Virulence mechanisms of the cystic fibrosis pathogen Achromobacter xylosoxidans

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Virulence mechanisms of the cystic fibrosis pathogen *Achromobacter xylosoxidans*

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Abstract

Cystic fibrosis (CF) is a genetic disorder caused by mutations in the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR). The defective ion transport causes systemic disease, most notably of the airways and pancreas, due to abnormal mucus viscosity. A major cause of disease burden is the acquisition of persistent bacterial airway infections. Species such as *Pseudomonas aeruginosa* are well-studied CF pathogens, and are known to cause lung damage and disease progression. In this thesis, we aimed to investigate potential virulence mechanisms of the emerging CF pathogen *Achromobacter xylosoxidans,* comparing it to *P. aeruginosa* and to other *Achromobacter* species found in CF sputum. *Achromobacter* infections have been correlated with worse lung disease in CF, but whether there is a causal relationship has been under debate due to limited research on its virulence.

In paper I, we compared inflammatory properties of exoproducts from *A. xylosoxidans*, *P. aeruginosa* and three other *Achromobacter* species, finding that all *Achromobacter* species studied were able to cause equal or stronger inflammatory responses as *P. aeruginosa*. In paper II we established the importance of the Tat secretion system by generating and characterising a Tat-deficient mutant strain. Paper III focused on identifying antigenic proteins of *A. xylosoxidans,* and paper IV describes *in vitro* polymicrobial interactions between *A. xylosoxidans* and *P. aeruginosa.* Taken together, our results indicate that *A. xylosoxidans* exhibits virulence mechanisms such as inflammatory exoproducts, Tat secretion, and polymicrobial interactions, and identified antigenic proteins which may be further studied as potential virulence factors.

Key words: Cystic fibrosis, airway infections, *Achromobacter xylosoxidans*, *Pseudomonas aeruginosa*

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To Mum

Look, I made it :)

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Characterisation of airway inflammation and airway proteomes associated with cystic fibrosis-related diabetes
Manuscript.

Abbreviations

ABC	ATP-binding cassette
ASL	Airway surface liquid
AMP	Antimicrobial peptide
ATP	Adenosine triphosphate
CF	Cystic fibrosis
CFRD	Cystic fibrosis-related diabetes
CFTR	Cystic fibrosis transmembrane conductance regulator
DDA	Data-dependent acquisition
EM	Electron microscopy
EPS	Extracellular polymeric substance
FCM	Flow cytometry
FEV_1	Forced expiratory volume in one second
IgG	Immunoglobulin G
IV	Intravenous
IVIG	Intravenous immunoglobulins
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LPS	Lipopolysaccharide
MS	Mass spectrometry
NET	Neutrophil extracellular trap
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
pwCF	Person/people with cystic fibrosis
SPM	Specialized proresolving lipid mediators
spp	Species (plural)
Tat	Twin-arginine translocation
T(X)SS	Type (X) secretion system
qPCR	Quantitative polymerase chain reaction

Populärvetenskaplig sammanfattning

Cystisk fibros (CF) är en av de vanligaste genetiska sjukdomarna i västvärlden. Ungefär 1 av 25 personer med europeiskt ursprung bär på anlaget, som beror på en mutation i genen CFTR (cystic fibrosis transmembrane conductance regulator). Personer som ärvt mutationen från båda föräldrar drabbas av rubbningar i cellernas salttransport, vilket leder till att kroppen producerar segt slem i framför allt bukspottkörteln och luftvägarna. Slemmet utgör en grogrund för bakterie- och svampinfektioner i luftvägarna, som kan vara mycket svåra att utrota med hjälp av antibiotika. Dessa långdragna luftvägsinfektioner leder ofta till permanenta lungskador och är den främsta orsaken till att personer med CF har haft en förväntad medellivslängd på ungefär 50 år, vilket väntas öka tack vare nya behandlingsmetoder. Sedan slutet av 2022 finns läkemedlet Kaftrio tillgängligt i Sverige, vilket kan korrigera effekterna av de vanligaste CFTR-mutationerna och minska slemproduktionen i lungorna. Behandlingen har förbättrat lungfunktion och livskvalitet för många som lever med CF, men problemet med kroniska infektioner kvarstår.

Den här avhandlingen fokuserar på infektioner med *Achromobacter*, ett bakteriesläkte som finns naturligt i fuktiga miljöer som t.ex. avloppsbrunnar. De orsakar vanligtvis inga infektioner hos friska personer. *Achromobacter*-infektioner hos personer med CF är förknippat med försämrad lungfunktion och svårare sjukdomstillstånd, men det har varit omdiskuterat huruvida detta beror på att bakterien orsakar skada eller om den tar fäste först efter att luftvägarna försvagats av andra orsaker. Den senaste forskningen pekar på att *Achromobacter* kan orsaka skada på egen hand, då de har ett flertal mekanismer som gör att de kan orsaka inflammation.

Olika *Achromobacter*-arter grupperas ofta ihop inom klinisk diagnostik, och det finns fortfarande mycket lite information om hur de skiljer sig från varandra. Vi jämförde utsöndrade produkter från fyra olika *Achromobacter*-arter och såg att alla kunde orsaka inflammation i odlade luftvägsceller på samma sätt som *Pseudomonas aeruginosa*, den vanligaste bakterien i CF som vi vet orsakar allvarliga infektioner.

Achromobacter har liksom *Pseudomonas* dubbla cellmembran, och använder sig av transportsystem för att flytta proteiner från cellens insida till utrymmet mellan membranen. Vi utforskade transportsystemet twin-arginine translocation (Tat) och upptäckte att *Achromobacter* som muterats för att sakna Tat fäste sämre till luftvägsceller och hade sämre upptag av järn från omgivningen, vilket gör att de får svårare att tillgodose sina näringsbehov. Tat-systemet är nödvändigt för att *P. aeruginosa* ska kunna orsaka skada, och våra resultat tyder på att *Achromobacter* fungerar på ett liknande sätt.

Personer med *Achromobacter*-infektioner kan utveckla antikroppssvar mot bakterien, men antikropparna är oftast otillräckliga för att bekämpa infektionen. Vi ville undersöka vad på bakterien de binder till, och hittade tre proteiner som mål för antikropparna. Om något av dessa proteiner är specifikt för *Achromobacter*-infektioner och inte binder till antikroppar mot t.ex. *Pseudomonas*, skulle de möjligtvis kunna användas som diagnostisk metod för att följa infektionen via ett blodprov istället för att ta odlingar från slemprov. Detta skulle underlätta uppföljningen av infektioner efter att behandling med Kaftrio minskat mängden slem i lungorna.

Eftersom *P. aeruginosa*-infektioner är vanliga bland personer med CF så är det sannolikt att personer med *Achromobacter* även är infekterade med *P. aeruginosa*. Vi studerade hur dessa bakterier interagerar med varandra och såg att samodling med *A. xylosoxidans* i vissa fall kan hindra *P. aeruginosa* från att bilda biofilm, en utsöndrad gel av proteiner och sockerarter som kapslar in bakterier på en yta för att skydda dem från immunförsvaret.

Sammanfattningsvis har vi identifierat flera sätt som *Achromobacter* liknar *Pseudomonas aeruginosa*, vilket stärker den numera mer och mer vedertagna uppfattningen att det finns ett orsakssamband mellan *Achromobacter*-infektion och försämrad sjukdomsbild i CF.

Popular science summary

Cystic fibrosis (CF) is one of the most common genetic diseases in the Western world. About 1 in 25 people of European descent is an unaffected carrier of the disease, which is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. People who have inherited the mutation from both parents sustain defects in cellular salt transport, which causes the body to produce thickened mucus particularly in the pancreas and airways. The mucus is a breeding ground for bacterial and fungal infections in the respiratory tract, which can be very difficult to eradicate through antibiotics. These long-term respiratory infections often lead to permanent lung damage and are the main reason people with CF have had a life expectancy of about 50 years, which is expected to rise thanks to new treatments. Since the end of 2022, the drug Kaftrio has been available in Sweden, which can correct the effects of the most common CFTR mutations and reduce mucus production in the lungs. This treatment has improved lung function and quality of life for many people living with CF, but the problem of chronic infections remains.

This thesis focuses on infections with *Achromobacter*, a bacterial genus that is found naturally in moist environments such as bathroom drains. They usually do not cause infections in healthy people. *Achromobacter* infections in people with CF are associated with decreased lung function and more severe disease, but it has been debated whether this is because the bacteria cause damage or if they infect only after the airways have been weakened by other causes. The latest research indicates that *Achromobacter* is likely able to cause tissue damage on its own, as it has several mechanisms that allow them to cause inflammation.

Different *Achromobacter* species are often grouped together in clinical diagnostics, and there is still very little information on how they differ from each other. We compared secreted products from four different *Achromobacter* species and found that all were able to cause inflammation in cultured airway cells in the same way as *Pseudomonas aeruginosa*, the most common bacterium in CF that we know causes serious infections.

Achromobacter, like *Pseudomonas*, have double cell membranes, and uses transport systems to move proteins from the inside of the cell to the space between the membranes. We explored the twin-arginine translocation (Tat) transport system and found that *Achromobacter* mutated to lack Tat could not attach as well to airway cells, and had an impaired uptake of iron from the environment, making it harder for them to meet their nutritional needs. The Tat system is necessary for *P. aeruginosa* to cause damage in humans, and our results suggest that *Achromobacter* functions in a similar way.

People with *Achromobacter* infections can develop antibody responses to the bacteria, but the antibodies are usually insufficient to clear the infection. We wanted to investigate what bacterial proteins the antibodies bind to, and found three proteins targeted by the antibodies. If any of these proteins are specific for *Achromobacter* infections and do not bind to antibodies against e.g. *P. aeruginosa*, they could possibly be used as a diagnostic method to follow the infection via a blood test instead of taking cultures from sputum samples. This would help in following up infections after treatment with Kaftrio reduces the amount of mucus in the lungs.

As *P. aeruginosa* infections are common among people with CF, it is likely that people with *Achromobacter* are also infected with *P. aeruginosa*. We studied how these bacteria interact with each other and saw that co-cultivation with *A. xylosoxidans* in some cases prevented *P. aeruginosa* from forming biofilm, a secreted gel of proteins and sugars that encapsulates bacteria on a surface to protect them from the immune system.

In summary, we have identified several ways in which *Achromobacter* is similar to *Pseudomonas aeruginosa*, which strengthens the now increasingly accepted viewpoint that there is a causal relationship between *Achromobacter* infections and CF disease progression.

Background

Introduction

Cystic fibrosis (CF) is a hereditary disease caused by an autosomal recessive mutation in the cystic fibrosis transmembrane conductance regulator (CFTR). Though systemic in nature, it most notably affects the airways and pancreas by causing a buildup of thick mucus due to dysfunctional salt transport [1]. The most common airway symptoms include persistent coughing, shortness of breath, and frequent infections. Other systems are also affected and may show symptoms such as salty-tasting skin, poor growth and/or weight gain, bowel obstructions, and infertility. The condition leads to a shortened life expectancy primarily due to bacterial infections progressively damaging the lungs [2]. Managing the disease consists of a multidisciplinary approach including pharmaceutical treatments, physical therapy, and diet.

As lung infections are a primary cause of disease progression in CF, following up and managing these is a cornerstone of CF care. Infections with *Staphylococcus aureus* and *Haemophilus influenzae* are common in children, while *Pseudomonas aeruginosa* is the most common infection in adulthood, affecting over half of people with CF (pwCF) [3]. These gram-negative bacteria are well-known to cause persistent infections that are very difficult to eradicate, and cause progressive lung damage due to long-term inflammation. In addition to these classical pathogens, infections largely unique to pwCF have been emerging in correlation with increased life expectancies. These include *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia* complex, and non-tuberculous mycobacteria [4].

This thesis focuses on elucidating virulence mechanisms of *Achromobacter xylosoxidans,* as well as its closely related species *Achromobacter insuavis, dolens,* and *ruhlandii*, which have all been found to cause lung infections in CF. Depending on geographical location, these infections affect around 5-10% of pwCF. Patients with *Achromobacter* colonisation often present with lower lung function and generally worse health status than those without this pathogen [5]. It has long been unclear whether this correlation is caused by the infection itself or not, instead being explained as an infection that takes hold only after the lung tissue has been weakened by other causes.

However, recent research including parts of this thesis has shown that *Achromobacter* is capable of causing inflammation similarly to *Pseudomonas aeruginosa*, one of the most well-known harmful infections in CF [6-8].

During the course of this thesis, CF care has changed dramatically with the introduction of CFTR modulators. By correcting the effects of the most common CFTR mutations, through a combination of drugs that help traffic the channel to the cell surface and keep it from prematurely closing, the mucus production characteristic to CF can be dramatically reduced [9]. This has led to an improved lung function and overall quality of life for the majority of persons with applicable mutations who are able to tolerate the treatment. After extended discussions due to the high cost of this treatment, it was approved for prescription in Sweden in December 2022.

Although the introduction of CFTR modulators has improved the lives of a large number of people with CF, CFTR modulation does not cure the underlying mutation, or correct lung injury already present at the time of starting treatment. At the time of writing, the future of CF care is hopeful but largely uncharted – with the hope that prophylactic intervention in children may prevent infections and tissue injury that is now too late to prevent in adults with CF, but a lot of uncertainty how this treatment will affect patients in the long term. We are likely going to see a reduced need for lung transplantations in this patient group, and a new need for CF care for health issues related to older age.

Current research on the effects of CFTR modulation on infection status indicate that treatment appears to reduce bacterial load in the short term, but that infections are not eradicated and may reemerge at a later time point [10]. CF lung infections can be persistent and may span across decades of subclinical colonisation, which has not yet been possible to study in relation to modulator therapy. In conclusion, additional research on the mechanisms and treatment of CF infection is still highly relevant in the age of CFTR modulation - both due to the uncertain effects of long-term modulation and for people who do not tolerate or respond to this treatment.

Cystic fibrosis

History

> "Woe to the child who tastes salty from a kiss on the brow, for he is cursed and soon will die". – European folklore

Before the introduction of medical interventions, children born with cystic fibrosis would rarely survive the first year of life. An infant could be "diagnosed" through a characteristic salty taste of the skin caused by the loss of sodium in sweat, and would often be born with a swollen belly due to intestinal obstructions caused by the increased viscosity of stool (meconium ileus).

In 1938, Dorothy Hansine Andersen first described the condition as cystic fibrosis [11], with the name referring to the fibrosis seen on the pancreas due to mucus cysts. By treating the pancreatic insufficiency with enzyme replacement, the malnutrition which had until then been the main cause of death could be better managed.

The diagnosis of CF was improved in 1952 when Paul di Sant'Agnese discovered the abnormal chloride levels in CF sweat [12], which could be implemented as a screening test [13] still used to this day, leading to improved care and survival. The first adult CF centre was established in London in 1965. Throughout the 60's and 70's, the main treatments used for CF lung disease were mist tents and postural drainage. Mist tents were plastic canopies used to let the patient breathe humidified air, helping with mucus clearance from the airways but at the cost of a high risk of droplet-borne infections. Postural drainage is a physiotherapy technique in which the patient assumes head-down positions which let gravity aid the mucus clearance while applying percussion to the chest walls. Though the improvements in diagnostics and antibiotic treatment increased survival, the average life expectancy was still under 20 years in 1970.

A major breakthrough in CF research was the discovery of the *CFTR* gene and its disease-causing ΔF508 mutation by Francis Collins and Lap-Chee Tsui in 1989 [14]. A vast amount of research into CFTR-modulating treatments and gene therapy has been made possible thanks to this discovery. Until the 2010s, CF care has relied on symptomatic treatments to manage the effects of mucus buildup and airway infections.

The life expectancy of pwCF increased to about 40 years through improved antibiotic protocols and possibilities of transplantation for end-stage lung disease.

The first disease-modifying drug on the market, the CFTR modulator Kalydeco (ivacaftor), was released by Vertex Pharmaceuticals in 2012. This treatment is primarily effective in persons with the G551D mutation, accounting for only about 5% of pwCF. The mutation causes a premature closing of the ion channel, which the drug targets by stabilising CFTR in its open state.

The release of the combination therapies Orkambi (ivacaftor/lumacaftor, 2015) and Symkevi (ivacaftor/tezacaftor, 2018), which help transport CFTR to the cell membrane in addition to keeping it open, expanded treatment possibilities to target the more common mutations. The triple modulator regimen Kaftrio (ivacaftor/tezacaftor/elexacaftor) was approved by the FDA and EMA in 2019. It was approved for prescription in Sweden in 2022 after extensive disputes between the Swedish Dental and Pharmaceutical Benefits Agency and clinicians and patient organisations due to the high cost of the treatment.

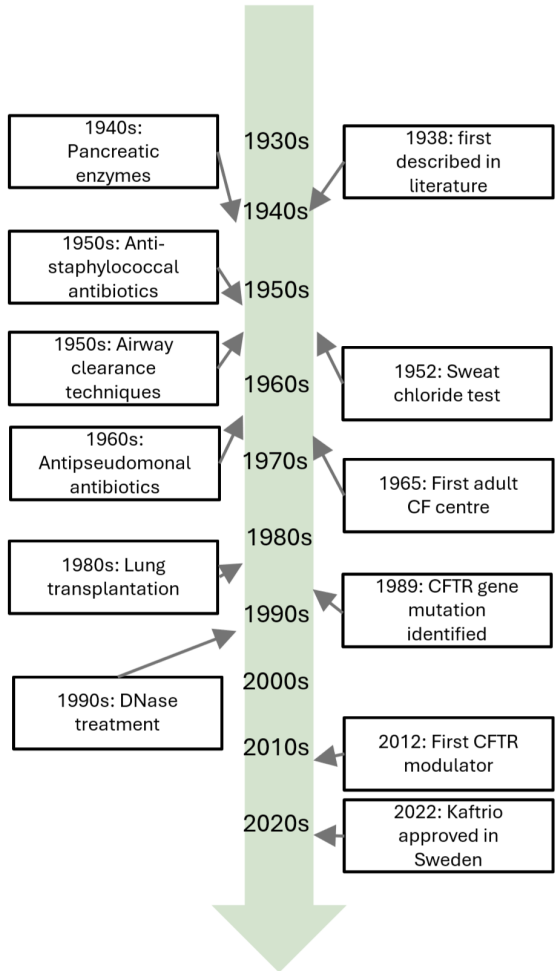

Figure 1: Developments in CF diagnosis and care over time.

With the recent advent of modulator therapies, the current state of CF care is at a point of rapid change and adaptation. While the available CFTR modulators are not curative for patients with previously sustained organ damage, and not effective in e.g. persons with certain rare mutations or those choosing to discontinue treatment due to side effects, the possibility of preventing disease progression in children and increasing the quality of life for adults is making the future of CF care look more hopeful than ever.

Genetics

Cystic fibrosis is one of the most common genetic disorders among people of European descent, with about 1 in 25 people in this group being an unaffected carrier. Around 1 in 2500-5000 persons of European heritage is born with CF [15]. It is less common in other ethnic groups, affecting for example 1 in 15 000 African-Americans and 1:35 000 Asian-Americans [16]. In Sweden, around 20 children per year are born with CF [17].

Although cystic fibrosis is always caused by a recessively hereditary CFTR mutation, different mutations in the *CFTR* gene give rise to varying protein defects. The most common mutation (ΔF508) accounts for 70% of all CF cases [18], but the resulting disease severity may still vary greatly from person to person. Reasons for this include exposure to different microbial pathogens, socioeconomic factors such as the availability of timely diagnosis and treatment, compliance with treatment, and the presence of comorbid conditions.

CFTR mutations can be divided into six classes with various outcomes on CF severity. In class I mutations, the *CFTR* gene contains premature termination or stop codons which cause a near-complete absence of CFTR protein which cannot be corrected with current modulators. These mutations is found in about 10% of CF patients [19]. Class I mutations are sometimes described as two separate classes: stop-codon mutations and mutations that cause no mRNA to be transcribed ("class VII" or "IA") [20]. Class II mutations are caused by misfolding of the CFTR protein, leading to insufficient trafficking to the cell membrane. ΔF508 is a class II mutation, and can be targeted by CFTR correctors. Class III mutations cause CFTR to be present at the cell membrane but in a closed conformation. CFTR potentiators may target this defect by maintaining the channel in its open state.

Persons with class IV-VI mutations express functional CFTR proteins, but to a lesser extent than persons without CF. These mutations often give rise to less serious symptoms as a limited amount of chloride transport is still possible. Channels affected by class IV mutations have a decreased permeability to ions, resulting in less effective conductance. Class V mutations cause normal CFTR to reach the cell surface but in lower copy numbers. In class VI mutations, the functional CFTR has an abnormal turnover and is degraded too rapidly.

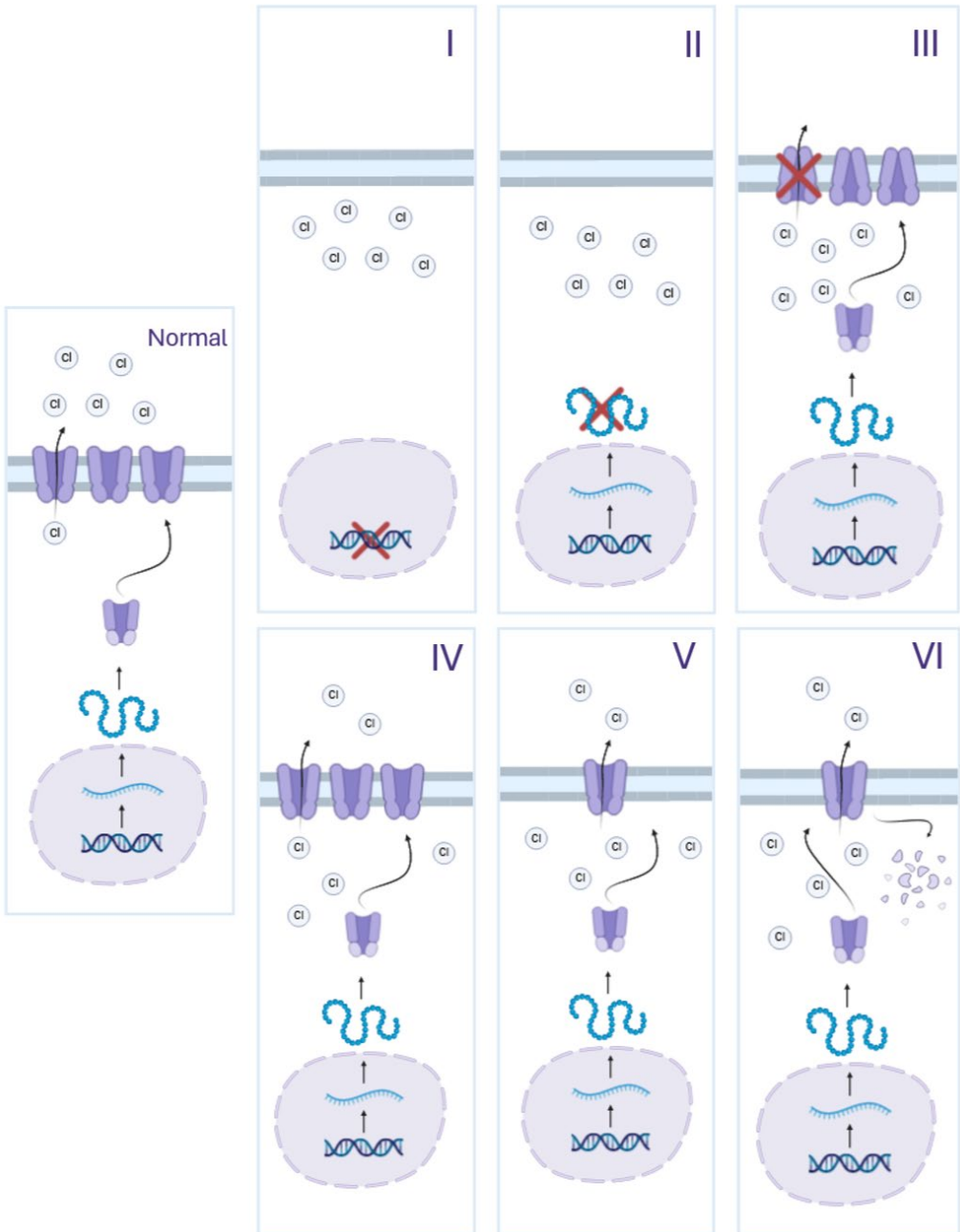

Figure 2: The six classes of CFTR mutations. Lack of CFTR transcription and translation (I), defects in protein folding and subsequent transport to the cell membrane (II), and gating mutations (III) lead to more severe disease. Defects in channel conductance (IV), copy number (V) and stability (VI) can cause less severe CF.

Symptoms and complications

Although all cystic fibrosis is caused by a mutation in one and the same gene, people with CF are a heterogeneous group when it comes to disease severity. This can be partially explained by differences in outcome of the six mutation classes, such as heterozygosity of severe and mild mutations, with some people having a reduced but closer to normal amount of functional CFTR protein. Exposure to microbial pathogens can also vary from person to person or between geographical locations, and has a strong effect on disease severity. Finally, lifestyle factors such as diet, exercise and adherence to treatment protocols influence the overall health outcomes.

Cystic fibrosis airway disease

Cystic fibrosis can manifest in all tissues where CFTR is expressed, but the most strongly affected system is usually the airways, with respiratory failure being the most common cause of death. The disease affects both the upper and lower airways, with nasal obstruction and sinusitis being reported by the majority of pwCF [21]. Briefly, the impaired CFTR function causes an imbalanced electrolyte gradient, leading to thickening of the airway mucus and providing an optimal environment for bacterial growth. Airway damage is then incurred by the persistent microbial colonisation, both by bacterial or fungal factors and by the persistent inflammation caused by immune responses towards the infection. This tissue injury further damages the cilia and the possibility to clear mucus from the airways, leading to permanent airway damage known as bronchiectasis.

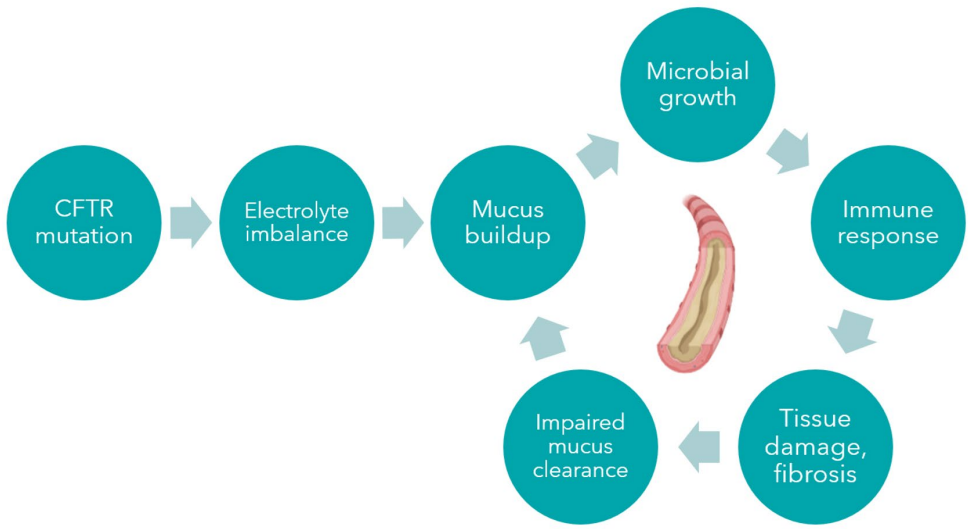

Figure 3: CF lung disease progression illustrated as a 'vicious circle', where lung damage caused by infection leads to further mucus accumulation and microbial growth.

CF lung disease presents as periods of symptomatic stability interspersed with exacerbations, during which symptoms intensify and may cause a need for hospitalisation. The definition of a pulmonary exacerbation varies between treatment centres. It is usually characterised by increased cough, sputum volume and shortness of breath, combined with a decrease in lung function as measured in forced expiratory volume (FEV_1) [22]. The cause of exacerbations is not fully understood, but has been associated with infection status. Possible causes can be the acquisition of new bacterial strains, viral infections, or a change in composition of the existing microbiome, e.g. an increased bacterial load of *Pseudomonas aeruginosa* [23]. The frequency of exacerbations also varies between people with CF. About one third of pwCF experience less than one exacerbation per year, one third 1-2 per year, and one third more than two per year [24]. With CFTR modulator therapy only being available for a few years at the time of writing, the long-term effects of these on the frequency and severity of exacerbations remains to be studied.

Cystic fibrosis of the pancreas

The pancreas has two important functions. The exocrine pancreas produces enzymes necessary for digestion: amylases, proteases and lipases. The endocrine pancreas produces hormones including insulin, glucagon and somatostatin, which are involved in regulating blood sugar levels. In CF, both the endocrine and exocrine pancreas can be affected.

Insufficiency of the exocrine pancreas (PI) affects over 85% of people with CF from birth [25]. The defective chloride transport hinders normal water transport in the epithelial cells of pancreatic ducts, leading to abnormal pancreatic secretions which can cause plugging and fibrosis of the gland [26]. PI needs to be managed through enzyme replacement therapy, in the form of capsules containing pancreatic enzyme extract from pigs.

Despite the extensive damage to the exocrine pancreas, making up 95% of the pancreatic tissue, the endocrine islets are relatively unharmed in most children with CF. Cystic fibrosis-related diabetes (CFRD) affects about 2% of children, 20% of adolescents and 50% of adults with CF [27], although abnormal glucose tolerance is present in a larger amount of persons who may or may not develop CFRD. The pathophysiology of CFRD is not fully understood, and there are two main hypotheses: Either that islet cells are damaged as a result of the fibrosis of surrounding exocrine tissue over time, or that the CFTR mutation may directly impair the insulin secretion of beta cells [28] and the glucagon secretion of alpha cells [29]. In addition to the additional treatment burden of requiring insulin therapy, CFRD also strongly correlates to poor lung function and increased mortality [30].

Other manifestations of CF

The increased viscosity of mucus affects people with CF on a systemic level, and can in addition to the symptoms described above also cause intestinal obstructions. One of the earliest signs of CF is meconium ileus, in which the viscosity of bowel movements makes them unable to pass through the infant's small intestine. Although it only affects 20% of infants with CF, 90% of all cases of are caused by CF, making the symptom a strong indicator towards further screening [31]. Later in life, intestinal complications can persist in the form of constipation, steatorrhea or distal intestinal obstruction syndrome (DIOS) [32].

Most men with cystic fibrosis are infertile due a congenital absence of the vas deferens. In these cases, biological paternity is only possible by using epididymal sperm aspiration and in vitro fertilisation. Women with CF may have reduced fertility due to abnormalities of the cervical mucus, but are largely able to become pregnant naturally [33].

The physical symptoms of CF as well as the rigorous treatment requirements can have a strong impact on mental health in both pwCF and their families. The prevalence of anxiety and depression is about 3 times higher among pwCF than in the general population, and the 2-4 hours a day required for management can lead to feelings of social isolation [34]. Although the availability of CFTR modulation may help reduce the psychological burden of living with CF, some people report choosing to cease the treatment due to adverse effects related to disordered sleep or increased anxiety [35].

The current standard of CF care

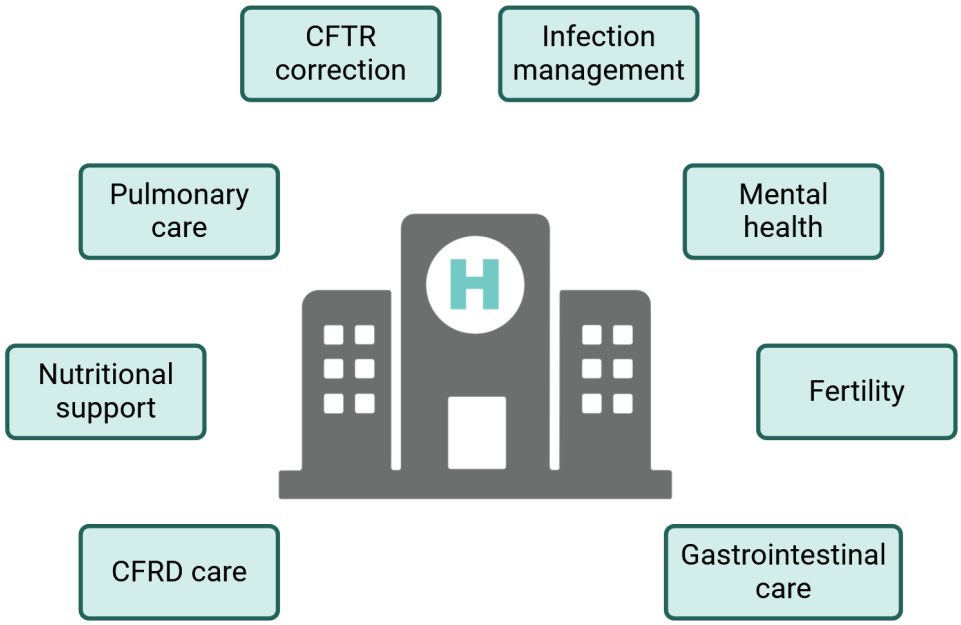

Figure 4: Healthcare aspects which may be applicable to people living with cystic fibrosis.

Due to the systemic nature of the disease, providing healthcare for people with cystic fibrosis generally requires a specialist CF team comprised of multiple specialties. In addition to CF doctors, nurses, and physiotherapists, patients may need to visit a dietitian, psychologist, mental health therapist, endocrinologist, otolaryngologist, and/or gastroenterologist. In Sweden, specialist CF centres are available in Stockholm, Uppsala, Gothenburg and Lund. Although the introduction of CFTR modulator therapies has drastically reduced the healthcare needs for eligible patients, the need for CF teams remains to follow up progression since modulation is not a curative treatment, as well as for those who do not tolerate or respond to modulators.

Screening and diagnosis

Cystic fibrosis in children is normally detected by symptoms such as meconium ileus at birth, diarrhoea, frequent coughing or lung infections, low weight gain or salty-tasting skin. Newborn screening can be performed by testing the level of immunoreactive trypsinogen (IRT) in blood, which is a marker of pancreatic function. In Sweden, there is no screening programme for asymptomatic newborns [36], which has come under debate due to the possibility of starting modulator treatment earlier if detected [37].

If CF is suspected, a sweat test is performed by measuring the chloride concentration in sweat using electrodes attached to the arm. In addition to this test, CF can be confirmed by genetic testing to identify the specific CFTR mutations.

Pulmonary care

Methods to lower the amount of mucus in the airways are a cornerstone of CF treatment. Physiotherapeutic methods such as postural draining, percussion and coughing techniques are used to loosen and evacuate sputum – sometimes for as much as several hours per day [17], which can be a significant burden to quality of life. These methods are often supplemented with inhalation therapies such as hypertonic saline, dornase alpha (a DNase aimed at cleaving viscous DNA released by neutrophils), or salbutamol (a β2-adrenergic bronchodilator) aimed at reducing sputum and bronchiectasis. During regular healthcare visits, lung health is continuously assessed by auscultation and spirometry. The volume of air exhaled during the first second of a maximal exhalation (forced expiratory volume, FEV_1) is an important parameter for monitoring lung function over time.

In the case of end-stage lung disease which has not been responsive to treatment, lung transplantation may become necessary. In this case, patients will require life-long immunosuppression therapy in order to combat organ rejection.

CFTR correction

Correcting the defective CFTR in CF can work in one of two ways: By improving cellular trafficking of CFTR from the cytosol to the cell membrane (correctors), or by keeping the channel from closing prematurely (potentiators). As of 2024, there are four CFTR-modulating therapies available in the European Union: Kaftrio (elexacaftor/tezacaftor/ivacaftor), Symkevi (tezacaftor/ivacaftor and ivacaftor), Orkambi (lumacaftor/ivacaftor), and Kalydeco (ivacaftor) [38]. Out of these combination therapies, elexacaftor, tezacaftor and lumacaftor are correctors while ivacaftor is a potentiator. Kaftrio is the most recent and is applicable for the broadest spectrum of CFTR mutations, making over 90% of people with CF eligible for this treatment [39, 40]. After its approval by the European Medicines Agency in 2019, a long debate took place between representatives of the CF community and The Dental and Pharmaceutical Benefits Agency of Sweden (TLV), with the latter arguing that the treatment was too expensive to be covered by subsidisation. This rejection was finally overturned in December 2022. Although CFTR modulation is not a cure for CF, and damage to e.g. the lungs or pancreas acquired prior to treatment will persist, many patients report a greatly improved quality of life on Kaftrio due to the reduced sputum production. Even though airway pathogens may persist through modulator treatment [41], the need for hospitalisations and intravenous antibiotics is greatly reduced [42]. Kaftrio can currently be prescribed from age 2 and up, with the hope that preventing lung damage early may enable people with CF to reach a normal life expectancy.

Infection management

As airway infections are a major cause of disease progression in CF, treating them appropriately is of central importance. Antibiotics used to treat bacterial infections (chapter 2.4: CF pathogens) may be administered in oral, inhaled, or intravenous formulations, with IV antibiotics being used primarily during exacerbations. Due to the establishment of biofilms in the CF airway, antibiotic treatment is in many cases insufficient to eradicate the infection [43, 44]. In these cases, antibiotics are used to reduce the bacterial load in order to reduce symptoms. Persistent infections are followed up during regular visits to the CF clinic, either by culturing of sputum samples or by throat swabbing if sputum cannot be produced.

Although primarily used as an antibiotic inhibiting bacterial ribosomes, the macrolide azithromycin can be used in CF for its anti-inflammatory properties. Treatment with azithromycin has been shown to reduce leukocyte migration to the lung, decrease proinflammatory cytokine secretion, and polarise macrophages towards the anti-inflammatory M2 phenotype [45, 46].

Vaccination against airway infections is especially important for people with CF. The Swedish vaccination programme for children covers 12 infections, including the airway pathogens *Bordetella pertussis* and *Streptococcus pneumoniae* [47]. In addition to these, it is highly recommended to vaccinate annually against influenza.

Nutritional support and gastrointestinal care

Due to the effects of CFRD mutation on the exocrine pancreas, most people with CF need to supplement pancreatic enzymes at every meal in order to digest food. The diet is often supplemented with CF-specific vitamins, i.e. the fat-soluble vitamins A, D, E and K [48]. Due to the viscosity of mucus in the intestines, faecal obstruction (distal intestinal obstruction syndrome; DIOS) may occur in about 10-20% of people with CF [49]. The first line of treatment is to use laxatives to relieve constipation, by increasing water retention in the bowel (osmotic laxatives) or increase motility (stimulant laxatives). If unsuccessful, surgical intervention may be required. The effect of modulator therapy on exocrine pancreatic function and gastrointestinal symptoms is inconclusive at the time of writing [50], but treatment appears to improve pancreatic function in some persons [51].

CFRD care

Cystic fibrosis-related diabetes (CFRD) of the endocrine pancreas normally appears later in life, and is screened for through oral glucose tolerance testing. The condition develops over time, with asymptomatic but impaired glucose tolerance preceding the development of CFRD. CFRD occurs in about 45% of pwCF over 30 years old [52]. The only recommended treatment for CFRD is insulin therapy. As the aetiology differs from both type 1 and 2 diabetes mellitus, other diabetes medications such as metformin are ineffective [53].

Fertility

The effect of CF on reproductive health varies greatly between men and women. Out of men with CF, over 95% are affected by infertility due to congenital absence of the vas deferens. As the cause of infertility is an obstruction and not due to defects in sperm quality, sperm extraction followed by in vitro fertilisation shows a similar success rate as in people without CF [54]. Women with CF are more likely to have decreased fertility than the general population [55] due to CFTR effects on the cervical mucus, but are often able to conceive naturally or with the help of intrauterine insemination or in vitro fertilisation [56]. The success of pregnancies in women with CF is dependent on the person's overall health, and pre-pregnancy consultation is recommended to improve the outcomes for mother and child. Partners or relatives of people with CF may choose to undergo genetic screening, as two copies of *CFTR* mutation is required to develop cystic fibrosis. A carrier couple may then choose preimplantation genetic diagnostics to select an embryo without CF-causing mutations [57].

Pathophysiology of the CF airway

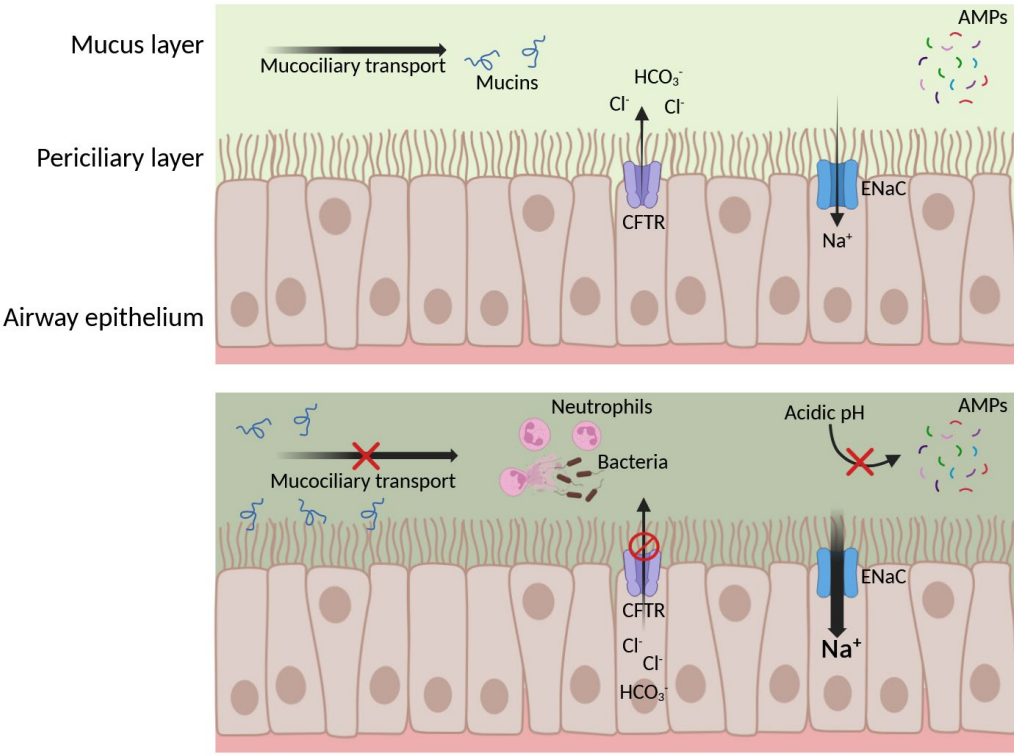

Figure 5: Overview of the effects of CFTR mutation on the airway epithelium. Compared to the healthy airway epithelium (above), the CF airway (below) is characterised by defects in chloride and bicarbonate transport by CFTR, increased sodium transport by ENaC, acidic pH, impaired mucociliary transport, bacterial infections, and neutrophilic inflammation.

CFTR is a transmembrane channel protein belonging to the ATP-binding cassette (ABC) transporter superfamily, and is the only known ABC transporter functioning as an ion channel [58]. As an active transporter, it requires ATP to transport anions across the cell membrane. Though its main function is as a chloride (Cl^-) channel, it also transports bicarbonate (HCO_3^-) to a lesser extent. CFTR plays an integral role in fluid homeostasis of epithelial cells, but the defect has also been implied to affect the function of other cell types, including immune cells, platelets, and endothelium [59, 60].

In the lung, the impairment in Cl^- secretion causes depletion of the airway surface liquid (ASL). The ASL is a thin layer of fluid and mucus that plays a role in early microbial defence, trapping pathogens and disposing them via mucociliary clearance. In CF, the lack of functional CFTR causes an acidification of the ASL due to the loss of HCO_3^- transport. This in turn causes an upregulation of epithelial Na^+ channel (ENaC), leading to increased flow of Na+ from the ASL into epithelial cells, altered pH and dehydration of the mucus layer.

The exact pathophysiology of the CF lung disease has been under debate. Although it is clear that CF airway mucus is abnormally viscous and prone to bacterial colonisation, the primary cause is not fully agreed upon [61, 62]. The high-salt hypothesis suggests that aberrant transport of Cl^- or HCO_3^- affects its osmolarity, and in turn the disrupted pH of the ASL impairs the function of other proteins such as antimicrobial peptides and mucins. The low-volume hypothesis instead proposes that the increased reabsorption of Na^+ due to lack of ENaC inhibition leads to a lowered ASL volume, which with its high viscosity can physically inhibit the normal movement of the cilia. Regardless of the exact mechanism, the central pathology of the CF airway eventually arises from the thickened mucus which leads to infection sensitivity and persistent inflammation.

CF can present very heterogeneously even among people with the same mutation. Although CF is a monogenic disease, its severity may be affected by polymorphisms in non-CFTR ('modifier') genes [63]. These include SNPs in other ion channels such as SLC9A4, a Na^+/H^+ exchanger correlated to meconium ileus and earlier acquisition of *P. aeruginosa* infection, and SLC26A9, a Cl^-/HCO_3^- transporter; correlated with CFRD. Polymorphisms in genes of other inflammatory mediators such as cytokines and TLRs may also play a role in the severity of CF.

Inflammation in CF

Persistent inflammation caused by immune responses against microbial pathogens is a major cause of airway damage in CF. In addition to this central concept, an increasing amount of evidence suggests that CFTR mutations can cause dysregulation of immune pathways even in the absence of infection. This can be observed in *CFTR*-knockout animal models, which develop progressive lung disease even in pathogen-free conditions [64]. Studies of bronchoalveolar lavage fluid from infants with CF have shown that although inflammatory markers are increased in infants not infected with CF pathogens, it could not be ruled out that bacteria were present below detectable limits [65]. NF-κB, a transcription factor complex present in most cells, is elevated in CF and mediates a chronic inflammatory state by e.g. increasing levels of IL-8, a potent neutrophil chemoattractant [66]. The anti-inflammatory cytokine IL-10, which can inhibit the activity of NF-κB, has also been found to be deficient in CFTR-deficient mouse models [67]. However, people with CF are not more susceptible to infection at other sites, and so the airway mucus appears to be the determining factor [68].

Neutrophils are the predominant cause of inflammation in the CF airway, making up the majority of cell content in sputum. CFTR mutation affects neutrophil function in multiple ways [69, 70]. The altered chloride concentrations lead to impaired production of reactive oxygen species, and subsequently a lower capacity to degrade microbes in the phagolysosome. The abnormal cellular pH can also cause excessive degranulation of elastase and a favouring of NETosis over apoptosis during cell death. This leads to a proinflammatory phenotype which e.g. causes a more severe lung inflammation upon LPS challenge [71] than wild-type neutrophils.

Despite the hyperactivated proinflammatory state of CF leucocytes, pathogen clearance is impaired due to pH imbalances in the phagolysosome. This defect also impairs the capacity of efferocytosis, i.e. the clearance of apoptotic cells by phagocytes [72].

In addition to neutrophils, the effects of CFTR dysfunction can also be observed in other cell types involved in immune responses: e.g. macrophages, lymphocytes, vascular endothelial cells, and platelets.

Alveolar macrophages are the primary immune cells of the healthy lung. They maintain the alveolar environment by clearance of apoptotic cells and inhaled debris, and play an important role in responding to airway pathogens by phagocytosis or secretion of cytokines, chemokines and reactive oxygen species. As in neutrophils, the defective CFTR ion transport leads to impaired phagocytic function, which can allow bacteria to survive intracellularly [73]. Exposure to bacterial lipopolysaccharide (LPS) causes abnormally prolonged proinflammatory responses in CF macrophages, leading to overproduction of cytokines such as the neutrophil chemoattractant IL-8 [74, 75].

CFTR defects in lymphocytes can cause a skew from Th1 towards Th2 responses [76], which can be increased further by *P. aeruginosa* infection and correlates with a worse lung function [77]. The role of CFTR in endothelial cells is not fully understood, but studies have described increased vascular permeability [78] and circulating endothelial adhesion molecules [79] in CF endothelium, furthering the process of neutrophil infiltration to the lung environment.

Platelets are a cell type with a main function of responding to vascular injury by forming thrombi, but they also play an important role in the immune system [80]. The cells respond to infection by secreting chemokines such as CXCL4, which can bind to bacteria after which neutrophils are recruited and activated [81]. A lack of CFTR in platelets causes a reduction in lipoxin A_4 [82], an anti-inflammatory lipid involved in the resolution phase of inflammation, and increased inflammatory responses to *P. aeruginosa* or LPS [59].

Finally, it has been implied that the mechanisms contributing to the resolution of inflammation are impaired in CF, resulting in prolonged inflammatory responses [83]. Specialised proresolving mediators (SPMs) are lipids which function to resolve inflammation by halting neutrophil infiltration and proinflammatory signals, and promote tissue regeneration [84]. Defects in SPM-mediated inflammatory resolution has been implicated in CF, such lower lipoxin concentrations in the airways [85, 86].

The CF airway microbiome

Introduction to bacteria

There are five main types of infectious agents: Bacteria, viruses, fungi, parasites (including amoebas, worms, and ticks), and prions (misfolded proteins which can spread its misfolding to normal proteins, such as in mad cow disease). Out of these, bacteria are the main cause of concern in CF, although viral and fungal infections are also able to cause airway inflammation [2].

Bacteria range in size from approximately 0.1 to 10 μM in length, with rod-shaped bacteria such as *Escherischia coli* and *Pseudomonas aeruginosa* measuring about 1.5-3 μM. In contrast, red blood cells measure about 7 μM and bronchial epithelial cells around 10 μM [87]. Along with archaea, bacteria are prokaryotic cells, which when compared to eukaryotic cells are characterised by their lack of a nucleus and membrane-bound organelles such as mitochondria, the endoplasmic reticulum, lysosomes, or chloroplasts. Their genomes are also simpler than those of eukaryotes, normally containing a single cyclical chromosome which lacks the histones used for coiling the chromosome structure [88]. Bacteria can have varying shapes and structures, and the only structures common to all bacteria are the **cytoplasm, ribosomes, chromosome,** and **plasma membrane**.

Most bacteria have a **cell wall**, which surrounds the plasma membrane and differs in structure between gram-positive and gram-negative cells. Gram-positive bacteria are defined by their thick peptidoglycan layer surrounding the plasma membrane, which cause them to retain a purple colour when stained with crystal violet. Gram-negative bacteria are instead surrounded by a second outer membrane. The outer membrane contains **lipopolysaccharides** (LPS). LPS is a molecule comprised of three parts: Lipid A, a toxin released from the membrane upon lysis of the cell, the core polysaccharide providing structural stability, and the O-antigen, a variable polysaccharide which can be recognised by host antibodies [89]. The space between the two membranes is called the **periplasm**, and contains a thinner peptidoglycan cell wall than in gram positives. This space contains proteins including virulence factors, such as secreted toxins. The double membrane also supports gram negatives by providing a compartmentalised space for protein folding, oxidation, and secretion [90], and allow the assembly of complex secretion systems spanning both membranes [91].

Other outer membrane proteins of gram negatives include the **porins**, which allow passive transport of hydrophilic compounds into the periplasmic space. As this includes hydrophilic antibiotics, the amount and type of porins on the outer membrane along with the presence of active efflux pumps affect the antibiotic sensitivity of gram negatives [92].

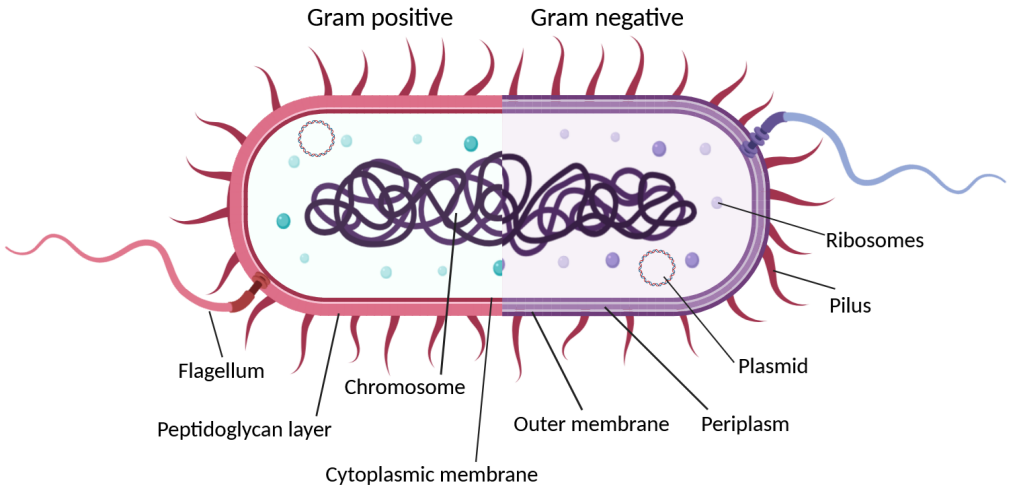

Figure 6: Bacterial structures in gram-positive and gram-negative cells.

Some bacteria employ additional protective mechanisms for survival. By secreting an external layer of polysaccharides or polypeptides – the **glycocalyx** – bacteria are able to evade host defences by hiding pathogen-associated molecular patterns (PAMPs) from recognition by immune cells, adsorb antimicrobial peptides (AMPs) and antibiotics, and adhere to surfaces to form biofilms [93]. A glycocalyx firmly attached to the cell wall is called a **capsule**, while an unorganised glycocalyx layer around the cell is called a **slime layer**.

Bacteria may use **flagella** for movement, which are comprised of an extracellular filament mainly consisting of the protein flagellin, attached to the cellular membrane by a flagellar hook complex. This structure can occur in both gram positives and gram negatives, and can be present on the cell surface in different amounts and arrangements [88]. Bacteria may also move by using **pili**, a smaller filamentous appendage consisting of pilin proteins. By extending and then retracting the pilus structure after contact with a surface, bacteria are able to use a mode of movement called twitching motility [94].

Pili can be divided into **fimbriae** and **conjugative pili**. Fimbriae may be distributed around the entire cell, and are involved in attachment and adhesion to surfaces. Conjugative pili are used in bacterial conjugation, i.e. the transfer of DNA such as plasmids between cells [95].

Plasmids are circular, double-stranded DNA molecules most commonly found in bacteria. As they are not part of the chromosome, they are able to replicate independently of chromosomal DNA. They can be transferred between cells using conjugation, and often contain genes responsible for providing an advantage to the bacteria, such as antibiotic resistances [96], but which are not necessary for survival under normal conditions.

In order to survive in low-nutrient conditions, some bacteria may accumulate proteins, lipids or polysaccharides in **inclusions**, which are densely aggregated structures which are stored as reserves during times of good nutrient availability [97].

In addition to their shape or cell wall structure, bacteria can also be classified based on their tolerance to oxygen, which affects their mode of growth and which metabolic pathways are used to generate energy in the form of ATP [88]. In order to utilise oxygen in aerobic respiration, the resulting reactive oxygen species need to be broken down using enzymes such as catalase or superoxide dismutase to prevent cell damage. Bacteria that use oxygen in respiration and have these enzymes are either obligate aerobes, which require oxygen for growth, or facultative anaerobes, which are also able to grow in conditions without oxygen by using anaerobic respiration or fermentation. On the other hand, bacteria without capabilities to neutralise ROS are not able to grow in the presence of oxygen, and are called obligate anaerobes. A limited amount of neutralising enzymes are present in aerotolerant anaerobes, which do not use oxygen in respiration but are able to grow in its presence. Finally, microaerophiles require oxygen for growth but only in low concentrations.

Host-pathogen interactions

Strategies of the immune system: Host defence mechanisms

The immune system is generally divided into three parts, playing roles at different stages of an infection. The first line of defence is the physical barriers, which simply refers to blocking a pathogen from entry through our skin, mucous membranes, and secretions such as the low-pH gastric juice or enzyme-rich saliva. The defences provided by immune cells are then divided into two parts: The innate and adaptive immune systems. The innate immune system includes e.g. the neutrophils and macrophages, which are able to rapidly recognise an infection using pattern recognition receptors. These patterns are not antigens against any specific pathogen, but rather general bacterial targets such as DNA, LPS or peptidoglycans [98]. The adaptive immune system becomes active several days after the initial infection, as these cells require activation by antigen-presenting cells. After antigen presentation, these cells then mount a defence specific to the infecting pathogen, which can be activated rapidly on re-exposure. These responses include the production of antibodies by B cells, and the killing of intracellularly infected human cells by cytotoxic T cells [99].

Neutrophils are the main drivers of inflammation in the CF lung [100]. They have four main functions: Phagocytosis of pathogens, secretion of cytokines, degranulation of antimicrobial proteins and reactive oxygen species, and NETosis. During NETosis, neutrophils release their DNA as neutrophil extracellular traps (NETs) during cell death in order to trap bacteria. The bacteria can then be killed due to antimicrobial proteases such as elastase in the NETs [101]. Another important cell type in CF inflammation is the macrophage, particularly the alveolar macrophages which are located in the airway lumen [102]. Macrophages are phagocytes, and are also able to produce macrophage extracellular traps (METs) [103]. They also serve an important function as antigen-presenting cells capable of activating T cells of the adaptive immune system.

Strategies of bacteria: Immune evasion and counterattack

In order to survive the initial stage of establishing an infection, bacteria employ a multitude of survival mechanisms. Virulence mechanisms employed by different bacteria include adhering to host cells [104], breaking down NETs through DNases [105], inhibiting opsonisation through inducing ineffective antibodies [106] or proteolysis of IgG [107], surviving phagocytosis [108], degrading cytokines [109], or intrinsic resistances to antibiotics and antimicrobial peptides [110], among others.

However, the most important mechanism used by bacteria to avoid host defences and establish a chronic infection in the CF airway is the production of **biofilms** [111].

These are microbial communities where bacteria gain an advantage by growing together, encased in a protective extracellular polymeric substance (EPS) of polysaccharides, lipids, proteins, and DNA [88]. The establishment of biofilms starts when bacteria adhere to a surface, such as a mucosal epithelial layer or an implanted medical device. This reversible attachment by pili or flagella is followed by irreversible attachment, where bacteria produce adhesins to strongly latch onto the surface and start forming a microcolony. As the biofilm matures, bacteria within the colony undergo phenotypic shifts, adapting to life in a multicellular colony by utilising cell-to-cell signalling (quorum sensing) to coordinate gene expression. Through this coordination, bacteria closer to the attachment surface can adapt to the lower availability of nutrients by slowing down metabolism.

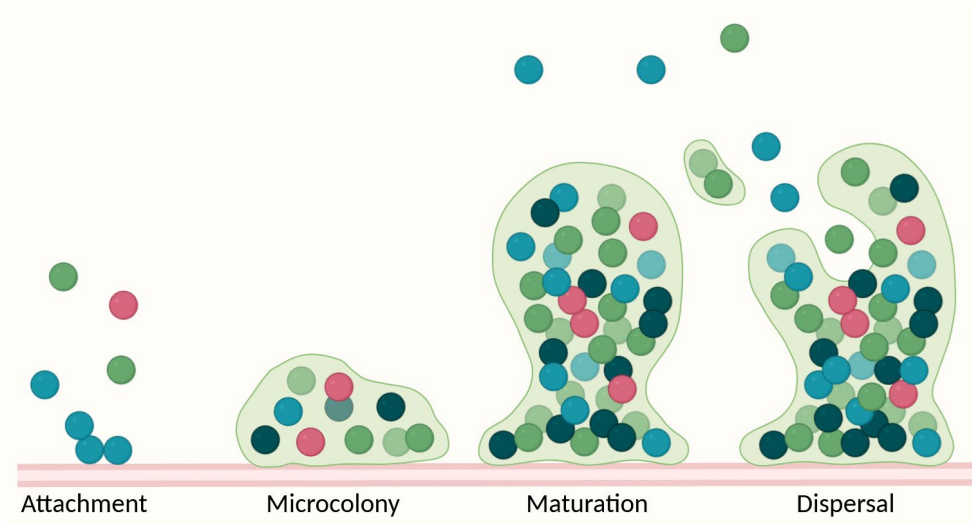

Figure 7: The stages of biofilm formation.

One of the main advantages of biofilm growth is the physical barrier function. By encasing the colony within EPS, bacteria decrease the risk of PAMPs or antigens being detected by host defences. The matrix also provides a protective effect against NETs [112]. Quorum sensing within the colony allows for communication to upregulate virulence factors in response to external factors, such as toxins targeted against neutrophils [112], or provide a survival advantage by downregulating metabolic activity in low-nutrient conditions. EPS restricts the penetration of antibiotics but is not inherently sufficient as protection [113], and quorum sensing appears to be the main mechanism with which biofilms are more resistant to antibiotics by upregulating e.g. efflux pumps [113, 114]. Reduced metabolic activity near the surface provides additional protection against antibiotics targeting bacterial metabolic processes [115].

If dispersed, planktonic bacteria originating from a biofilm do not retain the increased antibiotic resistance [116], presenting an interesting target for therapeutic development [117].

The biofilm mode of growth is used by multiple CF pathogens such as *P. aeruginosa* [118], *Staphylococcus aureus* [119] and nontypeable *Haemophilus influenzae* [120]. By growing in multi-species biofilms, bacteria can gain further advantages such as increased antibiotic resistance or synergistic metabolism, where one species utilises metabolites from another [121]. In CF, biofilms are not necessarily attached to a surface, and *P. aeruginosa* aggregate structures resembling biofilms have been identified [122]. As the presence of bacterial biofilms can trigger NETosis, but are concurrently protected from its effects, persistent biofilm growth in the CF lung gives rise to chronic neutrophilic inflammation [112]. As these infections become chronic, in-host evolution further favours biofilm-forming genotypes with strong capabilities of producing EPS components and lower metabolic activity and motility [123].

Polymicrobial interactions

A bacterial pathogen must survive many obstacles in order to colonise the human body. Not only do they need to evade host defences such as immune cells and antimicrobial proteins, they will also find themselves in the presence of other microorganisms. Most areas of the body which are in contact with the outside world, such as the skin, airways, genitalia, and gastrointestinal tract, contain a normal microbiota of non-pathogenic bacteria. These commensal bacteria live in symbiosis with their human host, protecting from pathogenic microorganisms by competing for the available attachment surfaces and nutrients, or even by secreting antimicrobial toxins called bacteriocins [124]. Some components of the normal microbiome can become pathogenic if the composition is altered, such as bacterial vaginosis caused by the replacement of *Lactobacillus* by other species [125]. Some microbes are pathogenic if transferred from their normal site, e.g. *S. aureus* carriage on the skin causing infections when entering a wound [126], or intestinal *E. coli* causing urinary tract infections [127].

Interactions between microorganisms can either positive or negative. In a cooperative relationship, bacteria can benefit each other by sharing nutrients, for example if one species can utilise the secondary metabolites of another [128]. Cooperation can also occur in multi-species biofilms, where cells can use quorum sensing signals, horizontal gene transfer and nutrient sharing to promote the survival of the entire colony [129, 130]. Bacteria are also able to use competitive mechanisms to suppress other microorganisms.

These interactions can be direct or indirect. An example of indirect inhibition is the alteration of pH by producing e.g. lactic acid, creating an unfavourable environment for other bacteria. Active inhibition includes the secretion of toxins, which can even be injected into competitor bacteria via a type VI secretion system [131]. Toxins can also be used to disrupt biofilms of competing microorganisms [129].

The interactions between the CF pathogens *P. aeruginosa* and *S. aureus* are well-studied, with the former being able to employ various antagonistic strategies in order to suppress its competitor. However, *P. aeruginosa* can either coexist or compete with *S. aureus* depending on length of infection or growth conditions, with e.g. low-iron conditions causing more antagonistic behaviours [132]. Inter-strain variation also affects the behaviour of *P. aeruginosa*. For example, some strains can detect peptidoglycans in gram-positive cell walls and respond by producing toxins such as elastase, pyocyanin and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) [133]. HQNO is able to kill *S. aureus* as an inhibitor of aerobic respiration. However, a low concentration of HQNO is insufficient to kill the bacteria, and instead exerts pressure towards anaerobic metabolism and the formation of small-colony variants [134]. These have a slower growth, but are also more resistant to antibiotics due to the shift in metabolism and favouring of biofilm growth [135]. Another mechanism of competition is the triggering of phospholipase A2 secretion from airway epithelial cells, which is toxic towards *S. aureus* but not *P. aeruginosa* [136].

As *P. aeruginosa* adapts towards the biofilm mode of chronic infection, virulence towards *S. aureus* is reduced [137]. The production of alginate, a major component of EPS in *P. aeruginosa* biofilms, promotes the survival of *S. aureus* [138], and mixed biofilms have a higher resistance against antibiotics [139]. On a cohort level, *P. aeruginosa* replaces *S. aureus* as the most common infection adults compared to children with CF [140]. However, co-infections are able to persist in individuals infected with both pathogens [141]. These co-infections correlate with a worse clinical outcome than *S. aureus* alone, but some studies have shown outcomes of co-infections as similar to *P. aeruginosa* mono-infections [142] while others have observed an additive effect [143, 144]. A suggested explanation for this discrepancy is whether the infections were chronic or sporadic, as some have even described chronic co-infections to be associated with less severe outcomes – perhaps due to coexistence being a trait of less virulent strains [145].

Cystic fibrosis pathogens

The composition of the microbiome in the CF lung is strongly linked to overall health. During early life, a greater diversity of bacteria is normally present in the airways, and infections with *S. aureus* or *H. influenzae* are common. In adults with more progressed CF airway disease, infections with *P. aeruginosa* and a loss of microbial diversity are associated with a poorer prognosis [146-148].

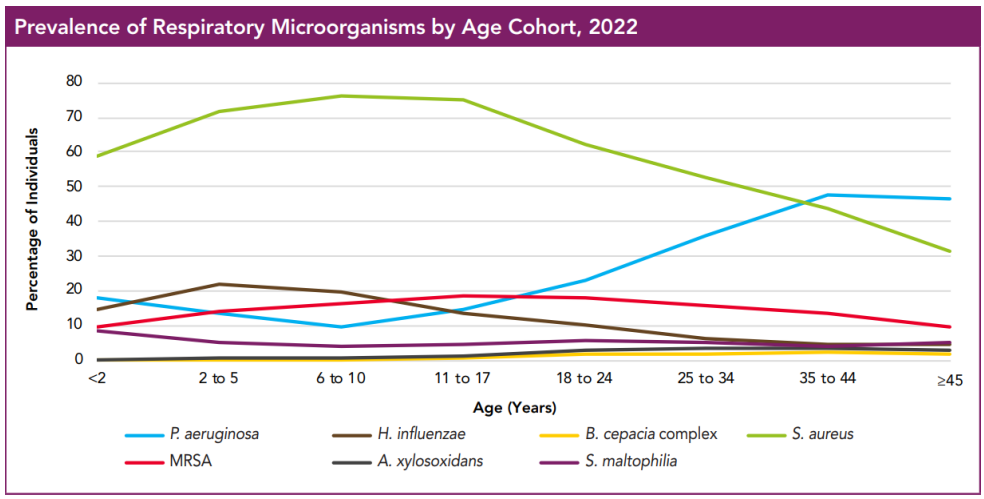

Figure 8: Prevalence of CF pathogens. Source: Cystic fibrosis patients under care at CF Foundation-accredited care centres in the United States, who consented to have their data entered. Reprinted with permission from the Cystic Fibrosis Foundation, Patient Registry 2022 Annual Data Report. ©2023 Cystic Fibrosis Foundation.

Pseudomonas aeruginosa is the most common bacterial CF pathogen in adults, affecting over 60% of this group [3]. It is one of the most aggressive CF pathogens, and is capable of dominating the airway microbiome [147]. A strong capacity to form biofilms, combined with antibiotic resistances and antagonistic mechanisms against other bacteria, help it survive both the immune system, antibiotic treatment and the microbiome.

P. aeruginosa can be found in wet environments such as sinks, drains, and freshwater. In most cases, especially in younger children, early-stage infections are caused by *P. aeruginosa* in a non-mucoid phenotype [149], more resembling the phenotype found in nature. In-host adaptations can then cause a conversion to the mucoid phenotype, leading to increased alginate production and improved biofilm formation [150].

Transition to the mucoid phenotype includes downregulating several virulence factors. This includes decreasing the production of flagella leading to a reduced motility [151], and less activation of the type III secretion system used to inject toxins into host cells [152]. Nevertheless, the upregulation of exopolysaccharides such as alginate is favourable to bacterial survival due to the biofilm mode of growth. Although the loss of flagella reduces the availability of PAMPs for detection by neutrophils, the evasion of phagocytosis appears to be dependent on nonmotility rather than flagella [153].

As biofilms have an increased resistance towards immunity and antibiotics, these infections lead to a more rapid and progressive decline in lung function, connected to increased risk of hospitalisation, worse quality of life, and increased mortality [149]. Treatment of chronic mucoid *P. aeruginosa* is rarely capable of eradicating it, but various antibiotics can be used to reduce bacterial load (Table 1). Aggressive antibiotic treatment of intermittent infection is recommended to prevent the establishment of chronic mucoid *P. aeruginosa* infections [154]. *P. aeruginosa* are normally resistant towards beta-lactam antibiotics through the use of beta-lactamases or efflux pumps [155], and further resistances can be spread using horizontal gene transfer [156]. This can be addressed through susceptibility testing, combination therapy with beta-lactamase inhibitors, and other antibiotic classes such as quinolones, aminoglycosides or monobactams, although resistance is possible towards these classes as well [157, 158].

Staphylococcus aureus is the most prevalent pathogen in children with CF, affecting around 80% [140]. It is found as part of the commensal flora of the skin and upper airways in about 30% of people, but can cause wound infections or even pneumonia in healthy people [159]. Its virulence mechanisms include the ability to form biofilms and persistent small-colony variants, superantigen toxins, immune evasion proteins such as inhibitors of immunoglobulins, complement proteins and chemotaxins, and staphylokinase which can cleave IgGs and activate fibrin degradation [160]. *S. aureus* can utilise horizontal gene transfer through bacteriophages integrated into the bacterial genome, encoding virulence factors which spread at bacteriophage release [161], a process which is enhanced by environmental stressors.

S. aureus infections in CF are associated with pulmonary inflammation and decreases in FEV_1, but to a lesser extent than *P. aeruginosa* [162]. However, the outcomes are worse in the case of methicillin-resistant *S. aureus* (**MRSA**). The prevalence of MRSA is increasing and is a cause for concern on a global level [163].

Chronic MRSA infection in CF is associated with accelerated decline in lung function, increased hospitalisations, use of IV antibiotics, and earlier mortality [164, 165]. As MRSA contain Penicillin-Binding Protein 2, they are resistant to most beta-lactam antibiotics and are treated with e.g. glycopeptide or aminoglycoside antibiotics (Table 1).

***Burkholderia cepacia* complex** (Bcc) is an umbrella term encompassing over 20 species of the gram-negative *Burkholderia* genus. Their prevalence in people with CF is around 4% [166]. The most common Bcc infection in people with CF, *Burkholderia multivorans*, is associated with lung function decline at a similar rate as *P. aeruginosa* [167]. However, *Burkholderia cenocepacia* infection carries a higher risk of cepacia syndrome, a rare but severe complication leading to rapid respiratory decline due to necrotising pneumonia and bacteremia. The mortality rate of cepacia syndrome is over 50% [168]. Its high virulence is caused by factors such as extracellular proteases, biofilm formation and quorum sensing, a type III secretion system promoting cellular invasion, siderophores used in iron uptake from the environment, enzymes inactivating reactive oxygen species, and antibiotic resistances caused by efflux pumps and betalactamases [169].

Haemophilus influenzae infections are found in about 10% of children with CF, with a prevalence that goes down with age [166]. Infections are caused by nontypeable *H. influenzae*, which are not prevented by immunisation against *Haemophilus influenzae* type B. As it is often replaced by other infections and is rare in adults, its impact as a CF pathogen has been under debate [170], but has been shown to cause airway inflammation during chronic infections [171].

Emerging pathogens is a term used to describe CF pathogens other than the four previously described 'classical' CF pathogens. Their prevalence varies greatly depending on geographical location, but are generally considered to be increasing. The term is loosely defined, and can encompass species such as ***Stenotrophomonas maltophilia***, ***Achromobacter xylosoxidans,*** **nontuberculous mycobacteria (NTM),** and various **anaerobes** colonising the CF airway [172]. A common trait of this group is the lack of consensus on their role as CF pathogens due to relatively recent identification and a lack of conclusive research on their virulence [43, 173], There are some exceptions – e.g. *Mycobacterium abscessus* is referred to an emerging pathogen due to its recent increase in prevalence, but is well-defined as a pathogen [174].

Fungal infections are frequently present in the respiratory tract of CF patients. The most common species causing infections is the filamentous fungus ***Aspergillus fumigatus*** [175]. *A. fumigatus* is normally present in the environment, and spores can enter the airways through inhaled air [176]. Colonisation is often asymptomatic, but *A. fumigatus* can sometimes cause allergic bronchopulmonary aspergillosis (ABPA). ABPA is difficult to diagnose in people with CF, as many symptoms overlap between ABPA and CF. Episodes of ABPA can lead to decline in pulmonary function and are typically treated with systemic steroids [177].

Although **viruses** do not directly benefit from the CF mucus environment, viral airway infections are still of concern as they are a common cause of pulmonary exacerbations. Viral infections are present in 13~60% of exacerbations, with the most common types being RSV (respiratory syncytial virus), rhinovirus, influenza and parainfluenza [2]. Viral infections could interact with bacterial species, causing alterations in the microbial community composition [178]. Viral infections have been linked to new acquisition of *P. aeruginosa* [179, 180], and may enhance its biofilm formation [181].

Table 1: Antibiotics used in the treatment of CF pathogens. Selection of antibiotic treatment protocols are based on the antibiotic sensitivity of each individual isolate, as the species listed may be resistant to one or more available options, and may require combination therapy of several antibiotics.

Antibiotic class	Mechanism of action	Infection
Penicillins ** In combination with betalactamase inhibitor*	Inhibit cell wall peptidoglycan synthesis (Beta-lactam)	*S. aureus* (MSSA) *P. aeruginosa** *S. maltophilia** *H. influenzae* *A. xylosoxidans**
Cephalosporins	Inhibit cell wall peptidoglycan synthesis (Beta-lactam)	*S. aureus* (MSSA) *P. aeruginosa* *B. cepacia* *S. maltophilia* *H. influenzae* *A. xylosoxidans*
Carbapenems	Inhibit cell wall peptidoglycan synthesis (Beta-lactam)	*S. aureus* (MSSA) *P. aeruginosa* Nontuberculous mycobacteria *B. cepacia* *A. xylosoxidans*
Sulfonamides (Sulfa)	Inhibit dihydropteroate synthase used in folate synthesis.	*S. aureus* (MSSA/MRSA) Nontuberculous mycobacteria *B. cepacia* *S. maltophilia* *A. xylosoxidans*
Tetracyclines	Inhibit protein synthesis by binding tRNA.	*S. aureus* (MSSA/MRSA) Nontuberculous mycobacteria *B. cepacia*
Vancomycin	Inhibition of cell wall peptidoglycan synthesis - Glycopeptide	*S. aureus* (MRSA)
Lincosamides	Inhibit protein synthesis by binding to ribosomes.	*S. aureus* (MRSA)
Oxazolidinone	Inhibit protein synthesis by binding ribosomes.	*S. aureus* (MRSA) Nontuberculous mycobacteria
Aminoglycosides	Inhibit protein synthesis by binding ribosomes.	*P. aeruginosa* Nontuberculous mycobacteria

Macrolides **As antiinflammatory*	Inhibit protein synthesis by binding ribosomes.	*P. aeruginosa** Nontuberculous mycobacteria
Quinolones	Inhibit bacterial DNA replication.	*P. aeruginosa* *S. aureus* (MSSA/MRSA) Nontuberculous mycobacteria
Monobactams	Inhibit cell wall peptidoglycan synthesis – Beta-lactam	*P. aeruginosa*
Colistin	Disrupt the outer membrane in gram negatives	*P. aeruginosa* *A. xylosoxidans*
Rifamycin	Inhibit bacterial RNA polymerase	*S. aureus* (MRSA) Nontuberculous mycobacteria
Clofazimine	Bind to bacterial DNA, blocking transcription and replication	Nontuberculous mycobacteria
Ethambutol	Inhibit arabinosyltransferase used in mycobacterial cell wall synthesis	Nontuberculous mycobacteria

Achromobacter xylosoxidans

Introduction

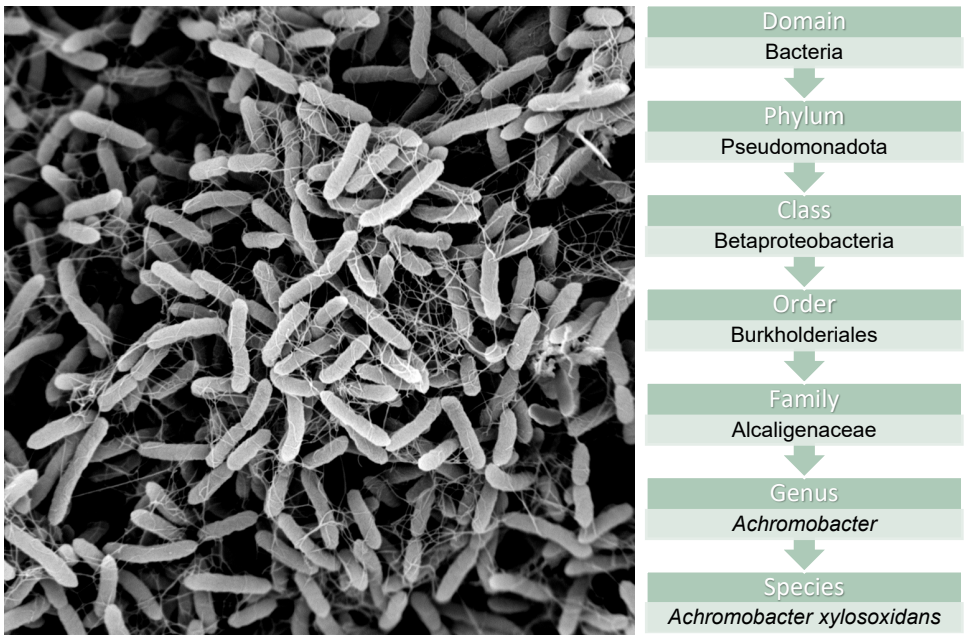

Figure 9: *A. xylosoxidans* imaged at 15000x using scanning electron microscopy (SEM). Image credit: M. Baumgarten 2022.

Achromobacter is a genus of gram-negative, nonfermenting, catalase- and oxidase-positive, rod-shaped bacteria which can be found in wet environments such as soil, freshwater, sinks and drains [182]. The genus is closely related to *Alcaligenes* and *Bordetella,* and contains 22 identified species [183]. Most *Achromobacter* infections in people with CF are caused by *A. xylosoxidans*, *A. ruhlandii, A. insuavis*, and *A. dolens* [184-186]. These infections are rare in immunocompetent persons, but can appear as opportunists in people with e.g. malignancies or immune deficiencies [187]. The largest risk factor of acquiring *Achromobacter* infection is cystic fibrosis.

In people without CF, they may cause pneumonia or bacteraemia, or infections of the ears or skin. The most common *Achromobacter* species to cause infections in humans, *A. xylosoxidans*, was first identified in 1971 by Yabuuchi and Oyama while studying ear infections [188].

Achromobacter spp infections affect around 5-10% of pwCF, out of which 35-70% are *A. xylosoxidans* [186, 189, 190]. The prevalence differs greatly based on geographical location and the definition of infection (sporadic presence of bacteria in a sputum sample vs. chronic colonisation persisting across 6-12 months of sampling, with the diagnosis of chronic colonisation lacking a standardised definition). The second most common species in Europe is *A. insuavis*, while *A. ruhlandii* is more common in the Americas [184, 185, 191].

As *Achromobacter* spp typically appear in persons with poor lung health at the start of the infection, their status as CF pathogens have been debated. Some studies have failed to find a correlation between *Achromobacter* spp and lung function decline [192-194], which has led to the hypothesis that they correlate to poor lung health solely due to opportunistic infection patterns, without being inherently pathogenic. However, other studies have called this viewpoint into question as *Achromobacter* infections have been shown to correlate with decreases in FEV_1 and increases in exacerbations, hospitalisations, IV antibiotic requirements and mortality [195-198]. Another factor implying *Achromobacter* as a CF pathogen is its capacity to, like *P. aeruginosa*, dominate the airway microbiome and sharply reduce its microbial diversity [199]. A study on bacterial community composition in 190 people with CF identified that *Pseudomonas, Staphylococcus, Achromobacter* and *Burkholderia* were often the most abundant genera and associated with low microbial diversity [147].

Recent studies have begun to elucidate the virulence factors of primarily *A. xylosoxidans*, identifying multiple mechanisms which make it capable of causing airway inflammation and tissue damage (Chapter 3.2). However, the understanding of *Achromobacter* pathogenicity remains rather limited, as e.g. the lower prevalence of non-*xylosoxidans* species makes it difficult to study their comparative virulence.

The emergence of *Achromobacter* spp as CF pathogens is contributed to by improvements in identification. Misidentification as *P. aeruginosa* or *B. cepacia* complex has been common due to similar phenotypes such as biochemical activity, antibiotic resistance profiles and colony appearance on agar plates [200]. Species identification by MALDI-TOF mass spectrometry is able to correctly identify *Achromobacter* spp infection, but has until recently been unable to distinguish between species [201]. A more sensitive method to distinguish between species is sequencing, such as PCR of the nrdA gene, multilocus sequence analysis (MLSA), or whole-genome sequencing (WGS) [189, 202]. These methods are not routinely performed in clinical laboratories.

Infections with *A. xylosoxidans* are difficult to treat primarily due to their capacity to form biofilms in the CF airway [203, 204], but also due to their wide range of antibiotic resistances. *Achromobacter* spp are innately resistant to aminoglycosides, tetracyclines, and multiple beta-lactams, and clinical isolates can display even broader antibiotic resistance likely acquired by horizontal gene transfer [205, 206]. Treatment is based on the antibiotic susceptibility of the individual isolate, and there is no standardised treatment protocol between CF centres. Although antibiotic susceptibility varies between isolates, the majority of *Achromobacter* isolates are sensitive to piperacillin–tazobactam (a penicillin/betalactamase inhibitor combination), meropenem and imipenem (carbapenems), and trimethoprim-sulfamethoxazole (folate synthesis inhibitors) [207, 208].

Virulence mechanisms

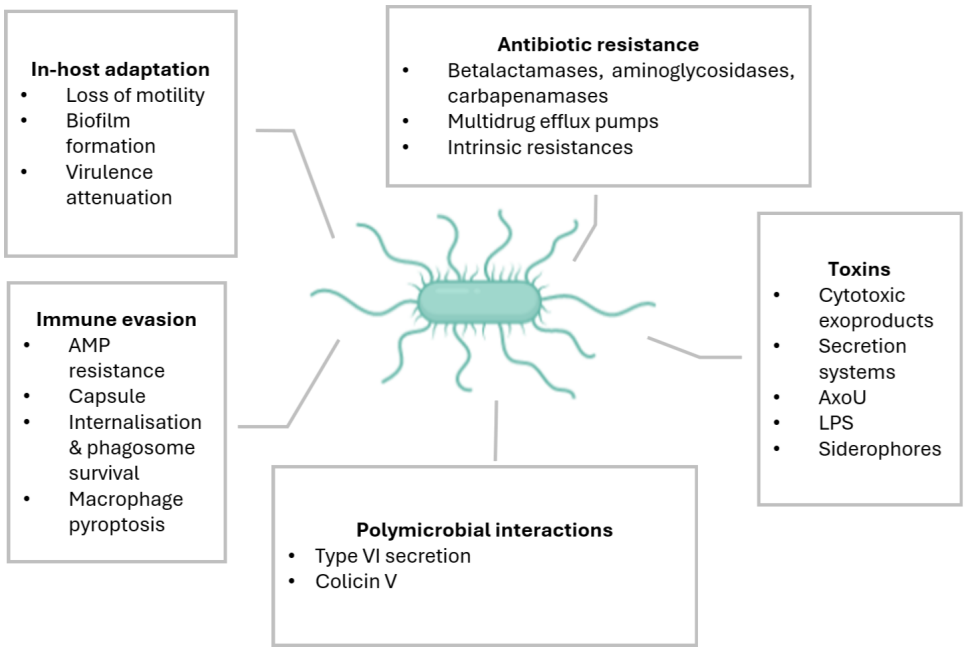

Figure 10: Overview of the known mechanisms of virulence in *A. xylosoxidans*.

As the recognition of *Achromobacter* spp as important CF pathogens is increasing, the field has attracted further interest and the amount of studies on its virulence mechanisms is increasing, particularly in the last five years. This chapter provides a brief summary of the current understanding of *Achromobacter* virulence mechanisms – particularly those of *A. xylosoxidans* – laying the foundation for this thesis.

Morphological features

A. xylosoxidans has been described to possess peritrichous flagella, providing the bacteria with swimming motility. As a gram negative, it has an outer membrane allowing the use of periplasmic transport and secretion systems anchored in both cellular membranes. Genomic analysis of an *A. xylosoxidans* isolate identified secretion systems of type II, III, VI and VII [209]. Type II secretion is primarily involved in exotoxin secretion, while type III and type VI are able to directly inject toxins into other cells through their needle structure. The type VII secretion system is poorly characterised at the time of writing, and has primarily been studied in the virulence of mycobacteria and gram positives [210].

In a comparative study of six *Achromobacter* genomes, including two CF-origin *A. xylosoxidans* and four non-CF *Achromobacter* spp strains, the genomes of CF origin contained unique genes likely responsible for their virulence: the type III secretion system, LPS O-antigen, capsular synthesis, and the siderophore alcaligin. CF isolates also display a higher binding affinity to mucin, collagen and fibronectin than non-CF isolates [211].

Biofilms of *A. xylosoxidans* has been observed in the CF airway [204, 212], although the capacity of biofilm formation varies greatly by isolate when studied *in vitro* [203]. *Achromobacter* biofilm formation leads to higher resistance towards antibiotics [203, 211, 213]. The ability to adhere and form biofilms has been attributed to genes involved in the synthesis of a Vi capsular polysaccharide [214] and the poly-β-1,6-N-acetyl-D-glucosamine polysaccharide [209]. Although only confirmed on a genomic level, the presence of a bacterial capsule protects the bacteria from phagocytosis, environmental toxins and desiccation.

Compared to *P. aeruginosa* and *S. maltophilia*, *A. xylosoxidans* is significantly more resistant to antimicrobial peptides. This may be due to a difference in surface charge, where *P. aeruginosa* is more negatively charged than *A. xylosoxidans*, causing it to be more susceptible to binding of the positively charged AMPs secreted by the airway epithelium [215, 216].

Metabolism

Although *Achromobacter* spp are primarily aerobic, they are able to survive in anaerobic environments as facultative anaerobes. *A. xylosoxidans* have been described to use anaerobic respiration through nitrate reduction, facilitating growth in the hypoxic CF mucus environment [217]. They do not form spores to persist on surfaces [218], but have been found as nosocomial pathogens due to their ability to survive in environments such as disinfectant solutions and IV fluids [219-221].

An important virulence factor for the survival in low-nutrient environments is the production of siderophores. These are secreted molecules which bind iron in the extracellular environment, and can then be targeted by receptors on the bacterial cell surface for uptake of the complex. An example of this is pyoverdine, which is responsible for the blue-green coloration of *P. aeruginosa*. In addition to sequestering iron, siderophores can have other effects on virulence such as supporting the formation of biofilms and cytotoxicity towards macrophages [222]. Siderophore production in *Achromobacter* spp has also been observed, with a significantly higher rate in clinical strains than environmental, implicating their role in pathogenicity [223].

In-host adaptation

As chronic infection takes hold, *Achromobacter* spp are able to undergo genetic adaptation and within-host evolution. This process involves the selection of beneficial mutations over time, rather than only affecting gene expression. Genetic adaptations have been observed in longitudinal isolates of *Achromobacter* spp from chronically infected CF patients. In late isolates, genes involved in the initiation of infection, such as type I and III secretion systems and genes related to pili and flagella, had been selected against [224]. This study also identified mutations in genes related to general metabolism, maintenance of the cell wall and capsule, antibiotic resistance, and iron uptake. The decrease in LPS production in chronic isolates has also been described in a study comparing the genomes of chronic and sporadic *Achromobacter* infections [225]. Another study on the genetic adaptations of *A. xylosoxidans* during long-term colonisation identified mutations in genes encoding signal transduction proteins and transcriptional regulators [226]. Interestingly, both studies on longitudinal CF isolates reported varying differences in biofilm formation, with some displaying increased biofilm formation over time and others decreased.

Although it is difficult to conclude whether the observed mutations caused a loss of function based on genomic analysis, it can be speculated that loss of function of redundant virulence factors promotes long-term colonisation and immune evasion. In an *in vivo* infection model of *G. mellonella* larva by *A. xylosoxidans*, sporadically infecting isolates caused a higher mortality than chronic isolates [227].

Some isolates which contain defects in the methyl-directed mismatch repair (MMR) system are considered hypermutators, which may be an advantage towards rapidly spreading pathogenic mutations [226, 228], a phenomenon which has also been observed in *P. aeruginosa* [229]. The hypermutator trait appears to correlate with chronic rather than sporadic *Achromobacter* infection [225].

Secretion systems and toxins

Until recently, the mechanisms of cellular toxicity in *Achromobacter xylosoxidans* have been largely unidentified. *A. xylosoxidans* had been shown to produce cytotoxic exoproducts, demonstrated to cause inflammation and cell death *in vitro* [230], which we further explored in paper I and found to be equally inflammatory as those of *P. aeruginosa* [6]. *Achromobacter* has also been confirmed to produce phospholipase C, which aids mucosal invasion by hydrolysing airway surfactant phospholipids [231, 232]. Although all gram-negative bacteria contain LPS anchored to the outer membrane, their potential as endotoxins vary by species, and the LPS of *Achromobacter* has been described to have proinflammatory properties [233].

Recent studies have started to elucidate the role of bacterial secretion systems in *Achromobacter* virulence. The type III secretion system (T3SS) is one of the most important determinants of virulent *Achromobacter* species. It is involved in infecting host cells by injecting bacterial proteins including toxins, and has been found in the genomes of the majority of clinical *Achromobacter* strains examined. The phospholipase AxoU is a T3SS substrate, and has been shown to cause toxicity towards phagocytic cells by cleaving membrane phospholipids [7]. The cytotoxic effect observed in this study was dependent on internalisation of *A. xylosoxidans*, suggesting that the bacteria use this mechanism as an additional immune evasion method. A follow-up study identified the ArtA adhesin, which facilitated internalisation into macrophages and was recognised by serum antibodies from pwCF infected with *A. xylosoxidans* [234]. The mechanism of cytotoxicity in macrophages has been shown to involve T3SS-mediated pyroptosis, a proinflammatory lytic cell death which can take place in response to intracellular pathogens [235]. This process was initiated by the *A. xylosoxidans* T3SS being detected by the NLRC4 or NLRP3 inflammasome sensors in macrophages, independently of the presence of AxoU and ArtA adhesin [236].

A study on *A. xylosoxidans* lung infection in mice identified type III secretion, the Vi capsule, antisigma-E factor and ArtA adhesin as determining virulence factors for severe disease and higher mortality [237]. The presence of these virulence factors were concurrent with an *in vitro* phenotype of acute macrophage cytotoxicity. This highly virulent strain was isolated from an ear infection and compared to a less-virulent CF strain, which could possibly be attributed to virulence attenuation during adaptation in chronic CF infections [224]. Genes involved in type III secretion have also been described as more common in chronic than sporadic isolates [225], although this comparison included early isolates of chronic infections.

Type II, VI and VII secretion systems has been identified on the genomic level [209], although the substrates of these secretion systems in *Achromobacter* have not yet been identified. T2SS is involved in exotoxin secretion, adhesion, biofilm formation, and phagosome survival in other bacteria [238, 239]. T6SS is particularly important in the context of polymicrobial infections, as this secretion system is able to inject toxins into other bacterial cells in order to exert competition [240]. The activity of a T6SS was confirmed in CF isolates of *A. xylosoxidans*, where the T6SS-encoding gene cluster TAX-1 was activated during culturing in synthetic CF sputum medium and triggered bacterial internalisation to lung epithelial cells as well as antagonism towards *E. coli* and *P. aeruginosa* [241]. Finally, the Tat secretion system is important to the virulence of e.g. *P. aeruginosa,* by transporting virulence factors such as toxins from the cytosol to the periplasmic space [242, 243]. It had been identified at a genomic level in *A. xylosoxidans*, and we explored its role in virulence in paper II.

Antibiotic resistance

The antibiotic resistances of *Achromobacter* spp are attributed to multidrug efflux pumps and beta-lactamases. Beta-lactamases are a group of enzymes which are able to cleave different beta-lactam antibiotics, and thus do not inherently cause full resistance against all antibiotics of this class. The types found in clinical strains of *Achromobacter* are OXA-114-like, extended-spectrum (ESBL), AmpC-type and metallo-beta-lactamases [244]. OXA-114-like beta-lactamases provide resistance towards penicillins, while ESBL and AmpC affect all beta-lactams except carbapenems. Some isolates have been identified with carbapenamases, which can be chromosomal or transferred through plasmid DNA [244]. It is important to note that not all *Achromobacter* isolates carry all listed types of beta-lactamases, although all isolates have beta-lactamase activity [245] and OXA-114-like beta-lactamase is considered to be naturally occurring in *A. xylosoxidans* [246].

Achromobacter antibiotic resistance is further strengthened through active efflux pumps. The AxyABM efflux pump is common to all characterised *Achromobacter* species and can facilitate efflux of cephalosporins and carbapenems [247]. AxyXY-OprZ can effectively extrude aminoglycosides, as well as to a lesser extent cefepime, carbapenems, fluoroquinolones, tetracyclines and erythromycin [248]. This efflux pump is not found in all *Achromobacter* species, but was identified in the most common CF pathogens: *A. xylosoxidans, A. ruhlandii, A. insuavis,* and *A. dolens.*

Resistance towards aminoglycoside and fluoroquinolone antibiotics through acetyltransferases have been identified in *Achromobacter* spp, particularly in clinical isolates [249-251]. Finally, the last-resort antibiotic polymyxin B, which binds and neutralises LPS, may be evaded through modification of the lipid A component of *Achromobacter* LPS [252].

Polymicrobial interactions

Although co-infections by multiple pathogens are common in people with CF, the interactions between *Achromobacter* spp and other microorganisms are poorly understood. As chronic *A. xylosoxidans* infections are often shown to dominate the microbiome when identified in the CF airway [147, 199], it can be speculated that it employs competitive mechanisms to outpersist other species.

Genomic analysis identified the presence of Colicin V [209], an antimicrobial peptide secreted by e.g. *Enterobacteriaceae* as an antagonistic mechanism against other bacteria competing for nutrients. The study also identified the *A. xylosoxidans* T6SS, which was further characterised in another study implicating it in antagonistic mechanisms against *E. coli* and *P. aeruginosa* [241]. The specific substrates of T6SS in *Achromobacter* are not yet known.

Co-culturing experiments on the CF pathogens *P. aeruginosa, A. xylosoxidans, S. maltophilia* and *S. aureus* revealed that the interactions between *A. xylosoxidans* and *P. aeruginosa* are highly variable and strain-dependent [253]. This study included isolates from six CF patients, retrieved from both sputum samples and the home environment. In co-cultures of *A. xylosoxidans* and *P. aeruginosa*, around half of the *P. aeruginosa* strains were affected in comparison to monoculture – but the effect of the interaction varied. Growth inhibition of *P. aeruginosa* by *A. xylosoxidans* was observed in a similar number of occasions as growth stimulation. Effects on motility and pigment production also varied. Some *A. xylosoxidans* strains were also shown to inhibit *S. maltophilia* growth and motility.

Another co-culturing study on *Achromobacter* and *P. aeruginosa* longitudinal pairs identified an early isolate of *A. insuavis* which secreted exoproducts inhibiting the adhesion ability of *P. aeruginosa* co-isolated from the same person, while the late isolate did not retain this ability [254]. It also inhibited the biofilm formation of *P. aeruginosa* when grown in co-culture. The inhibitory effect was not seen in the late *A. insuavis* isolate or in either the early or late isolate of *A. xylosoxidans*, although only one pair of each species was tested. This study showed that some *Achromobacter* may possess mechanisms to inhibit the adhesion and biofilm formation of other bacteria, but others grow synergistically in mixed biofilms. The effect appears to involve type VI secretion, as the VgrG gene encoding a T6SS tip protein was only present in the early *A. insuavis* isolate.

The clinical implications of these observed interactions remain to be understood. On one hand, one could speculate that competitive *Achromobacter* isolates are more virulent due to their implied ability to dominate the microbiome and reduce the presence of less-virulent species, an outcome which is strongly associated with a poor prognosis compared to having a higher airway microbial diversity [148]. On the other hand, co-infections with both *A. xylosoxidans* and *P. aeruginosa* appears to be correlated to a worse lung health than colonisation by one or the other [255]. In this case, less-competitive isolates which are able to coexist in mixed-species biofilms may lead to the worst outcomes.

Present investigations

At the time of initiating this thesis, there was very little knowledge on the virulence of *Achromobacter* species, and the genus' role as CF pathogens was less recognised than it is today. The viewpoint of *Achromobacter* as harmless opportunists has been challenged by multiple studies identifying pathogenic mechanisms, many of which have been published during the course of this project. At the start of the project, our general aims were to:

- Study whether *Achromobacter* species cause stronger or weaker inflammatory responses from host cells compared to *Pseudomonas aeruginosa*, the most well-known CF pathogen which causes lung function decline in adults.
- Study whether different *Achromobacter* species found in CF infections cause different responses from host cells, and by extension whether it is clinically important to define which species is found in a culture.
- Identify mechanisms of importance for *A. xylosoxidans* virulence, with focus the on secretion system Tat.
- Confirm the presence of antibody responses in persons with *Achromobacter* infections, and identify the targets of serum IgGs towards *A. xylosoxidans*.
- Investigate the interactions between *A. xylosoxidans* and other CF pathogens, particularly *P. aeruginosa*, by studying mixed-species liquid cultures and biofilms.

Paper I

Background

Although *A. xylosoxidans* is the most common *Achromobacter* infection, other species can also cause airway infections in CF. At the time of this paper, the identification methods used in routine diagnostics do not distinguish between species, and provide a result classified only as '*Achromobacter* spp'. As even the virulence capacity of *A. xylosoxidans* was under debate, and some disagreement still exists on whether it is a true pathogen, no research had been done on the comparative virulence between different *Achromobacter* species.

Aims

The overall aim of this paper was to contribute towards the knowledge of similarities or differences between *Achromobacter* species, in order to provide more information on whether distinguishing between them is clinically important. Towards this goal, we aimed to:

- Comparatively investigating the inflammatory properties of exoproducts from *A. xylosoxidans*, *A. insuavis, A. dolens* and *A. ruhlandii*, using *P. aeruginosa* as a positive control.
- Phenotypically and morphologically characterise the four *Achromobacter* species.

Methods

Cell-free bacterial supernatants from the four *Achromobacter* species were used to stimulate CF bronchial epithelial cells and whole blood from healthy individuals. Supernatants from the well-characterised CF-pathogen *Pseudomonas aeruginosa* were included for comparison. The release of IL-6 and IL-8 from the bronchial epithelial cells was analysed with ELISA. Leucocyte activation was assessed using flow cytometry, with CD11b used as a marker of neutrophil and monocyte activation. Finally, heparin-binding protein was measured by ELISA in peripheral blood stimulated with bacterial supernatants.

Phenotypic characterisation was performed by assessing biofilm formation with crystal violet assay, comparing motility using semi-solid agar plating, and visualising the four species in scanning electron microscopy (SEM).

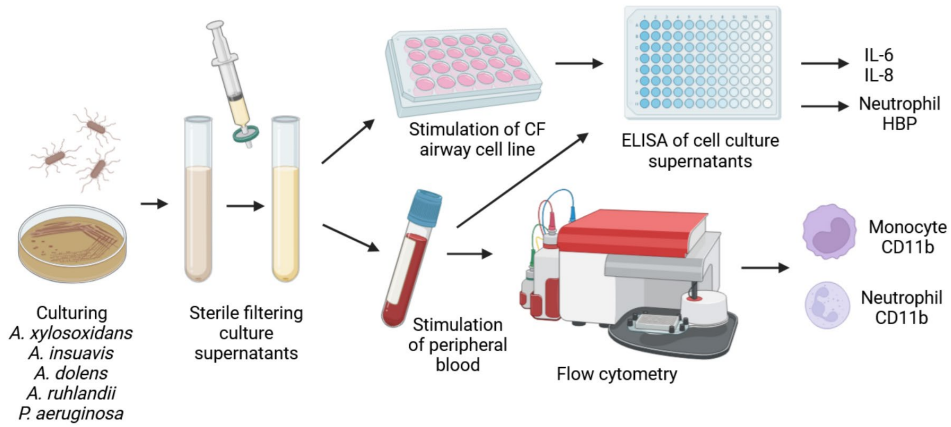

Figure 11: Workflow of Paper I.

Results

Out of 21 isolates identified as *A. xylosoxidans* by MALDI-TOF, six (29%) were determined by nrdA sequencing to be *A. insuavis, A. ruhlandii* or *A. dolens*. When stimulating bronchial epithelial cells with exoproducts from the different *Achromobacter* species, small variations of IL-6 and IL-8 release was observed between them. The only significant difference was between *A. xylosoxidans* and *A. insuavis*, where the latter had a weaker inflammatory effect. On the other hand, *P. aeruginosa* consistently gave rise to the lowest cytokine levels. *P. aeruginosa* also caused significantly lower HBP release than all *Achromobacter* species. CD11b as a marker of activation in neutrophils and monocytes was also higher after exposure to *A. xylosoxidans* and *A. insuavis* than *P. aeruginosa*. This effect did not appear to be caused by LPS, as inhibition of LPS by polymyxin B did not lower the inflammation.

Swimming motility varied greatly within groups but not between groups, suggesting that this property is more dependent on strain than species. Biofilm formation capacity also varied within groups, with e.g. two out of five *A. xylosoxidans* isolates being competent at forming biofilms, but all *Achromobacter* groups significantly less so than *P. aeruginosa*. Interestingly, all four *Achromobacter* species differed in morphology when visualised by SEM. *A. xylosoxidans* appeared as short rods with abundant flagella, *A. ruhlandii* as shorter rods with very few flagella, *A. insuavis* with a filamentous appearance without visible flagella, and *A. dolens* as filamentous with visible flagella.

Conclusion

Although we did not observe any consistent differences between *Achromobacter* species – for example, *A. insuavis* caused lower IL-8 secretion but higher neutrophil activation – *A. xylosoxidans* gave rise to consistently high inflammatory markers and was significantly more potent than *P. aeruginosa* under all tested conditions. This supports the hypothesis that *A. xylosoxidans* (and likely other *Achromobacter* species) have inherently pathogenic properties, rather than being correlated with a worse lung status purely due to opportunistic infection. Due to the low number of clinical isolates from non-*xylosoxidans* species and the scope of the study not including infection with live bacterial cells, further studies are required to validate the differences in virulence between species.

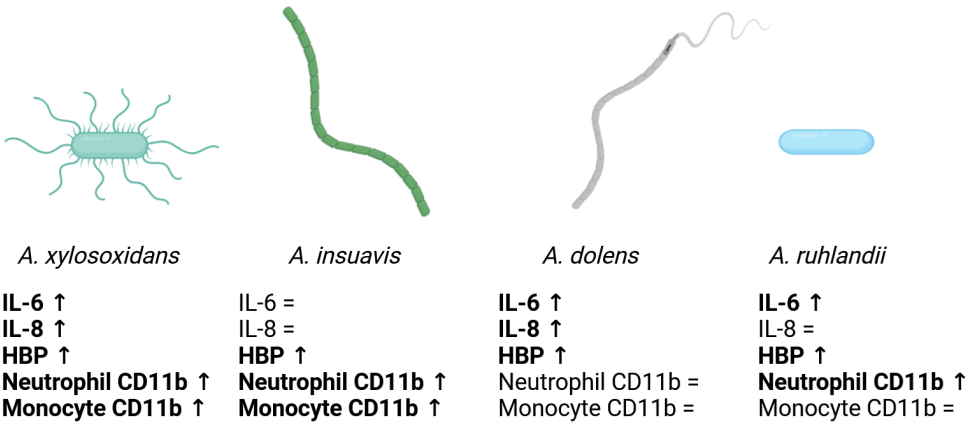

Figure 12: Conclusions of paper I. When compared to *P. aeruginosa*, supernatants of all *Achromobacter* species caused equal or higher inflammatory markers, and *A. xylosoxidans* caused higher responses than *P. aeruginosa* in all tested conditions.

Paper II

Background

Bacterial secretion systems are an important determinant of their virulence. For example, the type III secretion system has been determined to be a trait of clinical as opposed to environmental *Achromobacter* strains and necessary for their virulence. In addition to secretion into the environment or into recipient cells, secretion systems also exist to transport proteins between cellular compartments. The most common pathways are Sec (Secretory) and Tat (Twin-arginine translocation), which transport proteins across the cytoplasmic membrane in unfolded and folded states, respectively [91]. They exist in both gram negatives and positives as well as in eukaryotic cells, but are especially important in gram negatives as a means to transport proteins into the periplasmic space [256]. The Tat secretion system has been described as a necessary component of *P. aeruginosa* virulence [242, 243]. While it had also been identified in the *A. xylosoxidans* genome, its importance for virulence in this species was undetermined.

Aims

The aim of this study was to evaluate the Tat secretion system as a putative virulence determinant in *A. xylosoxidans*, by generating a ΔtatC mutant and characterising its phenotypic properties. We also aimed to identify Tat substrates through comparative proteomic analysis of the cytosolic, membrane and periplasmic fractions of mutant and wild-type cultures.

Methods

A ΔtatC mutant was generated using in-frame deletion. The pDM4-ΔtatC plasmid sequence was generated by amplifying and ligating *A. xylosoxidans* genomic DNA fragments upstream and downstream of the *tatC* coding region. The plasmid was first electroporated into *E. coli* and then transformed into *A. xylosoxidans* after sequence verification. Colonies were then plated on chloramphenicol selective agar several times until becoming susceptible. Deletion mutants were validated by sequencing.

Putative Tat substrates were first identified using the prediction tools TatFind, TatP, and PRED-Tat, and considered a candidate if identified by at least two. The mutant and wild-type *A. xylosoxidans* were then cultured and fractionated into cytosolic, membrane, and periplasmic fractions using osmotic shock fractionation. The fractions were analysed using LC-MS/MS, and the resulting spectral data analysed in Proteome Discoverer 2.5 against an *A. xylosoxidans* reference proteome.

The mutant was then compared to the wild-type by analysing growth rate, adhesion to bronchial epithelial cells, and growth in iron-limited conditions.

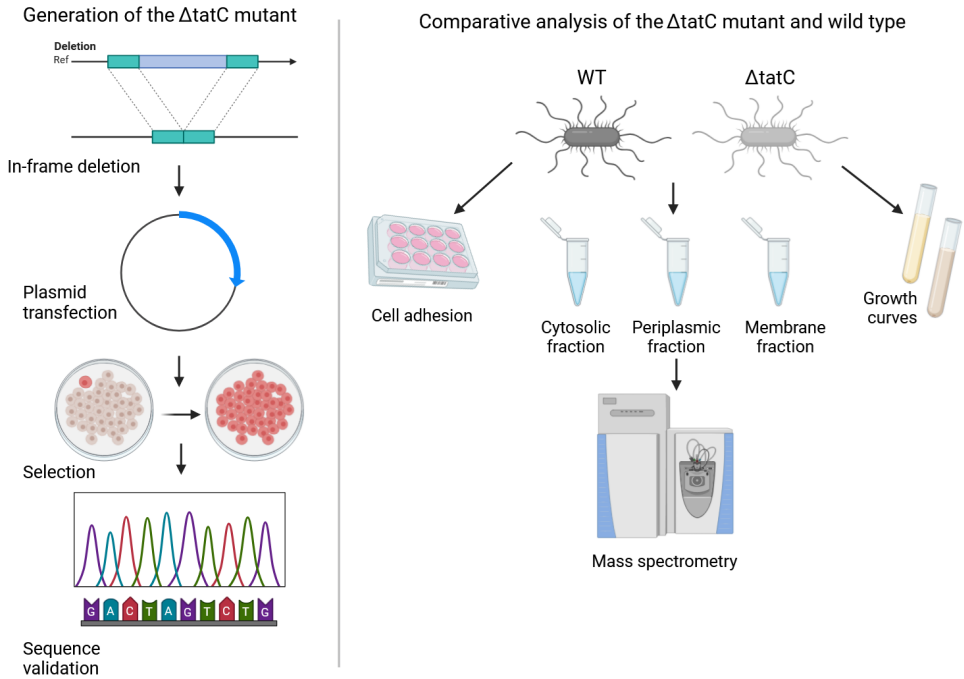

Figure 13: Workflow of Paper II.

Results

Using the *in silico* prediction tools TatFind, TatP, and PRED-Tat, 128 putative Tat substrates were identified by ≥2 tools and 56 by all three. Out of the 128 candidate substrates, 21 were identified by LC-MS/MS to be present in the wild type *A. xylosoxidans* isolate. All but one were found in the periplasmic fraction. When comparing the protein contents of the wild-type to the ΔtatC mutant, 9 of the 20 periplasm-associated proteins were significantly associated with the wild type, implying that the mutant was unable to transport them into the periplasm. The ferripyoverdine receptor FpvA was completely absent in the wild type under normal conditions, but was expressed during iron starvation.

When comparing the phenotype of the mutant to the wild-type, the ΔtatC mutant exhibited significantly reduced adhesion to bronchial epithelial cells (11.9% versus 3.5% binding), and significantly slower growth in liquid medium either with or without iron availability.

Conclusion

In this study, we identified 128 putative Tat substrates *in silico*, which was narrowed down to 21 proteins identified by LC-MS/MS and 9 which were identified less in the periplasm of a ΔtatC mutant compared to the wild type. As a comparison, we identified 44 only putative Tat substrate in a *P. aeruginosa* proteome using the same prediction tools, suggesting that Tat secretion plays an important role in *A. xylosoxidans*.

The ΔtatC mutant had significantly lower adhesion to bronchial epithelial cells, suggesting Tat secretion has an important role in the early establishment of infection. A role in iron acquisition was implied as the growth rate of the ΔtatC mutant under iron-limited conditions was significantly slower than that of the wild type, and while the addition of iron improved the growth rate of the wild type, it remained low in the mutant. The ferripyoverdine receptor FpvA was upregulated during iron-limited conditions in the wild type. As FpvA is used as a pyoverdine receptor in *P. aeruginosa*, it can be speculated to also be a siderophore receptor in *A. xylosoxidans*. It was expressed regardless of iron availability in the ΔtatC mutant, suggesting that the mutant may undergo iron starvation due to defective acquisition. Although the ΔtatC mutant showed reduced adhesion and iron acquisition, none of the nine periplasmic proteins associated with the wild type were involved in these processes, and further studies would be required to fully explain the observed phenotype.

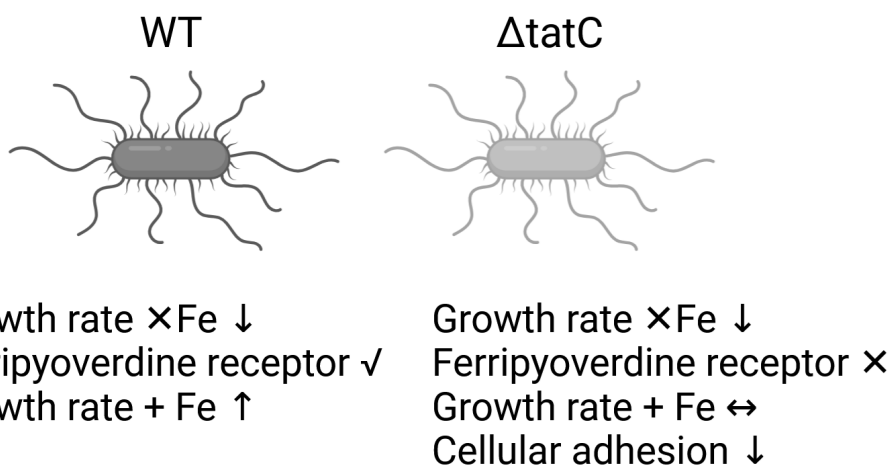

Figure 14: Conclusions of paper II. While growth rates in both cultures were inhibited in the absence of iron, the ΔtatC mutant was unable to retain a normal growth rate in the presence of iron. It also showed an inhibited adhesion capacity to bronchial epithelial cells, and lacked expression of a ferripyoverdine receptor which was upregulated by the wild type in low iron conditions.

Table 2: Tat substrates identified by ≥2 prediction tools and LC-MS/MS. Nine of the proteins were significantly associated with the wild type periplasm, suggesting they were unable to be transported to the periplasm in the ΔtatC mutant: ABC transporter substrate-binding protein A0A0D6IGC9 and A0A0D6IPK9, Argininosuccinate lyase A0A0D6I447 and A0A0D6IQY6, BMP family protein, BsSco, Extracellular solute-binding protein, Glutamine-binding periplasmic protein, and Neu5Ac-binding protein.

Protein description	Uniprot accession	Identified by prediction tools (n)	Expressed in periplasm
ABC transporter substrate-binding protein	A0A0D6IGC9	3	Yes
ABC transporter substrate-binding protein	A0A0D6GVW1	3	Yes
ABC transporter substrate-binding protein	A0A0D6H3R4	3	Yes
ABC transporter substrate-binding protein	A0A0D6IPK9	3	Yes
Argininosuccinate lyase	A0A0D6FWL1	2	Yes
Argininosuccinate lyase	A0A0D6I447	2	Yes
Argininosuccinate lyase	A0A0D6IQY6	3	Yes
OmpH family outer membrane protein	A0A0D6HF24	2	Yes
BMP family protein	A0A0D6FDV3	3	Yes
BsSco	A0A0D6FIS4	2	Yes
D-alanyl-D-alanine carboxypeptidase	A0A7T2RFI8	2	Yes
Extracellular solute-binding protein	A0A0D6H151	3	Yes
Ferripyoverdine receptor	A0A0D6IKG8	3	Yes
Fimbrial protein	A0A0D6G5C4	2	Yes
Glutamine-binding periplasmic protein	A0A0D6HE12	3	Yes
Neu5Ac-binding protein	A0A0D6I797	3	Yes
Nitrous-oxide reductase	A0A0D6IAU8	3	Yes
Succinate dehydrogenase flavoprotein subunit	A0A0D6GP36	2	Yes
Twin-arginine translocation pathway signal	A0A0D6H5H3	2	Yes
Ubiquinol-cytochrome c reductase iron-sulfur subunit	A0A0D6FH58	2	Yes
Uncharacterised protein	A0A0D6HQ03	2	No

Paper III

Background

Achromobacter spp infections in people with CF have been shown to give rise to serum IgG responses against the bacteria, with high variability between individuals. High titres have been correlated with a more rapid lung function decline, as the response is an insufficient response to clear the infection [257]. However, the available research on antibody responses to *Achromobacter* spp is very limited, and the targets of serum antibodies have not been identified.

Aims

In this study, we wanted to apply a novel affinity purification-mass spectrometry approach in order to study the IgG responses in serum of pwCF infected with *Achromobacter* spp. The main aims were to:

- Confirm whether people with *Achromobacter* spp have circulating IgG against whole bacteria, and if the response is specific by comparing serum from persons infected with *P. aeruginosa*.
- Identify the target antigens of IgG against *A. xylosoxidans* using affinity purification-mass spectrometry.
- Validate the identified antigens by using the recombinantly produced proteins in ELISA.

Methods

Serum IgG against *A. xylosoxidans*, *A. insuavis*, and *P. aeruginosa* was analysed in direct ELISA against whole bacteria. Sera were obtained from pwCF with *Achromobacter* spp infection (n=7), chronic *P. aeruginosa* infection (n=22), neither *Achromobacter* spp or *P. aeruginosa* (n=20), and healthy controls (n=4).

A serum with high titres against *A. xylosoxidans* infection was selected for further antigen analysis using affinity purification. Another serum with high titres against *A. xylosoxidans,* but from a person infected with *P. aeruginosa*, was selected as a control against cross-reactivity. Three negative controls were included: A healthy control serum, Omalizumab, and IVIG. Antigens were enriched by adhering immunoglobulins to protein G beads, incubating with surface and secreted proteins from *A. xylosoxidans*, and eluting the resulting IgG-antigen complexes. Samples were analysed using LC-MS/MS, and the data analysed in MaxQuant 2.2.0.0 and Perseus 2.0.7.0. Three candidate antigens were produced recombinantly in *E. coli* and used in coating ELISAs for screening antibody responses in the entire cohort. Finally, the three candidates were identified in a wider collection of *A. xylosoxidans* isolates using PCR, and investigated for sequence homology with *A. insuavis* and *P. aeruginosa* proteins using BLAST and CLUSTALW.

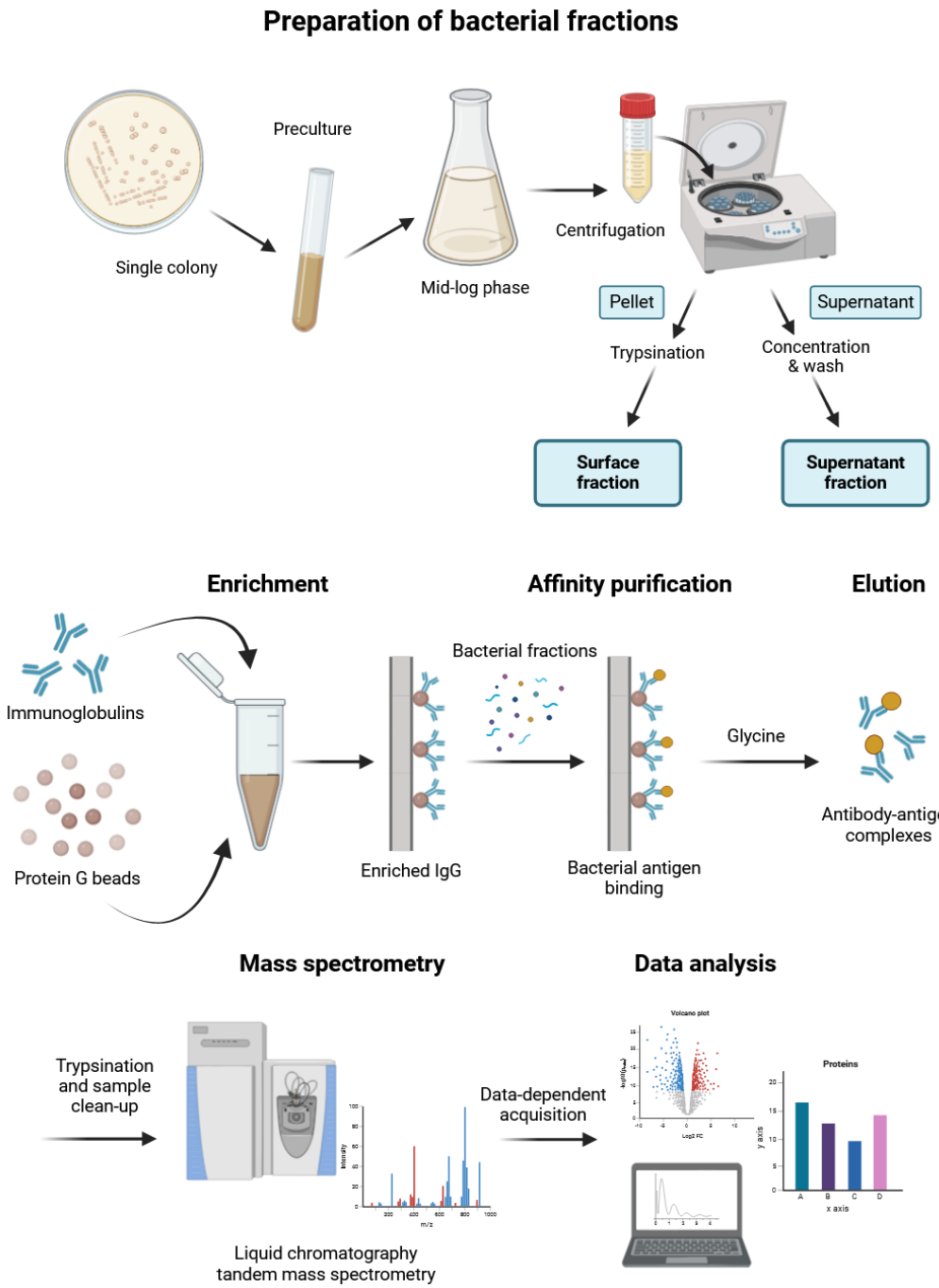

Figure 15: Workflow of Paper III.

Results

In ELISA against whole bacteria, 4 out of 7 pwCF with *Achromobacter* spp infections had IgG responses against *A. xylosoxidans*. Two out of the three non-responders were infected with *A. insuavis*, but had no IgG responses against this species either. Non-responding sera were also observed in people infected with *P. aeruginosa* when sera were tested against this species. Reactivity of *P. aeruginosa*-exposed sera against *A. xylosoxidans* bacteria was observed, but not vice versa.

After performing affinity purification-mass spectrometry, 28 antigens were found in complex with IgGs from a person infected with *A. xylosoxidans*. This was narrowed down to eight antigens, after ruling out unspecific bindings by selecting antigens significantly enriched compared to the negative control Omalizumab. When compared to serum from a *P. aeruginosa* infected person, four antigens were found specific to the *A. xylosoxidans* serum.

Type I secretion C-terminal target domain-containing protein (T1S-DCP), Dihydrolipoyl dehydrogenase (DLD), and Domain of uncharacterized function (DUF336) were produced recombinantly and used to coat ELISA for screening of IgG responses in all 53 sera. T1S-DCP was the least suitable candidate for a serological screening assay, with 71% of sera positive in the *Achromobacter* spp group but 82% positive sera in the *P. aeruginosa* group. DLD had the same amount of positive sera in the *Achromobacter* spp group but only 32% in the *P. aeruginosa* group. DUF336 ELISA had only one positive serum in the *P. aeruginosa* group (5%) and 43% in the *Achromobacter* spp group. The two sera from persons infected with *A. insuavis* continued to show among the lowest responses to all three antigens.

DUF336 showed a high similarity to heme-binding proteins in both *A. insuavis* and *P. aeruginosa*. DLD was found in all species and was considered homologous in *P. aeruginosa* but not in *A. insuavis*. No homologous proteins to T1S-DCP were identified in either species.

Conclusion

We identified eight putative IgG antigens of *A. xylosoxidans* using an affinity purification-mass spectrometry systems antigenomics workflow. Three of the candidate antigens were produced recombinantly and used to validate the method by serum ELISA, identifying two proteins with good specificity to sera from persons infected with *A. xylosoxidans*: Dihydrolipoyl dehydrogenase and Domain of uncharacterized function (DUF336). If validated in a larger cohort, it may potentially be possible to develop a serological ELISA as a complement to culture-based methods for the diagnosis of *Achromobacter* infections. Our results also warrant further studies on the lack of observed IgG responses against *A. insuavis*, to investigate whether these infections correlate with e.g. lower inflammatory responses than other *Achromobacter* spp.

Table 3: *Achromobacter xylosoxidans* antigens identified by LC-MS/MS and significantly enriched compared to the negative control IgG Omalizumab. Four of the proteins were also significantly enriched when compared to serum from a person with *P. aeruginosa* infection and high titres against *A. xylosoxidans*, affirming their specificity: Type I secretion C-terminal target domain-containing protein, Dihydrolipoyl dehydrogenase, Domain of uncharacterized function (DUF336), and Amino acid ABC transporter substrate-binding protein.

UniProt ID	Name	Fraction	Function
A0A7T2RJE8	Type I secretion C-terminal target domain-containing protein	Secreted, surface	Virulence
A0A0D6GPL2	Dihydrolipoyl dehydrogenase	Secreted, surface	Dehydrogenase activity
A0A0D6GMC1	Domain of uncharacterized function (DUF336)	Secreted, surface	Heme binding
A0A0D6HJI6	DNA protection during starvation protein 2	Surface	Iron binding
A0A0D6FRA6	Amino acid ABC transporter substrate-binding protein	Secreted, surface	Membrane transport
A0A0D6GVE5	Inorganic pyrophosphatase	Secreted, surface	Phosphatase activity
A0A0D6HFP0	Glutamate dehydrogenase	Secreted, surface	Dehydrogenase activity
A0A0D6GJM9	Alkyl hydroperoxide reductase AhpD	Secreted, surface	Hydroperoxidase activity

Paper IV

Background

In addition to surviving the immune defences and antibiotic treatments, pathogenic bacteria must also be able to persist in the presence of other microorganisms. The interactions between different bacterial species can be either in the form of competition or coexistence. Although infections by multiple airway pathogens are common in people with CF, most available studies are focusing on the interactions between *P. aeruginosa* and *S. aureus*. Only two studies had been performed on the polymicrobial interactions of *A. xylosoxidans* at the time of writing, indicating that some isolates exhibit competition and others coexistence with *P. aeruginosa* and *S. maltophilia* [253, 254].

Aims

As very little information was available on the interspecies interactions used by *A. xylosoxidans*, we wanted to confirm the data obtained by other studies that it may inhibit the biofilm formation of *P. aeruginosa*, and further characterise the mechanisms of this interaction. As one of the previous studies identified an *A. insuavis* isolate which exerted biofilm inhibition against *P. aeruginosa* in an early but not a late isolate, we also wanted to test the hypothesis that *A. xylosoxidans* early chronic isolates are more competitive due to virulence attenuation in late isolates.

Methods

Biofilm quantification of *P. aeruginosa* was performed by crystal violet assay in microtiter plates, both in monoculture and in coculture with *A. xylosoxidans*. The *P. aeruginosa* CF isolate was selected based on high biofilm formation, and the six *A. xylosoxidans* isolates were longitudinal pairs from three pwCF: One from a person never infected with *P. aeruginosa*, one where the *A. xylosoxidans* had outpersisted all other CF pathogens, and one where *A. xylosoxidans* and *P. aeruginosa* were persistently co-isolated. In co-cultures where biofilm inhibition was observed, growth conditions were modified in order to identify the mechanisms necessary for the inhibition to occur. Growth curves were studied in broth culture in order to evaluate whether the inhibition was caused by differential growth rates. Finally, the adhesion capacity to plastic was compared between the competitive *A. xylosoxidans* isolate and *P. aeruginosa*.

Results

Out of the six *A. xylosoxidans* isolates tested, all showed poor capacity for biofilm formation in microtiter plates. When cocultured with *P. aeruginosa*, the three early isolates were able to significantly inhibit its biofilm formation. Thus, we aimed to modify the growth conditions of one of these co-cultures until the inhibition no longer took place. The inhibitory effect was lowered by increasing the proportion of *P. aeruginosa* in the coculture, but was still in effect at a 4:1 ratio. No inhibition of biofilm was seen when adding *A. xylosoxidans* to an established *P. aeruginosa* biofilm, when adding heat-killed *A. xylosoxidans*, or when incubating *P. aeruginosa* with 10% *A. xylosoxidans* supernatant. The *P. aeruginosa* isolate had a steeper growth curve than *A. xylosoxidans,* which was not inhibited by *A. xylosoxidans* supernatants. When studying the adhesion capacity to plastic, the *A. xylosoxidans* isolate had a higher amount of remaining attached colonies after washing than *P. aeruginosa*.

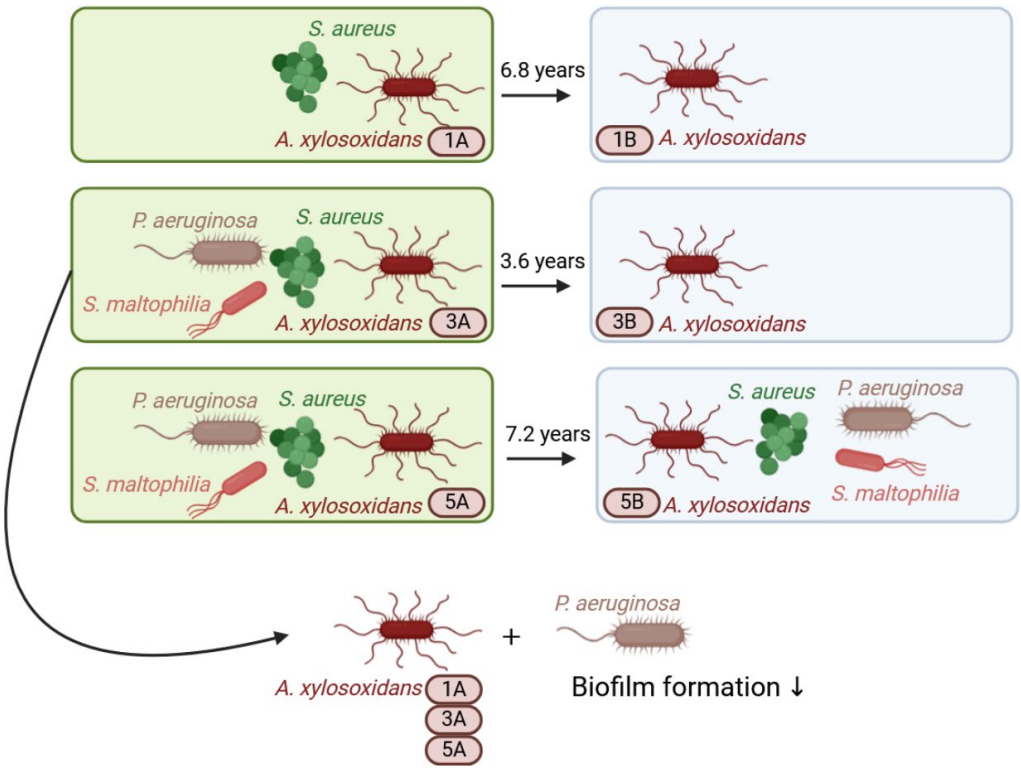

Figure 16: Selection of *Achromobacter xylosoxidans* CF isolates in paper IV. When co-cultured with *P. aeruginosa*, the early isolates 1A, 3A and 5A inhibited *P. aeruginosa* biofilm formation, while their corresponding late isolates did not.

Conclusion

In agreement with earlier studies, we found that some – but not all – isolates of *Achromobacter xylosoxidans* were able to inhibit the biofilm formation of *Pseudomonas aeruginosa*. This effect was observed in all early isolates of the three longitudinal pairs, but not in the late isolates.

The inhibition of *P. aeruginosa* biofilm by *A. xylosoxidans* was lost when incubating with only bacterial proteins (supernatant or heat-killed bacteria). Adding *A. xylosoxidans* to an established *P. aeruginosa* biofilm did not reduce its biomass, but *P. aeruginosa* added to a 24-hour culture of *A. xylosoxidans* was still able to produce biofilms. These results suggest that live cell-cell interactions during the early establishment stages of a biofilm are required for the competitive effect to take place.

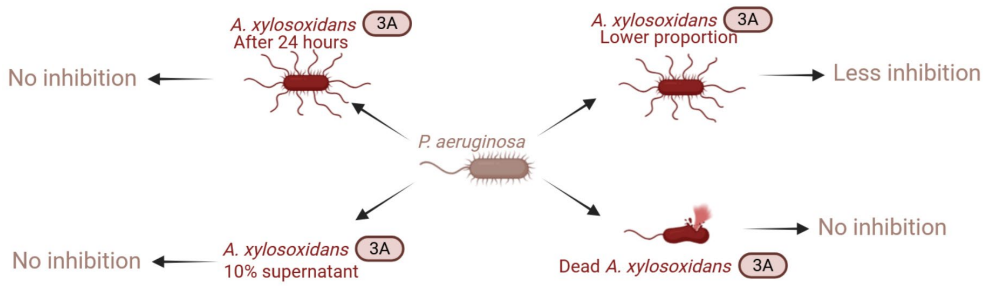

Figure 17: Conclusions of paper IV. Biofilm inhibition required the presence of live *A. xylosoxidans* cells before the establishment of the *P. aeruginosa* biofilm, and is correlated with the proportion of each bacteria.

The clinical implications of infection by bacteria with competitive traits are yet to be elucidated. Loss of microbial diversity is a hallmark sign of lung disease progression in CF, which is indicated by the persistence of a single species – often *P. aeruginosa* – dominating the microbiome [146-148]. Conversely, co-infection with both *A. xylosoxidans* and *P. aeruginosa* have been associated with worse outcomes than infection with one or the other [255]. Both species have been shown to undergo virulence attenuation during chronic infections [224, 226, 258], and the coexistence of both species in e.g. a multispecies biofilm could possibly lead to a worse prognosis than highly competitive isolates due to their improved persistence. Thus, it would be of interest to continue investigating the biofilms of *P. aeruginosa* in co-culture with the late non-competitive *A. xylosoxidans* isolates, with regards to survival benefits such as immune evasion or growth in nutrient-limited conditions.

General methodology

Enzyme-linked immunosorbent assay (ELISA)

One of the most commonly used methods in molecular biology, ELISA can be used to easily detect the presence of antigens or antibodies in a sample [259]. If compared to a standard sample with a known concentration, the amount of the detected protein can also be quantified. In this thesis, we use sandwich ELISA in paper I to quantify the amount of inflammatory markers in cell culture medium and peripheral blood, and indirect ELISA in paper III to detect antibodies against whole bacteria or bacterial proteins.

In a sandwich ELISA, the assay plate is coated with antibodies directed against a specific protein of interest, in our case e.g. IL-8. When incubating with a sample obtained from our cell culture supernatants, these capture antibodies bind IL-8 while other proteins in the sample can be washed away. The protein can then be detected by adding another antibody against IL-8, the detection antibody, which has been labelled with biotin. The biotin can then be bound to by streptavidin, which is conjugated to horseradish peroxidase (HRP). The concentration of IL-8 is then measured by adding tetramethylbenzidine (TMB), which turns blue in proportion to the amount of HRP in the well.

In an indirect ELISA, there is no capture antibody, and wells are coated directly with a protein sample. We used this method to study the presence of antibodies in serum, by coating plates with e.g. *Achromobacter* proteins. By adding a detection antibody targeted against human IgG, we were able to quantify the amount of IgGs in a sample relative to other samples such as negative controls. However, it was not possible to quantify the concentration against a standard curve, as there was no standard sample of purified anti-*Achromobacter* antibodies. The cutoff for serum positivity was instead determined by using the average optical density value of the negative control group + 3 standard deviations.

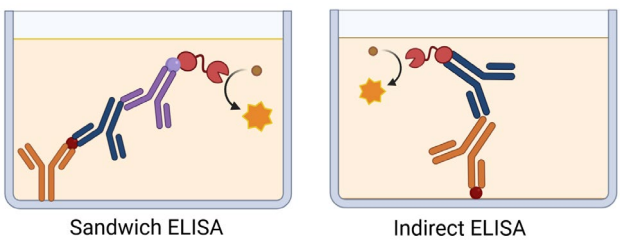

Figure 18: ELISA methods of detecting antigens (sandwich) and antibodies (indirect).

Flow cytometry

In paper I, we used flow cytometry in addition to ELISA to study human inflammation in response to stimulation with bacterial proteins. While ELISA was used to quantify cytokines secreted by airway epithelial cells, flow cytometry was used to measure the activation of neutrophils and monocytes in peripheral blood. Flow cytometry is based on the flow of cells passing by lasers, one at a time. It measures two fundamental parameters: Light scattering and fluorescence [260]. Light scattering occurs when cells pass by and occlude the laser. Forward scattering (FSC) of the light can estimate the size of the cells, while side scattering (SSC) is a measure of the granularity of the cell contents. The number of events passing by the laser is then graphed on a plot in real time. The FSC and SSC measurements can be used to identify the cell population of interest on the plot, which in our case was neutrophils and monocytes. As the cytometer measures all events flowing past the laser, FSC and SSC are important to distinguish live cells from e.g. apoptotic debris. By applying gates onto the plots in the software, one can choose to study only the relevant cell population by knowing their approximate FSC and SSC. The distinction between neutrophils and monocytes was done based on their different clustering patterns on the FSC/SSC plot.

At the same time as measuring the light scatter, the flow cytometer also measures fluorescence of the cells. While cells have a certain level of autofluorescence, the main utility of this function is the possibility to label cells through fluorescent antibodies or dyes. In this thesis, we used the fluorescent antibodies CD45-FITC and CD11b-PE-Cy5. The first antibody was used as a green-fluorescing pan-leucocyte marker in order to gate our cell population of interest. We then measured the amount of red fluorescence from PE-Cy5, which showed leucocyte activation determined by the upregulation of CD11b. The distinction between different colour wavelengths of fluorescence makes the flow cytometer a powerful tool to study multiple parameters of interest at once.

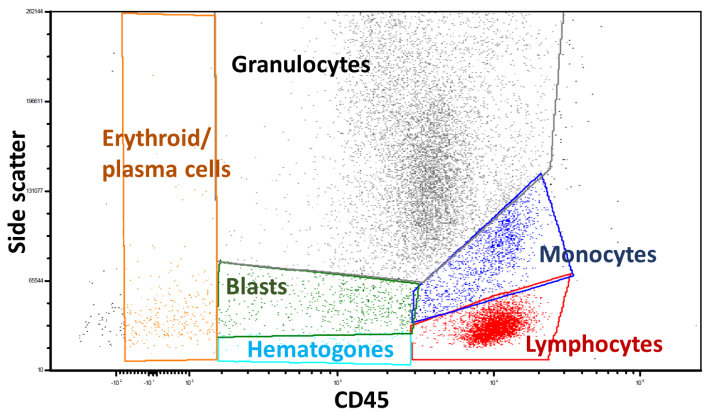

Figure 19: Example of cell population gating applied to a flow cytometer plot.
Image by Mikael Häggström, MD (Public Domain, CC0 1.0)

Mass spectrometry

In the simplest of terms, the fundamental concept behind all variants of mass spectrometry can be described as a "molecular scale". By measuring the weight of molecules at a resolution sensitive enough to distinguish between the atomic weights of different isotopes, it becomes possible to identify molecules by the weight of its component atoms [261]. The concept of identification by molecular weight can be used on a large variety of chemical substances ranging from inorganic compounds to large protein complexes. In this thesis, the method is used to identify proteins based on the molecular weights of their component amino acids. All 20 amino acids have a known weight from the sum of their atomic weights, with which their sequence in a peptide can be determined - with the caveat that molecular weight cannot distinguish between the structural isomers leucine and isoleucine.

Proteins can be analysed in two ways: as whole proteins which are subsequently fragmented by the mass spectrometer itself (top-down MS), or by first digesting the proteins using trypsin and injecting the resulting peptide fragments (bottom-up MS). These fragments are more easily ionised than in top-down MS, which makes it more suitable for proteomic identification and quantification as in this thesis. On the other hand, top-down MS is useful for studying the structure and modification of a specific known protein [262].

Mass spectrometry measures molecular weight as a mass-to-charge (m/z) ratio of ions. In order to achieve this, all analytes must be ionised using either electrospray ionisation (ESI) or matrix-assisted laser desorption (MALDI). The mass-to-charge ratio of the ions can then be detected. In this thesis, the method used combines liquid chromatography (LC) with tandem mass spectrometry (MS/MS). Samples are injected into the LC which separates peptides in a complex mixture based on hydrophobicity. After being ionised by the first MS, they are further fragmented before the m/z ratio is measured in a second MS. The additional fragmentation step is necessary to study samples containing a mixture of proteins, such as bacterial cultures or serum, in order to distinguish peptides with overlapping m/z ratios.

In tandem mass spectrometry, two approaches are possible with regards to data acquisition. When using data-dependent acquisition (DDA), only the most abundant peptides proceed past fragmentation to the second MS. This leads to a rapid workflow, and a sensitive quantification of abundant peptides. Data-independent acquisition (DIA) analyses all peptides in the second MS, which leads to a more complete and more easily reproducible dataset including low-abundance peptides [263].

The data obtained from an LC-MS/MS run is a spectrum of m/z ratios plotted against intensity. When using DDA, these spectra have to be analysed using a reference proteome from the species the sample was obtained from.

Bioinformatics software is then able to predict which proteins are present in the sample and its relative abundance, based on how much of a protein sequence is present as peptide fragments and how high their intensity is on the spectrum. As there is a significant risk of overlap with peptides fitting into different protein sequences, the data is usually filtered based on proteins identified with unique peptides.

In this thesis, the same methodology was used in paper II and III, i.e. bottom-up mass spectrometry with LC-MS/MS in DDA mode. This method selection is appropriate for the study of samples with unknown protein contents (e.g. cellular proteomes rather than purified proteins) but with an available reference proteome to map the spectral data against. By using DDA mode, we obtain an efficient and accurate quantification of the most abundant peptides, at the cost of possibly missing out on detecting very low-abundance peptides, which are likely to be less relevant for the purpose of our studies.

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a method used to amplify DNA sequences, most commonly a known gene. In paper I, we use PCR to amplify the *nrdA* gene, which was then sequenced externally in order to accurately identify *Achromobacter* species which had previously only been classified by genus. In paper II, PCR was used to amplify the DNA of the transfection plasmid used to generate the TatC mutant strain. PCR was used in paper III to confirm the genomic presence of our identified antigens in multiple *A. xylosoxidans* isolates, and in paper IV to quantify the ratio of *A. xylosoxidans* to *P. aeruginosa* DNA in a co-cultured biofilm.

In essence, PCR mimics the natural process of DNA replication, but is scaled up to produce millions of copies by the use of thermal cycling. The process is set up by adding DNA polymerase, nucleosides and a pair of primers to the DNA sample. Primer pairs are short DNA strands complementary to the start and end of the sequence targeted for amplification. The DNA polymerase needs to be heat-resistant in order to withstand the thermal cycling, such as the *Taq* polymerase isolated from thermophilic bacteria. The reaction starts by heating the sample to ~95 °C, which denatures the double-stranded DNA template. The temperature is then lowered to 50~65 °C, which allows annealing of the primers to the single-stranded DNA templates. The temperature is raised again to the optimal temperature for the polymerase, usually 72 °C. During this step, the polymerase uses nucleosides added to the reaction mix to elongate the complementary strand started by the primers. After one cycle consisting of denaturation, annealing, and elongation, 2^n copies of the target sequence are generated [264]. The cycling is usually repeated around 30 times.

After producing a sample of amplified DNA, it can be analysed using downstream methods such as sequencing or electrophoresis. Gel electrophoresis is the most common method of analysing amplified DNA, and is based on applying an electrical current to a gel containing DNA samples, which makes the strands migrate based on charge and size. The bands of DNA are visualised by fluorescent dye added to the samples before addition to the gel, and their base-pair length can be measured by comparing to a standard sample of DNA of known lengths. The method is used as quality control of amplified products, as a lack of bands would show that the amplification was unsuccessful. On the other hand, the presence of multiple bands or bands of unexpected sizes may be due to e.g. an inappropriate selection of primers or annealing temperature [265].

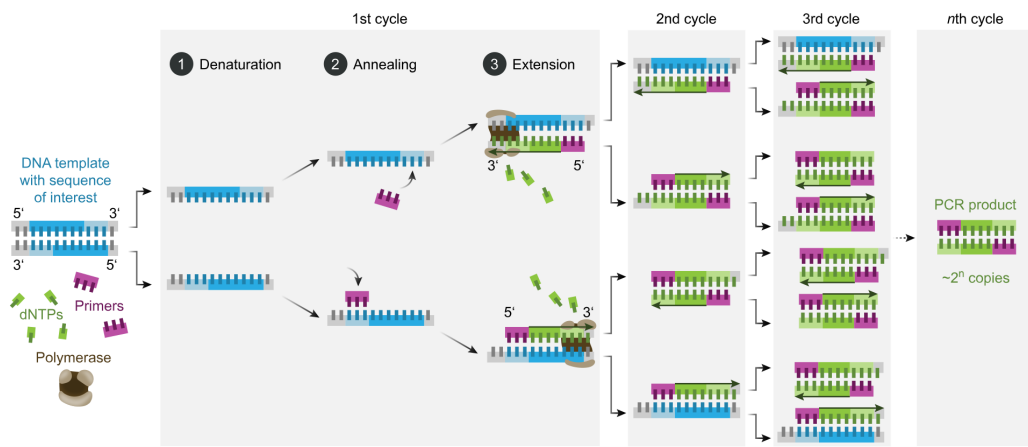

Figure 20: Schematic mechanism of PCR cycles. (Source: Enzoklop, CC BY-SA 4.0 https://creativecommons.org/licenses/by-sa/4.0, via Wikimedia Commons)

Another application of the PCR method is real-time or quantitative PCR (qPCR), which is used in this thesis for the quantification of bacterial DNA in biofilms (paper IV). This method applies the same cycling sequences as in a conventional PCR, but with the addition of fluorescent compounds measured after each cycle in order to quantify the amplification over time. In our experiments, we used a reaction mix containing the fluorescent dye SYBR Green, which binds to double-stranded DNA. As the amplified DNA becomes the majority of all DNA in the sample after several cycles, the total fluorescence in the sample can be used to quantify the amount of the target sequence. The fewer cycles it takes to reach a certain threshold level of fluorescence, the more of the target sequence was present in the original sample. This can be quantified in further detail by comparing to a standard curve of samples with known DNA concentrations.

Biofilm assays

One of the most commonly used methods to quantify the biofilm formation of bacteria is through crystal violet staining [266]. This method provides a simple measurement of the biomass attached to a well after washing, but can only provide a quantification relative to other samples as there is no universal standard to use as a reference. The dye, which is also part of the standard gram-staining protocol, binds negatively charged molecules such as DNA and extracellular polysaccharides. This method is very sensitive to differences in the performance of wash steps and pipetting, and special considerations were taken to make sure all repeats were performed by the same person and with the same technique. This is done to avoid variation due to e.g. leaving a small drop of dye remaining on the rim of the plate after wash steps and affecting the final colour of the sample. Despite this, variation was observed between repeats performed under exactly the same conditions. This can be due to day-to-day differences between biological replicates, or non-adhered but biofilm-like bacterial aggregates being washed off. Nevertheless, the method is reliable to differentiate between samples with large differences in biofilm formation. In order to optimise the method, we performed several pilot studies in order to find the optimal growth conditions with regards to incubation time, plate coverings to prevent evaporation but retain access to oxygen, and rotation in the incubator. While this method is useful to classify bacteria as strong or poor biofilm producers, other methods are available which would more closely resemble the *in vivo* conditions and could be of interest for future studies. For example, growing biofilms in a flow cell system allows for the continuous cycling of medium and the removal of planktonic cells from the biofilm [267]. This can then be combined with fluorescence microscopy to observe the structure of the biofilm non-invasively over time. Another quantitative method is colony counting, which depends on reliably dislodging the biofilm from the surface by scraping or sonication, after which the suspension can be plated or counted with flow cytometry [268]. As we grew mixed-species biofilms which could not be distinguished by selective agar due to similar antibiotic resistances, we opted to use qPCR of the DNA content of wells to obtain an indication of the biofilm composition.

Discussion

At the beginning of this thesis, the status of *Achromobacter* species as CF pathogens was less accepted than it is today. Several clinical retrospective studies had been conducted with sometimes conflicting results, with some finding a correlation between *Achromobacter* infection and e.g. decreases in FEV_1 and other studies being unable to replicate the findings. The identified correlations between infections and clinical parameters have also been inconclusive as evidence of bacterial virulence, as persons presenting with *Achromobacter* infections often have a worse lung status from the start of the infection due to more advanced CF or older age. For this reason, it has been unclear whether *Achromobacter* infections are causative of lung injury or appear as a marker of lung damage caused by other pathogens. During the 2020s, additional studies on the virulence factors of the *Achromobacter* genus have advanced our knowledge on its pathogenicity. These recent studies include e.g. mouse models of *A. xylosoxidans* lung infections [237], identification of siderophores [223], and characterising its type III and VI secretion systems [7, 236, 241], which had previously only been identified on the genomic level [209].

Another issue causing uncertainty regarding the pathogenicity of *Achromobacter* species is the lack of knowledge about the difference in virulence between isolates, strains, or even species. For example, CF isolates appear to have higher production of siderophores [223] and antibiotic efflux pumps [248] than environmental isolates. However, there was to our knowledge no available information on the comparative virulence of different *Achromobacter* species. Due to the relatively low prevalence and the frequent classification of *Achromobacter* infections only by genus, it would be difficult to draw conclusions on patient outcomes based on infecting species through retrospective studies. To this end, we wanted to investigate the differences or similarities between species *in vitro*. In **Paper I**, we selected the four most common species found in CF infections: *A. xylosoxidans*, *A. insuavis, A. dolens* and *A. ruhlandii*. Most isolates were obtained from CF sputum from persons attending the CF centre in Lund. The bacteria were isolated by the laboratory for clinical microbiology, thus removing all human biological material from the samples. As the bacterial samples did not require any additional sampling and could not be traced to any person, it was not necessary to obtain consent towards obtaining these samples. Patient data was only available to the treating physician and was confidential to the research laboratory personnel.

On the other hand, blood samples from healthy donors (paper I and III) were drawn after obtaining written informed consent and was approved by the regional Ethical Review Board in Lund.

In this study, we prepared sterile-filtered culture supernatants of *Achromobacter* spp and *Pseudomonas aeruginosa* in order to obtain cell-free exoproducts. These were then used to stimulate cultures of CFBE41o- cells (ΔF508 human CF bronchial epithelial cells) as well as neutrophils and monocytes from peripheral blood of healthy donors. The choice of using exoproducts as stimulant was taken after considering infection models using live bacteria, which gave rise to results that were not considered consistently reproducible enough for publication. One important drawback of live infection models was the difficulty in analysing cellular toxicity. While we saw some results that indicated a difference in cytokine production between cells infected with *Achromobacter* spp and *P. aeruginosa*, we were not able to draw a conclusion on whether lower cytokine levels indicated less inflammation or a more rapid cell death. We attempted to investigate this by using viability assays. For this purpose, the MTT assay (a colorimetric assay measuring metabolically active cells) was deemed unsuitable as it disproportionately stained the bacterial cells. We also attempted the LDH assay (a colorimetric assay measuring the amount of membrane damage markers in the medium), which was disrupted by the presence of bovine serum in the culture medium. In the future, it would be of interest to study differential cytotoxicity of *Achromobacter* species using microscopy-based methods such as a fluorescent live/dead stain or anti-*Achromobacter* rabbit serum with fluorescent conjugate antibodies [269].

A strength of this study is the selection of multiple inflammatory markers and the comparison to *P. aeruginosa* as a control condition. Although *P. aeruginosa* is a well-known CF pathogen which can cause airway inflammation and injury, its exoproducts did not cause higher responses than the *Achromobacter* groups in this study. Although the exoproducts may contain more than proteins (e.g. exotoxins), we ruled out LPS as the main driver of inflammation by inhibiting it with polymyxin B. LPS also appeared to have a stronger effect on monocytes than on neutrophils, as the positive control LPS was caused CD11b upregulation on monocytes but not neutrophils. This effect was inhibited by the addition of polymyxin B. Although the presence of functional CFTR on neutrophils and monocytes may have affected the results somewhat compared to CF cells, we opted to not study samples from pwCF as the treatment protocols with e.g. antibiotics may also affect the results and complicate reproducibility. It may be of interest in future studies to study the effects of bacteria on leucocytes after the addition of a CFTR inhibitor. It would also be of interest to culture alveolar macrophages, as monocytes were included in this study primarily as a precursor to this cell type. A recent study has also identified CD14 as an important marker of lung pathology in murine models of *Achromobacter* infection, which could be a useful target for studying differential virulence in the future [270].

In conclusion, rather than to identify any strong differences between *Achromobacter* species, this study indicates that all species' exoproducts were able to cause equal or stronger inflammation than *P. aeruginosa*, which further supports the consideration of *Achromobacter* species as CF pathogens.

In **paper II**, we aimed to further characterise *A. xylosoxidans* with regards to its Tat secretion system. Secretion systems are important virulence factors in bacteria, and especially the type III and type VI secretion systems have been recently characterised as vital factors to *A. xylosoxidans* virulence [7, 236, 241]. In contrast to these secretion systems used to transport proteins outside the cell, the Tat secretion system is used to transport proteins from the cytosol into the periplasmic space. Tat secretion is an important determinant of *P. aeruginosa* virulence, as it can transport exotoxins such as phospholipases for further secretion through type II systems [242, 243]. In this study, we wanted to investigate whether the same was true for *A. xylosoxidans*. After the generation of an *A. xylosoxidans* strain with a deletion mutation in *tatC*, a gene encoding the TatC subunit required for Tat function, we characterised the mutant compared to the wild type strain to determine the importance of Tat secretion. We studied three main endpoints: Attachment to CFBE41o- cells, growth curves with and without iron, and proteomic contents of cellular fractions with and without iron. We identified limitations in growth rate and cell attachment in the TatC mutant, and the growth rate was not improved in the presence of iron as in the wild type. As the mutant was specifically targeted towards *tatC* deletion using a plasmid validated by sequencing, it is reasonable to assume these defects were directly caused by the lack of Tat secretion. However, it is more difficult to draw conclusions based on the results of the proteomic analysis. Out of 20 Tat substrate identified both by *in silico* prediction tools and LC-MS/MS, 9 were significantly associated with the wild type periplasm when compared to the Tat mutant, implying that the mutant was indeed unable to transport them to the periplasm due to its lack of functional Tat secretion. The annotation of these proteins did not link them to cell adhesion or iron acquisition, but they may play a role in cellular processes not directly linked to these phenotypes, and their importance cannot be confirmed nor ruled out by this study alone. One significant limitation of this study was the contamination of the periplasmic fraction by cytosolic proteins. This was seen in both the wild type and mutant, but was more pronounced in the mutant. As the method is dependent on separating fractions through centrifugation and separation of the pellet and supernatant by careful pipetting, it is inevitable that some degree of contamination of the fractions can occur. However, the fact that more leakage occurred in the mutant lead us to speculate that Tat secretion may also play a role in bacterial membrane integrity. This was also considered when culturing the mutant strain, as it grew in a more lumpy, aggregated fashion compared to the wild type dispersing evenly throughout the broth. The effect on Tat secretion on membrane integrity would be an interesting direction to explore in future studies, as targeting the Tat pathway function or its substrates might become a venue to develop new antimicrobial compounds [271-273].

In **paper III**, we continue the exploration of the *A. xylosoxidans* proteome with a focus on identifying IgG antigens. The presence of IgG responses in people with CF infected with *A. xylosoxidans* is not enough to eradicate the pathogen, and rather seems to correlate with a worse clinical status [257]. This is likely due to the multiple mechanisms the bacteria employ in order to evade immune responses, most importantly the biofilm mode of growth. We wanted to investigate the targets of these antibodies primarily to further our understanding of proteins which may be important to bacterial virulence, but also with the possibility of developing a serological assay if we identified antigens with high enough sensitivity and specificity. As treatment with CFTR modulators has changed the landscape of CF treatment by dramatically reducing sputum in eligible patients, chronic infections may still persist and require regular diagnostic culturing. This process is made more difficult by the lack of sputum production, and could perhaps be complemented by serological assays in people who produce antibodies against CF pathogens.

To this end, we utilised a novel affinity purification-mass spectrometry method developed by S. Chowdhury, J. Malmström and colleagues for the purpose of studying streptococcal antigens [274]. Through this collaboration, we also aimed to validate the method for antigen identification in gram negatives for the first time. After screening our cohort of CF sera on indirect ELISA plates coated with *A. xylosoxidans*, we selected a serum with high titres against the whole bacteria. The serum IgGs were then affixed to magnetic protein G beads and used to bind *A. xylosoxidans* proteins, which were then eluted and analysed with LC-MS/MS. The same process was also performed with a serum from a *P. aeruginosa* infection and negative controls. With this method, we identified 28 antigens bound to anti-*A. xylosoxidans* serum, out of which 8 were not found in the negative control and 4 not found in the anti-*P. aeruginosa* serum. Out of the 4 candidate antigens specific to *A. xylosoxidans*, we selected 3 to produce in recombinant form and use to coat ELISA plates for screening our entire serum cohort. The 3 selected antigens were higher in abundance than the fourth candidate, making them more likely to be a true IgG target. During a pilot study not described in our manuscript, we had previously identified the 3 selected candidates as *A. xylosoxidans* antigens but not the fourth, which supported our selection of these targets. The most abundant protein in the sample was flagellin, which was ruled out as a candidate due to not being specific to anti-*A. xylosoxidans* serum.

An important limitation of this study is the identification of antigens using only one serum as a representative of *A. xylosoxidans* infection. We aimed to offset this by validating the identified antigens in the ELISA against recombinant antigens, making us able to screen the entire cohort of 53 sera without having to repeat the laborious LC-MS/MS analysis. One of the antigens, a type I secretion C-terminal target domain-containing protein (T1S-DCP), had a surprisingly low specificity for anti-*Achromobacter* sera, as our singled-out *P. aeruginosa* control serum remained negative to this antigen but other sera in the control groups had titres against the

protein. The antigen Domain of unknown function (DUF) 336 had a very high specificity to the anti-*Achromobacter* sera, making it the most interesting candidate for the future development of a serological assay. Although the majority of anti-*Achromobacter* sera were also negative towards this antigen, the two consistently high-titre sera had a response multiple times higher than the rest of the group. The validation of DUF336 as a useful target for serology is severely limited by our group size, as we only had access to seven sera from pwCF infected with *Achromobacter*. Out of these, three were co-infected with *P. aeruginosa*, and two were infected with *A. insuavis* rather than *A. xylosoxidans*, making the group rather heterogenous. In contrast to the bacterial samples used in paper I, the serum samples were donated with written informed consent and approval of the regional ethics board. Although it would be difficult to expand the group size at this point, one possibility to explore could be to collaborate with other CF research centres, e.g. by sharing antigen-coated ELISA plates for screening other local cohorts. Another consideration for this study is the continuous updating of proteome annotations, as evidenced by the names of proteins identified in this study changing during the course of the research process. For example, the protein we identified as DUF336 is currently labelled as a heme-binding protein. Nevertheless, the validation of *ex vivo* binding of serum IgG to these antigens suggest that the amino acid sequences are indeed present in *A. xylosoxidans*, although perhaps as part of a larger protein structure or with a different function than previously predicted.

Finally, we investigated the interactions between CF isolates of *A. xylosoxidans* and *P. aeruginosa* in **paper IV**. At the time of initiating this study, there had only been two publications on this topic, indicating that some *Achromobacter* isolates - but not all - could inhibit the biofilm formation of *P. aeruginosa*. Starting out with the very broad question of "How do these two species interact with other?", we started to approach it with methods such as cross-streaking assays, plating both bacteria in lines and inspecting whether there appeared to be growth inhibition in the area where they intersect. Finding no conclusive interactions in this assay, we continued to attempt co-culturing in liquid broth and plating on selective agar to whether co-culturing inhibited either species in comparison to monocultures. In this experiment, we were able to study the effects of *P. aeruginosa* on the growth of *A. xylosoxidans* – but the opposite could not be investigated as there was no antibiotic with sufficient effect on *A. xylosoxidans* that did not also affect *P. aeruginosa*. The use of chromogenic agar plates to separate the two species proved promising, but was placed on hold as the medium was obtained as a sample product not yet on the market, and thus not sufficiently reproducible. Finally, the bacterial composition of broth co-cultured was quantified using qPCR. When proceeding to study the effects of interspecies interactions on co-cultured biofilms, we wanted to study both the effects of *A. xylosoxidans* on *P. aeruginosa* and vice versa. In this study, none of the *A. xylosoxidans* isolates formed biofilms on plastic when cultured in 96-well plates and quantified with crystal violet.

Although *A. xylosoxidans* biofilm formation has been described in the CF lung [204], the species has been shown to display poor adhesion to surfaces *in vitro* [254]. The low quantification of biofilms by the crystal violet assay may be due to the formation of aggregates rather than adhesion to the surface, which would cause the biofilm to be removed during the wash steps.

The field of polymicrobial interactions in the CF airway has thus far mostly been focused on the interactions between *P. aeruginosa* and *S. aureus.* The effects of these interactions can be either positive or negative to human health and remain relatively unexplored. For example, one pathogen exerting competition to inhibit another may be detrimental to the person's health by leading to microbiome dominance by a single pathogen. On the other hand, coexistence between pathogens may also be a negative outcome as multi-species biofilms may confer survival advantages to each other. When it comes to *P. aeruginosa* and *A. xylosoxidans*, co-infection with both pathogens appears to cause worse outcomes than one or the other [255]. For this reason, it would be interesting to also study the biofilms of *P. aeruginosa* when co-cultured with late non-inhibitory *A. xylosoxidans* isolates, comparing the biofilm composition to monocultures through e.g. imaging with fluorescence *in situ* hybridization. It would also be relevant to compare the interactions between *A. xylosoxidans* and *S. aureus* to those exerted by *P. aeruginosa*. Understanding the dynamics of biofilm growth is important not only in the context of CF, but also towards developing new antimicrobial treatments in a future increasingly threatened by the spread of antibiotic resistances. For example, targeting biofilms and dispersing bacteria to be more readily targeted by the immune system or antibiotics is one venue being explored to combat this problem [117]. The polymicrobial interactions exerted by probiotic bacteria competing with pathogens is another mechanism which may be of use as a treatment option.

As the introduction of CFTR modulator therapy has rapidly changed the standard of CF treatment during the course of this thesis, the research on airway infections remains important. The effect of modulator treatment on chronic infections is inconclusive to date. Although the reduction in sputum volume decreases bacterial load initially, some studies have indicated that chronic infections persist and inflammatory markers can rebound during continuous treatment [41, 275]. Additionally, research towards improving care for people with CF unable to benefit from CFTR modulators must not be neglected.

In this thesis, we provide new insight into the properties of *Achromobacter xylosoxidans:* Its differences and similarities to other *Achromobacter* species, its protein secretion, antigen targets and polymicrobial interactions, indicating several similarities in virulence mechanisms to *P. aeruginosa* and supporting its recognition as a true CF pathogen.

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