and macrophages have recently been shown to also lack CFTR on their phagosomal membrane (Porto et al., 2011, Downey et al., 2009, White et al., 2015). Thus Cl^- anions needed to produce HOCl are not transported to the phagolysosome leading to a decreased production and reduced bacterial killing (Painter et al., 2008, Porto et al., 2011, Painter et al., 2006, Ng et al., 2014).

Neutrophils also use a specific death cell program called NETosis in order to bind and trap the bacteria. This innate response described first in 2004 by Brinkmann et al., leads to the excretion of DNA and granules that form Neutrophil Extracellular Traps (NETs) which can bind the bacteria (Williams and Davies, 2012, Branzk and Papayannopoulos, 2013, Brinkmann et al., 2004). Excessive NETosis can cause further thickening of the ASL as the extracellular DNA release gets entangled with the mucins and further obstructs the lungs (Lethem et al., 1990, Marcos et al., 2015, Gray et al., 2015).

The epithelial cells also generate antibacterial molecules following the infection, such as AMPs or hypothiocyanite ($OSCN^-$) which is produced by the dual oxidase/lactoperoxidase (DUOX/LPO) pathway. The DUOX on the apical membrane releases H_2O_2 into the ASL (Fischer, 2009). The H_2O_2 released generates HOSCN through the reaction with SCN- which is catalysed by LPO. SCN- is obtained from the patient's diet and is transported to the airway ASL through various channels, amongst which is the CFTR, thus potentially leading to lower levels of HOSCN in the CF lungs (Conner et al., 2002, Moskwa et al., 2007).

Various AMPs are produced by epithelial cells and neutrophils, such as lactoferrins, lysozymes, cathelicidins, β and α defensins and surfactant proteins A and D (SP-A and SP-D) (Rogan et al., 2006). Defensins and cathelicidins are both produced by epithelial cells and neutrophils, while BPI by neutrophils, and lysozymes and SPs by epithelial cells (Rogan et al., 2006). AMPs mostly target the outer layer of the bacterial membrane which is negatively charged due to phospholipids. The interaction with the membrane alters the membrane structure, which loses its permeability, integrity and depolarises, finally causing holes in the bacterial membrane and lysis (Zasloff, 2002, Ginsburg, 2004). Lysozymes can cause enzymatic lysis of the peptidoglycans of the cell membrane while SP-A and D bind to the bacteria helping phagocytosis (Dajani et al., 2005, Lawson and Reid, 2000). Finally, lactoferrins can deprive the bacteria of iron by strongly binding to it (Rogan et al., 2006). Several studies have found in human epithelial cells and porcine models that the acidification of the ASL in CF airways

may lead to an impaired function of most AMPs, as they rely on a neutral environmental pH (Berkebile and McCray, 2014, Pezzulo et al., 2012).

In the absence of a functional CFTR, several host-defence defects have been observed which are almost certainly the reason for the lack of eradication of the infections in the CF lungs. Thus, in patients with CF the inflammation is sustained and this prolonged inflammatory state coupled with mucus obstruction subsequently leads to the damaging of the lung tissue and, ultimately, to respiratory failure (Elborn et al., 1993).

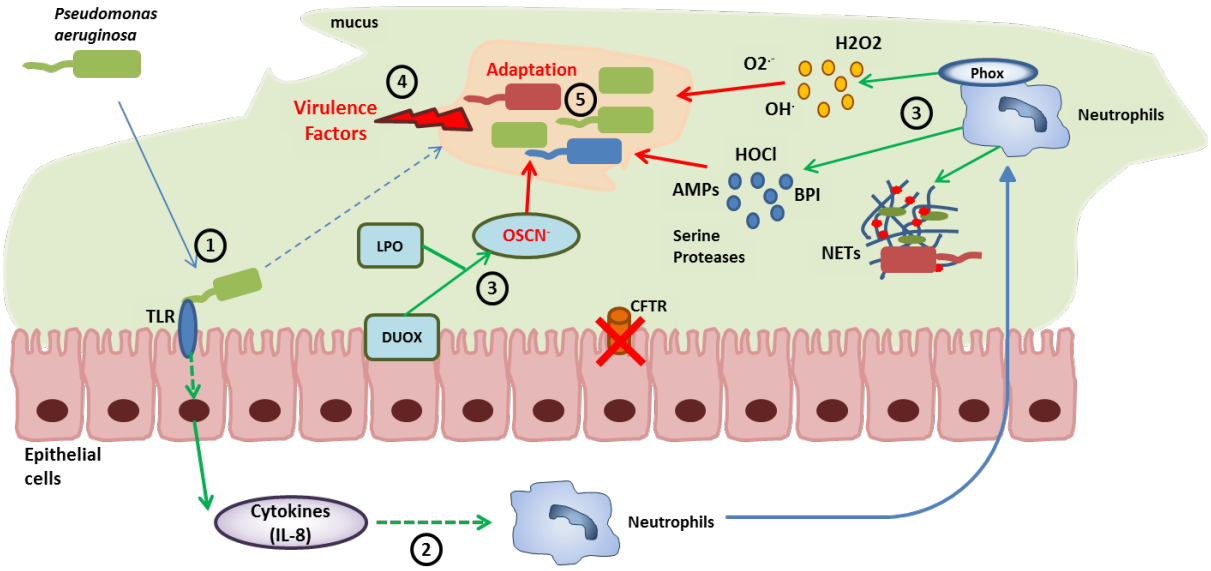


Figure 1-4: The innate immune response to Pseudomonas aeruginosa in the cystic fibrosis airway epithelium. 1. Initial colonisation of *P. aeruginosa* and recognition of the infection by the Toll-Like Receptors (TLR) of the epithelial cells **2**. Production of cytokines by the epithelial cells and the alveolar macrophages, in response to the presence of the bacteria, leading to the recruitment and influx of neutrophils to the epithelial airway surface. **3.** The innate immune system tries to eradicate the infection. The epithelial cells produce hypothiocyanate (OSCN-) through the DUOX/LPO system while the neutrophils excrete many antimicrobial molecules such as reactive oxygen species through the phagocytic oxidase (Phox), antimicrobial peptides (AMPs), bactericidal/permeability-increasing proteins (BPI), etc. The neutrophils also use the cell death program called NETosis to produce Neutrophil extracellular traps (NETs) that bind and try to kill the bacteria. The failure to eradicate the bacteria leads to inflammation of the airway leading to long-term damage. **4**. The bacteria may evade the innate system through the production of virulence factors such as pyocyanin, proteases, hydrogen cyanide and rhamnolipids but also protect themselves through the production of biofilm aggregates. **5.** Due to the environmental pressure from the lung environment and the innate immune system, the bacteria evolve over time leading to adaptations such as mucoidy through the production of alginate, the decrease of production of virulence factors, the loss of the flagella.

1.2.4 Eradication therapy and exacerbations

Chronic infections by *P. aeruginosa* are associated with worse clinical outcome, making early eradication treatments crucial to help the immune system's attempt to suppress early infections (Emerson et al., 2002). These eradication treatments involve aggressive use of antibiotics and have been used for decades and have become standard care (Frederiksen et al., 1997, Hewer and Smyth, 2017). Drugs are usually inhaled +/- oral; the intravenous (IV) approach is sometimes used, but was recently demonstrated as non-superior in a UK randomised controlled trial (Hewer et al., 2020). Many studies have reported varied efficacy of these eradication treatments, which could be partly linked to the differences in treatment procedure and patient population (Emiralioglu et al., 2016). Studies report that eradication treatments have an efficacy of around 70-80% but reinfection rate and recrudescence of only partly suppressed infections over long term follow up remain high (Van Stormbroek et al., 2019, Cohen-Cymberknoh et al., 2016, Ratjen et al., 2010). A recurrent finding amongst studies was the association between older age and higher failure rate of eradication (Van Stormbroek et al., 2019, Cohen-Cymberknoh et al., 2016). Nonetheless, some studies have found reinfections occurred for different *P. aeruginosa* genotypes suggesting eradication success preceding a new infection (Taccetti et al., 2005).

These eradication treatments are associated with slower decline in lung function and delay in the onset of chronic stages of infections leading to better clinical outcome (Blanchard et al., 2017, Frederiksen et al., 1997). Conversely, failure to eradicate *P. aeruginosa* might in fact lead to the bacterial community being modified as well as selecting for resistant strains (Tueffers et al., 2019, Fernandez-Barat et al., 2017). Although the factors affecting the success of the treatment are unclear, some studies found that *P. aeruginosa* exhibiting phenotypes associated with chronic infections, such as mucoidy, lack of motility and deficiency in quorum sensing production, were linked to eradication failure (Mayer-Hamblett et al., 2014a, Vidya et al., 2016). Mayer-Hamblett et al. found that failure to eradicate increases chances of patients having exacerbations in the future while successful eradication reduced the risk of future exacerbations (Mayer-Hamblett et al., 2012, Mayer-Hamblett et al., 2018).

Pulmonary exacerbation is the term used for periodic episodes of deterioration in patient symptoms; there is not one single clinical definition and the underlying mechanisms leading to

this state are still unclear (Goss and Burns, 2007). The acute symptoms during exacerbations have long term effects as patients do not always recover previous lung function; thus, exacerbations accelerate long term lung deterioration and worsen clinical symptoms (Sanders et al., 2011, Sanders et al., 2010, de Boer et al., 2011, Amadori et al., 2009). Waters et al. demonstrated that patients that had more regular exacerbations, especially with less than 6 months between them, saw worse lung function decline (Waters et al., 2012).

The treatment of exacerbations, most commonly with IV or oral antibiotics, has been extensively studied and had shown a varied amount of success (Heltshe et al., 2018, Hurley et al., 2015, Parkins et al., 2012). Parkins et al. found that a quarter of patients with exacerbations failed to see improvement with initial treatment (Parkins et al., 2012). This might partially be explained by the fact that eradication treatments have been found to have higher rate of failure in chronically infected patients who also tend to suffer more exacerbations (Vidya et al., 2016, Parkins et al., 2012, Foweraker et al., 2005). Although antibiotics, mostly anti-pseudomonals, are the standard of care to resolve exacerbations, many studies have questioned the effect on *P. aeruginosa*. While some studies found the density of *P. aeruginosa* decreases marginally in some patients, most studies suggest there is no difference in bacterial load before and after exacerbations (Tunney et al., 2011, Sibley et al., 2008, Workentine et al., 2013, Deschaght et al., 2013).

The cause of exacerbations remains unclear, especially regarding the role of *P. aeruginosa* in the onset of these. One study suggested the acquisition of new strains of *P. aeruginosa* was not found to be the probable cause of exacerbations with no significant difference in antibiotic susceptibility for the strains typed previously to the exacerbation (Aaron et al., 2004b). One hypothesis has been that exacerbations are due to an increase in virulence factor production from *P. aeruginosa*. A historic study showed higher exoenzyme (or protease) production from *P. aeruginosa* in patients experiencing pulmonary exacerbations and a decrease following antibiotic treatment (Grimwood et al., 1993). Several studies have since found associations between exacerbation periods and phenotypic changes in *P. aeruginosa* such as increase in pyocyanin production (Fothergill et al., 2010, Mowat et al., 2011). Other studies did not, on the other hand, find any associations with virulence factors but only with colony size (Manos et al., 2013, Workentine et al., 2013). These differences in findings could be explained by the fact the studies finding associations between exacerbations and changes in virulence factors

were using epidemic strains. Indeed, epidemic strains have been found to be responsible for more hospitalisations and exacerbations (Parkins et al., 2018, Jones et al., 2002).

Another hypothesis has been that changes in the CF microbiome could be the cause of exacerbations. Nonetheless, several studies have found that there were no clear changes in microbial community composition associated with periods of exacerbation (Fodor et al., 2012, Whelan et al., 2017, Price et al., 2013, Cuthbertson et al., 2015). Finally, a few studies have suggested the involvement of viruses in exacerbations, potentially through interactions with bacteria or their biofilms (Burns et al., 2012, Kiedrowski and Bomberger, 2018).

Both Fodor et al., and Whelan and Surette suggest an interesting alternative hypothesis where exacerbations may reflect a spread of infection to previously unaffected areas of the lungs rather than changes in microbial composition and density (Fodor et al., 2012, Whelan and Surette, 2015). This new expansion of infection causes inflammation and the onset of exacerbation symptoms. This could also explain the failure to regain pre-exacerbation lung function. Whelan and Surette suggest this spread of infection could be due to small active subpopulation (Whelan and Surette, 2015).

1.2.5 *P. aeruginosa* pathogenesis and virulence factors

Although the innate immune defences are weakened in CF lungs, *P. aeruginosa* still needs to evade and fight back. *P. aeruginosa* produces a multitude of virulence factors to survive and defend itself against the attacks of the host defence (Lyczak et al., 2000).

P. aeruginosa in turn attacks the host through the production of multiple virulence factors such as pyocyanin, rhamnolipids, hydrogen cyanide (HCN), siderophores (pyoverdine and pyochelin), elastase and alkaline protease (Williams and Davies, 2012, Van Delden and Iglewski, 1998, Gallagher and Manoil, 2001).

Pyocyanin

Pyocyanin is a green-blue phenazine, giving some *P. aeruginosa* colonies a characteristic colour. It has been shown to be critical in helping the bacteria establish infection (Lau et al., 2004). This virulence factor is derived from quinolone pathway, which also synthesises

quorum-sensing molecules used to regulate virulence in *P. aeruginosa*. Pyocyanin is specifically synthesised by the *phz* operons, and *phzM* and *phzS* catalyse the last two steps of the synthesis. A phzM mutant was found to decrease infection levels in chronic acute mouse models by 10 000 fold (Lau et al., 2004b).

This redox phenazine has a direct role in virulence through oxidative stress (Allen et al., 2005, Arora et al., 2018). It is able to oxidise NADH into NAD, and in cells produces reactive oxygen species (ROS) in the presence of oxygen (Lau et al., 2004a, Hall et al., 2016). These ROS are mainly superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Hall et al., 2016). These ROS lead to pro-inflammatory responses causing damage to the host cells (Allen et al., 2005). The effects of oxidative stress mediated killing has been studied for decades, and was first shown in models such as *C. elegans (Mahajan-Miklos et al., 1999)*. Pyocyanin can cross cell membrane and enter the epithelial cells in which it produces ROS (Arora et al., 2018). As well as epithelial cells, it can also induce neutrophil death (Manago et al., 2015). By lysing cells pyocyanin allows the release of extracellular DNA, which is beneficial for the formation of biofilms (Das and Manefield, 2012). The presence of pyocyanin also impairs phagocytosis of apoptotic cells leading to the accumulation of cell debris (Bianchi et al., 2008). It can also cause increased levels of cytokines (IL-8) (Rada et al., 2011, Denning et al., 1998), and through inducing the release of cytokines, pyocyanin promotes the release of mucins. The release of extracellular DNA and mucins leads to further increase in viscosity of the mucus. By also being able to slow down cilia beating, it participates in hindering the clearance of the bacteria by the host (Munro et al., 1989).

Hydrogen cyanide

P. aeruginosa is one of the few bacteria able to produce this toxic gas. HCN is synthesised from glycine through the catalysis by the HCN synthase enzyme, which is encoded by the *hcnABC* operon (Anderson et al., 2010, Blumer and Haas, 2000). The synthesis is tightly controlled by several regulatory systems, including the QS system, and the mutation of these regulators hinders the production of HCN (Pessi and Haas, 2000, Pessi and Haas, 2001). HCN inhibits aerobic respiration through its affinity to and inhibition of the cytochrome c oxidase, which is the terminal complex of the respiratory chain (Blumer and Haas, 2000). To protect itself from the toxic effects of the gas Pa it has developed protective mechanisms, such as the addition of a terminal complex to its respiratory chain, the cyanide insensitive oxidase (CIO)

(Williams et al., 2006). It is also able to convert cyanide to thiocyanate by the rhodanese sulfurtransferase (Williams et al., 2006).

The role of this gas in infection is unclear (Blumer and Haas, 2000, Leavesley et al., 2008). It has been shown to be lethal in *C. elegans* models and toxic for Drosophila melanogaster (Gallagher and Manoil, 2001, Broderick et al., 2008). However, a study has demonstrated that HCN inhibits ciliary beating when in contact with epithelial cells, with a reversible effect (Nair et al., 2014a). This study suggests that HCN may further impair MCC, thus limiting the natural elimination of sputum and bacteria and allowing their persistence in the lung.

HCN can be detected in exhaled breath of infected CF patients (Smith et al., 2013, Gilchrist et al., 2013, Gilchrist et al., 2015). It has also been detected in CF patients' sputum and its presence is associated with a decrease in lung function, further suggesting its role as a virulence factor (Sanderson et al., 2008, Ryall et al., 2008).

Proteases

As well as the host cells, *P. aeruginosa* also produces proteases, such as alkaline protease (APR) and elastase LasA and LasB. APR is synthesised by a 5 gene operon : *aprA* encoding for APR, *aprI* encoding an inhibitor, and *aprD*, *aprE* and *aprF* involved in the secretion of the protein (Duong et al., 1992).

Proteases hinder the innate immune system and damage host cells (Saint-Criq et al., 2018, Döring et al., 1985). Several studies have found that elastase degrades certain AMPs such as SP-A and SP-D (Mariencheck et al., 2003, Alcorn and Wright, 2004, Kuang et al., 2011). The degradation of these proteins enables the bacteria to avoid surfactant protein-mediated opsonisation and therefore phagocytosis (Kuang et al., 2011). *P. aeruginosa* also evades phagocytosis through the production of Apr of LasB, which is able to block complement activation (Laarman et al., 2012, Bastaert et al., 2018). LasB can also down regulate different cytokine pathways by degrading them, a process which can alter the immune response and inhibit host cell repair (Saint-Criq et al., 2018, Bastaert et al., 2018)

Pyoverdine

Pyoverdine is a green fluorescent siderophore produced by *P. aeruginosa* to help the bacterium survive in the lung by chelating iron present in the mucus (Cornelis and Dingemans, 2013, Cox

and Adams, 1985). The synthesis of pyoverdine is complex but four main genes are involved: *pvdL*, *pvdI*, *pvdJ* and *pvdD* (Visca et al., 2007). A tight iron homeostasis limits the iron availability in the lungs although it has been found that in the CF lung there are higher levels due to inflammation (Ghio et al., 2013, Stites et al., 1998). However, due to the presence of host iron sequestration molecules, such as lactoferrin or transferrin, it is vital for the bacteria to compete in chelating iron in order to survive in this environment (Tyrrell and Callaghan, 2016). Most of the iron is present under ferric form (Fe3+) to which pyoverdine can bind. Once bound to ferric iron, the complex is uptaken back into the bacterial cells through the FpvA transporter and pyoverdine is then recycled and excreted again by efflux pumps (Cézard et al., 2015). *P. aeruginosa* can also take up iron in haem form from hemoproteins or scavenge siderophores from other bacterial species (Cornelis and Dingemans, 2013, Tyrrell and Callaghan, 2016).

Pyoverdine also plays a virulence role as a study found that a pyoverdine mutant had decreased virulence and led to lower mortality in *C. elegans* and murine models (Minandri et al., 2016, Kang et al., 2019). Several studies have also shown the participation of pyoverdine and iron availability in the regulation of biofilms (Greenberg and Banin, 2008, Berlutti et al., 2005).

Biofilm

In the CF lung *P. aeruginosa* is known to demonstrate a biofilm mode of growth, a mechanism used by many bacteria to protect themselves from their environments. Biofilms are formed in several steps starting with the motile bacteria attaching themselves onto a surface. The first phase is a reversible attachment which then becomes irreversible. Studies suggest, however, that this attachment does not happen in the CF lung (Sriramulu et al., 2005). The biofilm complex then matures through the production of a matrix. This matrix is composed of exopolysaccharides (alginate, Psl, Pel), DNA and proteins (Costerton et al., 1995, Ryder et al., 2007).

Biofilm production enables the bacteria to persist in the lung by protecting themselves through forming a physical barrier to antibiotics and the immune system (Borriello et al., 2004). A study found that the presence of neutrophils enhanced the production of biofilm, strengthening the idea that bacteria use biofilm mode of growth to shield from the host defence cells (Walker et al., 2005). A number of studies have shown biofilms have a higher tolerance to antibiotics than planktonic organisms (Ciofu and Tolker-Nielsen, 2019, Stewart, 2015, Hoiby et al., 2010,

Macia et al., 2014). The oxygen limitation within the biofilms gives protection against anti-bacterial molecules by limiting their efficacy (Borriello et al., 2004).

A study showed that in vitro mucins can serve as attachment surface for bacteria to form biofilm aggregate through adhesion-mucin interaction, which also changed the structure of the biofilm making it more tolerant to tobramycin (Landry et al., 2006). However, a study in 2014 showed that mucins can excessively enhance twitching motility, which hinders the formation of surface attached biofilms (Haley et al., 2014). Interactions between the bacteria and the ASL components remain unclear, but this effect of mucins could further emphasise the potential biofilm aggregate growth in the CF lung. Several studies have suggested that, unlike surface attached biofilm within the thickened ASL, *P. aeruginosa* forms biofilm aggregates (Staudinger et al., 2014b, Sriramulu et al., 2005). These microcolony clusters have similar properties to surface attached biofilm but are embedded in the ASL mucus (Sriramulu et al., 2005).

The presence of biofilm can be a cause of more severe lung damage, as these structures induce hyper inflammation (Ciszek-Lenda et al., 2019). This underlines how lung damage is a vicious circle where bacteria protect themselves, in turn causing sustained inflammation and lung health degradation.

1.2.6 Quorum sensing regulation of virulence

The production of virulence factors is controlled by multiple interconnected regulation systems (Jimenez et al., 2012). The quorum sensing (QS) system is the most important in the regulation of production of these virulence factors.

The QS systems allow the bacteria from a community to communicate and coordinate the expression of genes. At least four quorum sensing systems co-regulate the virulence in *P. aeruginosa*, the Las, Rhl, Pseudomonas Quinolone Signal (PQS) systems and the newly discovered integrating quorum sensing system (IQS) (Dubern and Diggle, 2008, Lee and Zhang, 2015). Discovery of these different systems was progressive with the Las and Rhl systems discovered in the 1990s, the PQS system in 1999 and in 2013 the IQS system (Toder et al., 1991, Gambello and Iglewski, 1991, Pearson et al., 1995, Pesci et al., 1999, Lee et al., 2013).

All three main systems Las, Rhl and PQS are composed of a regulator and an inducer leading to the production of an autoinducer molecule. The autoinducers for these three systems are of two types, N-acyl homoserine lactones and quinolones. The autoinducer for the Las is N-3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-AHL), for the Rhl system it is N-butanoyl-homoserine lactone (C4-HSL), and 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) for the PQS system.
For all 3 systems the transcriptional regulators induce the production of the autoinducer signal and the subsequent complex between regulators and autoinducers in turn induces the regulation of other genes (Lee and Zhang, 2015). The complexes can activate their own expression through a feedback loop, as well as regulating the expression of different systems and virulence factors synthesis genes (Figure 1-3).

The Las system is at the top of the QS hierarchy system. The LasR-3-oxo-C12-AHL complex can activate both the Rhl and PQS systems. Similarly the PQS system can positively regulate the Rhl system, while RhlR-C4-HSL inhibits the expression of PQS (Cao et al., 2001, Gallagher et al., 2002). This QS network is itself also subject to alteration of its expression through the control of several other regulators, which can modulate the QS system (Jimenez et al., 2012, Williams and Camara, 2009).

The QS systems regulate directly or indirectly a wide range of genes, as around 10% of the *P. aeruginosa* genome was found to be potentially under AHL regulation (de Kievit, 2009). Another study, this time of the *P. aeruginosa* proteome, revealed that more than 20% of the proteome differed by at least 2.5 fold change in double AHLs mutants (Arevalo-Ferro et al., 2003). All the virulence factors and biofilm production cited previously are under the regulation of at least one or several of these QS systems (Figure 1-5).

The QS systems are activated under a number of environmental stresses such as phosphate-depletion, starvation, limited oxygen (Lee and Zhang, 2015). The QS regulation is also activated in response to components of the host immune system such as cytokines or antimicrobial proteins (Wu et al., 2005, Strempel et al., 2013). On the other hand, the autoinducer signals can also directly have effects on the host immune response such as the activation of cytokines (DiMango et al., 1995, Smith et al., 2001, Smith et al., 2002).

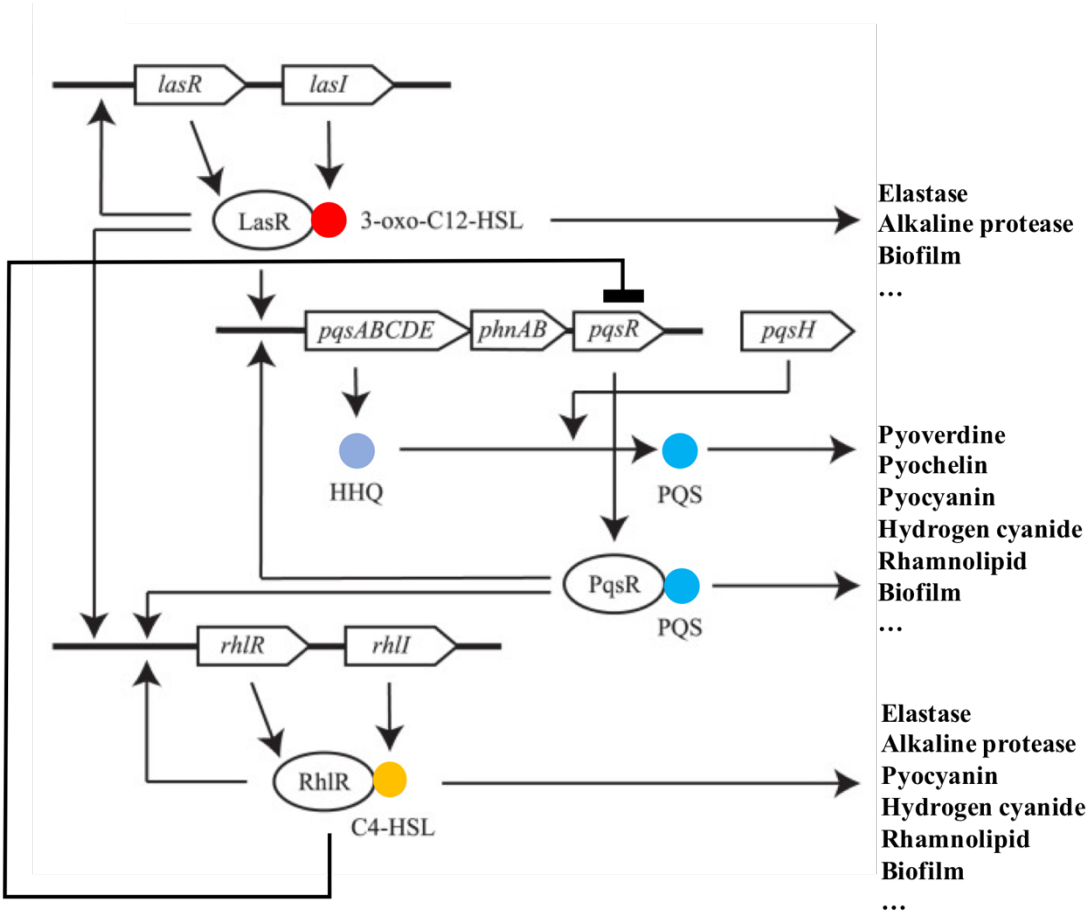


Figure 1-5: Network of Las, Rhl and PQS quorum sensing regulation systems. LasR is the global regulator, which can impact the regulation of both the rhl and pqs genes. The PQS regulator PqsR can also positively regulate the rhl system, while this rhl system negatively regulates the PQS one. These three systems possess a feedback loop on their own genes. All three systems promote the multiple virulence factors produced by *P. aeruginosa* as well as regulate biofilm growth. Image taken from Tashiro et al., and modified (Tashiro et al., 2013).

Multiple other systems are also involved in the regulation of the virulence factor, through interactions and modulation of the QS systems or independently (Jimenez et al., 2012, Moradali et al., 2017). These systems include for example a multitude of two-component systems and other nucleotide based signals (Jimenez et al., 2012). The GacA/GacS two-component system with the RsmA post-transcription factor is for example involved in the regulation of HCN and pyocyanin through the inhibition of RhlI (Reimmann et al., 1997). The nucleotide based signal ci-di-GMP is important in the regulation of biofilm production (Valentini and Filloux, 2016).

1.3 Adaptation to the CF lung

The CF lung is altered during infection due to sustained inflammation, to changes in microbiota, to long-term antibiotic treatment and to tissue damage (Hauser et al., 2011). Therefore, the CF lung is a highly complex and heterogeneous environment exerting variable selective pressures on the infecting bacteria.

1.3.1 Drivers of adaptation

Any bacteria colonising a novel environment will have to adapt to it to survive and thrive. In comparison to natural environments from which *P. aeruginosa* often originates, the CF lung environment is defined by specific characteristics.

The first specificities of this environment are directly linked to the lack of CFTR function and its effect on modifying the airway mucus and ASL. In fact, the CF lung presents several physical properties which can provide selective pressures to *P. aeruginosa*. These physical specificities comprise acidic ASL, high viscosity and shear stress. (Staudinger et al., 2014b, Dingemans et al., 2016, Tang et al., 2016). Another consequence of the thick layer of mucus is that the CF airway becomes a hypoxic environment to which the *P. aeruginosa* will have to adapt (Arai, 2011, Eichner et al., 2014). *P. aeruginosa* is very versatile and can use different metabolic pathways for either aerobic, micro-aerobic or anaerobic respiration (Arai, 2011, Alvarez-Ortega and Harwood, 2007). Secondly, the lung environment shortage of some nutrients and abundance of others is another selective pressure leading to modification in metabolic pathways (Schick and Kassen, 2018, Palmer et al., 2007). Indeed, many metabolic changes occur during *P. aeruginosa* adaptation to the CF lung (La Rosa et al., 2019, Behrends et al., 2013).

Besides adapting to the CF lung's physical and chemical properties, *P. aeruginosa* will also adapt in response to interactions with immune cells and other microorganisms. *P. aeruginosa* will change in response to attacks from the immune system to evade the host's response to infection (Malhotra et al., 2019, Amiel et al., 2010). Moreover, interactions with other microorganism can lead to adaptation of *P. aeruginosa* to establish itself in the CF lung. Interspecies communication and exploitation can indeed shape *P. aeruginosa*'s adaptation

(O'Brien and Fothergill, 2017, Sibley et al., 2008, Duan et al., 2003). *P. aeruginosa* has been found to modulate gene expression in the presence of other bacterial species (Duan et al., 2003, O'Brien and Fothergill, 2017). Viruses such as bacteriophages have also been found to be responsible for the shaping of *P. aeruginosa*'s genetic adaptation in the CF lung through horizontal gene transfer (O'Brien et al., 2019, Qiu et al., 2009).

Finally, on top of intrinsic characteristics of the CF lung environment, external factors such as antibiotic treatment used for early eradication or to resolve exacerbations as discussed previously, can also be important drivers of adaptation of *P. aeruginosa* (Wright et al., 2013).

1.3.2 Genotypic and phenotypic adaptation over length of infection

During the early stages of infection, *P. aeruginosa* uses its virulence factors to fight back the host defence mechanisms. This acute stage of infection is not sustainable as it leads to damage of the CF lung environment and cannot be a long-term strategy for the bacteria. In order to establish chronic infection of the CF lung, *P. aeruginosa* goes through extensive adaptation, certain mechanisms of which remain unclear as does its extent.

P. aeruginosa is a highly adaptable bacterium with a high genome plasticity, and is able to alter the regulation of its genes dependent on the environment (Mathee et al., 2008). A study found that 3% of genes were regulated differently in a CF-like medium in comparison to a regular laboratory growth medium (Palmer et al., 2005). Many studies have, therefore, investigated the genotypic and phenotypic changes that occur during the establishment of chronic infection in the CF lung.

In 2013, Behrends used untargeted metabolic NMR profiling of growth supernatants to compare the metabolic footprinting of a set of longitudinally acquired isolates. Over the course of infection. *P. aeruginosa* isolates seemed to more efficiently take up amino acids having a higher biosynthetic cost of production for the bacteria, proving a reduction in metabolism (Behrends et al., 2013). This emergence of auxotrophy is a common metabolic adaptation observed for *P. aeruginosa* to limit its energy expenditure (Hogardt and Heesemann, 2010, Hoboth et al., 2009, Marvig et al., 2015a, Barth and Pitt, 1996).

A number of longitudinal studies have sequenced several isolates from patients over years and observed the mutations that appear in chronic stages of infection (Smith et al., 2006, Bragonzi et al., 2009, Marvig et al., 2013, Marvig et al., 2015b, Feliziani et al., 2014). All these studies have found similar mutations in genes involving regulation including QS, virulence factors, mucoidy, motility, antimicrobial resistance and hypermutability. In a big study, Marvig et al. sequenced the genome for 474 isolates over 36 lineages and showed similar mutations over time in 52 genes, thus confirming common paths of adaptation (Marvig et al., 2015b). These 52 genes were involved antibiotic resistance, motility, DNA replication and repair systems, cell wall molecule synthesis, transcriptional regulators and virulence factors.

Some strains of *P. aeruginosa* develop a higher rate of mutation due to mutation in genes responsible in the mismatch repair system. It has been thought that there is an emergence of hypermutable strains in chronic stage of infection, which help the bacteria adapt (Mena et al., 2008). Mutations in genes involved in the mismatch repair system (*mutS*, *mutL*, *mutY)* have been observed (Marvig et al., 2013, Oliver et al., 2000). It has, however, been shown that hypermutability is also found in environmental strains and, on the other hand, is not more prevalent in epidemic strains (Kenna et al., 2007).

1.3.3 Quorum sensing mutations and virulence decrease

One of the major adaptations to the CF lung involves the appearance of regulatory mutants alongside an overall decrease in virulence. Mutations in sigma factors, as well as common mutations of *lasR* and *rpoN*, have been observed (Marvig et al., 2015b, Jorgensen et al., 2015, Feliziani et al., 2014). In an evolution experiment in *C. elegans* model, the appearance of *lasR* mutations was also observed (Jansen et al., 2015). A previous study showed that the *lasR* mutation brought a growth advantage in media rich in amino acids (D'Argenio et al., 2007). This again shows, alongside auxotrophy, an adaptation to the lung environment which is rich in amino acids from cell debris and proteins.

Mutations in these regulatory genes will have an important effect on virulence due to their pleiotropic effect.

This decrease in virulence has been observed in numerous studies, as in a study showing lower virulence of *P. aeruginosa* CF isolates than burn strains in a murine model (Luzar and Montie, 1985). Another study compared general virulence between CF isolates and environmental ones,

and found that CF strains demonstrate decreased virulence in a *G. mellonella* model (Cullen et al., 2015). In several non-mammalian and a murine model, chronic CF isolates were found to show reduced virulence in comparison to early strains (Lore et al., 2012). Several in vitro studies show the same trends regarding the production of specific virulence factors (Rau et al., 2010, Hu et al., 2013).

Also the high mutation frequency in pyoverdine genes as well as its receptor FpvA has been linked to the change in iron uptake mechanism over the course of infection (Tyrrell and Callaghan, 2016, Andersen et al., 2015). Studies have shown that in chronic infection *P. aeruginosa* starts preferentially using the iron in haem form found in the CF lung, using other systems like Has and Phu (Marvig et al., 2014, Konings et al., 2013).

A very large study on 649 children, followed over more than 5 years, observed a decrease in virulence between early, intermittent and chronic stages of infection (Mayer-Hamblett et al., 2014b). The phenotypes changing the most and best distinguishing the stage of infection were pyoverdine and protease production, while pyocyanin was the phenotype most produced in early stages. The phenotypic observation of virulence enables the study of large cohorts of patients.

All these studies suggest that the establishment of chronic infection goes hand in hand with decrease of virulence, with the bacterial aim of surviving long term undetected and evading the host defence.

Mucoidy

Mucoidy is a very common adaptation of *P. aeruginosa* during chronic infection, which bacteria use as a strategy to shield themselves from antimicrobials. This mucoid phenotype is characterised by the production of a specific polysaccharide, alginate (Deretic et al., 1993). This morphological adaptation occurs following the mutation in the anti-sigma factor MucA, which normally sequesters the sigma factor AlgT/U (Deretic et al., 1993). The mutations in *mucA* hinder its sequestration of ALgT/U, which is free to bind to the RNA polymerase and leads to the production of alginate. Like the biofilm matrix, alginate represents a physical barrier from the immune system and antimicrobials. Reversal of mucoidy can happen frequently in in vitro settings as producing alginate is energy intense for the bacteria (Schurr et al., 1994, Govan, 1975). This reversal is due to second-site mutations that have been identified in other genes of the pathway (Delgado et al., 2018, DeVries and Ohman, 1994).

Thus, the extent of mucoidy in vivo could be underestimated in vitro as the phenotype is lost on serial transfer on solid media (Govan, 1975).

The presence of mucoidy bacteria in biofilms was found to further increase their tolerance to antibiotics, increasing efficacy to protect and making biofilms better suited to the CF lung environment. The same study also demonstrated that biofilms of mucoid phenotype bacteria show microcolony structures (Hentzer et al., 2001). The increase in alginate production from chronic *P. aeruginosa* could thus further explain the aggregate type biofilm in CF lungs (Staudinger et al., 2014b, Hentzer et al., 2001). The added antibacterial tolerance could be caused by the alginate increasing viscoelasticity of the biofilm, which has been shown to cause higher antibiotic resistance (Rozenbaum et al., 2019).

The pathway involved in mucoidy seems to be involved also in the down-regulation of some of the virulence factors during the chronic stage of infection in the CF lung (Ryall et al., 2014). The mutation in *mucA* that causes mucoidy also influences the production of pyocyanin or HCN through effects on the quorum sensing systems. This study shows that mutations other than the common *lasR* mutation also have a role in the decrease in virulence during chronic stages of infection (Ryall et al., 2014).

Motile switch to a sessile lifestyle

P. aeruginosa is able to use three types of motility to colonise the lungs during acute infection. Swimming motility enables the bacteria to glide through a low viscosity environment using the rotation of the flagella (Doyle et al., 2004). Swarming motility involves the use of both the flagella but also the type IV pili (Kohler et al., 2000). Swarming motility is coupled with the production of rhamnolipids which are used as biosurfactants in which the bacteria glide (Deziel et al., 2003). Finally, twitching motility is dependent on the extension and retraction of type IV pili and allows the bacteria to pull itself across a surface (Burrows, 2012).

Motility is necessary for the initial colonisation of the lung and helps the formation of biofilms, which in turn aid persistence within the CF lung (O'Toole and Kolter, 1998, Feldman et al., 1998). However, both the flagella and type IV pili are PAMPS and induce phagocytosis and NET formation (Sonawane et al., 2006, Feldman et al., 1998). Thus, the switch into a sessile biofilm lifestyle to better evade the host defence has been commonly described (Wolfgang et al., 2004).

Nearly 30 year ago a study found that whereas early CF isolates from acute infection showed the same phenotype as environmental strains, chronic isolates were typically non-motile (Mahenthiralingam et al., 1994). Many other studies have subsequently also shown this decrease in motility in isolates during chronic CF infection (Mayer-Hamblett et al., 2014b, Marvig et al., 2015a, Marvig et al., 2015b).

Antibacterial resistance

Although biofilm growth and mucoidy act as physical barriers against antibacterial molecules, *P. aeruginosa* possesses intrinsic resistance to antibiotics. But most troubling is its ability to acquire new mechanisms during chronic infection triggered by courses of antibiotics (Dötsch et al., 2009, Strateva and Yordanov, 2009). These mechanisms of antibiotic resistance involve efflux pumps, changes to the membrane and its permeability, inactivation and genetic resistance through mutations or transfer of plasmids (Breidenstein et al., 2011, Moore and Flaws, 2011, Kapoor et al., 2017). Many studies have observed this increase in antibiotic resistance through the mutation of genes involving all mechanisms, for example in the gene for the efflux pump *mexB* (Breidenstein et al., 2011, Marvig et al., 2015a, Cramer et al., 2011, Bragonzi et al., 2009, Smith et al., 2006). Darch et al. showed recombination between isolates was linked to an increase in antimicrobial resistance (Darch et al., 2015). Another study showed that just a two-week course of antibiotics had increased bacterial antibiotic resistance (Fernandez-Barat et al., 2017).

Importance of phenotypic observation of adaptation

A study has described a different mechanism of motility loss involving the elastase LasB and APR degrading flagellin protein which composes the filament of the flagella (Casilag et al., 2015). This shows that although motility genes can still be expressed, the final phenotype of the bacteria will be non-motile.

A further study showed that even a quadruple QS knockout strain had the same capability for infection as wild-type, underlining complex regulation of virulence due to polygenic regulation (Lazenby et al., 2013). This was also found in another study where virulence was not correlated to the presence of LasR mutants (Lelong et al., 2011).

Although genotype brings valuable information on the adaptation of the bacteria, it does not lead, on its own, to the full understanding of the phenotypic changes occurring during the infection. Phenotypes are polygenic, often being determined by the action of many genes in concert. It is therefore also crucial to investigate adaptation on a phenotypic level, as several studies suggest that in some cases genetic changes do not correlate to phenotypic changes due to the complex expression of multiple genes, which lead to a single phenotype (Workentine et al., 2013). The multiplicity of complex regulation systems makes predicting the phenotypic outcome impossible.

Population diversity of *P. aeruginosa*

Many studies focus mainly on longitudinal samples over time of one isolate from one or several patients (Mena et al., 2008, Bragonzi et al., 2009). As discussed previously these were beneficial to understand the common adaptive mechanisms in the lung. Typically, as seen in a study from Bragonzi et al, one isolate from each of six patients was collected several times over the course of a year, or in one individual over 32 years for the study led by Markussen (Bragonzi et al., 2009, Markussen et al., 2014). These studies do not seek to assess within-patient diversity but rather the evolution over time of only one example of strain. This is not then a snapshot of the real diversity found in an individual at a certain point in time.

Nguyen et al., concluded in their 2006 study how surprised they were to have discovered several lineages of the same initial isolate within a single patient (Nguyen and Singh, 2006). This study mentions another, suggesting that this diversity could be due to it having an "insurance effect" enabling bacteria to survive through a wider set of conditions (Yachi and Loreau, 1999).

Since then, there has been a realisation of the coexistence of evolved isolates from the same initial strains, which can nonetheless present unique adaptations and different phenotypes. The importance of investigating the diversity within the lungs of individual patients has become apparent.

Recently several studies have found that the genotypic evolution leads to the coexistence of several lineages within individuals, through genetic transfers by homologous recombination (Darch et al., 2015, Williams et al., 2015, Waine et al., 2009). Nevertheless, this recombination

phenomenon between strains in the CF lung is controversial, some suggesting its importance while others disputing this and claiming that it is only a minor source of genotypic adaptation (Williams et al., 2016b). There is, however, a consensus on genetic adaptation through adaptive radiation caused by the various pressures from the multiple niches within the lung, such as differences in microbiome, nutrients and the presence of a gradient of oxygen between the top and bottom lobes of this organ (Williams et al., 2006, Palmer et al., 2007).

Several studies have highlighted the regional differences within the lungs, representing potential different niches for bacterial adaptation. A study showed with CT-scans that the severity of lung disease was patchy (Gurney et al., 1997). Another study used multiple bronchoalveolar lavage on over 33 children to observe the extent of differences between the two lobes (Gutierrez et al., 2001). Lobes were found to be different in bacterial loads and cytokine production. Significant bacterial presence was found in a portion of children in only one of the lobes. This study suggested heterogeneous bacterial presence in different niches of the lung.

A very thorough paper showed evidence of distinct adaptation patterns in various niches within the lung. Jorth et al. assessed 10 pairs of explanted lungs, with 1200 isolates collected for each pair (Jorth et al., 2015). Isolates were isolated for each of the lungs in three sites (upper, middle and lower lobes), some which were severely and others only mildly damaged. Firstly, all pairs of lungs were found to be largely infected by single lineages. The study found regional phenotypic, proteomic and genetic diversity, with the presence of 12 to 40 phenotypic subpopulations by lung. The proteome analysis showed that around 20% of proteins were differentially expressed between regions for a subset of 3 lungs. Although difference was observed between lobes, lower diversity was also found within each region, with one to three subpopulations. Overall obvious phenotypic diversity was present regarding nutritional uptakes, virulence and antibiotic resistance. Moreover, bacteria found in more damaged parts of the lungs were more cytotoxic to host defence cells and more virulent. Finally, the study did not find any consistent pattern between patients regarding a particular region of the lung, underlining the specific adaptation occurring in each individual patient. According to the study there is little mixing between the different niches of the lung and the regional bacterial communities evolved independently (Jorth et al., 2015). This suggests that compartmentalisation of the bacteria in different niches of the lung is a driver for divergent evolution, resulting in important diversity.

Schick et al. defend the idea in their study that adaptation and diversification within a niche is dependent on the nutritional complexity present (Schick and Kassen, 2018). They found in an in vitro evolutionary experiment that *P. aeruginosa* grown in a CF mimicking medium (Synthetic Cystic Fibrosis Medium or SCFM) demonstrated within-population diversity that was higher than when grown in simpler media. This could be due to competition for resources, thus generating a selection pressure.

Alongside this very complete study on transplanted lungs and in vitro evolution, a number of other studies have examined within-patient diversity in vitro from the collection of clinical strains. Several started investigating diversity within a very limited number of patients.

The first study investigating the diversity amongst patient isolates showed the presence of heterogeneity in virulence phenotypes and also of QS mutants within patients, ranging from 0 to almost 90% over the 8 patient samples investigated (Wilder et al., 2009).

In 2015 Clark et al. phenotypically studied one patient over a year, collecting multiple isolates per sample with a total of 235 isolates (Clark et al., 2015). Overall a high within sample polymorphism was observed over all 34 phenotypes tested, with no combination of phenotypes being found to be predominant. They also found colony morphology was not a good predictor of any other phenotype. Fothergill et al. had also previously found diversity in morphology, and that isolates with a similar morphotype still showed diversity regarding antibiotic resistance patterns (Fothergill et al., 2010).

Another group studied the genotypic diversity sequencing 11 isolates from a single sample for two patients. Although isolates were all clonal, within-patient diversity was observed for both patients (Feliziani et al., 2014). The isolates showed high diversity, with groupings into clusters. The coexistence of these clusters over many years, for example regarding hypermutability where both hypermutants and non-hypermutators are observed all along. Another paper also found the parallel coexistence of both hypermutators and non-hypermutators at all points showing the parallel evolution within the CF lung, giving different phenotypes (Ciofu et al., 2010). These findings further suggest adaptive radiation as no dominance appears from specific strains. Nonetheless, there seems to be common pressures as the two patients' isolates both had some similar adaptation trajectories.

A further study examining the diversity of a large number of phenotype regarding motility, auxotrophy, QS production, virulence factors and antibiotic resistance found extensive diversity (Workentine et al., 2013). The diversity was observed in one single patient over 34 samples of multiple clonally related isolates over a year. In addition to the significant diversity of all phenotypes, there was an interesting observation that isolates of similar morphotypes show different patterns of antibiotic resistance. Genotypic diversity was also measured, with the clustering of isolates into three groups, however the correlations between the clustering of the genotypes and phenotypic data were low. A further 88 supplementary isolates were collected from a single sample and these represented 2 morphotypes, for which extensive phenotypic diversity was also found, highlighting the issue of under-sampling.

Several studies have investigated both within-patient diversity and between-patient diversity. Mowat et al. investigated this in patients all chronically infected by a common epidemic strain, shown to be able to spread from patient to patient. Interestingly, having observed the phenotypes of 40 isolates of Liverpool Epidemic Strains (LES) for each of 10 patients, Mowat et al. showed that phenotypic within-patient diversity contributed more to the total diversity than did between-patient diversity (Mowat et al., 2011). Indeed, the study found that about 50% of the diversity observed could be attributed to the diversity between isolates of a same sample. In total they observed 75 sub-types, which each corresponded to a different combination of the 9 phenotypes tested, with an average of 10 sub-types observed per sample, thus showing an important diversity overall. The diversity within patients contributed to 81% of the total diversity.

Ashish et al. observed similar spread of diversity amongst non-LES strains (Ashish et al., 2013). They investigated diversity of *P. aeruginosa* isolates in 5 chronically infected patients through collecting 40 isolates for each patient during two stages of exacerbations, which corresponds to increased severity of symptoms. Within-patient diversity was found to also contribute more to overall diversity than between-patient diversity, with ~10 phenotypic sub-types per sample and 75 different in total across all samples.

All studies investigating diversity within individual patients found that it was significant, with some studies finding it to be more substantial than the diversity between patients. However, the chronology of the appearance of the diversity still remains unclear.

Another different mechanism could partly explain the diversity of *P. aeruginosa* within CF lungs. Studies have described how within a population of *P. aeruginosa* only a proportion of the bacteria lose the ability to produce a certain virulence factor or trait.

Indeed, although several studies such as Jorth et al. show that part of the diversity within the lungs stems from the geographic separation in different niches of this heterogeneous environment, diversity could also be due to complex bacterial cooperation and cheating (Jorth et al., 2015). This leads some bacteria to be social cheats, which lose the ability to produce a compound but count on other bacteria to survive (Andersen et al., 2015). For example, it is known that *P. aeruginosa* can scavenge pyoverdine from other species and thus pyoverdine mutants are common (Tyrrell and Callaghan, 2016, Harrison et al., 2006). Andersen et al. have shown that in the presence of extracellular pyoverdine the cheats keep the function of the FpvA pyoverdine receptor; however, in the absence of pyoverdine the rate of FpvA mutants increases (Andersen et al., 2015).

This social cheating is also common in the context of biofilm growth where QS mutants can still thrive thanks to co-operators and the continued presence of overall QS regulation (Köhler et al., 2009). Thus, the scale of this social cheating can affect the diversity of the overall population.

This social cooperation can only function if a sufficiently high percentage of the population still produces the compound needed for survival, similarly to the hypothesis of herd immunity in the context of vaccination. A study has unveiled a mechanism in which some bacteria fight the presence of social cheats (Wang et al., 2015). The co-operators that retain some virulence are able to produce HCN. Although the production of HCN is costly, it is used by co-operators to punish the social cheaters, which were found to be more susceptible to the gas. This reveals an interesting-regulation system to social cheating, with a new potential role for the production of HCN (Wang et al., 2015).

Conversely to social cheating a study found that a small portion of the population were over-producers of virulence factors. This hyper-virulent subpopulation persists during chronic infection along avirulent isolates (O'Brien et al., 2017). Fothergill et al. found the overproduction of pyocyanin and LasA in a sub-population of epidemic strains (Fothergill et al., 2007). Thus, in contrast to social cheating, some bacteria of the population could retain the

ability to produce virulence factors that are unnecessary in the context of the chronic infection. O'Brien et al. conclude that this sub-population could act as a reservoir of isolates able to cause patient to patient transmission through their sustained virulence and capacity of colonising new hosts.

1.4 Hypotheses & aims of the project

The three main hypotheses of the project are the following:

- *P. aeruginosa* modifies its phenotypic behaviour over the course of infection and is different between early and chronic stages of infection.
- *P. aeruginosa*'s phenotypes are correlated to one another through the regulation of quorum sensing molecules.
- The within-patient diversity of *P. aeruginosa* strains is greater than the diversity of strains between subjects.

To investigate these hypotheses, the aims of the project are to:

1- Obtain clinical strains isolated from patients attending the Royal Brompton Hospital who are a) infected for the first time (early stage isolates), b) chronically infected (chronic isolates).
2- Optimise high or medium through-put fully quantitative assays to then measure phenotypes involving key virulence traits in the strain set gathered and in a strain set previously obtained.
3- Undertake analysis to determine the changes in phenotypic measurements and their variation both within patients and between patients.

Chapter 2: Material and Methods

2.1 Strains and growth conditions

2.1.1 Control Strains

All the bacterial strains used were grown in Lysogeny Broth (LB) media or agar with the appropriate antibiotics added when necessary. The list of control strains used for the assays are listed in Table 2-1.

Strain	Description		Use	Source
PA14	Wild type, clinical isolate		Positive control for all assays	M. Ausubel (Rahme et al., 1995)
PA01	Wild type, clinical isolate		Positive control for motility and biofilm assays	Lab strain, original source B. Holloway (Holloway, 1955)
ΔhcnB	PAO6344 in-frame deletion in the PA01 background.	HCN synthase enzyme	Negative control for HCN assay	D. Haas, Université de Lausanne
ΔflgE	In-frame deletion in the PA14 background.	Flagellar hook protein	Negative control for swimming assay	Lab strain
ΔfliF		Flagellar ring complex subunit	Negative control for all motility assays	
pilB::MAR2xT7	PA14NR library transposon insertion mutants. Mariner-based transposon, MAR2xT7. (GmR)	Type IV pilus motor protein ; retraction ATPase	Negative control for twitching, swarming and biofilm assays	M. Ausubel (Liberati et al., 2006)
rhlA::MAR2xT7		Rhamnolipids biosynthesis gene	Negative control for swarming and protease assays	
rhlR::MAR2xT7			Negative control for swarming and protease assays	
LasA		Elastase biosynthesis gene	Negative control for the elastase assays	
phzM::MAR2xT7		Phenazine-specific methyltransferase	Negative control for pyocyanin assays	
phzS::MAR2xT7		Pyocyanin biosynthesis genes		
rsmA::MAR2xT7		Negative regulator of virulence	HCN overproducer	
pvdF::MAR2xT7		Pyoverdine synthesis gene	Negative control for pyoverdine assays	

Table 2-1: A list of strains used in this study.

2.1.2 Clinical strain isolation and curation of Royal Brompton Hospital strains

Sputum samples were diluted and plated on Pseudomonas Selective Agar (PSA) according to clinical Standard Operating Procedures (SOP) by staff of the Royal Brompton Hospital (RBH) (London, UK) microbiology laboratory. Plates of these strains were recovered and 24 single well separated colonies from each plate were isolated in order to represent the potential phenotypic variation within a single patient (Figure 2-1). The isolates were grown in 2.2 ml 96 deep-well plates (Corning, Netherlands) in 420 µl of LB, glycerol was then added to 15 % final concentration and plates were stored at -80 °C. Figure 2-1 presents a diagram of the collection strategy. Up to 420 isolates from 17 chronically infected patients and 221 isolates from 7 newly infected patients were assayed.

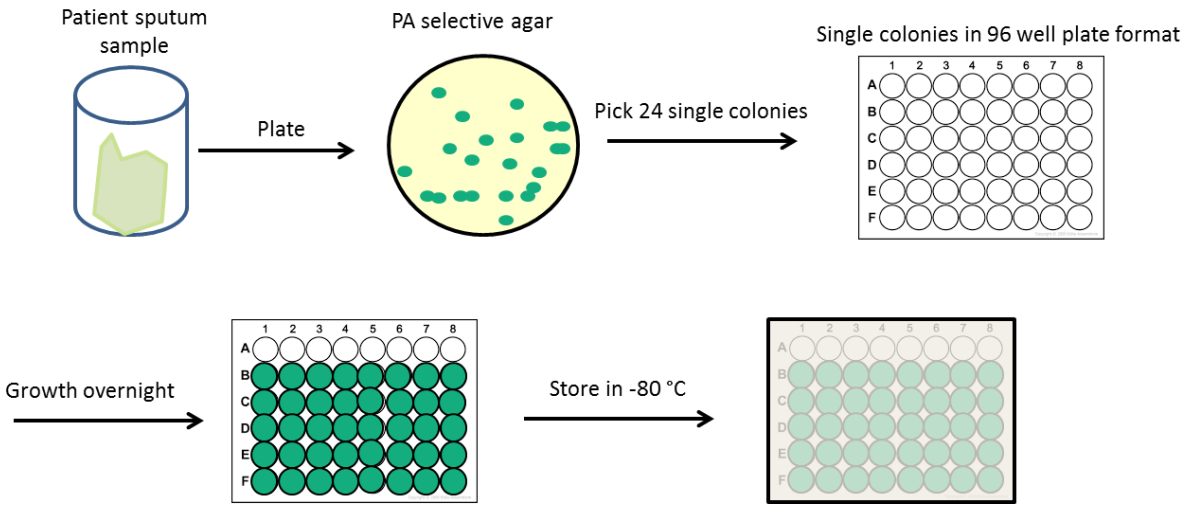


Figure 2-1: Collection strategy of clinical strains from the Royal Brompton Hospital.

2.1.3 University of British Columbia strains

A collection of strains already available in the laboratory were also used in this study. These strains were recovered from children and adults attending the British Columbia Children's hospital, the Shaughnessy or the St Paul's Hospital, Vancouver, British Columbia. The collection consists of 154 isolates from 16 patients harvested from either sputum samples or cough swabs as previously described (Mahenthiralingam et al., 1994). Strains were plated on Columbia agar with 5% sheep's blood (CBA) then grown in Mueller Hinton Broth with 8%

dimethylsulphoxide, before storage at -80°C. The strains were subsequently revived on CBA to be sent to the Williams lab on charcoal swabs. For this study the strains were grown in 2.2 ml 96 deep-well plates (Corning, Netherlands) in 420 µl of LB broth. Glycerol was then added to 15 % final concentration and plates were stored at -80 °C. The list of strains and further information is available in the Table 2-2.

Patient	Identification	Date of isolation	Patient	Identification	Date of isolation	Patient	Identification	Date of isolation
P01	C2773C	26/03/1988	P06	C7626M	03/06/1997	P12	C7399M	07/01/1997
P01	C2943C	02/09/1988	P06	C7625C	03/06/1997	P12	C7401C	07/01/1997
P01	C3639M	27/02/1990	P06	C7989M	01/04/1998	P12	C8584M	20/04/1999
P01	C3637C	27/02/1990	P06	C9660C	15/05/2001	P12	C8583C	20/04/1999
P01	C4023M	28/12/1990	P06	D1384C	06/12/2004	P12	D1029C	26/02/2004
P01	C4021C	28/12/1990	P06	D1385M	06/12/2004	P12	D1032M	26/02/2004
P01	C4278M	03/07/1991	P06	D2604E	27/08/2007	P12	D2561C	16/08/2007
P01	C4277D	03/07/1991	P06	D2631C	23/09/2007	P12	D2751M	19/12/2007
P01	C4276C	03/07/1991	P06	D2639M	01/10/2007	P13	C0803C	09/12/1983
P02	C3195C	07/03/1989	P07	C4303C	23/07/1991	P13	C2548E	15/09/1987
P02	C3380C	01/08/1989	P07	C4432C	22/10/1991	P13	C2757C	16/02/1988
P02	C6557C	16/05/1995	P07	C5656C	25/01/1994	P13	C3995R	20/11/1990
P02	C7632C	06/06/1997	P07	C5995C	19/07/1994	P13	C3994C	20/11/1990
P03	C4142C	19/03/1991	P07	C6631C	27/06/1995	P13	C3993M	20/11/1990
P03	C4299C	23/07/1991	P07	C9027C	02/03/2000	P13	C4976M	24/11/1992
P03	C4934M	03/11/1992	P07	D0006C	25/01/2002	P13	C4978C	24/11/1992
P03	C6942M	13/02/1996	P08	C7250C	06/09/1996	P13	C4977C	24/11/1992
P03	C6943C	13/02/1996	P08	C7464E	12/02/1997	P13	C7004M	19/03/1996
P03	C6941C	13/02/1996	P08	D1120E	13/05/2004	P13	C7005C	19/03/1996
P03	C6944D	13/02/1996	P08	D1121D	13/05/2004	P13	C9082M	20/04/2000
P03	C8596C	22/04/1999	P08	D1126C	13/05/2004	P13	D1204C	26/07/2004
P03	C8595M	27/04/1999	P08	D1157C	13/05/2004	P13	D1342M	08/11/2004
P03	D1701C	24/08/2005	P08	D2410E	12/04/2007	P13	D2982M	04/06/2008
P03	D1702M	24/08/2005	P08	D2980C	05/06/2008	P14	C5198D	11/05/1993
P03	D2938E	09/05/2008	P09	C4267C	28/06/1991	P14	C5203M	14/05/1993
P03	D2955D	09/05/2008	P09	C6676M	12/07/1995	P14	C5204C	14/05/1993
P03	D2956E	09/05/2008	P09	C6675C	12/07/1995	P14	C5733C	07/03/1994
P03	D2994M	02/06/2008	P09	C8341C	15/10/1998	P14	C5732C	07/03/1994
P04	C2684C	12/01/1988	P09	C8340R	15/10/1998	P14	C5731M	07/03/1994
P04	C3779C	15/05/1990	P09	C8339M	15/10/1998	P14	C5730C	07/03/1994
P04	C4652E	07/04/1992	P10	C4443C	29/10/1991	P14	C7025M	02/04/1996
P04	C6082C	30/08/1994	P10	C4678E	05/05/1992	P14	C7024C	02/04/1996
P04	C6617M	20/06/1995	P10	C4677C	05/05/1992	P14	C9661C	15/05/2001
P04	C6616M	20/06/1995	P10	C5724C	08/03/1994	P14	D2950M	08/05/2008
P04	C6618C	20/06/1995	P10	C5725M	08/03/1994	P14	D3010C	07/07/2008
P04	C6939M	21/02/1996	P10	C9805D	11/09/2001	P15	C3881C	28/08/1990
P04	C6938C	21/02/1996	P10	C9804M	11/09/2001	P15	C4197D	07/05/1991
P04	C6940C	21/02/1996	P10	D2750C	05/02/2007	P15	C4196D	07/05/1991
P04	C7471E	17/02/1997	P10	D2749M	05/02/2007	P15	C4198C	07/05/1991
P04	D1173M	24/06/2004	P10	D2326D	05/12/2007	P15	C6926M	06/02/1996
P04	D1172C	24/06/2004	P10	D2325M	05/12/2007	P15	C6927C	06/02/1996
P05	C7577M	07/05/1997	P11	C5858D	10/05/1994	P15	C7515D	18/03/1997
P05	C7660M	15/07/1997	P11	C6193D	25/10/1994	P15	D0305D	05/08/2002
P05	C9264E	31/08/2000	P11	C6192D	25/10/1994	P15	D0315M	20/09/2002
P05	C9263M	31/08/2000	P11	C6599D	25/05/1995	P16	C8415D	26/11/1998
P05	D1427E	13/01/2005	P11	C8168D	25/06/1998	P16	C8527D	26/02/1999
P05	D1426M	13/01/2005	P11	D2957D	07/05/2008	P16	D0198D	19/06/2002
P05	D2787M	17/01/2008	P12	C5800E	12/04/1994	P16	D0316M	20/09/2002
P05	D3025C	24/07/2008	P12	C5799C	12/04/1994	P16	D2554M	30/07/2007
P06	C3330C	12/07/1989	P12	C6065C	23/08/1994	P16	D2825M	04/02/2008
P06	C4658M	14/04/1992	P12	C7092M	14/05/1996			
P06	C4657C	14/04/1992	P12	C7091E	14/05/1996			

Table 2-2: List of UBC strains used in this study.

2.1.4 Growth media

For all assays, bacteria were grown in Lysogeny Broth (LB) broth or agar plates (5 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone, 1.5 % (w/v) agar for plates) at 37°C overnight (~16-18 hours).

The PA14NR library transposon mutants were grown overnight in LB supplemented with 15 μg/ml gentamicin (Gm).

2.1.5 Genotyping: Variable Number Tandem Repeat Analysis for typing of *P. aeruginosa*

A selection of isolates from the RBH set of strains was genotyped. The selection was made to investigate if patients were infected by several genetically distinct strains. Only chronically infected patients were selected for genotyping and half the isolates from half the chronic isolates were chosen.

The amplification at 9 Variable Number Tandem Repeat (VNTR) loci, allows description of strains by the number of repeats found at each locus. For loci with small repeat units, PCR was carried out using fluorescently labelled forward primers and products were sized on a sequencer; repeat numbers at the other loci were determined by sizing using agarose gel electrophoresis.

Preparation of Primers

Sterile water was added to give a stock primer concentration of 100 μM. Fluorescently labelled primers were protected from light and primer solutions were stored in the freezer. For non-fluorescently labelled primers, forward and reverse primer working solutions were combined for each locus to a final concentration of 10 μM. For the fluorescently labelled loci, forward and reverse primer solutions were prepared separately to give a 10 μM working solution.

DNA Extraction

Growth from a single colony on plates was suspended in 100 μl PCR water in a sterile 0.5 ml Eppendorf tube. The bacterial suspension was heat-treated by incubation at 95^{o}C for 10 minutes in a thermocycler. It was vortexed for approximately 10 seconds, then centrifuged at 15,500

g/rcf for 2 minutes. The extracted DNA was stored in the refrigerator at 2 – 8°C. A representative of the 'Liverpool' strain (or other strain of known VNTR profile) was included as control in each run.

Reactions set up in 20 µl volumes as follows the QIAGEN HotStarTaq® *Plus* Master Mix:
145.6 µl water
22.4 µl primer mix (10 µM F/R mix)
56 µl CoralLoad Concentrate (10x)
280 µl 2 x Master Mix

Add 18 µl mix to 2 µl extracted DNA.

For the loci using fluorescent primers (207,209,61):
145.6 µl water
11.2 µl forward primer (10 µM)
11.2 µl reverse primer (10 µM)
56 µl CoralLoad Concentrate (10x)
280 µl 2 x Master Mix

Add 18 µl mix to 2 µl extracted DNA.

PCR reaction conditions were as follows:
5 min 95°C (activation)
Then 34 cycles of:
94 °C 1 min
60 °C 1 min
72 °C 1 min
then a final extension
72 °C 10 min
Hold at 12 °C.

Agarose Gel Electrophoresis

PCR products were run on a 1.5% (w/v) agarose gel in 5% TBE buffer (445mM Tris base, 10mM EDTA (pH 8.0) and 445mM boric acid).

HyperLadder 50bp (Bioline, Memphis USA) was used as the PCR product size marker and the agarose gel was run at 170 VoIt was stained with GelRed (3X) (Biotium, Freemont USA) for 30 minutes and viewed under UV transillumination.

Size Tables from amplification with normal primers

The tables presented below were used to determine the number of repeats at each locus, according to sequence length of PCR products analysed by agarose gel electrophoresis.

ms172

Primers:	**Repeat number**	**Size (bp)**
ms172L GGATTCTCTCGCACGAGGT	**1**	**195**
ms172R TACGTGACCTGACGTTGGTG	**2**	**249**
	3	**303**
54bp repeat with a 141bp flanking sequence	**4**	**357**
	5	**411**
	6	**465**
	7	**519**
	8	**573**
	9	**627**
	10	**681**
	11	**735**
	12	**789**
	13	**843**
	14	**897**

ms211

Primers:	Repeat number	Size (bp)
ms211L ACAAGCGCCAGCCGAACCTGT	**1**	**259**
ms211R CTTCGAACAGGTGCTGACCGC	**2**	**360**
	3	**461**
101bp repeat with a 158pb flanking sequence	**4**	**562**
	5	**663**
	6	**764**
	7	**865**
	8	**966**

ms213

Primers:	Repeat number	Size (bp)
ms213L CTGGGCAAGTGTTGGTGGATC	**1**	**221**
ms213R TGGCGTACTCCGAGCTGATG	**2**	**324**
	3	**427**
103bp repeat with a 118bp flanking sequence	**4**	**530**
	5	**640 (633)**
	6	**743**
	7	**846**
	8	**949**
	9	**1052**
	10	**1155**
	11	**1258**

ms214

Primers:	Repeat number	Size (bp)
ms214L	**1**	**196**
ms214R	**2**	**311**
	3	**426**
115bp repeat with an 81bp flanking sequence	**4**	**541**
	5	**656**
	6	**771**
	7	**886**
	8	**1001**

ms217

Primers:	Repeat number	Size (bp)
ms217L TTCTGGCTGTCGCGACTGAT	**1**	**497**
ms217R GAACAGCGTCTTTTCCTCGC	**2**	**606**
	3	**715**
109bp repeat with a 388bp flanking sequence	**4**	**824**
	5	**933**
	6	**1042**

ms222

Primers:	Repeat number	Size (bp)
ms222L AGAGGTGCTTAACGACGGAT	**1**	**289**
ms222R TGCAGTTCTGCGAGGAAGGCG	**2**	**390**
	3	**491**
101bp repeat with an 188bp flanking sequence	**4**	**592**
	5	**693**
	6	**794**
	7	**895**
	8	**996**

Fluorescently labelled Fragment Analysis

10 µl of Hi-Di formamide (ThermoFisher Scientific, Waltham USA) and 0.5 µl of size standard GeneScan 600 LIZ (ThermoFisher Scientific, Waltham USA) was added to individual fluorescently labelled samples and mixed thoroughly. Samples were analysed on an ABI system by the Public Health England's Genomic Services and Development Unit (GSDU). Results were analysed using PeakScanner software (ThermoFisher Scientific, Waltham USA) to determine the size of each peak for each sample.

Size Tables from amplification with fluorescently Labelled Forward Primers:

ms207

Primers:	**Repeat number**	**Size (bp) (ABI)**
ms207L ACGGCGAACAGCACCAGCA	**1**	**-**
ms207R CTCTTGAGCCTCGGTCACT	**2**	**-**
	3	**123.8**
(ABI 6-FAM-ms207L (blue))	**4**	**130**
6bp repeat	**5**	**136**
	6	**142.1**
	7	**148.5**
	8	**154.7**
	9	**160.9**
	10	**167**
	11	**173**
	12	**180**
	13	**186.1**
	14	**192.1**
	15	**198.4**

ms209

Primers:	Repeat number	Size (bp) (ABI)
ms209L CAGCCAGGAACTGCGGAGT	**1**	**119.1**
ms209R CTTCTCGCAACTGAGCTGGT	**2**	**125.1**
	3	**131.1**
(ABI NED-ms209L (yellow/black))	**4**	**137.1**
6bp repeat	**5**	**143.1**
	6	**149.1**
	7	**154.1**
	8	**160.1**

ms61

Primers:	Repeat number	Size (bp) (ABI)
ms61F CTTGCCGTGCTACCGATCC	**1**	**-**
ms61R CCCCCATGCCAGTTGC	**2**	**-**
	3	**-**
	4	**76.7**
	5	**83.3**
	6	**89.28**
	7	**95.91**
	8	**102.8**
	9	**108.8**
	10	**114.8**
	11	**120.9**
	12	**126.9**
	13	**132.9**
	14	**138.9**
	15	**144.9**
	16	**151**

17	**157**
18	**163**
19	**169**
20	**175**
21	**181**

2.2 Phenotypic assays

2.2.1 Overnight cultures

For all assays, strains and appropriate controls were inoculated and grown overnight in 2.2 ml 96 deep-well plates in 420 µl of LB broth at 37°at 200 rpm. All assays were undertaken in LB broth or agar and all bacteria for assays was grown shaking (200 rpm) overnight (~16 hours) at 37°C.

2.2.2 Speed of growth

All isolates were grown overnight as detailed, and ~5 µl of the overnight cultured bacteria was used to subculture strains to an OD_{595} of ~0.05 into 200µl of LB in a flat bottomed 96-well polystyrene microtiter plate (Falcon Corning, Netherlands). The plate was shaken at 200 rpm and OD595 was measured every hour for 8 hours, and an additional time point was measured at 24 hours. The data of at least 3 biological replicates was transferred to Dr. Volker Behrends to calculate the t50 for each isolate. The t50 values correspond to the time at which the bacteria reach half of the maximal OD (from stationary phase). It is the time of "half growth" meaning that it will be lower for a fast-growing bacterium.

2.2.3 Swimming, swarming and twitching motility assays

For motility assays 125ml of 0.25%, 0.5% and 1% semisolid LB agar were prepared respectively for swimming, swarming and twitching assays in square petri dishes of 140mm diameter (VWR, UK) before being left to dry for 1 hour under a sterile flow hood. The plates were then inoculated with the overnight cultured broth of the strains by stabbing the agar plates

25 times in a regular manner, every 2.5cm, using sterile toothpicks. For the twitching assay the toothpick was held to the bottom of the plate for 2 seconds. To have controllable parameters plates were first left in an incubator at 20^{o}C but the air flow within dried out the plates and hindered natural motility. The assay was therefore optimised by leaving the plates at room temperature for 24 hours for the swimming and 48 hours for swarming and twitching assays on the lab bench. The lack of temperature control meant slightly more variation over the year but this was accounted for by using the means of all biological replicates for statistical analysis. Motility was measured with an electronic caliper as the diameter of the haze of growth in the agar for swimming, the colonial growth for swarming (Ha et al., 2014). For twitching motility, the agar was removed and 25ml of 0.1% crystal violet was added for 10 seconds before rinsing with water and the stained halo at the bottom of the plate was measured with an electronic caliper.

2.2.4 Protease and Elastase assays

These assays were performed similarly to the stabbing motility assays using 1.5% LB agar supplemented with 1.5% milk for the protease assay and 0.4% elastin from bovine neck ligament (Sigma-Aldrich) (Kessler and Safrin, 2014). The plates were incubated at 37^{o}C and the diameter of clearance was measured after 24 and 48 hours for the protease and elastase assays respectively.

2.2.5 Crystal violet biofilm formation assay

10 µl of overnight deep well plate cultured bacteria was used to subculture strains to an OD_{595} of ~0.1 into 140µl of LB in a flat bottomed 96-well polystyrene microtiter plate (Falcon Corning, Netherlands). The microtiter plate was left to incubate statically at 37 °C. After 6 hours the growth of the strains was recorded (OD_{595}). Strains are considered as having grown if $OD_{595} > 0.3$. The formation of biofilm was investigated with a crystal violet staining assay, quantifying the bacterial adherence by solubilising the 0.1% crystal violet stained biofilms in 95% ethanol and measuring the absorbance at 595nm after (Coffey and Anderson, 2014). In more detail, the 140 µl of LB was pipetted and discarded and 200 µl of 0.1% crystal violet was added to each well. After 5 minutes the crystal violet was pipetted and discarded, and the wells were washed 3 times with 250µl of water. Finally, 250µl of 95% ethanol was added and 200µl was transferred to a new microtiter plate and absorbance was measured.

2.2.6 Hydrogen cyanide paper assay

10 µl of overnight deep well plate cultured bacteria was used to subculture strains to an OD_{595} of ~0.1 into 190 µl of fresh LB broth in a 96-well polystyrene microtiter plate (Falcon Corning, Netherlands). The subcultured plate was stored in the fridge all day until evening when it was left to grow with the lid on overnight for 18 hours statically at 37°C. In parallel, hydrogen cyanide (HCN) detection paper was prepared by saturating Whatman paper using a pipette with 5 mg tetra base (4,4'-methylenebis(N.N-dimethylaniline)) and 5 mg copper (II) ethylacetoacetate diluted in 2.5 mL chloroform (Nair et al., 2014b). After 18 hours the detection paper was placed on top of the microtiter plate and secured with a breathable membrane (breathable sealing tape). The detection paper was left for 3 hours lidless and when removed the growth of the strains was recorded by the measurement of OD_{595} (Nair et al., 2014b). The cyanide detection paper was scanned at 400 dpi and saved as a PNG file. Quantitative analysis was realised through a MatLab program called CyQuant written by Dr. Volker Behrends, previous member of the Williams lab. The intensity of the blue colour, proportionate to the amount of cyanide, is quantified with this CyQuant program resulting in higher numbers for higher intensity and cyanide concentrations.

2.2.7 Pyocyanin measurements methods

Pyocyanin supernatant method

Overnight deep-well plates cultures (prepared as explained in Overnight Cultures section) were spun down at 20,000 g for 20 minutes and 200 µl of the supernatant was transferred to a 96-well polystyrene microtiter plate (Falcon Corning, Netherlands). The absorbance was measured at 691 nm to quantify pyocyanin.

This method was used to measure pyocyanin for both UBC and RBH sets of strains.

Pyocyanin chloroform extraction

Strains were grown overnight in 2 ml of LB broth in deep-well plates and incubated overnight at 37°C at 200 rpm. After using 200 µl to measure the optical density at 600 nm, 1.5 ml of the cultures were spun down at 20,000 g for 20 minutes. 1 ml of the supernatant was then mixed

with 1 ml of chloroform in 2ml Eppendorf centrifuge tube and vortexed thoroughly before being spun down at 20,000 g for 2 minutes. 800 µl of the bottom layer (chloroform + pyocyanin) was transferred to a clean 1.5ml Eppendorf tube. Then 400 µl of 0.2M HCl was added and the samples are vortexed to extract the PYO in the HCl layer. The tube was finally spun down (20,000 g for 2 minutes) before pipetting 200 µl of the top layer (HCl + pyocyanin) in 96-well polystyrene microtiter plate (Falcon Corning, Netherlands) and reading the absorbance at 520 nm. The readings were then normalised by multiplying by 17.072 (extinction coefficient) and by 1.25 (to compensate the use of only 800 µl out of the 1 ml of chloroform) (Essar et al., 1990).

This measurements method was used to quantify pyocyanin for the RBH isolates only.

Pyocyanin LC-MS method

See quorum sensing molecules quantification method.

2.2.8 Pyoverdine measurement

As in the pyocyanin supernatant method, the overnight deep-well plates were spun down at 20,000 g for 20 minutes. 200 µl of the supernatant was transferred to a 96-well polystyrene microtiter plate (Falcon Corning, Netherlands). The absorbance was measured at 405 nm to quantify pyoverdine.

2.2.9 Quorum sensing molecules quantification

Briefly, the whole 593 isolates from the RBH set of strains were grown for 24 hours in deep-well plates and 200 µl of the supernatant was recovered into 96-well polystyrene microtiter plate (Falcon Corning, Netherlands) and covered with aluminium foil seals before being stored at -80 °C. The supernatants were then transferred to the university of Roehampton for the QS molecules to be quantified using a mass spectrometry technique undertaken by Dr. Volker Behrends. All compounds were measured by mass spectrometry, with quality control every eight biological samples, integrated using the in-house pipeline that allows spectrum-level scrutiny and employs baseline correction, thus getting an integral only when there is a real

signal. The resulting data was spline-fitted on a per compounds basis using the QC to control for instrument drift.

The concentrations are expressed as a percentage of a PA01 24h culture used as internal quality control sample for all measurements. This had the advantage of being more reliable than pure standards and could be used for a larger number of compounds as there was no need to have a standard of the compounds.

2.3 Clinical information

All clinical data was retrieved from records by an authorised medical doctor belonging to the Royal Brompton Hospital team, and all files were anonymised before transfer for use in this study. At no time for this study were samples retrieved directly from patients. All biological material was transferred from the Royal Brompton clinical laboratory, and once again no patients' data revealed.

Clinical information for the patients attending the Royal Brompton Hospital (RBH) from which samples were collected and isolates recovered to build the RBH strain set, was collected from the microbiological laboratory records as well as the patients' records. These were gathered by a qualified doctor and information was anonymized before being transferred and used in the analysis of this study.

2.4 Data Analysis

2.4.1 Controls and replication

For all phenotypes biological replicates for each assay were undertaken with a minimum of n=3 to ensure statistical power and up to n=7 (Appendix 2-1). Means of all biological replicates were used to undertake statistical analysis.

For all assays PA01 and PA14 wild type strains were used as controls besides at least one negative control with the exception for the HCN production assay where only the PA14 wild type strain was measured (see table 2.1).

To account for variability in data, normalising it to the wild type strain controls was considered. The variation with data normalised to the PA01 control (ant to PA14 for HCN production) was observed against the raw non normalised data, and for half of the assays (growth, protease, HCN, pyoverdine and pyocyanin) the standard deviations and coefficients of variation increased when data was normalised (Appendix 2-2). It is hypothesised that this was due to PA01 biological variation not linked to external factors for most assays and thus not representing trends for all the data. The assays where external and environmental factors had the biggest effect on the variation overall were the motility assays as they were undertaken at room temperature which was not controlled (see 2.2.3). This was confirmed as swarming, swimming and twitching were the phenotypes (as well as biofilm and elastase) where normalising the data decreased the standard deviation for a majority of isolates. Thus, normalising was an option for certain phenotypes although the overall statistical trends and outcome were the same. Also, there was a smaller increase of standard deviation when not normalising the data for those phenotypes than when normalising the data for the other phenotypes which showed lower deviation with the raw data. Therefore, to not normalise only parts of the data to the controls it was decided that the raw data would be directly used for all analyses with no normalisation of the data.

2.4.2 Statistical analysis

The statistical analysis was undertaken using JMP 13 (SAS).

In Chapter 4, Mann-Whitney U tests were used to compare early and chronic stage of infections. Spearman correlations were used to correlate phenotypes with infection stages (RBH isolates) and infection length (UBC isolates).

In Chapter 5 mixed linear models were undertaken, and the model was:
Patient: random
Isolates: Random
and isolates nested in patient (Isolate[Patient]).

Nested ANOVAs were undertaken to test diversity for each RBH patient. Throughout the assays, p values < 0.05 were considered significant, except for correlations where p values of

<0.01 were deemed significant due to the large number of correlations and risk of false positives.

Chapter 3: Isolation and preliminary characterisation of pseudomonas aeruginosa strains for phenotypic analysis

3.1 Introduction

Previous studies of the adaptation of *P. aeruginosa* to the CF lung environment have focused on a range of different aspects of adaptation such a genotypic changes, recombination, phenotypic variations, and emergence of antibiotic resistance (Darch et al., 2015, Ciofu et al., 2010, Clark et al., 2018, Diaz Caballero et al., 2015). The overall aim of this project was to investigate phenotypic adaptations of *P. aeruginosa* to the CF lung through specific perspectives. The first was to investigate phenotypic changes of the bacteria over time and stages of infection to better understand the adaptation leading to the establishment of chronic infection, as well as the associations between various phenotypes. The second angle was to explore the diversity of *P. aeruginosa* between and within patients. Therefore, the approach in this study was to use two independent sets of strains to help investigate these different angles of adaptation. The first set was previously available in the laboratory and allowed observation of adaptation of the bacteria longitudinally over time. The second set was collected for the purpose of this study and allowed testing of multiple colonies within a sample to enable diversity of *P. aeruginosa* both within and between patients to be assessed.

The phenotypes of *P. aeruginosa* have roles in virulence during infection, facilitate colonisation of the lung or help establish chronicity (Heeckeren et al., 1997, Rau et al., 2010, Davies, 2002). In total ten phenotypes were selected that were linked to virulence such as speed of growth, motility, establishment of biofilm, production of protease, hydrogen cyanide, pyocyanin and pyoverdine. Additionally, several regulatory molecules linked to quorum sensing were quantified. All the assays used to measure phenotypes were optimised and several measurement methods were compared in the case of pyocyanin. All assays were optimised to be fully quantitative to observe the full range or measurements for each phenotype.

The specific aims of this chapter are to introduce:

- The selection and isolation process of sets of strains used for this study, as well as the relevant clinical information.
- The genotyping of the strains used.
- The quantitative phenotypic data obtained and the choice of methods, specifically for pyocyanin.
- The steps taken in order to get data ready for further analysis, through testing of statistical distribution.
- The importance of quantitative data to assess the full extent of the diversity of phenotypes.

3.2 Results

3.2.1 Strains from the University of British Columbia

This first set of strains used during this project was donated to the Williams laboratory by Drs James Zlosnik and David Speert and originated from the Cystic Fibrosis centre at the University of British Columbia (UBC), Canada. The set was previously utilised in a metabolomics study (Behrends et al., 2013). The data available on the isolates includes the date of sampling, the morphotypes and genotypes of most isolates. No supplementary clinical information is known about the patients such as their age, gender or FEV1 at the time of sampling. All the data available on the set of UBC strains is summarised in Appendix 3-1.

This set of strains was comprised of 154 isolates that were recovered from 104 samples collected from 16 different patients infected with *P. aeruginosa*. Isolates were longitudinally collected from first samples over various lengths of time, ranging from 5 to up to 24 years. The dates of the first samples and subsequent ones were available; thus, the length of infection was calculated. At each sampling date a single isolate was collected, unless if different morphologies had been observed in the sample, then up to 4 different colonies were isolated. Therefore, various numbers of isolates were recovered per patient over 3 to 10 samples, with a mean of 6.5 samples per patient. Between 4 and 15 isolates were collected for each of the 16 patients, with more than 10 isolates for 7 patients (Figure 3-1).

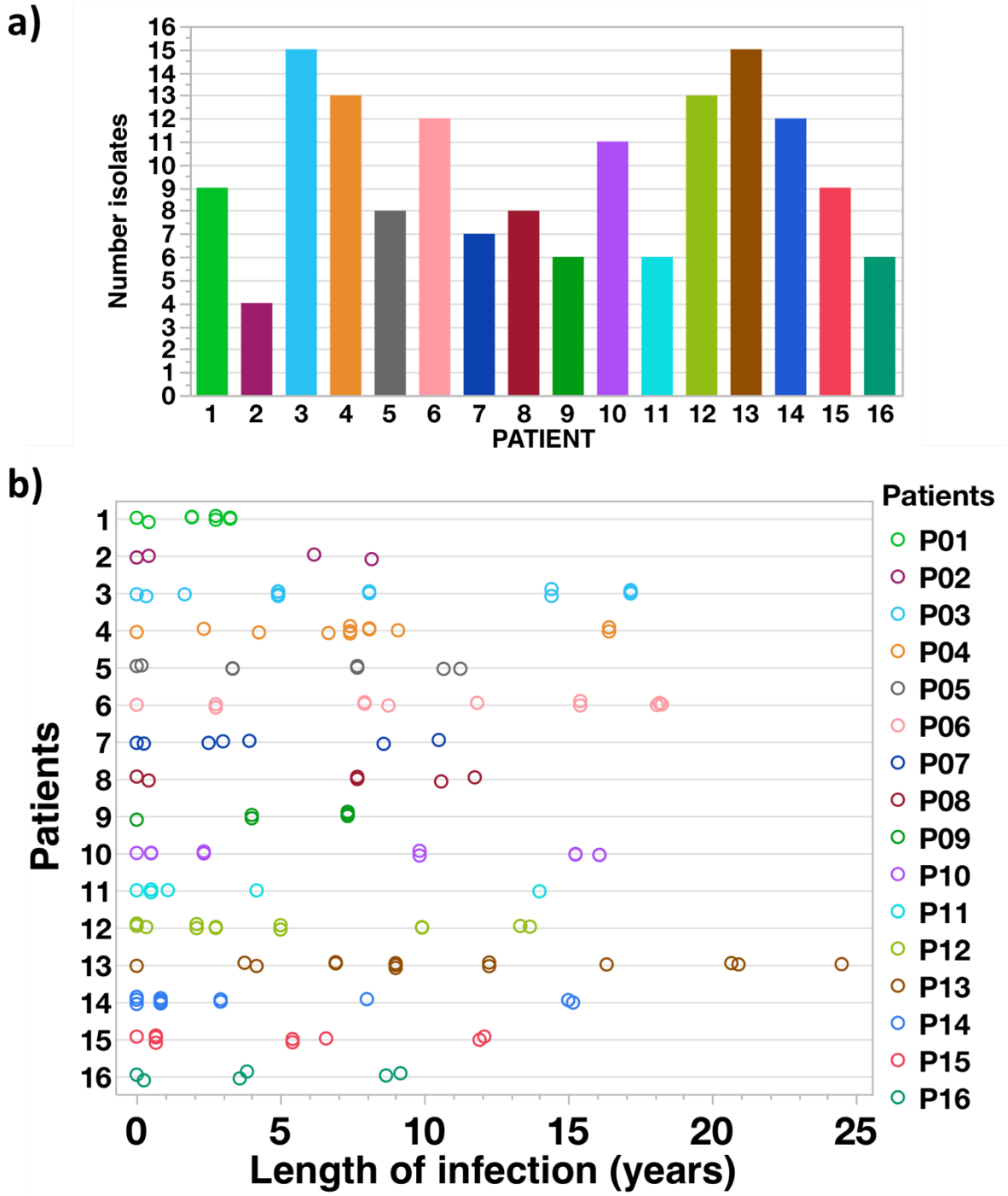


Figure 3-1: Number and timeline of collection of the UBC set of strains. Patients are represented by the same colour in both graphs. a) Total number of isolates recovered for each of the 16 patients. b) Timeline of the collection of strains for each patient. Each circle represents a time when isolates were recovered from patients. Overlapping circles correspond to samples where multiple isolates were recovered.

The samples from which the bacteria were isolated were either cough swabs or sputum samples. The majority (87 of 104, 84.5%) were from sputum (Figure 3-2). Most cough swab

samples were collected at the beginning of infections, for half the patients the first sample was a cough swab.

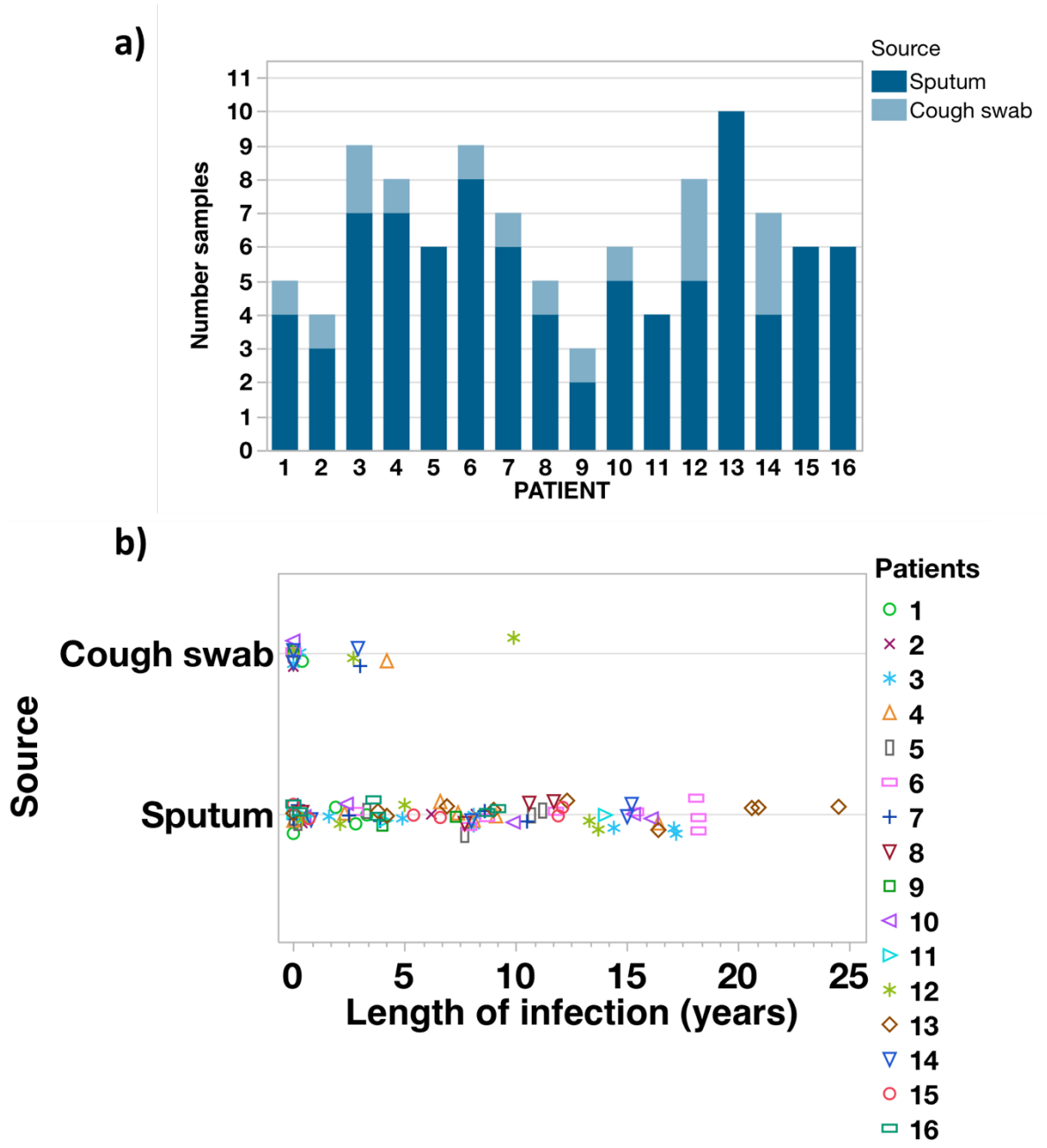


Figure 3-2: Sources of samples used to recover the strains. a) Proportion of cough swabs versus sputum samples for the 16 patients. b) Timeframe at which samples of each source were collected.

Morphological and genotypic information about the bacteria

Morphotypes were recorded at the time of isolation at the UBC according to the morphotype grouping from Wahba, A. and Darrell, J. in 1965 (Mahenthiralingam et al., 1996, Wahba, 1965). Five morphotypes were described, which were normal, mucoid, enterobacter-like, rough and dwarf. Out of a total of 154 isolates, 70 (45.4%) had normal morphotype, with colonies looking flat and smooth. The second most prevalent morphotype was mucoid, accounting for 48 (31.2%) isolates. These mucoid isolates were shiny with elevated and viscous growth. Another 20 (13%) isolates were of dwarf morphotype represented by small colonies, and 14 (9.1%) isolates were enterobacter-like which were translucent and circular. Finally, only 2 isolates (1.3%) were of rough morphotype with rough edged colonies. In 13 out of 16 patients (81%), multiple morphotypes were observed amongst the isolates collected. In a majority of patients (62.5.%), 3 morphotypes of *P. aeruginosa* were recovered over the sample collection period (Figure 3-3).

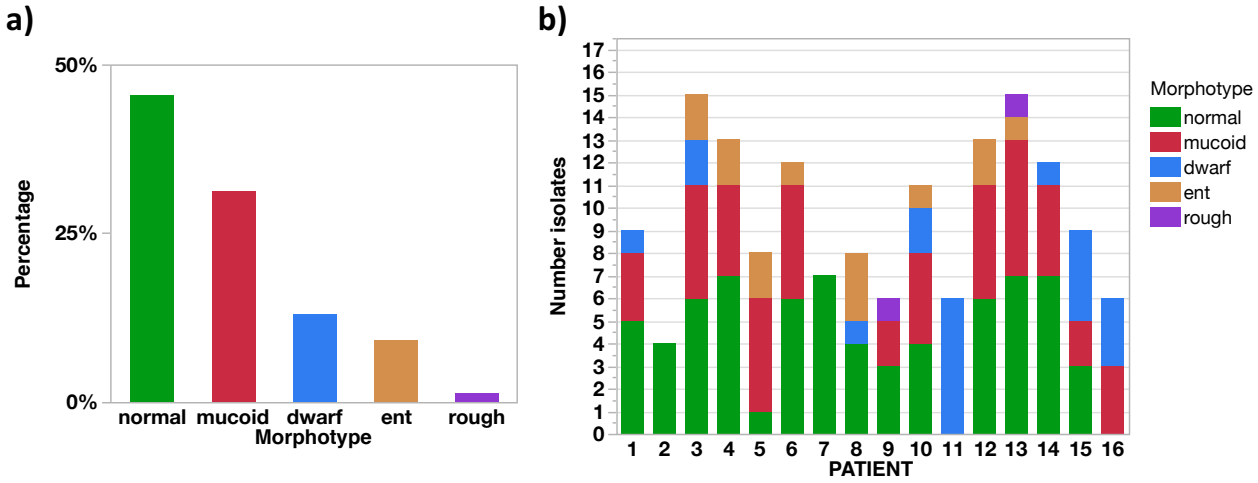


Figure 3-3: Classification of morphology types observed from the isolates of the UBC set of strains. Morphotypes were classified at the time of isolation. a) Total percentage of each of the morphotypes observed over all samples. b) Proportion of isolates of each morphology found within the isolates of each individual patient. morphology of the isolates recovered. normal: flat colony with a smooth surface, mucoid: shiny, elevated colony with a smooth edge, dwarf: small colony variant, enterobacter-like: short for translucent circular colony, rough: rough edged colony, according to (Mahenthiralingam et al., 1996).

A subset of 98 of the 154 isolates in total (64%), were genotyped by Rapid Amplified Polymorphic DNA typing (RAPD) at the UBC (Mahenthiralingam et al., 1996, Campbell et al., 2000). Between 4 and 11 isolates (on average 72%) were genotyped per patient, with the exception of patient 7 where none were typed. According to the RAPD results, in most patients the same strain was isolated over time. That was the case for all patients except patient 3 and 10 for which 2 and 3 different strains were observed respectively (Figure 3-4 and Appendix 3-1). In total 15 different RAPD type strains were found, with only 2 types shared between 2 and 3 patients (Figure 3-4).

Almost all patients' isolates showed different morphotypes over time. Nevertheless, in most cases this did not reflect a change in RAPD genotype. For example, in patient 9 normal, rough and mucoid isolates were recorded concurrently while still having the same RAPD type (Appendix 3-1).

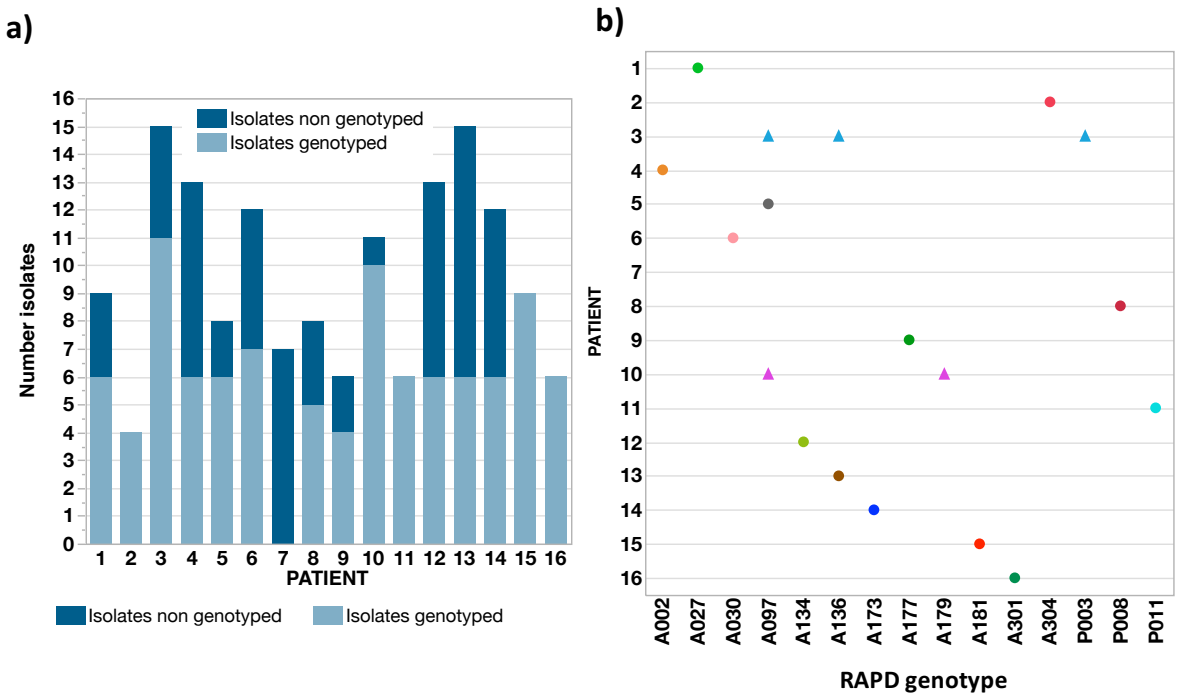


Figure 3-4: Genotyping of the UBC set of strains. a) Proportion of isolates genotyped per patients. 96 isolates were genotyped out of 154 total. b) RAPD types of isolates for each patient. RAPD types from patients 3 and 10 (triangles) are the two only patients where more than one genotype was found. RAPD types A097 and A136 were the only types found in 3 (patients 3, 5 and 10) and 2 patients (patients 3 and 13) respectively.

3.2.2 Strains from the Royal Brompton Hospital (RBH)

Isolation of the RBH set of strains

A second set of strains was specifically collected for this project to study and investigate both within-patient and between-patient diversity. The idea was to collect multiple isolates from each patient sample from both early and chronic infection stages. This collection strategy would allow us to observe the diversity within, as well as changes between these two stages of infection. Early and chronic stages of infection were categorised according to the Leeds criteria (Proesmans et al., 2006, Lee et al., 2003, Crull et al., 2018). The original agar plates inoculated with the patients' samples were retrieved from the Royal Brompton Hospital (RBH) microbiology laboratory. The overall isolation strategy process is shown in Figure 3-5. Chronic isolates were collected between April and July 2015, and first/new infection strains were specifically flagged by the RBH until April 2016.

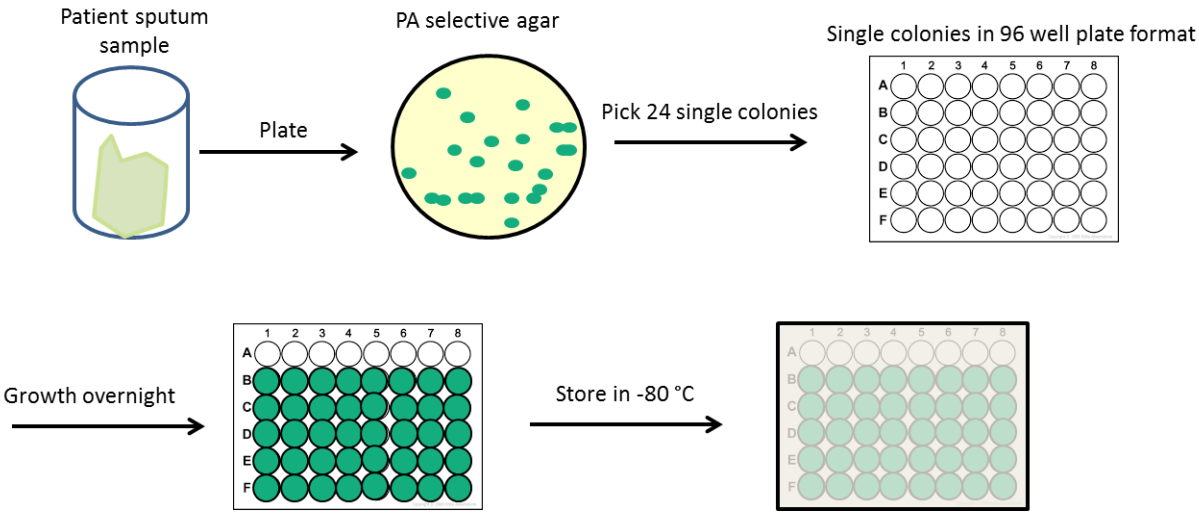


Figure 3-5: Collection strategy to generate the RBH set of strains. Patient samples (sputum or cough swabs) were plated on selective agar (refer to material and methods). Selective plates were retrieved and multiple colonies were collected from each plate. Colonies were grown in LB overnight in 96 well plates. Glycerol was added obtaining a 20% final solution and then plates were numbered and stored in a -80˚c freezer for future use.

Isolates were recovered from 25 patient samples. It was subsequently discovered that patient 17 was not *P. aeruignosa* but *Burkholderia cepacia* and removed from the study. Two other samples originated from the same patient, thus called chronic 8a and chronic 8b. In total,

isolates originated from from 16 chronic and 7 first (early) infected patients. For the chronic samples the aim was to recover 24 colonies from each sample, which was the case for all except 3 patients, where only 12 could be obtained. As early infections were relatively rare, as many isolates as possible were collected, between 13 and 60 isolates per sample (Figure 3-6). In total 593 isolates were recovered, 372 (62.7%) from chronic infections and 221 (37.3%) from early infections. Overall most samples were sourced from sputum (79%), and the remainder from cough swabs. All chronic isolates were from sputum samples, whereas the source of 5 out of 7 early samples were from cough swabs due to the young age of these patients most of whom could not yet expectorate.

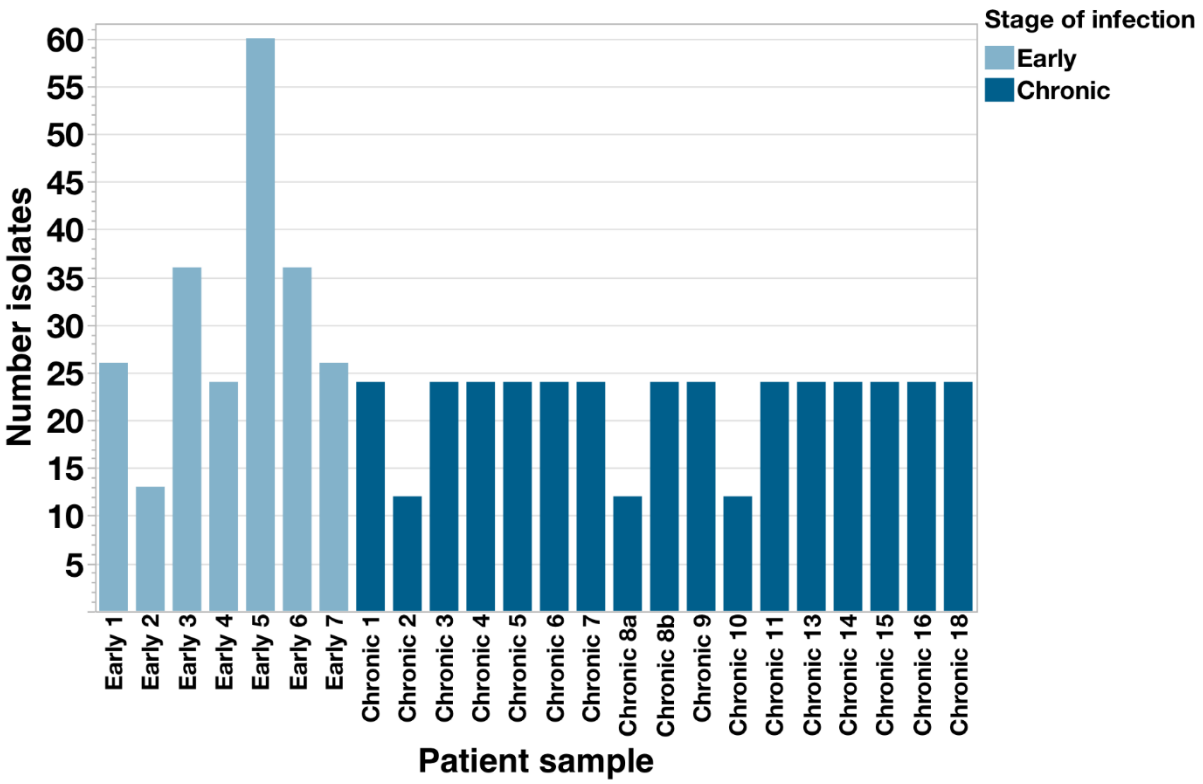


Figure 3-6: Stage of infection and number of isolates per patient for the RBH strain set. Number of isolates recovered from each patient sample according to infection status. Patient sample 8a and 8b stem from the same patient.

Patient clinical data

Amongst this set of patients, 11 (47.8%) were homozygous for the Phe508del (Figure 3-7a). Another 9 (39.1) were heterozygous for the Phe508del with various other second mutations and the genotypes of another one patient was not known. Two patients had 2 non-Phe508del

mutations (Figure 3-7a; Appendix 3-2). Samples were recovered from 15 male patients and 8 female patients of various ages, ranging from 1.3 to 58.1 years. The patients with first infections were significantly younger than chronically infected patients; the median age was 2.3 years (1.3 to 12.6) in the case of early infection and 22.3 years (15.1 to 58.1) for the chronic infections (Figure 3-7b).

The lung function of patients was measured in clinic using the Forced Expiratory Volume in the first second and corrected according to age, gender and height (FEV_1% predicted) following the Global Lung Function Initiative (GLI) equations (Quanjer et al., 2012). This corresponds to the volume of air that a patient can exhale forcefully in the first second after taking a full inhalation (Graham et al., 2019, Moore, 2012). Due to the young age of most of the early infected patients, the FEV1 was available for only 2 out the 7 early patients, and the value for both was above 100%. Regarding the chronic patients, the FEV1 measurements were available for all patients except one and measurements were significantly lower, with maximum FEV1 measurements of 83%. Indeed, over half of the chronic patients' FEV1 were under 50%, the overall mean being 52.8% (Figure 3-7c).

Finally, data on exacerbations was recovered from patients' files for chronic patients at the time of the sample. According to the treating clinicians, 5 patients were defined in a stable state, 7 patients defined as experiencing a pulmonary exacerbation. Finally, for another 5 patients the status was somewhat unclear from the records (Figure 3-7d). However, clinical information available suggested chronic patient 1 was experiencing the end of an exacerbation and was treated with IV antibiotics as an outpatient, while chronic patient 8 was in a stable state following an exacerbation.

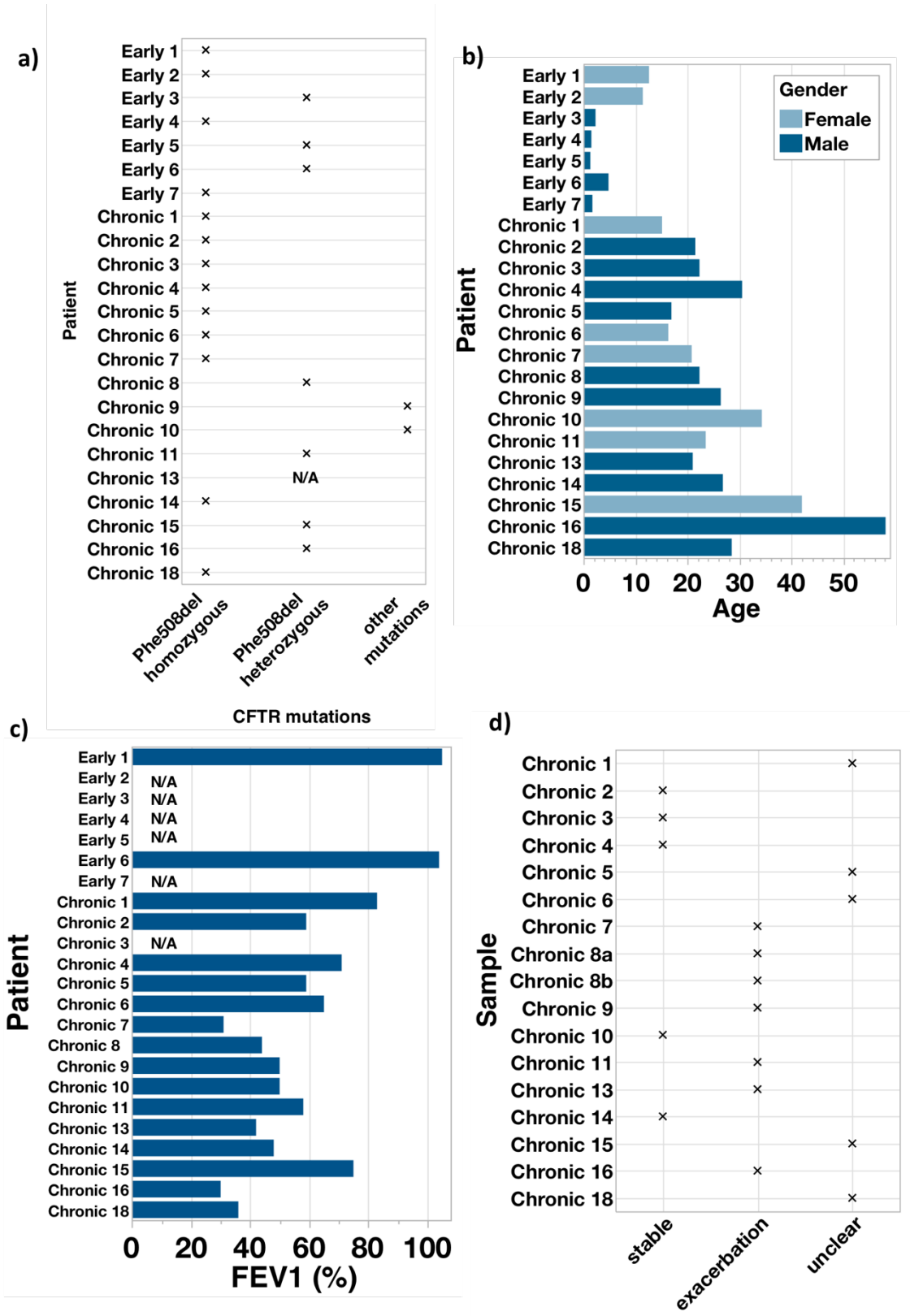


Figure 3-7: Clinical information about the RBH patients. a) CFTR genotypes of patients. Proportion of patients being homozygous or heterozygous for the Phe508del mutation, or having other mutations for both alleles. b) Age and gender of the patients in the study. c) FEV1 lung function measurements. FEV1: Forced Expiratory Volume in the first second, or volume exhaled out in one second after taking a full inhalation. d) CF genotype of patients. Proportion of patients being homozygous or heterozygous for the Phe508del mutation, or having other mutations. (All raw data available in Appendix 3-2).

Based on clinical laboratory records, in addition to the *P. aeruginosa*, 45.8% of this cohort were co-infected with other species. The most common co-infecting bacterial species was *Staphylococcus aureus*, some being Methicillin-resistant *S. aureus* (MRSA) and several patients also had a fungal infection from *Candida* species (Appendix 3-2). *Candida* species as a cause of lower respiratory tract infection are somewhat controversial. Indeed, their role as microbial bystanders or active participants in infection remains unclear (Gileles-Hillel et al., 2015, Williams et al., 2016a).

3.2.3 MLVA genotyping of RBH strains: clonality of isolates

Knowing how many different strains were infecting patients is essential for the type of study conducted here. Genotypic information not only enables identification of possible cross infections, observed for example in the case epidemic strains, but also investigation of the diversity of bacteria found within patients (Pitt, 2002, Cheng et al., 1996). In this study all genotyping was done using multi-locus variable number tandem repeat analysis (MLVA) typing method (Vu-Thien et al., 2007).

This method is PCR-based and amplifies 9 short repetitive DNA sequences called Variable Number Tandem Repeats (VNTR) (Turton et al., 2010). The number of repeats at each VNTR can be different in each strain. The MLVA profiles therefore correspond to the number of repeats at each of the 9 VNTR, thus defining a unique DNA fingerprint for each strain. The number of repeats are given in a specific order of VNTR loci which is 172, 211, 213, 214, 217, 222, 207, 209 and finally locus 61. The last locus, locus 61, is very variable and therefore the most discriminatory. Changes in this locus do not only indicate a different strain but are considered as evolution within a strain. Isolates that had up to 2 different number of repeats over the 9 VNTR are routinely still considered to be the same strain by PHE during their routine genotypic, which is the cut-off followed in this project.

Every infected patient at the RBH should have a *P. aeruginosa* isolate genotyped at least once a year. The yearly MLVA type saved in patient records was, in most cases, not acquired from the same sample as the ones used to isolate bacteria in this study. The MLVA information recovered from clinical records was therefore from genotyping undertaken at the closest date

before or after the sample used to recover isolates. As this routine MLVA is performed only sporadically and also on one isolate only, more typing was done specifically for this project. I undertook all the additional MLVA at the Public Health England (PHE) laboratories. This was enabled through the help of Prof. Jane Turton and Dr. Dervla Kenna from the antimicrobial resistance and healthcare associated infections department (ARHAI).

A subset of 108 isolates was selected for typing from only chronic isolates to check that phenotypic diversity measurements were not complicated by genotypic diversity. In total 12 randomly selected isolates from each of 9 chronic patients were typed, accounting for 50% of isolates from 50% of the chronic patients.

The clinical records showed the same MLVA type being recurrently identified suggesting that the vast majority of patients were chronically infected by one single strain. This was confirmed by the complementary MLVA undertaken for this project. This supplementary typing found the presence of one single lineage in each individual patient (Table 3-1). For 3 patients (numbers 4, 5 and 14) the records did not give a definite answer to the presence of a unique infecting strain, as different MLVA types were found over the years, but not close to the time of sampling (Table 3-2).

Both the RBH routine typing at PHE and the specific one for this study suggest that patients are overall infected by a single strain, which may adapt to various niches within the lung. Overall 10 patients out of the 23 were infected by a strain isolated in no other patient from the RBH (unique strain), while the others harboured epidemic strains or commonly observed ones (Table 3-2). It can be noted that early patients 3 and 5 are infected by the same strain, as are chronic patients 1 and 2, and 8 and 18.

Patient sample	locus 172	locus 211	locus 213	locus 214	locus 217	locus 222	locus 207	locus 209	locus 61
Chronic 2	8	3	-	5	2	3	5	2	9
Chronic 6	11	6	5/-	2	4	1	12/3	2	8
Chronic 7	13	4	3/-	2	3	1	9	6	11
Chronic 8a	11	4	5	2	3	2	8	4	12
Chronic 9	13	-	5	4	4	4	5	4	5
Chronic 10	11	-	5	4	3	2	6	1	8
Chronic 13	11	7	5/-	4	4	3	5	2	6
Chronic 15	12/13	2	1	5	2	3	6	5	6/7
Chronic 16	9	8	5	2	1	1	3	2	9/12

Table 3-1: MLVA for a selection of chronic stage RBH isolates. VNTR profiles were obtained through MLVA undertaken for this study on 12 isolates for the 9 chronic patients, presenting the number of repeats at each of the VNTR loci (172, 211, 213, 214, 217, 222, 207, 209 and 61). Numbers presented as "12/13" represent variations over the 12 isolates for specific VNTR loci. Up to 2 differences in the MLVA profile are accepted for bacteria being of a same strain (Vu-Thien et al., 2007).

Patient	VNTR profile	VNTR profile confirmed	Strain
Early 1	10,2,6,5,5,4,5,2,9		unique
Early 2	9,2,7,3,3,2,3,1,12		unique
Early 3	11,6,2,2,1,3,7,2,12		clone C (Römling et al., 2005)
Early 4	12,5,1,5,2,2,9,2,10		cluster H (Martin et al., 2013)
Early 5	11,6,-,2,1,3,8,2,10		clone C (Römling et al., 2005)
Early 6	-		
Early 7	12,2,-,5,3,3,8,2,10		unique
Chronic 1	8,3,-,5,2,3,5,2,10		cluster A (Martin et al., 2013)
Chronic 2	8,3,-,5,2,3,5,2,9	Confirmed	cluster A (Martin et al., 2013)
Chronic 3	10,3,4,2,2,2,10,2,14		Nottingham / Trent strain (Snyder et al., 2013)
Chronic 4	12,2,-,6,3,5,5,4,6		Midlands 1 strain (Scott and Pitt, 2004)
Chronic 5	-,3,5,5,4,1,3,7,8		cluster D

Patient	VNTR profile	VNTR profile confirmed	Strain
			(Martin et al., 2013)
Chronic 6	**11,6,5,2,4,1,12,2,8**	**Confirmed**	**unique**
Chronic 7	**13,4,3,2,3,1,9,6,11**	**Confirmed**	**unique**
Chronic 8	**11,4,5,2,3,2,8,4,12**	**Confirmed**	**Common** (Martin et al., 2013)
Chronic 9	**13,3,5,4,4,4,5,4,5**	**Confirmed**	**unique**
Chronic 10	**11,3,5,4,3,2,6,1,8**	**Confirmed**	**unique**
Chronic 11	**9,2,3,-,2,2,5,3,9**		**Manchester epidemic strain** (Scott and Pitt, 2004)
Chronic 13	**11,7,5,4,4,3,5,2,6**	**Confirmed**	**Liverpool epidemic strain** (Cheng et al., 1996)
Chronic 14	**10,5,-,-,4,1,15,4,10**		**unique**
Chronic 15	**13,2,1,5,2,3,6,5,7**	**Confirmed**	**unique**
Chronic 16	**9,8,5,2,1,1,3,2,12**	**Confirmed**	**unique**
Chronic 18	**11,4,5,2,3,2,8,4,11**		**Common** (Martin et al., 2013)

Table 3-2: VNTR profiles of the RBH strains. Documented VNTR profiles were recovered from the closest MLVA typing registered in patient's records; MLVA results are presented as 9 digits corresponding to the number of repeats at each of the VNTR loci (locus 172, 211, 213, 214, 217, 222, 207, 209 and 61). Genotyping undertaken in this study confirmed VNTR profiles for 8 chronic patients.

3.2.4 Phenotypic data: quantification, distribution and range

The objectives of this project were to look further into phenotypic adaptation over time, the associations between phenotypes and the diversity of these phenotypes. To do so multiple phenotypes were measured in high through-put assays using all 593 isolates of the RBH strain set and all the 154 isolates from the UBC strain set. The phenotypes measured were motility (swimming, swarming, twitching), production of protease and also specifically elastase, biofilm formation, pyocyanin, pyoverdine, and hydrogen cyanide. Moreover, the production of various quorum sensing molecules was quantified separately. The methods for all assays are detailed in Chapter 2.

Pyocyanin quantification methods

Pyocyanin was measured through different quantification methods, one method was done concomitantly to the measurements of the QS molecules and two other methods have been described in various studies. The first method to quantify pyocyanin is a quicker one-step method. It simply consisted of centrifuging bacterial cultures to get pure supernatants and measuring the absorbance at 691nm, as detailed in the previous chapter on methods. The second method is a chloroform extraction method. In short, pyocyanin present in bacterial culture supernatant was first extracted in chloroform, then reduced and extracted in hydrochloric acid (HCl), as detailed in the methods chapter. By its reduction in HCl, pyocyanin turned pink and the intensity of the colour was quantified through the absorbance at 520nm. The last method consists of measuring the exact concentration of pyocyanin by mass spectrometry (MS). This method was the only fully direct quantitative one out of the three carried out, as it is the method measuring directly pyocyanin and not the absorbance at a wavelength of the pyocyanin spectrum. Because of the cost only one biological replicate was measured using this method.

Pyocyanin was quantified through all three methods for the RBH strain set, but only through the supernatants and MS methods for the UBC strain set. The three methods could thus be compared to one another. This comparison consisted of correlating the methods to one another through Spearman correlations. The results from all three methods were strongly correlated. In the case of the RBH strain set, I found that all three correlations were highly significant ($p<0.0001$) and of similar strength. Strong positive correlations were seen for all three methods, with similar Spearman ρ coefficients of 0.73 to 0.79 (Figure 3-8). Results were similar for the UBC set, with the same strong positive correlation between the supernatant and mass spectrometry methods ($\rho=0.7412$; $p<0.0001$) (not shown).

These results show that all three ways of measuring pyocyanin give comparable results. Both of the more affordable methods, that is the chloroform extraction and the direct supernatant measurement, can thus be used as routine methods to measure pyocyanin. Throughout the rest of the study, only one method was used for all remaining analysis to avoid redundancy of results if all three were used. The MS method could not be chosen as there was only one biological replicate.

For all following analysis the chloroform method was used for the RBH set, and the supernatant method was used for the UBC set.

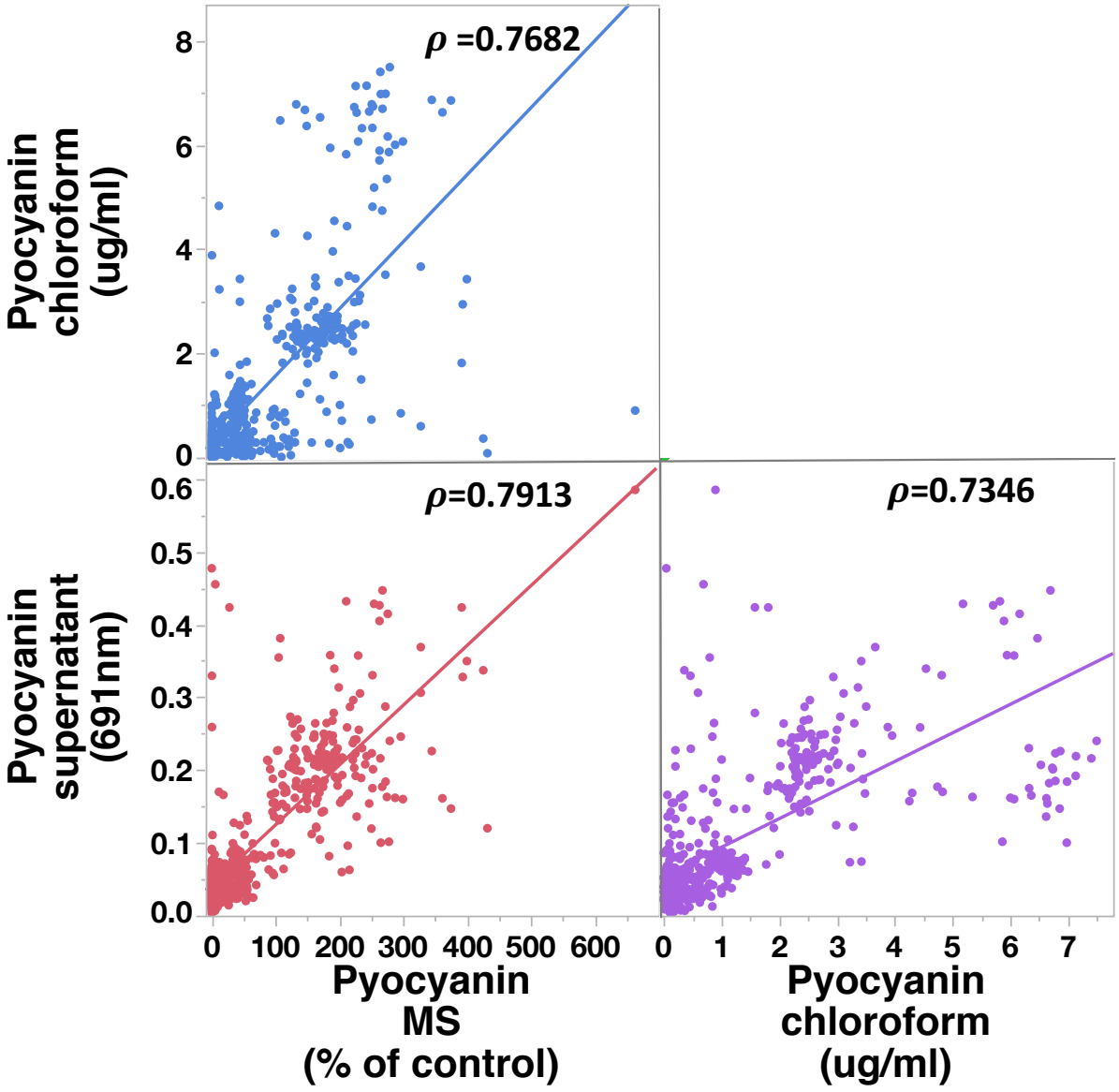


Figure 3-8: Non-parametric Spearman correlations were used to compare all three methods. The Spearman coefficient ρ expresses the strength of each correlation. All three correlations are highly significant ($p<0.0001$) and strongly positive to a similar degree. Each value: mean of ≤ 3 biological replicates.

Distribution of phenotypic data

To statistically analyse the phenotypic measurements, the appropriate statistical tests are decided upon consideration of the probability distribution of the data. Different distribution profiles were observed between patients and phenotypes. To select the correct statistical approach, I first determined whether the phenotypic data was normally distributed using the Shapiro-Wilk test. The hypothesis of the test implies that data is naturally skewed from being normally distributed. This hypothesis means that normally distributed data rejects this null hypothesis. Thus, a significant p value ($p<0.05$) means the data is considered not normally distributed. If the data is normally distributed more stringent tests can be applied whereas different analysis strategies are used if the data is not normally distributed.

This test was performed on the RBH isolates separately for each phenotype and patient. The data could be normally distributed within each patient but not if all patients were considered together, as they were infected by different strains. The complete results are presented, but are found in Appendix 3-3. An overall summary of the percentage of normally distributed data for all phenotypes is presented in Figure 3-9.

The results of the test showed that regarding QS molecules, except C4-HSL, a majority of patients' measurements were not normally distributed. Growth and motility phenotypes showed overall more data that was normally distributed, especially for twitching where only 2 early patients (12%) showed non-normal data. Concerning the other phenotypes, the data for protease, elastase, pyocyanin, biofilm, HCN and pyoverdine showed a majority of non-normally distributed data. On average over all phenotypes, data was found to be non-normally distributed in over 60% of patients. The data from the UBC strain shows the same trends, with phenotypes and QS molecules showing highly significant p values ($p<0.003$) for the Shapiro-Wilk test, with the exception of HCN ($p=0.1786$). Therefore, as a whole, the data for both strain sets was not treated as normally distributed for the subsequent analysis, and was analysed using non-parametric tests.

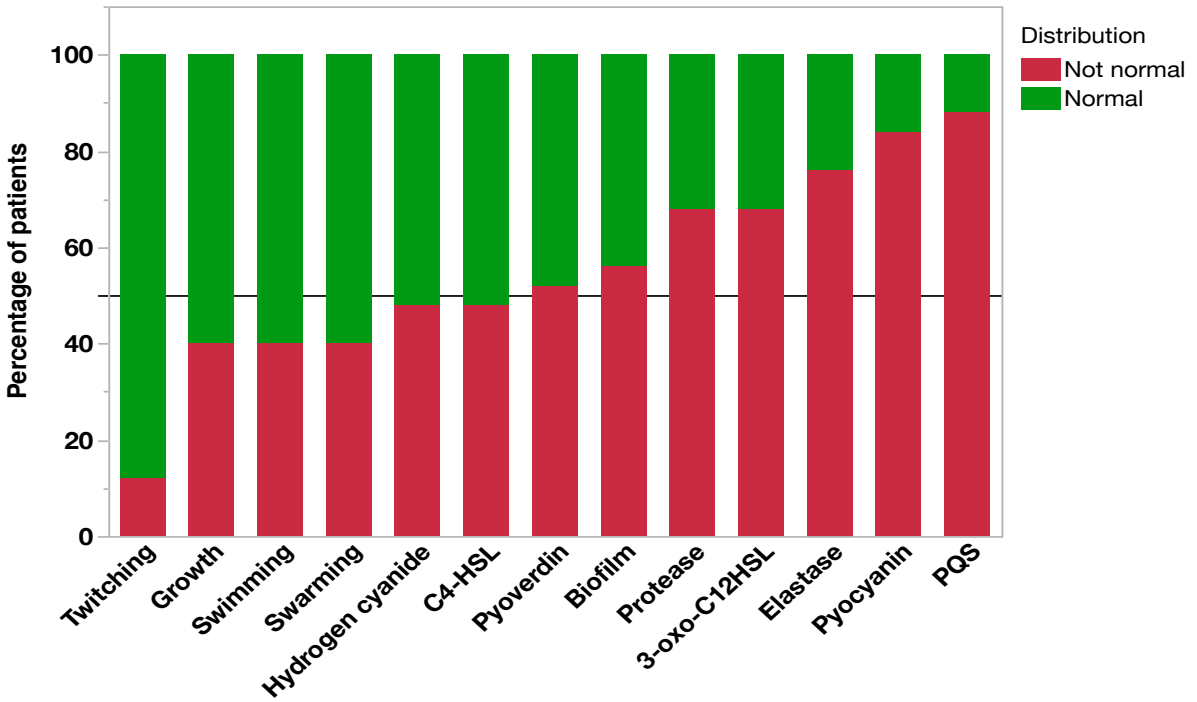


Figure 3-9: Normality of the phenotypic data. The graph shows phenotypes in order of decreasing proportion of normal data. All phenotypes demonstrate a proportion of data that does not follow normal distribution according to the Shapiro-Wilk test. Shapiro-wilk test : normal hypothesis is that data is drawn from normal distribution. If the p value is significant ($p < 0.05$) the null hypothesis is then rejected meaning the data does not follow a normal distribution. The p values obtained for each phenotype and patient are available in Appendix 3.

Range of phenotypic measurement

To study the extent of adaptation and diversity all phenotypic assays of *P. aeruginosa* strains were optimised to be fully quantitative. The rich diversity of phenotypic range of *P. aeruginosa* is illustrated by plotting the phenotypic measurements from the RBH set of strains in ascending order for all 593 strains in Figure 3-10 to Figure 3-19. The phenotypes were calibrated with the laboratory strain PA01 (and PA14) and in some cases with mutants known to be defective in the specific phenotype being measured. All phenotypes appeared to be continuous, according to Figure 3-10 to Figure 3-19, with no phenotypes clearly being binary (on/off) or having only a limited number of categorical phenotypic states. Indeed, values were spread across the entire range of measurement, with no obvious grouping of the data at high or low values. For some phenotypes, such as elastase production and swimming motility, there was a clear division between "non producers" and "producers", but producers then had a range of different levels of production. Similarly, if active twitching motility is defined as strains that show a value greater than a *pilA* mutant then once this threshold is crossed there is a continuous increase in twitching motility amongst clinical isolates. The same observations were found when the values from the UBC set of strains were plotted in a similar fashion (Appendix 3-4). A general observation is that the lab strains PA01 and PA14 had higher quantitative phenotype levels than clinical strains, but in almost all cases there were isolates with values higher than in lab strains. These isolates scoring the highest values, which are more scarce, are overproducers of the virulence factor in question (O'Brien et al., 2017).

This spread of the data observed for all phenotypes underlines the importance and need for the use of quantitative methods to accurately gauge the full extent of diversity. Indeed, using an arbitrary cut-off might in turn lead to underestimating or on the contrary overestimating the true biological diversity.

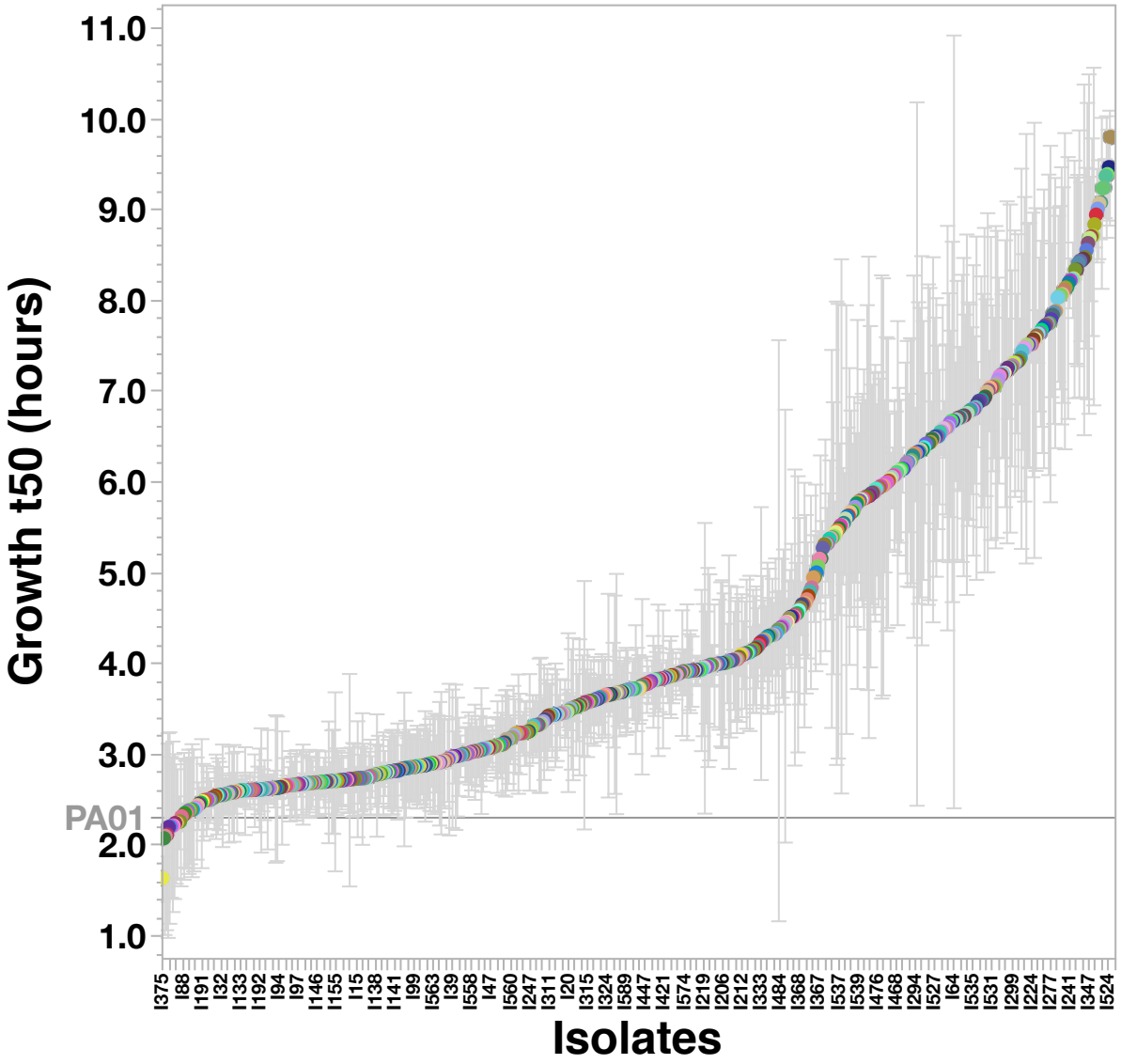


Figure 3-10: Measurements of growth by ascending order for all RBH isolates. All 593 isolates were ordered by ascending order according to the measurements frome the growth assays. Every point represents the mean value from at least 3 biological replicates for every phenotype. The error bars represent the standard deviation for each isolate. The horizontal reference line represents the mean measurement for the laboratory PA01 control.

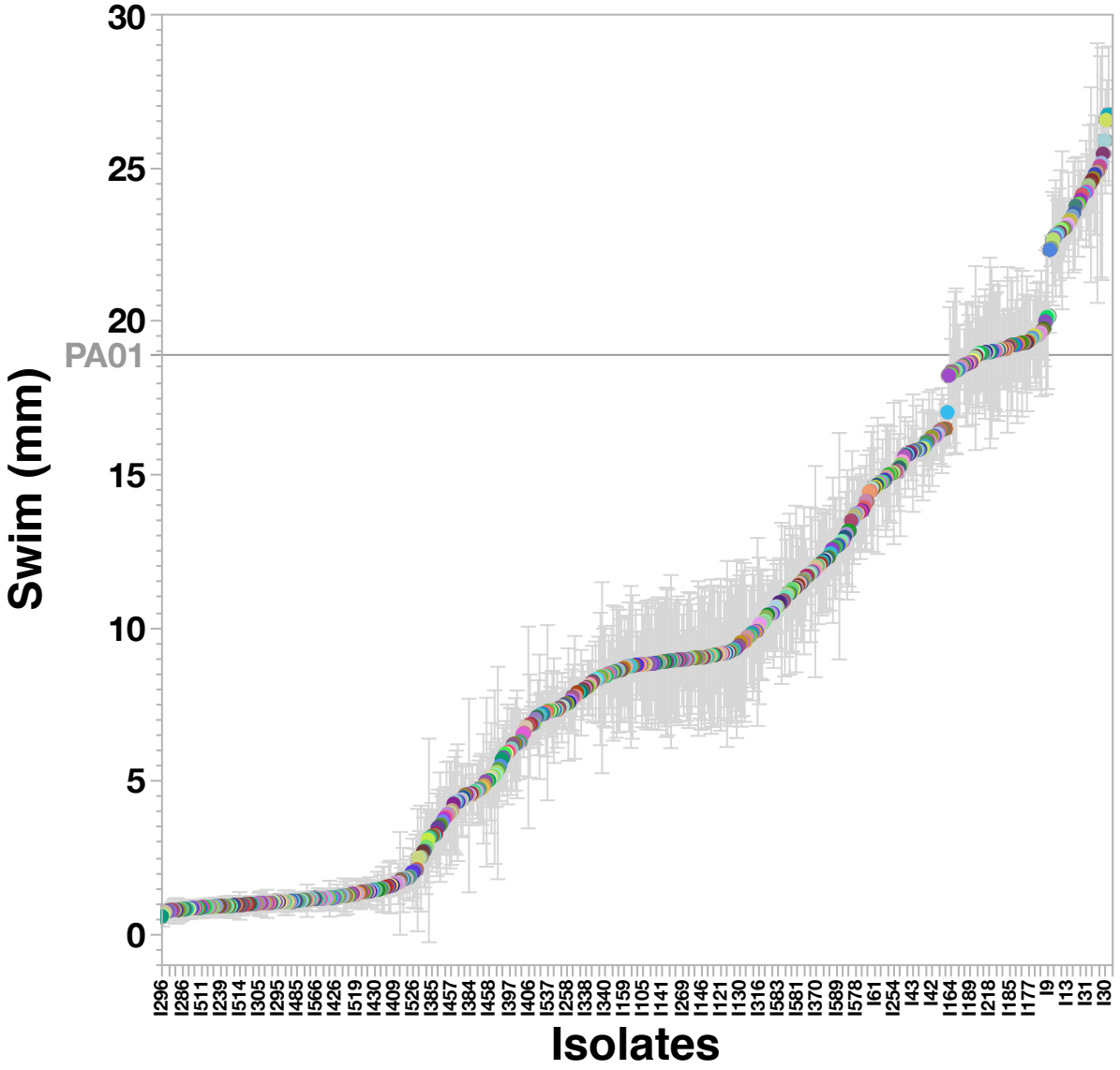


Figure 3-11: Measurements of swimming by ascending order for all RBH isolates. All 593 isolates were ordered by ascending order according to the measurements from the swimming assays. Every point represents the mean value from at least 3 biological replicates for every phenotype. The error bars represent the standard deviation for each isolate. The horizontal reference line represents the mean measurement for the laboratory PA01 control.

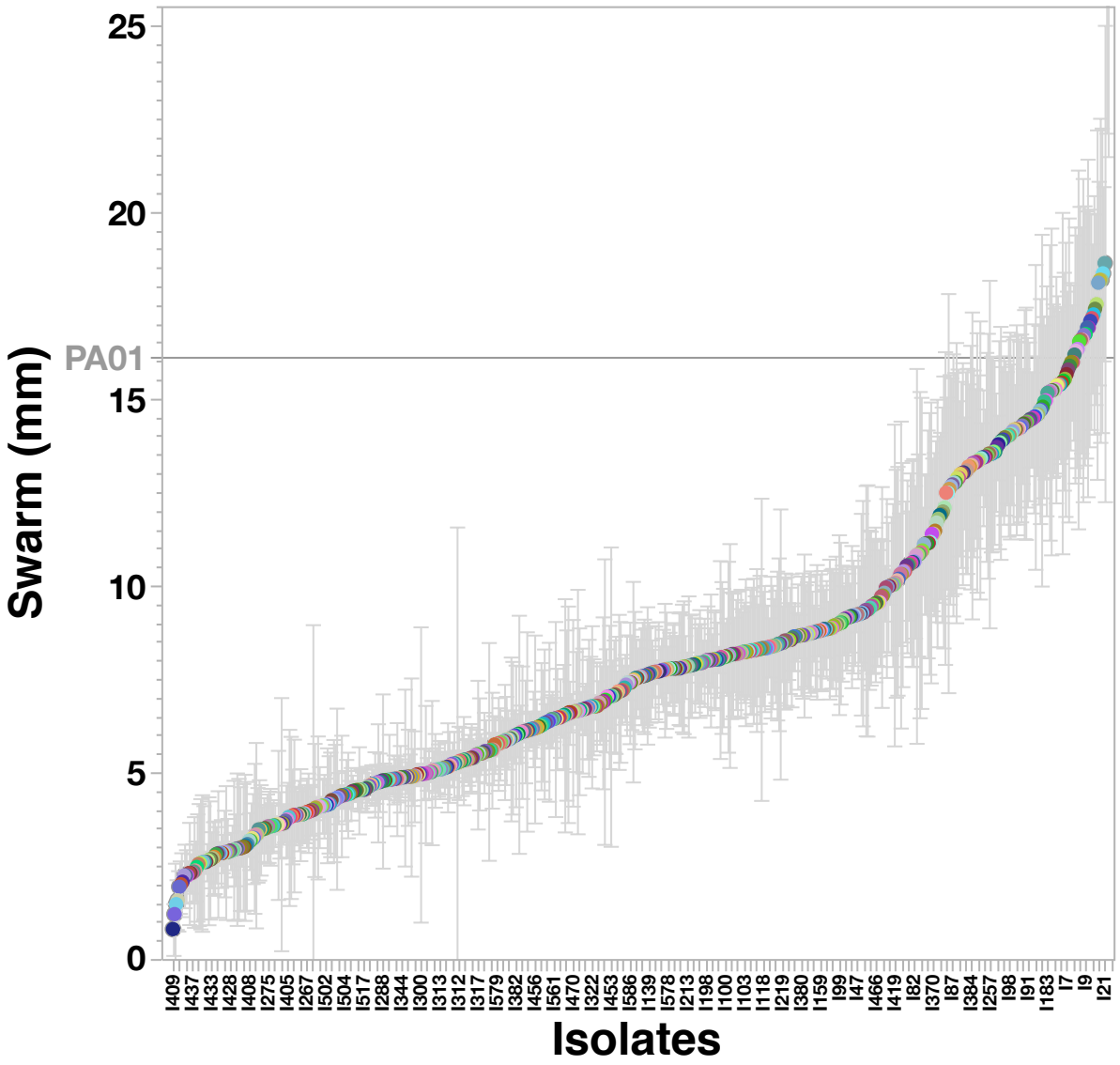


Figure 3-12: Measurements of swarming by ascending order for all RBH isolates. All 593 isolates were ordered by ascending order according to the measurements from the swarming assays. Every point represents the mean value from at least 3 biological replicates for every phenotype. The error bars represent the standard deviation for each isolate. The horizontal reference line represents the mean measurement for the laboratory PA01 control.

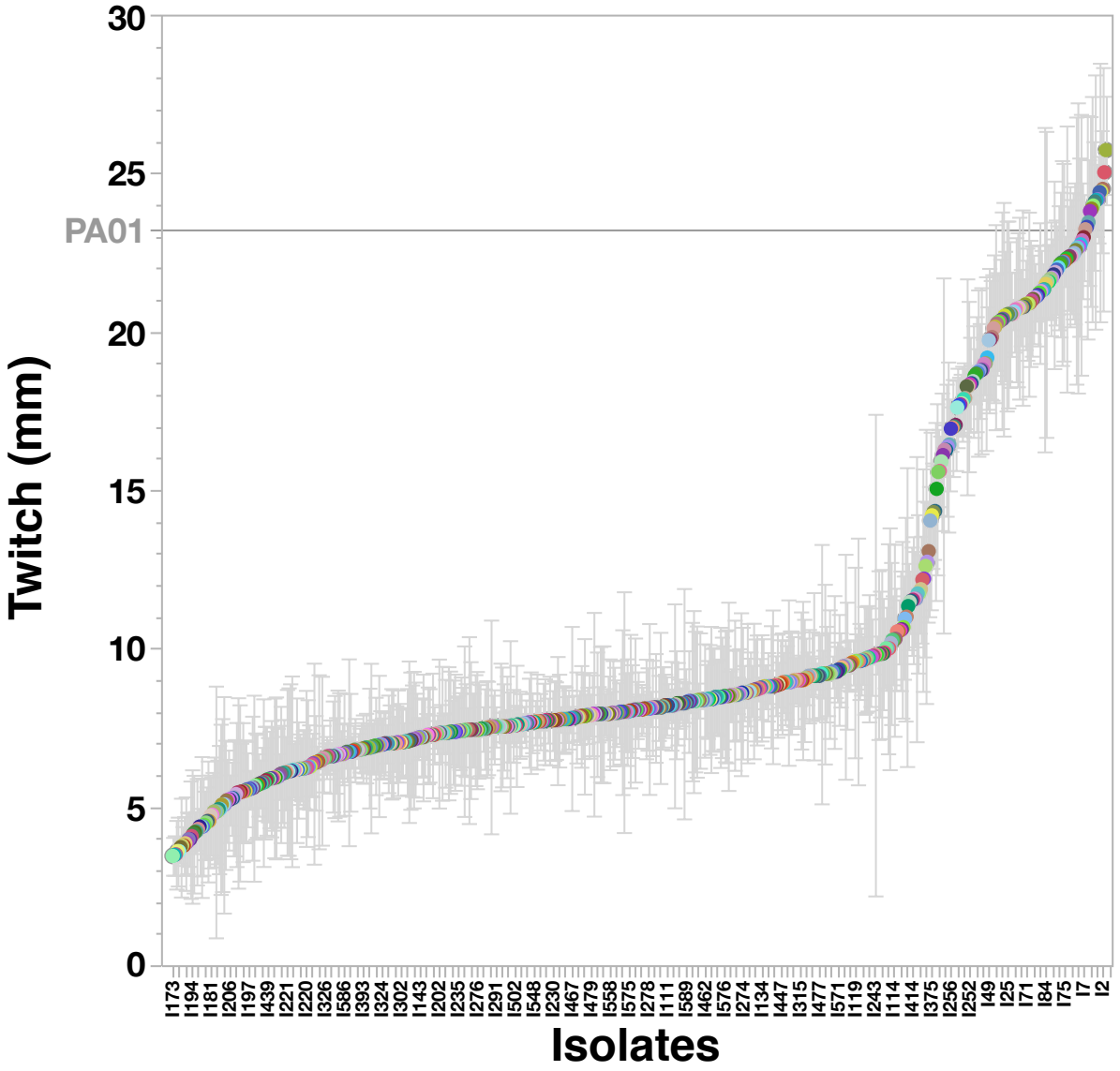


Figure 3-13: Measurements of twitching by ascending order for all RBH isolates. All 593 isolates were ordered by ascending order according to the measurements from the wtitching assays. Every point represents the mean value from at least 3 biological replicates for every phenotype. The error bars represent the standard deviation for each isolate. The horizontal reference line represents the mean measurement for the laboratory PA01 control.

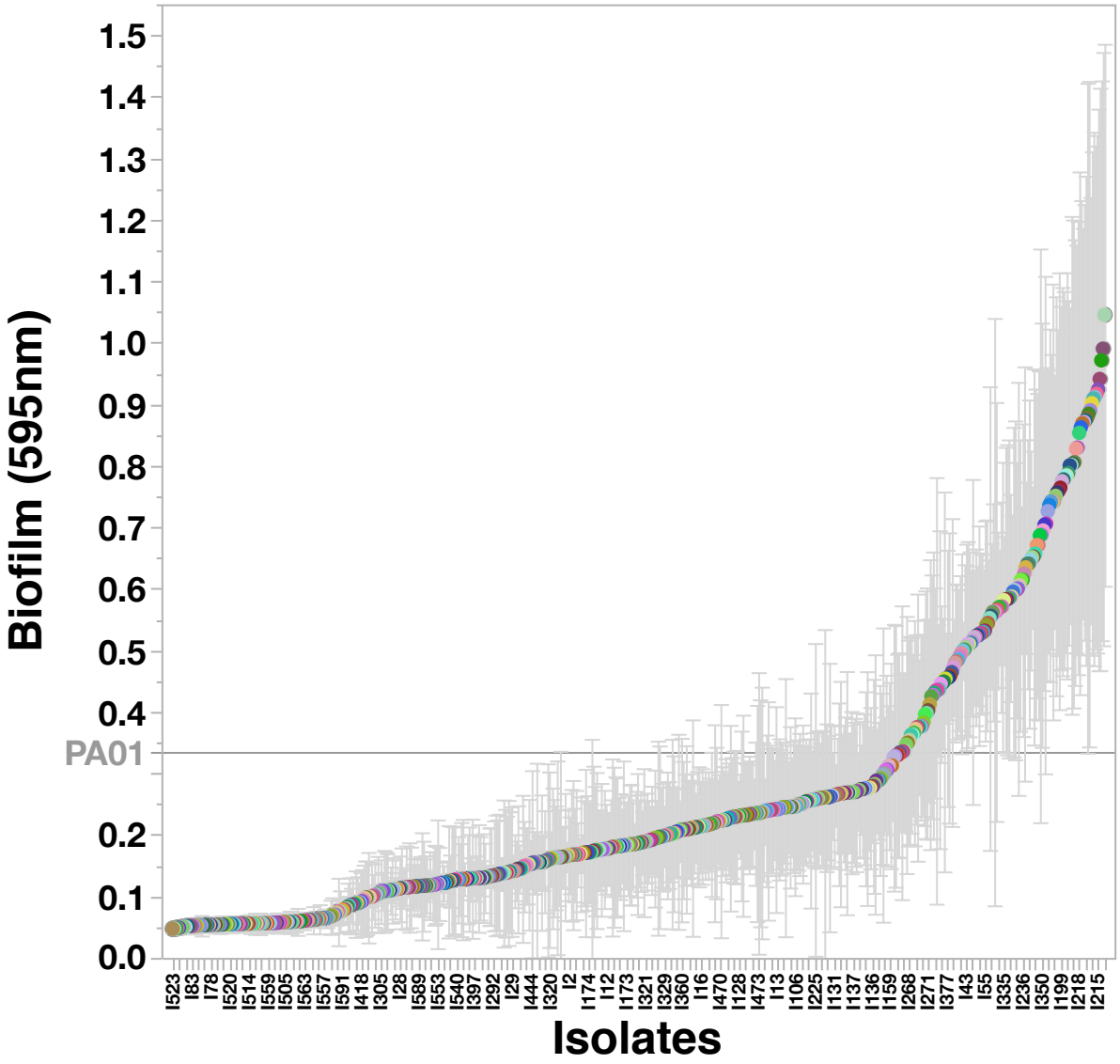


Figure 3-14: Measurements of biofilm by ascending order for all RBH isolates. All 593 isolates were ordered by ascending order according to the measurements from the biofilm production assays. Every point represents the mean value from at least 3 biological replicates for every phenotype. The error bars represent the standard deviation for each isolate. The horizontal reference line represents the mean measurement for the laboratory PA01 control.

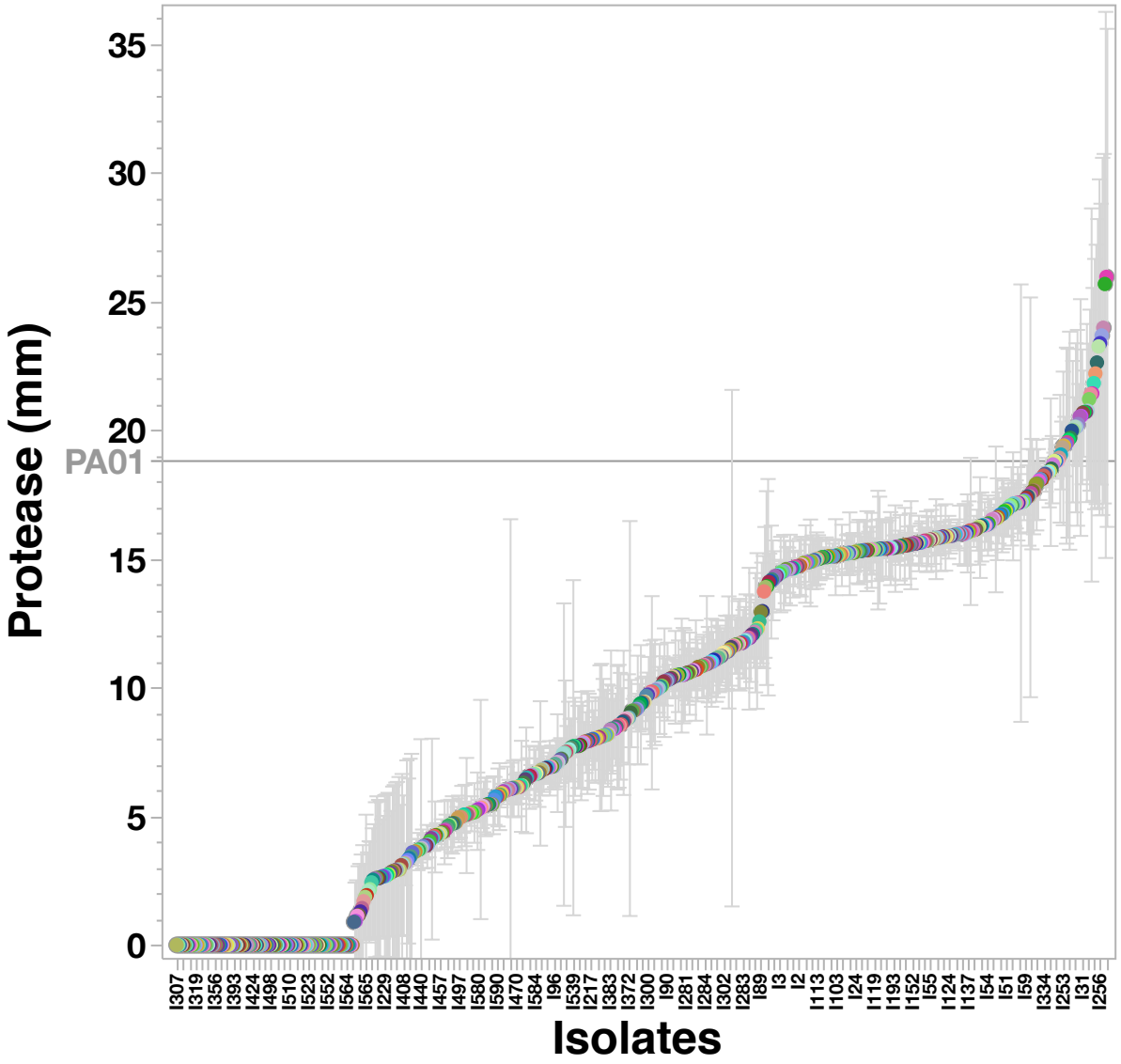


Figure 3-15: Measurements of protease by ascending order for all RBH isolates. All 593 isolates were ordered by ascending order according to the measurements from the protease production assays. Every point represents the mean value from at least 3 biological replicates for every phenotype. The error bars represent the standard deviation for each isolate. The horizontal reference line represents the mean measurement for the laboratory PA01 control.

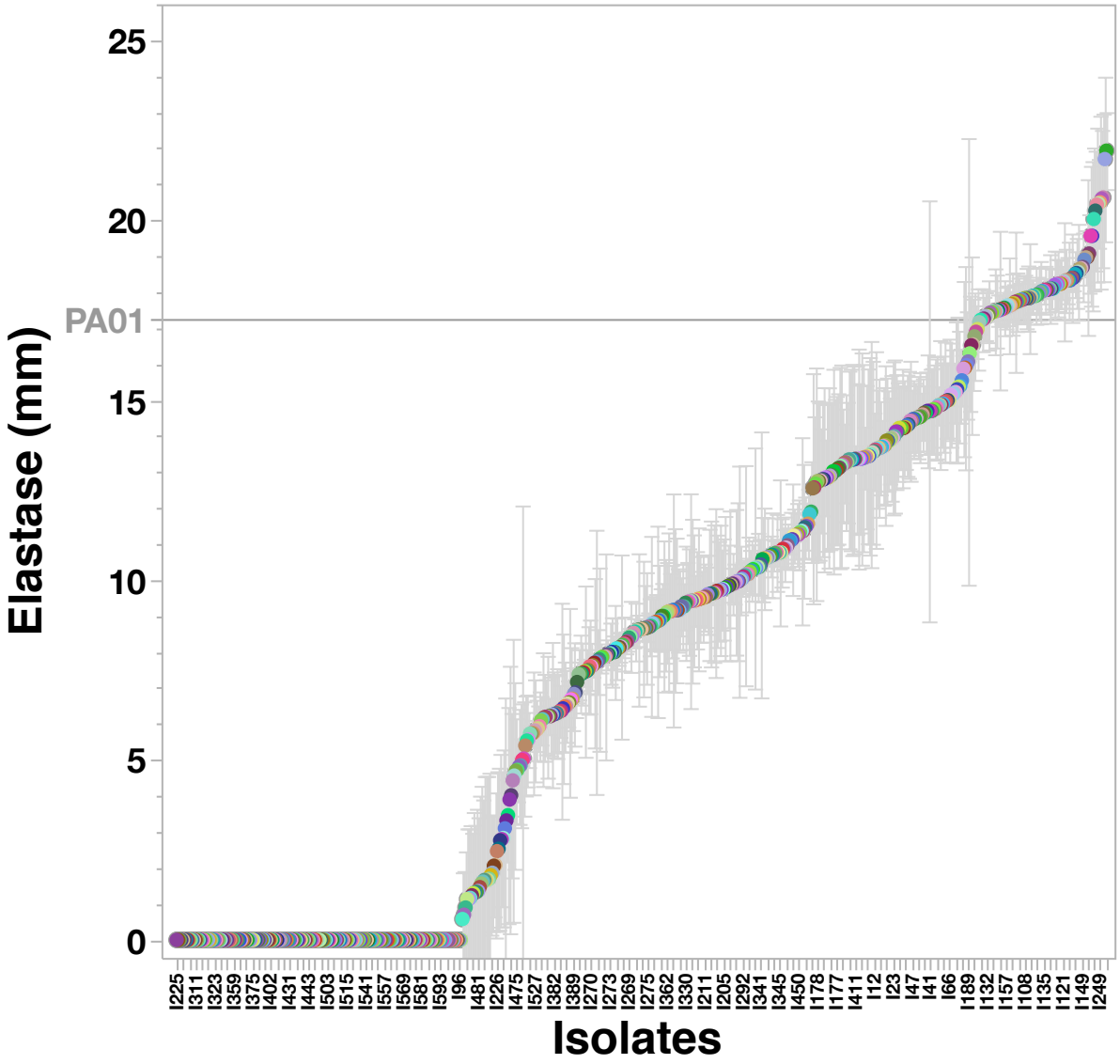


Figure 3-16: Measurements of elastase by ascending order for all RBH isolates. All 593 isolates were ordered by ascending order according to the measurements from the elastase production assays. Every point represents the mean value from at least 3 biological replicates for every phenotype. The error bars represent the standard deviation for each isolate. The horizontal reference line represents the mean measurement for the laboratory PA01 control.

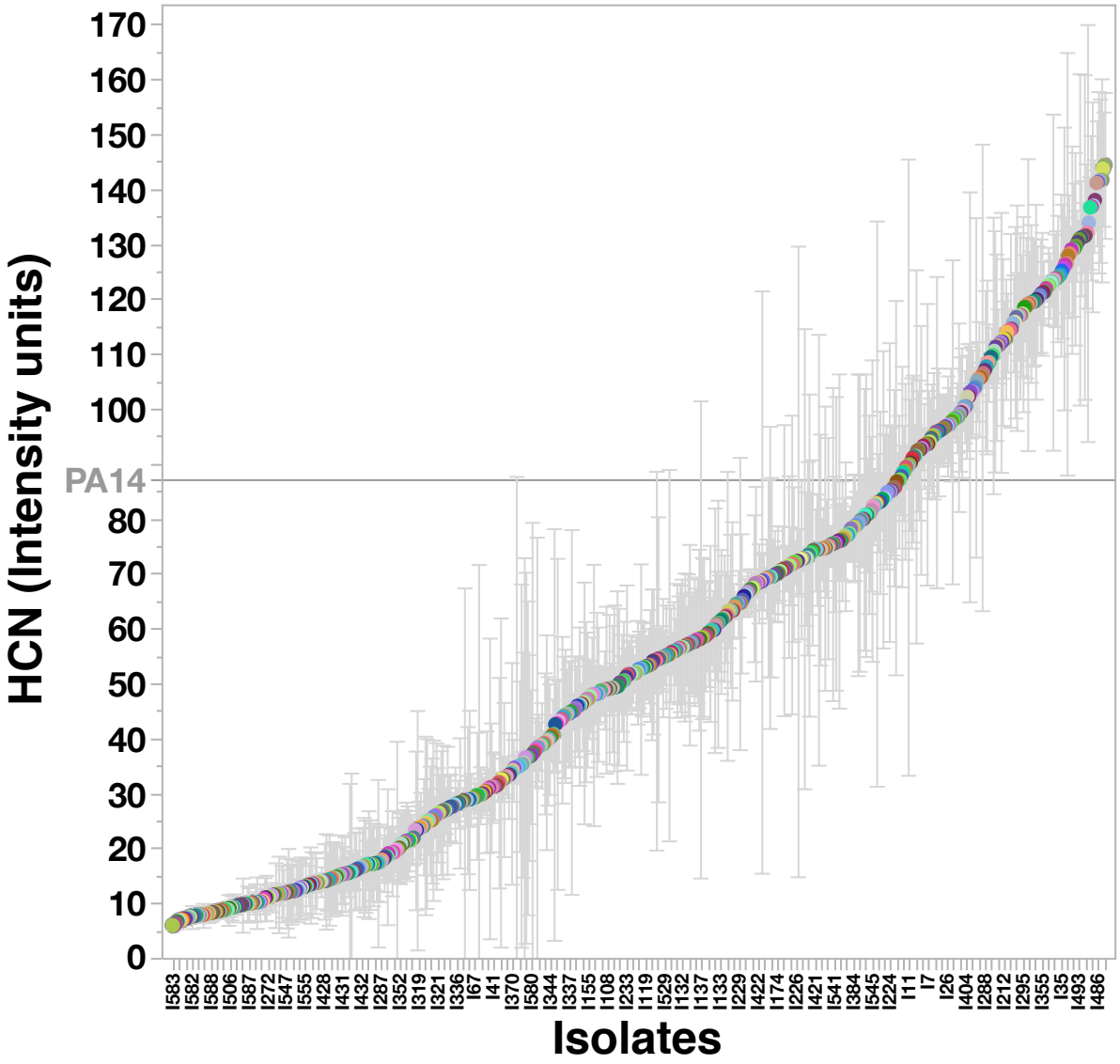


Figure 3-17: Measurements of hydrogen cyanide by ascending order for all RBH isolates. All 593 isolates were ordered by ascending order according to the measurements from the HCN production assays. Every point represents the mean value from at least 3 biological replicates for every phenotype. The error bars represent the standard deviation for each isolate. The horizontal reference line represents the mean measurement for the laboratory PA14 control.

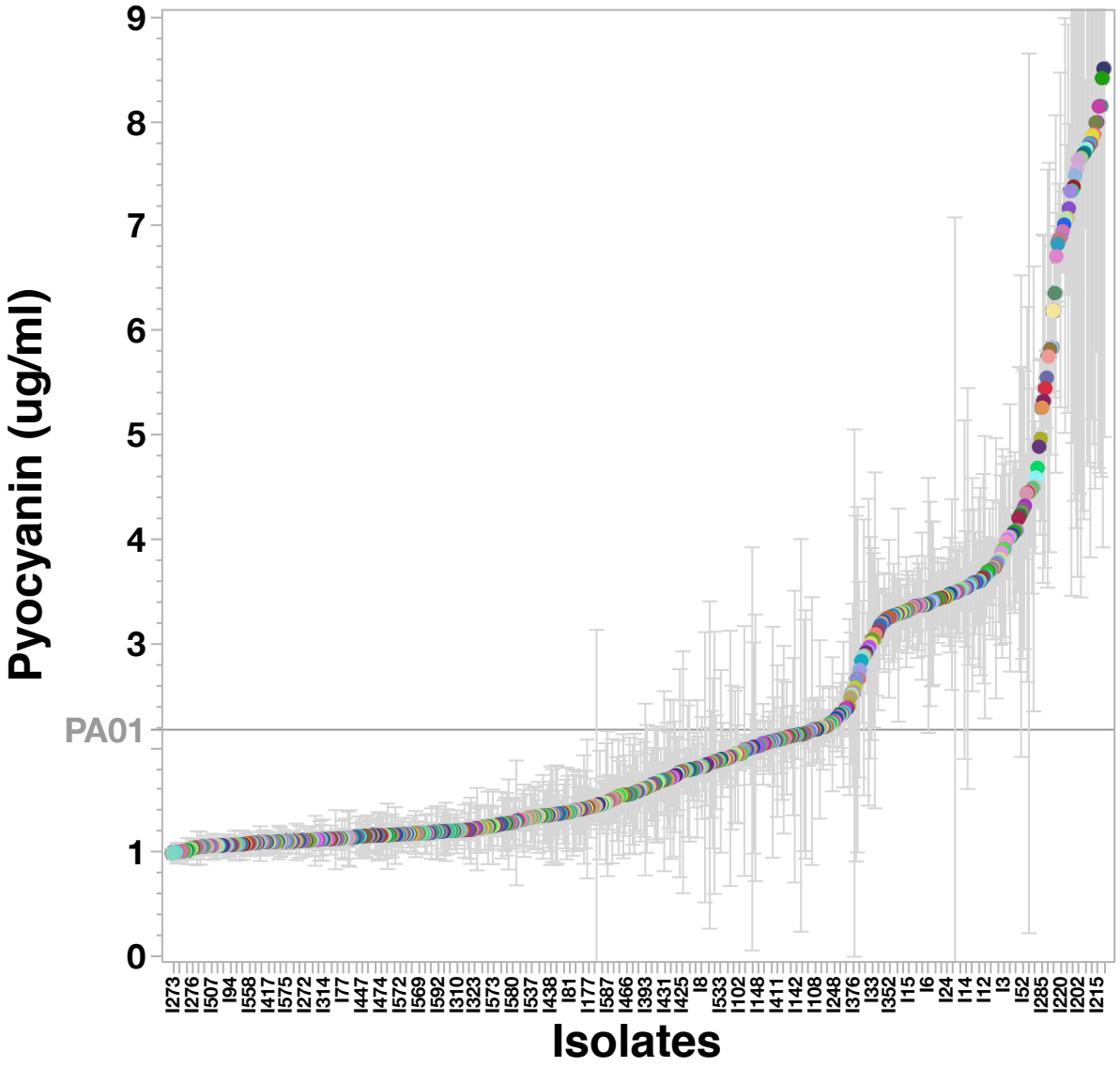


Figure 3-18: Measurements of pyocyanin by ascending order for all RBH isolates. All 593 isolates were ordered by ascending order according to the measurements from the pyocyanin production assays. Every point represents the mean value from at least 3 biological replicates for every phenotype. The error bars represent the standard deviation for each isolate. The horizontal reference line represents the mean measurement for the laboratory PA01 control.

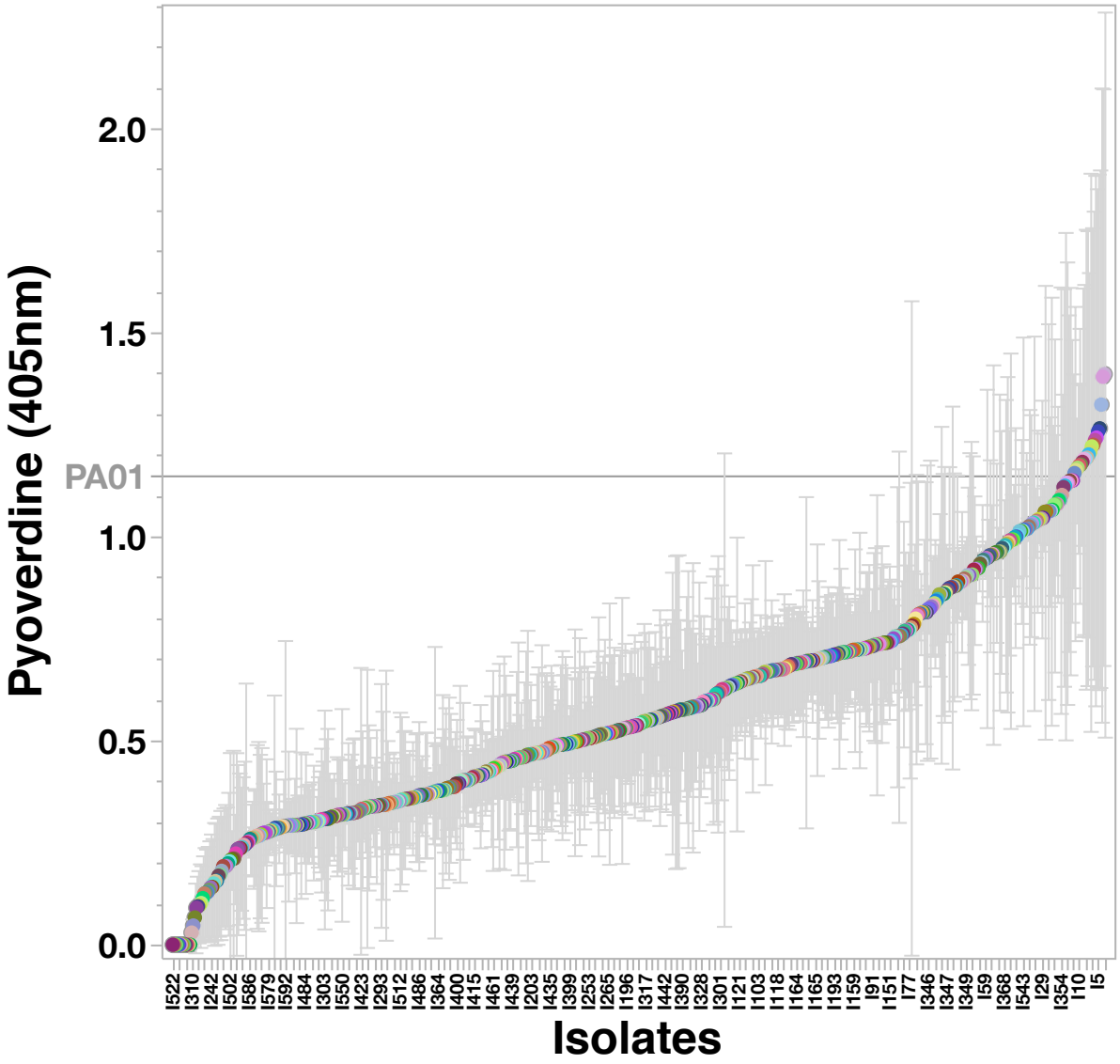


Figure 3-19: Measurements of pyoverdine by ascending order for all RBH isolates. All 593 isolates were ordered by ascending order according to the measurements from the pyoverdine production assays. Every point represents the mean value from at least 3 biological replicates for every phenotype. The error bars represent the standard deviation for each isolate. The horizontal reference line represents the mean measurement for the laboratory PA01 control.

3.3 Discussion

In this chapter the main aim was to introduce the clinical sets of strains used in the study as well as the phenotypic assays carried out using those isolates. The first section presented the two independent clinical sets of strains used in this study and their characteristics regarding the isolation strategy, the clinical information available about the patients and infection, and genotyping . The second part of this chapter presented the phenotypic data gathered. The need for data normalisation was considered, and the distribution of the data was analysed to understand how to handle this big data set. Finally, the validation of the various quantification methods used to measure pyocyanin was investigated.

3.3.1 Strengths and limitations of the UBC and RBH sets of strains

All clinical isolates used in this study, from both sets of strains, were retrieved either from sputum samples or cough swabs. In both sets of strains, sputum samples represented around 80% of the total samples collected. Both sampling methods used to isolate the bacteria used here are considered as indirect methods. Indeed, the bacteria can also be recovered directly from explanted lungs or bronchoalveolar lavages (Nixon et al., 2001, Jorth et al., 2015, Bjarnsholt et al., 2005). Although sputum samples are an indirect method of sampling, they have been shown to be as efficient to detect bacteria as the more invasive bronchoalveolar lavage method (Aaron et al., 2004a). Therefore, this sampling method is routinely practised as it presents the advantages of being straightforward and non-invasive.

In the case of young children, sputum samples can often not be obtained as these patients cannot expectorate. The available methods of sampling available then are either invasive bronchiolar lavages or cough swabs. Cough swabs can have a lower sensitivity for detecting bacteria than the other methods as they are less direct (Equi et al., 2001). All these indirect methods have the flaw of being unable to identify the region of the lung from which the sample originated. However, they remain the only way to collect samples from first-time infections and serially, as samples from explanted lungs can only be sampled at a single time point always characterised by late stage disease.

The first set of strains used in this study was already available in the laboratory, as the isolates were collected at the University of British Columbia (UBC) and previously sent. This UBC set consisted of 154 isolates from 16 patients collected in a longitudinal way for up to 24 years. It is rare to be able to have data collected over such a long time-span and for as many patients. Indeed, some studies have also used longitudinally collected sets of strains but often over shorter amounts of time, and also in only a few patients (Clark et al., 2015, Workentine et al., 2013).

The downside is that this long period of collection implies different people being involved in the isolation and all information was not shared. Therefore, the clinical information available was scarce. The information available concerned the length of infection and original morphotypes of the bacteria. This information showed that over 75% of isolates were either normal or mucoid, the two most common morphotypes respectively. Mucoid morphotype is a known adaptation of *P. aeruginosa* to the Cystic Fibrosis lung (Fegan et al., 1990). The other less common morphotypes were enterobacter-like, dwarf and rough. The dwarf, also called small colony variant (SCV), has been described in literature and seems to show enhanced virulence and antibiotic resistance (Häußler et al., 1999, Malone, 2015).

Morphotypes are a commonly recorded phenotype in studies (Lozano et al., 2018, Workentine et al., 2013, Vidya et al., 2016). However, the reproducibility of this descriptive trait can be questioned as the morphological classification will be highly subjective. Indeed, morphological characteristics used to classify isolates include, for example, the colour of the colony, its shape, its size, its surface texture, its opacity, which are all qualitative and descriptive (Vidya et al., 2016, Workentine et al., 2013, Mayer-Hamblett et al., 2014b). Overall mucoidy is the only morphotype clearly defined and easily identifiable. This multitude of categories to describe and separate morphotypes only increases the risk of subjective classification of morphologies which have no biological relevance, such as differentiating a tan versus a grey colony. Some studies have found morphotypes to be poorly predictive of other phenotypic traits (Clark et al., 2015, Ashish et al., 2013). Although different morphotypes suggest diversity, the same morphotype does not mean a lack of diversity, with studies finding isolates of the same morphotypes showing a range of different phenotypes (Workentine et al., 2013, Ashish et al., 2013). Therefore, no conclusions can be drawn from morphotype observation. Thus for the second set of strains collected specifically for this study, morphology was not selected as a phenotype and not recorded.

The second set of strains was, indeed, collected specifically for this study, through the Royal Brompton Hospital microbiology laboratory and with the help of Neil Madden, Senior Biomedical Scientist at RBH. The process of collection and storage was carried out to keep as much as possible the in vivo characteristics of the bacteria. To do so the bacteria were isolated with as little manipulation as possible as passaging bacteria several times is known to result in cumulative mutations (Kugelberg et al., 2005). By limiting the steps of collection, the chances of any changes in the in vivo phenotypic traits were reduced. A limitation of the collection process was, however, the manual picking of single well defined and separated colonies, so as to get isolates stemming from one original bacterial cell. This inherently introduced bias as it meant colonies that were overlapping or merged were not selected. This may have led overall to the selection of more similar isolates, which, for example, grow slower or are smaller and have more chance not to merge with others on a plate culture. Merged colonies could have been picked and restreaked to be better separated, but this would have introduced the issue of passaging described above.

From the 24 samples which constitute the RBH set of strains, 7 samples detected *P. aeruginosa* for the first time in those patients, therefore allowing the collection of early isolates. All other isolates were collected from chronically infected patients. It was impossible to obtain a balanced number of early and chronic isolates due to the relative rarity of newly infected patients at the RBH clinics. The early isolates collected are the first time *P. aeruginosa* had been recorded in these patients. Nevertheless, there is a low possibility that the strain could have been missed in the previous routine check-up due to the low sensitivity of sampling. For each sample, multiple isolates were collected to enable the observation of the diversity within patients. Also, having both early and chronic isolates enabled the examination of the phenotypic changes between stages of infection but also the changes regarding diversity. Other studies have also studied diversity but only in chronically infected patients (Jorth et al., 2015).

Clinical information about the patients was gathered from the database of the RBH, giving insight into the patients' health as well as their infection status. The clinical information confirmed that the chronically infected patients were significantly older than patients infected for the first time, with the average age of chronically infected patients of 26.6 years. According to the CF Trust annual report 2018, over 50% of patients between 24 to 27 years are chronically infected. Regarding the mutations causing CF, 47.8% of patients were homozygous for

phe508del CFTR mutation, and another 39.1% of patients were heterozygous. Thus, the proportion of each mutation as well as the ages observed in the population selected is a good representation of what has been found in general CF patients (Tsui, 1992).

The FEV1% predicted is a good measure of the pulmonary health of patients. The mean of the FEV1 for chronically infected patients was around 52%, showing the severity of their disease. Also, the older were the patients, the lower was their FEV1, which is commonly found as disease progresses and often worsens with age (Corey et al., 1997).

Finally, it is known that persistent *P. aeruginosa* infections can lead to increased risk of exacerbations in turn leading to inflammation and damage in the lungs (Mayer-Hamblett et al., 2012, Brockbank et al., 2005, Goss and Burns, 2007). The majority of chronic patient samples were recovered during or just after an exacerbation.

The clinical data presented in this chapter sets the picture of the range of patients from which bacteria was collected. Nevertheless, patients' parameters linked to overall health such as FEV1 and number of exacerbations result from a multitude of factors, some unrelated to infection such as the type of genotypic mutation causing CF, age of diagnosis, prescription of and adherence to therapies. However, studies have found direct links between infection status and some other clinical features, such as gender (Chotirmall et al., 2012, Demko et al., 1995, Wang et al., 2010). Due to the relatively small number of patients, no reasonable conclusions can be drawn here regarding the clinical information. Nevertheless, the patients' clinical data shows that the set of strains used in this study stem from a group of patients which are representative of the general CF population.

3.3.2 Genotyping outcomes and meaning

A proportion of both the UBC isolates and RBH isolates were genotyped. The UBC strains typing was undertaken using RAPD typing whereas the RBH isolates were MLVA typed. Many studies used RAPD typing on *P. aeruginosa* isolates a few decades ago but it is becoming less widely used today (Ghazi et al 2016; Waters et al 2012). Indeed, in comparison to other techniques it is not very discriminatory between strains (Waters et al 2012). The MLVA typing technique used for this project was optimised by PHE to amplify 9 VNTR regions (Turton et

al., 2010). This is the technique routinely used by PHE to type *P. aeruginosa* and it is recognised as being highly reproducible and easily interpreted (Chen et al., 2018, Maâtallah et al., 2013, Libisch, 2013).

In total 96 isolates of the 154 from the UBC set were genotyped at the UBC. Between half and all isolates were typed for 15 out of 16 patients. Over time the same strains were repeatedly isolated from individual patients, with the same strain type identified for all isolates in 13 patients. In only 2 patients several different strains were genotyped over time. A large variety of strains between patients was identified, with 15 different strains identified and only 2 found in more than one patient.

Regarding the RBH strains, half of the chronic isolates were typed specifically for this project. Only chronic strains were typed with the assumption that, most likely, only one strain is responsible for early stage infections. Indeed previous studies have found that most early stage infections are caused by a single strain (Hall et al., 2014). In addition, records were reviewed to find the routine MLVA typing of a patient's *P. aeruginosa* isolates, closest to the samples taken specifically for this study. All typing undertaken specifically for this study concurred with the data recovered from records for each patient. The same strain type was consistently found for all 12 isolates from 9 chronic patients specifically typed. Also, clinical records from the RBH showed that the majority of patients were infected by the same strain at each routine typing year after year.

In conclusion, the typing of isolates from both sets of strains showed that the vast majority of patients with *P. aeruginosa* are chronically infected by a single strain. This finding of only one lineage per patient was consistent with several studies (Jorth et al., 2015). This was also supported by the PHE database on all VNTR typing undertaken for the RBH patients, with most patients being recurrently infected by the same VNTR type over multiple years.

Many individual strains were identified, with a high proportion of unique ones as well as a few well documented ones such as epidemic strains (Jones et al., 2001, Al-Aloul et al., 2004, Martin et al., 2013, Wright et al., 2015). Previous studies on the other hand have focused on sets of strains composed only of epidemic *P. aeruginosa* (Mowat et al., 2011, Williams et al., 2015). Having a wide range of unique strains was advantageous for the subsequent phenotypic investigation. Indeed, any commonalities in results observed would be due to common

adaptation linked to the pressures of the CF lung, and not due to specificities of a certain type of strain. In the UBC strain set the same genotypes were found over time, but various different morphotypes were nevertheless recorded. This shows that although isolates stem from the same original strain, individual adaptations do occur. These differences amongst the same strains highlight the importance of phenotypes, as many genes are involved in the regulation and expression of certain traits.

Overall MLVA genotyping is a useful technique to know if isolates have in fact evolved from a common single ancester. Nonetheless, this technique does not enable us to delve into important mutations that accumulate during the adaptation to the CF lung. Indeed, next generation sequencing is more costly but would give further information about SNPs and mutations differentiating isolates of a same strain. Further genotyping, using for example NGS, would be beneficial in the continuation of this project to bring more valuable insight regarding the link between genotypic and phenotypic adaptation of *P. aeruginosa*.

3.3.3 Phenotypic measurements

In this study 10 phenotypic assays were undertaken using the RBH set of strains, as well as the measurement of a few key quorum sensing regulatory molecules. All the phenotypes tested were carefully chosen as they are involved in establishing infection, chronicity and linked to virulence leading to lung health degradation. The phenotypes measured were motility (swimming, swarming and twitching), speed of growth, ability of attachment to produce biofilms, production of HCN, proteases and specifically elastase, pyocyanin and pyoverdine. No previous study has focused on such a wide range of different phenotypes linked to virulence and chronicity. Studies looking at phenotypic adaptation have often focused instead on antibiotic resistance, limiting the knowledge about the changes in other phenotypes. Indeed, there has been very little focus on the importance of the speed of growth as well as the production of HCN in the CF lung. The phenotypes chosen for this study represent a good range of potential biomarkers to better follow the path to chronic infection and to situations of poor outcome. On the other hand, not measuring antibiotic susceptibility can also be considered a limitation, restricting the possibility of comparing results with other studies and building on the body of work already known about antibiotic resistance. In addition to the 10 phenotypes measured, testing antiobitc susceptibility to several antibiotics in a fully quantitative manner

would have added a significant amount of work. This could, however, be a next step for the continuation of this study.

3.3.4 Validation of measurements: Pyocyanin can be readily measured using low-cost high through-put methods.

Pyocyanin is a blue phenazine molecule produced by *P. aeruginosa* having been extensively studied as it has a direct role in virulence. This phenazine has been shown to be critical in helping the bacteria establish infection (Lau et al., 2004). When produced in the CF lung by the bacteria, pyocyanin causes oxidative stress leading to damage to host cells (Allen et al., 2005, Arora et al., 2018, Usher et al., 2002).

The production of pyocyanin by the bacteria can be measured through various methods. Three methods were undertaken in this study to be cross validated, as many studies have been using one or another of these methods. Firstly, pyocyanin can be measured using mass spectrometry (Wilson et al., 1988). This first method is the most quantitative due to the direct measurement of the pyocyanin itself. Nevertheless, this method is not cost efficient as it involves the use of expensive equipment. Also, access to a mass spectrometer and the expertise to analyse the results is not available in all laboratories. In this study these measurements were undertaken by Dr. Volker Behrends from the University of Roehampton. Due to the previously mentioned cost limitations, only one biological replication for each isolate was possible. Therefore, it is a good method to validate others which can be routinely and more readily used.

The second method involved the extraction of the pyocyanin in chloroform. This method is a standard benchmark method that has been used for many years (Diggle et al., 2003, Ugurlu et al., 2016, Rada and Leto, 2013, Wilson et al., 1988, Wilson et al., 1987). The drawback is the need to use chloroform, which is a harmful and carcinogenic chemical needing to be handled under a chemical flow hood. This method does, however, allow concentrations of pyocyanin in μg/ml to be obtained (see Chapter 2).

Finally, pyocyanin production was quantified from a direct absorbance method of bacterial supernatant. This method is the quickest and easiest to perform and therefore has been used in

several studies in recent years (Reszka et al., 2004, Das and Manefield, 2012, O'Brien et al., 2017, Bragonzi et al., 2009). The drawback of this method could be the lack of an extraction phase and the direct measurement from the bacterial supernatant. Indeed, due to the abundance of other molecules in the supernatant, an overlap of the spectrum of pyocyanin and of other molecules such as pyoverdine or pyochelin is possible.

Overall the results obtained for all three methods strongly correlated with one another. This cross-validation showed that any of these methods were suitable for the measurement of pyocyanin. Thus, only one set of data was needed for the rest of this study. Due to the lack of replication the MS data could not be chosen. The chloroform method was chosen for the RBH strain set, while the supernatant had to be used for the UBC isolates, for which the chloroform method was not measured.

3.3.5 Normality of the distribution of the data

To be able to use the phenotypic data for analysis, its statistical distribution needed to be understood. Indeed, the analysis approach of data is dependent on several factors such as the sample size tested, the number of tests, the replicability of the measurements, and the distribution of the data. In many cases normality is often presumed, which can lead to the misuse of statistical tests and skewed conclusions. In this study several isolates were available for each of the 24 patients, thus normality for each patient and phenotype was tested separately. Testing normality can be done visually or by several tests; a well recognised test used here is the Shapiro-Wilk test (Ghasemi and Zahediasl, 2012).

The data is overall not normally distributed and this needs to be accounted for by using non-parametric tests for analysis. It is important to note that although all the data is not spread similarly, non-parametric tests are suitable for normally distributed data as well as non-normal (Cleophas and Zwinderman, 2011). Therefore, the same analysis strategy can be used for all the data irrespective of how it is spread. Nevertheless, it has been shown that in sets of data with large samples classical t-tests and linear regressions can still safely be used (Lumley et al., 2002).

3.3.6 Importance of quantitative measurements

Measuring phenotypes in bacteria can be done through categorising measurements as positive or negative, or through quantitative measurements. When all phenotypes were displayed in ascending order, they showed a continuous range of measurements, underlining that none of these phenotypes could be categorised satisfactorily in a binary way. Another study on one single patient also found that phenotypes could not be categorised as either present or absent (Workentine et al., 2013). As precious information and data would be lost, not quantitatively measuring phenotypes could lead to underestimating changes in phenotypes which could be important biologically in the CF lung.

Phenotypes such as pyocyanin, pyoverdine, HCN and proteases are regulated by the quorum sensing system. These intricate regulatory systems can be envisaged as leading to complex regulation of these phenotypes through several pathways causing different expression levels and varying extents of the phenotypic traits (Pessi and Haas, 2001, Pessi and Haas, 2000, Reimmann et al., 2003, Reimmann et al., 1997, Pearson et al., 1997, Dietrich et al., 2006, Stintzi et al., 1998) .

Studies have shown a decrease in swimming motility of *P. aeruginosa* in chronic infections (Hogardt and Heesemann, 2010). The current common hypothesis is that flagellin expression decreases over time as it activates the signalling pathway for immune activation and phagocytosis (Balloy et al., 2007). However, recent studies have underlined the importance of motility itself in the evasion of the innate immune system (Amiel et al., 2010, Arora et al., 2005). *P. aeruginosa* flagella possess two reversible motors MotAB and MotCD (Doyle et al., 2004). These two motors work together to ensure swimming and swarming although the MotCD motor specifically enables motility in more viscous environments such as those encountered at higher agar concentrations (Toutain et al., 2005).

Studies have shown that the loss of motility and not the loss of the flagellum itself is critical for the resistance to phagocytosis (Arora et al., 2005). Another study showed that the amount of phagocytosis observed was proportional to the rotary force of the flagellum motors. Thus the loss of motility in a step-wise manner progressively gives increased ability to *P. aeruginosa* to evade phagocytosis (Lovewell et al., 2011). We can hypothesise that the partial motilities

observed over time could be due to the presence of only one functional set of the flagellar motors. Therefore, the intermediate level of swimming motility may have a biological importance for the survival of the bacteria.

These examples underline the risk of categorising phenotypes, which would lead to underestimating again the true diversity of the phenotypic traits. Therefore, the choice of developing all the phenotypic assays to be quantitative was validated by the fact all phenotypic readings were found to be continuous throughout the measurement range. On the other hand, the decision to only include quantitave results to this study while investigating a large number of isolates introduces a limitation as to the number of time points used to measure the phenotypes, as well as the type of assays.

Chapter 4: Evolution of quantitative *P. aeruginosa* phenotypes over stages of infection and associations between phenotypes and clinical data

4.1 Introduction

As introduced in Chapter 3, this study used two different set of strains in order to investigate the phenotypic adaptation of *P. aeruginosa* to the CF lung. The first set of strains was longitudinally collected over time and the second set consisted of single time-point isolates stemming from either first-time infection or chronic infections. For all isolates 10 phenotypic traits associated with colonisation and chronic infection of the CF lung were assayed. Levels of regulatory QS molecules were also measured and the evolution of these traits and their correlations with the phenotypes they regulate were investigated.

It has been widely accepted for some time that a decrease in virulence occurs in chronic infection, however the extent of the change and differences between phenotypes remain unclear (Luzar and Montie, 1985, Luzar et al., 1985, Burke et al., 1991). Thus, in this chapter we use the phenotypic data set available to further investigate the key phenotypic changes that occur over the course of *P. aeruginosa* infections. Through the investigation of correlations between phenotypes and between phenotypes and clinical data we unveil some associations, which help further understand the underlying pressures leading to these changes and their effects. Understanding the patterns of phenotypic changes that lead to the establishment of long- term infections is critical in order to try and prevent the chronicity of the infections and thus improve clinical outcome.

The aims of the chapter are to:

- Assess the changes and evolution of phenotypes over the length and stages of infection.
- Investigate correlations between phenotypes.
- Investigate correlations between phenotypes and the levels of quorum sensing molecules.

- Investigate links between phenotypes and patient clinical data.

4.2 Results

4.2.1 Evolution of phenotypes over stages and length of infection

We first looked at whether there were any correlations between phenotypes and length of infection using the UBC strain set. Spearman correlations were undertaken to evaluate this evolution over time. The correlations were evaluated using 132 isolates out of the 154 available in total from 15 out of the 16 patients. As presented in the previous chapter, several patients were infected with multiple strains over the course of infection. As patient 3 in this data set was infected with multiple strains over the time course of infection their data was excluded. Similarly, patient 10's data was only analysed for the time period the patient was infected with the initial infecting strain.

The results show an overall decrease of speed of growth, motility and production of virulence factors and biofilm with length of infection. Spearman coefficients ranged from -0.08 to -0.31 with correlations being significant ($p<0.05$) for all but 3 phenotypes, with changes in the production of hydrogen cyanide, pyocyanin and biofilm formation not being significant over the length of infection. The other 7 phenotypes showed a significant correlation with coefficients between -0.18 and -0.31. (Table 4-1).

Phenotypes	Spearman coefficient	p value
Growth	0.212	*
Swim	-0.1852	*
Swarm	-0.23	**
Twitch	-0.2285	**
Protease	-0.3093	***
Elastase	-0.2497	**
Hydrogen cyanide	-0.1292	NS
Pyocyanin	-0.0389	NS
Pyoverdine	-0.2157	*
Biofilm	-0.0846	NS

Table 4-1: Spearman correlations between all phenotypes and length of infection using the UBC set of strains. The correlations were evaluated over 132 isolates from 15 different patients. NS: non significant; * $p<0.05$; ** $p<0.01$; ***$p<0.001$. $n\geq3$ for phenotypic assays.

Next the RBH strain set was investigated comparing phenotypes of early and chronically infected stage isolates. In this set multiple isolates of *P. aeruginosa* were collected from each patient sample with a range of 12 to 60 isolates per patient sample, so in statistical terms, the isolates were nested within patients. Also, all isolates from a patient resulted from the same original strain. In the context of looking only at the overall difference between early and chronic stages of infection, these multiple isolates from the same patients might be considered as repeated measures if differential adaptation within the lung is overlooked.

Because of these multiple isolates two analysis approaches were undertaken in parallel. The first approach (full data approach) was to use the data from all isolates stemming from all patients (Figure 4-1). The second (means approach) was to use only the means of all isolate measurements for each patient and phenotype (Figure 4-2). This second approach limits the number of data points, with only 7 early stage infection and 17 for chronic stage infection, but prevents data resembling repeated measures.

Both approaches show the same trends with regard to phenotypic differences between stages of infection. All median phenotype values are lower in the chronic group than the early, except for growth rate where the higher median equals slower growth. Nonetheless some differences

are observed, for example regarding the medians, which seems most obvious in the case of twitching.

Figure 4-1 and Figure 4-2 present quartile boxplots for each phenotype with the early stage isolates plotted alongside the chronic infection isolates. The boxes themselves represent the data between the 25th and 75th percentile, meaning 50% of the data is found within the box. Both figures show that for all phenotypes the range of measurements for early and chronic stages of infection overlaps. Moreover, most phenotypes show the presence of outliers, indicating some phenotypic diversity both in early and chronic isolates. Nonetheless, half the phenotypes do not show an overlap of the boxes between early and chronic stages of infection. The biggest overlap in early and chronic measurements seems to be for twitching and production of hydrogen cyanide.

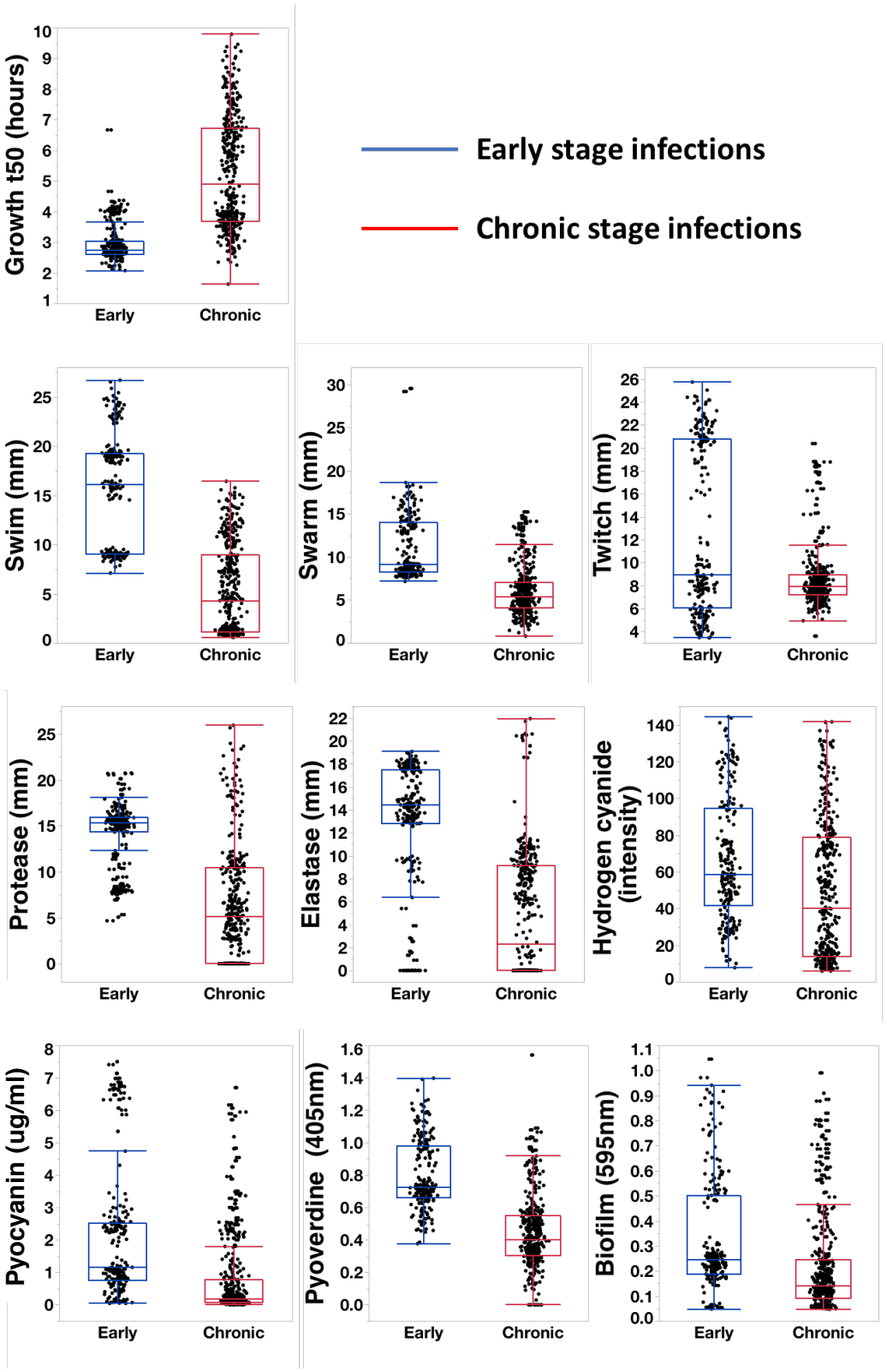


Figure 4-1: Evolution of phenotypes between early and chronic stages of infection of RBH isolates using the full data approach. Each dot represents the mean of at least 3 biological replicates (n≥3) for each individual isolate from all patients infected either for the first time (early: in blue) or chronically (chronic: in red). Boxes and whiskers represent the median and interquartile range for each phenotype and stage of infection.

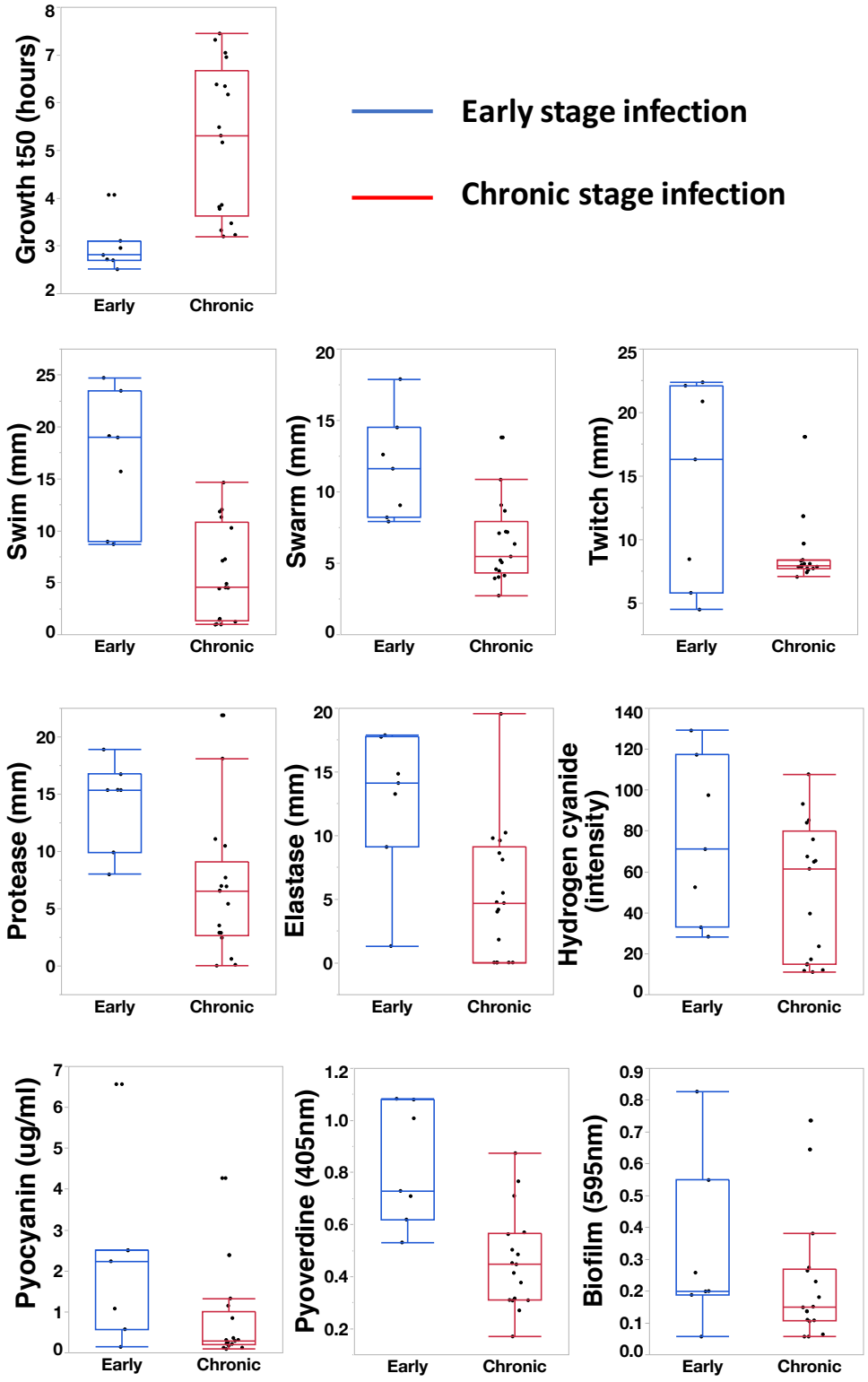


Figure 4-2: Evolution of phenotypes between early and chronic stages of infection of the RBH isolates using the patients means approach. Each dot represents the mean of all measurements of the isolates of a single patient. Each individual measurement corresponds to the mean from at least 3 biological replicates (n≥3) for each individual isolate from all patients infected either for the first time (early: in blue) or chronically (chronic: in red). Boxes and whiskers represent the median and interquartile range for each phenotype and stage of infection.

In order to quantify the changes observed, statistical analysis was done and both data sets and approaches were undertaken.

In the previous chapter the distribution of the data was tested. Given that most of the data seemed not to follow normal distribution the non-parametric Mann-Witney U test was used for statistical analysis.

In the case of the full data approach, all phenotypes showed a highly significant change between early and chronic stages of infection (Table 4-2). The results using the means approach showed a similar trend, but the change was not statistically significant for twitching motility, production of HCN and biofilm formation (Table 4-3). Overall a decrease in speed of growth, motility and production of virulence factors was observed, corresponding to what was visually observed. Although the results conclusions are similar over both approaches, the values for the full data approach were higher. Z scores were 3- to 7-fold higher for the full data approach than the means approach. This is due to the means approach being underpowered due to the much smaller number of data points, especially for the early infection isolates.

Phenotypes	median early	median chronic	Mann-Whitney U	
			z score	p value
Growth	2.73	4.88	16.61	***
Swim	16.13	4.30	-16.30	***
Swarm	9.14	5.33	-15.94	***
Twitch	8.97	7.93	-4.43	***
Protease	15.35	5.13	-14.33	***
Elastase	14.43	2.27	-15.34	***
Hydrogen cyanide	58.40	40.03	-5.98	***
Pyocyanin	1.16	0.18	-11.91	***
Pyoverdine	0.72	0.40	-15.59	***
Biofilm	0.24	0.14	-8.12	***

Table 4-2: Statistical analysis using the "full data approach" of early stage infection in comparison to chronic stage infection for all 10 phenotypes tested. All statistical analysis was undertaken on phenotypic data which corresponded to the mean n≥3 biological replicates for phenotypic assays. *** $p < 0.0001$.

Phenotypes	median early	median chronic	Mann-Whitney U	
			z score	p value
Growth	2.80	5.30	3.30	**
Swim	18.96	4.51	-3.11	**
Swarm	11.60	5.45	-2.92	**
Twitch	16.28	7.88	-1.33	NS
Protease	15.33	6.53	-2.67	**
Elastase	14.10	4.67	-2.42	*
Hydrogen cyanide	70.96	61.34	-1.52	NS
Pyocyanin	2.23	0.28	-2.03	*
Pyoverdine	0.73	0.45	-2.92	**
Biofilm	0.20	0.15	-1.14	NS

Table 4-3: Statistical analysis using the "means" approach of early stage infection in comparison to chronic stage infection for all 10 phenotypes tested. For each patient and phenotype, the mean of all isolate readings was averaged and the mean was used here. The analysis used 7 data points for early and 17 for chronic stage infection. All statistical analysis was undertaken on phenotypic data which corresponded to the mean of n=3 at minimum. NS: non-significant; * p value <0.05; ** p value <0.01; *** p value <0.0001. n≥3 for phenotypic assays.

Finally, a multivariate method involving Principal Component Analysis (PCA) was also used to analyse the data, which allowed the data to be visualised globally. PCAs were performed using the data from all 10 phenotypes for both sets of strains.

According to the Kaiser criterion, dimensions having eigenvalues greater than 1 were selected for further analysis using their eigenvectors (Kaiser, 1960). For both sets of isolates, the first three principal components were selected, representing together 67.2% and 72.9% of the total variance for the UBC and RBH set respectively (Table 4-4). The first component seemed to best separate the isolates based upon the length or stage of infection. The first component explains the most variance and thus shows the most important phenotypes explaining the phenotypic changes over time and stages of infection. This first component in both cases involves motility and proteases, and these phenotypes seem grouped according to loading plots (Figure 4-3 and Figure 4-4). The second and third components together are explained overall by the same phenotypes which are speed of growth, pyocyanin, biofilm and HCN production. Both speed of growth and HCN seem to mostly not be grouped to other phenotypes.

	RBH			UBC		
	PC1	**PC2**	**PC3**	**PC1**	**PC2**	**PC3**
Eigenvalue	4.618	1.609	1.064	3.745	1.743	1.235
Proportion of variance (%)	46.179	16.088	10.635	37.452	17.427	12.349
Cumulative variance (%)	46.179	62.266	72.901	37.452	54.879	67.227
Eigenvectors:						
Growth	-0.278	**0.403**	0.039	-0.194	0.180	**0.623**
Swim	**0.394**	-0.101	0.182	**0.320**	-0.083	-0.293
Swarm	**0.341**	-0.315	**0.327**	**0.386**	-0.007	-0.045
Twitch	0.282	-0.272	-0.025	**0.337**	-0.227	0.301
Protease	**0.387**	0.051	-0.240	**0.466**	-0.087	0.066
Elastase	**0.366**	0.100	-0.141	**0.435**	-0.040	0.134
Pyocyanin	0.251	**0.554**	0.062	0.231	**0.580**	0.151
Pyoverdin	**0.377**	-0.061	-0.105	0.119	**0.639**	0.143
Biofilm	0.250	**0.449**	**-0.426**	0.268	-0.278	**0.370**
HCN	0.143	**0.360**	**0.764**	0.228	0.278	**-0.480**

Table 4-4: Principal component analysis for RBH and UBC isolates. PCA performed on 10 phenotypes. The eigenvectors in bold represent the highest for each principal component. All phenotypes with eigenvectors under 0.3 are considered as having a low contribution in the principal component.

The results for the RBH isolates show an obvious grouping of the early isolates towards the right hand side of the score plot in the case of the plots for PC1-PC2 and PC1-PC3 (Figure 4-3). The only overlap observed is for a few chronically infected patients which shows the phenotypic pattern of early patients. This is particularly the case for chronic patients CP2 and CP6 while early patients EP4 is the closest to chronic strains on the score plots. Other chronically infected patients show more similar phenotypic patterns.

For the UBC strains (Figure 4-4), the score plot using PC1 and PC2 and similarly for PC1 and PC3 show that the isolates infecting patients for < 2.5 years are in majority found on the right part whereas those present the longest show a shift towards the left. Indeed, in the isolates of <2.5 years old 65% are on the right side of the graph and 35% on the left side whereas over time the isolates infecting for >17 years show the opposite pattern, 67% on the left and 33% to the right of the graph. The isolates infecting for 2.5 to 17 years are equally distributed between

both sides of the graph. Nonetheless, all the isolates the furthest to the right side remain the ones having been infecting patients for < 2.5 years.

Overall the observation of the loading and score plots show the shift that occurs between early and chronic stages of infection corresponded to a general loss of virulence, particularly of proteases and motility and a slower growth. For both the UBC and RBH strains, a shift was observed. In both sets of PCAs early isolates mostly separated according to motility, whereas chronic mostly according to growth, which is slower.

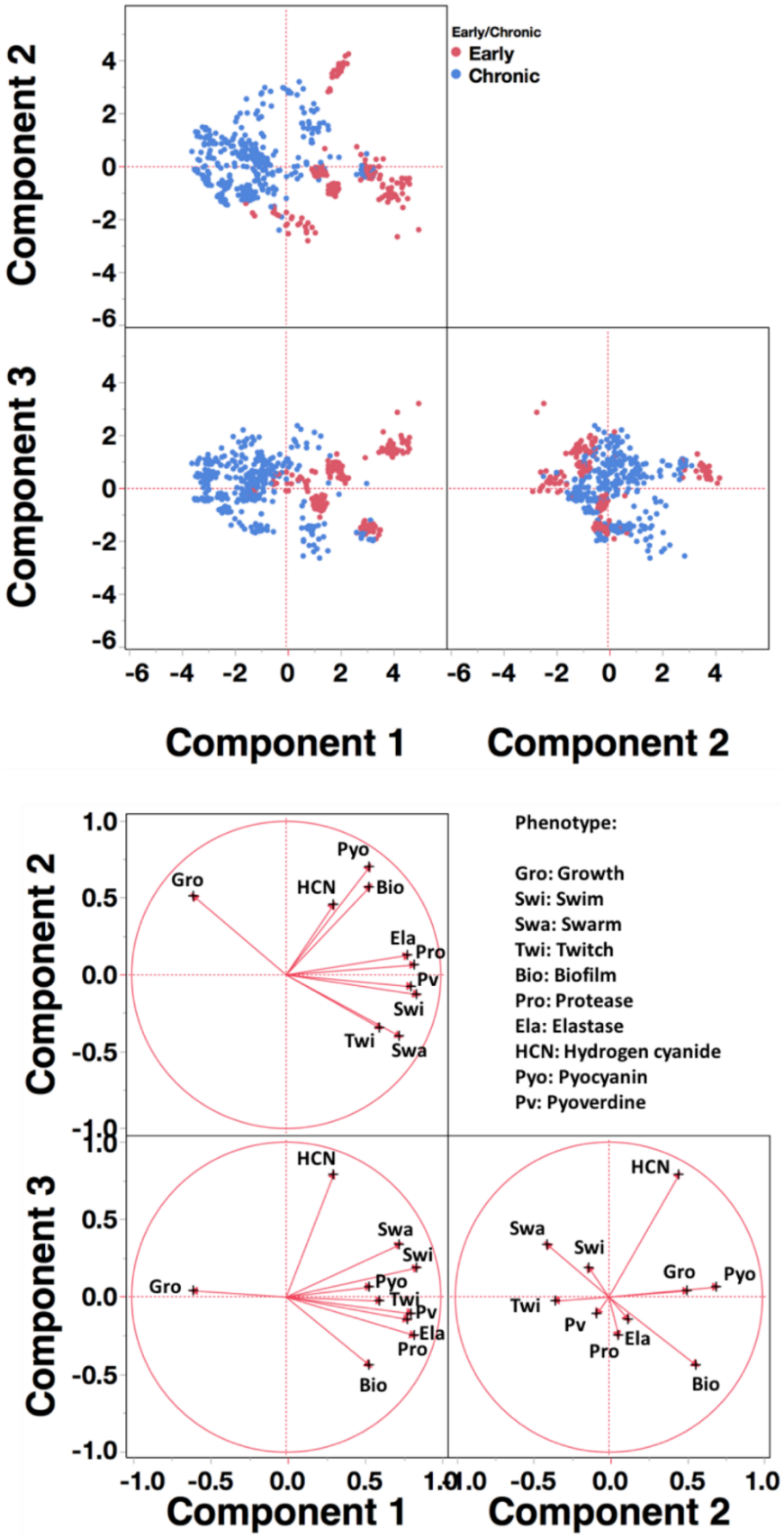


Figure 4-3: PCA score plots and loading plots from the RBH data set. The data from all 10 phenotypes was used for the PCA and the 3 first components were retained explaining overall 72.9% of the total variance. Component 1, 2 and 3 explained respectively 46.2%, 16.1% and 10.6% of the total variance.

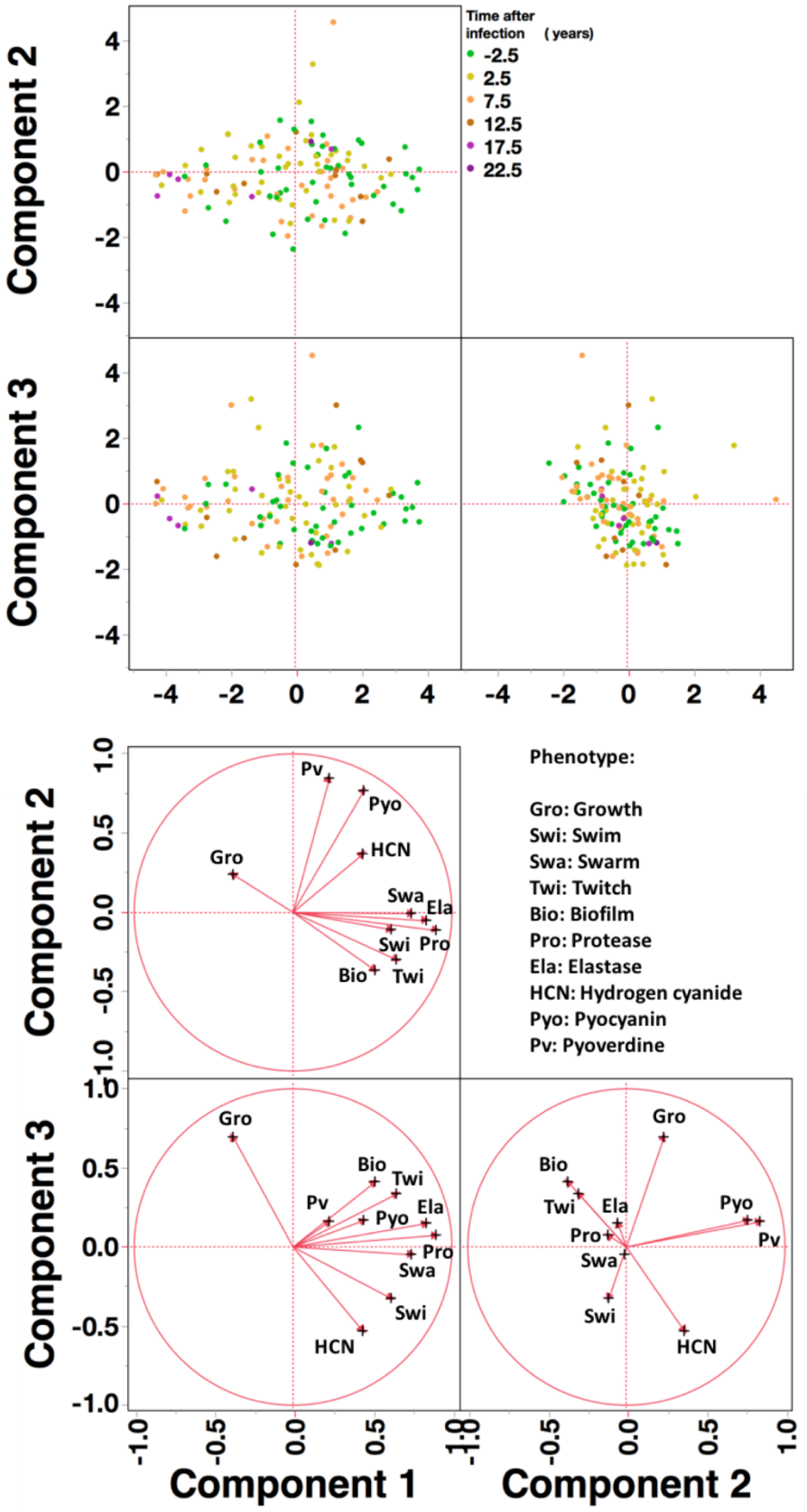


Figure 4-4: PCA score plots and loading plots from the UBC data set. The data from all 10 phenotypes was used for the PCA and the 3 first components were retained, explaining overall 67.2% of the total variance. Component 1, 2 and 3 explained respectively 37.4%, 17.4% and 12.3 % of the total variance.

4.2.2 Associations between phenotypes

After having investigated the changes in phenotypes over length and stages of infection, links between phenotypes themselves were then examined for both sets of strains. Spearman correlations were used due to the data not having a normal distribution overall. In total 45 correlations were observed for each set of strains over the 10 phenotypes. As so many correlations were performed on this data set, for all further correlation, I elected to state as significant only relationships where $p<0.01$.

For the RBH and UBC isolates the majority of correlations had Spearman coefficients above 0.3, with 28 and 30 correlations respectively out of 45 in total reaching this level (Appendix 4-1). Conversely only 3 correlations were non-significant in the case of RBH and 11 in the case of the UBS isolates ($p<0.01$). Between the sets of isolates only one correlation was non-significant, HCN-Twitch, involving the phenotypes showing the weakest associations with other phenotypes.

Overall stronger correlations were observed in the RBH set of strains (Figure 4-5), with 17 correlations having Spearman coefficients of above 0.5, compared to just 7 in the case for the UBC strains. Five correlations showed Spearman ρ coefficients above 0.5 for both sets: elastase and protease, pyoverdine and pyocyanin, protease and swarming, elastase and swarming, and swarming and swimming.

The strongest correlations were observed between a subset of the virulence factors regulated by the quorum sensing (QS) system. Indeed, protease, elastase, pyoverdine and pyocyanin strongly correlated with one another, with Spearman coefficients between 0.8 and 0.5 (Figure 4-5). Biofilm was less strongly associated with other phenotypes, while HCN stood out in correlating very weakly with other phenotypes whether these were regulated or not. Other strong correlations were observed between the speed of growth and flagellar motility swimming and swarming. Moderately strong correlations involving flagellar motility and the QS regulated virulence factors were observed ($\rho=0.3$-0.5). Finally, weak correlations ($\rho<0.3$) mainly involved HCN, twitching and growth.

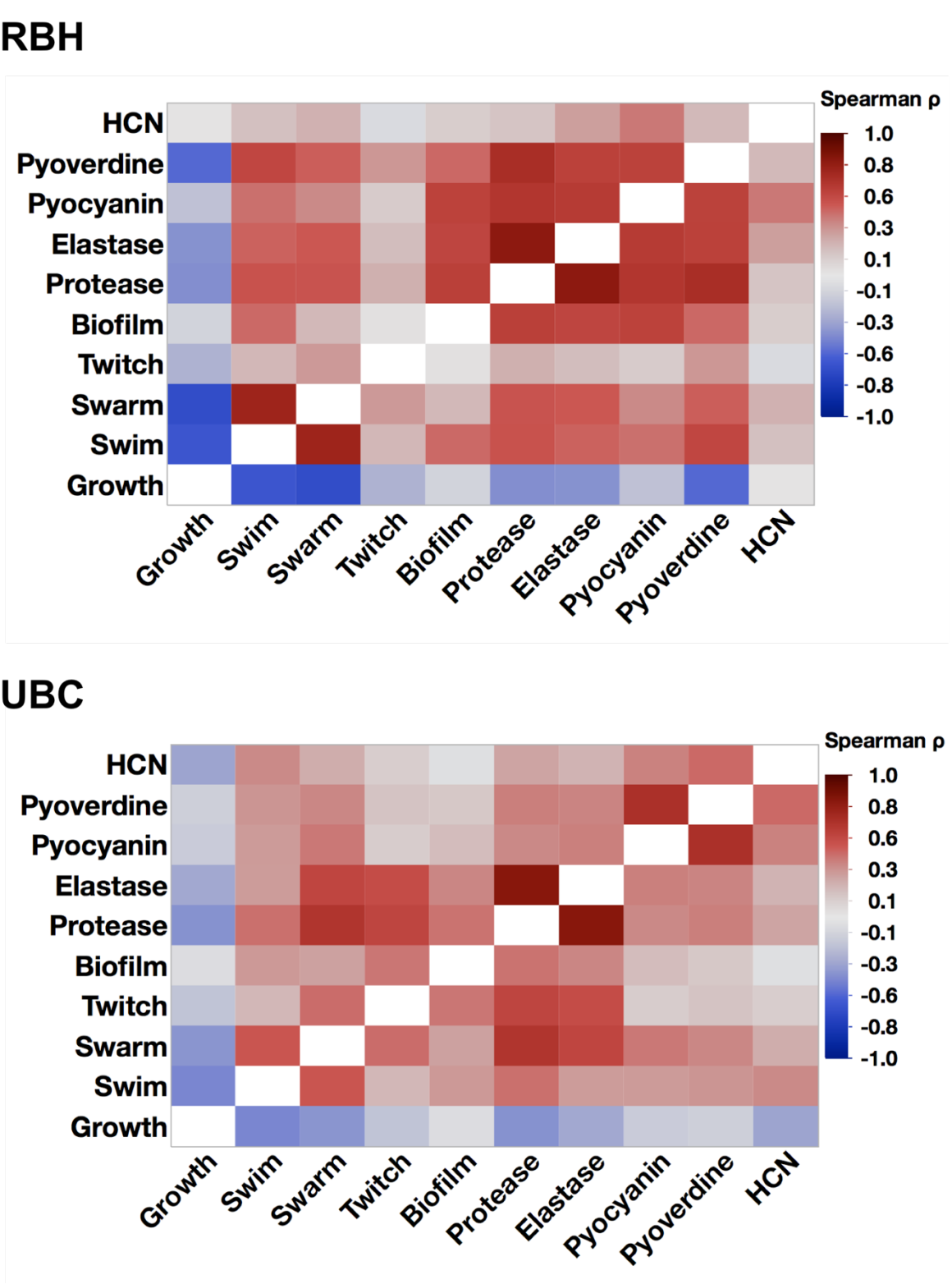


Figure 4-5: Heat maps representing the strength and intensity of correlations between all phenotypes for RBH and UBC set of strains. Correlations in red are positive and those in blue negative with the colour intensity being proportionate to the strength of the correlations. n≥3 for phenotypic assays.

4.2.3 Regulation of phenotypes: quorum sensing molecules

In addition to the 10 phenotypes previously discussed, the levels of 4 quorum sensing (QS) molecules were measured using LC-MS at Roehampton University by Dr. Volker Behrends. These molecules are involved in the direct regulation of the production of several of the other virulence factors tested, such as proteases (including elastase), pyocyanin, pyoverdine, HCN and biofilm formation (Lee and Zhang, 2015).

These QS molecules were only measured once per isolate, as replication was not possible due to costs of analyses. Four QS molecules were measured, including two homo serine lactones (HSLs), C4-HSL (N-butanoyl-l-homoserine lactone) and 3-oxo-C12-HSL (N-(3-oxododecanoyl)-l-homoserine lactone), and two quinolones, PQS (Pseudomonas Quinolone Signal or 2-heptyl-3-hydroxy-4-quinolone) and its precursor HHQ (2-heptyl-4-quinolone).

Timing of synthesis of the QS molecules

The QS molecules were measured from filtered bacterial supernatant cultures for both sets of strains.

The samples for QS extraction were collected at exponential growth phase after 4 hours and to stationary phase 24 hours for the UBC strains. For this set, between the 4-hour and 24-hour time point, the concentrations of C4-HSL and PQS increased in 73% and 91% of isolates respectively. The opposite was observed for 3-oxo-C12-HSL with 83% of isolate supernatants showing a decrease in concentrations between the two time points (Figure 4-6). Finally, for HHQ half the isolates demonstrated an increase (53%) and the other half a decrease in concentrations.

These observations were corroborated with a Mann Whitney U test and confirmed those trends. A highly significant ($p<0.0001$) increase for both C4-HSL ($z=6.70$) and PQS ($z=9.94$) and decrease for 3-oxo-C12-HSL ($z=-8.13$) were recorded, whereas the change for HHQ was not significant.

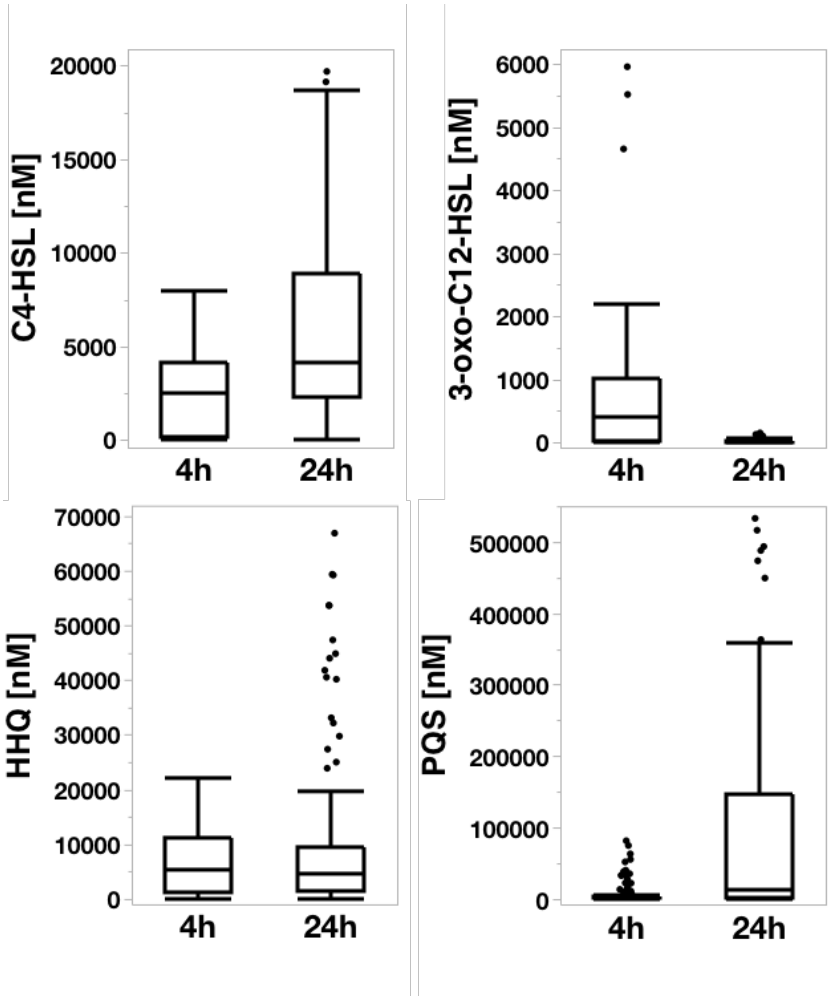


Figure 4-6: Levels of HSLs and quinolones at 4-hour and 24-hour time points for UBC isolates. The difference between time points was analysed using a Mann Whitney U test and the difference is significant ($p<0.0001$) for C4-HSL ($z=6.70$), 3-oxo-C120HSL ($z=-8.13$) and PQS ($z=9.94$)). The difference between the two groups was not significant for HHQ ($p>0.05$).

Correlation between QS molecules

Due to the fact that most QS molecules were present in increased concentrations at 24 hours, this time point was then used also to measure the larger RBH set of isolates. Again, only one measurement for each isolate was performed due to the cost of the quantification method. These results were compared with the 24-hour UBC time point and used to determine associations with stage of infection, and the other phenotypes.

Firstly, the QS molecules were all significantly correlated ($p<0.05$) to one another to various extents, except for the two HSLs in for the UBC isolates (Table 4-5). Indeed, several moderately strong ($\rho>0.3$; $p<0.0001$) correlations were observed. For the RBH isolates, HSLs correlated moderately strongly to one another ($\rho=0.49$; $p<0.0001$). While 3-oxo-C12-HSL was also strongly associated to PQS ($\rho=0.72$; $p<0.0001$), this was not the case for C4-HSL ($\rho=0.28$; $p<0.0001$). Finally, PQS and its precursor HHQ correlated moderately too ($\rho=0.48$; $p<0.0001$).

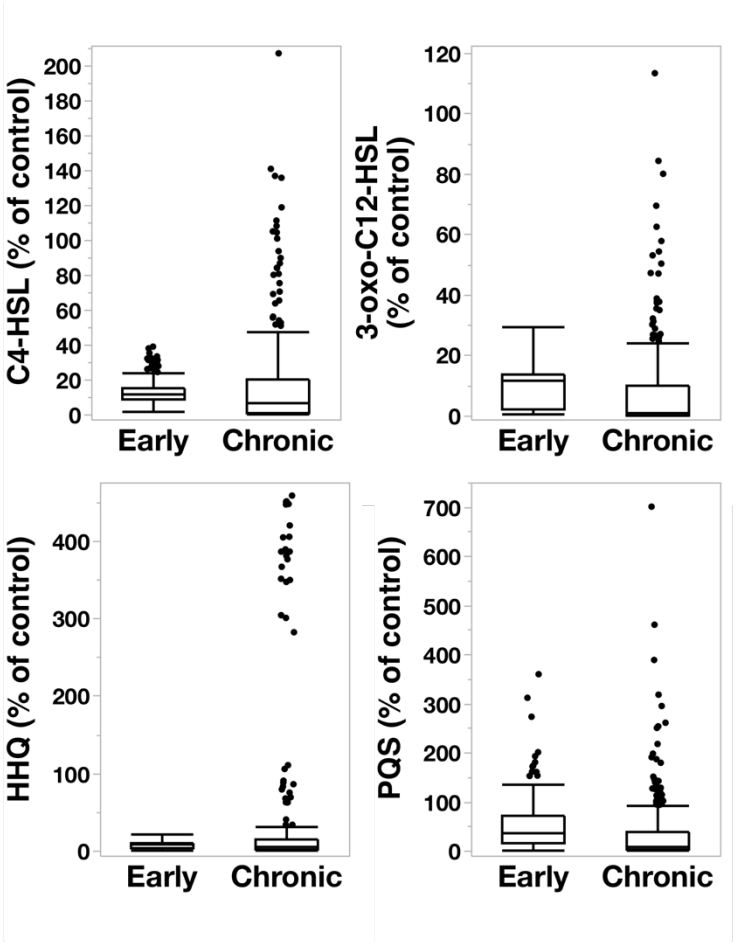
Correlation		RBH		UBC	
Variable 1	Variable 2	Spearman coefficient	p value	Spearman coefficient	p value
3-oxo-C12-HSL	PQS	0.7249	***	0.401	***
3-oxo-C12-HSL	C4-HSL	0.4886	***	0.0005	NS
PQS	HHQ	0.4809	***	0.6432	***
C4-HSL	PQS	0.2782	***	-0.2474	**
3-oxo-C12-HSL	HHQ	0.0879	*	0.2503	**
C4-HSL	HHQ	-0.123	**	-0.4331	***

Table 4-5: Spearman correlations between the 4 quorum sensing molecules tested. ***: $p<0.0005$; **: $p<0.005$; *: $p<0.01$; NS: non-significant. For the RBH the units were % of the PA01 control, the units for the UBC strains were the original concentration in the LC-MS plate.

Changes of QS molecules concentrations over time

The association with the stages and length of infection was also investigated. All QS molecule concentrations were lower in samples from chronic isolates than those from first-time early infections. The results showed a highly significant ($p<0.0001$) decrease in concentrations for C4-HSL ($z=-5.63$), 3-oxo-C12-HSl ($z=-8.98$) and PQS ($z=-6.86$), whereas no significant change was observed for HHQ. Spearman correlations undertaken for the UBC isolates with length of infection showed no significant association (data not shown). Despite this overall decrease in QS molecule production in chronic isolates, the presence of marked overproducers was clearly evident in a subset of chronic isolates, that was not observed in early isolates (Figure 4-7).

a)



b)

Phenotypes	median early	median chronic	Mann-Whitney U	
			z score	p value
C4-HSL	11.45	6.63	-5.63	***
3-oxo-C12-HCL	11.51	0.73	-8.99	***
PQS	34.51	7.74	-6.86	***
HHQ	7.31	4.97	-0.65	NS

Figure 4-7: Level Differences between early and chronic RBH isolates for 4 quorum sensing molecules. a) Boxplots of isolates grouped in early and chronic stages of infection. All data points are measured as a percentage of the PA01 control used on each LC-MS plate measured. b) The difference between early and chronic were analysed using a Mann-Whitney U test. ***: $p<0.0005$; NS: non-significant.

QS associations with virulence factors

Associations between QS molecule levels and the other phenotypes are reported in Table 4-6. The highest correlations ($\rho>0.3$; $p<0.05$) were between protease, elastase, pyoverdine, pyocyanin, and to a lesser extent biofilm with both HSLs and PQS. 3-oxo-C12-HSL correlated relatively strongly with protease, elastase, pyocyanin and pyoverdine in both sets of isolates and PQS also correlated moderately with protease and elastase. These correlations are between QS molecules, and known QS regulated virulence factors strikingly unveil that of all the phenotypes regulated by QS HCN was the least associated with the QS molecules with the strongest correlation being with HSLs with coefficients of only 0.2-0.3. Overall the HSLs seemed to show slightly stronger associations than PQS, while HHQ associate only weakly with phenotypes, with the highest association having a spearman coefficient of 0.2.

HSLs have further associations with flagellar motility than twitching, particularly for C4-HSL. Swarming and swimming correlated significantly though weakly, to a greater extent with HSLs rather than PQS. Conversely, PQS mainly correlated with twitching in both sets. The speed of growth did not demonstrate any associations with any of the QS molecules.

As a final point, although concentrations were only measured at exponential phase only for the UBC isolates, the correlations observed generally corroborated and confirm the associations observed at 24 hours. Nonetheless, when comparing correlations with other phenotypes at both time points, two differences can be observed (Appendix 4-2 and Table 4-6). These differences concern correlations with speed of growth and those involving HHQ. Regarding HHQ, correlations are significant at 4 hours but no longer at 24 hours similarly to that observed with the speed of growth. This is probably due to the fact that HHQ is the precursor to PQS so HHQ is produced prior to being turned into PQS.

Correlation		RBH		UBC	
Variable 1	Variable 2	Spearman coefficient	p value	Spearman coefficient	p value
3-oxo-C12-HSL	Protease	0.5839	***	0.3454	***
3-oxo-C12-HSL	Elastase	0.5638	***	0.3512	***
C4-HSL	Elastase	0.5365	***	0.2167	NS
C4-HSL	Protease	0.5075	***	0.2265	*
3-oxo-C12-HSL	Pyoverdine	0.4812	***	0.4919	***
C4-HSL	Pyocyanin	0.447	***	0.2441	**
C4-HSL	Biofilm	0.4392	***	0.2572	**
3-oxo-C12-HSL	Pyocyanin	0.4093	***	0.4618	***
3-oxo-C12-HSL	Biofilm	0.4029	***	0.1786	NS
PQS	Protease	0.3932	***	0.377	***
PQS	Elastase	0.3826	***	0.326	***
PQS	Pyoverdine	0.3522	***	0.3279	***
PQS	Pyocyanin	0.3456	***	0.2321	*
C4-HSL	HCN	0.3376	***	0.12	NS
C4-HSL	Pyoverdine	0.305	***	0.2313	*
PQS	Biofilm	0.3029	***	-0.0322	NS
PQS	Twitch	0.2568	***	0.191	NS
3-oxo-C12-HSL	Twitch	0.2394	***	0.1495	NS
C4-HSL	Swim	0.209	***	0.3028	***
C4-HSL	Swarm	0.2032	***	0.1463	NS
HHQ	Twitch	0.2023	***	0.1322	NS
3-oxo-C12-HSL	Swarm	0.1936	***	0.2843	**
HHQ	Pyocyanin	0.1506	***	0.0288	NS
3-oxo-C12-HSL	Swim	0.1451	***	0.2231	NS
PQS	HCN	0.0674	NS	0.1889	NS
3-oxo-C12-HSL	HCN	0.0613	NS	0.2588	**
HHQ	Growth t50	0.0409	NS	-0.0415	NS
HHQ	Pyoverdine	0.0367	NS	0.0344	NS
PQS	Swarm	0.0151	NS	0.2545	**
PQS	Swim	0.0137	NS	0.0287	NS
PQS	Growth t50	0.0057	NS	-0.192	NS
HHQ	Protease	-0.0058	NS	0.05	NS
HHQ	HCN	-0.0145	NS	0.1695	NS
HHQ	Elastase	-0.0337	NS	0.0392	NS
C4-HSL	Growth t50	-0.0371	NS	-0.0272	NS
HHQ	Swarm	-0.0638	NS	0.05	NS
HHQ	Biofilm	-0.1101	*	-0.0485	NS
3-oxo-C12-HSL	Growth t50	-0.135	**	-0.066	NS
HHQ	Swim	-0.1726	***	-0.0178	NS
C4-HSL	Twitch	-0.1937	***	-0.0311	NS

Table 4-6: Spearman correlations between the 4 quorum sensing molecules and all other phenotypes tested. Coefficients were sorted from highest to lowest according to the data from the RBH set of isolates, coefficients in red are the strongest and blue the lowest of negative. ***: $p<0.0005$; **: $p<0.005$; *: $p<0.01$; NS: non-significant. $n\geq3$ for phenotypic assays. For the RBH the units were % of the PA01 control, the units for the UBC strains was the original concentration in the LC-MS plate.

4.2.4 Bacterial phenotypes and clinical implications

These prolonged infections lead to chronic inflammation and potential deterioration of the lungs and of the general health of the patients (Bragonzi et al., 2018). Thus, changes of the phenotypes of *P. aeruginosa* within the CF lung could influence the outcome for patients and so correlation between phenotype of strain associated from patients and their clinical status was investigated.

The potential associations between the bacterial phenotypes and clinical data were undertaken for the RBH strains for which clinical data was available, data which included: gender, age, FEV1(% predicted), length of infection and exacerbation status. Finally, we tried to collect information from the microbiological lab about co-infection status, but only one early *P. aeruginosa* infection and three chronic were concomitant with another pathogen, thus due to low numbers of co-infection this information was not used further. FEV1 data was missing for 7 patients out of 24, mostly for early patients due to their young age.

Firstly, and unsurprisingly, length of infection and age were highly positively correlated ($\rho=0.72$; $p<0.0001$). All correlations involving FEV1, age and length of infection were statistically significant, except FEV1 and growth (Table 4-7). These correlations with FEV1 were positive, except a weak negative correlation with twitching. In the case of age and length of infection all correlations were negative, except for growth.

In absolute values associations with age and length of infection were all moderately strong, though weaker correlations were observed for twitching and HCN. On the other hand, FEV1 was least associated to motility and growth, however swimming was still moderately correlated ($\rho=0.31$; $p<0.0005$).

Overall HCN was the least associated QS regulated virulence factor for all correlations, while motility and growth were more weakly correlated to FEV1 (% predicted value) than for age and length of infection. Nonetheless for all three clinical variables associations with twitching were low.

Variable	by Variable	Spearman ρ	p value
Age	Length of infection	0.7207	***
FEV1	Length of infection	-0.3364	***
FEV1	Age	-0.5374	***
FEV1	Elastase	0.585	***
FEV1	Protease	0.532	***
FEV1	Pyocyanin	0.431	***
FEV1	Biofilm	0.387	***
FEV1	Pyoverdine	0.379	***
FEV1	Hydrogen cyanide	0.336	***
FEV1	Swim	0.311	***
FEV1	Swarm	0.167	**
FEV1	Growth t50	-0.049	NS
FEV1	Twitch	-0.165	**
Age	Growth t50	0.569	***
Age	Twitch	-0.183	***
Age	Hydrogen cyanide	-0.223	***
Age	Pyocyanin	-0.447	***
Age	Swarm	-0.490	***
Age	Biofilm	-0.515	***
Age	Swim	-0.534	***
Age	Protease	-0.572	***
Age	Elastase	-0.580	***
Age	Pyoverdine	-0.620	***
Length of infection	Growth t50	0.6058	***
Length of infection	Biofilm (595nm)	-0.2277	***
Length of infection	Twitch	-0.2559	***
Length of infection	Hydrogen cyanide	-0.3008	***
Length of infection	Pyocyanin	-0.4083	***
Length of infection	Protease	-0.4972	***
Length of infection	Swim	-0.5038	***
Length of infection	Pyoverdin	-0.5491	***
Length of infection	Elastase	-0.5658	***
Length of infection	Swarm	-0.6573	***

Table 4-7: Spearman correlations for FEV1, age and length of infection, with 10 phenotypes. ***: $p<0.0001$; **: $p<0.001$; NS: non-significant. $n\geq3$ for phenotypic assays.

As previous studies have shown differences in gender and CF symptoms, the potential impact of gender on the behaviour of the bacteria was determined (Table 4-8) (Demko et al., 1995, Wang et al., 2010). There were fewer female patients than male. Eight samples were recovered from female patients and sixteen from male and, if only chronic infections are considered, then 6 patients were female and 11 male. The results indicated that phenotypic values were significantly lower in male chronically infected patients for 5 of the 10 phenotypes, which were twitching, protease, HCN, Biofilm and pyoverdine.

Phenotypes	median female	median male	Mann-Whitney U	
			z score	p value
Growth	4.36	5.37	-0.18	NS
Swim	6.08	3.80	-1.94	NS
Swarm	5.28	5.34	-0.09	NS
Twitch	8.28	7.77	-3.05	**
Protease	4.25	5.40	-2.58	*
Elastase	1.78	3.06	0.42	NS
HCN	51.75	24.56	-3.35	**
Pyocyanin	0.22	0.16	-1.05	NS
Pyoverdine	0.48	0.38	-1.71	NS
Biofilm	0.18	0.13	-4.90	***

Table 4-8: Phenotypic differences between chronic infection in male patients and female patients. The data for the patients infected for the first time (early patients) were not included in this analysis. Data analysed using a Mann-Whitney U test. ***: $p<0.0005$; **: $p<0.005$; *: $p<0.05$; NS: non significant. $n\geq3$ for phenotypic assays.

4.3 Discussion

In this chapter the main aim was to investigate the links and associations between the phenotypes themselves but also with the length of infection or stage of infection. Furthermore, the degree of association with QS regulatory molecules and with patients with clinical data was also investigated.

Overall the results obtained using the data from the UBC and the RBH isolates presented differences, but the overall trends and strength of specific associations were similar.

The RBH strains were isolated specifically for this project and the rigorous isolation method means the conclusion using the results from this set will be (or are likely to be) more robust. The UBC set of strains was collected over many years, but this also means that different people isolated them, including potentially another level of experimental variation.

Regarding the UBC strains the length of infection and the number of strains isolated per time point and per patient varied. For some patients more isolates were recovered at different time points and were available, whereas for other patients only a few time points were available (see Chapter 3). Also, the routine of the hospital at the time is unknown and thus it is hard to be sure that the first isolates mentioned for each patient correspond truly to the first time the patients were found to be infected with *P. aeruginosa*.

Nevertheless, the use of two very different sets with overall common conclusions strengthens these results and observations concerning associations of the phenotypes with themselves and other factors. Only a few studies have investigated the phenotypic evolution and associations of *P. aeruginosa* over time and stages of infection over so many years but also having several isolates per patient (Workentine et al., 2013, Smith et al., 2006, Darch et al., 2015, Jiricny et al., 2014).

4.3.1 Rhythm of virulence decrease over time

The first part of the chapter investigated the changes in the phenotypes over the length of infection and between the early and chronic stages of infection.

When comparing all the isolates solely by stage of infection using the RBH data set, we found that all phenotypes showed significant decrease in virulence, motility and growth between early and chronic stages of infection. Similar results were observed to a lesser extent for the UBC set of strains where the change over the course of infection was significant for 7 of the 10 phenotypes. Indeed, the changes over time were not significant for HCN, pyocyanin and biofilm production in the UBC set and in the RBH set for the means approach. The general trends over both set of strains showed that for 6 phenotypes there was a significant change over time and stages of infection. The 6 phenotypes are growth, swim, swarm, protease, elastase and pyoverdine. On the other hand, HCN, twitching, biofilm, and to a lesser extent pyocyanin, showed only little change or a decrease. With regard to twitching, although the mean values remain similar between early and chronic isolates, an obvious change is observed between stages of infections, with the diversity diminishing drastically in chronic isolates, where all the isolates become low or non twitchers.

A small study of 40 isolates in total found similar results concerning some of the phenotypes (Jiricny et al., 2014). As with this study, they observed a significant decrease in protease, elastase and pyoverdine production but interestingly also found a lack of change in pyocyanin and biofilm production (Jiricny et al., 2014). Another bigger study looked at 600 patients, representing over 2500 isolates (Mayer-Hamblett et al., 2014b). The study found that the phenotypes that best correlated with the different stages of infection were pyoverdine and protease, with over double the proportion of chronic isolates showing a decrease in protease and pyoverdine production in comparison to newly cultured *P. aeruginosa* (Mayer-Hamblett et al., 2014b).

The general decrease in virulence in chronic stages of infection, meaning less ability to damage and cause inflammation in vivo, has been shown in several host models (Lelong et al., 2011, Lore et al., 2012). This decrease has been studied more in detail both genotypically and phenotypically. Indeed, the reduction in production of specific virulence factors and the decrease in motility has been observed previously in several studies as well as mutations in the genes involved in those phenotypes (Harmer et al., 2013, Smith et al., 2006, Bragonzi et al., 2009, Nguyen and Singh, 2006). One study suggests that some of these changes could occur in as little as 10 days in vitro (Azimi et al., 2019). The exact speed and time frame at which the decrease in virulence appears in the CF lungs remains unknown.

The strength of the phenotypic decrease could be a translation of the amount of pressure that the bacteria is under to alter that phenotype. This could unveil the most important phenotypic changes necessary in order to enable the establishment of chronicity. On the other hand, phenotypes lost at slower rates might be less relevant to the survival in the CF lung environment, and thus disappear due to the lack of need. Indeed, traits that could have been useful to the bacteria in the environment might no longer necessary in the context of CF infection. The different degrees of pressure to adjust specific phenotypic behaviours can be due to different reasons and causes.

For example, it is known that proteases, pyocyanin are involved in the fight against the immune system and inflammation in initial colonisation of the lungs (Kuang et al., 2011, Manago et al., 2015, Saint-Criq et al., 2018, Laarman et al., 2012). In the context of chronic infection thus these virulence exoproducts are no longer needed as the bacteria aim to evade the immune system to establish long term infection. Pyoverdine might also be another exoproduct being less needed once in the CF lung, as studies suggest that the bacteria adapt to the lungs and become less dependent on pyoverdine to scavenge iron due to different types available in the lungs (Nguyen et al., 2014, Marvig et al., 2014). While its main role is getting iron necessary for growth, pyoverdine also has a pathogenic role similar to the phenotypes cited, which could also explain its loss (Minandri et al., 2016, Granato et al., 2016).

Thus, for some phenotypes the drive for the loss of the virulence trait can be multifactorial. For phenotypes such as motility it is a question of evading the immune system as both the flagella and pili can both be recognised by the innate immune system (Balloy et al., 2007, Mahenthiralingam et al., 1994).

This suggests that the decrease in motility over time would be linked to evading the immune system. Nonetheless, studies have also shown a decrease in motility in vitro in media mimicking the CF mucus, but without immune cells, which might be due to viscosity changes (Wong et al., 2012, Staudinger et al., 2014b, Schick and Kassen, 2018). Interestingly, the loss of the flagella involved in swimming and swarming motility has been shown to also help resistance to antibiotics (Rundell et al., 2019).

It has been argued that there is a pressure for the biofilm production to persist, as it is serves as a physical barrier from antimicrobials and helps survival (Macia et al., 2014, Nickel et al.,

1985, Tseng et al., 2013, Jensen et al., 2010, Leid et al., 2005). Nonetheless in the study we found a small decrease in biofilm production in the RBH set of strains. This may be due to the unsuitable measurement method. The assay should have been undertaken for longer, as biofilm was only left to grow for 6 hours. There is also a real difficulty to measure true biofilm production capability through a rapid and high through put assay. The observation and measurement of biofilm biomass should rather be done by confocal microscopy, but this is not feasible for a large number of isolates for obvious reasons of time and cost. Instead the crystal violet assay commonly used in many studies only truly measures the ability of the bacteria to attach rather than form a mature biofilm (Staudinger et al., 2014a).

The reason why HCN does not decrease drastically with time of infection remains unclear, as the expression of biosynthetic enzyme hcnB and hcnC was shown to be lost through the changes in QS expression and *lasR* mutants (D'Argenio et al., 2007). This virulence factor has rarely been investigated phenotypically, with this being the first study looking at the changes of HCN over the course of infection. This gas might therefore be a good general marker of *P. aeruginosa* infection as it seems to be produced at every stage of infection, as has been suggested by several studies (Gilchrist et al., 2015, Gilchrist et al., 2013, Smith et al., 2013). A multivariate method was also used to look at changes in phenotypes. This principal component analysis method enabled to separate successfully chronic and early isolates. Early isolates were grouped on one side of the loading plots whereas chronic were more spread, showing a potential difference in diversity, which is investigated in detail in the next chapter. The results showed the shift that occurs between early and chronic stages of infection corresponded to a general loss of virulence, particularly of proteases and motility, and to a lesser extent pyoverdine. These phenotypes correspond to the ones identified as showing the biggest decrease between stages of infection. Thus, the changes in these phenotypes seem to be the most important for the establishment of chronicity and therefore could be good biomarkers to measure the infection stage and try to prevent chronic infections. On the other hand, HCN seemed to stand out in relation to other phenotypes.

Separating isolates per patient on a score plot also underlined that some chronic isolates had closer phenotypic behaviours to the majority of early isolates. This was the case for chronic patient 2 and chronic patient 6, while early patient 4 isolates had phenotypes closer to chronic ones. The reasons why the isolates in some patients have adapted more than others is unknown.

To investigate this the first step would be to see if this is related to important differences in the frequency of antibiotic treatments, as well as exacerbation symptoms.

4.3.2 Phenotypic associations

Next we investigated the associations between phenotypes to identify significant links, and Spearmen correlations between all phenotypes assayed were tested. First of all, it has to be noted that all phenotypes will be inherently linked to the growth rate of the bacteria. The growth rates of the isolates differed significantly, with some isolates reaching t50 in under 2 hours and others up to 6 hours. This growth rate difference will in turn have an impact on all the other phenotypes measured. Although it is possible to account for the final growth extent, this is based on the final OD and will not take into account the other phases of growth such as the lag and exponential phase. For other assays involving growth on agar it was difficult to account for growth. Nonetheless this can have an impact on all assays as slower dividing cells or longer lag phase will mean slower production of exoproducts and motility. This is, however, a phenotype rarely measured and acknowledged as having an important impact. It is known that some virulence factors such as HCN and pyocyanin are produced at the beginning of stationary phase (Cody et al., 2009, Nair et al., 2014b, Lundgren et al., 2013, Price-Whelan et al., 2007, Dietrich et al., 2006). Therefore, if the bacteria take longer to get to stationary phase the production will be delayed, even if a strain proportionately produces high amounts of virulence factors but will be underestimated in assays which measure production at a single final time point. Studies have found that patterns of production of HCN are unique to strains and for different lengths of time (Neerincx et al., 2015, Gilchrist et al., 2011). Thus, information about the period of production will not be captured with the method of measurement used in this study, which only measured the cumulative production of HCN but also the other virulence factors. Ideally all phenotypes would have therefore been measured such as growth, at regular intervals, to see evolution over time and evolution of production over the stages of growth. This could have also potentially explained some links between virulence factors if they were produced simultaneously or with different patterns. Single time point measurements for the phenotypes can therefore be considered a limitation of this study, although undertaking different time points would be difficult to achieve on such a big number of isolates. This could

be undertaken in the future on a small subset of strains, for example to delve further into differences amongst high HCN producers.

Regarding associations between all phenotypes, a challenge was to identify to what degree the correlations were biologically relevant according to the strength of the correlations. Different disciplines would consider correlations to be meaningful at different thresholds. Most studies seem to agree than in biological sciences associations with coefficients of 0.3 and above should be accepted of fair or moderate strength (Akoglu, 2018, Chan, 2003, Xiao et al., 2016).

Although the UBC set of strains showed correlation coefficients between phenotypes of under 0.3, some were of high significance and the trends of associations were similar to those found in the RBH set of strains.

Most phenotype couples tested were found to be positively correlated, or negatively in the case of growth as an increase in t50 means a decrease in speed of growth. As measurements decreased over time it makes sense that overall phenotypes all correlated to some extent to one another, but the strength of those correlations gives more information about the importance of those phenotypes and their associations to one another.

Swimming and swarming were both highly correlated, as was protease and elastase, which were both expected. Indeed, elastase was involved in the full proteolytic activity assay as one of several proteases produced by *P. aeruginosa* (Kohler et al., 2000, Galdino et al., 2017, O'Toole and Kolter, 1998). The strong correlation between flagellar motilities was confirmed in some studies whereas others have in fact found a lack of correlations between the different types of motility, especially with swarming (Murray et al., 2010, Workentine et al., 2013, Clark et al., 2015, Bernier et al., 2014).

Other phenotypes were strongly associated, all involving QS regulated virulence factors. Indeed, protease, elastase, pyoverdine and pyocyanin all showed strong associations to one another, with Spearman coefficient between 0.5 and 0.8. These associations were also observed in much smaller studies (Clark et al., 2015, Fothergill et al., 2010, Schick and Kassen, 2018, Workentine et al., 2013). Conversely this was not the case for other QS regulated phenotypes, with correlations involving biofilm production and HCN being considerably lower, especially regarding HCN. Associations between biofilm production and other virulence phenotypes were

nonetheless significant (Schick and Kassen, 2018). This again underlines the fact that HCN is produced and its regulation adapts in an unusual way in comparison to other QS regulated phenotypes.

Regarding associations between motility and other phenotypes, once again flagellar motilities were found to be most associated with the QS regulated phenotypes mentioned, which has been confirmed in some studies (Murray et al., 2010, Workentine et al., 2013), and disputed in others (Clark et al., 2015).

Positive correlation between motility phenotypes and biofilm was also found, the correlation being highest for swimming. Some previous studies found negative correlation (Murray et al., 2010, Clark et al., 2015, Schick and Kassen, 2018), whereas another study found positive correlation (Vidya et al., 2016). The role of motility in biofilm formation has been studied extensively and shows that motility is linked to biofilm biomass but not the ability to form a biofilm (Deligianni et al., 2010), the converse of what had been previously found (O'Toole and Kolter, 1998).

Finally, speed of growth seemed to be only moderately associated to a few phenotypes which were both flagellar motilities and proteases. On the other hand, growth was not associated to biofilm production, in accordance with the findings of Deligianni et al. (Deligianni et al., 2010). Another study found a negative correlation between growth and pyocyanin production, while in this study we found a very low positive correlation (Schick and Kassen, 2018).

4.3.3 QS Regulation

All of the virulence factors assayed in this study are regulated directly or indirectly by *P. aeruginosa's* QS systems, thus being expected to correlate to some extent to the QS signal molecules measured (Toder et al., 1991, Pessi and Haas, 2000, Stintzi et al., 1998, Shrout et al., 2006, de Kievit, 2009, Deziel et al., 2003).

For the UBC isolates the QS molecules were quantified at 4-hour and 24-hour time points. For C4-HSL and PQS overall concentrations increased between the two time points, whereas they decreased for 3-oxo-C12-HSL and remained similar for HHQ. The las QS system is the first to

be activated and in turn activates the Rhl and Pqs systems (Antunes et al., 2010, de Kievit, 2009). 3-oxo-C12-HSL is the signalling molecule of the Las system and is produced earlier than C4-HSL and PQS, and so shows as expected higher concentrations at the 4-hour time point.

With regards to changes between early and chronic stages of infection, a statistically significant decrease in concentration was observed for all molecules, with the exception of HHQ. The HSL 3-oxo-C12-HSL showed the biggest decrease, which was also found in a 2015 study (Darch et al., 2015). Although the decrease in the mean values was significant for the RBH strains, the highest concentrations were nonetheless observed in chronic isolates due to a small number of overproducers. This might have an important role in maintaining the ability of virulence for future colonisation and would need to be further investigated. Two chronic patients, numbers 11 and 12, had higher PQS concentrations than all others, whereas chronic patient 4 had higher concentrations for HHQ and both HSLs. This divergence between PQS and HHQ is interesting and might be due to HHQ also being able to activate directly the PQS system regulator pqsR (Rampioni et al., 2016). This decrease in QS concentrations in chronic isolates has been observed in other studies (Jiricny et al., 2014, Bjarnsholt et al., 2010). Conversely once again the changes were lesser for the UBC set, with no significant correlations between the length of infection and the QS molecules. The decrease in concentrations between the stages of infection has been linked to the common occurrence of *lasR* mutants (D'Argenio et al., 2007, Smith et al., 2006).

The first associations observed were those between the QS molecules themselves. The strongest correlation observed was between 3-oxo-C12-HSL and PQS. This correlation is expected as the Las system controls the production of PQS (Pesci et al., 1999). Conversely the other HSL, C4-HSL, is only weakly correlated to PQS, although it is known that PQS can regulate the Rhl system (McKnight et al., 2000). The two AHLs correlated only moderately, as did PQS and its precursor HHQ, while the precursor did not correlate with the AHLs. It is surprising that 3-oxo-C12-HSL does not correlate as strongly with C4-HSL as with PQS, as the Las system with its 3-oxo-C12-HSL signal also activates the Rhl system and its C4-HSL signal (Pesci et al., 1999, Diggle et al., 2003).

The moderate correlations between the AHLs, and between PQS and HHQ, were confirmed in other studies, although they observed higher associations between C4-HSL and PQS

(Workentine et al., 2013, Darch et al., 2015). These regulatory QS systems are interlinked and complex, and in addition it has also been shown that rhl can escape the las regulation system (Medina et al., 2003, Diggle et al., 2003, Kostylev et al., 2019, Feltner et al., 2016).

The regulation of the virulence factors by the QS molecules was revealed by significant correlations between QS molecules and phenotypes assayed. Overall phenotypes showed marginally stronger associations with AHLs than PQS. The PQS precursor HHQ did not show any correlations with any of the phenotypes, as a precursor it might not directly regulate virulence factors although it can also activate directly the PQS system in some cases (Rampioni et al., 2016).

The phenotypes showing the strongest correlations with QS were protease, elastase, pyoverdine and pyocyanin. Overall there seems to be a recurrent emphasis on proteases, that appear to have a central role, being the most associated both to other phenotypes and here to the QS molecules. Once again HCN stood out amongst the QS regulated phenotypes, with weaker associations to HSLs and no significant correlation to PQS. This is unexpected as it has been shown that PQS can regulate HCN (Gallagher et al., 2002). However, HCN is also known to be regulated by the anaerobic regulate ANR (Pessi and Haas, 2000).

Overall motility did not show any strong links to any QS molecules, which is unexpected in the case of swarming as this type of motility is known to be dependent on biosurfactants regulated by QS systems (Deziel et al., 2003). Another study also showed only low QS association with swarming and not with the other motilities (Workentine et al., 2013).

Finally, growth did not show any significant associations to the QS molecules, except at the 4-hour time point, as QS activation is dependent on cell density but not the speed of growth itself (de Kievit and Iglewski, 2000, Fuqua et al., 1994).

Although significant, the correlations all showed coefficients under 0.6, underlining the fact that some virulence factors were produced without the presence of QS molecules. This once again underlines the complexity of the regulation of virulence factors in *P. aeruginosa* (Lelong et al., 2011).

In conclusion, although phenotypes are regulated by QS as shown in this study and others by clear associations with the AHLs and PQS, these correlations remain moderate, showing the potential for other potential regulation systems involved in some cases (Workentine et al., 2013, Cullen and McClean, 2015). These multifactorial regulations underline the complexity in predicting the effect of changes in the QS systems and QS mutants on virulence factors (Kostylev et al., 2019, Lee and Zhang, 2015, Feltner et al., 2016).

4.3.4 Clinical data and potential phenotypic associations

Clinical data regarding the RBH set of isolates was gathered from hospital records and we investigated the potential associations with the phenotypic behaviours of the isolates infecting individual patients.

The first associations observed were those regarding the age of patients, their FEV1 and the length of infection. The length of infection was potentially not very accurate, due to adult patients not having always been followed by the same hospital. This is why the RBH isolates were categorised as only early or chronic. Nonetheless, it was found that age of patients and length of infection correlated strongly, which would be expected, and would suggest that the length of infections recorded are relatively accurate. FEV1 also correlated moderately with both the length of infection and age, and is known to indeed decrease in CF patients and more so when infected by *P. aeruginosa* (Konstan et al., 2012, Konstan et al., 2007, Coburn et al., 2015, Que et al., 2006). These moderate associations with FEV1 may be due to other factors than time, such as exacerbations which can lead to further decrease in lung function (Amadori et al., 2009, Sanders et al., 2011, Ramos et al., 2017).

Regarding associations with phenotypes, age and length of infection show similar trends, with length of infection corresponding even closer to the findings when isolates were separated into early versus chronic. HCN, biofilm and twitching were found to be the phenotypes which least changed over time. Similar results are found with FEV1 regarding the QS regulated phenotypes. On the other hand, motility and growth were only weakly or not significantly correlated to FEV1. This potentially gives further insight into the role of these phenotypes, being linked primarily to survival and adaptation to the lung environment. The numbers of

patients remain nonetheless too low to make any assumptions as to the particular role of individual phenotypes on the patient's health regarding the FEV1.

Several studies have shown that gender is linked to the clinical outcome of the patients. Indeed, the median survival of female patients is lower than for male CF patients (Demko et al., 1995, 2019). According to the Cystic Fibrosis Trust registry report of 2018, the median predicted survival age was 44 for female and 51 for male CF patients. A study found increased inflammation markers in explanted lungs from female patients, while another study suggested oestrogen could be responsible as it was found to increase inflammation as well as *P. aeruginosa* burden in mice (Lammertyn et al., 2017, Wang et al., 2010). Oestrogen has also been found to directly impact *P. aeruginosa*, one study finding it caused the mucoid switch in the bacteria while another study finding female patients acquired mucoid *P. aeruginosa* earlier than male counterparts (Chotirmall et al., 2012). Interestingly in this study *P. aeruginosa* from female patients were more virulent than those isolated from male patients. In another phenotypic study of *P. aeruginosa*, Manos et al., found lower pyoverdine production and swimming in isolates from male subjects (Manos et al., 2013). The role of oestrogen in CF infections underlines the necessity to take into consideration all characteristics of individuals as CF patients, like the infecting bacteria, are not a homogeneous population.

No definitive conclusion can be made regarding the links between the clinical data and the phenotypes due to the small number of patients in the study. However, there are potential associations which could be further investigated to understand better the role of the virulence factors not only on the chronicity of the infection in the CF lung but also on the effects on the patients' general health.

Chapter 5: Within and between patient phenotypic diversity

5.1 Introduction

Adaptation of *P. aeruginosa* to the CF lung leading to long term chronic infections has been extensively researched for many years both phenotypically and genotypically (Darch et al., 2015, Clark et al., 2015, Workentine et al., 2013, Burke et al., 1991, Hogardt and Heesemann, 2010, Govan and Harris, 1986, Mena et al., 2008, Smith et al., 2006). In the last decade, studies have started revealing the diversity of *P. aeruginosa* isolates responsible for CF infections (Mowat et al., 2011, Jorth et al., 2015). However, the large majority of studies have focused on a certain subgroup of patients, for example those chronically infected, during exacerbations or patients infected by a specific epidemic strain (Fothergill et al., 2010, Mowat et al., 2011, Ashish et al., 2013). Therefore, there is a need for further investigation to unveil the extent of the diversity and understand its biological implications during chronic infection and the potential repercussions on the patients' overall condition.

In order to investigate the diversity of the bacteria within the CF lung, multiple isolates were collected from 23 early and chronic patient samples, resulting in a set of close to 600 isolates. These isolates were assayed for 10 phenotypic traits associated with colonisation and chronic infection, as well as to measure the production of 5 quorum sensing (QS) regulatory molecules. All phenotypes were assessed through fully quantitative measurements.

It was shown in the previous chapter that these 10 phenotypes exhibited lower measurements in chronic stage than in early stages of infection as well as various associations between each other. This chapter describes the extent of the quantitative phenotypic heterogeneity of these 593 isolates by testing both the within-patient and between-patient phenotypic diversity.

The aims of this chapter are to:

- Evaluate the overall diversity of the phenotypes assayed.
- Determine whether within-patient or between-patient phenotypic diversity is the greatest.
- Assess the differences in diversity between early and chronic stage isolates as a surrogate to understand the evolution of diversity over time.
- Determine the diversity of regulatory quorum sensing molecule levels.

5.2 Results

5.2.1 Between-patient diversity is higher than within-patient diversity

Previous observations in Chapter 3 demonstrated that the measurements for every phenotype cannot be rationally categorised into groups, but rather spread over a range of quantities and concentrations. Thus, knowing that for all phenotypes the data shows linear variation along the range of measurement, the next step is to investigate further the observed variation and assess both the between-patient or within individual patient diversity. To investigate both types of diversity multiple isolates of *P. aeruginosa* were retrieved from samples originating from 23 individual patients. A minimum of 12 isolates, and a maximum of 60, were collected from each patient sample.

All patients' data was observed as a whole through the visual representation of the full data set in Figure 5-1, in the format of box plots for each phenotype. Box plots allow one to see all phenotypes side by side, presented with appropriate units for each one of them. The presence of variation within each phenotype is evident; the array of different box sizes shows the clear presence of variation of the diversity between patients. For all phenotypes the range of measurement is extensive, with at least one patient per phenotype having its isolates' values spread across the whole range of measurement. Conversely for most phenotypes several patients show very low readings for all their isolates. Generally, patients with the highest phenotype values also show the highest diversity, with a few exceptions. For some phenotypes a pattern of low phenotype value/level and low diversity are present in the majority of patients,

for instance with twitching, pyocyanin and biofilm. On the other hand, a few patients for each phenotype show high measurements coupled with high diversity.

Also, for most phenotypes although some patients show varying amounts of diversity, the overall mean of all the isolates' phenotype values is similar, and this is visible through the mean of the boxes represented in Figure 5-1. Indeed, patients that have very different individual diversity nonetheless have similar means over all isolates. This is obvious, for example, in the case of HCN levels between several patients having very different diversity patterns but same mean value.

The final observation about the diversity between patients involves difference observed between the early and chronic isolates. In the case of growth, all early isolates grew amongst the fastest whereas many chronic patients had all their isolates grow slower than any of the early isolates. For the assays of swimming and twitching motility, pyocyanin and HCN early stage patients' isolates were more motile or produced more virulence factors than any isolates from chronically infected patients. In parallel, for 6 of the 10 phenotypes, that is except for twitching, elastase, pyocyanin and biofilm production, the lowest expression of the phenotypes was observed in chronically infected patients. These differences between stages follow the results of the previous chapter.

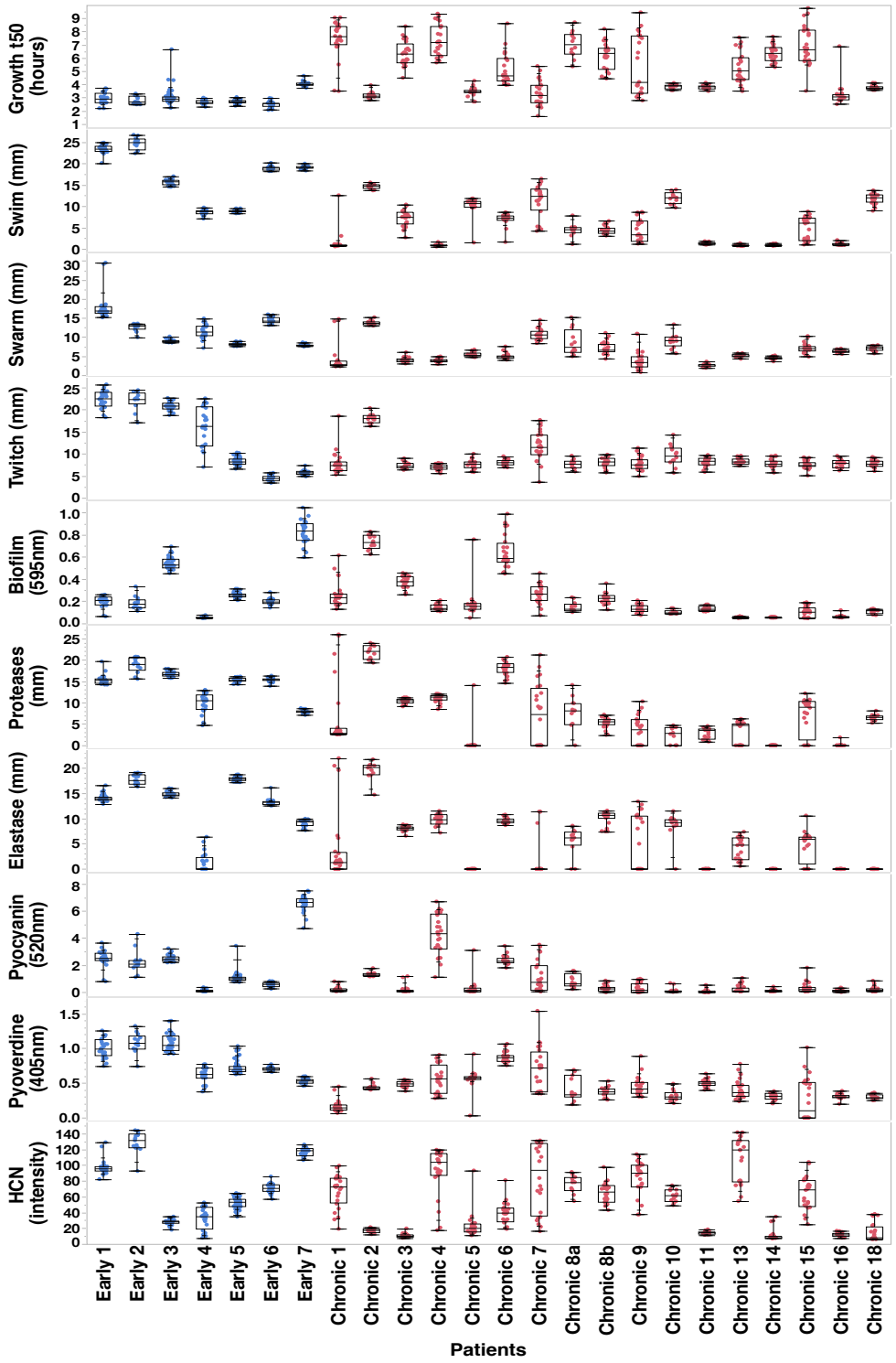


Figure 5-1: Box plot visual presentation of variation for all phenotypic data for all patients. Each box represents the interquartile range. The box plots show the extent of the diversity for each patient taking into account all the single isolates from that patient, dots representing each isolate. For each patient a minimum of at least 12 isolates were used in the test of phenotypes. All values used to make plots are means of at least 3 biological replicates for each phenotypic assay. Early= Isolates from early stage infections; Chronic= Isolates from chronic stage infections. All measurements in mm correspond to the diameter of the bacterial spread for motilities and zone of clearance for proteolytic activity and elastase production. Hydrogen cyanide was measured as a relative intensity. Proteases corresponds to the overall full proteolytic activity.

To determine the extent of behaviour within patient variation, the statistical strategy was to use a mixed linear model approach, which takes into account the several levels of the data. Indeed, as multiple isolates originated from each individual patient, the isolates ought to be considered as nested within patients. The model was built using 'Patient' and 'Isolate (Patient)' as random factors, isolates thus being nested in each 'Patient' category. The percentage of diversity explained by between-patient diversity and within-patient diversity was calculated for each phenotype. One of the outputs of the analysis was the R square, which represents the proportion of variation explained by the variables used in the analysis. The variables here are the patients' and the individual isolates, indicative of between- and within-patient diversity respectively. The R square values showed that the model explained at least 79% of the overall variation. The between-patient diversity explained between 78 to 96% of the variation within the model. Conversely, the within-patient diversity represented at maximum only 21.6% of diversity for speed of growth and then decreased down to a minimum of 3.9% for swimming motility. The percentage for within-patient diversity for each phenotype decreased, while it increased for between-patient diversity. The percentage increased in the following order: growth, pyoverdine, HCN, protease, swarming, elastase, pyocyanin, twitching, biofilm and finally swimming. Thus, for all phenotypes between-patient diversity was considerably greater than within-patient diversity (Table 5-1).

When analysing the data by stage of infection the same observations were made, with between-patient diversity being higher for both early and chronic isolates (Table 5-2). It should be noted that in early stages of infection the between-patient diversity was not significant for all phenotypes, with p values all being around 0.08, which may be influenced by the smaller numbers of isolates than for chronic isolates. There does not seem to be any specific grouping of phenotypes showing specifically higher or lower of either types of diversity. Motility seems to show relatively low within-patient diversity, while QS regulated phenotypes show variable amounts of diversity.

Overall these results confirm what had been observed visually, that diversity was observed for all phenotypes to different degrees. The extent of the between-patient diversity in comparison to the within-patient diversity therefore suggests the importance of the specific type of initial infecting strain.

Phenotypes	R Square	Between-patient diversity (%)	Within-patient diversity (%)
Growth	0.79	78.38	21.62
Swim	0.96	96.09	3.91
Swarm	0.86	86.56	13.44
Twitch	0.91	91.69	8.31
Protease	0.84	84.53	15.47
Elastase	0.89	88.61	11.39
HCN	0.82	83.53	16.47
Pyocyanin	0.91	90.51	9.49
Pyoverdine	0.79	78.96	21.04
Biofilm	0.92	92.36	7.64

Table 5-1: Within- and between-patient diversity for individual phenotype. All phenotypes show significant between- and within-patient diversity. Between-patient diversity corresponded to the random factor 'Patient' from the mixed linear model, and within-patient diversity from the nested factor 'Isolate(Patient)'.

5.2.2 Diversity differences between early and chronic stage isolates

The between-patient diversity was shown to represent by far the greatest source of variation overall, regardless of stage of infection. The next step was to investigate the evolution of diversity between early and chronic stages of infection both within and between patients as it could reveal more. The data was, therefore, further analysed separating the data into two groups, according to the two stages of infection of the patients.

As mentioned previously, all between-patient diversity in early infections has associated p values of 0.08. This is not the case for chronic stage of infection, where all values are significant, most likely due to the fact that there are three times more chronic isolates than early ones. Thus, for the analysis of the data these early between-patient diversity values are still considered. All other values for diversity were highly significant ($p<0.005$) for within-patient diversity in early stage and for both types of diversity in chronic stage.

Moreover, the R square values were lower when looking at chronic stage of infection, with 59 to 87% of the variation explained by the variables, whereas in early infection they were of 61 to 99% (Table 5-2). Thus, in order to compare the diversity over stages of infection, between- and within-patient values were adjusted according to the R square value (Table 5-2).

Although between-patient diversity was higher than within-patient diversity in both stages of infection, differences between infection-stage groups were apparent. For almost all phenotypes similar differences in measurements of diversity were observed between early and chronic stages of infection. The contribution of between-patient diversity to total variation was lower in the chronic stage of infection than in early. This is with the exception of the speed of growth, which showed a similar and even slightly higher between-patient diversity in chronically infected patients' samples. Between-patient diversity in early stages of infection varied between 66 and 98%, except for growth where it was only 40%. In the chronic stage of infection between-patient diversity was lower, between 34 and 77%. The phenotypes showing the highest difference in between-patient diversity were twitch, elastase, HCN and pyoverdine in descending order. Unlike the other 3 phenotypes that showed also an important increase in within-patient diversity, twitching showed a smaller increase proportionally.

On the other hand, the contribution of within-patient diversity to total diversity was greater in chronic stages of infection but to different extents for the phenotypes. In early infected patients, the within-patient diversity of the bacteria represents 0.9 to 13% of total variation, with only growth higher at 20.8%. Conversely in chronic stage the within-patient diversity was higher, with values between 9.7 and 25.4%. The 4 phenotypes showing the highest within-patient diversity change are HCN, elastase, pyocyanin and pyoverdine.

At chronic stage of infection, several phenotypes, namely swarm, twitch, protease, elastase, HCN and pyocyanin, show similar ratios of diversity. These 6 phenotypes all show 50 to 60% between-patient diversity and 15 to 20% within-patient diversity at chronic stage. Thus within-patient diversity is relatively homogeneous between phenotypes. In early stage an overall higher variation in within-patient diversity was observed. Nevertheless, 7 phenotypes showed within-patient diversity between 0.93 and 6%, except for growth, swarm and pyoverdine with respectively 21%, 11% and 13%. Another 2 phenotypes, swimming and biofilm also showed similar ratios, 10% more or less than the other 6 phenotypes, with higher between-patient

diversity and lower within-patient diversity. Finally, growth and pyoverdine have the lowest difference between both types of diversity in chronic stages.

Growth is the only phenotype showing slightly different trends than for the other phenotypes. This phenotype shows a small increase in between-patient diversity, smaller than the decrease for all other phenotypes. It also shows by far the smallest increase of within-patient diversity, which can be considered insignificant. As for pyoverdine it shows similar values for both types of diversity at chronic stages, showing that between-patient diversity is less important in overall variation than for other phenotypes.

The phenotypes showing the highest changes are the same for both between-and within-patient diversity, except for twitching which shows more change in within-patient diversity. Finally, swimming and biofilm show the least changes in diversity between stages of infection. Indeed, at early stage both between- and within-patient diversity are some of the highest and lowest values respectively, and at chronic stage their values are the two highest between-patient diversity and the two lowest within-patient diversity values.

To conclude between-patient diversity is greater in early than chronic stages of infection. This might be due to the fact that as within-patient diversity increases and is different from patient to patient, the overall mean for each patient is quite similar in chronic patients thus limiting between-patient variation. Nevertheless, it is important to remember that the between-patient remains significantly higher than within-patient diversity for almost all phenotypes.

Phenotypes	Early					Chronic				
	R square	Between-patient diversity (%)	Within-patient diversity (%)	**Between-patient diversity (adjusted %)**	**Within-patient diversity (adjusted %)**	R square	Between-patient diversity (%)	Within-patient diversity (%)	**Between-patient diversity (adjusted %)**	**Within-patient diversity (adjusted %)**
Growth	0.61	65.92	34.08	**40.21**	**20.79**	0.67	67.51	32.49	**45.29**	**21.80**
Swim	0.99	99.06	0.94	**97.92**	**0.93**	0.86	86.90	13.10	**74.37**	**11.21**
Swarm	0.85	86.72	13.28	**74.13**	**11.35**	0.72	76.15	23.85	**54.90**	**17.19**
Twitch	0.94	95.22	4.78	**89.74**	**4.51**	0.67	75.89	24.11	**51.16**	**16.26**
Protease	0.90	92.84	7.16	**83.23**	**6.42**	0.73	75.84	24.16	**55.39**	**17.65**
Elastase	0.97	98.02	1.98	**95.51**	**1.93**	0.74	77.93	22.07	**58.01**	**16.43**
HCN	0.94	96.10	3.90	**90.70**	**3.68**	0.75	74.40	25.60	**55.90**	**19.23**
Pyocyanin	0.95	96.36	3.64	**91.93**	**3.48**	0.80	78.95	21.05	**63.23**	**16.86**
Pyoverdine	0.79	83.60	16.41	**66.04**	**12.96**	0.59	57.23	42.77	**33.98**	**25.40**
Biofilm	0.96	96.56	3.44	**92.22**	**3.28**	0.87	88.91	11.09	**77.39**	**9.66**

Table 5-2: Proportion of between- and within-patient diversity adjusted according to R square values. Values in the table correspond to percentages of the types of diversity with respect to the whole phenotypic variation. All early between-patient diversity was not significant, for all phenotypes the p values being of 0.08. All other diversity was statistically significant ($p<0.05$). between- and within-patient diversity for all phenotypes in early and chronic stages of infection. Diversity is determined using a mixed linear model approach. The R square represents the proportion of variation explained by the variables. All early between-patient diversity was not significant, for all phenotypes the p values being of 0.08.

5.2.3 Individual within-patient diversity

Although within-patient diversity was shown to be smaller than the diversity between patients this type of diversity is still significant for all phenotypes and is greater at chronic than early stages of infection. Thus, I considered it important to assess the diversity for each phenotype between isolates from each individual patient.

For all patient samples a minimum of 12 isolates were available, but for most samples at least 24 single isolates were collected. Phenotypes were measured in different units. To observe the phenotypic diversity present at patient level, values were transformed. This transformation consisted of recalculating values in comparison to the maximum value for each phenotype. Doing this resulted in a scale spreading from 0 to 100, with 100 corresponding to the maximum value observed for a particular phenotype. As faster speed of growth meant lower values, the percentage values obtained were subtracted from 100 for easier visualisation. All individual

patients are presented in four figures according to the diversity patterns observed. All patients infected by early isolates are shown in Figure 5-2. All chronic patients showing particularly overall high diversity are presented in Figure 5-3 while Figure 5-4 and Figure 5-5 show chronic strains having low or varying degrees of diversity for certain phenotypes. These figures are a visual representation of within-patient diversity for all phenotypes.

As discussed in the first results chapter, half of the isolates from half of the chronic patients were genotyped and one unique strain seemed to infect each patient. Therefore, on top of grouping patients by their visual diversity pattern, they were also grouped according to whether they had been genotyped or not. None of the isolates presented in Figure 5-2 and Figure 5-5 have been genotyped whereas in Figure 5-3 and Figure 5-4 patients presented had half their isolates genotyped. This is with the exception of chronic patient 1 (CP1) and CP8a in Figure 5-3, which also showed high diversity and both samples from CP8 are presented side by side. Any diversity observed is considered likely to be due to adaptation of the bacteria within the CF lung and not to parallel infecting strains with different phenotypic behaviours.

This type of visual representation shows again obvious variation between isolates of individual patients. The diversity observed seems to differ both from patient to patient and between phenotypes. None of the patients show readings that are altogether high or low for all phenotypes measured. Indeed, while the results show low readings for one phenotype, the measurements can be high for others, with no overall obvious trend between phenotypes. High readings for one phenotype do not mean high production of all virulence factors or motility.

This underlines a first type of diversity regarding phenotypes themselves, these links and correlations between phenotypes having been discussed in the previous results chapter. For example, protease and elastase were highly correlated, nonetheless patients 7,10, 12 for example show low protease but higher elastase values. However, some patients do show overall low readings for all phenotypes, this is the case for chronic patients 16, 3, 11, 14, and to a lesser extent CP18 and CP2.

Early patients show relatively low variation for each phenotype, confirming within-patient variation being lower in early stage than chronic (Figure 5-2). One early patient, number 4 (EP4), shows higher variation than any other. Early patients are presented together, and few seem to group by pattern. Isolates from patients EP2, EP3, EP5 and EP6 seemed to differ only

in pattern regarding twitching, swimming and HCN production, while EP1, EP4 and EP7 had unique patterns. According to the genotyping information EP3 and EP5 were infected by the same strain, and in pattern only differ regarding twitching motility.

Chronic isolates were sorted according to patterns and genotyping. Figure 5-3 shows the patients for which isolates show high variations in readings for the majority of phenotypes. The isolates from CP7 show particularly high variation, and that for all 10 phenotypes. The colours representing each isolate also show that it is not always the same isolates that are responsible for high or low measurements. Figure 5-4 displays all the remaining chronic patients for which isolates were genotyped and Figure 5-5 those remaining for which no isolates were genotypically checked. A couple of patients, such as CP4 and CP13, have similar patterns with very low diversity for half the phenotypes and quite high for others, which all seem to be QS regulated.

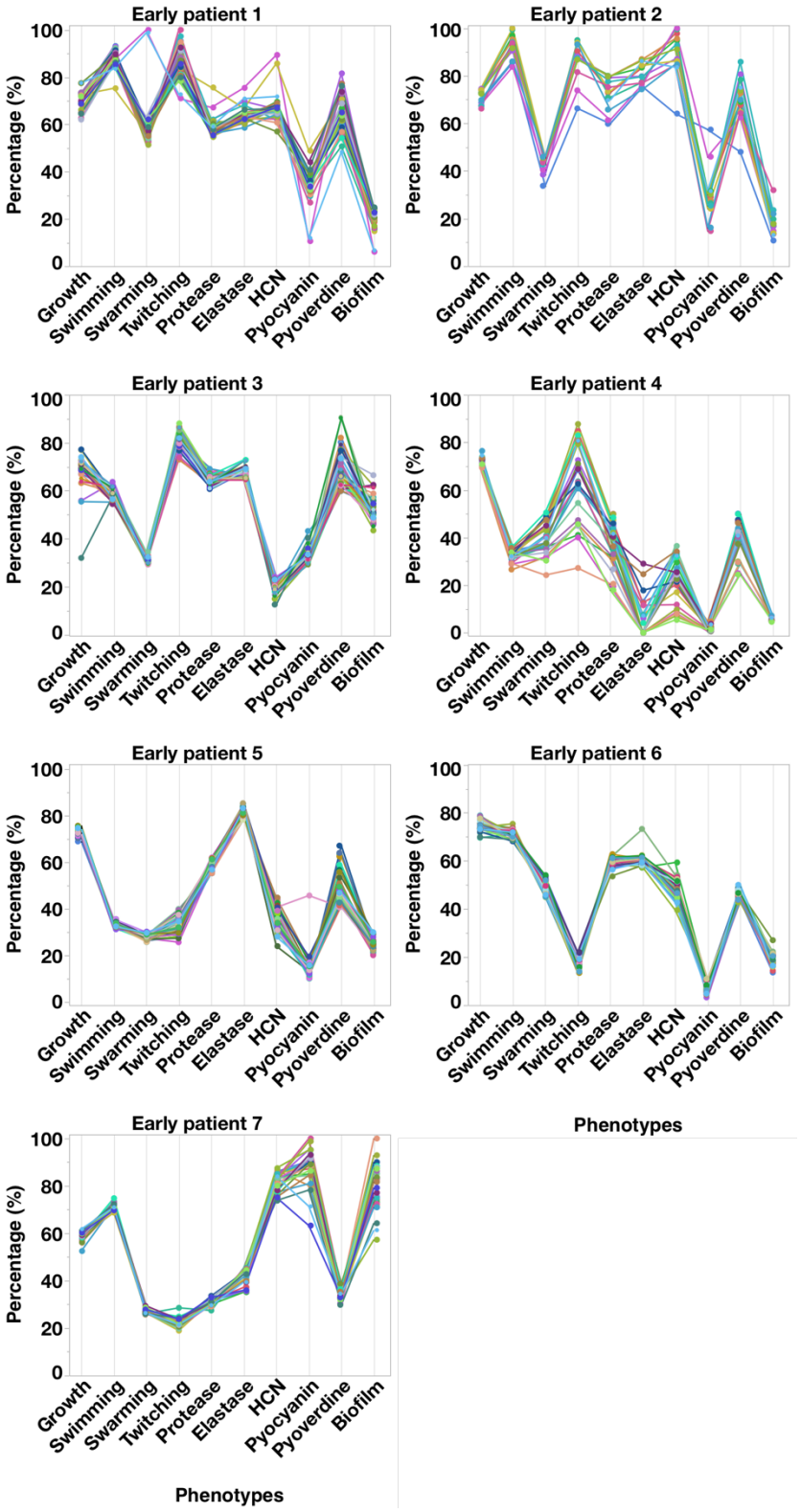


Figure 5-2: Individual phenotypic profiles for all isolates from 7 early stage infection patients. Profiles made using all isolates available for each patient. The number of isolates vary for early patients (EP) that is: EP1: 26 isolates; EP2: 13 isolates; EP3: 36 isolates; EP4: 24 isolates; EP5: 60 isolates; EP6: 36 isolates; EP7: 26 isolates. All phenotypic measurements were converted to percentages in comparison to the highest value for each phenotype. Original values were calculated from a mean of a least 3 biological replicates for all assays. For all phenotypes the closer the ratio is to 100 the higher the reading.

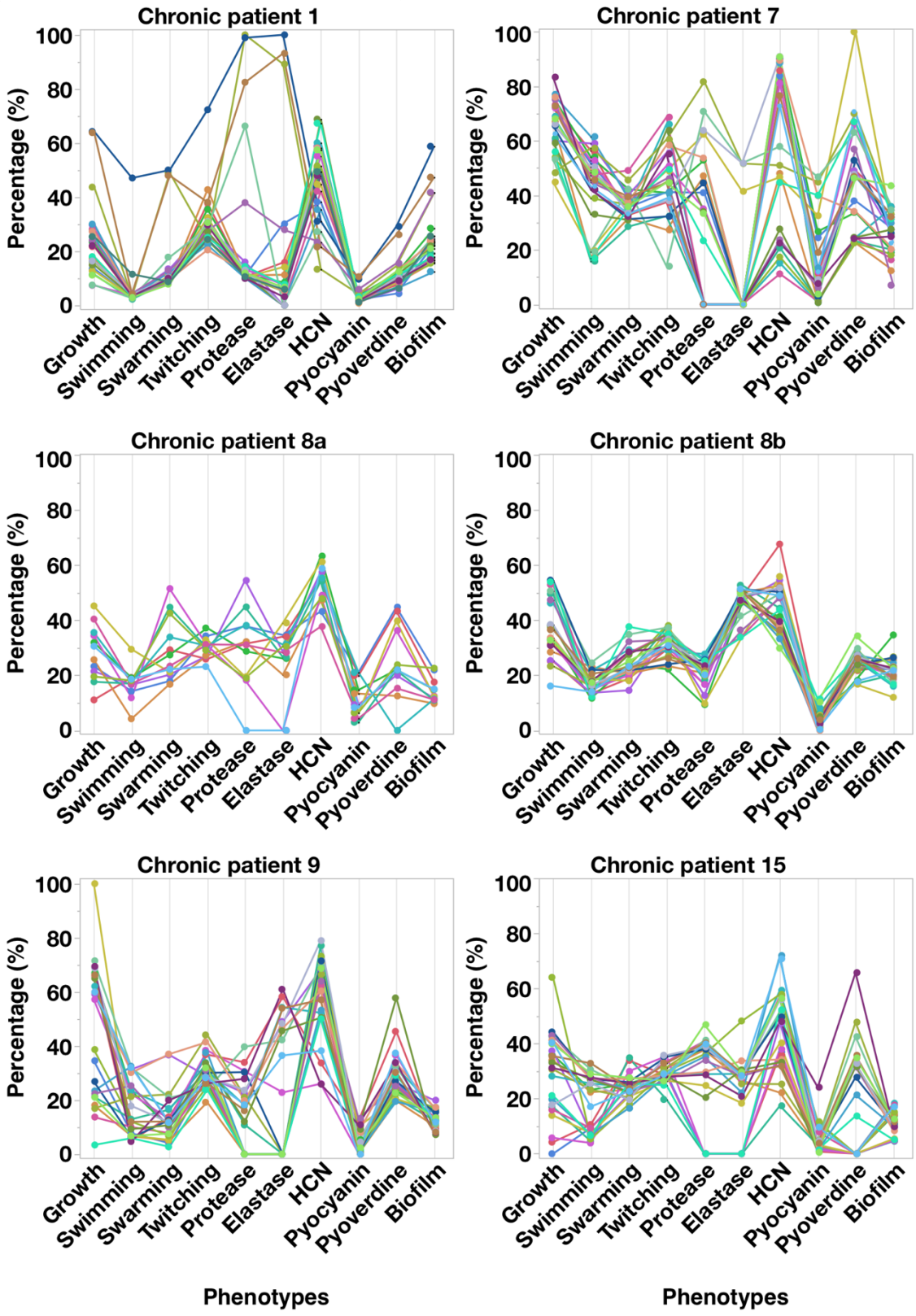


Figure 5-3: Individual phenotypic profiles with high variation for all isolates from 6 chronically infected patients. Profiles made using all isolates available for each patient. CP1, CP7, CP8b, CP9, CP15: 24 isolates presented; CP8a: 12 isolates presented. All phenotypic measurements were converted to percentages comparatively to the highest value for each phenotype. Original values were calculated from a mean of a least 3 biological replicates for all assays. For all phenotypes the closer the ratio is to 100 the higher the reading. All patients, except CP1 and CP8b, had half their isolates genotyped and only one strain was found per patient.

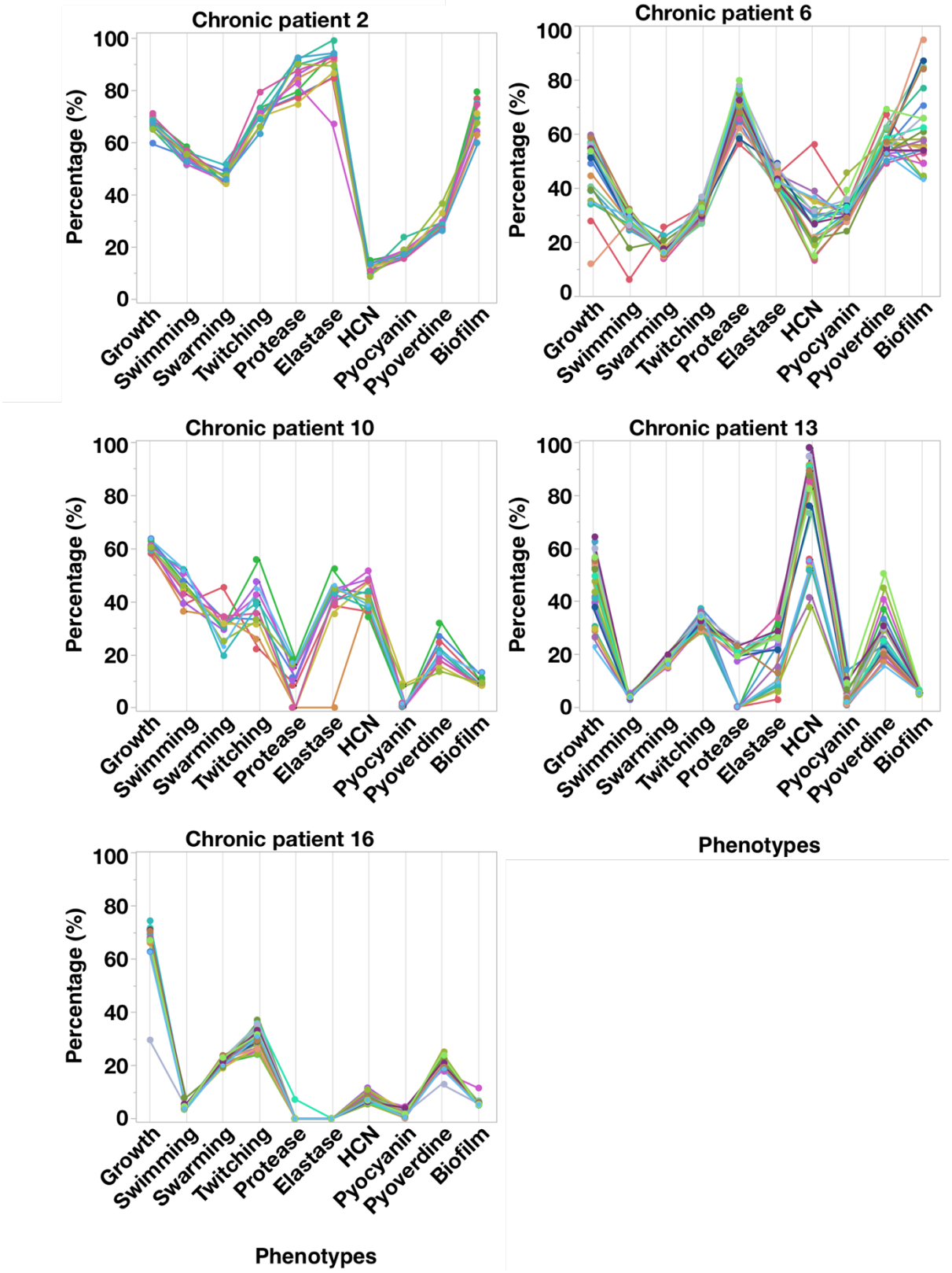


Figure 5-4: Individual phenotypic profiles with mixed variation for all isolates from 5 chronically infected patients. Profiles made using all isolates available for each patient. CP6, CP13, CP16: 24 isolates presented; CP2, CP10: 12 isolates presented. All phenotypic measurements were converted to percentages comparatively to the highest value for each phenotype. Original values were calculated from a mean of a least 3 biological replicates for all assays. For all phenotypes the closer the ratio is to 100 the higher the reading. All patients had half their isolates genotyped and only one strain was found per patient.

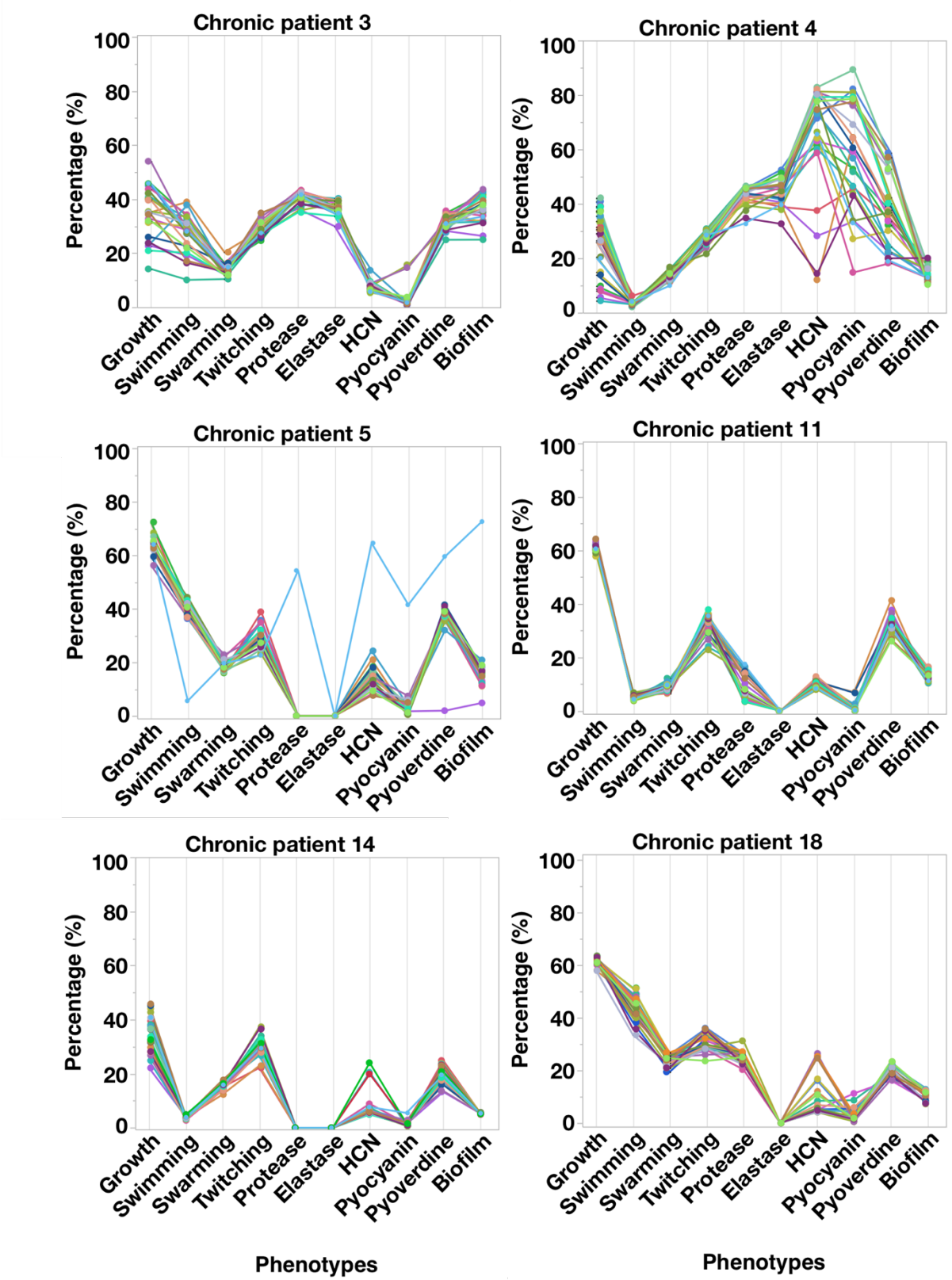


Figure 5-5: Individual phenotypic profiles with low or mixed variation for all isolates from 6 chronically infected patients. Profiles made using all isolates available for each patient; 24 isolates presented per patient. All phenotypic measurements were converted to percentages comparatively to the highest value for each phenotype. Original values were calculated from a mean of a least 3 biological replicates for all assays. For all phenotypes the closer the ratio is to 100 the higher the reading. None of the patients had any isolates genotyped.

Visual observation of the data gives a first insight into the phenotypic variation within individual patients, but quantitative statistical analysis is required. A one-way ANOVA was used in order to identify patients for which the isolates show significant variation and thus diversity. The entire detailed results can be found in Appendix 5-1.

Results in Table 5-3 showed that 10 chronically infected patients presented a significant variation for at least half of the 10 phenotypes (CP1, CP7, CP15, CP9, CP5, CP8b, CP13, CP4, CP6 and CP8a in descending order). The diversity found within these 10 chronic patients is obvious when observing the F ratios, as well as within CP10 which was significant for 4 phenotypes (Figure 5-6). Only 2 early stage patients, EP1 and EP4, display significant diversity for 6 and 8 phenotypes respectively. This again underlines that the isolates of chronically infected patients tend to show higher within-patient diversity than isolates from early stage infections.

The visual observations are also confirmed regarding the patients showing the least amount of diversity. Indeed, the isolates of patients EP5, EP6, EP7, EP3 and CP16, CP18, CP11, CP14 and CP2, showing low diversity visually, had significant variation for only 2 phenotypes or less.

For most phenotypes a minority of patients are responsible for the diversity. With the exception of proteolytic activity and HCN, fewer than half of the patients show significant diversity for each phenotypic assay (Figure 5-6). The only 2 phenotypes exhibiting significant diversity over half the patients are HCN and protease. HCN is also the only phenotype with significant diversity in over half the early patients, most others were in only 0 to 2 patients. Chronic stage patients demonstrated the opposite, with a minority of phenotypes, pyoverdine, pyocyanin, twitching and biofilm, exhibiting significant diversity in 7 or less of the 17 chronic patients (Table 5-3). These results confirm that some patients show low diversity across all phenotypes. Conversely, only 3 chronic patients show high diversity for at least 9 phenotypes and all display varying amounts of diversity.

Moreover, some patients show high diversity for phenotypes such as proteases, pyocyanin and pyoverdine which are all regulated by quorum sensing molecules, confirming correlations observed in the previous chapter.

Finally, according to genotyping data introduced in the first results chapter, certain patients were infected by the same strains. As mentioned previously EP3 and EP5 are infected by the same strains, as are patients CP1 and CP2, and CP8a, CP8b and CP18 respectively. Although infected by the same strains, the chronic patients all show different and individual patterns of diversity. Indeed, differences in diversity between CP1 and CP2 are obvious, CP1 exhibiting significant diversity for all phenotypes while CP2 for none (Table 5-3). CP8a and CP8b also demonstrate different diversity patterns and both show significantly higher diversity than CP18.

	Growth	Swimming	Swarming	Twitching	Protease	Elastase	Hydrogen cyanide	Pyocyanin	Pyoverdine	Biofilm
EP1	***		***	*			**	*		*
EP2				***	*		*			
EP3										
EP4	*		**	***	***	***	***	**	**	
EP5							***		*	
EP6							*			
EP7							*			
CP1	***	***	***	***	***	***	***	***	*	**
CP2										
CP3	*	***				*				
CP4	***					***	***	***	*	
CP5		**		*	***		***	***		***
CP6		***	***		***		***	*		
CP7	***	***		***	***	***	***	***	***	*
CP8a		**	***		***	***			*	
CP8b	***	**			**	***	**		*	
CP9	***	***	***		***	***	**		***	
CP10		**		*	***	***				
CP11					**					
CP13	***		**		***	***	***	*		
CP14			*							
CP15	*	***	**		***	***	*	**	***	***
CP16	***		*							
CP18		*					*			

Table 5-3: Significance from ANOVA for all phenotypes and individual patients. P values were obtained from one-way ANOVA analysis using all data available. ***(red): $p<0.0005$; **(orange): $p<0.005$; *(yellow): $p<0.05$.

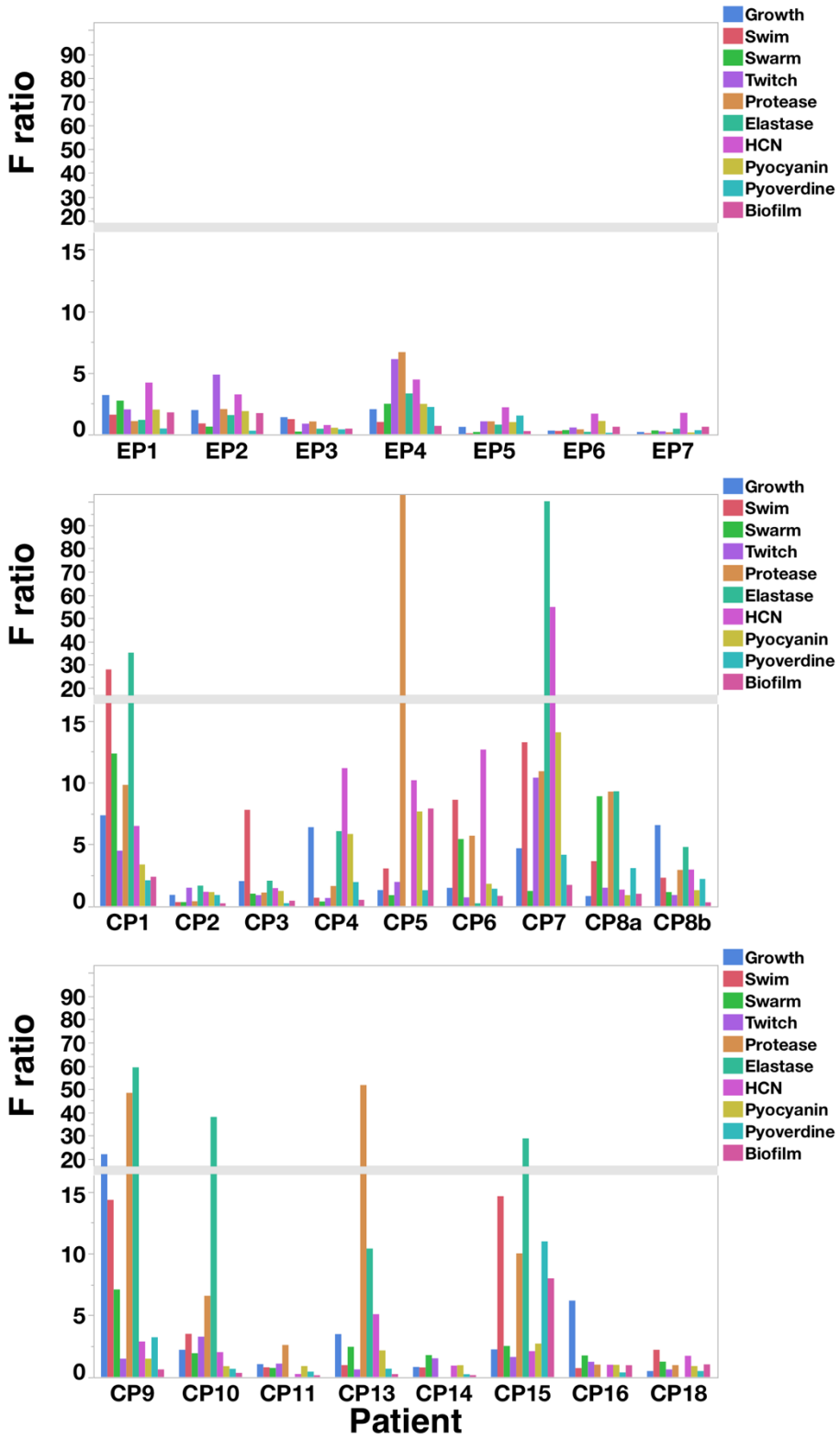


Figure 5-6: F ratios from ANOVA analysis for all phenotypes and individual patients. One-way ANOVA analysis was undertaken using all the individual patient data. All analysis was done taking into account all isolates for each patient. The number of isolates per patient vary between 12 and 60. The number of isolates vary for early patients that is: EP1: 26 isolates; EP2: 13 isolates; EP3: 36 isolates; EP4: 24 isolates; EP5: 60 isolates; EP6: 36 isolates; EP7: 26 isolates. All chronic patient data are based on 24 isolates except for CP2, CP8a and CP10 with only 12 isolates. For all phenotypic assays the number of replicates is n≥3.

5.2.4 Diversity amongst QS molecules production

Quorum sensing (QS) molecules are responsible for the regulation and expression of a number of phenotypes measured in this study and were found to be associated to various extents with those in the previous chapter. Briefly, two classes of these signalling molecules, homoserine lactones and quinolones, were quantified by mass spectrometry undertaken by Dr. Volker Behrends at the University of Roehampton. As well as the 2 quinolones mentioned in the previous chapter (PQS and precursor HHQ), another quinolone was quantified in the isolates from the RBH set. This molecule, 2-heptyl-4-hydroxyquinolone-N-oxide (HQNO), is a precursor of PQS, earlier than HHQ (Déziel et al., 2004). HQNO showed the same levels of association with the other phenotypes as the associations found for PQS.

The data obtained is represented visually in Figure 5-7. The concentrations are expressed as a percentage of the levels of a PA01 control sample. This had the advantage of being more reliable than pure standards and could be used for a larger number of compounds as there was no need to have a standard of each of the compounds. Due to the analysis cost only one single biological replicate was measured per isolates and per type of molecule. Further analysis on the individual patient using one-way ANOVA was, therefore, not possible due to the lack of replication.

As for the other phenotypes, the presence of both within- and between-patient diversity was evident through visual observation. However, unlike with the other phenotypes only a small number of patients displayed any obvious variation for most QS molecules tested; this is with the exception of HQNO where a higher number of patients show diversity. In order to observe more closely the different diversity between patients showing lower measurement a zoomed in box plot of QS molecule concentrations with the maximum equivalent to PA01 levels for all phenotypes and patient is shown in Appendix 5-2. This low diversity is coupled with very low concentrations in comparison to the maximum value. Chronic patients 4, 11 and 12 seem to show the highest concentrations for several QS molecules as well as the most important variation amongst their isolates. Overall HSLs show lower concentrations and diversity than quinolones in comparison to PA01 control levels. It is important to note that for quinolones the highest concentrations measured were for PQS.

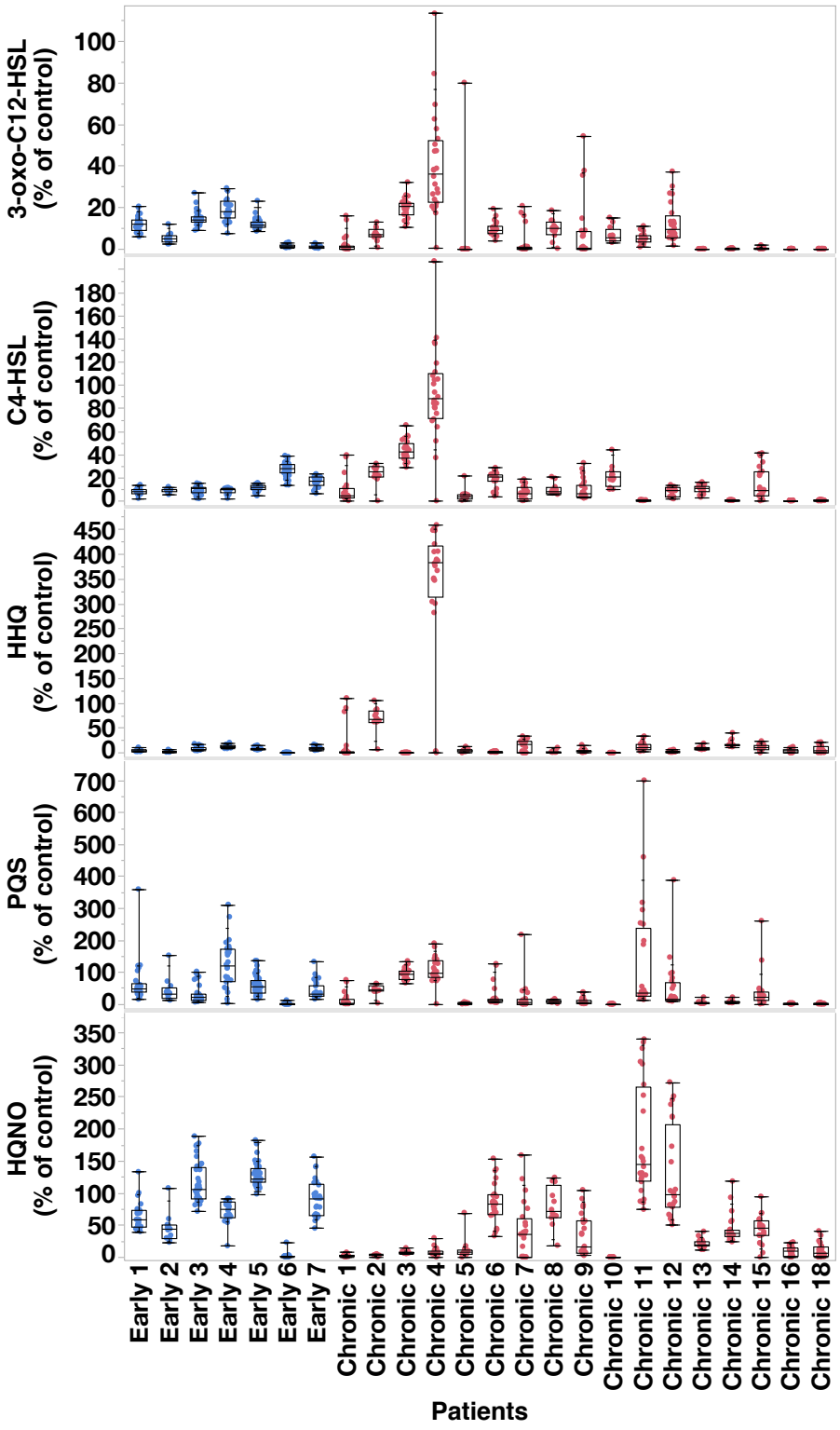


Figure 5-7: Visual representation of QS data for all phenotypes and patient. Each box represents the extent of the diversity for each patient taking into account all the single isolates from that patient, dots representing each isolate. The unit corresponds to the proportion in comparison to the PA01 control. For each patient a minimum of at least 12 isolates were used in the test of phenotypes. All values used to make plots are means of at least 3 biological replicates for each phenotypic assay. Early= Isolates from early stage infections; Chronic= Isolates from chronic stage infections. 100% corresponds to PA01 control sample levels.

The same statistical analysis was undertaken as for the other phenotypes using a mixed linear model approach. This enabled determination of the extent of both the between- and within-patient diversity (Table 5-4). The percentage of diversity explained by between-patient diversity and within-patient diversity was between 39% and 79% when not taking into account stages of infection. The between-patient diversity explained most of the variation, between 72% and 85%, for C4-HSL, HHQ and HQNO. On the other hand, for 3-oxo-C12-HSL the between-patient diversity explained only 60% of the overall diversity, and for PQS only 38%. Therefore, similarly to the other phenotypes, overall the between-patient diversity seems more important than the within-patient diversity, with an exception regarding PQS. The same overall results were witnessed when reanalysing the data by stages of infection (Table 5-5). As observed previously, the between-patient diversity in early stages of infection was not significant for all 5 molecules, with p values all being around 0.08.

QS	R square	Between-patient diversity	Within-patient diversity
3-oxo-C12-HSL	0.60	59.57	40.43
C4-HSL	0.79	79.21	20.79
HHQ	0.84	84.64	15.36
PQS	0.39	38.28	61.73
HQNO	0.75	72.31	27.69

Table 5-4: Within- and between-patient diversity for individual phenotype regardless of stage of infection. Diversity is determined using a mixed linear model approach. The R square represents the proportion of variation explained by the variables. Values in the table correspond to percentages of the types of diversity with respect to the variation indicated by the R square. All phenotypes show significant ($p<0.05$) between- and within-patient diversity.

The changes in diversity between stages of infection fit the results found with the phenotypic data regarding within-patient diversity but are less clear cut regarding between-patient diversity. The proportion of variation explained by within-patient diversity increases for all QS molecules except HHQ, for which it slightly decreases. The biggest increase is seen for 3-oxo-C12-HSL, then HQNO and PQS, and finally stays practically equal for C4-HSL. The importance of the between-patient diversity decreases for 3-oxo-C12-HSL, PQS and HQNO. On the other hand, it stays constant for C4-HSL and increases for HHQ.

Overall 3-oxo-C12-HSL, HQNO and PQS, and to a lesser extent C4-HSL, are the only QS molecules showing a decrease in between-patient diversity and increase in within-patient diversity between both stages of infection. However, for PQS in both stages of infection the importance of within-patient diversity surpasses that of between-patient diversity. In early stage infection the diversity for PQS is almost split equally between both types of diversity, with within-patient diversity slightly higher and this difference becomes accentuated in the chronic stage.

In conclusion, the results are less clear cut than for the other phenotypes, although, overall, the between-patient diversity is the most important. Its importance decreases in chronic stages of infection, while that of within-patient diversity increase.

QS	Early			Chronic		
	R square	Between-patient diversity % (adjusted)	Within-patient diversity % (adjusted)	R square	Between-patient diversity % (adjusted)	Within-patient diversity % (adjusted)
3-oxo-C12-HSL	0.81	84.34 (68.44)	15.66 (12.71)	0.57	54.11 (30.66)	45.89 (26.00)
C4-HSL	0.77	78.3 (60.31)	21.70 (16.71)	0.79	77.59 (61.26)	22.413 (17.70)
HHQ	0.74	77.79 (57.54)	22.21 (16.43)	0.84	82.83 (69.41)	17.17 (14.39)
PQS	0.41	46.30 (19.10)	53.7 (22.16)	0.37	34.37 (12.66)	65.63 (24.17)
HQNO	0.82	80.36 (65.64)	19.64 (16.04)	0.68	66.89 (45.32)	33.11 (22.43)

Table 5-5: Within- and between-patient diversity for individual phenotype according to early and chronic stages of infection. Diversity is determined using a mixed linear model approach. The R square represents the proportion of variation explained by the variables. Values in the table correspond to percentages of the types of diversity with respect to the variation indicated by the R square. The values were adjusted, in brackets, to show the percentage according to the total variation. All early between-patient diversity was not significant, for all phenotypes the p values being of 0.08. All other diversity was statistically significant ($p<0.05$).

5.3 Discussion

In this chapter the main aims were to assess both the within-patient and between-patient diversity for all phenotypes and the changes in diversity between early and chronic stages of infection. The phenotypes used to measure diversity were a set of 10 virulence factors, but also 5 QS regulatory molecules. Also, multiple isolates from individual patients genotyped showed patients to be only infected by a single strain, thus any phenotypic diversity observed here should not be due to several different infecting strains but adaptation within the CF lung.

5.3.1 Between-patient diversity is higher than within-patient diversity

Both the between-patient diversity and within-patient diversity of isolates stemming from both early and chronic stages of infection were examined and compared. Visually, diversity was obvious and statistical analysis indicated that between-patient diversity weighed significantly more in the overall variation than within-patient diversity for all. The between-patient diversity represented over 75% of the diversity for all phenotypes, and this difference was observed over both stages of infection. Although between-patient diversity is higher than within-patient diversity, it is nonetheless significant for all phenotypes and stages of infection.

These findings are in contradiction with the results from a study concentrating on patients all infected by Liverpool Epidemic Strains, where the within-patient diversity was found to be more important (Mowat et al., 2011, Williams et al., 2015, Ashish et al., 2013). The LES strain is a transmissible strain with enhanced virulence which has become one of the most prevalent strains isolated from CF patients in the UK (Salunkhe et al., 2005, Winstanley et al., 2009). The LES strains isolated from the patients in Liverpool most probably originated from the same initial strain with the deriving lineages having the same genotypic background. Therefore, these studies concentrated on a very different set of isolates than in this study.

In this present study, the 23 patients from which samples were gathered were infected by a more varied selection of strains. Indeed, 10 strains were unique in this context, as they were each found in only one patient attending the Royal Brompton Hospital. Another 12 patients were infected by 9 different common strains (amongst which 5 different epidemic strains) (Williams et al., 2018, Martin et al., 2013). The information was missing for one early patient

and two pairs of patients were each infected by the same strain. Several other studies have also found high genetic diversity of *Pseudomonas aeruginosa* strains (Johansson et al., 2015, Vosahlikova et al., 2007). A 2015 study found that amongst 100 patients in a CF centre, most patients were infected by different unique strains (Johansson et al., 2015). The fact that the between-patient diversity is the highest diversity further underlines the importance of the diversity of infecting strains.

Although the 2011 study from Mowat et al. found within-patient diversity being responsible for most of the diversity, between-patient diversity of *Pseudomonas aeruginosa* was nonetheless significant. In addition to the specific strain infecting, the between-patient diversity is most probably also impacted by the individual patients. The patients' characteristics and health will lead to a lung environment with particular specificities in turn impacting the bacteria, as described in the previous chapter. Indeed, the patients' gender, age and health can impact the immune system of patients. The innate immune system is the first line of defence against bacterial infection in the CF lung. Different levels in immune cells were found to be gender-specific in several studies (Demko et al., 1995, Corey et al., 1997, Lammertyn et al., 2017), and also correlate to FEV1 (Corey et al., 1997, Ramos et al., 2017).

Moreover, the immune system contributes to inflammation in the CF lung. Thus the different levels of immune cells lead to changes of the lung environment and different outcomes for CF patients (Hausler et al., 2002, Moser et al., 2002, Mayer-Hamblett et al., 2007). This and the varying concentrations of nutrients and oxygen levels found in the CF lung will result in the bacteria adjusting once more to this complex environment (Worlitzsch et al., 2002, Yoon et al., 2002, Bhagirath et al., 2016). Nonetheless, the extents to which the sputum specificities might impact the bacteria are still unknown. The bacteria thus adapt to the various niches and environmental pressures and this will lead to both within- and between-patient diversity.

As presented in previous Chapters 3 and 4, in this study we only possessed limited clinical information about the patients and their treatment regimen, the impact of which was not accounted for. Moreover, further analysis of the patients' sputum at the time of bacterial isolation could potentially unveil specific characteristics such as differences in nutrients or immune cells present. Gathering further clinical information could enable delving deeper into the impact of patients' individual characteristics with respect to phenotypes and diversity of isolates.

5.3.2 Differences in diversity between early and chronic stages of infection

Previously in the chapter it was shown that although the initial infecting strain and individual patient account for most of the diversity overall, adaptation within the lung is nevertheless observed. It has been described how recombinations and hypermutations lead to a multiplication of sub-populations evolving in parallel while originating from a same common ancestor strain (Huse et al., 2010, Workentine et al., 2013).

Although between-patient diversity accounts for most of the diversity overall, observing the differences between the stages of infection nonetheless reveals the importance of within-patient diversity. We found that overall within-patient diversity was greater in chronic stages of infection than early and was significant for all phenotypes. This within-patient diversity is lowest for biofilm and swimming. Regarding biofilm production, this might be explained by the fact that the assay measures the capacity of attachment to form a classical biofilm, whereas in the CF lung biofilms resemble aggregates; measurements were, as a result, low with little diversity (Staudinger et al., 2014a). For speed of growth, the diversity remains the same between stages of infection, and for pyoverdine both diversities are nearly equal in chronic stage of infection. However, for the majority of phenotypes, that is, swarming, twitching, protease, elastase, HCN and pyocyanin, it is interesting to note that the proportion of within-patient diversity in chronic stages of infection is similar. In the chronic stage of infection all these phenotypes show a ratio of around 20% for within-patient diversity.

For several phenotypes the within-patient diversity was already significant in early stages of infection, which might be due to the fact that it is impossible to get a snapshot of the bacteria as it enters the lung, as patients have routine samples once every 3 months. Thus, certain phenotypic modification most critical to install infection might have already occurred.

The time frame in which changes occur remains unclear and needs to be investigated more closely. Indeed, knowing the exact chronology of adaptation and increase in diversity could give us clues on the importance of various phenotypes regarding initial colonisation but also installation of chronic infection. Some of the data in this chapter gives, however, some insight into the chronology of change. The isolates from EP3 and EP5 were found to correspond to the same initial strain. When looking at the levels of the phenotypic readings and the overall

pattern, they seemed similar with the exception of twitching. As both patients had not been infected for long, this observation indicated that in some cases twitching decreases instantly when colonising the lungs. Indeed, the fact that certain early isolates already present a lack of twitching suggests that there is a higher pressure to lose the use of the pili, in the same way that it is well known that the loss of the flagella is a way to evade phagocytosis (Wilder et al., 2009, Palmer et al., 2005, Amiel et al., 2010). Twitching also shows the most binary type phenotype; thus, we can presume there is strong pressure for all isolates to lose this type of motility. Unlike for twitching, other phenotypes may be very diverse and advantageous for survival, or simply due to less common pressure to evolve, although distinguishing between the two remains complex.

P. aeruginosa having never been isolated from these patients previously, the early isolates were considered as first-time infections. Nonetheless, the bacteria could have been previously present but simply not detected in prior samples. The significant within-patient diversity of isolates in patients infected for the first time might also be due to the bacteria already being present in the paranasal sinuses. Indeed, several studies suggest that *P. aeruginosa* infect the sinuses prior to colonising the lungs of CF patients, and already exhibit adaptation in this upper airway niche (Hansen et al., 2012, Ciofu et al., 2013, Mainz et al., 2009, Johansen et al., 2012, Fothergill et al., 2014). Several of these studies show similar adaptations to those observed in the CF lung, such as loss of motility (Hansen et al., 2012, Fothergill et al., 2014). Therefore, it would have been interesting at the time of first isolation of *P. aeruginosa* in the lower respiratory tract of the early patients of this study to also try to isolate bacteria from an upper airway swab to compare strains found and their phenotypic characteristics.

The appearance of sub-populations has been observed to happen fairly rapidly, and recently a study has implied that most of the adaption occurs within 2-3 years after colonisation (Bartell et al., 2019, Clark et al., 2015). But the exact time in which the phenotypic diversity significantly increases leading to subpopulations remains unknown. Also adaptation within the CF lung has been observed in many longitudinal studies but mostly only on a single isolate or a limited number (Smith et al., 2006). Other studies have concentrated solely on diversity in chronic infection (Ashish et al., 2013, Mowat et al., 2011, Fothergill et al., 2010). Though, as mentioned previously, looking also at early stage isolates is essential to better identify the most critical phenotypic changes necessary for the bacteria to try installing long term infection.

5.3.3 Individual within-patient diversity patterns

As discussed previously, within-patient diversity is biologically relevant and is higher in chronic stages of infection. Although the results do show significant within-patient diversity, we might still be slightly underestimating this diversity due to our sampling method from sputum samples. The best approach is to sample directly from explanted lungs but this limits the number of patients (Jorth et al., 2015). Nevertheless, it has been shown that the diversity and type of sub-population observed are constant from several samples recovered from an individual patient the same day (Mowat et al., 2011). This underlines that this method of sampling also used in this study is sufficiently robust.

The statistical analysis also needs to be taken into consideration when trying to quantify accurately the extent of within-patient diversity. Although the statistical analysis correlates with the trends observed, with the ANOVA approach the range of variation does not seem to be the only factor weighing in diversity. Indeed, the values of only a single or a few isolates seem to increase the diversity significantly, when the rest of the isolates in the individual patient make for an otherwise much less diverse population. Protease production in CP5 is a good example where all values are null except for one isolate, resulting in a high statistically significant diversity. Thus, outliers or technical mistakes could easily skew the data. In several patients (CP5, CP10, CP13), although the range of variation is smaller for a certain phenotype the diversity can be higher than for another due to the isolates being separated in two groups rather than spread across the range.

It is also important to remember the difference between what statistics identify as significant diversity and what is biologically relevant. For example, there is most probably a biological difference between high diversity due to even spread of measurements and high diversity due to clusters of high and low readings, though both cases lead to statistical high diversity.

The results demonstrated that all patients showed very individual patterns of phenotypic diversity and measurements. The diversity fluctuates from one phenotype to another for all isolates, though some show constant low diversity. Thus, there was a lack of overall pattern in chronic stages of infection, confirming why the phenotypic associations found in the previous chapter were only of moderate strength.

Interestingly, none of the patients infected by epidemic strains showed the highest diversity in phenotypes. Most patients showing the highest diversity were infected by unique strains. A couple of patients were infected by the same strains but nonetheless presented very different phenotypic patterns and diversity. This could be due to the length of infection or differences in overall clinical health of the individual patients. Patients CP1 and CP2 were found to be chronically infected by the same strain; however, they presented different levels of diversity with CP1 exhibiting significantly higher diversity. This could be due to the fact that patient CP1 has been chronically infected for 5 years, whereas CP2 for only 6 months, suggesting yet again that within-patient diversity increases over time through adaptation. But other factors than length of infection could also explain the difference in diversity. The sample from patient CP1 was recovered at the end of an exacerbation, whereas patient CP2 was in a stable condition. Similarly, patients CP8 and CP18 were infected by the same strain and had both been infected for a relatively similar amount of time, around 4 and 5 years respectively.

Two samples from CP8 were recovered, both during the same exacerbation but 2 weeks apart. The isolates from CP8a recovered first showed higher diversity than the second sample CP8b. On the other hand, the sample from patient CP18 was collected during a visit after the end of IV antibiotic treatment after the end of an exacerbation. The isolates recovered from CP18 showed lower diversity than both of those from CP8. It is also interesting to note that CP7, who is the patient showing the higher diversity overall, is also one of only 2 patients at the beginning of a pulmonary exacerbation. The other patient is CP14 also showing particularly high diversity for HCN production, with both these patients being infected with *P. aeruginosa* isolates producing the highest amount of the gas. Finally, the patients for which the isolates are showing significant diversity for the highest number of phenotypes are patients CP1, CP7, CP9 and CP15, who happen to all have been experiencing pulmonary exacerbations.

No conclusions can be drawn from these observations on only a few patients but these potential links between exacerbation and diversity follow the results shown from previous studies. Indeed, these observations suggest that exacerbation could be due to a transient increase in virulence for some isolates, which in turn leads to increased diversity (Fothergill et al., 2010). However, the role of *P. aeruginosa* remains very unclear, as some studies did not find any specific fluctuations in phenotypes during exacerbations while others did (Workentine et al., 2013, Mowat et al., 2011). Some studies suggest that these exacerbations could be due to the spread of the infection to new areas of the lungs (Whelan and Surette, 2015, Fodor et al., 2012).

This new spread of infection could occur through a small active subpopulation (Whelan and Surette, 2015). This active subpopulation could be similar to the overproducers described by O'Brien et al. and could translate in isolates within patients showing high diversity as some show low virulence while others become more active and virulent (O'Brien et al., 2017). As exacerbations have a detrimental effect on patients' symptoms it is important to further explore the role of *P. aeruginosa* in these adverse events. These preliminary observations on a small cohort of patients further suggest the implication of the bacteria and the importance to further investigate the differences in phenotypic behaviours and within-patient diversity during these exacerbation periods. Although the isolates from these patients were not all genotyped, the acquisition of a new strain was seemingly not the cause for the exacerbation (Aaron et al., 2004b). In the future, better coordination with clinicians could enable getting multiple isolates from samples clearly identified as coming from stable patients and others stemming from patients experiencing the beginning of a pulmonary exacerbation before any IV antibiotic treatment. Indeed, it is a limitation of this study that bacterial isolation took place before selecting patients for clinical characteristics.

Most patients stay infected by the same unique strain for an extended period of time (Hall et al., 2014). Thus, the diversity observed within the isolates of a same patient is a direct consequence of adaptation to the CF lung of each patient. A number of studies have also observed the presence of many phenotypic sub-populations despite a single clonal lineage (Workentine et al., 2013, Marvig et al., 2015b, Williams et al., 2018). The appearance of diversity is thought to happen through multiple routes of adaptation individual to each patient (Bartell et al., 2019).

It is also important to understand the manner in which the bacteria adapt and the pressures and reasons why they do so in these specific ways. Diversity seems to be mainly driven by hypermutation and recombination (Darch et al., 2015, Mena et al., 2008, Huse et al., 2010). The reasons why are less well understood but it is thought that it is for the benefit of the global bacterial *P. aeruginosa* community. By social cheating self-generated diversity thus secures communities (Smith et al., 2006, Boyle et al., 2017, Boles et al., 2004). The different sub-populations therefore could have different roles in the community, for example evading the immune system (Lovewell et al., 2011). This seems to lead, for example, to the presence of a small minority of strains that retain their virulence and can be considered overproducers (O'Brien et al., 2017). Indeed in an environment that fluctuates heavily, for example due to

courses of antibiotics, having communities of mixed phenotypes is an advantage to the bacteria (Markussen et al., 2014). Therefore, the idea that a diverse phenotypic community fares better could be tested in an animal infection model, with the use of isolates from individual patients showing high or on the contrary low diversity.

5.3.4 Diversity of the production of regulatory quorum sensing molecules

In addition to the 10 phenotypes tested, the concentrations of 7 regulatory QS molecules were measured by mass spectrometry. These QS molecules regulate directly the expression of several of the phenotypes tested such as HCN, proteolytic activity, pyocyanin and pyoverdine production (Dubern and Diggle, 2008). Therefore, the observation of similar diversity trends as with the QS regulated phenotypes could be expected. The main results were similar to those found for the other phenotypes, with the between-patient diversity being more important to entire diversity than the within-patient, except for PQS. Regarding changes, the importance of within-patient diversity increased from early to chronic stages of infection and the between decreased. HHQ was an exception with within-patient diversity slightly lower in chronic stages and between-patient diversity higher. Thus the patterns of change were different for all quinolones, which was unexpected, as HHQ, PQS and HQNO all have a common precursor (Déziel et al., 2004).

Overall quinolones presented higher concentrations and higher diversity for more patients than the two HSLs. A previous paper also found similar results regarding the low concentrations of HSLs, finding that isolates tested produced less HSLs than the PA01 control strain, which was also observed in our study (Wilder et al., 2009). The paper also showed that the lack of HSL production was consistent with mutations in either the *lasI* and *rhlI* or *lasR* and *rhlR* genes while normal levels of 3-oxo-C12-HSL but low levels of C4-HSL would be the profile for *lasR* mutant. It would, therefore, be interesting to look into the genetic background of the isolates used in this study.

Regarding individual patients, both final products of the quinolone synthesis pathway, PQS and HQNO, have similar patterns and higher diversity than the two HSLs (Nguyen et al.,2016). On the other hand, HHQ showed closer similarities to the two HSLs when looking at individual patients. In the case of PQS and HQNO the patients showing the highest diversity are CP11

and CP12 and to a lesser extent CP6, CP7 and CP15, whereas for the other QS molecules it is by far CP4. The 2 patients, CP4 and CP11, which show the highest diversity and concentrations, are both epidemic strains. A study found that epidemic strains showed over-production of pyocyanin and LasA elastase (Fothergill et al., 2007). However, although these patients produced higher amounts of QS molecules, CP4 produced high pyocyanin concentrations while CP11 produced very little. In fact, 2 other patients (CP3 and CP13) were also infected by epidemic strains and showed conflicting patterns. Patient CP3 exhibited the second highest concentrations of HSLs but low production of virulence factors. Conversely, CP13 had some high diversity and measurements for virulence factors such as HCN, but low QS concentrations. In fact, all patients showing highest diversity for virulence factors were not epidemic.

In the previous chapter, statistically significant correlations of moderate strength revealed associations between QS molecules and phenotypes. None, however, were perfect correlations. Thus, these diversity results also suggest that other regulatory systems might also be involved in the modification of the phenotypic levels.

The ability to conclude firmly about the results concerning the diversity of QS molecules is however limited due to the lack of replication of the measurements.

To conclude, for all phenotypes and regulatory molecules measured the importance of the between-patient diversity was the more important than the within-patient diversity. However, this between-patient diversity decreases between early and chronic stages of infection while within-patient diversity increases. The significance of within-patient diversity thereby confirms recent studies which highlight its importance. These results help to further understand its extent. Indeed, although the same pressures lead to some common adaptation and decrease in between-patient diversity, the adaptation to the different niches of this heterogeneous and complex environment is obvious (Jorth et al., 2015). This diversity undoubtedly brings survival advantages to the bacteria and helps them establish long-term chronic infection.

Chapter 6: General discussion

6.1 Main findings of this thesis

The main aim of this study was to further investigate the phenotypic adaptation of *P. aeruginosa* to the CF. The hypotheses were that changes in phenotypes would be observed between early and chronic stages of infection as well as links between phenotypes. Another hypothesis involved the diversity of *P. aeruginosa* isolates within and between patients.

The RBH and UBC set of strains presented in Chapter 3 were investigated and used to measure 10 quantitative phenotypes linked to virulence within the CF lung. All phenotypic results from both strain sets were used in Chapter 4 to unveil what changes occurred over the length of infections and between early and chronic stages of infection, the extent of correlations between phenotypes and with the regulatory QS molecules and with patients' clinical data. Finally, in Chapter 5 adaptation of the RBH strains by measuring phenotypic diversity was investigated. The phenotypic diversity of RBH strains was analysed from isolates within patients and between the patients, revealing the changes in the extent of diversity over early and chronic stages of infection.

The main findings of this work on phenotypic adaptation of *P. aeruginosa,* following the order of chapter in which they were presented, were:

- The RBH set of strains consisted of a small cohort of 7 patients infected for the first time and 16 patients chronically infected by *P. aeruginosa.* This small number of patients were however found to be representative of the wider populations according to the clinical data.

- Most patients were infected by clonally related isolates, all descending from a single original infecting strain. All variation observed amongst these isolates was, thus, due to adaptation during the time of infection within the lungs. This was the case for both

RBH and UBC set of strains where for most patients the same strain was isolated again over time.

- For all phenotypes, lower phenotypic diversity was observed in chronic stages of infection. This supports data from the literature presented in the introduction according to which, virulence decreases over the course of infection and is minimal when compared to bacterial strains from early infection (Rau et al., 2010, Mayer-Hamblett et al., 2014b). However, the extent of the difference between stages of infection was phenotype specific. The phenotypes showing the least variation between stages of infection were HCN concentration, biofilm accumulation, twitch response and pyocyanin concentration.

- The majority of phenotypes tested correlated with all other phenotypes with varying intensity, the strongest correlations were between the virulence factors under QS regulation. However, biofilm and especially HCN showed the least strong correlations of all QS regulated virulence factors. The quorum sensing molecules measured were found to be positively correlated to all phenotypes to varying extents, with the exception of speed of growth. The moderate correlations with some virulence phenotypes suggest that other mechanisms of regulation might also be involved.

- Unlike other QS regulated phenotypes, HCN measurements showed little decrease between stage of infections and correlated only weakly to other phenotypes. When considering exacerbations, it was the only phenotype of those tested showing an increase in production during an exacerbation.

- Between-patient diversity was greater than within-patient diversity overall and remains true for both early and chronic stages of infection separately. This does not substantiate my hypothesis which was based on the findings of two previous studies, which found within-patient diversity to be greater than that between patients (Mowat et al., 2011, Ashish et al., 2013). However, the diversity was measured differently, considering a combination of phenotypes as a specific sub-type, while in this study diversity was analysed for each phenotype separately.

- Within-patient phenotypic diversity was nonetheless significant for all phenotypes and was higher in the chronic stage of infection than in the early one.

- Results suggest that patients experiencing pulmonary exacerbation exhibited higher within-patient diversity than those in a stable state. No firm conclusion can be drawn from such a small cohort of patients but these potential links between exacerbation and diversity follow the results shown from previous studies (Whelan and Surette, 2015, Fothergill et al., 2010, O'Brien et al., 2017).

6.2 Study strengths and weaknesses

To investigate the phenotypic adaptation of *P. aeruginosa* two different sets of strains were used. The UBC set of strain enabled investigation of changes over a prolonged period of time, up to 24 years, for 16 patients. Previous longitudinal studies were undertaken on shorter time spans as well as smaller cohorts of patients (Clark et al., 2015, Workentine et al., 2013). The second set RBH was composed of nearly 600 isolates from 7 early stage infections and 16 chronic infections. The use of these two very different sets of strains to study phenotypic adaptation was an asset, as similar results were found, thus producing more robust conclusions. Indeed, these similar results were found when investigating the decrease of virulence over the length of infection and between early and chronic stages of infection, as well as when investigating correlations between phenotypes. The results for both sets of strains confirmed previous literature regarding decrease in virulence over early and chronic stages of infection, as described in Chapter 4 (Harmer et al., 2013, Bragonzi et al., 2009, Mayer-Hamblett et al., 2014b). My methodology seemed therefore reliable, thus enabling well founded and credible conclusions about diversity in Chapter 5.

In addition to the important of numbers of isolates assayed, a wide selection of types of strains were used. Overall 12 patients from which bacteria were isolated to compose the RBH set, were infected by common epidemic strains and 10 patients by uncommon strains isolated in no other patient attending the hospital centre. This contrasted with studies which only assayed LES isolates, and enabled this study to investigate the common adaptations of different strains to the CF lungs (Mowat et al., 2011, Fothergill et al., 2010, Williams et al., 2015). Two couples of patients were also infected by the same strains, which also allowed the observation of

individual differences of adaptation specific to individual patients and clinical status. Another strength of the study is not only observing diversity in chronic isolates but also amongst early isolates, which shows that adaptation happens very quickly as diversity was smaller but already present in early strains.

Although the sets of strains available for this study were a significant asset, the method of isolation of those was also a limitation to investigate diversity of *P. aeruginosa* isolates within the CF lung. Indeed, one limitation to the current study was the isolation strategy of bacteria, which meant we did not know the sputum's exact spatial provenance from within the lung, unlike the study of Jorth et al. which used explanted lungs, or others using bronchoalveolar lavages (Jorth et al., 2015, Bjarnsholt et al., 2005, Nixon et al., 2001). A study nonetheless suggested that sputum samples remained efficient at detected bacterial infection within the CF lung (Aaron et al., 2004a). Also, a similar strategy used here was used previously by Mowat et al. in which they sampled a patient several times in a day. The multiple sampling showed recurrence of the same sub-populations and strain, therefore somewhat validating the method (Mowat et al., 2011). Ideally, the bacteria would be extracted from precise lobes within the lung in vivo through bronchoalveolar lavage but this is not feasible as too intrusive for patients. This would be especially useful regarding the early isolates as they often originate from young patients, incapable of expectorating and providing sputum samples. Thus, for the majority of early patients cough swabs were used, which have been shown to be less sensitive than sputum samples to identify infections (Equi et al., 2001). The use of cough swabs means that a limited amount of bacteria might have been identified leading to misrepresentation of the infecting strain and potentially underestimating the phenotypic diversity of *P. aeruginosa* in these patients.

The manual isolation of well-defined colonies from agar plates could also be a cause of bias, with the choice of colonies to pick from being limited. As a result, isolates which were more homogeneous, might have been picked unwittingly.

Although having fully quantitative data on over 600 isolates was a clear asset, the completion of the assays for all isolates was time consuming, thus, limiting the measurement of further phenotypes such as antibiotic susceptibility, which would have been needed to be done for several compounds. Also, adding more genotypic data to this large phenotypic study could further help to explain some aspects of adaptation and links between phenotypes. Moreover,

the multiple isolates recovered per patients to investigate diversity was at the expense of the number of patients. This compromise allowed for interesting results on the diversity within and between patients, but did not enable to draw firm conclusions about correlations between clinical status of patients and the phenotypic changes and evolution of the bacteria. Nonetheless, for such a small cohort of patients, data suggested a reasonable representation of the CF population. Having further clinical information could unveil more about specific adaptation, as it is suggested that the evolution of the bacteria might vary depending on health of the CF lungs of the individual patients (Klockgether et al., 2018).

6.3 Relevance and future directions

This study helps to further understand the adaptation of *P. aeruginosa* to the lungs in the establishment of long-term chronic infections.

The first crucial aspect of adaptation to be investigated in this study was the transition between the early stage of infection and the long-term chronic stage. All phenotypes showed a decrease in measurements over the length of infection and between early and chronic stage of infection for both sets of strains. The phenotypes showing by far the biggest decrease between early and chronic stages of infection were flagella motility swimming and swarming, protease production (including elastase) and pyoverdine production. We can then question whether important decrease in these phenotypic measurements is due to the fact that the behaviour is no longer needed in this new environment or that this loss of expression is needed for chronicity. Loss of motility might be used to evade the immune system, but also might no longer be needed when bacteria grow in biofilm like aggregates, as suggested by Staudinger et al. (Staudinger et al., 2014b). The same might be true about the decrease in pyoverdine production as the bacteria no longer needs pyoverdine to scavenge for iron which is available in different forms (Marvig et al., 2014, Nguyen et al., 2014). Concerning proteases, they are known to participate in helping in immune attack and evasion, therefore, being needed less in chronic stages of infection (Kuang et al., 2011, Laarman et al., 2012, Bastaert et al., 2018).

One study has also previously found protease production to decrease the most over the course of infection (Jiricny et al., 2014). Another study found pyoverdine to be most correlated with stages of infection (Mayer-Hamblett et al., 2014b). Interestingly, in this study, these two

phenotypes also stand out as the only exoproducts linked to virulence with all early isolates having some levels of production present. Protease was also found to be one of the phenotypes most associated to others as well as to the production of QS molecules. Although protease production decreases significantly between stages of infection it nonetheless shows important diversity in some chronic patients. Therefore, protease production should be further investigated for the complex role it seems to play in infection and adaptation. It may be a good biomarker to transition towards chronicity.

However, the phenotype that stood out the most during this study was the production of hydrogen cyanide. Firstly, HCN production seemed to be sustained over all stages of infection, as it is the exoproduct showing the smallest decrease in production between early and chronic stages of infection. Secondly, HCN production also stood out when looking at associations with other phenotypes. Indeed, HCN production was only weakly correlated to all other phenotypes but also to QS molecules although it is one of the QS regulated exoproducts. PQS has been shown to be involved in the regulation of HCN. However, it can also be regulated by other regulators (Pessi and Haas, 2000). Finally, when investigating within- and between-patient diversity HCN production also displayed an unusual trend with some of the highest within-patient diversity. Although definite conclusions cannot be drawn due to the small number of patients, HCN production also seemed to display high within-patient diversity in patients experiencing the beginning of a pulmonary exacerbation.

Based on all these results regarding changes over time, the role of HCN during infection, including during chronic stage, should be further investigated. Indeed, as mentioned in the introduction to this thesis, HCN is the virulence factor least studied at present. Thus, in the same way that protease production could be a good marker to monitor stages of infection, HCN could potentially be a good biomarker for the prediction of exacerbations. Additionally, HCN can be directly measured through breath condensate (Gilchrist et al., 2013, Enderby et al., 2009). Therefore, further investigation of levels of HCN over the course of exacerbations should be undertaken and could be done both on a patient level through breath condensate as well as through direct measurement from isolated bacteria.

The shift from stable asymptomatic clinical state to a worsening of symptoms during times of exacerbation is indeed also an important change in infection status, as is the shift to chronicity. It would thus be beneficial to focus future investigations on the fluctuations of diversity around

these times of exacerbations through longitudinal studies using multiple isolates per sample. This would be a long-term strategy, similar to that for the UBC set of strains.

Continuing to better our understanding of the extent and dynamics of the within-patient diversity remains crucial, especially during these pivotal phases of infection. This could provide further biomarkers to monitor the changes in infection and aggravation of conditions such as exacerbations. Understanding the rate of adaptation through these biomarkers would give a clearer idea to the window of opportunity for treatment before the establishment of chronicity. Overall having robust biomarkers could help be able to identify which patients are most at risk.

The virulence factors measured in this study are the tools with which the bacteria cause inflammation. To increase understanding of the interactions with the host, isolates could be investigated in their survival levels to various immune cell molecules such as ROS ($OSCN^-$, HOCl, H_2O_2), and to neutrophil killing and NETosis. This could be undertaken with the same strain set to be correlated with the phenotypic traits already measured and give a fuller picture of adaptation and diversity. This could demonstrate whether diversity of survival capacity also exists alongside virulence diversity, and how they correlate, leading to further understanding of the adaptation strategies of the bacteria.

In addition to looking at adaptation through different angles and phenotypes, other types of groups of strains should also be investigated. Indeed, to better understand the early adaptation it would be good to further investigate the phenotypic diversity of *P. aeruginosa* within the upper airway. Indeed, *P. aeruginosa* has been detected in patients' sinuses before being isolated in the CF lungs, and isolates from the upper airway already exhibit similar adaptation (Hansen et al., 2012, Ciofu et al., 2013, Mainz et al., 2009, Johansen et al., 2012, Fothergill et al., 2014). Therefore, it would be very interesting to collect longitudinally upper airway swabs at regular intervals in children with no *P. aeruginosa* infection in the CF lungs and follow them over years to compare any upper airway isolates collected to any new infection in the lower airway. Also including the same environmental strains as those involved in early infections could also help to demonstrate the initial adaptations.

To conclude, this study adds to the body of work showing the complexity of adaptation of *P. aeruginosa* and brings further insight into the extent of diversity within and between patients.

As between-patient diversity is substantial, this study's results suggest it is important to take into consideration the specificities of individual patients. They also further underline the need for precision medical treatments, tailored to each patient and their isolate diversity. Previous studies have demonstrated contradictory results, showing greater within-patient diversity, especially in the context of studying epidemic strains (Mowat et al., 2011, Ashish et al., 2013). However, it is now clearer than ever that within-patient diversity is also important to take into consideration when testing patients' samples in clinical setting, and it is naïve of clinicians to still too often treat *P. aeruginosa* infections as a single entity. Indeed, through showing the extent of phenotypic diversity, this study further demonstrates the need to collect multiple isolates per sample to test for antimicrobial resistance as diversity is present in all patients. Underestimating bacterial diversity leads to the inability to address the complexity of individual infections and provide the correct treatment.

Continuing to investigate the extent and chronology of changes and diversity of phenotypic behaviours will lead to further understanding of the critical factors enabling survival in the CF lung. Studies including this one have already uncovered a handful of potential biomarkers to predict important changes occurring amongst the bacterial community and find alternative strategies to prevent the establishment of chronic infection or the recurrence of exacerbations. Preventing the chronicity of *P. aeruginosa* infections would in turn participate in preventing the decline of patient's health by limiting lung damage due to inflammation caused by infection. The same would be true in the prevention of exacerbations, which worsen people's long term lung function and quality of life (Waters et al., 2012, Britto et al., 2002, Sanders et al., 2010).

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APPENDICES

Appendix 2-1: Number of biological replicates for each patients isolates for each phenotypic assays.

Patient	Growth t50	Swimming	Swarming	Twitching	Protease	Elastase	Pyocyanin	Biofilm	Hydrogen Cyanide	Pyoverdine	QS molecules
Early 1	3	3	4	4	4	4	3	4	3	3	1
Early 2	3	3	4	4	4	4	3	4	3	3	1
Early 3	3	3	4	4	4	4	3	4	3	3	1
Early 4	4	6	5	3	3	3	3	5	5	6	1
Early 5	4	6	5	3	3	3	3	5	5	6	1
Early 6	3	3	4	4	3	4	4	7	3	3	1
Early 7	3	3	4	4	3	4	4	7	3	3	1
Chronic 1	6	3	5	3	4	3	3	5	4	5	1
Chronic 2	6	3	4	3	4	3	3	5	4	5	1
Chronic 3	5	3	4	3	4	3	3	5	4	5	1
Chronic 4	5	3	4	3	4	3	4	5	4	5	1
Chronic 5	3	3	5	3	3	3	3	4	4	4	1
Chronic 6	3	3	5	3	3	3	3	4	4	4	1
Chronic 7	3	3	5	3	3	3	3	4	4	4	1
Chronic 8	3	3	5	3	3	3	3	4	4	4	1
Chronic 9	3	4	3	3	3	3	4	4	3	4	1
Chronic 10	3	4	3	3	3	3	4	4	3	4	1
Chronic 11	3	4	3	3	3	3	4	4	3	4	1
Chronic 12	3	4	3	3	3	3	4	4	3	4	1
Chronic 13	4	3	6	3	3	5	4	4	4	3	1
Chronic 14	4	3	6	3	3	5	4	4	4	3	1
Chronic 15	4	3	5	3	3	5	4	4	4	3	1
Chronic 16	4	3	5	3	3	6	3	3	4	3	1
Chronic 18	4	3	5	3	3	6	3	3	4	3	1

Appendix 2-2: Standard deviation and Coefficient of Variation for each phenotypic assay for the RBH set. For each isolate the standard deviation (STDEV) and coefficient of variation (CV) between the biological replicates for each phenotypic assay was calculated for both non normalised and normalised data. The normalised data was obtained by normalising the raw data to the measurement of PA01 (or PA14 for HCN) for each assay. The table presents the sum of all STDEV and CV of all isolates for both non normalised and normalised data. The number of individual isolates for which the STDEV and CV was higher or lower when data was not normalised comparatively to the normalised data was counted and the percentage of total isolates is presented in the table.

STDEV: Standard Deviation ; CV : Coefficient of variation.

Phenotypic assays	Growth	Swim	Swarm	Twitch	Biofilm	Elastase	Protease	HCN	Pyoverdine	Pyocyanin
STDEV data not normalised	418.0	714.3	984.0	1068.5	64.6	569.6	745.3	6117.4	139.3	314.3
CV data not normalised	9360.9	11502.7	13408.0	11115.7	24259.8	8920.7	10414.5	14385.8	14147.6	11713.7
STDEV data normalised	568.3	676.1	879.5	1052.5	74.5	521.8	837.5	8707.9	160.2	421.9
CV data normalised	12064.3	10857.3	11493.6	10724.1	23123.8	8395.6	11277.2	17325.8	16457.1	18280.0
Lower variation when not normalised (% of total isolates)	71	44	32	49	49	40	65	68	76	76
Higher variation when not normalised (% of total isolates)	29	56	68	51	51	60	35	32	24	24

Appendix 3-1: Information on the UBC strains. Morphotypes distinctions - normal: flat colony with a smooth surface, mucoid: shiny, elevated colony with a smooth edge, dwarf: small colony variant, ent: short for enterobacter- like or "entire", translucent circular colony, rough: rough edged colony.

Patient	Identification	Date of isolation	Length infection	Morphology	Source	RAPD type
P01	C2773C	26 March 1988	0.0	normal	Sputum	A027
P01	C2943C	02 September 1988	0.4	normal	Cough swab	A027
P01	C3639M	27 February 1990	1.9	mucoid	Sputum	A027
P01	C3637C	27 February 1990	1.9	normal	Sputum	
P01	C4023M	28 December 1990	2.8	mucoid	Sputum	
P01	C4021C	28 December 1990	2.8	normal	Sputum	
P01	C4278M	03 July 1991	3.3	mucoid	Sputum	A027
P01	C4277D	03 July 1991	3.3	dwarf	Sputum	A027
P01	C4276C	03 July 1991	3.3	normal	Sputum	A027
P02	C3195C	07 March 1989	0.0	normal	Cough swab	A304
P02	C3380C	01 August 1989	0.4	normal	Sputum	A304
P02	C6557C	16 May 1995	6.2	normal	Sputum	A304
P02	C7632C	06 June 1997	8.2	normal	Sputum	A304
P03	C4142C	19 March 1991	0.0	normal	Cough swab	
P03	C4299C	23 July 1991	0.3	normal	Cough swab	
P03	C4934M	03 November 1992	1.6	mucoid	Sputum	
P03	C6942M	13 February 1996	4.9	mucoid	Sputum	A097
P03	C6943C	13 February 1996	4.9	normal	Sputum	P003-3
P03	C6941C	13 February 1996	4.9	normal	Sputum	A136a
P03	C6944D	13 February 1996	4.9	dwarf	Sputum	A097
P03	C8596C	22 April 1999	8.1	normal	Sputum	A097
P03	C8595M	27 April 1999	8.1	mucoid	Sputum	A097
P03	D1701C	24 August 2005	14.4	normal	Sputum	A097
P03	D1702M	24 August 2005	14.4	mucoid	Sputum	A136
P03	D2938E	09 May 2008	17.1	enterobacter-like	Sputum	
P03	D2955D	09 May 2008	17.1	dwarf	Sputum	A097
P03	D2956E	09 May 2008	17.1	enterobacter-like	Sputum	A136
P03	D2994M	02 June 2008	17.2	mucoid	Sputum	A097
P04	C2684C	12 January 1988	0.0	normal	Sputum	
P04	C3779C	15 May 1990	2.3	normal	Sputum	A002
P04	C4652E	07 April 1992	4.2	enterobacter-like	Cough swab	A002
P04	C6082C	30 August 1994	6.6	normal	Sputum	
P04	C6617M	20 June 1995	7.4	mucoid	Sputum	A002
P04	C6616M	20 June 1995	7.4	mucoid	Sputum	
P04	C6618C	20 June 1995	7.4	normal	Sputum	
P04	C6939M	21 February 1996	8.1	mucoid	Sputum	
P04	C6938C	21 February 1996	8.1	normal	Sputum	A002
P04	C6940C	21 February 1996	8.1	normal	Sputum	
P04	C7471E	17 February 1997	9.1	enterobacter-like	Sputum	
P04	D1173M	24 June 2004	16.4	mucoid	Sputum	A002
P04	D1172C	24 June 2004	16.4	normal	Sputum	A002
P05	C7577M	07 May 1997	0.0	mucoid	Sputum	A097
P05	C7660M	15 July 1997	0.2	mucoid	Sputum	
P05	C9264E	31 August 2000	3.3	enterobacter-like	Sputum	A097
P05	C9263M	31 August 2000	3.3	mucoid	Sputum	A097
P05	D1427E	13 January 2005	7.7	enterobacter-like	Sputum	
P05	D1426M	13 January 2005	7.7	mucoid	Sputum	A097
P05	D2787M	17 January 2008	10.7	mucoid	Sputum	A097
P05	D3025C	24 July 2008	11.2	normal	Sputum	A097
P06	C3330C	12 July 1989	0.0	normal	Cough swab	
P06	C4658M	14 April 1992	2.8	mucoid	Sputum	A030
P06	C4657C	14 April 1992	2.8	normal	Sputum	A030
P06	C7626M	03 June 1997	7.9	mucoid	Sputum	A030
P06	C7625C	03 June 1997	7.9	normal	Sputum	
P06	C7989M	01 April 1998	8.7	mucoid	Sputum	
P06	C9660C	15 May 2001	11.8	normal	Sputum	A030
P06	D1384C	06 December 2004	15.4	normal	Sputum	A030
P06	D1385M	06 December 2004	15.4	mucoid	Sputum	
P06	D2604E	27 August 2007	18.1	enterobacter-like	Sputum	
P06	D2631C	23 September 2007	18.2	normal	Sputum	A030
P06	D2639M	01 October 2007	18.2	mucoid	Sputum	A030
P07	C4303C	23 July 1991	0.0	normal	Sputum	
P07	C4432C	22 October 1991	0.2	normal	Sputum	
P07	C5656C	25 January 1994	2.5	normal	Sputum	
P07	C5995C	19 July 1994	3.0	normal	Cough swab	
P07	C6631C	27 June 1995	3.9	normal	Sputum	
P07	C9027C	02 March 2000	8.6	normal	Sputum	
P07	D0006C	25 January 2002	10.5	normal	Sputum	
P08	C7250C	06 September 1996	0.0	normal	Cough swab	
P08	C7464E	12 February 1997	0.4	enterobacter-like	Sputum	
P08	D1120E	13 May 2004	7.7	enterobacter-like	Sputum	
P08	D1121D	13 May 2004	7.7	dwarf	Sputum	P008-2
P08	D1126C	13 May 2004	7.7	normal	Sputum	P008-2
P08	D1157C	13 May 2004	7.7	normal	Sputum	P008-2
P08	D2410E	12 April 2007	10.6	enterobacter-like	Sputum	P008-2a
P08	D2980C	05 June 2008	11.7	normal	Sputum	P008-5

P09	C4267C	28 June 1991	0.0	normal	Cough swab	
P09	C6676M	12 July 1995	4.0	mucoid	Sputum	A177a
P09	C6675C	12 July 1995	4.0	normal	Sputum	
P09	C8341C	15 October 1998	7.3	normal	Sputum	A177
P09	C8340R	15 October 1998	7.3	rough	Sputum	A177
P09	C8339M	15 October 1998	7.3	mucoid	Sputum	A177
P10	C4443C	29 October 1991	0.0	normal	Cough swab	A179
P10	C4678E	05 May 1992	0.5	enterobacter-like	Sputum	A179
P10	C4677C	05 May 1992	0.5	normal	Sputum	A179
P10	C5724C	08 March 1994	2.4	normal	Sputum	A179
P10	C5725M	08 March 1994	2.4	mucoid	Sputum	A097
P10	C9805D	11 September 2001	9.9	dwarf	Sputum	A097
P10	C9804M	11 September 2001	9.9	mucoid	Sputum	A097
P10	D2750C	05 February 2007	15.3	normal	Sputum	A097
P10	D2749M	05 February 2007	15.3	mucoid	Sputum	
P10	D2326D	05 December 2007	16.1	dwarf	Sputum	A097
P10	D2325M	05 December 2007	16.1	mucoid	Sputum	A097
P11	C5858D	10 May 1994	0.0	dwarf	Sputum	P011-1
P11	C6193D	25 October 1994	0.5	dwarf	Sputum	P011-1
P11	C6192D	25 October 1994	0.5	dwarf	Sputum	P011-1
P11	C6599D	25 May 1995	1.0	dwarf		P011-1
P11	C8168D	25 June 1998	4.1	dwarf	Sputum	P011-1
P11	D2957D	07 May 2008	14.0	dwarf	Sputum	P011-1
P12	C5800E	12 April 1994	0.0	enterobacter-like	Cough swab	
P12	C5799C	12 April 1994	0.0	normal	Cough swab	A134
P12	C6065C	23 August 1994	0.4	normal	Sputum	A134
P12	C7092M	14 May 1996	2.1	mucoid	Sputum	A134
P12	C7091E	14 May 1996	2.1	enterobacter-like	Sputum	A134
P12	C7399M	07 January 1997	2.7	mucoid	Cough swab	
P12	C7401C	07 January 1997	2.7	normal	Cough swab	
P12	C8584M	20 April 1999	5.0	mucoid	Sputum	A134a
P12	C8583C	20 April 1999	5.0	normal	Sputum	A134a
P12	D1029C	26 February 2004	9.9	normal	Cough swab	
P12	D1032M	26 February 2004	9.9	mucoid	Cough swab	
P12	D2561C	16 August 2007	13.3	normal	Sputum	
P12	D2751M	19 December 2007	13.7	mucoid	Sputum	
P13	C0803C	09 December 1983	0.0	normal	Sputum	
P13	C2548E	15 September 1987	3.8	enterobacter-like	Sputum	
P13	C2757C	16 February 1988	4.2	normal	Sputum	A136
P13	C3995R	20 November 1990	6.9	rough	Sputum	
P13	C3994C	20 November 1990	6.9	normal	Sputum	
P13	C3993M	20 November 1990	6.9	mucoid	Sputum	
P13	C4976M	24 November 1992	9.0	mucoid	Sputum	A136
P13	C4978C	24 November 1992	9.0	normal	Sputum	
P13	C4977C	24 November 1992	9.0	normal	Sputum	A136
P13	C7004M	19 March 1996	12.3	mucoid	Sputum	
P13	C7005C	19 March 1996	12.3	normal	Sputum	
P13	C9082M	20 April 2000	16.4	mucoid	Sputum	A136
P13	D1204C	26 July 2004	20.6	normal	Sputum	A136
P13	D1342M	08 November 2004	20.9	mucoid	Sputum	A136
P13	D2982M	04 June 2008	24.5	mucoid	Sputum	
P14	C5198D	11 May 1993	0.0	dwarf	Cough swab	A173
P14	C5203M	14 May 1993	0.0	mucoid	Cough swab	A173
P14	C5204C	14 May 1993	0.0	normal	Cough swab	
P14	C5733C	07 March 1994	0.8	normal	Sputum	
P14	C5732C	07 March 1994	0.8	normal	Sputum	A173
P14	C5731M	07 March 1994	0.8	mucoid	Sputum	A173
P14	C5730C	07 March 1994	0.8	normal	Sputum	
P14	C7025M	02 April 1996	2.9	mucoid	Cough swab	
P14	C7024C	02 April 1996	2.9	normal	Cough swab	
P14	C9661C	15 May 2001	8.0	normal	Sputum	
P14	D2950M	08 May 2008	15.0	mucoid	Sputum	A173
P14	D3010C	07 July 2008	15.2	normal	Sputum	A173
P15	C3881C	28 August 1990	0.0	normal	Sputum	A181
P15	C4197D	07 May 1991	0.7	dwarf	Sputum	A181
P15	C4196D	07 May 1991	0.7	dwarf	Sputum	A181
P15	C4198C	07 May 1991	0.7	normal	Sputum	A181
P15	C6926M	06 February 1996	5.4	mucoid	Sputum	A181
P15	C6927C	06 February 1996	5.4	normal	Sputum	A181
P15	C7515D	18 March 1997	6.6	dwarf	Sputum	A181
P15	D0305D	05 August 2002	11.9	dwarf	Sputum	A181
P15	D0315M	20 September 2002	12.1	mucoid	Sputum	A181
P16	C8415D	26 November 1998	0.0	dwarf	Sputum	A301
P16	C8527D	26 February 1999	0.3	dwarf	Sputum	A301
P16	D0198D	19 June 2002	3.6	dwarf	Sputum	A301
P16	D0316M	20 September 2002	3.8	mucoid	Sputum	A301
P16	D2554M	30 July 2007	8.7	mucoid	Sputum	A301
P16	D2825M	04 February 2008	9.2	mucoid	Sputum	A301

Appendix 3-2: Clinical information about the RBH set of strains collected. Information recovered from the patients' record from the RBH.

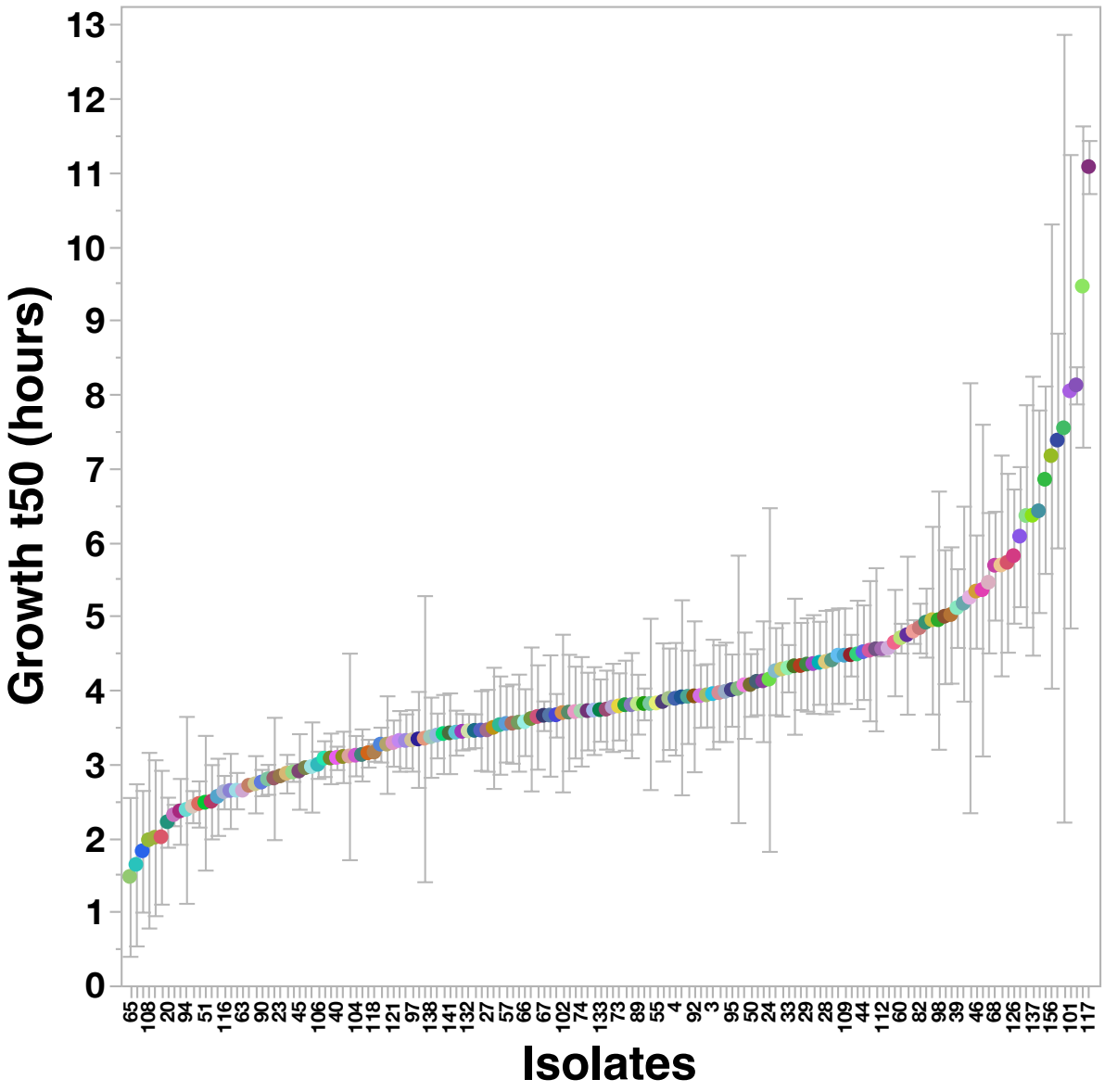
	Clinical information					Infection and sample information				
Patient	Gender	Age (years)	FEV1	CFTR mutation 1	CFTR mutation 2	Length of infection (years)	Co-infection	Sample source	Number of isolates recovered	Exacerbation status
Early 1	F	12.6	105%	Phe508del	Phe508del	<0.25	-	Cough Swab	26	Stable
Early 2	F	11.4	-	Phe508del	Phe508del	<0.25	*S. aureus*	Spontaneous Sputum	13	Stable
Early 3	M	2.3	-	Phe508del	621+1G>T	<0.25	-	Cough Swab	36	Stable
Early 4	M	1.5	-	Phe508del	Phe508del	<0.25	-	Cough Swab	24	Stable
Early 5	M	1.3	-	Phe508del	Asn1303Lys	<0.25	-	Cough Swab	60	Stable
Early 6	M	4.8	104%	Phe508del	Pro67Leu	<0.25	-	Spontaneous Sputum	36	Stable
Early 7	M	1.7	-	Phe508del	Phe508del	<0.25	*E. coli*	Cough Swab	26	Stable
Chronic 1	F	15.1	83%	Phe508del	Phe508del	5	*E. dermatitidis; S. apiospermum*	Spontaneous Sputum	24	End
Chronic 2	M	21.5	59%	Phe508del	Phe508del	0.5	-	Spontaneous Sputum	12	Stable
Chronic 3	M	22.3	NA	Phe508del	Phe508del	5.3	*A. fumigatus; Candida species*	Spontaneous Sputum	24	Stable
Chronic 4	M	30.5	71%	Phe508del	Phe508del	5.1	-	Spontaneous Sputum	24	Stable
Chronic 5	M	16.9	59%	Phe508del	Asn1303Lys	5.2	-	Spontaneous Sputum	24	End
Chronic 6	F	16.3	65%	Phe508del	Pro67Leu	4.8	-	Spontaneous Sputum	24	End
Chronic 7	F	20.8	31%	Phe508del	Phe508del	1.6	*S. aureus*	Spontaneous Sputum	24	Beginning
Chronic 8 a	M	22.3	44%	Phe508del	2118del4	3.8	*Candida species*	Spontaneous Sputum	12	Middle
Chronic 8 b								Spontaneous Sputum	24	Middle
Chronic 9	M	26.4	50%	E60X	Q493X	5	*S. apiospermum*	Spontaneous Sputum	24	Middle
Chronic 10	F	34.3	50%	3849+10kbC>T	W1282X	5	-	Spontaneous Sputum	12	Stable
Chronic 11	F	23.5	58%	Phe508del	2184delA	1.5	-	Spontaneous Sputum	24	Middle
Chronic 13	M	21	42%	-	-	1.3	*MRSA*	Spontaneous Sputum	24	Beginning
Chronic 14	M	26.8	48%	Phe508del	Phe508del	3.1	*MRSA*	Spontaneous Sputum	24	Stable
Chronic 15	F	42	75%	Phe508del	R117H	1.3	-	Spontaneous Sputum	24	Middle
Chronic 16	M	58.1	30%	Phe508del	G551D	4	*Candida species*	Spontaneous Sputum	24	Middle
Chronic 18	M	28.5	36%	Phe508del	Phe508del	5.4	*Candida species*	Spontaneous Sputum	24	End

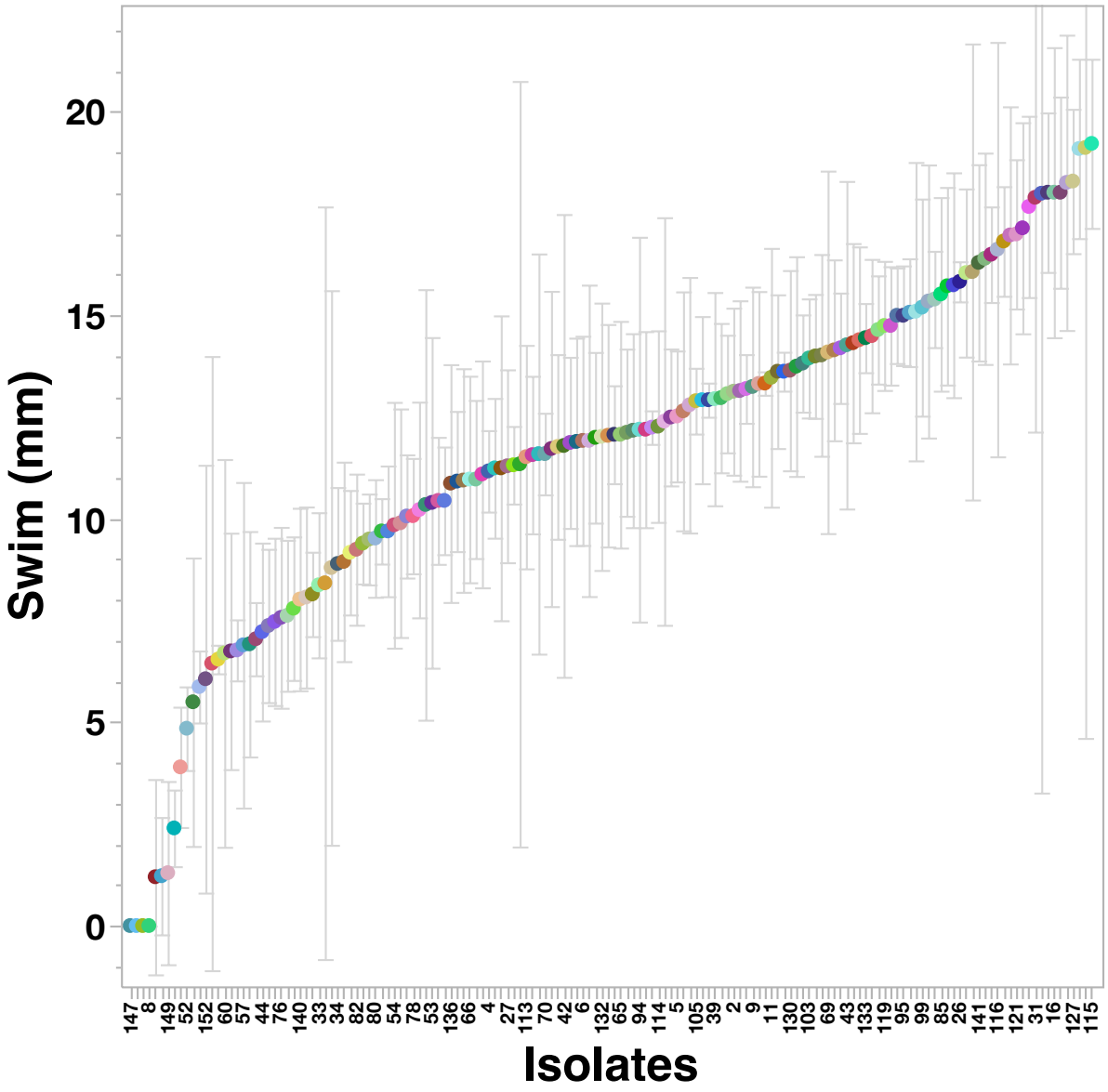
Appendix 3-3: Probability results from the Shapiro-Wilk test for the RBH patients. The hypothesis is that data is drawn from normal distribution. If the p value is significant ($p < 0.05$) the null hypothesis is then rejected meaning the data does not follow a normal distribution (in pink).

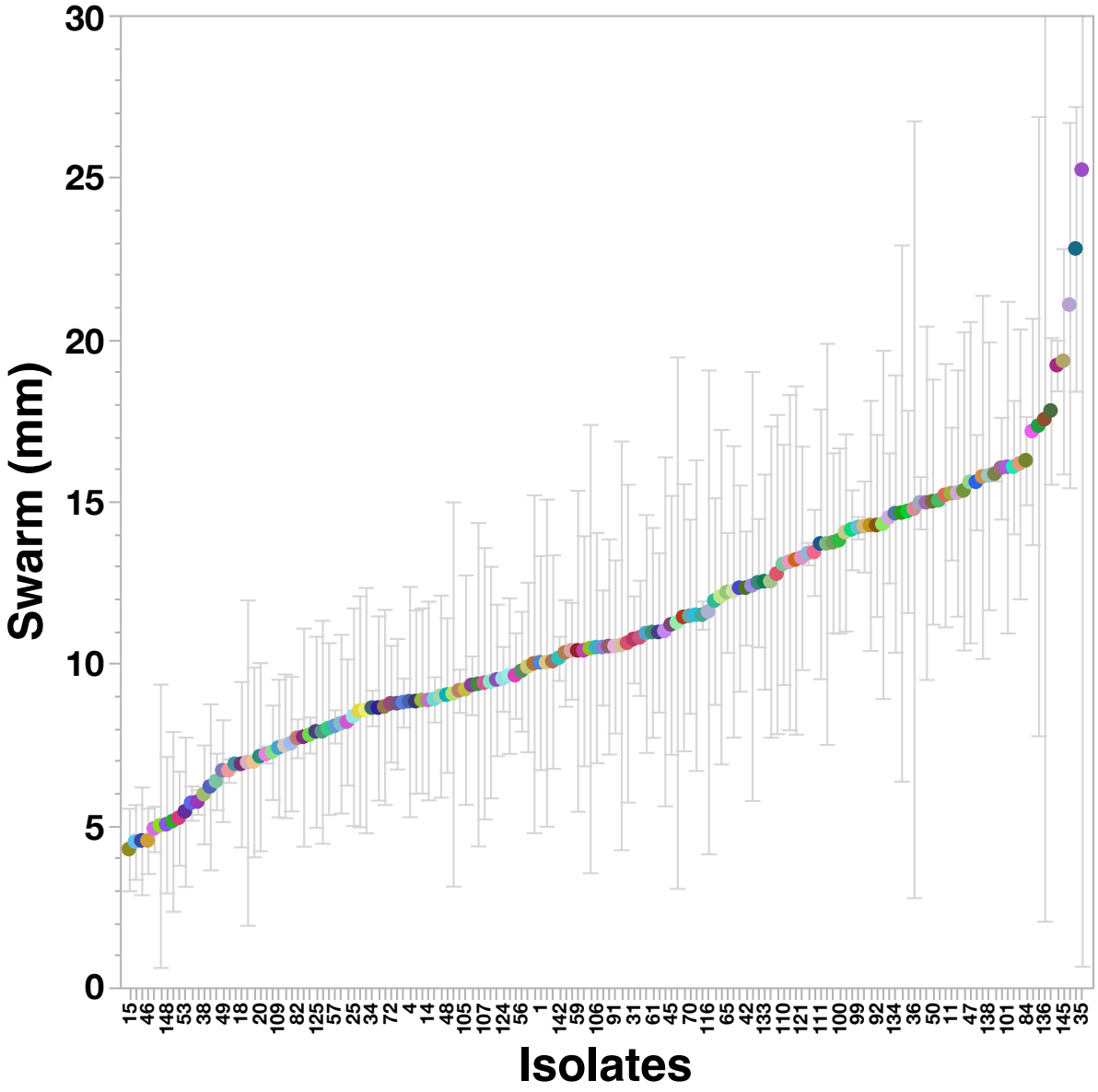
Phenotypes	Growth	Swimming	Swarming	Twitching	Proteolytic activity	Elastase	Pyocyanin 520	Pyocyanin 691	Biofilm	Hydrogen cyanide	Pyoverdin 405
EP1	0.2309	0.0136	0.0001	0.7861	0.0001	0.0108	0.0021	0.0001	0.0043	0.0001	0.8638
EP2	0.0487	0.5295	0.0010	0.0405	0.1094	0.2017	0.0383	0.8880	0.3497	0.0429	0.7711
EP3	0.0001	0.0945	0.0187	0.4909	0.3350	0.0302	0.0022	0.0269	0.0161	0.2600	0.0107
EP4	0.1852	0.2122	0.9262	0.2389	0.0403	0.0001	0.0113	0.8930	0.0109	0.0512	0.0337
EP5	0.8156	0.6086	0.4766	0.0922	0.1488	0.5078	0.5133	0.0132	0.0768	0.3028	0.0001
EP6	0.1205	0.5831	0.2973	0.4033	0.0293	0.0001	0.1204	0.5233	0.3283	0.2690	0.8970
EP7	0.0331	0.9582	0.0012	0.1976	0.5860	0.5536	0.0262	0.6236	0.6402	0.3331	0.7515
CP1	0.0005	0.0001	0.0001	0.0001	0.0001	0.0001	0.0034	0.0001	0.0006	0.0023	0.0002
CP2	0.0638	0.8209	0.4116	0.7053	0.6420	0.0120	0.0363	0.1604	0.5646	0.8815	0.0136
CP3	0.6490	0.5757	0.1496	0.1721	0.0092	0.0776	0.0001	0.5369	0.2469	0.2321	0.7212
CP4	0.0854	0.0004	0.7789	0.4993	0.0022	0.7362	0.5658	0.0811	0.0006	0.0021	0.1045
CP5	0.0150	0.0001	0.0298	0.8830	0.0001		0.0001	0.0001	0.0001	0.0001	0.0001
CP6	0.0033	0.0001	0.0001	0.9671	0.3062	0.2551	0.0093	0.0001	0.0141	0.0523	0.0882
CP7	0.4724	0.0075	0.3449	0.8728	0.0002	0.0001	0.0016	0.0140	0.9937	0.0167	0.0001
CP8	0.9972	0.0635	0.0814	0.9997	0.8871	0.0093	0.1864	0.0064	0.0146	0.8453	0.0001
CP9	0.0039	0.0044	0.0021	0.7289	0.0032	0.0001	0.0001	0.0003	0.5492	0.0470	0.0004
CP10	0.2275	0.6674	0.3266	0.9994	0.0189	0.0003	0.0442	0.7788	0.0153	0.8980	0.1181
CP11	0.8874	0.8355	0.3075	0.0685	0.0040		0.0001	0.0016	0.3299	0.6889	0.0001
CP12	0.2904	0.1644	0.0903	0.3995	0.0248	0.0001	0.0098	0.1025	0.5194	0.4340	0.8723
CP13	0.2047	0.2625	0.5370	0.3466	0.0001	0.0193	0.0001	0.0008	0.0146	0.0037	0.0150
CP14	0.8274	0.3858	0.6560	0.2841			0.0001	0.7528	0.2043	0.0001	0.1749
CP15	0.4555	0.0045	0.0428	0.1196	0.0002	0.0004	0.0001	0.0259	0.0004	0.6589	0.8204
CP16	0.0001	0.0006	0.9510	0.5993	0.0001		0.0080	0.0001	0.0001	0.0326	0.0140
CP17	0.0001	0.6841	0.0387	0.0585	0.6444		0.0003	0.2754	0.0001	0.0004	0.0012
CP18	0.0343	0.4982	0.2870	0.8785	0.2182		0.0001	0.9709	0.0128	0.0001	0.1303
All patients	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

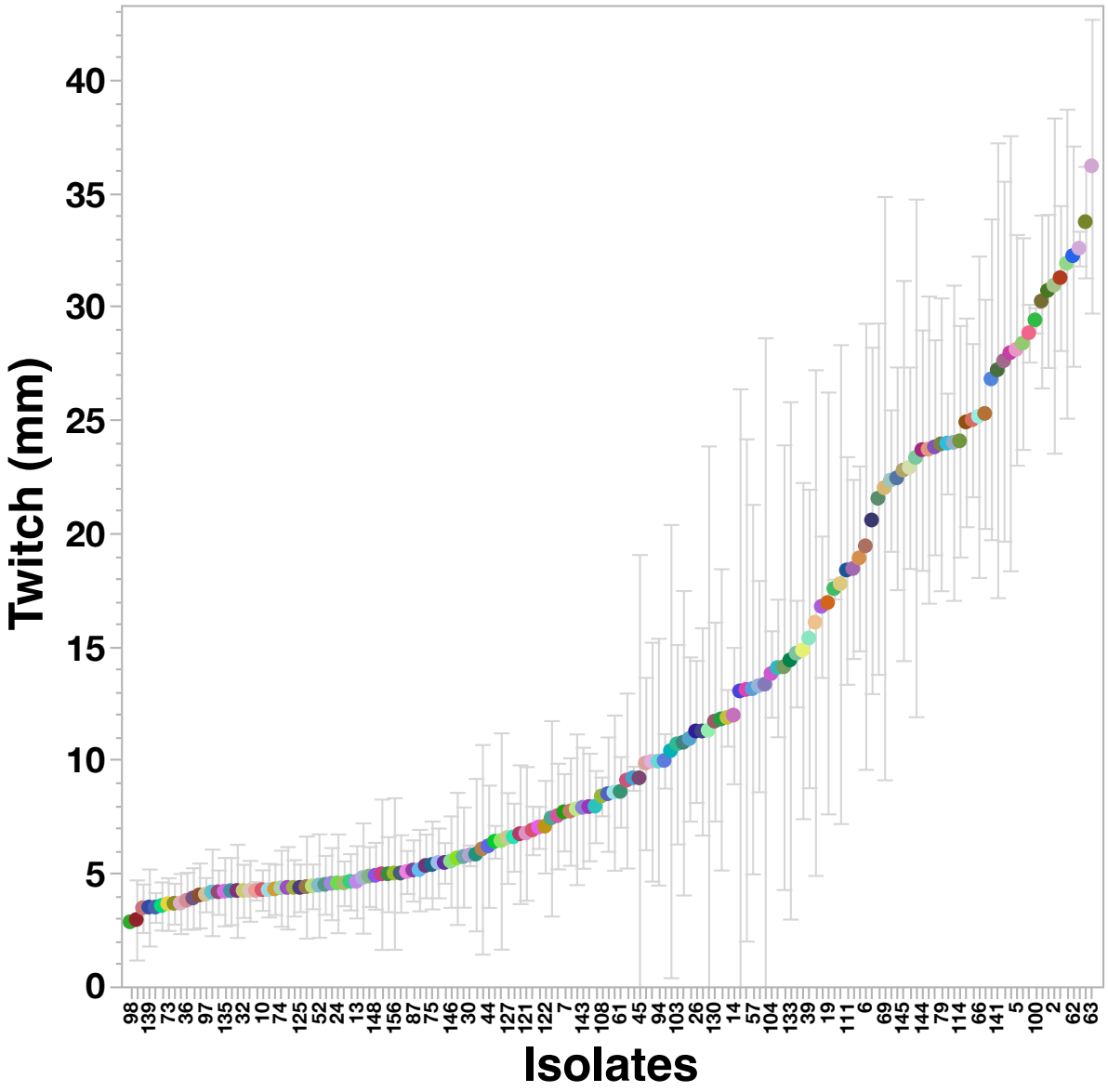
Phenotypes	Growth	Swimming	Swarming	Twitching	Proteolytic activity	Elastase	Pyocyanin 520	Pyocyanin 691	Biofilm	Hydrogen cyanide	Pyoverdin 405	C4-HSL	PQS_alt	Pycyanin_ MS	HHQ	HQNO	PQS	NHQ	NQNO	3-oxo-C12HSL	3-oxo-C12HSL _alt
EP1	0.2309	0.0136	0.0001	0.7861	0.0001	0.0108	0.0021	0.0001	0.0043	0.0001	0.8638	0.5635	0.0001	0.0829	0.0125	0.0034	0.0001	0.0001	0.0001	0.0001	0.4712
EP2	0.0487	0.5295	0.0010	0.0405	0.1094	0.2017	0.0383	0.8880	0.3497	0.0429	0.7711	0.4204	0.0009	0.6832	0.0257	0.0043	0.0011	0.0044	0.1882	0.7945	0.0760
EP3	0.0001	0.0945	0.0187	0.4909	0.3350	0.0302	0.0022	0.0269	0.0161	0.2600	0.0107	0.0067	0.0001	0.1803	0.0002	0.0066	0.0001	0.0001	0.0435	0.0006	0.0004
EP4	0.1852	0.2122	0.9262	0.2389	0.0403	0.0001	0.0113	0.8930	0.0109	0.0512	0.0337	0.0042	0.5951	0.2925	0.5950	0.0037	0.7165	0.4992	0.0044	0.5859	
EP5	0.8156	0.6086	0.4766	0.0922	0.1488	0.5078	0.5133	0.0132	0.0768	0.3028	0.0001	0.0011	0.0051	0.6607	0.0003	0.0032	0.0031	0.0014	0.0175	0.0001	
EP6	0.1205	0.5831	0.2973	0.4033	0.0293	0.0001	0.1204	0.5233	0.3283	0.2690	0.8970	0.0948	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0979	
EP7	0.0331	0.9582	0.0012	0.1976	0.5860	0.5536	0.0262	0.6236	0.6402	0.3331	0.7515	0.0207	0.0001	0.1633	0.5536	0.1770	0.0001	0.4513	0.2377	0.1613	
CP1	0.0005	0.0001	0.0001	0.0001	0.0001	0.0001	0.0034	0.0001	0.0006	0.0023	0.0002	0.0001	0.0001	0.0001	0.0001	0.0020	0.0001	0.0001	0.0001	0.0001	
CP2	0.0638	0.8209	0.4116	0.7053	0.6420	0.0120	0.0363	0.1604	0.5646	0.8815	0.0136	0.0760	0.0119	0.0357	0.0266	0.1897	0.0470	0.5738	0.3021	0.8344	
CP3	0.6490	0.5757	0.1496	0.1721	0.0092	0.0776	0.0001	0.5369	0.2469	0.2321	0.7212	0.6242	0.4092	0.6591	0.3524	0.0014	0.0027	0.0069	0.7596	0.7326	
CP4	0.0854	0.0004	0.7789	0.4993	0.0022	0.7362	0.5658	0.0811	0.0006	0.0021	0.1045	0.2571	0.1531	0.0003	0.0001	0.0001	0.0025	0.0012	0.0009	0.0390	
CP5	0.0150	0.0001	0.0298	0.8830	0.0001		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0365	0.0001	0.0635	0.0001	0.8833	0.3030	0.0001	0.0001	
CP6	0.0033	0.0001	0.0001	0.9671	0.3062	0.2551	0.0093	0.0001	0.0141	0.0523	0.0882	0.0052	0.0001	0.0001	0.0216	0.4024	0.0001	0.0001	0.0003	0.0286	
CP7	0.4724	0.0075	0.3449	0.8728	0.0002	0.0001	0.0016	0.0140	0.9937	0.0167	0.0001	0.1603	0.0001	0.0001	0.0030	0.0033	0.0001	0.0093	0.0020	0.0001	
CP8	0.9972	0.0635	0.0814	0.9997	0.8871	0.0093	0.1864	0.0064	0.0146	0.8453	0.0001	0.0057	0.7142	0.0017	0.0001	0.5344	0.0095	0.0008	0.0575	0.8700	
CP9	0.0039	0.0044	0.0021	0.7289	0.0032	0.0001	0.0001	0.0003	0.5492	0.0470	0.0004	0.0003	0.0001	0.0001	0.0002	0.0020	0.0001	0.0001	0.0004	0.0001	
CP10	0.2275	0.6674	0.3266	0.9994	0.0189	0.0003	0.0442	0.7788	0.0153	0.8980	0.1181	0.1303	0.3416	0.0009	0.0015	0.2475	0.2436	0.0191	0.1549	0.0245	
CP11	0.8874	0.8355	0.3075	0.0685	0.0040		0.0001	0.0016	0.3299	0.6889	0.0001	0.8763	0.0001	0.0557	0.0088	0.0034	0.0001	0.0112	0.0362	0.0478	
CP12	0.2904	0.1644	0.0903	0.3995	0.0248	0.0001	0.0098	0.1025	0.5194	0.4340	0.8723	0.0082	0.0001	0.1379	0.0043	0.0008	0.0001	0.0001	0.0001	0.0020	
CP13	0.2047	0.2625	0.5370	0.3466	0.0001	0.0193	0.0001	0.0008	0.0146	0.0037	0.0150	0.6290	0.0001	0.0442	0.0001	0.0789	0.0001	0.0001	0.0001	0.0101	
CP14	0.8274	0.3858	0.6560	0.2841			0.0001	0.7528	0.2043	0.0001	0.1749	0.8625	0.0001	0.2996	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	
CP15	0.4555	0.0045	0.0428	0.1196	0.0002	0.0004	0.0001	0.0259	0.0004	0.6589	0.8204	0.0039	0.0001	0.0513	0.9834	0.6996	0.0001	0.0965	0.0001	0.0001	
CP16	0.0001	0.0006	0.9510	0.5993	0.0001		0.0080	0.0001	0.0001	0.0326	0.0140	0.1059	0.0493	0.0001	0.1999	0.1811	0.0214	0.0995	0.0001	0.1426	
CP17	0.0001	0.6841	0.0387	0.0585	0.6444		0.0003	0.2754	0.0001	0.0004	0.0012	0.8603	0.0001	0.0178	0.0001	0.0001	0.0006	0.0001	0.0001	0.0007	
CP18	0.0343	0.4982	0.2870	0.8785	0.2182		0.0001	0.9709	0.0128	0.0001	0.1303	0.4350	0.0040	0.0999	0.0073	0.0040	0.0001	0.0027	0.0002	0.0488	
All patients	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	

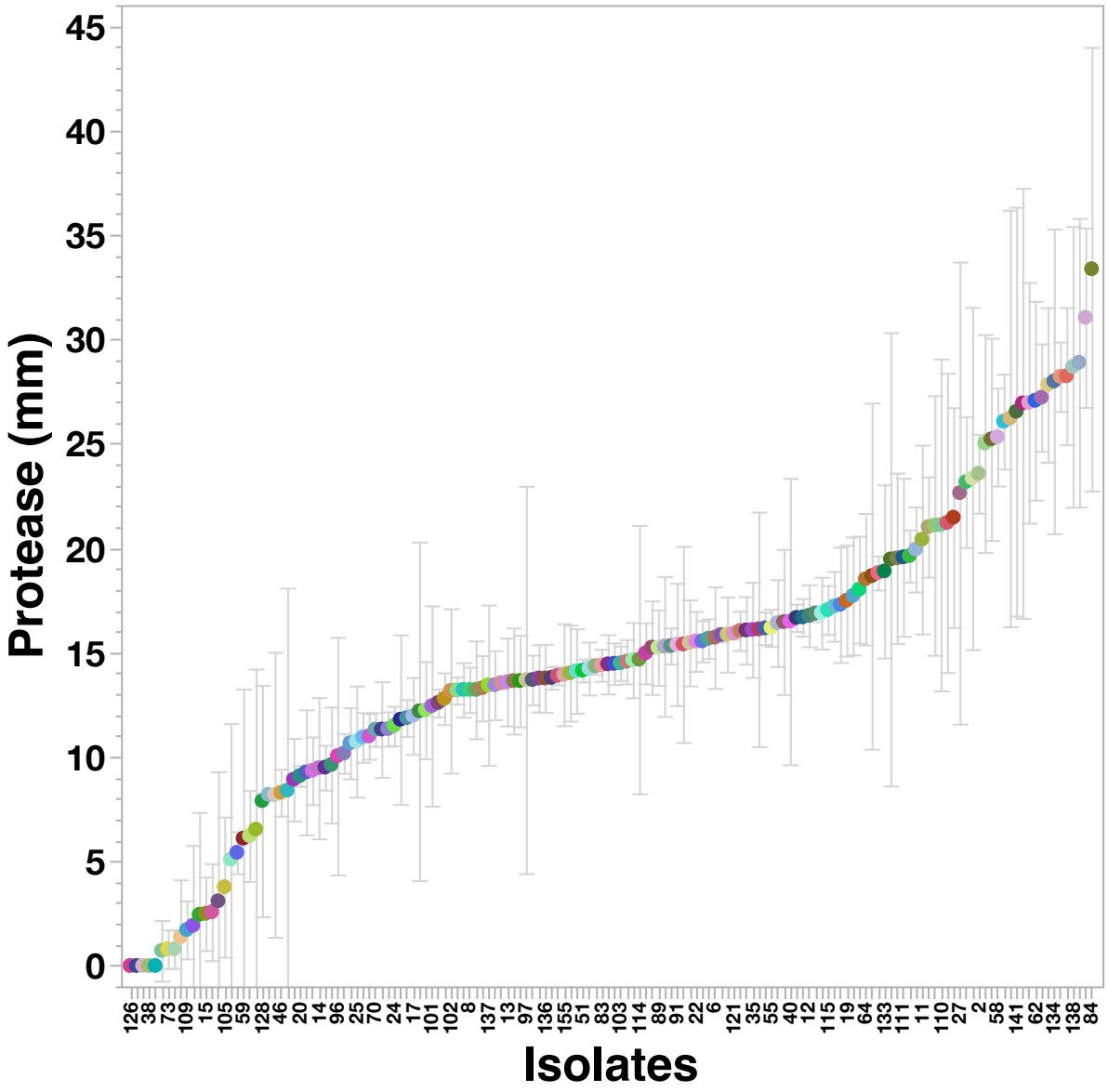
Appendix 3-4: Measurements of phenotypes by ascending order for all UBC isolates. All 154 isolates were ordered by ascending order according to the measurements for each of the 10 phenotypes. Every point represents: mean of ≤ 3 biological replicates. The error bars represent the standard deviation for each isolate.

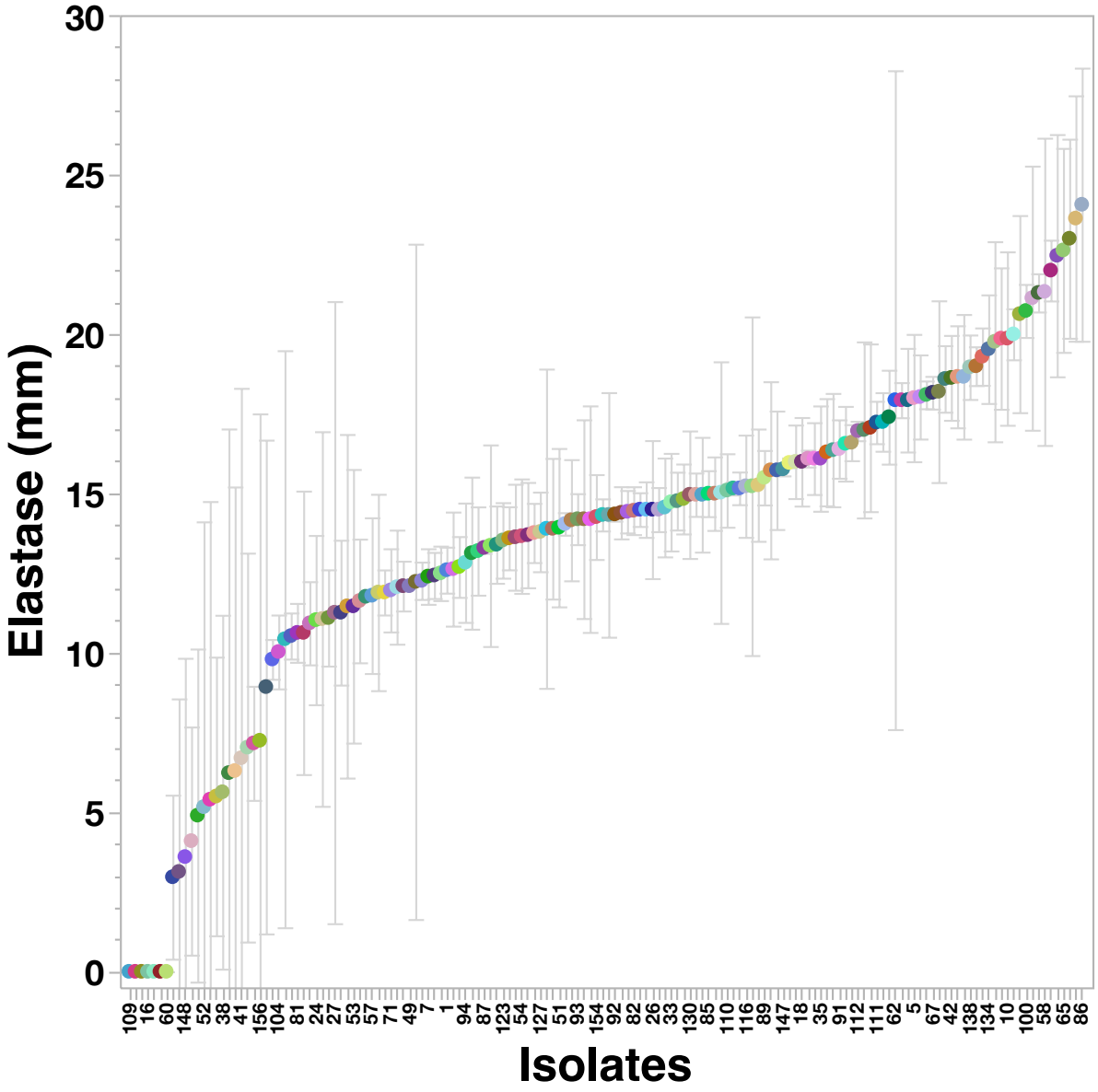


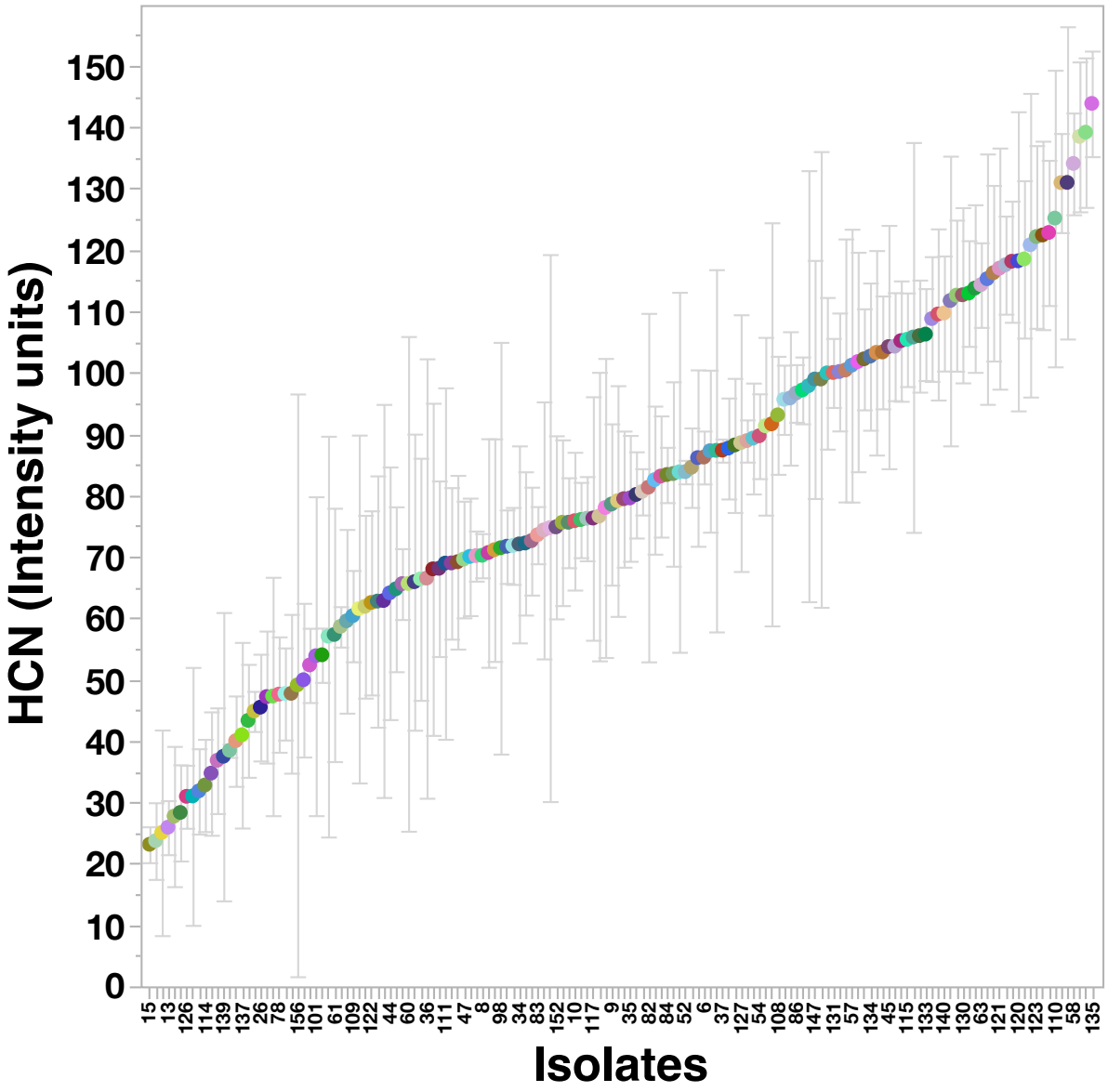

Swim (mm)
20
15
10
5
0
147 8 149 52 152 60 57 44 76 140 33 34 82 80 54 78 53 136 66 4 27 113 70 42 6 132 65 94 114 5 105 39 2 9 11 130 103 69 43 133 119 95 99 85 26 141 116 121 31 16 127 115
Isolates

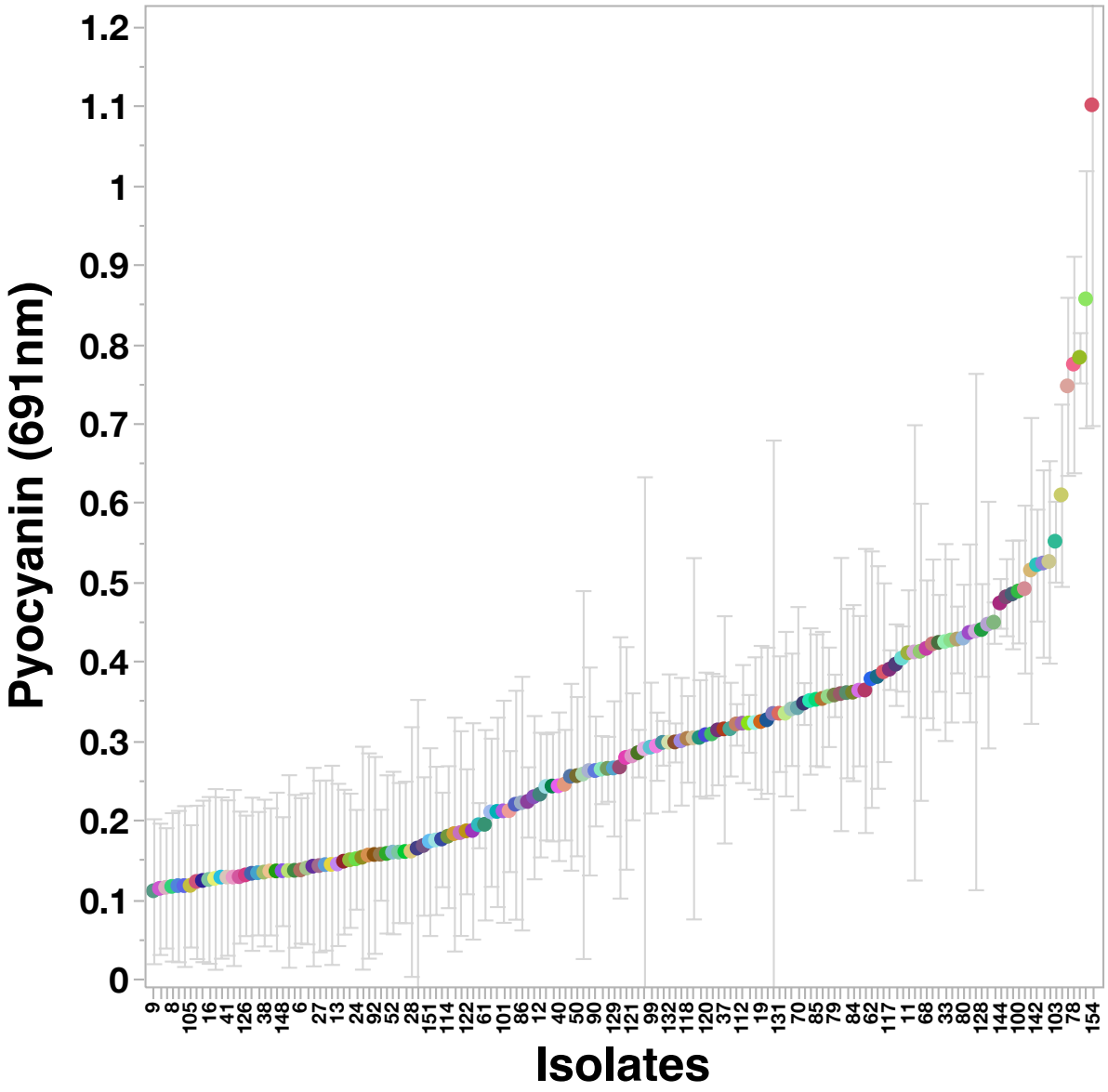

Swarm (mm)
30
25
20
15
10
5
0
15 46 148 53 38 49 18 20 109 82 125 57 25 34 72 4 14 48 105 107 124 56 1 142 59 106 91 31 61 45 70 116 65 42 133 110 121 111 100 99 92 134 36 50 11 47 138 101 84 136 145 35
Isolates

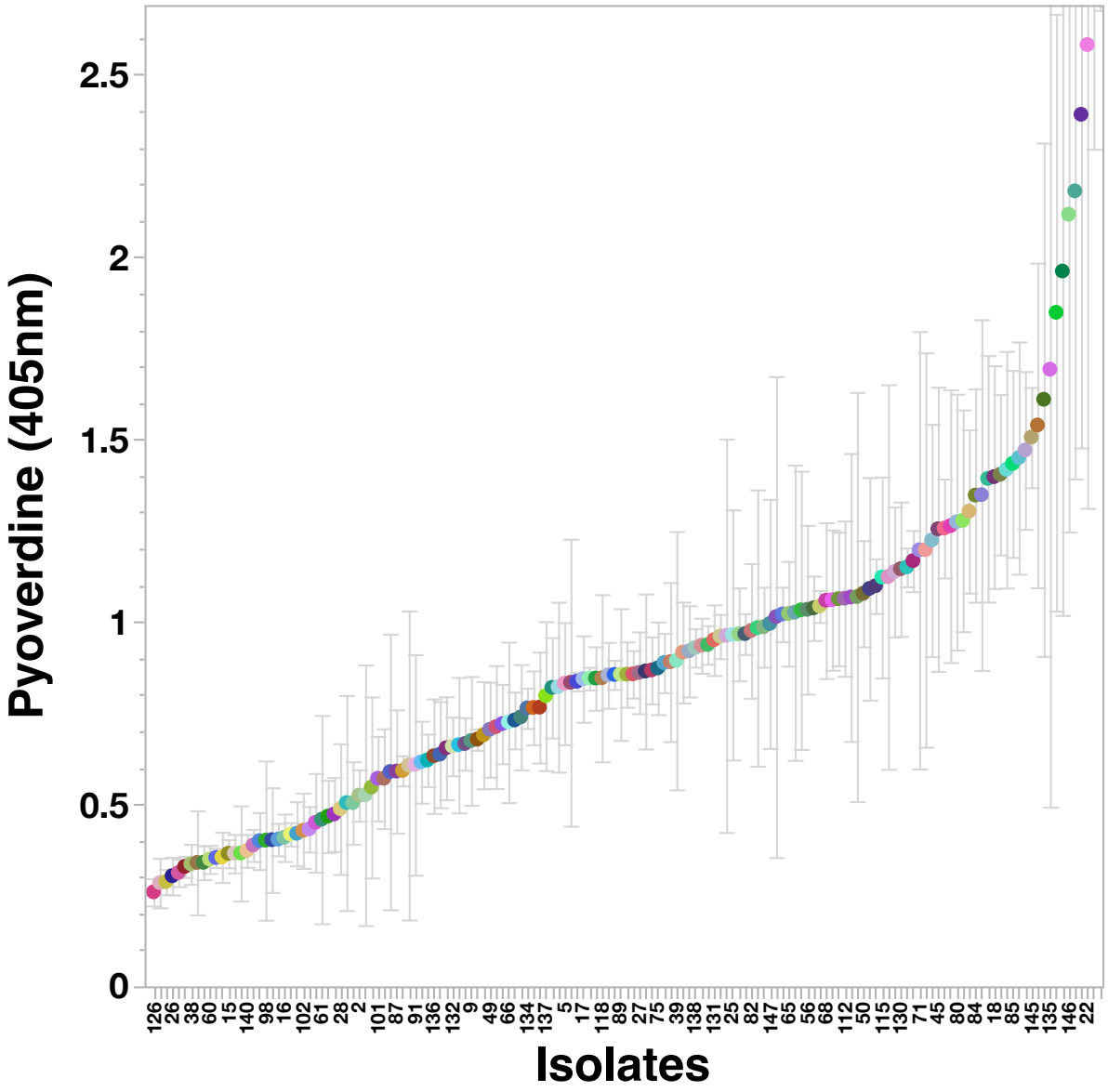

Twitch (mm)
0
5
10
15
20
25
30
35
40
98 139 73 36 97 135 32 10 74 125 52 24 13 148 156 87 75 146 30 44 127 121 122 7 143 108 61 45 94 103 26 130 14 57 104 133 39 19 111 6 69 145 144 79 114 66 141 5 100 2 62 63
Isolates

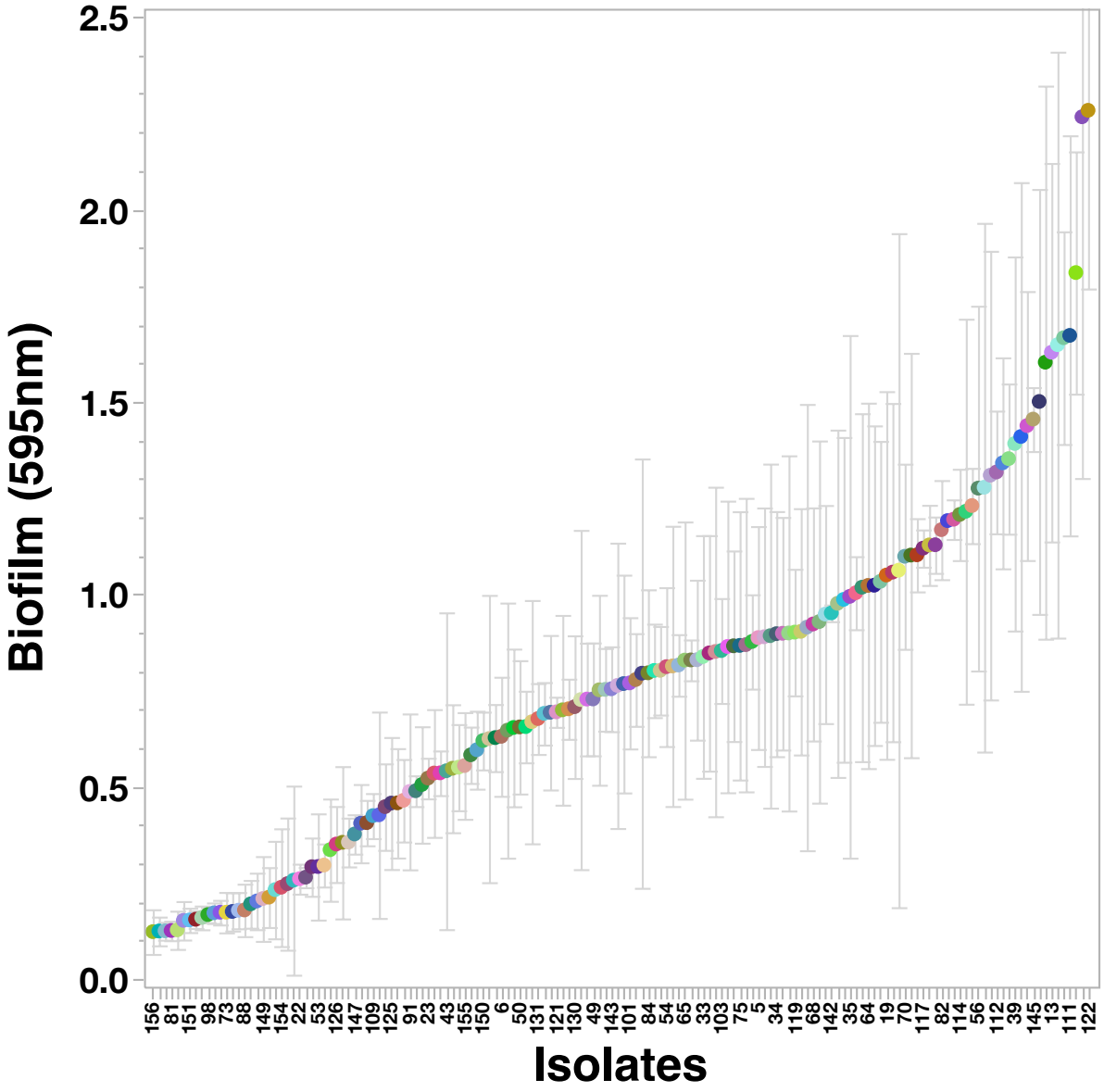

Protease (mm)
45
40
35
30
25
20
15
10
5
0
126 38 73 109 15 105 59 128 46 20 14 96 25 70 24 17 101 102 8 137 13 97 136 155 51 83 103 114 89 91 22 6 121 35 55 40 12 115 19 64 133 111 11 110 27 2 58 141 62 134 138 84
Isolates


Elastase (mm)
30
25
20
15
10
5
0
109 16 60 148 52 38 41 156 104 81 24 27 53 57 71 49 7 1 94 87 123 54 127 51 93 154 92 82 26 33 130 85 110 116 89 147 18 35 91 112 111 62 5 67 42 138 134 10 100 58 65 86
Isolates


HCN (Intensity units)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
15 13 126 114 139 137 26 78 156 101 61 109 122 44 60 36 111 47 8 98 34 83 152 10 117 9 35 82 84 52 6 37 127 54 108 86 147 131 57 134 45 115 133 140 130 63 121 120 123 110 58 135
Isolates


Pyocyanin (691nm)
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
9 8 105 16 41 126 38 148 6 27 13 24 92 52 28 151 114 122 61 101 86 12 40 50 90 129 121 99 132 118 120 37 112 19 131 70 85 79 84 62 117 11 68 33 80 128 144 100 142 103 78 154
Isolates


Pyoverdine (405nm)
2.5
2
1.5
1
0.5
0
126 26 38 60 15 140 98 16 102 61 28 2 101 87 91 136 132 9 49 66 134 137 5 17 118 89 27 75 39 138 131 25 82 147 65 56 68 112 50 115 130 71 45 80 84 18 85 145 135 146 22
Isolates


Biofilm (595nm)
2.5
2.0
1.5
1.0
0.5
0.0
156 81 151 98 73 88 149 154 22 53 126 147 109 125 91 23 43 155 150 6 50 131 121 130 49 143 101 84 54 65 33 103 75 5 34 119 68 142 35 64 19 70 117 82 114 56 112 39 145 13 111 122
Isolates

Appendix 4-1: Table of all correlation for both RBH and UBC sets of strains. Correlations are ordered from strongest to lowest according to results from the RBH set of strains. Correlation signs correspond to: +: positive correlation; -: negative correlation; +/- and -/+: sign of correlation for RBH and UBC respectively. p value: *** p <0.0005; ** p <0.005; * p <0.01; p values that are different for the RBH and UBC strains are marked as p value RBH/ p value UBC. Correlations rank corresponds to the rank of the correlation according to the spearman coefficient out of 45 correlations. The difference corresponds to the absolute difference in correlation coefficient or rank between the RBH and UBC sets of strains.

Correlation	Correlation sign	Spearman ρ				Correlation rank (/45)		
		RBH	UBC	Difference	p value	RBH	UBC	Difference
Elastase-Protease	+	0.8133	0.8315	0.0182	***	1	1	0
Swarm-Swim	+	0.7463	0.5112	0.2351	***	2	7	5
Pyoverdine-Protease	+	0.6956	0.4009	0.2947	***	3	16	13
Swarm-Growth	-	0.6695	0.3936	0.2759	***	4	18	14
Pyocyanin-Protease	+	0.6543	0.3699	0.2844	***	5	23	18
Pyocyanin-Elastase	+	0.631	0.3982	0.2328	***	6	17	11
Swim-Growth	-	0.6218	0.4453	0.1765	***	7	10	3
Protease-Biofilm	+	0.609	0.4305	0.1785	***	8	12	4
Pyoverdine-Elastase	+	0.5998	0.3823	0.2175	***	9	20	11
Pyoverdine-Pyocyanin	+	0.599	0.6835	0.0845	***	10	2	8
Biofilm-Pyocyanin	+	0.5962	0.1929	0.4033	***/NS	11	37	26
Elastase-Biofilm	+	0.5832	0.3774	0.2058	***	12	21	9
Pyoverdine-Swim	+	0.5755	0.3335	0.242	***	13	26	13
Pyoverdine-Growth	-	0.5481	0.1362	0.4119	***/NS	14	41	27
Protease-Swim	+	0.5205	0.4391	0.0814	***	15	11	4
Protease-Swarm	+	0.5192	0.6611	0.1419	***	16	3	13
Elastase-Swarm	+	0.5017	0.5807	0.079	***	17	5	12
Pyoverdine-Swarm	+	0.4842	0.3746	0.1096	***	18	22	4
Elastase-Swim	+	0.4754	0.3092	0.1662	***	19	30	11
Pyoverdine-Biofilm	+	0.4592	0.149	0.3102	***/NS	20	40	20
Swim-Biofilm	+	0.4573	0.3204	0.1369	***	21	27	6
Pyocyanin-Swim	+	0.4398	0.3113	0.1285	***	22	29	7
Protease-Growth	-	0.4204	0.4074	0.013	***	23	15	8
Pyocyanin-HCN	+	0.4181	0.3922	0.0259	***	24	19	5
Elastase-Growth	-	0.406	0.3151	0.0909	***	25	28	3
Pyocyanin-Swarm	+	0.367	0.419	0.052	***	26	14	12
Pyoverdine-Twitch	+	0.3281	0.1662	0.1619	***/NS	27	38	11
Twitch-Swarm	+	0.32	0.4463	0.1263	***	28	9	19
HCN-Elastase	+	0.2994	0.2299	0.0695	***/*	29	34	5
Twitch-Growth	-	0.2716	0.1948	0.0768	***/NS	30	36	6
Protease-Twitch	+	0.2427	0.5826	0.3399	***	31	4	27
HCN-Swarm	+	0.2355	0.2507	0.0152	***/**	32	33	1
Twitch-Swim	+	0.2137	0.2123	0.0014	***/NS	33	35	2
Swarm-Biofilm	+	0.2086	0.292	0.0834	***	34	31	3
HCN-Pyoverdine	+	0.206	0.4584	0.2524	***	35	8	27
Pyocyanin-Growth	-	0.2035	0.1569	0.0466	***/NS	36	39	3
Elastase-Twitch	+	0.1913	0.5499	0.3586	***	37	6	31
HCN-Swim	+	0.1789	0.3674	0.1885	***	38	24	14
HCN-Protease	+	0.1619	0.2831	0.1212	***	39	32	7
Pyocyanin-Twitch	+	0.1318	0.1277	0.0041	**/NS	40	42	2
HCN-Biofilm	+/-	0.1252	0.0477	0.0775	**/NS	41	45	4
Biofilm-Growth	-	0.1137	0.0593	0.0544	*/NS	42	44	2
HCN-Twitch	-/+	0.0754	0.1211	0.0457	NS/NS	43	43	0
Twitch-Biofilm	+	0.0321	0.4237	0.3916	NS/***	44	13	31
HCN-Growth	+/-	0.0187	0.3353	0.3166	NS/***	45	25	20

Appendix 4-2: Spearman correlations between QS molecule 4-hour time point and 10 phenotypes.

The data stems from the UBC sets of strains. Correlations are in order of strength from strongest to lowest. p value: *** p <0.0005; ** p <0.005; * p <0.01.

Correlations		UBC	
QS (4h)	Phenotype	Spearman ρ coefficient	p value
C4-HSL	Protease	0.6056	***
C4-HSL	Elastase	0.542	***
HHQ	Protease	0.5035	***
3-oxo-C12-HSL	Pyoverdine	0.4783	***
PQS	Protease	0.4745	***
HHQ	Elastase	0.4542	***
C4-HSL	Pyoverdine	0.4529	***
3-oxo-C12-HSL	Protease	0.4498	***
PQS	Elastase	0.4313	***
PQS	Pyoverdine	0.4195	***
3-oxo-C12-HSL	Elastase	0.4165	***
3-oxo-C12-HSL	Pyocyanin	0.4117	***
HHQ	Swim	0.3814	***
PQS	Swarm	0.3658	***
C4-HSL	Swarm	0.362	***
PQS	Pyocyanin	0.3607	***
HHQ	Pyoverdine	0.3413	***
HHQ	Swarm	0.34	***
C4-HSL	Pyocyanin	0.3399	***
C4-HSL	Swim	0.3356	***
PQS	HCN	0.3315	***
PQS	Swim	0.3041	***
3-oxo-C12-HSL	Swarm	0.3037	***
C4-HSL	HCN	0.301	***
HHQ	HCN	0.289	**
HHQ	Pyocyanin	0.285	**
3-oxo-C12-HSL	Swim	0.2684	**
C4-HSL	Biofilm	0.2342	*
3-oxo-C12-HSL	HCN	0.2182	NS
HHQ	Biofilm	0.2163	NS
3-oxo-C12-HSL	Biofilm	0.2083	NS
HHQ	Twitch	0.1742	NS
C4-HSL	Twitch	0.1729	NS
PQS	Twitch	0.1494	NS
3-oxo-C12-HSL	Twitch	0.1023	NS
PQS	Biofilm	0.0515	NS
3-oxo-C12-HSL	Growth	-0.2197	NS
C4-HSL	Growth	-0.3689	***
PQS	Growth	-0.3969	***
HHQ	Growth	-0.4616	***

Appendix 5-1: Analysis of variance for phenotypes and individual patients. A one-way ANOVA approach was used to estimate the amount of variation between the multiple isolates of each patients. EP2: 13 isolates; EP4: 24 isolates; EP1 and EP7: 26 isolates; EP3 and EP6: 36 isolates; EP5: 60 isolates; All CP patients: 24 isolates, except CP2, CP8a and CP10: 12 isolates. ***: <0.0005 ; **: <0.005; *:<0.05; NS: non significant.

Phenotypes	Growth		Swimming		Swarming		Twitching		Protease		Elastase		HCN		Pyocyanin		Pyoverdine		Biofilm	
Patient	F ratio	P value	F ratio	P value	F ratio	P value	F ratio	P value	F ratio	P value	F ratio	P value	F ratio	P value	F ratio	P value	F ratio	P value	F ratio	P value
EP1	3.1753	***	1.5835	NS	2.7201	***	2.0092	*	1.0567	NS	1.1587	NS	4.1921	***	1.9883	*	0.4559	NS	1.7714	*
EP2	1.9557	NS	0.8708	NS	0.6108	NS	4.8423	***	2.0388	*	1.5514	NS	3.2259	*	1.8656	NS	0.2796	NS	1.7102	NS
EP3	1.3754	NS	1.2224	NS	0.2145	NS	0.854	NS	1.0349	NS	0.4395	NS	0.7399	NS	0.5194	NS	0.3882	NS	0.4457	NS
EP4	2.0316	*	0.9869	NS	2.4676	**	6.1017	***	6.6678	***	3.3145	***	4.439	***	2.4649	**	2.2144	**	0.67	NS
EP5	0.5946	NS	0.069	NS	0.1816	NS	1.0379	NS	1.0371	NS	0.7653	NS	2.178	***	0.9813	NS	1.5083	*	0.2479	NS
EP6	0.2902	NS	0.2643	NS	0.3322	NS	0.5423	NS	0.3853	NS	0.1907	NS	1.6638	*	1.0688	NS	0.1113	NS	0.5968	NS
EP7	0.1845	NS	0.0811	NS	0.2992	NS	0.2304	NS	0.1452	NS	0.4433	NS	1.7338	*	0.1446	NS	0.323	NS	0.5955	NS
CP1	7.335	***	27.8694	***	12.3353	***	4.4839	***	9.8029	***	35.1127	***	6.4777	***	3.3633	***	2.0812	*	2.3745	**
CP2	0.9029	NS	0.3183	NS	0.3174	NS	1.4803	NS	0.3863	NS	1.6452	NS	1.15	NS	1.1276	NS	0.8852	NS	0.2214	NS
CP3	2.0164	*	7.7785	***	1.001	NS	0.8796	NS	1.0893	NS	2.0452	*	1.4485	NS	1.2191	NS	0.2306	NS	0.4317	NS
CP4	6.3846	***	0.6733	NS	0.3722	NS	0.6491	NS	1.6225	NS	6.0593	***	11.1636	***	5.8263	***	1.9377	*	0.4956	NS
CP5	1.2891	NS	3.0414	**	0.8804	NS	1.9455	*	462.35	***			10.1823	***	7.6454	***	1.2803	NS	7.8947	***
CP6	1.471	NS	8.5983	***	5.4195	***	0.6981	NS	5.6897	***	0.2181	NS	12.6643	***	1.7992	*	1.3953	NS	0.8203	NS
CP7	4.6717	***	13.2514	***	1.2246	NS	10.3925	***	10.9138	***	100.26	***	54.7805	***	14.0579	***	4.147	***	1.7147	*
CP8a	0.8121	NS	3.6255	**	8.8842	***	1.476	NS	9.2553	***	9.2767	***	1.33	NS	0.8764	NS	3.0656	*	0.9879	NS
CP8b	6.5544	***	2.2907	**	1.1349	NS	0.8875	NS	2.9164	**	4.7838	***	2.9507	**	1.278	NS	2.1942	*	0.3025	NS
CP9	21.9355	***	14.3257	***	7.0836	***	1.4783	NS	48.3556	***	59.3155	***	2.8669	**	1.477	NS	3.208	***	0.6093	NS
CP10	2.1965	NS	3.4876	**	1.9221	NS	3.2648	*	6.5712	***	37.996	***	2.011	NS	0.87	NS	0.6571	NS	0.3298	NS
CP11	1.0427	NS	0.7799	NS	0.726	NS	1.0815	NS	2.5904	**			0.2365	NS	0.8848	NS	0.4309	NS	0.1405	NS
CP13	3.4706	***	0.9586	NS	2.4377	**	0.6122	NS	51.7052	***	10.3867	***	5.0791	***	2.1516	*	0.6692	NS	0.2306	NS
CP14	0.8051	NS	0.7686	NS	1.765	*	1.51	NS					0.9216	NS	0.9501	NS	0.2202	NS	0.1472	NS
CP15	2.2308	*	14.6327	***	2.5131	**	1.6174	NS	9.9989	***	28.731	***	2.0856	*	2.6991	**	10.9716	***	7.9776	***
CP16	6.1827	***	0.7156	NS	1.735	*	1.2289	NS	1	NS			0.9891	NS	0.9821	NS	0.3729	NS	0.9545	NS
CP18	0.4843	NS	2.1976	*	1.2373	NS	0.6132	NS	0.9568	NS			1.7145	*	0.8833	NS	0.4765	NS	1.0182	NS

Appendix 5-2: Zoomed in box plot of QS molecule concentrations with the maximum equivalent to PA01 levels for all phenotypes and patient. 100% correspond to the PA01 control sample mean concentration. Each box represents the extent of the diversity for each patient taking into account all the single isolates from that patient, dots represent each isolate. The unit corresponds to the proportion in comparison to the PA01 control For each patient a minimum of at least 12 isolates were used in the test of phenotypes. All values used to make plots are means of at least 3 biological replicates for each phenotypic assay. Early= Isolates from early stage infections; Chronic= Isolates from chronic stage infections.

