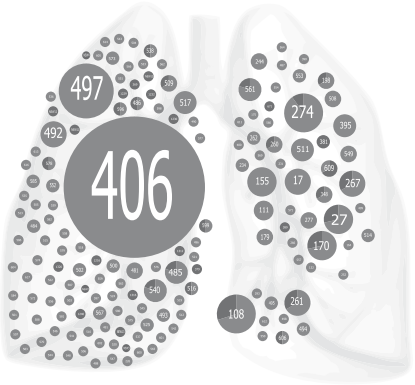

***Pseudomonas aeruginosa* colonization in patients with cystic fibrosis;**
population structure, the Dutch clone and effects of segregation

Rosa van Mansfeld

Pseudomonas aeruginosa colonization in patients with cystic fibrosis

PhD thesis, University of Utrecht, The Netherlands

ISBN: 978-94-6169-574-1

Layout and printed by: Optima Grafische Communicatie, Rotterdam, The Netherlands

Printing of this thesis was financially supported by Astellas Pharma B.V. , BD, ChipSoft B.V., Infection & Immunity Centre Utrecht, Forest Pharma B.V., The Netherlands Society of Medical Microbiology (NVMM) and Novartis Pharma B.V.

Pseudomonas aeruginosa colonization in patients with cystic fibrosis;
population structure, the Dutch clone and effects of segregation

Pseudomonas aeruginosa kolonisatie in patiënten met cystische fibrose;
populatie structuur, de Nederlandse kloon en effecten van segregatie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 27 november 2014 des middags te 4.15 uur

door

Rosa van Mansfeld
geboren op 12 februari 1978 te Woerden

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	Prof.dr. C.K. van der Ent
Copromotor:	Dr. R.J.L Willems

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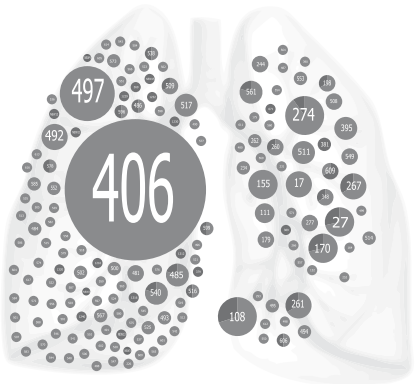
General introduction



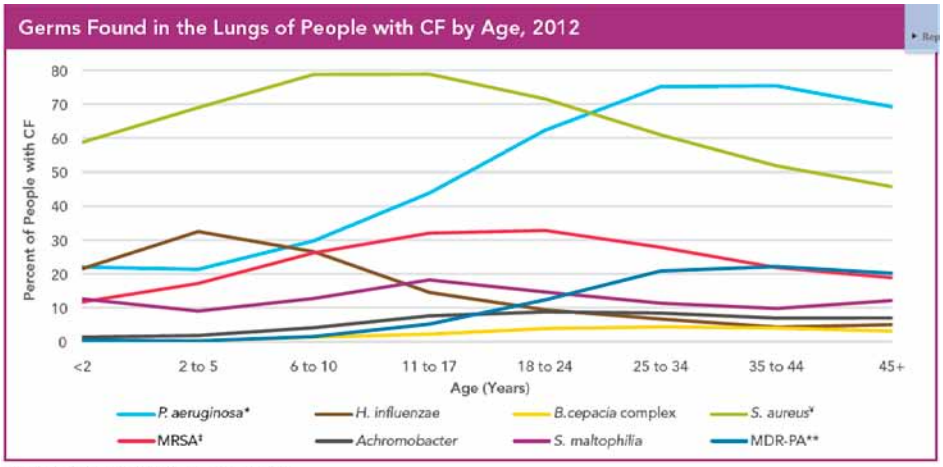
General introduction

Cystic Fibrosis

In the Netherlands one of every 4750 newborns has cystic fibrosis (CF)[1]. Cystic fibrosis is an inherited disease that is caused by mutations in the gene that encodes the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) that is located on human chromosome 7. This protein is a chloride ion channel that enables transport of water and electrolytes over epithelial cell surfaces and mutations in the *CFTR* gene affect functioning of this chloride channel. The main clinical symptoms in CF patients are inflammation and infection of the lungs and the disruption of passage through the gastro-intestinal tract that can lead to obstruction and pancreatic dysfunction[2]. The exact mechanism of how the defective CFTR causes increased susceptibility to infection in the lungs remains unclear. The defective chloride ion transport results in very viscous mucus in the lungs that interferes with the mucocilliary clearance of inhaled microorganisms and obstruction by mucus plugs[3]. However, it has been shown that CFTR dysfunction also directly affects host-pathogen interaction through different components of the innate mucosal immune system[4]. This indicates that the pathogenesis of pulmonary disease in CF patients is likely to be multi-factorial[2]. Inflammation and chronic infection of the lungs lead to tissue destruction and fibrosis which leads to decreased lung function and respiratory failure which is the main cause of death in CF patients[5]. More intensive monitoring of patients with CF and early start of aggressive antibiotic treatment have contributed to a steady increase in life expectancy. The predicted median survival of CF patients in the United States of America has increased from 29,4 years in 1992 to 41.1 years in 2012[6]. In the Netherlands the median age of patients that died in 2012 was 38 years[7]. Since 2012 CF screening is performed in all children in the Netherlands in the first week after birth.

Respiratory infections

Many different bacteria but also viruses and fungi can be found in the CF lungs. In the respiratory tract of one CF patient multiple niches exist, even within one mucus layer, with different oxygen levels, nutrient availability, components of the immune system and concentration of antibiotics, when administered. Culture independent techniques have shown that more than 1300 bacterial species can exist in the respiratory tract of one CF patient[8]. In this highly populated niche microbial interactions between the different bacterial species have been documented. For instance, virulence of *Pseudomonas aeruginosa* can be modified by resident bacteria like streptococci[9;10] and antibiotic resistance and growth characteristics of *Staphylococcus aureus* can be influenced by *P. aeruginosa*[11;12]. This indicates that the respiratory tract of CF patients forms a complex and dynamic ecosystem of which many aspects are still unknown. However, thus far, a



*P. aeruginosa *includes people with MDR-PA.*
***MDR-PA is multi-drug resistant* Pseudomonas aeruginosa (P. aeruginosa).*
*¥ S. aureus *includes people with MRSA.*
‡ MRSA is methicillin-resistant Staphylococcus aureus (S. aureus).

Figure 1. Percentage of different microorganisms found in patients with cystic fibrosis under care at CF Foundation-accredited care centers in the United States, who consented to have their data entered in 2012[6]. Reproduced with permission.

relatively small number of bacteria have been associated with CF lung disease. Early in childhood *Haemophilus influenzae* and *S. aureus* can cause symptomatic infections. Later in life *P. aeruginosa* causes up to 75% of lung infections and over 50% of adult patients is chronically infected with *P. aeruginosa*[6;13] (Fig. 1). These chronic infections are almost impossible to eradicate, even with aggressive antibiotic therapy, and are often maintained throughout the lifetime of the individual patients[14].

Pseudomonas aeruginosa

P. aeruginosa is a gram negative, rod-shaped bacterium that is well adapted to live in moist environments[15]. This versatile bacterium grows in (surface) water and can infect plants and animals as well as humans. *P. aeruginosa* can use a wide array of compounds for its growth which enables it to grow in diverse ecological niches including those where nutrients are limited[16]. Although, *P. aeruginosa* is an aerobic microorganism, when oxygen is limited (e.g. in biofilms), *P. aeruginosa* is able to use nitrogen or arginine as electron acceptor for its metabolism[17]. Its ubiquitous growth capacity combined with a high intrinsic resistance against antibiotics and disinfectants and the ability to readily acquire resistance mechanisms makes *P. aeruginosa* an important pathogen for humans[18]. Healthy humans are rarely infected, but this pathogen is infamous for causing nosocomial infections in immunocompromised patients, ventilated patients in the intensive care unit and patients with burn wounds. It can also cause chronic

infections like otitis media, chronic ulcers and chronic lung infections in patients with cystic fibrosis[16].

P. aeruginosa genome

Whole genome sequencing revealed that *P. aeruginosa* has a very large genome varying from 5.5 to 7 million nucleotides that codes for more than 5500 genes of which approximately 10% consists of regulatory genes[16;19]. This large and complex genome probably permits *P. aeruginosa* to adapt to and thrive in diverse environmental niches as different as river water and human lungs. Approximately 90% of the genome is highly conserved with low sequence diversity of 0.5-0.7%[19]. This sequence diversity is similar along the core genome with only a few highly divergent hotspots in genes coding for pyoverdine synthesis and uptake (iron-scavenging siderophores), flagella and O-antigen (the polysaccharide part of LPS). These proteins are important for virulence and are immunogenic and therefore under diversifying selection in patients[20;21]. The highly variable accessory genome consists of extrachromosomal elements like plasmids and blocks of DNA inserted at various loci in the chromosome called "regions of genome plasticity" (RGP). So far 89 of these regions have been described and most sequenced strains carry about 40 RGPs with insertions[22]. Comparison of 28 whole genome sequences (WGS) of different CF-related strains and 4 non-CF-related strains demonstrated that the accessory genome content was often shared among isolates with closely related core genomes[20]. However, another WGS study showed that even within one sputum sample, two *P. aeruginosa* isolates with almost identical core genome had different prophages as part of their accessory genome[23].

P. aeruginosa virulence

Many virulence determinants are part of the *P. aeruginosa* core genome[18;20]. For initial establishment of acute respiratory tract infection flagella are important for motility and chemotaxis, as well as type IV pili (adhesins) for adhesion and twitching motility and LPS (O-antigen) for adhesion and interaction with TRL4 and CFTR[18]. These are all highly immunogenic components, which implies that they are under selection pressure by the human immune system in chronic infections. Several virulence factors that cause cytotoxicity and tissue destruction are proteins that are excreted by the type III secretion system (T3SS) like ExoS, ExoU, ExoT and ExoY. These proteins are injected directly into the cytoplasm of the host cell by this secretion apparatus and disturb host cell signaling pathways[24]. *P. aeruginosa* has many other secretion systems, like secretion system type I, II, IV, V and IV that secrete other proteins like exotoxin A, pyoverdin, pyocyanin, elastases, rhamnolipids and phospholipase C that cause host cell damage or alveolar surfactant degradation[18;24]. Many of the virulence factors are under regulation of quorum sensing systems. Quorum sensing is a process of bacte-

rial communication whereby the bacteria excrete signal molecules called autoinducer molecules. When proliferation of bacteria results in a critical concentration of these molecules, expression of genes is changed in response. There are at least three different quorum sensing systems present in *P. aeruginosa*[25]. Quorum sensing also regulates biofilm formation, which is another important virulence factor that plays a major role in chronic infections, protecting the bacteria against antibiotics and components of the host immune system. In addition, *P. aeruginosa* can also harbor genomic islands harboring genes which can increase its pathogenic potential. These islands can be transferred by horizontal gene transfer[20].

P. aeruginosa adaptation to the CF lung

Compared to the environment the human body is a warm, humid and nutritious niche[14]. However, in the CF lungs *P. aeruginosa* also encounters many stressful conditions like osmotic stress from the high viscosity of the mucus, different levels of oxygenation, other resident microorganisms, antibiotics, neutrophils that release ROS (reactive oxygen species) and other components of the immune system[5]. *P. aeruginosa* can express a variety of virulence factors that play a role both in the initial infection and chronic colonization of CF lungs as described above. First colonization of CF patients by *P. aeruginosa* is often by strains that seem to originate from the environment[26]. A period of intermittent infections that can still be eradicated by antibiotic therapy eventually leads to a chronic infection in the CF patient. In 2012 in the UK 35% of CF children up to 16 years of age had a lung infection with *P. aeruginosa* with only 10% being chronically colonized, while in adult patients 68% was infected of which 54% chronically[13]. *P. aeruginosa* isolates from chronically infected CF patients have adapted to the specific niche of the CF lung and show specific phenotypic traits. Immunogenic factors like flagella, type III secretion proteins, O-antigen, exotoxin and protease are lost during adaptation to the CF lung[27;28]. The mucoid form of growth caused by excessive alginate production is an important phenotypic trait of chronic isolates[14]. The alginate forms a slime layer around the bacteria which protects them against antibiotics and components of the immune system. Mucoid growth has been a marker for chronic infection although this trait can also be lost again during chronic colonization[5;14]. Analysis of the accessory genome of 28 CF related strains showed enrichment of genes involved in oxidation-reduction processes, regulation of transcription, pathogenesis, transmembrane transport and metabolic processes[20]. Genes involved in redox functionality in the core genome contained most mutations. Besides, genes involved in redox functionality were also highly present in the accessory genome. This indicates that these genes, that are necessary for surviving oxidative stress caused by macrophages and neutrophils and toxic nitric oxide that is produced during anaerobic respiration, are important inside the CF lungs[20]. Presence of certain genomic prophages have shown to be critical

for in vivo competitiveness of a CF- related epidemic *P. aeruginosa* clone[29]. In general, however, mutations in specific "pathoadaptive genes" as driving force for pathogen fitness seems to be more important in the pathogenesis of chronic CF infection than the acquisition of virulence genes through horizontal gene transfer[30;31]. The important role of mutation in lung adaptation is illustrated by the fact that about half of the clonal isolates in CF patients became hypermutators in one study, with a presumed selective advantage, not only because of a higher evolution rate but also differential mutagenesis in homopolymer containing genes possibly affecting immunogenicity[30].

Antibiotic treatment and resistance

P. aeruginosa is intrinsically resistant to various classes of antibiotics like some beta-lactam antibiotics due to a chromosomally encoded AmpC cephalosporinase, and tetracyclines, chloramphenicol, macrolides, trimethoprim and sulfonamide due to less permeability and effluxpumps[32]. Resistance to other antibiotics like carbapenems, aminoglycosides and fluoroquinolons can be acquired. These resistance mechanisms include mutations that influence expression of efflux pumps, mutations in target sites, membrane modification and expression of enzymes that inhibit or modify antibiotics. In addition, the biofilm mode of growth in the lungs also impairs antibiotic activity. CF patients are treated with repeated oral, intravenous and inhaled antibiotics during their lifetime. Antibiotics that are used for therapy are beta-lactams, aminoglycosides, fluoroquinolons and colistin. However, resistance can occur against all of these antibiotics and this is reason for concern[33]. Eradication of early infection with *P. aeruginosa* in CF patients and prevention of chronic infection has been associated with clinical benefit and is nowadays standard of care. Successful Antibiotic Eradication Therapy (AET) has shown to preserve lung function compared to chronically infected patients[34-36] and failure of early eradication of *P. aeruginosa* is associated with an increased risk of exacerbation[37]. A commonly used AET is 28 days of tobramycin inhalation, however other treatment protocols have also shown to be beneficial[38]. For chronically infected patients suppression therapy is considered beneficial[39]. Antibiotics that are used for suppression are tobramycin, aztreonam and colistin. These antibiotics are administered though inhalation or nebulization often in alternating cycles of 28 days on and off treatment. Exacerbations in chronically infected CF patients are treated with intravenous antibiotics like aminoglycosides, fluoroquinolones or beta-lactam antibiotics.

Typing methods

Pulsed Field Genome Electrophoresis (PFGE) has long been the gold standard for typing of *P. aeruginosa*. This method has a good discriminatory value and is suitable to study local and short term epidemiology but due to the lack of reproducibility not suitable to be used for library typing, i.e. to combine typing data from different research groups to

study the global and long-term epidemiology and population structure of *P. aeruginosa*. Therefore, a multi-locus sequence typing (MLST) scheme for *P. aeruginosa* was developed[40;41]. MLST is a robust, reproducible and portable typing method and therefore perfectly suitable for library typing. For *P. aeruginosa* seven fragments of housekeeping genes are sequenced and alleles assigned to analyze genetic relatedness.

In addition to MLST different 'Multi-locus variable number of tandem repeats analysis' (MLVA) schemes have been developed to study genetic relatedness and population structure of *P. aeruginosa*. MLVA relies on indexing the variation in numbers of tandem repeats in multiple loci in the genome of *P. aeruginosa*. Since the biological clock of these loci runs fast, this provides a highly discriminatory typing scheme. However, since the number of tandem repeats is determined by estimating sizes of PCR products on an agarose gel and not by sequencing, the robustness and reproducibility of MLVA is less of that of MLST.

Finally, the last 2-3 years, whole genome sequenced (WGS) based epidemiology has started to replace the other typing methods. In 2001 the first whole genome of *P. aeruginosa* was published[16], in 2011, 2012 and 2013 respectively 9, 30, and 168 whole genome sequences of different *P. aeruginosa* isolates became available in the NCBI database[42]. Because of costs and complexity of data analysis WGS has not yet fully replaced all the other typing methods to study the short-term local as well as long-term global epidemiology of *P. aeruginosa*. However, since costs for sequencing have dropped considerably and more and more tools have become available for rapid WGS data analysis[43], it is to be expected that WGS will become the preferred method to study genetic relatedness also for *P. aeruginosa*.

In this thesis both MLST and MLVA have been used to study the *P. aeruginosa* population structure, transmission and persistence in CF patients and other patient groups.

Molecular epidemiology

Studies of *P. aeruginosa* isolates from the environment, CF patients and other clinical specimens have revealed that the genetic diversity found in a certain geographical setting is indistinguishable from the genetic diversity found worldwide[44-46]. For example, *P. aeruginosa* isolates cultured from a Belgian river show as much genotypic diversity by AFLP typing as the diversity worldwide. Similarly, the diversity of clinical and environmental *P. aeruginosa* isolates found in Queensland, Australia was similar to the genetic diversity found worldwide using MLST. Furthermore, genotyping of *P. aeruginosa* isolates from wastewater lagoons in France by PFGE and MLST showed genotypes that were also found in clinical samples in the same area of which some belonged to prevalent clones described worldwide[47]. All these studies indicate that there is little association between genotype and ecological niche[20;44]. In contrast, other studies have shown that certain clonal complexes seem to be overrepresented in particular

populations that sometimes occur worldwide, suggesting evolutionary advantage of these clones[48;49]. An important example of this is that in CF patients different successful epidemic clones have been described, some of which seem to occur only in local settings while others are dispersed around the world[50-53]. Furthermore, isolates from the ocean near Japan were found to cluster distinct from other *P. aeruginosa* strains indicating distinction between different habitats[54]. Also, the role of recombination as driver for genetic diversity is disputed in literature. Some claim that recombination is more important than mutation[45;49] and describe the population structure of *P. aeruginosa* as non-clonal epidemic[44;45], while others hypothesize that the population structure of *P. aeruginosa* is likely shaped in the environment and dominated by several "extended clonal groups" that are selected in different ecological niches with only limited recombination[15]. Future WGS-based analyses of a geographically and ecologically diverse set of *P. aeruginosa* strains may provide more insight in the exact population structure and the drivers of genetic diversity of this species.

Epidemic clones and segregation

Since *P. aeruginosa* is omnipresent in our environment it has long been thought that CF patients acquire their own *P. aeruginosa* strain from the environment and that sharing of genetically identical strains between CF patients only happens between household contacts[55]. However, in 1996 an alarming report was published of an antibiotic resistant *P. aeruginosa* strain that had spread amongst CF children visiting a clinic in Great Britain[56]. Other reports of specific *P. aeruginosa* clones that were found amongst CF patients followed[57-59], which suggested a role of cross-transmission of these 'epidemic' strains between patients. Different countries described different high-prevalent clones

Table 1 Prevalent or epidemic clones harbored by CF patients.

	year first published	distribution	isolated	Ref
Clone C	1994	world	environment , clinical isolates, CF lungs	66
Liverpool epidemic strain (LES)	1996	world	CF lungs, clinical isolates	56
Manchester	2001	Great Britain	CF lungs	57
Norway	2001	unknown	unknown	67
Midlands	2004	Great Britain	CF lungs	53
AES-1 (Melbourne)	2000	Australia	CF lungs	59
AES-2 (Brisbane)	2004	Australia	CF lungs	64
AES-3 (Tasmania)	2008	Australia	CF lungs	68
Denmark red	2007	Denmark	CF lungs	69
Denmark blue	2007	Denmark	CF lungs	69
Netherlands	2007	Netherlands	unknown	65

among CF patients (table 1). There are indications that these clones are so successful because they are well adapted to the CF lung niche. Some clones like Clone C can be found worldwide both in the environment as well as in clinical isolates, indicating abundance as key factor for its success[60] while the Melbourne epidemic clone was not detected outside CF patients, which might indicate that adaptation to the CF lung or enhanced transmission is responsible for its success[59;61]. Some studies described an association of these CF related clones with an unfavorable clinical outcome[62-64]. Although these studies were cross-sectional and often prompted by the finding of resistant *P. aeruginosa* isolates, they did raise major concerns about cross-transmission of *P. aeruginosa* between CF patients. A study on the transmission of *P. aeruginosa* among patients attending a CF summer camp in the Netherlands in 2001 showed the occurrence of a dominant clone and three cases of probable transmission of *P. aeruginosa* strains between children that visited the camps[65].

Based on the presence of these epidemic clones among CF patients and presumed detrimental effects of these clones on disease progression segregation policies have been implemented for CF patients worldwide. Some countries implemented cohort segregation where patients with positive *P. aeruginosa* cultures are separated from negative patients[70;71]. Other countries implemented stricter segregation policies by separating patients harboring clonal or resistant *P. aeruginosa* strains in a distinct cohort or by separating all CF patients[72]. In the Netherlands segregation was implemented in 2006, and consisted of strict hygiene rules and the recommendation to avoid all contact between individual CF patients in inpatient and outpatient clinics, as well as outside the hospital setting[1;73]. Outside hospital settings it was recommended not to meet with other CF patients, or to adhere to strict hygiene rules (keep distance, don't touch, hand hygiene, cough hygiene, don't share utensils or medication) in case of contact. However, the population structure of *P. aeruginosa* in Dutch CF patients and the possible occurrence of epidemic clones were not known at the time this segregation policy was implemented. Therefore, we initiated a series of studies investigating the *P. aeruginosa* population structure and the effect of segregation on prevalence of *P. aeruginosa* among Dutch CF patients.

Aims and outline of this thesis

The major aims of this thesis were to investigate the population structure of *P. aeruginosa* among CF patients in the Netherlands and the effects of the segregation policy on this population structure. More specifically we aimed to:

- Investigate the population structure of *P. aeruginosa*, specifically in CF patients, with a focus on prevalence and genetic relatedness of dominant genotypes.
- Gain insight in transmission and persistence of the different *P. aeruginosa* genotypes.
- Evaluate the segregation policy as implemented in the Netherlands to answer the questions whether segregation has led to reduction of the prevalent clones and less acquisition of chronic *P. aeruginosa* infections.
- Investigate the high-prevalent *P. aeruginosa* CF clone both on phenotypic traits and on genomic level to gain insight in the specific link between this clone and the CF lung.

In chapter two we studied the population structure of *P. aeruginosa* in Dutch CF patients by MLST-based genotyping of *P. aeruginosa* isolates from respiratory cultures of approximately 45% of all Dutch CF patients. We describe two prevalent specific Dutch clones; ST406 and ST497 which seem age related. In chapter three we evaluated different typing techniques, presented a newly developed MLVA genotyping protocol and investigated the population structure of *P. aeruginosa* in different Dutch patient populations. In chapter four we assessed transmission of the different genotypes of *P. aeruginosa* during a one day open air event where CF patients meet in spite the segregation policy. In chapter five we investigated the persistence of the different *P. aeruginosa* genotypes during a nine year time interval and the clinical relevance of harboring the Dutch prevalent clone (ST406) in patients that went to CF summer camp in 2001. In chapter six we evaluated the segregation policy and the effect it has had on the population structure, acquisition of chronic *P. aeruginosa* infection and clinical relevance of harboring ST406 in a larger longitudinal cohort. In chapter seven we analyzed the high-prevalent *P. aeruginosa* clone ST406 and investigate its pathogenicity using whole genome sequencing, transcriptomics, biofilm formation assays and other phenotypic assays. In chapter eight the findings described in this thesis are discussed.

This thesis provides a description of the population structure of *P. aeruginosa* in clinical isolates and specifically in the lungs of Dutch CF patients, a quantification of the effects of segregation on the population structure and acquisition rates of chronic *P. aeruginosa* infection, a description of the specific traits of a Dutch high-prevalent CF clone, and a quantification of the clinical effects of chronic infection with this clone.

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Chapter 2

Pseudomonas aeruginosa genotype prevalence in Dutch cystic fibrosis patients and age dependency of colonization by various *P. aeruginosa* sequence types

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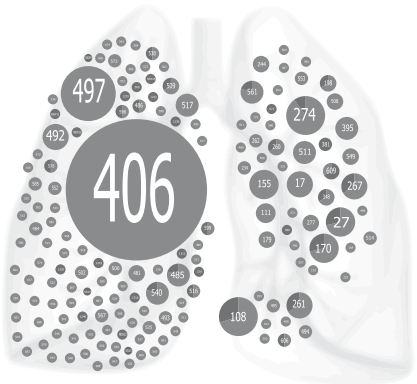
Journal of Clinical Microbiology, Dec. 2009, p.4096-4101



Abstract

The patient-to-patient transmission of highly prevalent *Pseudomonas aeruginosa* clones which are associated with enhanced disease progression has led to strict segregation policies for cystic fibrosis (CF) patients in many countries. However, little is known about the population structure of *P. aeruginosa* among CF patients. The aim of the present cross-sectional study was to determine the prevalence and genetic relatedness of *P. aeruginosa* isolates from CF patients who visited two major CF centers in the Netherlands in 2007 and 2008. These patients represented 45% of the Dutch CF population. *P. aeruginosa* carriage in the respiratory tract was determined by standard microbiological culture techniques, and all phenotypically different isolates in the first specimens recovered in 2007 and 2008 were genotyped by multilocus sequence typing. A total of 313 (57%) of 551 patients whose samples were cultured carried *P. aeruginosa.* Two sequence types(STs), ST406 and ST497, were found in 15% and 5% of the patients, respectively, and 60% of the patients harbored a strain that was also found in at least 2 other patients. The risk ratios for carrying ST406 and ST497 were 17.8 (95% confidence interval [CI], 7.2-43.6) for those aged between 15 and 24 years and 6 (95% CI 1.4-26.1) for those aged >25 years. ST406 and ST497 were not genetically linked to previously described epidemic clones, which were also not found in this CF population. The population structure of *P. aeruginosa* in Dutch CF patients is characterized by the presence of two prevalent STs that are associated with certain age groups and that are not genetically linked to previously described epidemic clones.

Introduction

Pseudomonas aeruginosa is a ubiquitous, versatile bacterium that can infect humans as well as plants and animals. The species is infamous for causing nosocomial infections in immunocompromised patients and patients in intensive care units and is a major cause of morbidity and mortality in patients with cystic fibrosis (CF)[26]. The widely held belief that CF patients acquire *P. aeruginosa* strains mainly from their inanimate environment, with most patients being colonized by unique strains, has been challenged by reports indicating that *P. aeruginosa* clones may frequently be transmitted between CF patients[3;6;18;19;23;24]. Some of these clones, such as the Liverpool epidemic strain and the Melbourne epidemic strain, have been associated with enhanced disease progression and higher rates of mortality, respectively[1; 13]. In the Netherlands, the patient-to-patient transmission of *P. aeruginosa* was documented during a summer camp[4]. These findings have led to strict segregation policies for CF patients in many countries, including the Netherlands. However, despite these studies, there is little information on the population structure of *P. aeruginosa* within populations of CF patients. We therefore investigated the prevalence and genetic relatedness of *P. aeruginosa* isolates compared to those of the international known genotypes in an unbiased cohort representing 45% of the CF population in the Netherlands in 2007 and 2008.

Materials and methods

Patients and design

All CF patients who visited the Wilhemina Children's Hospital/University Medical Centre Utrecht (UMCU), The Netherlands in 2007 or the Haga Teaching Hospital (Haga) in The Hague, The Netherlands, from August 2007 until June 2008 were included in this cross-sectional study.

Samples

All sputum and throat swab samples were cultured by the standard diagnostic laboratory protocols of each hospital. At UMCU, the samples were plated on Trypticase soy agar II with 5% sheep blood (TSA-blood) plates and MacConkey agar plates (Becton, The Netherlands) to detect *P. aeruginosa*. The plates were incubated at 37°C for 2 days, after which the isolates were identified on the basis of the oxidase reaction and Gram stain. At Haga, sputum samples were washed in phosphate buffer (pH 7.2 to 7.4) and plated on Columbia agar–5% sheep blood–colistin–aztreonam, chocolate, cystine lactose electrolyte deficient, and cepacia agar plates (Oxoid, The Netherlands). On the second day, all nonfermenters were plated on a cystine lactose electrolyte-deficient

plate(Oxoid, The Netherlands) and grown at 37°C in 5% CO2 for 36 h. All lactose negative colonies were plated on Columbia agar with C-390 and phenanthroline (Oxoid) and were grown at 37°C for a maximum of 3 nights. In addition, oxidase, acetamide, and antibiotic resistance tests (BBL discs; Becton Dickinson, Belgium) were performed. MICs were determined by using the CLSI breakpoints for colistin, tobramycin, ciprofloxacin, ceftazidime, and piperacillin-tazobactam in both hospitals[7]. Furthermore, the susceptibilities to piperacillin, amikacin, and meropenem were determined at UMCU and the susceptibility to imipenem was determined at Haga. One *P. aeruginosa* colony of each different colony morphology (according to rough, smooth, and mucoid characteristics and colony size) per sample was randomly picked and stored at -70°C. These isolates from the first respiratory tract culture that yielded *P. aeruginosa* from each patient during the study period were genotyped. Multilocus sequence typing (MLST) was used to confirm the correct species identification and to study the genetic relatedness of the *P. aeruginosa* isolates recovered.

MLST

MLST was performed according to the protocol of Curran *et al.*[8]. Some adjustments were made, including the use of newly designed primers (Table 1), because the initial protocol did not yield PCR products in all cases. The isolates were recultured on TSA-blood plates (Becton) overnight at 37°C, suspended in 20 µl lysis buffer (0.25% sodium dodecyl sulfate, 0.05 N NaOH), and incubated at 95°C for 20 min. The cell lysate was centrifuged and diluted with 180 µl buffer (10 mM Tris-DCl, pH 8.5). After thorough mixing of the solution, another centrifugation at 16,000 X g for 5 min was performed

Table 1: Primers used for MLST (both amplification and sequencing).

acsA for:	aagggcgtgctgcatacca
acsA rev:	cggccaggagtcgaggatc
aroE for:	atgtcaccgtgccgttcaag
aroE rev:	gcgccagaggaagaatgcc
guaA for:	actacggcgtgcaattccac
guaA rev:	gaacgggtggcggtagacc
mutL for:	agcctggcaggtggaaacc
mutL rev:	ctctccagcacgctctcgg
nuoD for:	gggacatgtacggcatcacct
nuoD rev:	gcgcaggatgctgttcttca
ppsA for:	cggtcaaggtagtggacgtcg
ppsA rev:	ttcttgcgcacatcgaaacc
trpE for:	cgcgaggactatgaaaacgc
trpE rev:	cgcttgttgatggtttctt

to remove the cell debris. The supernatants were frozen at -20°C until further use. A total of 2.5 µl of the lysate was used in a touchdown PCR, according to the protocol of Curran *et al.*[8]. Q buffer (Qiagen Benelux B.V., Venlo, The Netherlands) was added to the PCR mix. The PCR was conducted as follows: 10 min at 96°C and then 10 cycles of 30 s at 95°C, 30 s at 65°C with the use of a temperature 1°C lower every cycle, and 1 min at 72°C. This was followed by 25 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C, after which a final step of 7 min at 72°C was used. A check for the presence of PCR products was performed by electrophoresis on a 1% agarose gel. The PCR products were sequenced (BaseClear, Leiden, The Netherlands) with the same primers used for amplification. The sequences were analyzed by using the Bionumerics (version 5.1) program (Applied Maths, St-Martens-Latem, Belgium).

Sequence types (STs) were compared to those on the *P. aeruginosa* MLST website (http://pubmlst.org/paeruginosa/) developed by Keith Jolley[17], and new alleles and profiles were sent to the curator (A. Baldwin). MLST data on genetic similarity were also confirmed by PFGE (data not shown).

Statistics

Calculations and analyses were performed with the SPSS (version 12.0) program (SPSS, Chicago, IL). Genetic diversity was calculated as described previously by using the EpiCompare (version 1.0) program[14]. The eBURST algorithm[10], available at the eBURST (version 3) website (http://eburst.mlst.net), was used to display the *P. aeruginosa* population snapshot of 591 *P. aeruginosa* STs available at http://pubmlst.org/paeruginosa/ (September 2009). The following settings for the creation of the eBURST-based snapshot were used: number of loci per isolate, 7; minimum number of identical loci for group

definition, 0; minimal single-locus variant count for subgroup definition, 3; and number of resamplings for bootstrap analysis, 1,000.

Results

Patient characteristics

There are an estimated 1,300 CF patients in the Netherlands, and 596 (46%) of these patients visited UMCU (n=386) or Haga (n=210) during the study period. The average ages of the study population were 19.5 years (standard deviation [SD], 11.8 years; range, 1.7 to 55.9 years) for those visiting UMCU and 30.7 years (SD, 14.2 years; range, 0.9 to 69.1 years) for those visiting Haga (P = 0.001). The majority of patients (53%) were male, with the gender distributions in both centers being similar. During the study

period, respiratory tract samples (sputum or throat swab samples) were cultured from 551 (92%) patients, and *P. aeruginosa*

was identified in 313, yielding a prevalence in the sampled patients of 57% (55% at UMCU and 60% at Haga). Forty percent of the children (age, <18 years; n = 249) were colonized, as were 70% of the adults (age, ≥18 years; n = 302). A total of 443 *P. aeruginosa* isolates from 265 (85%) of 313 patients were genotyped.

Prevalence of *P. aeruginosa* genotypes

Among the 443 isolates that were genotyped, 142 different STs were identified, of which 43 were shared by patients. Although 135 (51%) colonized patients were simultaneously colonized with two or more phenotypically different isolates, isolates with different STs were found in only 28 (11%) patients. A separate analysis of throat swab versus sputum specimens did not yield a different interpretation (data not shown).

ST406 was found in 41 patients and ST497 was found in 14 patients, yielding prevalence rates of 15% (41/265) and 5% (14/265), respectively. Other genotypes were found among nine patients (ST274), seven patients (STs 108 and 155), six patients (STs 17, 492, and 511), five patients (STs 27, 170, 395, and 517), four patients (STs 111, 261, 267, 485, 540, and 561), and three patients (STs 179, 244, 277, 481, 508, and 549); and 19 STs were shared by two different patients. The *P. aeruginosa* population in Dutch CF patients is highly diverse, with the genetic diversity being 97.3% (95% confidence interval [CI], 96.2% to 98.4%). Among 173 patients, 99 different STs were found at UMCU, and 70 different STs were found among 92 patients at Haga. This results in a lower level of genetic diversity among the isolates from patients from UMCU (96.2%; 95% CI, 94.3% to 98.1%) than among the isolates from patients from Haga (98.5%; 95% CI, 97.8% to 99.3%). In total, 70% of all colonized patients harbored *P. aeruginosa* STs that were also found in other patients and 30% were colonized with isolates with unique STs. Seven percent of all patients were colonized with both isolates with unique STs and isolates with shared STs.

The most prevalent ST, ST406, had a higher prevalence in the UMCU population (20%) than in the Haga population (7%) (risk ratio [RR], 2.6; 95% CI, 1.2 to 5.6). ST497 had a comparable prevalence in both hospital populations (5% and 6% in the UMCU and Haga populations, respectively). The location of the patient's residence was not correlated with the carriage of ST406 or ST497 (Fig. 1).

The mean age of the patients colonized with ST406 strains was 19.8 years (SD, 3.6 years), whereas the mean ages were 32.8 years (SD, 8.6 years) for those colonized with ST497 strains and 26.8 years (SD, 14.1 years) for those colonized with non-ST406 and non-ST497 strains (P = 0.001). The prevalence of ST406 was 40 to 53% among colonized patients between 15 and 24 years of age (Fig. 2), yielding an RR of 17.8 (95% CI, 7.2 to 43.6) for ST406 carriage in this age group compared to the other age groups. The

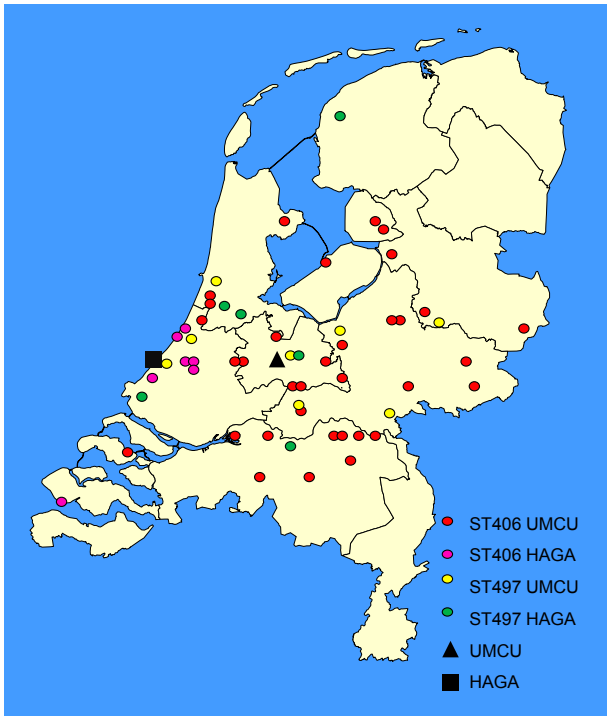


Figure 1. Locations of residences in the Netherlands of CF patients harboring the predominant Dutch *P. aeruginosa* clones ST406 and ST497.

prevalence of ST497 was as high as 14% among colonized patients between 25 and 35 years of age (Fig. 2), and the RR for the carriage of ST497 in CF patients older than 25 years was 6.0 (95% CI, 1.4 to 26.1). Similar to the two highly prevalent clones, other genotypes shared by at least three patients also appeared to be associated with age, with some STs (STs 27, 108, 540, 17, 481, and 492) being almost exclusively present in patients younger than 25 years of age and others (STs 549, 274, 511, and 508) being almost exclusively present in those older than 25 years of age (Fig. 3). Most of these age-associated STs were present in patients from both hospitals. The unique STs were found in patients of all ages.

Compared to strains of all other STs, strains of ST406 and ST497 could not be characterized by a specific antibiotic resistance profile. In general, ST406 strains were more frequently resistant to carbapenems and susceptible to ciprofloxacin. Both ST406 and ST497 strains were more often resistant to amikacin (data not shown).

Among our patient population, there were 28 sibling pairs and 5 sibling trios. Of these 33 sibling sets, both or all three siblings were colonized with *P. aeruginosa* in 13 instances, and in 10 cases all siblings shared strains of identical STs. In another 13 cases, all siblings had negative cultures. Thus, 79% of siblings had identical (or

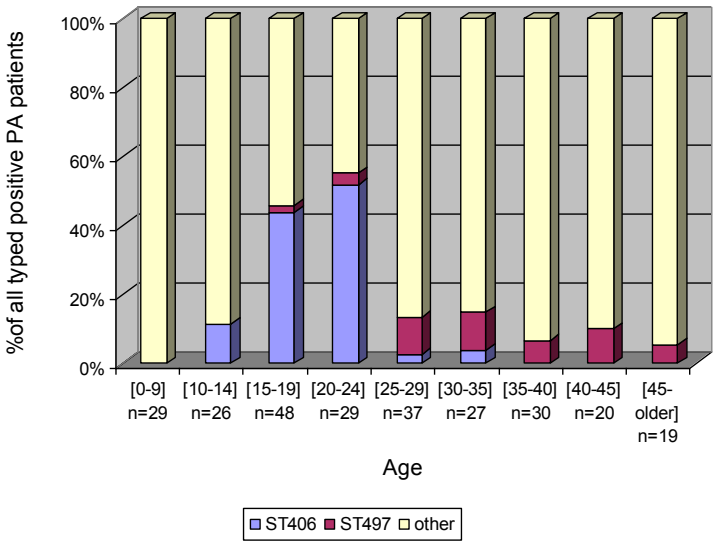


Figure 2. Patients colonized with *P. aeruginosa* ST406 and ST497 strains as a percentage of all patients colonized with *P. aeruginosa* by age group (n = total number of patients who were positive for *P. aeruginosa* [PA] in each group). The age categories are indicated in brackets.

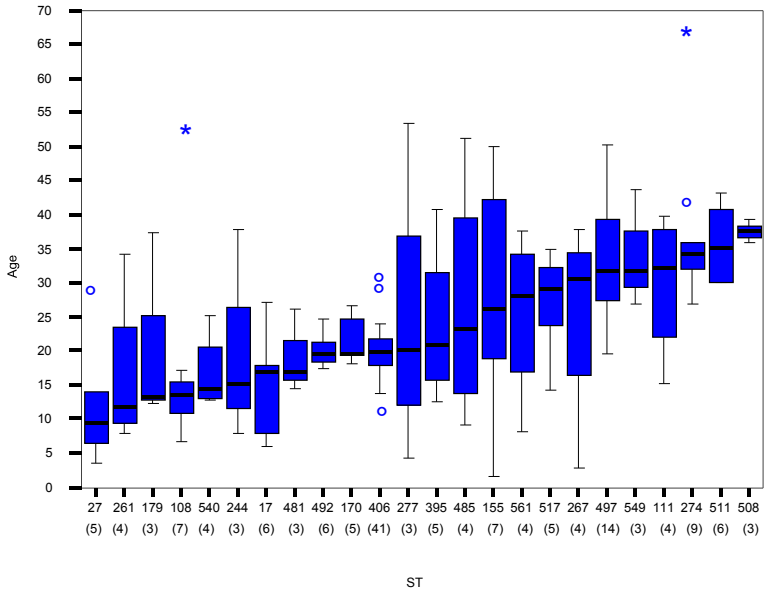


Figure 3. Age per sequence type. Black line, mean age; box, 25 and 75 percentile; °, outliers between 1.5 and 3 box lengths from either end of the box; *, cases more than 3 box lengths from either end of the box. The number of patients colonized by each ST is noted in parentheses. STs 27, 261, 108, 540, 17, 492, 170, and 406 were almost exclusively present in patients younger than 25 years of age; and STs 497, 549, 274, and 511 were almost exclusively present in those older than 25 years.

concordant) colonization status (negative for carriage or carriage of strains of the same genotypes).

A total of 126 STs from the present study had not been described before and were added to the existing 524 genotypes in the international *P. aeruginosa* MLST database. The eBURST picture of 591 STs from the available isolates in the MLST database revealed a high number of singletons, with only a few, small clonal complexes typical for species with a panmictic population structure being detected (Fig. 4). ST406 and ST497 appeared to be genotypically different from all known STs in the international database, indicating that they are most likely unique for the Dutch CF population. Moreover, they are not genetically linked to each other, nor are they linked to any of the known epidemic strains, such as the Midlands, the Liverpool, the Melbourne, and the Manchester clones and clone C. Furthermore, none of these clones were detected among this cohort of Dutch CF patients (Fig. 4).

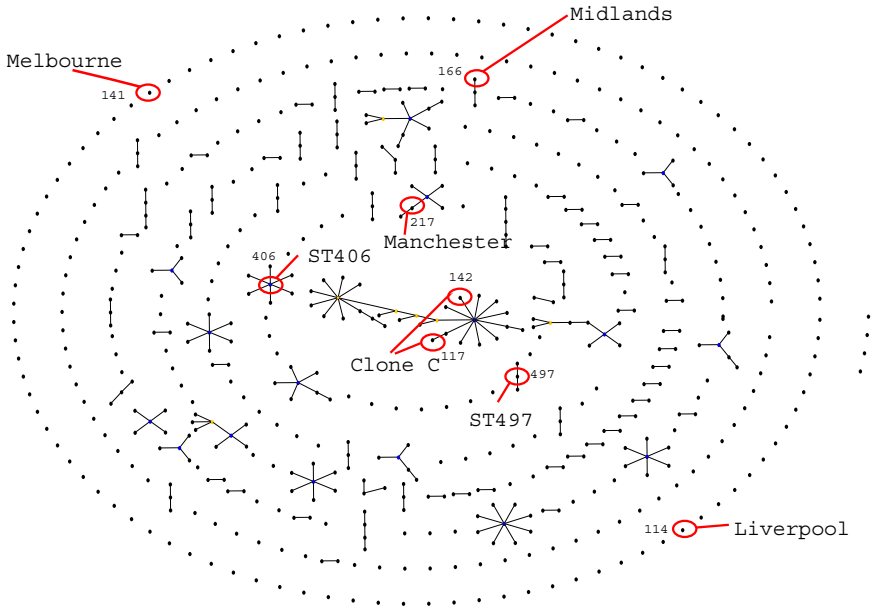


Figure 4. Snapshot of population of 591 *P. aeruginosa* STs on the basis of MLST allelic profiles using the eBURST algorithm10. Numbers and dots represent STs. Lines connect single-locus variants, which are STs that differ in only one of the seven housekeeping genes. The snapshot shows all clonal complexes (connected STs), singletons (unconnected STs), and patterns of evolutionary descent. The placement of singletons as well as the length of the lines is arbitrary. Blue dots indicate probable ancestors of clonal complexes. The two highly prevalent Dutch clones as well as the previous described epidemic clones are indicated.

Discussion

In this cross-sectional study, which included individuals representing about 45% of the Dutch CF population, the prevalence of *P. aeruginosa* colonization was 57%, and the individuals were colonized with a genetically highly diverse bacterial population. In all, 70% of the patients were colonized with strains that had genotypes that were also found in other patients, and strains with two highly prevalent STs, ST406 and ST497, were identified. Colonization with STs shared by at least three patients, including ST406 and ST497, was age dependent. ST406 and ST497 appeared to be genotypically different from all known STs in the international database and are not genetically closely linked to each other or to any of the known epidemic strains, such as the Midlands, the Liverpool, the Melbourne, and the Manchester clones and clone C. Besides, none of these clones were detected among the cohort of Dutch CF patients evaluated in this study.

The observed prevalence of *P. aeruginosa* carriage in the Dutch CF population of 57% (the prevalence is 40% in children and 70% in adults) is similar to prevalence rates reported among CF populations in the United Kingdom, the United States, and Australia, which range from 40 to 85%[9;13].

In the Dutch CF population, two *P. aeruginosa* clones are present in 20% of all patients colonized. Although CF patients mainly harbored unique *P. aeruginosa* strains, with infrequent transmission events occurring in some studies, others reported that specific *P. aeruginosa* strains were shared by from 14% to 60% of CF patients, with the rates of patient-to-patient transmission being presumed to be high[3;5;6;11;19;25;28;29]. In a cohort of 849 CF patients colonized with *P. aeruginosa* from 31 CF centers in the United Kingdom, 11% and 10% carried the so-called Liverpool and Midlands 1 genotypes, respectively[24]. In an Australian cohort of 100 CF patients from two clinics, 39 patients shared strains of one common genotype[23]. This indicates that the presence of specific predominant clones circulating in the CF patient population is not unique to the Dutch situation.

The reported proportions of CF patients colonized with genotypes also found in other CF patients were 28%, 30%, and 59% in studies from the United Kingdom, Belgium, and Australia, respectively[23;24;29]. The fact that 70% of our patients were colonized with genotypes also detected in other CF patients, indeed, suggests an important role for patient-to-patient transmission. The high level of congruence of colonization status and Pseudomonas genotype between tested siblings, which has also been found in other studies, is also an indication that patient-to-patient transmission plays an important role[20;25;29].

The number of shared STs might be related to the typing technique used, as the use of a detection method with a higher discriminatory power will result in more unique

types. In the current study, we used MLST to infer the clonality of strains, which was shown to be slightly less discriminatory than pulsed field gel electrophoresis (PFGE)[16]. Considering the small difference in discriminatory power between PFGE and MLST, it is unlikely that the high proportion of patients carrying shared genotypes results from a discriminatory power of MLST that is too low. We have chosen MLST because of its high discriminatory index, its use in revealing clonal relatedness between isolates when this is not readily apparent by PFGE, and its unambiguous, reproducible results that are easily electronically portable between laboratories, allowing the international comparison of genotypes[12].

Colonization with ST406 and ST497 was associated with the patient's age. ST406 was the dominant genotype in patients between 15 and 24 years of age, while ST497 was more prevalent in older patients. In retrospect, our findings confirm those from a previous study of 80 CF patients between 6 and 20 years old attending four Dutch CF summer camps in 2001.

MLST of the isolates in that study revealed that the most prevalent amplified fragment length polymorphism (AFLP) type represented ST406 and was found equally frequently among those between 6 and 15 years of age but less frequently among the oldest CF patients (ages, 15 to 19 years). An AFLP type representing ST497 was present only in these older patients[4]. Furthermore, in a study involving 157 pediatric patients in the Netherlands, a clone detected in CF patients on the basis of PFGE (the strain was ST406 when it was typed by MLST; C. van der Ent, personal communication) was not found in other non-CF patient groups[27].

The observed association with age, as determined in this study, is much stronger than that detected in previous studies of epidemic clones. For instance, both the Melbourne clone (pulsotype I) and the Liverpool epidemic strain were first identified in pediatric clinics[3;6], but later they were also identified in adult patients[2;22;23]. The Australian pulsotype 2 (PT2) strain was also found both in pediatric patients and in adults. However, almost all adults who harbored PT2 strains could be linked to the pediatric clinic where PT2 was found in abundance. In retrospect, this might also indicate an association of PT2 to a specific age group[23].

The absence of ST406 in patients older than 31 years of age is, as yet, unexplained, but several mechanisms could be hypothesized. Initial *P. aeruginosa* colonization in usually young CF patients is mostly transient and is followed by sustained colonization with a particular genotype in later years[21;29]. ST406 is possibly lost after a certain age, perhaps due to competition with other strains like the clonal strain of ST497, which is found almost exclusively in older patients. Superinfections with a transmissible *P. aeruginosa* strain have been demonstrated to occur in CF patients who were already colonized by other strains[15;22]. It is also possible that ST406 has only recently (in the last one or two decades) been introduced into the CF population and is therefore

linked to the younger cohort of patients who may have participated in activities with other CF patients. Finally, the carriage of ST406 in younger patients could be an effect of host tropism, in which ST406 favors the colonization of young patients above the colonization of older patients. The fact that ST406 was more prevalent in patients from UMCU than those from Haga might be related to differences in the patient population, such as age (with the UMCU patients being younger; 19.4 years versus 30.6 years for the Haga patients), which might have led to more frequent and more intense contacts with each other and thus an increased risk of transmission. In fact, host tropism, competition between strains, and age-related association patterns between patients could all interact. Quantification of these different aspects is needed to elucidate the underlying mechanisms of our observations. In addition, longitudinal studies are needed to determine the clinical consequences of colonization with certain genotypes.

ST406 and ST497 are different from other known (international) clones, and neither of these STs has been found in other countries so far, indicating that these clones are specifically linked to Dutch CF patients. Until now, there has been only limited evidence of the international spread of the known CF-related epidemic strains. In one study, a number of major *P. aeruginosa* clones appeared to be widespread in clinical and environmental habitats and were also isolated from CF patients in different countries[30]. Although this observation could have been related to the global environmental spread of these strains and the local acquisition by CF patients, some of the CF-related epidemic strains were found in other habitats as well. On the basis of strain typing by use of an ArrayTube chip, the Midlands epidemic strain was assigned to the same clonal group as strains from a German intensive care unit and one strain found in a German river, and the Liverpool epidemic strain belonged to the same type as an isolate from a patient with bacteremia in a Swiss clinic[30].

Rapid evolution of *P. aeruginosa* clones might decrease their genetic relatedness and obscure their epidemiological linkage, since the timed spread, genome plasticity, and discriminatory power of the typing method determine whether one will find a local, regional, or global spread of clones.

In this report, we describe a population structure of *P. aeruginosa* in Dutch CF patients that is characterized by the presence of two prevalent STs that are associated with certain age groups and that are not genetically linked to previously described epidemic clones.

In 2006, a segregation policy for CF patients was implemented in the Netherlands. Many CF patients find it difficult to adhere to these strict rules. The population structure of *P. aeruginosa* and the prevalence rates of genotypes in the Dutch CF patient population in 2007 to 2008 may now serve as a reference point for the future evaluation of the efficacy of this segregation policy. If it is successful, such a policy should reduce, or at least stabilize, the prevalence of dominant *P. aeruginosa* genotypes.

Acknowledgements

This publication made use of the Pseudomonas aeruginosa MLST website (http://pubmlst.org/paeruginosa/) developed by Keith Jolley[17] and sited at the University of Oxford. The development of the Pseudomonas aeruginosa MLST website has been funded by the Wellcome Trust.

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Chapter 3

The population genetics of *Pseudomonas aeruginosa* isolates from different patient populations exhibits high-level host specificity

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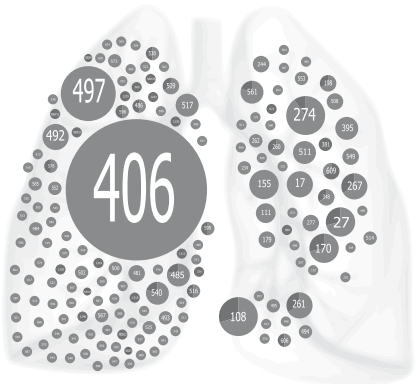
PLoS ONE 2010 5(10): e13482



Abstract

Objective

To determine whether highly prevalent *P. aeruginosa* sequence types (ST) in Dutch cystic fibrosis (CF) patients are specifically linked to CF patients we investigated the population structure of *P. aeruginosa* from different clinical backgrounds. We first selected the optimal genotyping method by comparing pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and multilocus variable number tandem-repeat analysis (MLVA).

Methods

Selected *P. aeruginosa* isolates (n = 60) were genotyped with PFGE, MLST and MLVA to determine the diversity index (DI) and congruence (adjusted Rand and Wallace coefficients). Subsequently, isolates from patients admitted to two different ICUs (n = 205), from CF patients (n = 100) and from non-ICU, non-CF patients (n = 58, of which 19 were community acquired) were genotyped with MLVA to determine distribution of genotypes and genetic diversity.

Results

Congruence between the typing methods was >79% and DIs were similar and all >0.963. Based on costs, ease, speed and possibilities to compare results between labs an adapted MLVA scheme called MLVA9-Utrecht was selected as the preferred typing method. In 363 clinical isolates 252 different MLVA types (MTs) were identified, indicating a highly diverse population (DI = 0.995; CI = 0.993–0.997). DI levels were similarly high in the diverse clinical sources (all >0.981) and only eight genotypes were shared. MTs were highly specific (>80%) for the different patient populations, even for similar patient groups (ICU patients) in two distinct geographic regions, with only three of 142 ICU genotypes detected in both ICUs. The two major CF clones were unique to CF patients.

Conclusion

The population structure of *P. aeruginosa* isolates is highly diverse and population specific without evidence for a core lineage in which major CF, hospital or community clones co-cluster. The two genotypes highly prevalent among Dutch CF patients appeared unique to CF patients, suggesting specific adaptation of these clones to the CF lung.

Introduction

Pseudomonas aeruginosa can cause nosocomial infections in immuno-compromised patients and patients in intensive care units (ICUs), and is a major cause of morbidity and mortality in patients with cystic fibrosis (CF)[1]. Molecular typing studies revealed the presence of so-called epidemic strains, frequently transmitted between CF patients and associated with higher morbidity and mortality[2-6]. As a consequence, many countries implemented segregation policies for CF patients[7].

In a previous cross-sectional study, investigating the population structure of respiratory *P. aeruginosa* isolates among Dutch CF patients by using multilocus sequence typing (MLST), we described two sequence types (ST), ST406 and ST497 in 15% and 5% of all patients infected with *P. aeruginosa*, respectively[8]. Both STs were not genetically linked to previously described international epidemic clones, which were not detected in this CF population.

In order to determine whether these prevalent STs are specifically linked to patients with CF, or ubiquitously present in other patient populations, we aimed to investigate the genetic relatedness and population structure of *P. aeruginosa* isolates from CF and non-CF patients. To do so, a highly discriminatory, cheap and easy to perform typing scheme, which also allows results to be easily compared with international databases, is required. Pulsed-field gel electrophoresis (PFGE) has been the most widely used typing method, but does not allow easy comparison of results of different origin because of a relatively high degree of inter-performer variation and lack of an international comparative database. MLST provides sequence-based, and thus unambiguous, results, but is rather expensive. We, therefore, first determined whether multi-locus variable number tandem-repeat analysis (MLVA) could fulfill these criteria required for library typing, by comparing a new MLVA scheme, adjusted from the published *P. aeruginosa* MLVA scheme[9], to PFGE and MLST. After identifying the optimal typing scheme, based on discriminatory power, typeability, time, ease of interpretation and of international comparability and costs, we determined the population structure of multiple *P. aeruginosa* isolates from different epidemiological backgrounds.

Materials and Methods

Genotyping

To determine the optimal molecular typing method, 60 *P. aeruginosa* isolates from sputum or throat swab cultures obtained from 58 different CF patients visiting the University Medical Centre Utrecht (UMCU) in 2007, were typed by PFGE, MLST and MLVA9-Utrecht. This selection represented the genotypes and genetic diversity found

in the Dutch CF patients as shown in a previous cross-sectional typing study. The Discriminatory Indices (DI) and the 95% confidence intervals (CI) were calculated as described before[10;11] using Bionumerics 5.1 (Applied Maths, St-Martens-Latem, Belgium). Criteria to assign isolates to clonal clusters (CCs) were defined as follows: PFGE types (PT) >80% similarity in band patters, MLVA types (MTs) with identical number of repeats in 8 out of 9 loci (single locus variants) and MLST types (STs) with identical sequence in 6 out of 7 loci. CCs were named after their presumed founder MT/ST, based on eBURST criteria[12]. The quantitative level of congruence between typing methods was calculated using the adjusted Rand and Wallace coefficients, available at http://www.comparingpartitions.info/. The adjusted Rand coefficient quantifies the global agreement between two methods, whereas the Wallace coefficient indicates the probability that two isolates classified as the same type by one method are also classified as the same type by another method[13]. MLVA9-Utrecht profiles were clustered with Bionumerics software (version 5.1) by using a categorical coefficient and a graphing method called minimum spanning tree[14].

PFGE

For PFGE, 2% agarose plugs were made with equal volume bacterial suspension of 3 McFarland. Plugs were incubated overnight with 0.5 mg/ml lysozyme (Sigma-Aldrich, Zwijndrecht, Netherlands) at 37°C. Next 1 mg/ml proteinase K (VWR, International, Amsterdam, Netherlands) was added and plugs were incubated overnight at 56°C. Plugs were washed for 30 min at 37°C once with 10 mM tris/1 mM EDTA (TE) buffer, then 0.75 mM phenyl-methyl-sulfonyl-fluoride (PMSF) in TE buffer, and again with TE buffer. Plugs were digested with SpeI 5 µl (50 U) in 25 µl NE buffer2 (Westburg, Leusden, Netherlands) and 220 µl water overnight at 37°C. Electrophoresis was performed with 1% agarosegel for 20 h at 6 V/cm with initial switch of 5.8 s and final switch of 38 s. *P. aeruginosa* strain ATCC 27853 was used as reference at minimal 5 lanes in each gel. The gels were stained with ethidiumbromide and bands were analysed with Bionumerics 5.1 (Applied Maths, St-Martens-Latem, Belgium). The band patterns were compared using the Dice-coefficient by using the unweighted pair group method to determine band similarity. Band patterns that were more than 80% identical were considered related conform the Tenover criteria[15;16], which state that a 2–3 band difference indicates related strains. On average we observed 16 bands in our *P. aeruginosa* PFGE gels, resulting in the 80% cut-off. Typeability was defined as all isolates that produced a band pattern divided by all isolates tested.

MLST and MLVA

Isolates were taken from the freezer and cultured on Trypticase Soy Agar II +5% sheep blood plates (Becton, The Netherlands) overnight at 37°C. A loop (few colonies) of

bacterial cells were suspended in 20 µl lysis buffer (0.25% SDS, 0.05 M NaOH) and incubated at 95°C for 20 min. The cell lysate was spun by short centrifugation and diluted with 180 µl buffer (10 mM Tris-HCl, pH 8.5). After thoroughly mixing, another centrifugation for 5 min at 16,000×g was performed to remove cell debris. Supernatants were frozen at −20°C until further use. Two and a half µl of the lysate was used in the PCR reactions for MLST and MLVA. For MLST a touchdown PCR was performed as described before, adapted from the protocol by Curran *et al*[17] with HotStarTaq Mastermix (Qiagen, Valencia, CA,USA). PCR products were sequenced (BaseClear, Leiden, The Netherlands) with the same primers as used for amplification. Sequences were analyzed using Bionumerics 5.1 (Applied Maths, St-Martens-Latem, Belgium). Sequence types (STs) were compared to the *P. aeruginosa* Multilocus Sequence Typing website (http://pubmlst.org/paeruginosa/) developed by Keith Jolley[18] and new alleles and profiles were sent to the curator A. Baldwin. For MLVA typing a touchdown PCR was performed adapted from the protocol by Vu-Thien *et al*[9] adding Q-buffer (Qiagen Benelux B.V., Venlo, The Netherlands) using the published primers for the following variable-number-of-tandem-repeats (VNTRs): ms77, ms127, ms142, ms211, ms213, ms215, ms216, ms217 and ms223 (called MLVA9-Utrecht). The PCR was conducted as follows: 10 min at 96°C, then 10 cycles of 30 s at 95°C, 30 s at 65°C with 1°C less every cycle and 1 min at 72°C. This was followed by 25 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C, and a final incubation of 10 min at 72°C followed. PCR products were separated on a 2% agarose gel by electrophoresis next to 100 bp DNA ladder (Invitrogen, The Netherlands). The size of each amplicon was measured using Bionumerics 5.1 (Applied Maths, St-Martens-Latem, Belgium) and the number of repeats was deduced by using the MLVA alleles assignment table on the *Pseudomonas aeruginosa* genotyping site (http://minisatellites.u-psud.fr/MLVAnet/). PA01 (ATCC BAA47) was used as control for checking consistency of allele assignments. Loci that repetitively did not yield a PCR product were assigned allele "99" to be able to include these isolates in subsequent cluster analysis. MLVA9-Utrecht types (MTs) were compared with the international database "pseudomonas2007" created by Gilles (http://minisatellites.u-psud.fr/MLVAnet/). Typeability was defined as the number of isolates for which repeat numbers could be inferred for all 9 loci divided by all isolates tested.

Patients and bacterial isolates

To determine the *P. aeruginosa* population structure, 363 isolates were collected from four different patient populations: 100 respiratory isolates from 90 CF patients who either were cultured because of an exacerbation or screened for their annual check-up (group I) and 205 *P. aeruginosa* isolates from aspirate, sputum or throat swab screening cultures from patients admitted to intensive care units (ICU) (one isolate per type per patient) in two hospitals in the Netherlands (126 isolates from 97 patients in hospital 1

(group IIa) and 79 isolates from 64 patients in hospital 2 (group IIb). Screening cultures were executed on admission, twice weekly thereafter and on discharge during a period of 14 months in both hospitals. Hospital 1 is a tertiary referral (university) hospital and patients were included in two ICUs (10 and 8 beds, of which 6 and 7 beds on a ward, respectively) harboring a mixed adult patient population. Hospital 2 is non-university teaching hospital, located 80 kilometers from hospital 1, and here patients from two ICUs (8 and 8 beds, single rooms) also harboring a mixed adult patient population were included. In both ICUs, CF patients were excluded. In total, 1200 patients were admitted for more than 24 hours and screened (cultures were not available of 113 patients). Isolates of 161 of 194 colonized patients were typed and included in this study. Group III consisted of 39 non-respiratory clinical isolates from 38 non-CF patients and non-ICU patients admitted to hospital 1. These 38 patients were mostly long-stay patients (admitted > one month) in different wards, including surgery, neurology, oncology and internal medicine. Group IV consisted of 19 isolates from 19 non-CF and non-ICU patients obtained within 48 hours after admission or during out-patient clinic visits at hospital 1. These isolates are considered "community acquired". The community acquired isolates were cultured mainly from eyes, ears, wounds and screening cultures of patients admitted for stem cell transplantation. The ethical committees (METC) of both the University Medical Center Utrecht and the St Elisabeth Hospital Tilburg approved this study and waived the requirement for informed consent (METC Utrecht protocol number: 05/311, METC Tilburg protocol number: 0655), since cultures were obtained as part of the hospital surveillance program or clinical practice.

Calculation of expected DI and MT distribution

The median value and the 95% confidence intervals for the DIs and the overlap in types between different clinical sources was calculated using Mathematica 7.0.1.0, (Wolfram Research, Champaign, Ill), by distributing the isolates 100,000 times, randomly, over the different clinical sources under the assumption that genotypes do not cluster. The number of isolates per group and the prevalence of the different MTs were considered fixed and only the distribution over the different groups was randomized.

Results

Adjusted MLVA9-Utrecht scheme

We first adjusted the published *P. aeruginosa* MLVA scheme, as originally described by Vu-Thien *et al.* The original scheme contained 15 variable number tandem-repeat (VNTR) loci, of which some, due to small repeat sizes, required analysis on a DNA sequencer. To create a robust MLVA scheme that was easy to perform without the need

for a DNA sequencer, we tested different combinations of the original 15 VNTR loci and calculated the DIs for the different combinations in a set of 101 *P. aeruginosa* isolates (the 100 selected CF isolates plus PA01; ATCC BAA47). VNTR loci that were not selected in the final scheme were loci with too small repeat size (<15 nt) and loci that could not be amplified in >10% of the isolates. Based on these criteria we selected a subset of nine MLVA loci, ms77, ms127, ms142, ms211, ms213, ms215, ms216, ms217, and ms223. This scheme yielded a PCR product in 91-100% of the isolates and a high discriminatory index of 0.984 (CI 0.972–0.996).

Comparison of typing methods

Subsequently we compared the adjusted MLVA9-Utrecht scheme with PFGE and MLST by typing 60 *P. aeruginosa* isolates from CF patients with the three methods. Typeability was 100% for MLST and MLVA9-Utrecht, but only 91.7% for PFGE as 5 isolates yielded, repeatedly, no banding patterns with this technique (Table 1). PFGE, MLVA9-Utrecht, and MLST distinguished 52, 45, and 36 types, respectively, which could be grouped in 33, 35, and 33 CCs. The DIs with 95% confidence intervals (CI) were comparable, although PFGE was slightly more discriminatory than MLST (Table 1). The three typing methods were highly congruent at the CC level with an adjusted Rand coefficient of 0.84 for PFGE vs. MLVA9-Utrecht, 0.91 for PFGE vs. MLST and 0.90 for MLST vs. MLVA9-Utrecht. Moreover, two strains that are of the same MT have a high probability of belonging to the same ST on the level of clonal clusters, as indicated by the Wallace coefficients (Table 2), which was highest between MLVA9-Utrecht and MLST (0.969).

Based on the high DI of MLVA9-Utrecht, the high congruence between this MLVA scheme and the other typing methods and the fact that MLVA is considerably cheaper

Table 1: Typing characteristics of the genotyping methods for the 60 isolates typed with all 3 methods.

	PFGE	MLVA9-UTRECHT	MLST
Typeability	91.7%	100%	100%
Costs[a]	€5.78	€7.21	€121.60
Time[b]	5 days	2 days	7 days
Ease of interpretation	-	+	++
International comparison[c]	-	+	++
Discriminatory Index	0.998 [0.995 – 1.0]	0.982 [0.968 – 0.998]	0.963 [0.936 - 0.991]

[a]:Cost per isolate tested, including materials, excluding labour and equipment depreciation since that is similar in all methods. MLST costs can be lower when not using outsourced sequencing.
[b]: Time can be shorter with MLST. In this study we outsourced sequencing that took extra time.
[c]: Comparison with international data in database on http://pubmlst.org/paeruginosa/ for MLST and http://minisatellites.u-psud.fr/MLVAnet/ for MLVA.

Table 2: Wallace coefficients, indicating congruence between the different typing methods.

	MLVA9-UTRECHT	MLST	PFGE
MLVA9-UTRECHT	NA	0.969	0.917
MLST	0.845	NA	0.918
PFGE	0.793	0.910	NA

than MLST, rapid to perform and allows data comparison with other datasets (Table 1), we selected MLVA9-Utrecht as the preferred typing method to determine the population structure of *P. aeruginosa* isolated from different epidemiological backgrounds in the Netherlands.

Population biology of *P. aeruginosa* clinical isolates

All 363 isolates were typed with the adjusted 9 loci-MLVA scheme and 252 different MLVA9-Utrecht types could be discerned (typing data available in supplement Data S1). Typeability was 91% and ranged from 87% to 95% in the different patient groups (Table 3). In 22 and 10 isolates one or two loci could not be amplified, respectively, and these were assigned allele "99". Of the loci that could not be amplified in all isolates, ms217, ms215 and ms77 could not be amplified in 12, 8 and 7 isolates, respectively. Ms127 was the only locus that could be amplified in all isolates. The genetic diver-

Table 3 *P. aeruginosa* MLVA9-UTRECHT typing results of four different patient populations.

	group I	group IIa	group IIb	group II	group III	group IV	
	CF	ICU -I	ICU-II	ICU total	Hospital acquired Non-CF/non-ICU	Community acquired Non-CF/non-ICU	Total
source	respiratory	respiratory	respiratory	respiratory	non-respiratory	non-respiratory	diverse
# isolates (from # pat)	100 (90)	126 (97)	79 (64)	205 (161)	39 (38)	19 (19)	363 (308)
typeability	88%	93%	100%	95%	87%	89%	91%
# types	72	82	63	142	33	17	252
Index of diversity-(CI)	0.984 (0.971-0.996)	0.981 (0.97-0.992)	0.991 (0.983-0.999)	0.991 (0.987-0.996)	0.989 (0.977-1.0)	0.988 (0.969-1.0)	0.995 (0.993-0.997)
Prevalent types (≥5%)	MT27(11%) MT11(5%)	MT44 (10%) MT68 (6%)	MT161 (8%)	NA	MT255 (5%) MT261 (5%)	MT276 (11%) MT212 (11%)	NA

CF: cystic fibrosis patients, ICU: intensive care unit patients, MT: MLVA9-UTRECHT type, #: number

sities in these four populations were similarly high, with an overall DI of 0.995 (CI 0.993–0.997) (Table 3).

The population structure of *P. aeruginosa* in this strain set based on MLVA9-Utrecht is characterized by a high level of host-specificity. Between 82% and 91% of MTs are unique for the different patient populations. Only 11 (4%) of the 252 MTs were detected in two different patient populations studied. These MTs represented 11% of the CF related types (group I), 6% of the ICU related types (group II), 9% of the non-ICU hospitalized patients (group III) and 18% of the community acquired types (group IV), respectively (table 4). The MTs found in groups III and IV (hospitalized, non-ICU patients and patients with community acquired isolates) were not found in groups I (CF patients) or II (ICU patients). When comparing MTs from the ICU populations in both hospitals, most MTs appeared to be ICU-specific (fig. 1/table 4). Only three (2%) of 142 MTs were detected in samples from patients in both ICUs indicating specific clustering in both location and patient group. The DIs of CF group and ICU-1 group appeared significantly lower than what would have been expected in case of random distribution of MTs (table 5). Furthermore, calculation of expected unique and shared types between the five groups in case of random distribution revealed that the observed numbers of shared types between all the different groups was significantly lower than what would

Table 4: Numbers (%) of shared and unique MLVA9-UTRECHT types (MTs) in the four groups of clinical sources compared to the numbers of expected values based on 100.000 permutations (median, range and 95% confidence interval (CI)) when assuming random distribution of types.

			MTs shared (percentage of total MTs) [95% CI]				
		Unique	CF	ICU-I	ICU-2	HA	CA
Source			group I	group IIa	group IIb	group III	group IV
CF	Observed	64 (89%)		6 (8%)[a]	2 (3%)[a]	0[a]	0[a]
	Expected	58 [50-66]		18 [13-23]	13 [8-18]	8 [4-12]	4 [1-7]
ICU-1	Observed	72 (88%)	6 (7%)[a]		3 (4%)[a]	0[a]	0[a]
	Expected	74 [66-82]	18 [12-23]		15 [10-20]	9 [5-13]	5 [2-8]
ICU-2	Observed	58 (92%)[b]	2 (3%)[a]	3 (5%)[a]		0[a]	0[a]
	Expected	46 [38-53]	13 [8-18]	15 [10-20]		7 [3-11]	3 [1-7]
HA	Observed	30 (91%)[b]	0[a]	0[a]	0[a]		3 (9%)
	Expected	23 [17-28]	8 [4-12]	9 [5-13]	7 [3-11]		2 [0-5]
CA	Observed	14 (82%)	0[a]	0[a]	0[a]	3 (18%)	
	Expected	10 [6-14]	4 [1-7]	5 [2-8]	3 [1-7]	2 [0-5]	

CF: cystic fibrosis patients, ICU: intensive care unit patients, HA: non-CF, non-ICU patients with hospital acquired *P. aeruginosa*, CA: non-CF, non-ICU patients with community acquired *P. aeruginosa.*
[a] : Value lower than expected within 95% CI range, i.e. less overlap of types between sources than in the case of random distribution of types. [b]: More unique genotypes per source than expected, i.e. high level of source-specificity rather than random distribution.

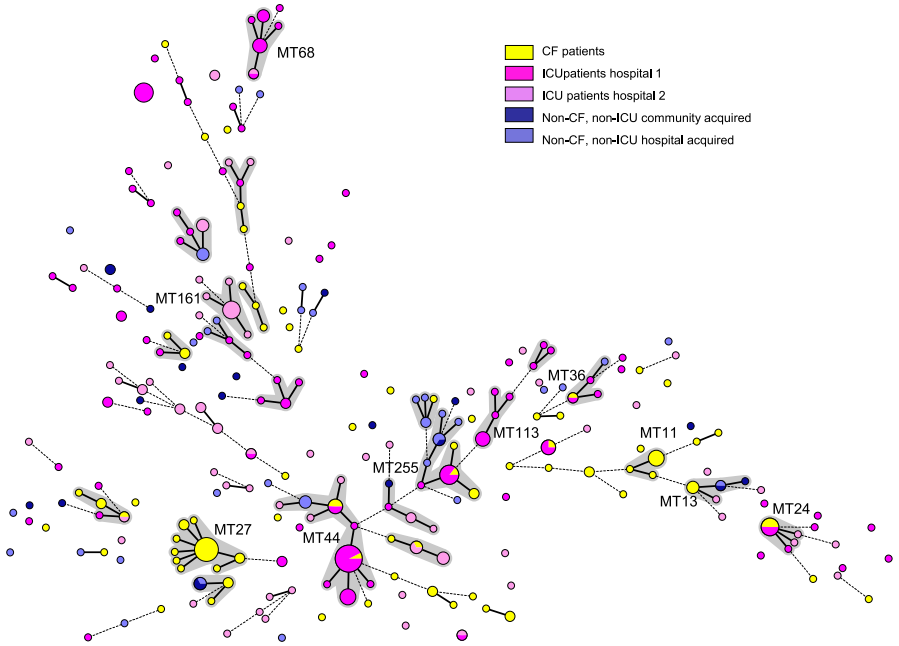


Figure 1. Minimum spanning tree of 363 *P. aeruginosa* isolates from different patient populations typed by MLVA9-UTRECHT. Circles represent MTs, the size of the circle is related to the number of isolates with that specific MT in this collection. Fat lines between the circles represent single locus variants (SLVs), differing only in one loci. Dotted lines represent double locus variants. Yellow color represents CF isolates, pink and purple are ICU respiratory isolates from two different hospitals, blue are non-CF non-ICU isolates (dark blue are "community acquired" isolates and light blue "hospital acquired"). Grey shading indicates clonal complexes.

Table 5: Expected Indices of Diversity (DI) and 95% confidence intervals based on 100.000 permutations based on random distribution of genotypes compared to observed DI.

	CF	ICU-1	ICU-2	HA	CA
Observed DI	0.984 *	0.981*	0.991	0.989	0.988
expected DI [95% CI]	0.995 [0.991-0.998]	0.995 [0.992-0.997]	0.995 [0.991-0.998]	0.996 [0.987-1.0]	1.0 [0.982-1.0]

* not within the 95% confidence interval (CI) range; i.e. diversity in that specific group is lower than would be expected on random distribution of type

have been expected, except between group III and IV (table 4). This proves non-random clustering of MTs and the presence of patient group-specific types.

The 252 MTs could be grouped in 22 CCs, defined as clusters of three or more types that share at least 8 out of 9 loci (fig. 1). The minimum spanning tree revealed that specific clustering in both location and patient group did not result in grouping of isolates from a single patient population in one genetic lineage or genetic subpopulation. In contrast, isolates belonging to a single patient population are scattered over the minimum spanning tree. In agreement with the observed host-specificity, only three CCs contained isolates from four of the five populations studied. These three CCs (CC44, CC255 and CC13) contain CF isolates that were also typed by MLST allowing comparison with other isolates in the MLST database[19]. These "mixed" CCs, detected in each patient population, are closely related to *P. aeruginosa* clones that had been detected up to 7 countries on 4 continents.

Three CCs contained isolates from CF patients only. Two of these, CC27 and CC11, contained the two previously reported high prevalent genotypes in CF-patients, ST406 and ST497, represented in this study by MLVA9-Utrecht types MT 27, 32, 52 and 238 (CC27) and MT 11 and 38 (CC11), respectively. This means that these two high prevalent CF clones are exclusively found in CF patients (fig. 1).

Discussion

Using a simplified MLVA scheme for genotyping we have demonstrated that the population structure of *P. aeruginosa* isolates is highly diverse and population specific. This implies that most clones specific for CF patients, including the highly prevalent Dutch clones MT27, 32, 52, 238 (ST406) and MT 11, 38 (ST497), are genetically distinct from clones from non-CF patients. The high prevalence of these clones in CF patients, therefore, is unlikely to result from transmission of particular dominant clones from the non-CF reservoir. Moreover, ICU-wards from different hospitals appeared to have location specific *P. aeruginosa* populations.

MLVA9-Utrecht revealed that the *P. aeruginosa* population in the different clinical settings is highly diverse with a DI of 0.995 with no difference in diversity between hospital acquired and community acquired strains. This corroborates with previous findings in ICU patients[20;21].

Studies in the last decade have proposed different types of *P. aeruginosa* population structures, ranging from panmictic in the early nineties[22;23] to more clonal in 2007[24]. The latest reports however, summarized by Pirnay in 2009, point towards a nonclonal epidemic population structure, with no distinction between clinical or environmental isolates[25]. In particular, the lack of distinction in genotype, function and chemotax-

onomy between clinical and environmental *P. aeruginosa* isolates has been reported by different research groups[26;27]. Based on FAFLP, gene sequencing and virulence gene profiling Pirnay *et al* described that strains which clustered in the same clonal complexes could have been isolated from inanimate environments, animals and humans, sometimes separated by thousands of miles. They concluded that there was no correlation between the clonal complexes and geographical origin or habitat. We also found that the three clonal complexes that were present in the four epidemiological backgrounds had been detected previously in up to seven other countries on four continents, indicating their global presence[28].

However, in contrast to previous research that suggested no correlation between *P. aeruginosa* clones and diseases or environmental habitats[29], we found genotypes to be highly specific for the different patient groups with only a relatively small number of clones distributed across patient population boundaries. However, since the MLVA database[28] does not provide data on the source of the isolate we cannot elaborate on the association between these types and epidemiological background. Our findings of high specificity of different sets of genotypes, not only in the various patient groups but also between ICUs in the different hospitals, are remarkable. Thus, discordant to the proposed consensus of a non-clonal epidemic population structure with some dominant clonal complexes, which are just as versatile in their habitat and geographic origin as the whole *P. aeruginosa* population, we found that both patient population and geographical origin appeared to be correlated to the prevalence of certain genotypes and that transmission of *P. aeruginosa* clones between ICUs, hospital wards and CF patients is rare.

The limited overlap between isolates from CF and non-CF patients also fails to support findings reported by Lanotte *et al*, who described, based on random amplification of polymorphic DNA (RAPD), a non-random distribution of isolates but with a subpopulation of isolates originating from patients with lung disease, both CF and non-CF. This could result from low discriminatory power of RAPD.

Pirnay *et al* also concluded that, based on typing of 328 unrelated isolates including 43 CF isolates, all CF isolates clustered into a "core lineage" that is predominant in both disease and environmental habitats across the world. Consequently, CF isolates belonging to the so-called "successful core lineage" are ubiquitous in the natural environment and are, therefore, more likely to infect CF patients. We failed to confirm such a level of "relatedness" in our populations, as CF isolates, as well as the ICU isolates and other clinical isolates were dispersed over the entire minimum spanning tree. Moreover, the two most successful CF clones in our country were not detected in other patient populations and they are not genotypically closely related to non-CF isolates. This suggests no common evolutionary background of *P. aeruginosa* isolates from CF patients nor of *P. aeruginosa* isolates from the other analyzed patient groups.

Our findings are more in line with the observation that the Australian Epidemic strains I and III (AESI and AESIII) could not be isolated from the environment[30;31]. These findings suggest selection of multiple specific clones with a distinct evolutionary background that are better equipped to adapt to and survive in the specific conditions in the CF lung. This also indicates that *P. aeruginosa* from many different lineages can adapt to all kinds of niches. This concurs with data from Pirnay *et al*, who found that a *P. aeruginosa* community in a Belgian river contained members of nearly all successful clonal complexes and was almost as diverse as the global population, represented by 73 clinical and environmental isolates from a previous study[32].

The strength of our study is the large and well-defined collection of isolates and the ability of MLVA9-Utrecht to show, highly reproducible, genotypic relatedness, with the possibility of comparing the genotypes to results contained in international databases via the internet, and that can be performed under point-of-care conditions. One should be aware that the MTs assigned in this study only refer to the MLVA9-Utrecht scheme. We did not include isolates from the environment in our study, shown to contain similar genotypes as clinical isolates in other studies, which may change our findings of specificity.

We conclude that the population structure of *P. aeruginosa* from different patient populations is highly diverse and characterized by high-level host-specificity and by the presence of many unique and only a limited number of more prevalent genotypes. The two genotypes (MT27/ST406 and MT11/ST497), frequently found in the Dutch CF patients, appear to be unique to CF patients and are not found in other clinical patients. Further studies are needed to elucidate the specific adaptations and survival strategies that these strains have adopted to survive in this special niche.

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Chapter 4

Possible cross-transmission of *Pseudomonas aeruginosa* genotypes between patients with cystic fibrosis during a one-day open air event

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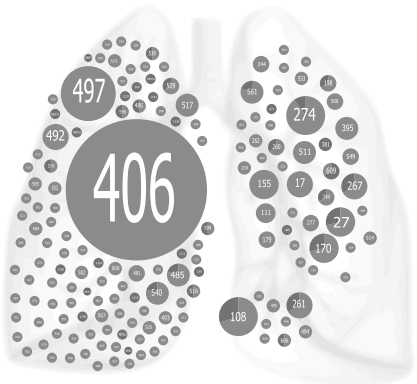

Abstract

Background

Segregation of patients with cystic fibrosis (CF) to prevent transmission of *Pseudomonas aeruginosa* is advised in many countries including the Netherlands. Despite this advice, a one-day open air event is yearly organized in the Netherlands.

Methods

Cross-transmission of *P. aeruginosa* between CF patients participating in this event was studied by molecular typing of isolates obtained before, directly after and three months after the event using Multiple-locus variable number tandem repeat analysis (MLVA). Surveys were done to assess whether risk factors other than genotype could be identified.

Results

In 16 of 22 participating patients *P. aeruginosa* was found, belonging to 18 genotypes. In total, 36% of these patients harbored MLVA type (MT)-27 that was previously linked to the highly prevalent Dutch CF clone ST406. One case of possible cross-transmission was detected, in which the acquired genotype (MT27) found immediately after the event, was also present in other participants. Eleven of 16 patients carried the same strain (MT) during the study period, while 7 patients (32%) acquired an (additional) *P. aeruginosa* MT during the event. Adherence to hygiene rules during the event was reported by 77% of the CF patients, while 50% of CF patients reported physical contact with other CF patients not belonging to their household in the three months after the event.

Conclusions

Transmission of *P. aeruginosa* between CF patients during a one-day open-air event is possible and general compliance to advices about hygiene and contact for CF patients is not high.

Introduction

In the first two decades of life more than 50% of patients with cystic fibrosis (CF) become chronically infected with *Pseudomonas aeruginosa*. It was previously thought that patients with CF acquired individual *P. aeruginosa* strains from the environment[1] and sharing of *P. aeruginosa* genotypes between patients occurred only in patients within the same household[2]. In the 1990's however, several studies suggested that cross-transmission could also take place between non-related patients who visited camps for CF patients. A retrospective study in Denmark showed that five *P. aeruginosa* negative children, who participated in a CF summer camp together with 17 *P. aeruginosa* positive children, became colonized with *P. aeruginosa*. These authors advised segregation of patients according to *P. aeruginosa* infection status[3]. In the Netherlands Brimicombe *et al*. performed a study in 80 children visiting a CF summer camp in 2001. AFLP typing showed 18 cases of possible transmission of *P. aeruginosa* strains between patients[4]. In addition, several studies from CF centers in the UK and Australia reported the occurrence of highly transmissible strains of *P. aeruginosa* and a possible detrimental effect on CF lung disease[5-8]. In the Dutch CF centers infection control policies aimed at limiting the spread of such strains were implemented in 2006 which included, besides a segregation policy of all CF patients in the clinical setting, the advice to discontinue the CF summer camps and to avoid meeting other CF patients[9].

In 2005 a group of Dutch teenage CF patients started organizing the CF Beach Dance event (CFBD), an opportunity for CF patients to continue meeting each other. This 1-day open-air event at the beach in Bloemendaal, the Netherlands, has been organized once yearly ever since and consists of an afternoon part with physical activities and an evening part with music and dancing. The organizers chose an event of short duration in the open air based on the idea that this would minimize the risk of cross-transmission. Besides, the organization advises to adhere to the hygiene rules recommended by the national health advisory board, e.g. no physical contact, coughing in a tissue, hand hygiene, no sharing of food, no use of medication or devices of other patients. Whether or not cross-transmission in such a one-day setting occurs has not yet been investigated, as previous studies only investigated the risk of camps of several days' duration[3;4]. In this study we aimed to determine the rate of cross-transmission during the CFBD by genotyping *P. aeruginosa* isolates obtained from patients who visited the event. Furthermore, we aimed to study if *P. aeruginosa* genotype determines the occurrence of transmission and whether other risk factors for transmission could be identified.

Methods

Participants

This study was performed during CFBD 2009, which took place on July 18th 2009. CFBD 2009 was promoted through a website (http://www.stichtingfok.nl/2009/06/18/123/), which also gave information about the background of the event and hygiene precautions. Potential visitors were informed of the study via the event's website (http://www.cfbeachdance.nl), on which CF patients are encouraged to register for the event. The event was also open to non-CF patients, but they were not asked to register. Those who registered online were asked to participate in the study, and informed consent from the patients (and their parents if applicable) was obtained. Patients who had not registered online, but who had been informed about the study online, were recruited upon arrival to the event and informed consent was obtained on site. The protocol for this study was approved by the institutional review board.

Sampling

Upon arrival at the beach, before being transported to the festival terrain, patients were asked to fill out a short questionnaire with their personal details and some information on their current treatment. Also a sputum sample was collected by the patients themselves (t1). If patients were unable to produce sputum, a cough swab was collected by a researcher. Sampling was repeated when patients left the festival terrain (t2), and the patients filled out a short questionnaire on their familiarity with and adherence to the hygiene rules. Three months after CFBD (t3), a sampling package was sent to the participants via mail. The package included material for sputum and cough swab collection and an instruction manual on how to obtain the samples and a short survey on whether they had met up with other CF patients since CFBD.

Isolation of bacteria and typing

Cough swabs that were obtained at the event were immediately streaked on agar plates and sputum samples were processed the next day. All respiratory samples were cultured according to standard laboratory practice and all morphologically different (rough/smooth, mucoid, size) *P. aeruginosa* isolates were frozen for storage. Multiple locus variable number tandem-repeat analysis (MLVA) was performed according to the "MLVA9-Utrecht" typing scheme as described before[10]. This typing method was chosen since MLVA is well suited to identify micro-variation because the variation that is indexed by MLVA (change in number of direct repeats through slipped-strand mispairing or homologous recombination) evolves rapidly. Identification of micro-variation is needed to distinguish between strains circulating within a confined geographic area during a short time period, like in this study.

Transmission

A case of cross-transmission was defined as the finding of a *P. aeruginosa* MT at t2 that was not cultured from the sputum of the patient upon arrival to the event (t1) while this MT was cultured from another patient at t1.

Results

Participants and samples

Out of 25 CF patients visiting CFBD, 22 CF patients agreed to participate in this study. Table 1 displays baseline characteristics of the participants. These patients live in different regions in the Netherlands and receive treatment in different CF centers. From all participating patients samples were obtained upon arrival at the event (t1), upon leaving (t2) and three months after the event (t3). In 14 of 22 patients (64%) cough swabs and or sputum samples were culture positive for *P. aeruginosa* at t1 while this was 68% (15/22 patients) at t2 and t3 (Table 2). This yielded a total of 131 *P. aeruginosa* isolates. When asked about the hygiene rules, all patients were familiar with these rules and 17 (77%) patients indicated they adhered to these rules to some extent (1 "strictly" and 16 patients "more or less") while 5 (23%) patients indicated they completely ignored the rules. Of the 22 participants, there were 3 pairs of patients who shared a household and there were 3 patients who shared a household with (an)other CF patient(s) not present at the event. Three months after the event, when asked about contact with other CF patients, 11 (50%) patients indicated they had had physical contact with another CF patient other than their sibling or household contact.

Table 1. Participant characteristics at day of event.

age, mean (SD)	28 (11)
male, n (%)	10 (45)
using antibiotics, n (%)	18 (82)
inhalation	9 (41)
oral	18 (82)
history of lung transplant, n (%)	6 (27)
time since last hospitalization*, n (%)	
less than 3 months	4 (18)
between 3 months and 1 year	7 (32)
more than 1 year	10 (46)

* data available for 21 patients

Table 2. Number of samples and number (%) of *P. aeruginosa* positive samples.

	t1(%)	t2(%)	t3(%)
cough swab	11	13	8
positive	3	6	3
sputum	11	9	14
positive	11	9	12
total	22	22	22
positive	14 (64)	15 (68)	15 (68)

MLVA types

In total, 131 morphologically different *P. aeruginosa* strains were isolated from 16 patients at three time points. After exclusion of identical MTs in each culture, 59 strains remained belonging to 18 different MTs (Table 3 and Supp. Table 1). Three MTs were shared between patients (MT11, MT27 and MT98) (Table 3). MT27 was the most prevalent type in 36% (8/22) of the patients. This MT represents the highly prevalent *P. aeruginosa* clone ST406 reported previously among CF patients in the Netherlands[10;11]. Most *P. aeruginosa* positive patients (11/16; 69%) were colonized by the same MT during the entire study period. Four of these patients acquired an additional *P. aeruginosa* MT during the event (t2). In total there were 10 occurrences in 9 patients (patient 1, 2, 3, 4, 5, 7, 9, 14 and 19) of a newly acquired MT, i.e. *P. aeruginosa* isolates with MTs not cultured at t1. In seven of these cases (in six patients) the new MTs found were single locus variants (SLVs) of MTs already present at t1. In eight cases (in seven patients), the newly acquired MTs (MT11, MT27, MT295, MT392, MT395, MT400 and MT410) were first cultured at t2, while in two cases the newly acquired MTs (MT13 and MT98) were first cultured at t3. MT11, MT13, MT98 and MT410 were the acquired types during the study-period, unrelated (not SLVs) to previously found MTs in the same patient.

There was one case in which a patient (patients 14) acquired a MT during the CFBD that was also found in other patients at t1, thus fulfilled the criteria for cross-transmission at the event. Patient 14 acquired the high prevalent MT27 at t2 found at t1 in seven other patients. The newly acquired MT was also isolated at t3 suggesting that *P. aeruginosa* acquisition resulted in sustained and not only transient colonization. In patient 4 and patient 5 MT11 was found at t2. This MT was not cultured from the 22 participating patients at t1, although patient 4 did harbor the SLV of MT11 (MT298) at t1. In one patient (patient 2) a new MT (MT98) was cultured at t3 that was found in another patient during the event. This does not fulfill our strict definition of possible cross-transmission since there is no control of what happened during the months after the event. Nevertheless, transmission during the event is a possibility in this case.

Table 3. MLVA types (MT), adherence to hygiene rules and contact with other CF patients in the three months after the event.

Patient*	MT at Time Point			Hygiene rules **	Household contacts	Other CF patients
	T1	T2	T3			
~2	27	27	98	2	+	+
3	98	98/395	98	2	-	+
4	27	27	27	2	-	-
	298[#]	11	11			
~5	27	27	27	2	-	+
		11	11			
11	27	27	27	1	-	-
13	27	27	27	2	+	+
14	230 [&]	230/27	27	2	+	+
15	27	27	27	2	+	+
22	27	27	27	2	+	+
7	44/296	295/392 [$]	296/392	2	-	-
9	300	300/400	300/400	2	-	+
17	293/403	293/403	293	2	-	-
20	399	399	399	2	-	-
21	398	398	398	1	-	-
6				1	-	-
8				2	+	+
10				2	+	+
12				1	+	-
16				1	-	+
18				3	-	-
~19			13	2	-	-
~1		410		2	-	-

Grey squares represent negative cultures. Colored squares represent shared MTs, orange: clonal MT27, green: MT11 and yellow: MT98. Multiple MTs in one square represent single locus variants (SLV). #: MT298 is single locus variant (SLV) of MT11, &: MT230 is SLV of MT27, $: MT295 and MT392 are SLVs of MT296. ~patients with new MTs, not SLV of MTs already present.

*Underscored patient numbers represent patients with a history of lung transplant.

**Their adherence to hygiene rules was indicated by the patients as follows: 1 Hygiene rules were ignored completely; 2 Hygiene rules were more or less followed; 3 Hygiene rules were very strictly adhered to.

Discussion

Molecular epidemiological analysis of *P. aeruginosa* isolated from CF patients attending the CFBD identified one case of possible cross-transmission during the event. There were 10 other cases of newly acquired MTs in nine patients, but these MTs could not be identified in other participants before the event or were first cultured 3 months after the event (1 case), which makes epidemiological linkage with the CFBD less likely. In six patients, including one possible transmission case, the new MTs found were SLVs of types already found in that patient. Possibly, instead of true transmission, this represents in-patient evolution of the chronically colonizing *P. aeruginosa* strains with SLVs of the initial colonizing strain simultaneously present. This is supported by the fact that in 9 cultures (20% of all positive cultures) SLVs were present simultaneously.

The five patients in which a novel MT, not seen in other patients before the event (t1), was cultured at the end of the CFBD (t2) could represent transmission of a strain acquired from a CF patient who did not participate, although there were only three non-participating patients. Besides, it cannot be excluded that *P. aeruginosa* strains from the environment or healthy visitors to CFBD were transmitted to CF patients. It is known that healthy people sometimes can be carrier of *P. aeruginosa* temporarily during e.g. respiratory virus infections[12]. Since our primary aim was to assess the risk cross-transmission between CF patients, we did not sample and analyze the environment or healthy persons attending the CFBD. It is conceivable that cross-transmission will be missed in the samples at t2 due to low load of *P. aeruginosa* types in comparison with the previous existing *P. aeruginosa* clones. In that case, the t3 samples could be indicators to evaluate a cross-transmission which then would show a possible transmission event in patient 2.

Eleven patients (50%) indicated to have had contact with other CF patients (beside their household contacts) against medical advice, in the three months after CFBD. This may also explain the fact that in two patients a new MT was detected at t3.

Among the participants, there was a relatively high number of patients (six) from which we could not culture *P. aeruginosa*. This was unexpected since these patients are particularly at risk of acquiring *P. aeruginosa* and we anticipated that these patients would be less willing to meet with other CF patients. Two out of the six culture negative patients had had a lung transplant, which may explain why they were no longer carriers of *P. aeruginosa* in their lungs. Besides, the different patients were using different antibiotic treatments which can influence culture yield. Another explanation could be that culture yield was not optimal in this research setting.

Whether *P. aeruginosa* genotype influences the occurrence of transmission can not be determined from these results because of the small study size. However, possible

cross-transmission occurred with the most prevalent MT among CF patients in the Netherlands[10].

Seven of the 22 participating patients were colonized with MT27 at the start of the event and one patient possibly acquired this clone possibly during the event. This clone has previously been identified as a highly prevalent clone in Dutch CF patients and is named, based on MLST, ST406[10;11]. This *P. aeruginosa* clone is thus far only found in CF patients and not in patients in the ICU or on other wards[10;13]. Previous transmission of this clone at social events (like summer camps) seems likely considering the evidence Brimicombe *et al* collected at the summer camps in 2001[4]. It is possible that several potential cases of transmission are missed; especially when MT27 clones were cross-transmitted between patients who already harbored this clone. Improved genotyping methods with increased resolution like MLST or MLVA schemes with an increased number of loci, preferably including loci encoding for virulence or resistance genes with an expected faster biological clock, or whole genome sequencing, may provide increased insight into frequency and extent of transmission of particular clones like *P. aeruginosa* MT27.

The results from this study should be interpreted with care because of the low number of patients who visited this edition of the event and predominance of one MT which precludes drawing definite conclusions on the risk of *P. aeruginosa* transmission during the 1 day open-air CF Beach Dance. Nevertheless, our data suggest possible transmission of *P. aeruginosa* between CF patients during a one-day open-air event. In addition we found the compliance to hygiene rules to be quite low. Therefore we advise against participation in these events at all, especially for patients without chronic *P. aeruginosa* colonization. For those CF patients who want to visit these events against medical advice, we recommend stricter adherence to hygiene rules when visiting such an event.

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Supplementary data

Supplementary Table 1. Results of MLVA typing.

patient	time point#	material*	ms77	ms127	ms142	ms211	ms213	ms215	ms216	ms217	ms223	MLVA Type (MT)	SLV of MT
1	2	C	3	9	3	4	1	5	1	3	5	410	
2	1	S	4	8	4	7	5	5	2	3	2	27	
2	2	C	4	8	4	7	5	5	2	3	2	27	
2	3	S	3	8	1	3	5	6	1	2	4	98	
3	1	C	3	8	1	3	5	6	1	2	4	98	
3	2	C	3	8	1	3	5	6	1	2	4	98	
3	2	C	3	8	1	3	5	99	1	2	4	395	SLV98
3	3	S	3	8	1	3	5	6	1	2	4	98	
3	3	S	3	8	1	3	5	6	1	2	4	98	
4	1	S	4	8	4	7	5	5	2	3	2	27	
4	1	S	3	8	4	2	1	7	2	4	3	298	SLV11
4	2	S	4	8	4	7	5	5	2	3	2	27	
4	2	S	3	8	4	2	1	6	2	4	3	11	
4	3	S	3	8	4	2	1	6	2	4	3	11	
4	3	S	4	8	4	7	5	5	2	3	2	27	
5	1	S	4	8	4	7	5	5	2	3	2	27	
5	2	S	4	8	4	7	5	5	2	3	2	27	
5	2	S	3	8	4	2	1	6	2	4	3	11	
5	3	S	3	8	4	2	1	6	2	4	3	11	
5	3	S	4	8	4	7	5	5	2	3	2	27	
7	1	S	3	8	5	4	5	4	2	3	2	44	slv296
7	1	S	3	8	3	4	5	4	2	3	2	296	
7	2	S	99	8	5	4	5	4	2	3	2	392	slv296
7	2	S	3	8	3	4	4	4	2	3	2	295	slv296
7	3	S	99	8	5	4	5	4	2	3	2	392	slv296
7	3	S	3	8	3	4	5	4	2	3	2	296	
9	1	C	3	8	4	3	7,5	1	2	3	2	300	
9	2	C	3	8	99	3	7,5	1	2	3	2	400	slv300
9	2	C	3	8	4	3	7,5	1	2	3	2	300	
9	3	C	3	8	99	3	7,5	1	2	3	2	400	slv300
9	3	C	3	8	4	3	7,5	1	2	3	2	300	
11	1	S	4	8	4	7	5	5	2	3	2	27	

Supplementary Table 1. *Continued*

patient	time point#	material*	ms77	ms127	ms142	ms211	ms213	ms215	ms216	ms217	ms223	MLVA Type (MT)	SLV of MT
11	2	S	4	8	4	7	5	5	2	3	2	27	
11	3	S	4	8	4	7	5	5	2	3	2	27	
13	1	S	4	8	4	7	5	5	2	3	2	27	
13	2	S	4	8	4	7	5	5	2	3	2	27	
13	3	S	4	8	4	7	5	5	2	3	2	27	
14	1	S	4	8	4	7	99	5	2	3	2	230	slv27
14	2	S	4	8	4	7	99	5	2	3	2	230	slv27
14	2	S	4	8	4	7	5	5	2	3	2	27	
14	3	S	4	8	4	7	5	5	2	3	2	27	
15	1	S	4	8	4	7	5	5	2	3	2	27	
15	2	S	4	8	4	7	5	5	2	3	2	27	
15	3	S	4	8	4	7	5	5	2	3	2	27	
17	1	S	99	7	3	3	5	3	3	3	3	403	slv293
17	1	S	3	7	3	3	5	3	3	3	3	293	
17	2	S	3	7	3	3	5	3	3	3	3	293	
17	2	S	99	7	3	3	5	3	3	3	3	403	slv293
17	3	S	3	7	3	3	5	3	3	3	3	293	
19	3	S	3	8	4	8	2	4	2	4	3	13	
20	1	S	3	8	6	3	3	4	1	2	5	399	
20	2	S	3	8	6	3	3	4	1	2	5	399	
20	3	S	3	8	6	3	3	4	1	2	5	399	
21	1	C	3	8	5	4	4	1	3	4	3	398	
21	2	C	3	8	5	4	4	1	3	4	3	398	
21	3	C	3	8	5	4	4	1	3	4	3	398	
22	1	S	4	8	4	7	5	5	2	3	2	27	
22	2	C	4	8	4	7	5	5	2	3	2	27	
22	3	C	4	8	4	7	5	5	2	3	2	27	

*material for culture: C=cough swab, S=sputum; ms=mini-satellite, locus for MLVA typing
time point 1= before event, 2=when leaving the event, 3=three months after the event

Chapter 5

High persistence of a Dutch *Pseudomonas aeruginosa* clone found in cystic fibrosis patients

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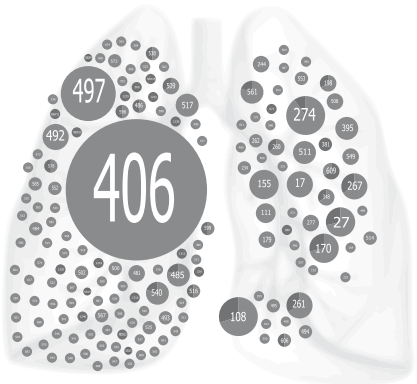

Abstract

Background

Some highly prevalent clones of *Pseudomonas aeruginosa* in patients with cystic fibrosis (CF) have been associated with adverse clinical outcome. Patient-to-patient transmission or enhanced persistence can contribute to the success of these highly prevalent *P. aeruginosa* clones. In this follow-up study we aimed to investigate whether the Dutch highly prevalent clone (ST406) is associated with enhanced persistence and whether infection with ST406 is associated with adverse clinical outcome.

Methods

Clinical data was collected from all participants of a *P. aeruginosa* transmission study during a CF summer camp in 2001. Sputum samples from living participants were collected and isolates were typed using multi locus variable-number tandem-repeat analysis (MLVA). In a previous study it was shown that ST406 is represented by MLVA-based clonal complex 27 (CC27). The risk of lung transplantation or death was investigated using a Cox proportional hazard model and lung function decline was investigated using a linear mixed model analysis.

Results

Of the 80 participants in 2001, 66 patients were alive in 2010 and from 41 a sputum sample was collected. CC27 was found in 44% (18/41) in 2001 and in 49% (20/41) in 2010. Persistence of CC27 was higher than that of other types (84% and 53% respectively, p=0.03). Association of CC27 with lung transplant-free survival or an increased lung function decline was not detected.

Conclusions

CC27 displays enhanced persistence over a nine-year period when compared to other *P. aeruginosa* types. However, no association with adverse clinical outcome could be detected.

Introduction

Patients with cystic fibrosis (CF) become chronically infected with specific pathogens, predominantly *Pseudomonas aeruginosa*, which infects over 50% of adult patients[1]. Although many patients are infected with unique strains that are presumably acquired from an environmental source[2-4], the occurrence of shared *P. aeruginosa* clones between unrelated patients at CF centres has been reported since the 1990's[5-7]. The published literature on these clones has recently been reviewed in detail[8]. The first reports of these highly prevalent clones showed an association with an unfavourable clinical outcome[9-12] but many of these studies were cross-sectional, and recent longitudinal studies have shown conflicting results on the effect of these clones[13;14]. Infection control policies were implemented in many countries to limit the spread of these clones. Whereas in the United Kingdom and Australia cohort segregation was implemented[12;15], in the Netherlands individual patient segregation was implemented in clinical settings in 2006, as well as the discontinuation of summer camps and advising against physical contact between all CF patients outside the hospital[16].

A study on the transmission of *P. aeruginosa* among patients attending a CF summer camp in 2001 already showed the occurrence of a dominant *P. aeruginosa* clone[17]. This was confirmed by a large scale cross-sectional analysis of the *P. aeruginosa* population structure of patients in the two largest Dutch CF centres in 2007, using multi-locus sequence typing (MLST), which showed a highly prevalent clone designated ST406[18]. This clone was not genetically linked to any of the reported highly prevalent clones and was not characterized by a specific antibiotic resistance profile or by a risk of adverse clinical outcome[19]. This clone is not detected in other MLST studies of clinical isolates and the environment which suggests a role for patient-to-patient transmission[20-22].

Several reports suggested that highly prevalent *P. aeruginosa* clones are sometimes characterized by specific virulence factors that are thought to contribute to their success, e.g. factors that might promote transmission[8;23]. Besides patient-to-patient transmission, another factor that could contribute to the success of highly prevalent clones is an enhanced capacity to persist. Because the 2007 study showed that despite implementation of individual segregation in 2006 in the Netherlands, the majority of patients were colonized with a genotype that was also found in other patients[18], we aimed to investigate the persistence of the different *P. aeruginosa* strains. We hypothesized that the Dutch clone (ST406) shows enhanced persistence over a nine-year period when compared to sporadic genotypes. Furthermore, we aimed to study whether ST406 is associated with a more severe course of CF lung disease longitudinally, since in contrast to other highly prevalent clones, cross-sectional data revealed no association with adverse clinical outcome[19].

Methods

Patients

This study was a follow-up of a study performed in 2001 that investigated transmission of *P. aeruginosa* between patients during a summer camp for CF patients. In 2001 sputum samples were obtained from 80 children between 6-19 years of age that visited one of four different camps. Samples were taken on day 1 of the camp, and 3 and 6 months after the camp[17]. The participants were treated in 24 different hospitals in 2001 and six of them had no prior history of *P. aeruginosa* infection. In 2010 all participants of the 2001 study who were still alive were invited to participate in this study. Those who agreed to study participation received a sample collection package mail and were asked to send a sputum or cough swab sample to the investigators. Patient instructions on how to collect the sputum sample or cough swab were included in the collection set. Clinical data of all participants of the 2001 summer camps were retrospectively collected from the medical records of the patients. The institutional ethical review board waived the need for individual informed consent for retrospective collection of clinical data.

Samples

All sputum and throat swab samples were cultured by standard diagnostic laboratory protocols. *P. aeruginosa* isolates were identified on the basis of morphology, oxidase and C390 resistance. One *P. aeruginosa* colony of each different colony morphology (rough, smooth, mucoid characteristics and colony size) per sample was randomly picked and stored at -70°C for typing with Multiple-Locus Variable number tandem repeat Analysis (MLVA). The *P. aeruginosa* strains that were isolated and typed with Amplified Fragment Length Polymorphism (AFLP) in 2001 were retyped using MLVA.

MLVA typing

MLVA was performed according to the "MLVA9-Utrecht" typing scheme as described before[24]. MLVA types (MTs) with identical number of repeats in 8 out of 9 loci (single locus variants; SLVs) were considered to belong to the same clonal complex (CC). MLVA9-Utrecht profiles were analysed using Bionumerics software (version 5.1).

Statistical analysis

Persistence was calculated for each CC by calculating the percentage of clones of that type that were present in 2001 and still persisted within the same patient in 2010, irrespective of whether a patient had two or more clones. A comparison of persistence was made between CC27 and non-CC27 clones using Fisher's exact test.

For the survival analysis, a Cox proportional hazards model based on n=77 subjects was used to evaluate association between lung transplant-free survival and presence of CC27 in 2001. The composite endpoint was the date of lung transplantation or death (whichever came first). The robustness of these results was tested by also performing these analyses without excluding the 3 patients with unknown lung transplantation data.

A linear mixed-effects model for FEV_1 (percent of predicted scale) was used to evaluate association between decline in lung function and persistent CC27 infection during 2001-2010. Lung function measurements were collected between 2001 and 2010 and if more than 1 measurement per three months was available, the highest FEV_1 % of predicted was included in the analyses. FEV_1 values after lung transplantation were excluded. Persistence of CC27 (yes or no), age at camp (years), elapsed time since arrival at camp and the CC27-by-time interaction were included as fixed effects. The model assumed patient-specific random effects for the intercept and the slope for time. Statistical computations were performed using IBM SPSS statistics, version 20.0.

Results

Patients

The original study in 2001 included 80 participants that were distributed over four camps based on their age. In 2010, 14 out of the 80 participants had died, and 13 had undergone lung transplantation, and of three patients it was unknown whether they had undergone a lung transplantation. Therefore, these three patients were not included in the survival analysis. In total, 41 of the 66 patients that were included in 2001 and that were still alive in 2010, participated in this study and delivered one or two respiratory samples. In total, 52 sputum samples and 19 cough swab samples were collected. Cultures were negative in four patients in 2010. Of the participants in this follow-up study, nine received a lung transplant sometime during the study period.

MLVA types

A total of 233 isolates were collected from 37 *P. aeruginosa* positive patients and typed using MLVA. This yielded 41 MLVA-genotypes (MTs) that were grouped into 9 clonal complexes (CCs), consisting of 26 different MTs, and 14 singletons, i.e. MTs not belonging to CCs (see supplement table S1). The CCs or singleton MTs found in each patient are displayed in Table 1. The isolates that had AFLP type 18 in 2001 all belonged to CC27 when retyped by MLVA. An earlier typing study revealed that isolates belonging to CC27 are ST406 based upon MLST[24]. CC27, was found in 49% of patients (n=20) in 2010, clearly demonstrating dominance of CC27 among these CF patients. MT11 was

Table 1. Distribution of CCs and MTs among *P. aeruginosa* recovered from CF patients in 2001 and 2010.

Patient	MT or CCs 2001	MT or CCs 2010
1	27	27, 9
2	27	27
3	27	27
4	27	27, 391
5	27	27
6	27	27
7	27	27
8	27	27
9	27	27
10	27	27
11	27	27
12	27	27, 176
13	27	27, 11
14	27	27
15	27, 225	27, 225
16	27	11
17	27, 346, 343	11, 346
18	27, 13	385
19	9	27
20	9	9
21	9	9
22	9, 11	9
23	9, 388	11, 388
24	11	345, 21
25	11	300
26	11	11, 78
27	11, 337	11
28	11, 44	44, 295
29	44	44
30	341	27
31	98	98
32	13	27
33	300	300
34	24	11
35	338	27
36	neg	2
37	neg	27

detected in 15% of patients (n=6) in 2010 and represents the second major clone also found in the previous studies (AFLP type 23 and ST497). Upon studying the distribution of CC27 in the participants and non-participants, there was no clinically relevant difference (in 2001 it was found in 47 (59%) out of all 80 participants and in 18 (44%) out of the 41 participants in the present study, p=0.12).

Persistence of clonal complexes

In 2001, MTs belonging to a total of 15 different singleton MTs or CCs were found in 37 *P. aeruginosa* positive participants, while in 2010 17 different singleton MTs or CCs were detected. Table 2 shows the prevalence of the detected CCs and singleton MTs

Table 2. Clonal complexes or singleton MTs and persistence within patients.

clonal complex or singleton MT		2001		2010		persistence in patient*	
		frequency (no of patients)	% of patients	frequency (no of patients)	% of patients	frequency	percent
MT	2	0	0.0	1	2.7		
CC	9	5	13.5	4	10.8	3	60
MT	11	6	16.2	7	18.9	2	33.3
CC	13	2	5.4	0	0.0	0	0.0
MT	21	0	0.0	1	2.7		
MT	24	1	2.7	0	0.0	0	0.0
CC	27	18	48.6	20	54.1	15	83.3
CC	44	2	5.4	2	5.4	2	100.0
CC	78	0	0.0	1	2.7		
MT	98	1	2.7	1	2.7	1	100.0
MT	176	0	0.0	1	2.7		
CC	225	1	2.7	1	2.7	1	100.0
MT	295	0	0.0	1	2.7		
MT	300	1	2.7	2	5.4	1	100.0
MT	337	1	2.7	0	0.0	0	0.0
CC	338	1	2.7	0	0.0	0	0.0
MT	341	1	2.7	0	0.0	0	0.0
MT	343	1	2.7	0	0.0	0	0.0
MT	345	0	0.0	1	2.7		
CC	346	1	2.7	1	2.7	1	100.0
MT	385	0	0.0	1	2.7		
CC	388	1	2.7	1	2.7	1	100.0
MT	391	0	0.0	1	2.7		

*Persistence is defined as the percentage of patients with the same CC or MT in 2001 and in 2010.

and their persistence in individual patients (persistence ranging from 0% to 100%). CC27 is by far the most prevalent clonal complex in both 2001 and 2010 (found in 18 and 20 patients respectively), followed by CC11 (found in 6 and 7 patients respectively). CC27 is more persistent than other clones (84% vs 53% p=0.03).

Clinical characteristics

Table 3 displays the baseline characteristics of the study participants (n=35) who had a *P. aeruginosa* positive sputum sample in 2001 and in 2010. Patients were grouped by whether CC27 was present in their 2001 samples. Although patients with CC27 in 2001 were on average 3.7 years younger than patients harbouring sporadic clones, there was no statistically significant difference in clinical parameters like pulmonary function. Patients with sporadic clones were more likely to use inhaled antibiotics in 2001.

A survival analysis including all 80 patients was performed to study whether patients who harboured CC27 in 2001 had an increased risk of lung transplantation or death (Fig. 1 shows the survival curve).

The total at risk time was 3016 months for patients who did not have CC27 in 2001, and 4467 months for patients who did have CC27 in 2001. During at risk time there were 25 events (lung transplantation=12, death=13). The unadjusted hazard ratio from the Cox proportional hazards model was 0.72 (95% CI [0.33, 1.58], p 0.41) for patients with CC27 in 2001. Covariate adjustment was achieved by including age and sex in the regression equation. Adjusted for age and sex, the test of association of CC27 with transplant-free survival was inconclusive (HR 1.01 [0.41, 2.49], p 0.99). Association of

Table 3. Baseline characteristics of participants at first sample in 2001.

	patients without CC27 (n=17)	patients with CC27 (n=18)	p
age, mean	16.6 (SD 3.8)	12.9 (SD 2.5)	0.002[a]
male, n	9 (52.9%)	9 (50.0%)	0.86[b]
dF508 homozygosity, n	10 (58.8%)	8 (61.5%)	0.67[c]
FEV_1 % of predicted, mean	75.0 (SD 28.3)	86.3 (SD 23.8)	0.25[a]
FVC % of predicted, mean	88.0 (18.1)	91.1 (19.3)	0.65[a]
previous camps visited, mean	6 (SD 3.5)	5 (SD 2.7)	0.51[a]
use of inhaled antibiotics, n	11 (65%)	4 (22%)	0.03[b]
use of rhDNase, n	12 (70.6%)	8 (47.1%)	0.16[b]
IV antibiotics courses in 2001, median	1 (IQR 0-2)	0 (IQR 0-1)	0.16[d]
CFRD*, n	3 (17.6%)	2 (12.5%)	1.0[c]

[a]t-test, [b]Chi-square, [c]Fischer's exact, [d]Mann-Whitney U, *CF-Related Diabetes

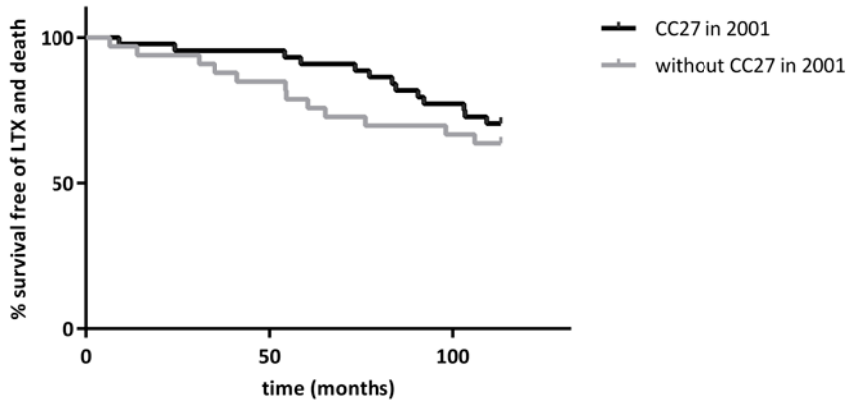

Figure 1. Survival free of lung transplantation or death. Results from a Cox proportional hazards analysis including n=77 subjects.

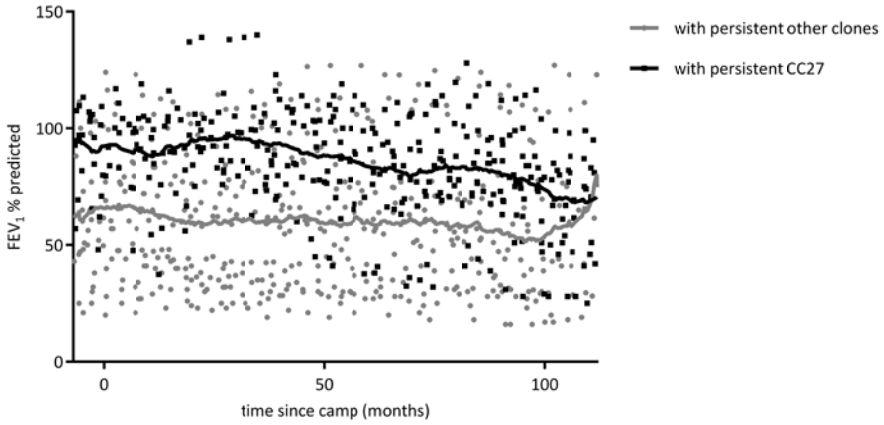

Figure 2. Lung function over time in relation to persistence of CC27. Graph represents the highest FEV1% of predicted values per three months, for each patient (845 measurements in total). Trend lines (Lowess curves) are shown.

survival with age and sex was also not detected. Inclusion of the patients with missing lung transplantation status did not change the results.

For the analysis of lung function decline, patients were divided into two groups: patients who were persistently infected with CC27 (n=14), and patients who were not persistently infected with CC27 (n=20). A total of 845 lung function measurements were included (mean 25 measurements per patient). Figure 2 shows the FEV_1% of

predicted values by time since the camp. Patients who did not have persistent CC27 infection had a lower estimate of lung function throughout the study period (-20.1, 95% CI [-40.1, -0.17], *p*=0.048). Association of CC27 persistence with rate of decline in FEV_1 was not detected: the test of the null hypothesis "the CC27-by-time interaction is zero" was inconclusive (p=0.79).

Discussion

The present study shows that the highly prevalent *P. aeruginosa* clone in the Dutch CF population (CC27/ST406) displays enhanced persistence over a nine-year period. Association of CC27 with survival was not detected. Patients who were persistently infected with CC27 did not have a statistically significant difference in lung function decline compared to patients infected with other clones. This suggests that although CC27 is able to frequently colonize lungs of CF patients and able to persist for prolonged periods it does not appear to be a highly pathogenic clone, compared to more sporadic clones. This has also been shown for some of the other reported "successful" CF clones[25]. Jelsbak *et al* also showed that a highly prevalent *P. aeruginosa* clone in CF patients in Copenhagen displayed decreased virulence in a *C. elegans* model[26]. And the Liverpool epidemic strain showed variable killing in a Drosophila model for acute infection between the different isolates of this clone[27]. The enhanced persistence of CC27 suggests that although its virulence is probably decreased, this clone is possibly better adapted to maintain in the CF lung. This would be in concordance with observations by Bragonzi *et al*, who show that CF isolates can show attenuated acute virulence, while still maintaining the capacity of causing chronic infection[28]. A future analysis based on whole genome sequencing and phenotypic traits might elucidate why this epidemic Dutch clone is well adapted to the CF lung. It can be speculated that the attenuated virulence in CC27 strains results in a less outspoken host immune response towards the pathogen, hereby contributing to its enhanced persistence.

Although we have shown an important role for persistence in the success of CC27, we can only speculate about the transmissibility of CC27. The 2001 study did show that several cases of probable transmission involved CC27, but many of the patients with this clone were already infected upon entering the camp, suggesting that it was already present before the camp[17]. Indeed transmission during a previous camp is plausible (patients were treated in different centres all over the country so transmission in a CF centre is less likely), but another possibility is the presence of an environmental source e.g. a tap, shower or other water source at the campsite from which several patients could have acquired the same clone. The widespread presence of certain *P. aeruginosa* clones in the environment has been suggested to contribute to the high prevalence of

clones[3;29]. However, the Dutch highly prevalent clone ST406 / CC27 could not be found in a study analysing *P. aeruginosa* isolates from non-CF patients from different wards and out-patient clinics in one hospital and patients on ventilation in the ICUs of two different hospitals, or in a study among patients with otitis media or urinary tract infections, which suggests that a widespread prevalence of this clone in the environment is not likely[24;30]. This is also supported by absence of ST406 isolates in the international MLST database and no detection of this sequence type in environmental studies [20-22]. Also, CC27/ST406, identified as type A418 by the AT-chip typing in a previous study[31] was only found once in a German CF patient in a comprehensive study of *P. aeruginosa* amongst environmental and clinical isolates from different countries. In comparison, Clone C, also a high prevalent clone among CF patients, is widely distributed in the environment and also found to cause infections in non CF patients[32;33]. However, there are no systematic genotyping data of environmental *P. aeruginosa* isolates in the Netherlands and the possibility that this clone is both prevalent in the environment and has a specific tropism for CF lungs without causing infections in non-CF patients cannot be certainly excluded.

One of the strengths of this study is the follow-up time of nine years which allowed for a longitudinal analysis of lung transplantation and death risk. Also, the initial evaluation of the *P. aeruginosa* genotype was performed without the knowledge of the occurrence of a highly prevalent clone as the 2001 study was performed with the intention of investigating *P. aeruginosa* transmission between patients. Many studies that investigate highly prevalent *P. aeruginosa* clones in CF are prompted by the occurrence of such clones and are prone to ascertainment bias, since in those cases where transmission within CF centres was observed, the detection of highly prevalent clones is more likely in patients who are more severely diseased, and visit the hospital more often[5]. The use of MLVA for typing has several advantages as genotyping method. MLVA offers high typeability, ease of interpretation and the possibility of comparison with international typing data and a high discriminatory index[24]. Furthermore, the MLVA9-Utrecht typing scheme used in this study was extensively compared to MLST in a previous study, which included several ST406/CC27 isolates, and showed a very high congruence on the level of clonal complexes (CC) indicated by a Wallace coefficient of 0.969[24]. In that study we showed that isolates belonging to CC27 are ST406. Because of the high discriminatory index of MLVA and because most of the single locus variants were found to co-exist in the lung of one patient at the same time (58%), we hypothesize that SLV's within a single patient represents in-patient evolution.

A limitation to our study is the participation of 62% of all eligible patients, which could have led to a selection bias, if those with CC27 would have been more likely to participate in the follow-up study. However the fact that the distribution of the highly prevalent clone in participants and non-participants was similar, makes this less likely.

Since segregation measures were already implemented in the Netherlands before the detection of a highly prevalent clone, it is important to study its clinical impact. This study shows enhanced persistence of CC27 compared to sporadic clones over a period of nine years, while an association with an increased risk of lung transplant or death could not be detected. This was also the case for our previous cross-sectional study and this should be taken into account when evaluating the current infection control policies. At least further prospective studying, in a large CF population, of the longitudinal effects of this clone is warranted. Possibly, future results could lead to a revision of the segregation policy in the Netherlands.

Acknowledgements

The authors would like to thank Dorien Holtslag, Els van der Wiel, Jane de Vries, Irma Bon and Maaike van Brederode for their help in collecting patient data and Bertie Dekker for her help in performing MLVA typing experiments.
Source of funding for this study: this research was supported by a grant from the Dutch Cystic Fibrosis Foundation (NCFS).

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Supplementary data

Supplemetary table S1

		MLVA alleles (MS=mini satelite)										
patient ID	culture date	MS77	MS127	MS142	MS211	MS213	MS215	MS216	MS217	MS223	MLVA TYPE	CC (S=singleton)
1	2010	4	8	4	7	1	5	2	3	2	351	27
1	2010	3	8	4	2	3	2	2	4	6	9	9
1	2010	4	8	4	7	99	5	2	3	2	230	27
1	2001	4	8	4	7	5	5	2	3	2	27	27
2	2010	4	8	3	7	5	5	2	3	2	349	27
2	2001	4	8	4	7	5	5	2	3	2	27	27
3	2010	4	8	4	7	5	5	2	3	2	27	27
3	2001	4	8	4	7	5	5	2	3	2	27	27
4	2010	4	8	4	7	5	5	2	3	2	27	27
4	2010	4	8	4	7	99	5	2	3	2	230	27
4	2010	4	8	4	5	99	5	2	3	2	391	S
4	2001	4	8	4	7	5	5	2	3	2	27	27
5	2010	4	8	4	7	5	5	2	3	2	27	27
5	2001	4	8	4	7	5	5	2	3	2	27	27
6	2010	4	8	4	7	5	5	2	3	2	27	27
6	2001	4	8	4	7	5	5	2	3	2	27	27
7	2010	4	8	4	7	5	5	2	3	2	27	27
7	2001	4	8	4	7	5	5	2	3	2	27	27
8	2010	4	8	4	7	5	5	2	3	2	27	27
8	2010	4	8	4	7	99	5	2	3	2	230	27
8	2001	4	8	4	7	5	5	2	3	2	27	27
9	2010	4	8	4	7	5	5	2	3	2	27	27
9	2001	4	8	4	7	5	5	2	3	2	27	27
10	2010	4	8	4	7	99	5	2	3	2	230	27
10	2010	4	8	4	7	5	5	2	3	2	27	27
10	2001	4	8	4	7	5	5	2	3	2	27	27
10	2001	4	8	4	7	99	5	2	3	2	230	27
11	2010	4	8	4	7	5	5	2	3	2	27	27
11	2001	4	8	4	7	5	5	2	3	2	27	27
12	2010	4	8	4	7	5	5	2	3	2	27	27

Supplemetary table S1 *(continued)*

patient ID	culture date	MLVA alleles (MS=mini satelite)										
		MS77	MS127	MS142	MS211	MS213	MS215	MS216	MS217	MS223	MLVA TYPE	CC (S=singleton)
12	2010	3	8	4	4	3	5	2	3	3	176	S
12	2001	4	8	4	7	5	5	2	3	2	27	27
13	2010	3	8	4	2	2	6	2	4	3	387	11
13	2010	4	8	4	7	5	5	2	3	2	27	27
13	2001	4	8	4	7	5	5	2	3	2	27	27
14	2010	4	8	4	7	5	5	2	3	2	27	27
14	2001	4	8	4	7	5	5	2	3	2	27	27
15	2010	3	9	1	3	3	25	1	99	2	344	225
15	2010	4	8	4	7	5	5	2	3	2	27	27
15	2010	4	8	4	7	99	5	2	3	2	230	27
15	2001	3	9	1	3	99	25	1	99	2	225	225
15	2001	4	8	4	7	5	5	2	3	2	27	27
16	2010	3	8	4	2	1	6	2	4	3	11	11
16	2001	4	8	4	7	5	5	2	3	2	27	27
17	2010	3	8	4	2	1	6	2	4	3	11	11
17	2010	3	8	5	2	1	6	2	4	3	340	11
17	2010	3	99	99	5	1	4	1	2	5	347	346
17	2001	4	8	4	7	5	5	2	3	2	27	27
17	2001	3	99	5	5	1	4	1	2	5	346	346
17	2001	3	8	6	3	99	4	2	2	2	343	S
18	2010	3	8	4	3	5	1	2	4	3	385	S
18	2001	3	8	4	8	2	4	2	4	3	13	S
18	2001	4	8	4	7	5	5	2	3	2	27	27
19	2010	4	8	4	7	99	5	2	3	2	230	27
19	2010	4	8	4	7	5	5	2	3	2	27	27
19	2001	3	8	4	2	3	2	2	4	6	9	9
20	2010	3	8	4	2	3	2	2	4	6	9	9
20	2010	3	8	4	2	2	2	2	4	6	380	9
20	2001	3	8	4	2	3	2	2	4	6	9	9
21	2010	3	8	4	2	3	2	2	4	6	9	9
21	2001	3	8	4	2	3	2	2	99	6	390	9
21	2001	3	8	4	2	3	2	2	4	6	9	9
22	2010	3	8	4	2	3	2	2	4	6	9	9
22	2001	3	8	4	2	3	2	2	4	6	9	9

Supplemetary table S1 *(continued)*

		MLVA alleles (MS=mini satelite)										
patient ID	culture date	MS77	MS127	MS142	MS211	MS213	MS215	MS216	MS217	MS223	MLVA TYPE	CC (S=singleton)
22	2001	99	8	4	2	1	6	2	4	3	315	11
23	2010	4	8	7	5	5	4	3	2	99	389	388
23	2010	3	8	4	2	2	6	2	4	3	387	11
23	2001	4	8	5	5	5	4	3	2	4	393	388
23	2001	4	8	7	5	5	4	3	2	4	388	388
23	2001	3	8	4	2	3	25	2	4	6	386	9
24	2010	3	9	5	4	4	1	2	3	2	345	S
24	2010	3	8	6	6	5	2	1	3	2	21	S
24	2001	3	8	4	2	1	6	2	4	3	11	11
25	2010	3	8	4	3	75	1	2	3	2	300	S
25	2001	3	8	4	2	1	6	2	4	3	11	11
26	2010	35	9	1	5	99	2	1	6	3	360	78
26	2010	35	9	1	5	4	2	1	6	3	78	78
26	2010	3	8	4	2	1	6	2	4	3	11	11
26	2001	3	8	4	2	1	6	2	4	3	11	11
27	2010	3	8	4	2	1	6	2	4	3	11	11
27	2001	3	8	4	5	10	5	2	4	5	337	S
27	2001	3	8	4	2	1	6	2	4	3	11	11
28	2010	3	8	3	4	4	4	2	3	2	295	S
28	2010	3	8	5	4	5	4	2	3	2	44	44
28	2001	3	8	5	4	5	4	2	3	2	44	44
28	2001	3	8	4	2	1	6	2	4	3	11	11
29	2010	3	8	5	4	5	4	2	3	2	44	44
29	2001	3	8	5	4	5	4	2	3	2	44	44
29	2001	99	8	5	4	5	4	2	3	2	392	44
30	2010	4	8	4	7	5	3	2	3	2	50	27
30	2001	3	8	5	4	4	6	2	4	5	341	S
31	2010	3	8	1	3	5	6	1	2	4	98	S
31	2001	3	8	1	3	5	6	1	2	4	98	S
32	2010	4	8	4	7	5	5	2	3	2	27	27
32	2001	3	8	4	8	2	4	2	4	3	13	S
33	2010	3	8	4	3	75	1	2	3	2	300	S
33	2001	3	8	4	3	75	1	2	3	2	300	S
34	2010	3	8	4	2	1	6	2	4	3	11	11

Supplemetary table S1 *(continued)*

patient ID	culture date	MLVA alleles (MS=mini satelite)										CC (S=singleton)
		MS77	MS127	MS142	MS211	MS213	MS215	MS216	MS217	MS223	MLVA TYPE	
34	2001	3	9	4	6	2	5	2	1	3	24	S
35	2010	4	8	4	7	5	5	2	3	2	27	27
35	2001	3	8	4	7	5	1	1	4	2	338	338
35	2001	3	8	4	99	5	1	1	4	2	339	338
36	2010	3	8	1	6	10	3	1	3	3	2	S
37	2010	4	8	4	7	5	5	2	3	2	27	27

Chapter 6

The effect of strict segregation on *Pseudomonas aeruginosa* acquisition in cystic fibrosis patients

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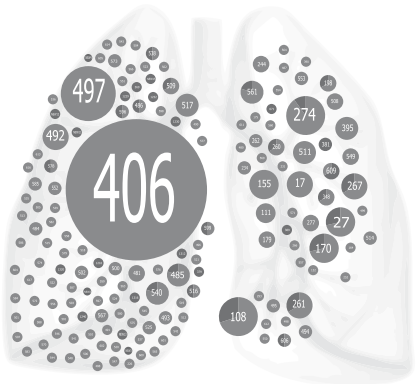
manuscript submitted



Abstract

Importance: Segregation of patients with cystic fibrosis (CF) was implemented in many countries to prevent chronic infection with epidemic *Pseudomonas aeruginosa* strains with presumed detrimental clinical effects. The effectiveness of this measure, though, has not been carefully evaluated.

Objective: To investigate the effect of strict segregation on the incidence of chronic *P. aeruginosa* infection in CF patients (primary objective), and on *P. aeruginosa* population structure and clinical parameters in patients infected with and without the Dutch epidemic clone (ST406) (secondary objectives).

Design: Incidences of chronic *P. aeruginosa* infection before (2005 and 2006) and after implementation of segregation (2007-2011) were determined through longitudinal protocolized follow-up of respiratory tract infection. The *P. aeruginosa* population structure was investigated in two cross-sectional studies in 2007 and 2011 using multi-locus sequence typing. Clinical effect of infection with ST406 was investigated longitudinally between 2007 and 2010.

Setting: A tertiary and secondary care referral center for CF patients in the Netherlands.

Participants: 784 CF patients that visited the hospitals between 2005 and 2011 (effects of segregation and *P. aeruginosa* population structure) and 219 patients with chronic *P. aeruginosa* infection (clinical relevance of ST406).

Exposure: Segregation was implemented during 2006.

Outcome: In all, 315 and 382 patients were at risk for acquiring chronic *P. aeruginosa* infection before and after segregation, and acquisition rates were 0.14 and 0.05 per 1,000 days at risk before and after implementation of segregation (HR: 0.66, 95% CI [0.2548-1.541]; p=0.28). In subgroup analysis acquisition was lower after segregation in children < 15 years of age (HR: 0.43, 95% CI[0.21-0.95]; p=0.04). Prevalence of chronic *P. aeruginosa* infection was 57% in 2007 and 52% in 2011. Proportions of patients infected with ST406 were 15% and 14% in 2007 and 2011, respectively. Chronic *P. aeruginosa* infection was highly persistent for all genotypes. There was no statistical association between ST406 and lung function decline, death or lung transplantation. Patients infected with ST406 had increased use of inhaled antibiotics (p=0.036).

Conclusions and relevance: In this cohort strict segregation was associated with lower acquisition of chronic *P. aeruginosa* infection in patients under 15 years of age. No association was detected between infection with the Dutch CF clone (ST406) and adverse clinical outcome.

Introduction

Pseudomonas aeruginosa can chronically infect the lungs of patients with cystic fibrosis (CF), contributing to disease progression and death[1]. Based on the presence of so-called epidemic *P. aeruginosa* clones among CF patients[2-6] and presumed detrimental effects of these clones on disease progression[7;8] segregation policies have been implemented for CF patients worldwide. It is thought that segregation prevents acquisition of chronic infections by CF adapted *P. aeruginosa* strains that are sometimes multi-resistant to antibiotics, and that strains acquired from the environment can be more easily eradicated[9]. In the Netherlands segregation was implemented in 2006, and consisted of strict hygiene rules and the recommendation to avoid all contact between individual CF patients in inpatient and outpatient clinics, as well as outside the hospital setting[10;11].

The effects of strict segregation of CF patients on *P. aeruginosa* acquisition and transmission have not been determined rigorously. Although reduced prevalence of epidemic strains[12-14], decreased incidence and prevalence of chronic *P. aeruginosa* infection[15] and less cross-infection between CF-patients after implementation of cohort segregation[16] have been reported, the dependency between prevalence observations, the dynamics of the population at risk and detailed analyses of pulmonary function in time were not included.

We have performed a longitudinal study in 784 CF patients attending care in two CF centers in the Netherlands to investigate the effects of segregation on acquisition of chronic *P. aeruginosa* colonization between 2005 and 2011. During this study period two cross-sectional molecular typing studies were performed, just after implementation of segregation in 2007 and five years after segregation in 2011, to determine the *P. aeruginosa* population structure, and prevalence of shared genotypes and epidemic clones. The first molecular cross-sectional study, performed in 2007, included 595 CF patients of whom 150 of 265 patients colonized with *P. aeruginosa* harbored *P. aeruginosa* Multi Locus Sequence Typing (MLST) genotypes that were shared by three or more patients[17]. Fifteen percent of the patients (n=41) were infected with clone ST406, which appeared unrelated to other international CF clones, was not detected in non-CF patients[18], and was not associated with unfavorable clinical outcome in a cross-sectional study[19]. Here we report on the effects of segregation on *P. aeruginosa* acquisition, on changes in molecular epidemiology by comparing the results of identical cross-sectional studies in 2007 and 2011, and on associations between infection with ST406 and decline in lung function and death in a nested cohort study of 219 patients.

Materials and methods

Patients and design

Standard of care for CF patients in the Netherlands consist of at least three-monthly visits to the out-patient clinic for physical examination, pulmonary function tests and microbiological cultures of sputum or throat swabs. However, some patients visit less regularly or visit a local hospital instead of the CF clinics for check-ups. Segregation measures were implemented in the Netherlands during 2006, and included strict segregation of all CF patients in clinical wards and outpatient clinics. Outside hospital settings it was recommended not to meet with other CF patients, or to adhere to strict hygiene rules (keep distance, don't touch, hand hygiene, cough hygiene, don't share utensils or medication) in case of contact[10;11]. All CF patients that visited the Wilhelmina Children's Hospital/University Medical Centre Utrecht (UMCU) or the Haga Teaching Hospital (Haga) in The Hague between 2005 and 2011 that had at least 4 years of culture data available (not necessarily consecutive), or were born after 1994, were included in the longitudinal study from the first known culture until the last if this was not performed in 2011 (otherwise included until 31-12-2011). Furthermore, two cross-sectional typing studies were performed, which included all patients visiting either hospital in 2007 and 2011. Persistence of *P. aeruginosa* genotypes was investigated in chronically colonized patients with *P. aeruginosa* isolates available for genotyping in both 2007 and 2011. All chronically infected patients who did not undergo lung transplantation and were included in the 2007 cross-sectional study, excluding patients with *Burkholderia species* (n=10), were included in the nested longitudinal follow-up study. The use of inhaled antibiotics was assessed per year, and expressed as a dichotomous value (yes or no) per year. To account for differences in frequency of pulmonary function measurement, only the highest Forced Expiratory Volume in one second (FEV_1) per three months was included. Lung function values were converted into percent of predicted values for FEV1 based on reference values for either adults[20] or children[21] where appropriate (using only one set of reference values per patient, i.e. the reference set that was appropriate for the majority of measurements). Based on the results of the first cross-sectional study, in 2007, patients were grouped according to *P. aeruginosa* genotype in group "ST406" or "other ST" [17]. Patients gave written informed consent for data collection in a database and for the use of this data for scientific purposes.

Definitions

"Chronic colonization" was defined as the detection of *P. aeruginosa* in ≥50% of the respiratory tract cultures per year for two consecutive years with at least three positive cultures. "Intermittent" colonization was defined as *P. aeruginosa* isolation from the respiratory tract in <50% of cultures in the previous year, with at least one positive

P. aeruginosa culture in the past. The date of acquisition of chronic *P. aeruginosa* colonization was defined as the date of the first *P. aeruginosa* isolate within three months before which chronic colonization was established. Chronic *P. aeruginosa* acquisition was investigated for the years 2005-2006 (before segregation) and 2007-2011 (after segregation) and expressed as the number of new chronic infections per day among patients at risk (i.e. not chronically infected). Two independent experts and two investigators analyzed culture data, blinded for patient identifier and calendar dates, to determine acquisition of chronic infection. Discrepancies were reevaluated by all experts and final decisions were reached by consensus.

Samples

All sputum samples and throat swabs were cultured according to standard diagnostic laboratory protocols of each hospital as described before [17]. In addition the UMCU also used C-390 disk diffusion (9mm Diatabs™, 40mcg) for determination of *P. aeruginosa*. One colony of each different colony-morphology (rough/smooth/mucoid and colony size) per sample was randomly picked and stored at -70˚C. Isolates of the first stored respiratory tract culture yielding *P. aeruginosa* of each patient, during 2007 and 2011, were genotyped with Multi Locus Sequence Typing (MLST). MLST was used to confirm correct species identification and study genetic relatedness of *P. aeruginosa*. Susceptibilities were determined by disk diffusion using EUCAST breakpoints for colistin, tobramycin, ciprofloxacin, ceftazidime, piperacillin + tazobactam in both hospitals[22] and amikacin and meropenem in the UMCU and imipenem in the Haga.

MLST

MLST was performed according to the protocol by Curran *et al.*[23]. Some adjustments were made, including the use of lysates of the isolates, newly designed primers, adding Q-buffer (Qiagen Benelux B.V., Venlo, the Netherlands) and the use of a touchdown PCR program as described before[17]. PCR products were sequenced on a 3730 DNA Analyzer with the same primers as used for amplification. Sequences were analyzed using Bionumerics 5.1(Applied Maths, St-Martens-Latem, Belgium).

Sequence types (STs) were compared to the *P. aeruginosa* Multi Locus Sequence Typing website (http://pubmlst.org/paeruginosa/) developed by Keith Jolley and new alleles and profiles were sent to the curator E. Pinnock[24]. STs that were detected in three or more unrelated patients are named "frequently shared STs". Relationships between STs were estimated using a Minimum Spanning Tree, based on allelic profiles using the goeBURST-based distances[25], as contained in PHYLOVIZ software[26]

Statistics

Calculations and analysis were performed using SPSS 20 (SPSS, Chicago,USA) and R (version 2.13.1). Genetic Diversity was calculated by Simpson's Index of Diversity (ID) as previously described using Bionumerics 5.1 (Applied Maths, St-Martens-Latem, Belgium). A Cox-proportional hazard model with segregation as time-dependent variable with time from birth as time scale was performed to determine the association between segregation and acquisition of chronic colonization with age as covariate. For continuous data the Student's t-test or Mann-Whitney U-test was used where appropriate. For binominal data the Chi-square test or Fisher's exact test were used where appropriate. A Cox proportional hazards model was used to study the association between ST406 and time to death or lung transplantation (combined endpoint, whichever occurred first). In case of death following lung transplantation, the date of lung transplantation was designated time of censoring. For longitudinal analyses of lung function, a linear mixed model was used. The model assumed a linear trend in FEV_1 over time for each patient, and allowed for random patient-specific slope and intercept. Several possible confounding factors were added to the model and tested for significance. A difference in decline of FEV_1 (slope analysis) between the two groups (ST406 or other clones) was tested by examining the improvement in model fit after adding an interaction term to the model (time*ST group). This interaction term would allow for different slopes over time for the two groups. For analysis of resistance to antibiotic classes between different genotypes the Mann-Whitney U test was used.

Results

In total, 784 patients were included in the longitudinal follow-up study between January 1st 2005 and December 31st 2011. Of these 117 (15%) were excluded because of insufficient culture results. At the start of the follow-up (1-1-2005) 217 patients were chronically infected with *P. aeruginosa*, 39 were infected with *Burkholderia species* or had already received lung transplantation leaving 411 at risk of acquiring chronic *P. aeruginosa* infection. Of the 411 patients, 315 were at risk for acquisition of chronic *P. aeruginosa* infection between 1-1-2005 and 31-12-2006, while after implementation of segregation (1-1-2007) until end of follow-up (31-12-2011) 382 patients were at risk for acquisition of chronic *P. aeruginosa* infection (Table 1 and suppl. Table 1). There were 11, 17, 13, 5, 5, 3, 2 acquisitions of infection with *P. aeruginosa* per year from 2005 till 2011, respectively. The acquisition rate of chronic infection with *P. aeruginosa* per year at risk was 0,051 and 0,018 before and during segregation, respectively. The hazard ratio for acquisition of chronic infection during segregation was 0.66 (CI [0.2548-1.541]; p=0.28), as compared to the risk before segregation. Exploration of the

Table 1. Patient characteristics, microbiological and genotypic characteristics of *P. aeruginosa* isolates associated with chronic infection of411 patients at risk for acquiring chronic infection before (2005-2006) and during (2007-2011) strict segregation of CF patients.

	Patients at risk for chronic infection before segregation (2005-2006) N=315	Patients at risk for chronic infection during segregation (2007-2011) N=382
Age at end of follow-up (average and SD)	21.9 (SD 13.2)	18.9 (SD14.0)
male/female	0.55/0.45	0.54/0.46
Patient days at risk	196,881	551,490
Number of patients with acquisitions of chronic *P. aeruginosa* infection	28	28
Rate of acquisitions with *P. aeruginosa*/1000 patient days at risk	0.14	0.05
Number of patients with chronic *P. aeruginosa* acquisition and isolates genotyped (%)	20 (71%)	18 (64%)
Number of different sequence types	18	17
Number of patients with shared sequence types (%)	12 (60%)	7 (38%)
Number of patients with acquisition of chronic *P. aeruginosa* ST 406 infection (%)	3 (15%)	0 (0%)

data to explain the wide confidence interval displayed different effects in different age groups with most effect of segregation occurring on younger age groups. Subsequent subgroup analysis of patients <15 years and >15 years of age resulted in HR of 0.43, (95% CI [0.21-0.95] p=0.04) and 0.88, (95% CI [0.23-3.34]; p=0.85), respectively.

Population structure *Pseudomonas aeruginosa*

The results of the first cross-sectional study (isolates from 2007) have been published[17]. The second cross-sectional study, of isolates obtained in 2011, included 631 patients (97% of all patients visiting either hospital) of which respiratory tract cultures were available (391 of 405 in UMCU (97%) and 240 of 244 in Haga (98%)). *P. aeruginosa* was detected in the respiratory tract of 326 of 631 patients (52%), and was more prevalent in adults (234 of 371, 63%) than in patients <18 years (92 of 260, 35%) (Table 2). From 280 (86%) patients with *P. aeruginosa* isolated from respiratory tract cultures 414 *P. aeruginosa* isolates were genotyped by MLST, yielding 157 different STs. The prevalence of ST406 and ST497 among patients infected with *P. aeruginosa* was 14% and 5%, respectively.

The prevalence of patients infected with *P. aeruginosa* was 52% in 2011 (versus 57% in 2007), the prevalence of patients with multiple genotypes was 8% (versus 11% in 2007) and the proportion of patients harboring STs that were shared with more than two unrelated patients was 50% (versus 57% in 2007) (Table 2). The prevalence

Table 2. Demographic and microbiological characteristics of cystic fibrosis patients included in cross-sectional studies in 2007 and 2011. (*= p<0.05)

	cohort 2007 (n=551)	cohort 2011 (n=631)
Average age (standard deviation) range	23.4 (13.8) 1.0-69.1	22.6 (14.0) 0-72.2
Proportion age <18/≥18	0.42/0.58	0.41/0.59
Proportion male/female	0.53/0.47	0.51/0.49
Number of respiratory tract cultures/year , [median, interquartile range]	3 [2-4]	4 [2-6] *
Number of *P. aeruginosa* isolates	443	414
Number of patients with intermittent or chronic infection with *P. aeruginosa* in the respiratory tract (%)	313 (57%)	326 (52%)
Number of patients with *P. aeruginosa* genotyped (% of infected)	265/313 (85%)	280/326 (86%)
Number of different sequence types	143	157
Number of patients with multiple phenotypically different *P. aeruginosa* isolates (% of patients with *P. aeruginosa* genotyped)	135/265 (51%)	113/280 (40%)
Number of patients infected with multiple *P. aeruginosa* sequence types (% of patients with *P. aeruginosa* genotyped)	28/265 (11%)	22/280 (8%)
Number of patients with frequently shared *P. aeruginosa* sequence types in more than two patients (number of different sequence types)	150 (57%) [24]	140 (50%) [22]
Number of patients infected with *P. aeruginosa* ST406 (% of patients with *P. aeruginosa* genotyped)	41 (15%)	38 (14%)
Number of patients infected with *P. aeruginosa* ST497 (% of patients with *P. aeruginosa* genotyped)	14 (5%)	13 (5%)
Simpson Index of diversity [95%CI]of *P. aeruginosa* isolates based on MLST	97.3 [96.2-98.4]	97.8 [96.9-98.7]

of ST406 was 15% in 2007 and 14% in 2011, but the average age of patients infected with ST406 increased from 19.8 years [range 11.0-30.5] in 2007 to 23.4 years [range 16.7-38.3] in 2011. In 2011, 109 patients (39%) harbored a unique ST, as compared to 81 patients (31%) in 2007. The genetic diversity of *P. aeruginosa* isolates from CF patients recovered in 2007 and 2011, expressed as the Simpson Index of diversity based on MLST, was 97.3 (95% CI 96.2-98.4) in 2007 and 97.8 (95% CI 96.9-98.7) in 2011.

There were 49 patients, not chronically infected with *P. aeruginosa* in 2007, who had "new" *P. aeruginosa* isolates genotyped in 2011 and thus acquired during segregation (mostly resulting in intermittent colonization). These 49 patients harbored 41 STs of which 20 were not detected in 2007 (15 new STs, and 5 STs previously described in the MLST database). The most prevalent among the detected ST, acquired after segregation, was ST108. The newly acquired STs had a comparable distribution of unique genotypes and shared genotypes as STs detected in 2007 (Fig. 1). Also the distribution among

three defined sources (i.e. sputum isolates from CF patients in the Netherlands, respiratory tract cultures in multiple countries, and other sources from multiple countries) was comparable for the STs detected in 2007 and 2011. A Minimum Spanning Tree based on 211 STs representing 599 isolates from Dutch CF patients revealed no specific sub-clustering according to year of isolation. STs only detected in 2007, detected both in 2007 and 2011, or detected only in 2011 were all dispersed similarly over the minimum spanning tree (suppl Fig 1). In the group of 49 "newly" infected patients there were no acquisitions of ST406 and ST497 genotypes, the most prevalent STs in 2007, during segregation (Fig 1). However, there were three acquisitions of chronic infection with ST406 after segregation in patients already chronically infected with non-ST406 *P. aeruginosa* in 2007.

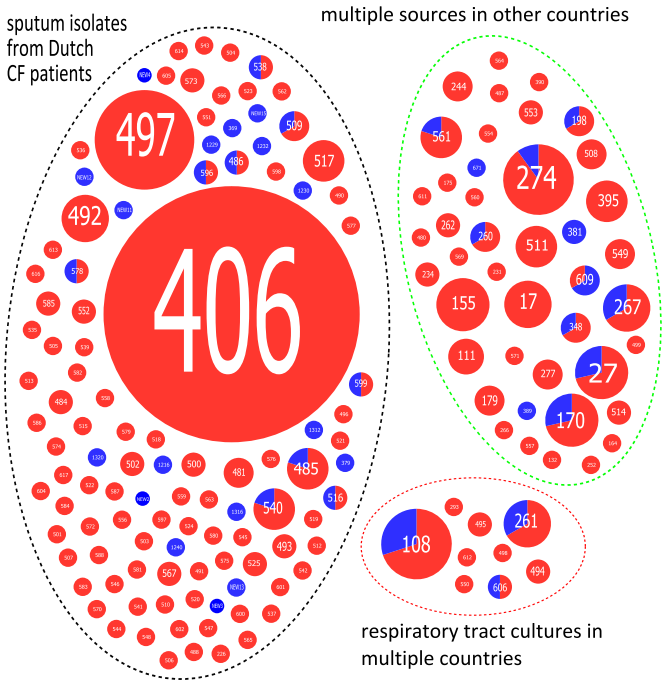


Figure 1. Distribution of *P. aeruginosa* 163 STs detected in 265 Dutch CF patients in 2007 and 49 patients with acquisition after segregation in 2011 among defined source groups. The 41 STs detected in 49 patients with acquired infection during segregation are indicated in blue and STs detected in the cross-sectional study in 2007 are depicted in red. Each circle represents an ST and size of circles represent number of isolates. Dotted circles enclose defined source groups. Assignment of STs to source groups is based on source group definition of STs, contained in the international pseudomonas MLST database (http://pubmlst.org/paeruginosa/), that are identical to the 163 STs identified in Dutch CF patients.

Antibiotic Resistance

P. aeruginosa ST406 isolates (n=51) were resistant to a median of 3 (inter quartile range (IQR) 2-3, maximum 5) of five antipseudomonal antibiotic drug classes (aminoglycoside, betalactam/betalactamase inhibitor combination, antipseudomonal cephalosporin, carbapenem and fluorochinolon), as compared to 1 (IQR 1-2, maximum 5) for other STs (n=302) (p<0.0005). Frequently shared STs were more resistant than non-frequently shared STs (n=181) (median 2, [IQR 1-3]and 1 [IQR 1-2]for STs (n=172) (p=0.003)).

Persistence of *P. aeruginosa* infection

In 158 chronically infected CF patients for which *P. aeruginosa* isolates were available for genotyping in both 2007 and 201, 173 different STs were detected, and 156 STs (90%) were detected in the same patient in 2007 and 2011. Clonal persistence was 89% and 83% for ST406 and other STs, respectively (p=0.4). Fifteen patients (9%) acquired a new ST, which occurred in addition to persistence of another ST in six patients.

Clinical effects of ST406

In 2007 219 patients had chronic *P. aeruginosa* infection with genotype data available and all were included in the nested longitudinal analysis of clinical outcome, yielding a total follow-up of 693.3 patient years. In 2007 the 40 patients infected with ST406 were, as compared to those infected with other STs, younger and used inhaled antibiotics more frequently (Table 3). Lung function measurements between 2007 and 2010 were available for 201 (92%) patients (n=1903, mean 9.5 measurements per

Table 3. Characteristics of patients infected with ST406 and non-ST 406 *P. aeruginosa* genotypes.

	ST 406 (n=40)	other clones (n = 169)	*p*
age, mean (SD)	18.9 (3.7)	25.0 (13.5)	0.005[a]
male, n (%)	20 (50)	91 (51)	0.92[b]
homozygosity dF508, n (%)	30 (75)	98 (61)	0.10[b]
ABPA, n (%)	3 (7.5)	34 (19)	0.08[b]
CFRD, n (%)	9 (23)	40 (22)	0.98[b]
hospitalizations, median (IQR)	0 (0-1)	0 (0-1)	0.20[c]
FEV1, mean (SD)	67.3 (27.3)	61.9 (23.5)	0.35[a]
BMI z-score, mean (SD)	-0.5 (1.2)	-0.4 (1.1)	0.53[a]
pulmozyme, n (%)	18 (45)	86 (48)	0.73[b]
inhaled antibiotics, n (%)	32 (80)	114 (64)	0.048[b]
age at diagnosis, median (IQR)	0 (0-3)	0 (0-4)	0.25[c]

[a]t-test [b]Chi^2 [c]Mann Whitney U

Abbreviations: SD=standard deviation, ABPA= Allergic bronchopulmonaryaspergillosis, CFRD= cystic fibrosis related diabetes, IQR= interquartile range, FEV1=Forced Expiratory Volume in one second.

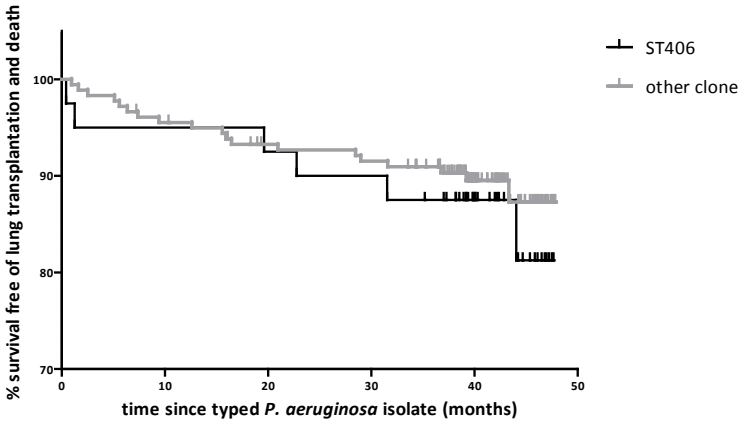


Figure 2. Survival free of lung transplantation or death.
Survival free of lung transplantation or death for CF patients with ST406 and CF patients with other *P. aeruginosa* clones (total n=219, lung transplantation n=11, died n=14).

patient); seven were too young for pulmonary function testing, seven underwent lung transplantation or died shortly after 2007 and four moved to another center or had irregular visits. The means of FEV_1 percent of predicted for patients with ST406 and with other STs did not decline and was not significantly different between both groups (Suppl. Fig.2). Including age and use of inhaled antibiotics or time on inhaled antibiotics in the linear mixed model did not change interpretation (Suppl. Table 2). Between 2007 and 2011, 14 patients died and 11 received a lung transplant. The hazard ratio (HR) for dying or receiving a transplant was not significantly different for patients with ST406 or other STs (HR 2.41, 95% CI 0.85-6.88, p=0.10) (Fig. 2).

Discussion

This longitudinal study of seven years demonstrated -in post hoc analysis- a statistically significant reduction in acquisition of chronic *P. aeruginosa* infection in Dutch CF patients under 15 years of age during the first five years after implementation of strict segregation. In two nested cross-sectional studies the molecular epidemiology of *P. aeruginosa* among CF patients remained largely unchanged with a high genetic diversity. Prevalence of *P. aeruginosa* infection decreased slightly from 57% in 2007 to 52% in 2011, as did the prevalence of frequently shared strains (from 57% to 50%), albeit not statistically significant. ST406, the most prevalent ST in this patient cohort, was acquired after implementation of segregation by three patients previ-

ously infected with other STs. Although ST406 infection appeared highly persistent and associated with resistance to more antibiotic classes it was not associated with a detectable decline in pulmonary function or a statistically significant reduced survival free of transplantation.

ST406 is the dominant clone in this Dutch CF cohort and our findings suggest that new acquisitions with ST406 in patients not chronically infected with *P. aeruginosa* did not occur since strict segregation was implemented. Yet, new acquisition of ST406 (and other strains) was documented in nine percent of chronically infected patients. The clinical relevance of these events is unknown, but it demonstrates the occurrence of super-infections with genotypes being resistant to more antibiotics despite the segregation policy.

In crude analysis there was a clear reduction in the incidence of acquisitions of chronic *P. aeruginosa* infection after segregation, with a trend towards a statistically significant reduction in Cox regression analysis. Yet, the effects of segregation were different in different age groups, with a more pronounced effect in children. A possible explanation for a larger effect in younger patients (who did not acquire new ST406 infections) is the absence of close contacts between CF patients during CF summer camps, that were no longer organized[27]. Lack of adherence to hygiene rules outside the hospital setting by adolescent and adult patients, is a plausible alternative also to explain the lack of effect of segregation in this age group. Indeed, some CF patients still visit annual CF beach dance parties or continue to meet other CF patients, against medical advice[28]. Another explanation could be chronic colonization of the sinuses with *P. aeruginosa* genotypes acquired before segregation[29-31]. The association of ST406 with a specific age category that was described before[17] is likely a cohort phenomenon, indicating that cross-transmission of ST406 in a certain age-group had occurred as the average age of patients infected with ST406 increased with time.

Our data demonstrate that frequently shared strains, especially ST406, are resistant to more antibiotics, which has also been described for the Liverpool Epidemic Strain (LES)[32] and the Australian Epidemic Strain (AES-1)[33]. In our cohort, patients infected with ST406 more frequently received inhaled antibiotics. As clinicians were not aware of *P. aeruginosa* genotypes, prescription of inhaled antibiotics cannot be confounded by prior knowledge. Therefore, it is possible that ST406 infection leads to more frequent antibiotic prescription, thereby increasing antibiotic resistance. However, comparison of resistance data from 2007 and 2011 failed to demonstrate an increase in antibiotic resistance during four years (data not shown). Another possibility is that ST406 was already more resistant when introduced into the CF population.

There are no randomized controlled trails in which the effectiveness of segregation measures for CF patients is evaluated. Yet, many countries have implemented cohort-segregation and in several reports reductions in transmission of epidemic clones, of

P. aeruginosa prevalence and of acquisition of chronic *P. aeruginosa* infection at an older age have been reported[12;13;15;16]. As, in addition to cohort-segregation, other interventions were implemented, such as earlier and more stringent antibiotic treatment, more frequent monitoring and screening of newborns for CF, it remains difficult to accurately quantify the effect of segregation from these studies.

There were several reasons for implementing strict segregation, rather than cohort-segregation, in the Netherlands. First, patients are not only colonized by *P. aeruginosa* but also harbor other CF associated pathogens that could cause clinical deterioration and might be transmitted though the hospital environment or patient contacts[34]. Second, the diagnostic delay of culture data creates a time window in which newly infected (but yet unidentified) patients may transmit *P. aeruginosa* (or other pathogens). Third, because of frequent hospital visits CF patients are at increased risk of acquiring multidrug resistant pathogens from non-CF patients[35]. Segregation based on antibiotic susceptibilities was not recommended because of the observed variability of antibiotic susceptibilities in strains over time. Others have proposed to perform segregation on infection status and antibiotic susceptibility patterns (non-*P. aeruginosa*, intermittent *P. aeruginosa*, chronic *P. aeruginosa* susceptible, chronic *P. aeruginosa* resistant and patients with *Burkholderia cepacia complex*) [36], which is more complicated and also suffers from the variability in antibiotic susceptibility. Complete segregation, therefore, seemed more feasible, but the impact of this regime on daily lives of CF patients should not be underestimated.

Strengths of our study include the longitudinal design, the large patient cohort, comprising half of the Dutch CF population, the completeness of clinical and microbiological data collection and the use of MLST for genotyping. The five year period of segregation might be considered a study limitation, but the (limited) magnitudes of the effects indicate that much longer follow-up periods would have been needed to reach statistical significance in any of the study endpoints for the total cohort. Only post hoc analysis, based on the finding of unequal distribution of effect amongst different age groups, yielded an effect of strict segregation in young CF patients. The cut-off point for implementation of segregation on 1-1-2007 is in practice probably an ongoing process of better adherence to the regulations that started to be implemented during 2006. The steep decline from 13 chronic acquisitions to 5 in 2007 and 2008 respectively might indicate ongoing improvement of segregation. Furthermore we have no data on patient behavior and adherence to hygiene rules in their private lives or during hospitalization. Yet, segregation measures have the highest priority amongst hospital staff during hospitalization of CF patients and separate hospital rooms and accommodating segregation in out-patient clinics is rigorously planned.

The conclusion of this study is dual; on the one hand we did not detect new acquisitions with the epidemic ST406 strain (which is a strictly CF related strain) among non-

infected patients after segregation. Although the clinical consequences of infection with ST406 seem marginal, it has a more extensive resistance profile and is associated with higher use of inhaled antibiotics. Besides, patients under 15 years of age had a significant decrease in chronic acquisition of *P. aeruginosa* after implementation of segregation. On the other hand, a reduction of acquisition of chronic infection with *P. aeruginosa* in all included Dutch CF patients could not be demonstrated and the molecular epidemiology of *P. aeruginosa* from CF patients hardly changed. Perhaps the effects of segregation are obscured due to less than strict adherence to the segregation policy by individual patients or colonization of paranasal sinuses but more likely there is also acquisition of chronic *P. aeruginosa* infection with unique strains from the environment.

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Supplementary data

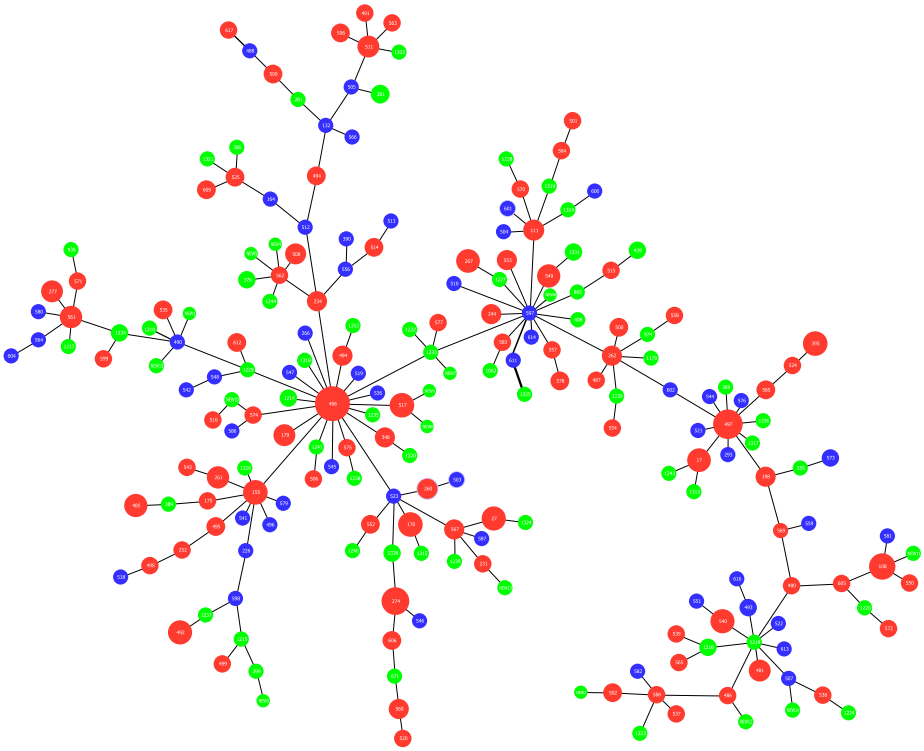
Supplementary Table 1. CF patients at risk for chronic colonization with *P. aeruginosa* between 2005-2011.

Year	At risk for chronic infection PA on 1stof January(n)	New patients at risk for chronic infection with PA (n)	Acquisitions of chronic infection with PA(n)	Lost to follow-up (n)
2005	232	57	11	0
2006	278	26	17	1
2007	286	20	13	2 (LTX, n=1)
2008	291	18	5	4 (LTX, n=1, death n=1)
2009	300	25	5	6 (death, n=2)
2010	314	21	3	10
2011	322	12	2	0

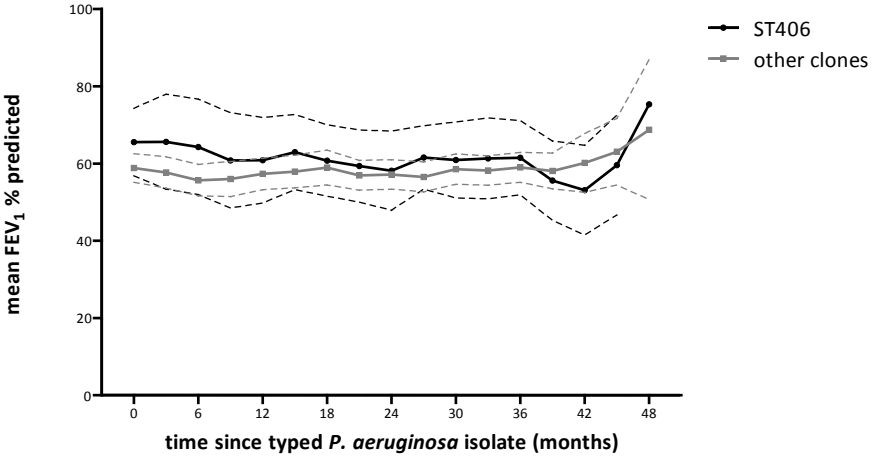
PA=*P. aeruginosa*, LTX= lung transplantation

Supplementary Table 2. Estimates of FEV_1 percent of predicted based on mixed model analysis.

variable	unadjusted estimate	95% CI	adjusted estimate	95% CI
intercept	59.17	[55.75, 62.58]	85.32	[77.45, 93.20]
ST406	3.60	[-4.35, 11.54]	-0.43	[-7.84, 6.98]
slope (ST406*time)	-0.59	[-1.80, 0.61]	-0.57	[-1.77, 0.63]
using inhaled antibiotics			-10.05	[-15.94, -4.16]
time (in years)	-1.19	[-1.71, -0.67]	-0.45	[-1.02, 0.12]
age (in years)			-0.75	[-1.00, -0.51]



Supplementary Figure 1. Minimum spanning tree based on allelic profiles of 211 STs representing 599 *P. aeruginosa* isolates from Dutch CF patients. Genetic linkage was done using the goeBURST distances[25]. Each circle represents a sequence type, the size of the circle represents the number of isolates (non-linear). Color of the circle indicates year of origin; blue: 2007 only, green: 2011 only, red: isolated in both 2007 and 2011. Lines represent possible genetic linkage between the STs and can be single of multiple locus variants.



Supplementary Figure 2. Mean FEV_1 % of predicted during longitudinal follow-up. Dotted lines represent 95% CI's. Longitudinal lung function measurements (mean FEV_1 as percentage of predicted) for patients with ST406 and patients with other clones. There was no significant difference in lung function for patients with or without ST406 (estimate -0.43, 95% CI -7.84 – 6.98). Adding an interaction term between ST406 and time (slope analysis, not shown in figure) did not significantly improve the model (p=0.35) indicating no difference in decline.

Chapter 7

Within-host evolution of the Dutch high-prevalent *Pseudomonas aeruginosa* clone ST406 during chronic colonization of a patient with cystic fibrosis

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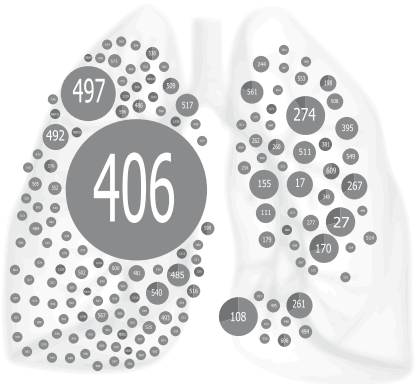
manuscript in preparation



Abstract

It has previously been demonstrated that adaptation of *P. aeruginosa* to the CF lung niche involves similar genes or functions in genetically different strains or in different chronically infected CF patients which suggests adaptation of *P. aeruginosa* to CF lungs through parallel evolutionary trajectories.

This study investigates adaptation of ST406, present in 15% of chronically infected CF patients in the Netherlands, in a newly infected CF patient during three years using whole genome sequencing (WGS), transcriptomics, and phenotypic assays, including biofilm formation.

WGS-based phylogeny demonstrates that ST406 is genetically distinct from other reported CF related strains including other CF related high-prevalent or epidemic clones. Comparative genomic analysis of the early (S1) and late (S2) isolate yielded 42 SNP differences and 10 indels. Furthermore, there was a single 7 kb genomic fragment only found in the late isolate, which was apparently acquired during chronic colonization of the CF lung. One of the SNPs was located in the *bfmS* gene, a gene that encodes one of the polypeptides of a two-component regulator involved in biofilm maturation (BfmR/S). Concurrent with this SNP there was a significant lower level of transcription of both *bfmR* and *bfmS* and reduced biofilm formation in the late isolate. Another non-synonymous SNP was observed in the *mexT* global regulator gene, which coincided with differential gene expression between isolate S1 and S2 of *mexE* and *mexF*, encoding the MexE/F efflux pump, and genes encoding the type six secretion system (T6SS). Furthermore, a non-synonymous SNP was detected in *exsA*, the main regulator of a type three secretion system (T3SS), which coincided with differential expression of genes encoding T3SS. In general, most SNPs and most differentially expressed genes encoded proteins involved in metabolism, secretion and signal transduction or transcription.

These data suggest that the high-prevalent Dutch CF clone ST406 displays adaptation to the CF lung niche which involves a limited number of mutations affecting regulators controlling biofilm formation and secretion and genes involved in metabolism. Some of the affected genes in ST406, like *mexT* and *exsA*, have also been previously implicated in adaptation of other *P. aeruginosa* strains during chronic infection of CF lungs supporting the concept of adaptive parallel evolution of *P. aeruginosa* that chronically colonizes lungs of CF patients.

Introduction

Pseudomonas aeruginosa is a versatile gram negative rod with a relatively large genome of more than 6 million base pairs that can thrive in many different niches[1]. *P. aeruginosa* chronically infects the lungs of patients with cystic fibrosis (CF) and lung deterioration due to inflammation is the major cause of death in these patients[2]. More than half of the CF patients become chronically infected during their lifetime and most chronically infected patients harbor the same *P. aeruginosa* strain for many years or even life-long[3-5]. Several studies have investigated adaptation of *P. aeruginosa* trying to understand what the essential factors are for colonization and persistence in the lungs of CF patients despite the host immune system and aggressive antibiotic treatment[2;6-12]. Genome analysis of *P. aeruginosa* strains demonstrated that genes related to CF lung adaptation included genes involved in biofilm formation, transmembrane transport, hemolysis, secretion systems and resistance to oxidative stress and antibiotics[6]. Phenotypically adaptation often involves a reduced growth rate, loss of motility, loss of substantial catabolic activities and inactivation of important regulatory functions[13]. Several studies demonstrated that parallel evolutionary events in these adapted strains do not seem to be due to loss or gain of certain genes[14], but rather to pathoadaptive mutations[10;15]. However, some CF adapted strains share accessory genomic elements like the LES-prophage-1, LESGI-2, and LESGI-4, that may contribute to increased competitiveness in the CF lung[16]. Some of the adapted strains are transmitted between CF patients and these epidemic *P. aeruginosa* clones seem associated with worse clinical outcome[17-22]. In the Netherlands, an epidemic *P. aeruginosa* clone with MLST type ST406, MLVA type CC27 and AT-chip SNP-type A418 or E418 was found in up to 50% of CF patients between 15 and 25 years of age in 2007[23-25]. This clone was not found in clinical cultures from non-CF patient and was genotypically different from the epidemic CF strains found in other countries[25;26]. Studies of clinical and environmental *P. aeruginosa* strains from all over the world using SNP-typing did not detect this genotype except in Dutch CF patients[27]. The fact that this clone is specifically linked to the CF lung niche suggests this genotype is very well adapted to the CF lung. In contrast to some other epidemic clones an association with clinical deterioration could not be detected for ST406[28]. Another clone (ST497) was found in 5% of Dutch CF patients and was associated with older age (>25 years)[24]. Likewise this genotype is thus far only isolated from Dutch CF patients.

Insights into the evolutionary dynamics during chronic colonization of the lungs of CF patients and genetic relatedness of epidemic CF clones that are specifically adapted to the cystic fibrosis lung niche might give further clues for new eradication therapies or preventive measures. To investigate the traits that may have contributed to adaptation of the *P. aeruginosa* ST406 clone to the CF lung we indexed genome-wide alteration

by performing comparative genomics in combination with comparative transcriptomics and cataloging phenotypic differences between two ST406 strains obtained from the same patient within a timespan of three years. The first isolate was cultured one month after a patient became first colonized with *P. aeruginosa* ("early isolate" or S1) while the second isolate was recovered from the same patients after three years of chronic colonization ("late isolate" or S2).

Methods

Strains and primers

The first, early, *P. aeruginosa* ST406 isolate (S1) was cultured from a sputum culture from a CF patient in 2004, one month after the first detection of *P. aeruginosa* in sputum. The second, late, *P. aeruginosa* ST406 isolate (S2) was cultured in 2007 from sputum from the same CF patient. ST497 isolate (S3) was cultured from a CF patient that was chronically infected with *P. aeruginosa* for more than seven years. *P. aeruginosa* strain PA01 and *E. coli* strains that harbor *lasB-gfp* (MH155) or *rhlA-gfp* fusion for quorum sensing detection were kindly provided by Søren Molin from DTU, Denmark. Primers used for PCR amplification of S2 specific genes and non-ST406 *P. aeruginosa* strains used to test presence of these genes are shown in Suppl. Table 1.

Whole genome sequencing and SNP analysis

Genomic DNA was prepared from *P. aeruginosa* isolates using a Wizard Genomic DNA Purification kit (Promega). Whole genome sequencing was done on an Illumina MiSeq platform. Raw 2×250 bp paired-end Illumina reads were quality-filtered using Nesoni 0.109 [Victorian Bioinformatics Consortium: http://www.vicbioinformatics.com/software.nesoni.shtml] (with following options: adaptor-clip (yes), adaptor-match (10), adaptor-match max error (1), clip ambiguous (yes), quality cutoff (10), minimum read length (150): non-default). De novo assembly was done using SPAdes 2.5.1[29]: kmers used (21, 33, 55, 77, 99, 127), with "careful" option turned on and cutoffs for final assemblies: minimum contig/scaffold size = 500 bp, minimum contig/scaffold average Nt coverage = 10-fold. Gene prediction and annotation was done using Prokka[30] (default options; using a Pseudomonas-specific BLAST database) and RAST[31]. Additional functional annotation of protein-coding genes was done using the COG database[32]. Protein sequences were scanned against the COG myva database using BLAST 2.2.29+[33] and received the same COG as their best BLAST hit (E-cutoff ≤ 1e-10).

To compare differences between the ST406 S1 and S2 isolate and investigate within-patient adaptation an in-house read-mapping pipeline was used to detect SNPs and small indels between strains S1 and S2. Nesoni-filtered reads of S2 were mapped

against the S1 assembly using Bowtie2[34]. To filter for genomic repeats, we removed reads that mapped to multiple positions in the S1 assembly. SAMtools 0.1.18[35] was used to call SNPs and indels with following conditions: Qscore ≥ 50, mapping quality ≥ 30, and calls were required to be homozygous (under diploid model). Additional criteria included a mapping depth ≥ 10 reads, a consensus of ≥ 75% to support a call, and ≥ 1 supporting read in each direction. To correct for potential assembly errors, we also performed the SNP/indel-calling procedure described above after mapping Nesoni-filtered reads of S1 against its own assembly. Genomic positions containing SNPs and indels in the S1 vs S1 comparison were ignored in the S2 vs S1 comparison. Identified SNPs and indels were linked to features (i.e. genes) and were inspected for synonymous vs non-synonymous mutations (in case of SNPs).

To compare gene content between S1 and S2 we used Inparanoid v4.129. All S1 genes that did not have a predicted orthologous relationship with an S2 gene and vice versa were considered as potentially unique for that given strain. To further verify these potential strain-specific genes, S2 reads were mapped against the S1 assembly and vice versa as described above, but without filtering for reads mapping to multiple genomic positions. Genomic regions significantly covered by mapped reads were identified by using the same criteria as described above for finding SNPs and small indels. Potentially strain-specific genes identified using Inparanoid were considered to be truly strain-specific if ≤ 10% of their entire length was covered by reads.

Publicly available *P. aeruginosa* WGSs with <750 contigs were downloaded from GenBank on 12 Feb 2014. The genome sequences of these publicly available strains and of strains S1, S2 and S3 were aligned using an in-house pipeline that makes use of the NUCmer v3.23 alignment algorithm[36]. All genomes were aligned against the completed genome sequence of *P. aeruginosa* PA01 (i.e. used as reference strain). Repetitively aligning genomic regions were removed from each pairwise alignment after which all pairwise alignments were merged into one *P. aeruginosa* core genome alignment. A phylogenetic tree was built from the polymorphic sites in the core genome alignment using RAxML[37]. The tree was built under the GTR model. Confidence was inferred by running 1000 bootstrap replicates under the same model. The tree was visualized using MEGA6[38]. Association between source and clade were calculated using Chi squared test.

Transcriptome analysis

Transcriptome analysis using GeneChip *P. aeruginosa* genome arrays (Affymetrix) was performed as described previously[13]. In short, *P. aeruginosa* strains were grown aerobically in LB medium starting from OD_{600} = 0.01 and harvested at OD_{600} =0.5. RNA was isolated using RNeasy Mini Purification kit (QIAGEN) and transcribed into cDNA using random primers (Invitrogen Life Technologies). Subsequently, cDNA was

purified (QIAquick, QIAGEN), fragmented and labeled and hybridized on an Affymetrix *P. aeruginosa* PA01 gene chip. The probe arrays were scanned with a GeneChip Scanner 3000 and the raw data was obtained using the Affymetrix GeneChip Operating System 1.4. Microarray data analysis was performed as described before[23] using bioconductor in R environment (http://www.bioconductor.org). Normalisation and expression index calculation was done with rma function[39]. The fold change was calculated using the average expression levels of three replicates. A cut off p-value < 0.05 and fold change > 2 between transcriptome levels of S1 and S2 was considered significant. The annotations and functional classes were assigned according to the Pseudomonas Genome Database[40].

Amplification of S2 specific genes

Isolates were cultured on TSA-blood plates (Becton, The Netherlands) overnight at 37˚C, suspended in 20 µl lysis buffer (0.25% SDS, 0.05 N NaOH) and incubated at 95˚C for 20 min. The cell lysate was centrifuged and diluted with 180 µl buffer (10 mM Tris-DCl, pH 8.5). After thoroughly mixing, another centrifugation for 5 min at 16,000 x g was performed to remove cell debris. Supernatants were frozen at -20˚C until further use. Two µl of the lysate was used in a touchdown PCR using Hotstar Taq DNA polymerase (Qiagen Benelux B.V.), and 5 µl Q-buffer (Qiagen Benelux B.V., Venlo, The Netherlands). The PCR was conducted as follows: 10 min at 96˚C, then 10 cycles of 30 sec at 95˚C, 30 sec at 65˚C with 1˚C less every cycle and 1 min at 72˚C. This was followed by 25 cycles of 30 sec at 95˚C, 30 sec at 55˚C and 1 min at 72˚C, after which the final step of 10 min at 72˚C followed. Presence of PCR products was checked by electrophoresis on 1% agarose gel. S2 was used as positive control.

Phenotypic experiments

Swimming, swarming and twitching motility and protease assays were performed in triplicate. Bacteria were grown overnight on Luria-Bertani (LB) agar plates. Swimming, swarming and twitching motility assays were performed on respectively 0.3%, 0.6% and 1,5% agarose plates containing AB minimal medium with glucose (0.5%) and casamino acids (0.5%). One bacterial colony was inoculated in, on and through the agar by sterile toothpick, respectively. Swimming ability was assessed after 24 hours incubation at 30°C by measuring the maximum diameter of the zone of growth. Swarming and twitching was assessed after 48 hours of incubation at 37°C by measuring zones of growth. Protease production in skim milk was measured by inoculating 100 µl of supernatant of overnight culture (after spinning at 7000 g for 5 min) on a LB-agar plate with 10% skim milk concentration. Clear zones of protease production were measured after 48 hours of incubation at 37°C.

Growth curves were made by measuring OD_{600} every 50 min, starting with an OD_{600} of 0.01. Doubling time was calculated in Microsoft Excel using the exponential trend line (tD=(ln2/μ)).

Quorum sensing signal production was tested by streaking isolates and a positive and negative control near two *E. coli* monitor strains that harbor *lasB-gfp* (MH155) or *rhlA-gfp* fusion products on a plasmid[41] that can be induced by the Las (3-O-C12-HSL) and Rhl signal molecule (C4-HSL), respectively. GFP (green fluorescent protein) production was visualized after 24 hours incubation at 37°C using a Zeiss Axion2 microscope with an integrated Coolsnap color cf camera at 2.5x magnification and 200ms exposure time.

Phenotype MicroArrays

Quantitative measurements of 1821 phenotypes of S1, S2 and of *P. aeruginosa* strain PA01 was determined using phenotype MicroArrays (Biolog, Inc Hayward CA, USA). In this assay cell respiration, measured by color change caused by reducing a tetrazolium dye, is used as a universal reporter for both amplification and precise quantitation of phenotypes. The measured average height of two replicates of each phenotype was compared between S1 and S2. Phenotypes with more than two-fold difference were considered different phenotypes.

Biofilm experiments

A semi-static biofilm model was used to assess biofilm formation of S1 and S2, as described before[42]. Overnight bacterial cultures were diluted to an OD660 of 0.01 in 6 ml LB medium with 1% glucose and added to a coverslip coated with poly-L-lysine (0.45 μm; diameter, 12 mm; Becton Dickinson) inside a well from a six-well polystyrene plate (Corning Inc.). Biofilms were grown at 30°C for 48 h at 120 rpm. After 48 h, the coverslips were washed with 0.85% NaCl and the biofilms were chemically fixed with 8% glutaraldehyde (Merck) for 20 min. Subsequently, the biofilms were stained with 15 μg/ml propidium iodide (PI) in 0.85% NaCl that was removed after 15 min. The coverslips were transferred to glass microscope slides and analyzed by a confocal laser scanning microscope (CLSM) (Leica SP5), equipped with an oil plan-Neofluar ×63/1.4 objective. PI was excited at 633 nm. Z stacks were taken with an interval of 0.42 μm. Pictures were analyzed with LAS AF software (Leica), and biofilm thickness and biomass were quantified using Comstat[43]/Matlab R2013b software (the MathWorks). The average thickness and biomass of the biofilms were measured at ten randomly chosen positions. Experiments were performed twice in duplicate.

Results and discussion

Whole genome sequencing and phylogenetic inferences

Illumina sequencing and subsequent assembly of the three *P. aeruginosa* isolates S1, S2 and S3 yielded draft genomes with an average assembly size of 6.32, 6.32, and 6.28 Mbp, consisting of 45, 42, and 48 scaffolds with a nucleotide coverage of 56.4, 55.9 and 81.1, respectively. To assess the phylogenetic relatedness of the three *P. aeruginosa* isolates from Dutch CF patients with an international collection of *P. aeruginosa* strains, publicly available genome sequences of 37 *P. aeruginosa* strains were downloaded and aligned together with the three Dutch *P. aeruginosa* isolates S1, S2, and S3 (Suppl. Table 2). This resulted in a core genome alignment of 3.27 Mb, containing 258,000 SNPs. An initial SNP-based phylogenetic tree showed that strains PA7 & VRFPA01 clustered far away from the other *P. aeruginosa* strains. To increase resolution, PA7 & VRFPA01 were left out and the remaining 38 strains were re-aligned. Identical strains were removed from the tree for clarity (MPA01P1, MPA01P2 and PA0579 represented by PA01 and PADK2CF510 represented by DK2). The final core genome alignment was 4.02 Mb and a phylogenetic tree including 34 strains was constructed using the 121,244 SNPs contained in this alignment (Fig. 1).

The phylogenetic tree demonstrated that the Dutch high-prevalent *P. aeruginosa* CF strains, S1/S2 and S3 were evolutionary not closely related to other epidemic CF related clones. Also the Liverpool epidemic strain clustered separately, while the Manchester and Danish epidemic CF strain shared a common ancestry. Two distinct clades (A and B) were identified in the tree that contain both environmental and clinical isolates as well as isolates from CF patients. However, clinical isolates were significantly associated with clade B (OR: 8.0; 95% CI: 1.5-43.7; $p<0.01$), while CF isolates were enriched in clade A, although this was only marginally significant (OR: 7.4; 95% CI: 0.8-68; $p=0.08$). This suggests that clinical non-CF isolates have an evolutionary background that is distinct from isolates colonizing CF patients.

In contrast to these observations, several studies investigating both clinical and environmental isolates have demonstrated that *P. aeruginosa* has a non-clonal epidemic population structure with no clear association between specific lineages and certain niches[22;27;44-49]. However, these studies indexed only a limited number of loci providing limited power for inferring phylogenetic relationships. The fact that the majority of CF isolates group in one clade does not mean that these CF isolates form one monophyletic group, thus that all share a recent common ancestor. Clearly, the Manchester, Danish, Liverpool, and both Dutch epidemic CF strains form a polyphyletic group in which phylogenetically different sequences have converted into a successful epidemic phenotype in CF patients.

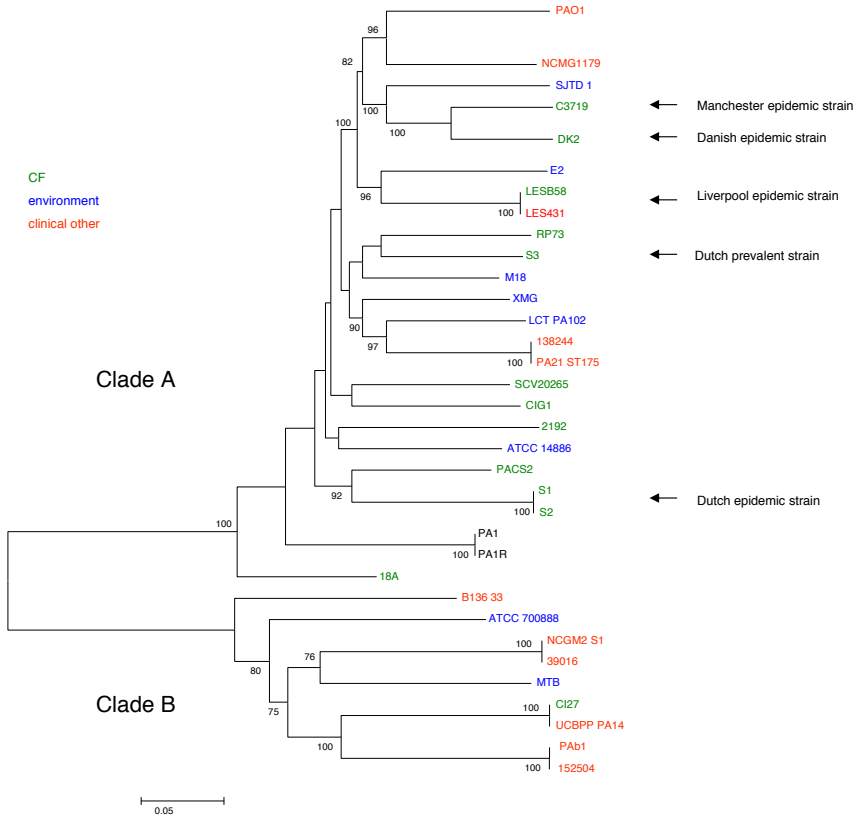


Figure 1. Phylogenetic tree including 34 *P. aeruginosa* strains built from an alignment of 121k core SNPs. Numbers along the branches indicate bootstrap values and only bootstrap values >75% are shown. Green = CF sputum isolate, red = clinical isolate, not CF, blue = environmental isolate. The Dutch high-prevalent CF strains and internationally known epidemic CF strains have been indicated.

Comparative genomics and transcriptomics of the early (S1) and late (S2) ST406 isolate

To investigate genomic differences between S1 and S2, 6,284,869 bases (99.4 - 99.5% of total assembly size) of the two isolates were aligned and this yielded 42 high quality SNPs and 10 indels. Twelve SNPs were synonymous SNPs, while 24 were non-synonymous. Five SNPs were located in intergenic regions and one occurred in an rRNA encoding gene (Table 1 and Table 2). Most (29%) non-synonymous mutations were in genes coding for COG categories involved in metabolism, followed by non-synonymous SNPs in COG category transcription and signal transduction (21%) and category unknown (25%) (Fig. 2).

Table 1. 24 non-synonymous SNPs detected between early (S1) and late (S2) ST406 isolate.

ST406 gene	SNP [aa change]a	PA01 annotation	gene	COG function classification	SNP in literature
3848	AAC[N] > ATC[I]	PA3545	*algG*	Inorganic ion transport and metabolism	[9], [13], [50]
5027	AAC[N] > ATC[I]	PA4102	*bfmS*	Signal transduction mechanisms	[13]
3581	AAG[K] > GAG[E]	PA1782		General function prediction only, Signal transduction mechanisms,Transcription, Replication, recombination and repair	
5598	AAT[N] > GAT[D]	PA4276	*secE*	Intracellular trafficking, secretion, and vesicular transport	
5091	ACC[T] > GCC[A]	PA4039		Cell motility	
1522	ACG[T] > GCG[A]	PA2727 + PA2728		Replication, recombination and repair	
4626	AGC[S] > AGA[R]	PA2385	*pvdQ*	General function prediction only	
4879	ATC[I] > AGC[S]	PA0690		secretion	[13]
0345	ATC[I] > GTC[V]	PA1147		Amino acid transport and metabolism	
2241	ATG[M] > AAG[K]	PA4519	*speC*	Amino acid transport and metabolism	[9]
3651	CCG[P] > CTG[L]	PA1713	*exsA*	Transcription	[8], [9], [10],[50]
5490	CTG[L] > ATG[M]	PA4163		Translation, ribosomal structure and biogenesis	
0807	GCC[A] > ACC[T]	PA0794		Energy production and conversion	
3973	GCC[A] > GGC[G]	PA3280	*oprO*	Inorganic ion transport and metabolism	
5588	GCC[A] > GTC[V]	PA4266	*fusA1*	Translation, ribosomal structure and biogenesis	[10], [50]
2194	GCG[A] > GTG[V]	PA4562		General function prediction only	
0321	GGC[G] > GTC[V]	PA1171		Cell wall/membrane/envelope biogenesis	
4666	GTC[V] > ATC[I]	PA2346		Lipid transport and metabolism	
4610	GTC[V] > GCC[A]	PA2402		Secondary metabolites biosynthesis, transport and catabolism	
4794	GTC[V] > GCC[A]	PA2552		Lipid transport and metabolism	
2089	TAG[stop] > TGG[W]	PA4661	*pagL*	no COG	[13]
4856	TTC[F] > TTG[L]	PA2492	*mexT*	Transcription	[8]
0194	TTG[L] > TTT[F]	PA1300		Transcription	
5101	TTG[M] > CTG[L]	PA4029		Function unknown	

[a] A=Alanine, D=Aspartic acid, E=Glutamic acid, F=Phenylalanine, I=Isoleucine, K=Lysine, L=leucine, M=methionine, N=asparagine, P=proline, R=arginine, S=serine, T=treonine, V=valine, W=tryptophan

Table 2. Indels in isolate S2 relative to isolate S1.

Indel in S2	PA01 annotation	gene	Gene product annotation
GC > GCC (+1C)	PA1302		putative heme utilization protein
GGC > GCGGTCCTGCAACTGC (+13CGGTCCTGCAACT)	PA4967	*parE*	topoisomerase IV subunit B
GCTGCGGCGC > GCTGCGGCGCGGCCGCCTG CGGCGC (+15CTGCGGCGCGGCCGC)	PA2727		histidine kinase
TGGG > TGG (-1G)	PA4661	downstream of *pagL*	lipid A 3-O-deacylase
GGGT > G (-3GGT)	PA4379		methyltransferase domain-containing protein
TGGTAGGTA > TGGTA (-4GGTA)	PA3806		putative Fe-S-cluster redox enzyme
TGCCGGCCG > TGCCG (-4GCCG)	PA0347	downstream of *glpQ*	glycerophosphoryl diester phosphodiesterase
T > TG (+1G)	PA1713	upstream of *exsA*	AraC family of transcriptional regulators
GT > G (-1T)	PA2172		putative cellulase
CT > C (-1T)	PA0705	*migA*	alpha-1,6-rhamnosyltransferase

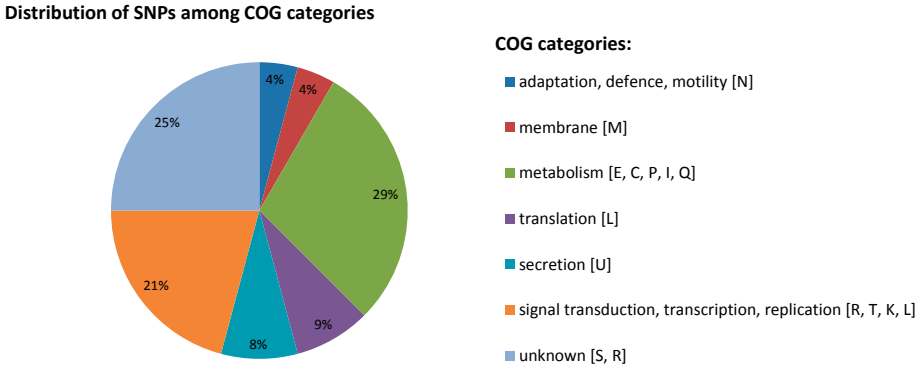


Figure 2. COG categories of non-synonymous SNP differences found between S1 (early isolate) and S2 (late isolate) isolated three years apart from the same patient. Percentage per category of total non-synonymous SNPs is indicated.

Metabolic alterations and changes in regulatory functions have also been described for other CF adapted clones. Whole genome sequencing of various isolates from different CF patients that harbored the DK2 clone for many years revealed loss of catabolic activities and inactivation of important regulatory systems[13]. Furthermore, analysis of highest relative rates of non-synonymous SNPs in core genes of 32 CF related strains including LES (Liverpool epidemic strain) isolates revealed SNPs in genes encoding proteins involved in oxidoreductase activity, secretion and heterocycle metabolism[6].

Comparative transcriptomics between ST406 S1 and S2 yielded 179 genes with different transcription levels ($p < 0.05$), of which 110 genes had a more than 2-fold difference in gene expression (Suppl. Table 3). Gene ontology enrichment analysis revealed overrepresentation of differentially expressed genes involved in metabolism

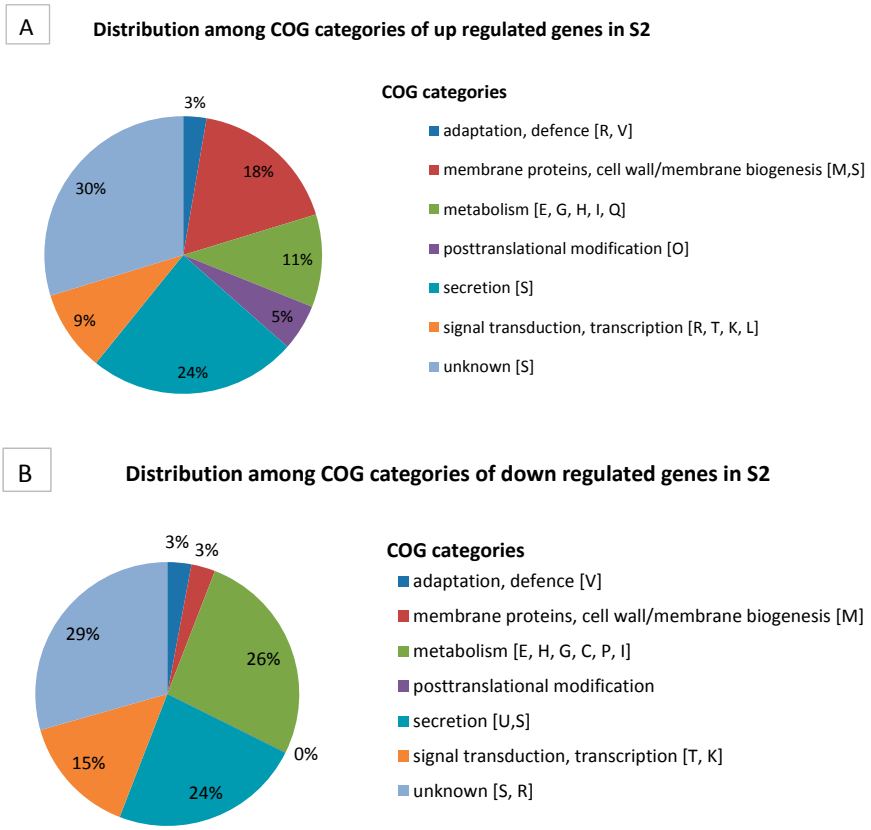


Figure 3. Functional categories of up- and down-regulated genes in S2 relative to S1 detected with Affymetrix transcriptome analysis. A, indicates the percentage of genes per COG category that were higher expressed in S2 compared to S1, while B, indicates the percentage of genes per COG category that were lower expressed in S2 compared to S1. COG category is indicated between brackets.

and posttranslational modification that were higher expressed in S1 and genes encoding membrane proteins or involved in membrane or cell wall biogenesis and proteins involved in secretion that were higher expressed in S2 (Fig. 3). Approximately 92% of all the genes in strains S1 & S2 were covered by uniquely and perfectly matching probes on the Affymetrix *P. aeruginosa* PA01 GeneChip when using a cutoff of at least 4 probes per gene (~96% in PAO1). This indicates that only a small proportion of S1 and S2 genes was not analyzed for transcription differences.

Several SNPs were located in genes that have been previously implicated in adaptation of *P. aeruginosa* to the lung of CF patients and were therefore designated patho-adaptive SNPs. These SNPs as well as SNPs and transcriptome differences between S1 and S2 in genes that were deemed important for observed phenotypic differences are described in more detail below.

ExsA and the type III secretion system (T3SS)

A non-synonymous SNP was identified in *exsA* (Table 1). Furthermore, a one nucleotide insertion was also detected upstream in the promoter region of this gene (Table 2). ExsA is a member of the AraC family of transcriptional regulators and is the primary regulator of the type III secretion system (T3SS). ExsA controls expression of genes implicated in T3SS biogenesis by directly binding to promoter sequences of these genes leading to activation of transcription[51]. Transcriptome analysis indicated differential expression of T3SS genes, with a significant lower level of expression in S2 (Suppl. Table 3). Also *exoS*, encoding the ExoS toxin, which is secreted by T3SS was expressed at a lower level in S2. Type III secretion mediated translocation of exoenzymes is used by *P. aeruginosa* to deliver exoenzyme effector molecules, like ExoS, into the eukaryotic cell. Our findings of higher expression of genes encoding T3SS in S1 relative to S2 suggest that T3SS may play an important role in acute CF infection but not or to a lesser extent during chronic infection. This would be consisted with previous findings that also demonstrated down regulation of ExoS in chronically infected CF patients[52]. On the other hand, a recent report also found up regulation of *exoS* during chronic infections[53]. These conflicting data indicate that further studies are needed to identify the exact role of ExoS during acute and chronic infections.

MexT, the MexEF efflux pump and the type VI secretion system (T6SS)

Another non-synonymous SNP was located in *mexT*, which encodes the transcriptional regulator MexT, that positively regulates the MexEF-OprN efflux pump and negatively regulates genes encoding T6SS[54-57] (Table 1). Transcriptome analysis revealed that the *mexEF* genes encoding the MexEF efflux pump were expressed at a higher level in the early S1 isolate, while genes encoding T6SS were expressed at higher levels in S2 (Suppl. Table 3). This suggests that the non-synonymous mutation in *mexT* affects some of

the effector functions of MexT. The Mex E/F-oprN efflux operon also confers resistance to quinolones, chloramphenicol and trimethoprim. Down regulation of *mexEF* genes in S2 coincided with less resistance against chloramphenicol and trimethoprim/sulfamethoxazol, while both isolates are resistant to quinolones and trimethoprim (Suppl Table 4). It is interesting to note that *mexT* has been described as a mutational "hot spot," where mutations can contribute to global phenotypic changes in *P. aeruginosa*[58].

MigA, PagL and LPS

The early and late isolates displayed different colony morphology with a more rough colony morphology of S2 compared to S1 (Fig. 4). Interestingly, a single bp deletion and a SNP between S1 and S2 were also detected in two genes, *migA* and *pagL* respectively, implicated in LPS biogenesis. Furthermore, S2 contains a single bp deletion just downstream of *pagL*, which may explain the observed differences in colony morphology (Table 1 and Table 2). The *migA* gene encodes an Alpha-1,6-rhamnosyltransferase involved in producing uncapped core oligosaccharide[59] while *pagL* encodes a lipid A 3-O-deacylase, which recognizes either 3-OH C10 or 3-OH C14 moieties of the lipid A component of LPS and adjusts the structure of lipid A. Mutations in genes affecting LPS biogenesis have also previously been described in *P. aeruginosa* during CF lung adaptation[9;10;13]. In *P. aeruginosa* isolates from CF respiratory infections the O-antigen is not produced in high amounts. Since O-antigen is highly immunogenic this adaptation possibly facilitates chronic persistence[12]. PagL is overexpressed in *P. aeruginosa* isolates isolated from CF infants compared to those from acute infections or environmental isolates. However, it has also been shown before that in chronic infection with severe lung disease PagL function is lost[60]. This together with our findings indicate that LPS

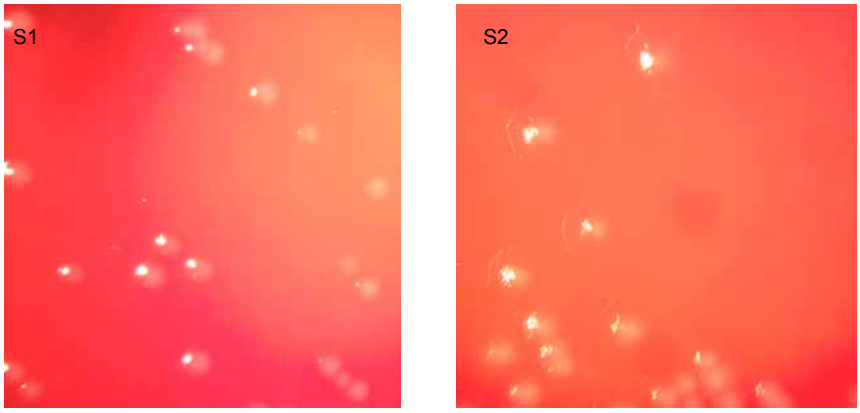


Figure 4. Colony morphologies on sheep blood agar. S1 (left) has a "smooth" colony morphology, while S2 (right) has a more "rough" colony morphology.

is under selective pressure in the CF lung and that LPS modifications contribute to adaptation of *P. aeruginosa* to the CF airway.

BfmR/S and biofilm formation

Analyzing biofilm formation of S1 and S2 demonstrated that, in a semi-static model of biofilm formation, S1 forms significantly thicker biofilms with more biomass than S2 (Fig. 5). The observed difference in biofilm maturation between S1 and S2 might be explained by a non-synonymous SNP in the *bfmS* gene, coinciding with lower expression levels of *bfmR* and *bfmS* genes (Suppl. Table 3) in S2. BfmS is part of the two-component regulatory systems named BfmR/S (PA4101/PA4102) that regulates biofilm maturation in *P. aeruginosa*. BfmR/S has been described as essential for biofilm maturation in *P. aeruginosa* by limiting bacteriophage-mediated lysis and thus, eDNA release[61;62]. This suggests that reduced expression of this two-component may explain the observed reduced biofilm maturation in S2 relative to S1.

In a Danish CF clone there is also a SNP described in *bfmS* that is fixed in the DK2 lineage in different patients[13]. Relation with biofilm maturation was, however, not

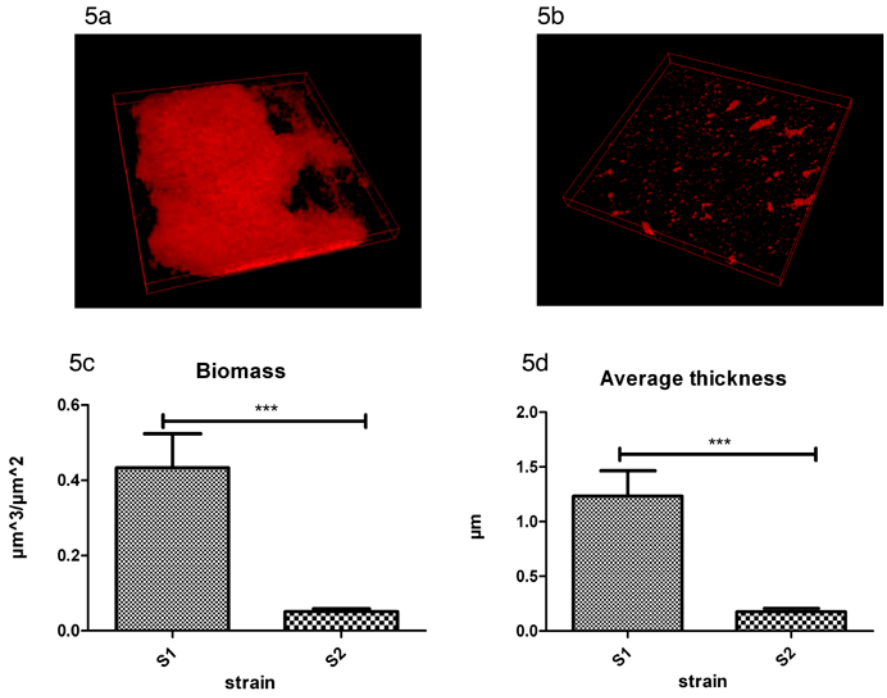


Figure 5. Biofilm formation in semi-static model of S1 (a) and S2 (b) after 48 hours in LB+1% glucose; Difference in total biomass (c) and average thickness (d) with Standard deviation error bars. *** P<0.005

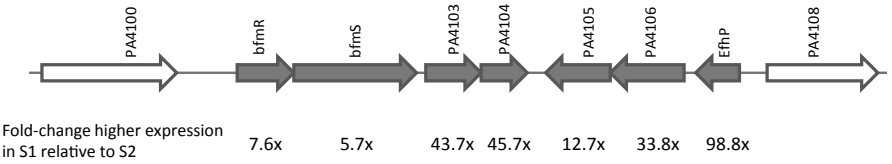


Figure 6. Overview of the gene cluster encompassing genes PA4101-4107 in grey with differences in transcription level between S1 and S2 and adjacent genes in white. Fold changes in expression level in S1 relative to S2 are indicated underneath. PA4103 is a hypothetical gene encoding a putative ferric reductase transmembrane component superfamily, PA4104 is a hypothetical gene encoding a polypeptide with similarities to the DoxX superfamily, PA4105 and PA4106 are hypothetical genes encoding DUF 2063 and DUF 692 superfamily protein, respectively. *EfhP* is the gene originally named PA4107. Arrows indicate direction of transcription.

demonstrated. Therefore, we postulate that the SNP in *bfmS* may represent a novel pathoadaptive mutation that has not been previously investigated in isolates from CF patients. At this moment, however, we can only speculate why loss of biofilm maturation is beneficial in the CF lung niche. Possibly the late isolate is a so-called "cheater strain" using the biofilm matrix produced by other clonal isolates for protection against the host immune system and antibiotics. It has been reported before that other "cooperative traits" like secretion of pyoverdin, elastase, protease and some quorum sensing molecules are also down-regulated in chronic CF isolates, indicating loss of social behavior of isolates that profit from products produced by cooperative strains[63]. Lower-level biofilm formation was not found in that study but may save energy, or even may increase transmissibility.

Directly adjacent to *bfmRS* is a cluster of 5 genes (*PA4103-PA4107*) that were, like *bfmRS*, also significantly lower expressed in S2 (Fig. 6). The function of these genes is largely unknown with the exception of *PA4107*, which was recently renamed *efhP* and found to be important for Ca^{2+} homeostasis and virulence[64]. Interestingly Ca^{2+} levels are increased in CF lungs[65] and in presence of high Ca^{2+} strains lacking functional EfhP were unable to produce pyocyanin, developed less biofilm, and had decreased resistance to oxidative stress (H_2O_2)[64]. Whether BfmRS is also implicated in regulation of expression of these adjacent located genes remains to be investigated.

In contrast to our finding this gene cluster was upregulated in a set of CF isolates during late stage of adaptation[66]. Most of these strains represent the Danish clone DK2 which also has a missense mutation in *bfmS* that was fixed in this lineage in different patients [13]. Biofilm formation was not investigated in this study. These contradicting results may indicate that also other regulators might be involved in expression of these genes located downstream of *bfmR/S*.

Table 3. Phenotypic traits of S1 and S2 compared to PA01.

phenotypic assay	S1	S2	PA01
swim	+	+	++
swarm	-	-	++
twitch	-	-	++
protease	+	++	+++
quorum sensing	-	-	+
growth rate (doubling time in minutes)	50	46	29

Other phenotypic differences between the early and late ST406 isolate

Besides differences in colony morphology as described above, other phenotypic characteristics like swimming, swarming, twitching, production of quorum sensing molecules and growth rate were comparable between S1 and S2 (Table 3). Protease production was slightly higher in S2. Both strains displayed a slow growing phenotype with lack of motility and lower protease production, compared to PA01. This is something that was also reported for other CF adapted strains[63;67;68]. Both S1 and S2 did not exhibit a mucoid phenotype, which is not unexpected. In general chronically infected CF patients harbor mucoid *P. aeruginosa*, however, mucoidity can be lost by secondary site mutations and mucoid and non-mucoid isolates are often found to co-exist in chronic lung infections[69]. The mucoid phenotype is caused by production of alginate, a viscous extracellular polymer. Alginate protects *P. aeruginosa* against antibiotics, ROS, opsonizing antibodies and phagocytes by forming a slimy capsule and promoting biofilm formation and is therefore considered an important virulence factor[11]. AlgG is a periplasmic C-5 mannuronan epimerase, that plays an important role in aliginate biosynthesis and in protecting alginate from degradation by the periplasmic alginate lyase AlgL[70;71]. A SNP was detected in *algG* but this nucleotide difference between S1 and S2 did not result in different mucoidity between the two isolates.

To further quantitatively measure phenotypic traits of S1 and S2, growth rates of S1 and S2 were measured on different substrates using Phenotype MicroArrays. Relative to S1, S2 demonstrated enhanced metabolism in the presence of toxic substances like chloride and bromide detergents, and diverse oxidizing agents, while the S2 isolate displayed decreased growth capabilities on various nutritional supplements and different Carbon, Phosphor and Nitrogen sources (Fig. 7). This indicated that the transition from early to late isolate was accompanied with major metabolic changes.

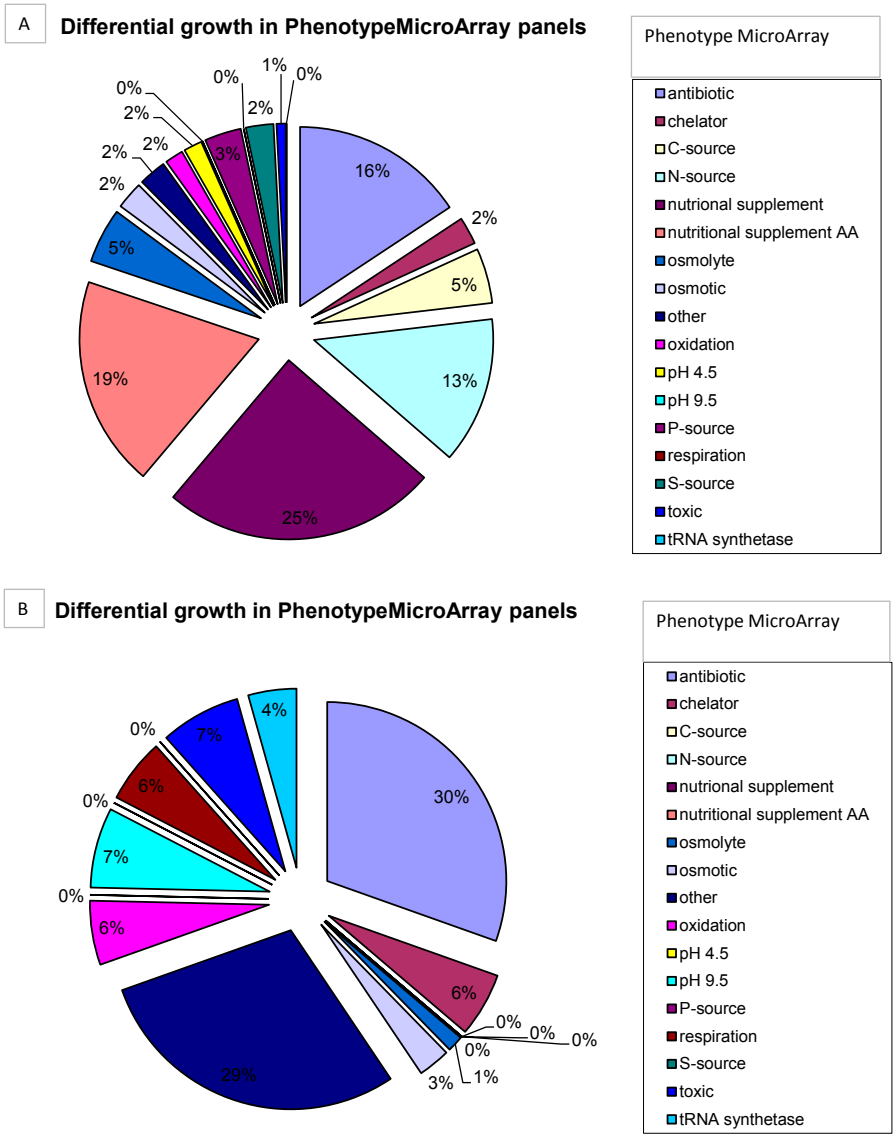


Figure 7. Phenotype MicroArray analysis of the early (S1) and late (S2) ST406 isolate. Indicated are the Phenotype MicroArray panels for which at least a two-fold difference in metabolism (=respiration) between S1 and S2 was detected. A, indicates the percentage of Phenotype MicroArray panels for which the growth rate of S2 was at least two-fold lower than that of S1, while B indicates the percentage of Phenotype MicroArray panels for which the growth rate of S2 was at least two-fold higher than that of S1. Phenotype MicroArray panel categories are color-coded and indicated on the right (C=carbon, S=Sulphur, N=Nitrogen, P=Phosphor).

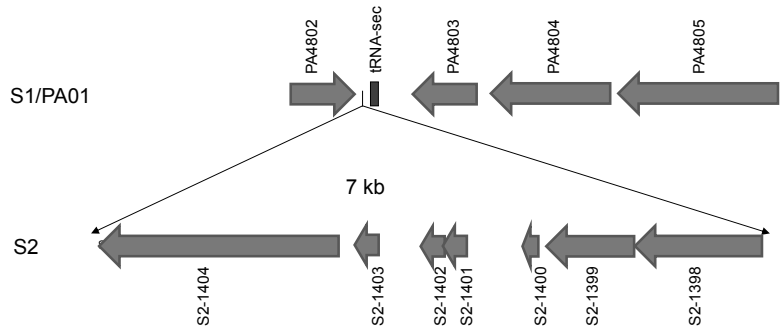


Figure 8. Overview of the seven gene cluster acquired by the S2 ST406 isolate. The 7 kb insert next to tRNAsec and upstream of gene PA4802 detected only in S2 includes a phage integrase gene (S2-1398) and a gene encoding a putative replication initiation factor (S2-1399) and five genes encoding hypothetical proteins.

Differences in gene content

Annotation of the S1 and S2 genomes revealed 5766 protein-encoding genes in S1 and 5772 protein-encoding genes in S2. A cluster of seven genes in S2 was absent in S1, thus apparently acquired during chronic colonization. These seven genes encode five hypothetical genes, a putative replication initiation factor and a phage integrase, suggesting incorporation of phage-associated genes in S2. This seven gene cluster is located next to a tRNA-SeC (p)-TCA gene and does not seem to disrupt a coding region or a predicted operon. On both sides of the 7 genes there is an identical stretch of 36bp. This partly overlaps with the tRNAgene on the 3' side (Fig. 8). PCR performed on three targets of this 7 kb DNA islet in 29 non-ST406 clinical isolates (6 ICU, 6 community acquired, 6 CF, 6 other clinical isolates from a previous study[25] and 3 isolates of the Liverpool epidemic strain, PA01 and the Midlands epidemic strain) did not result in a positive PCR product, indicating that this insertion is specific for the ST406 S2 and, therefore, has probably no general role in adaptation of *P. aeruginosa* to the lung of CF patients.

Concluding remarks

This study characterized genome-wide changes that have occurred in the Dutch high-prevalent clone ST406 after three years of chronic carriage. The data demonstrate that within-host evolution of the Dutch ST406 clone within a CF patient during three years was not driven by major gene acquisition or gene loss but mostly by point mutations that coincided with differences in transcriptome levels and phenotype, most notably with differences in biofilm formation. In general, clone ST406 seems to have a phenotypic

profile of low virulence. Known virulence-associated phenotypes that are important for *P. aeruginosa* colonization and infection, like motility and quorum sensing are not displayed in both the early and late ST406 isolate. Reduced virulence in CF adapted strains is also described in other studies[72;73]. Since the early isolate was recovered from a CF patient just one month after first time colonization with *P. aeruginosa*, this low virulence profile suggest that at the time of colonization the early isolate had already undergone a certain level of adaptation to the CF lung.

The CF lung contains many different niches, with varying levels of oxygen, nutrients, antibiotic concentrations and components of the host immune system, which all impose a strong selective pressure. Therefore, chronic colonization of the CF lung is often associated with clonal diversification of the infecting strain in which SNPs accumulate, while the gene content of chronically infecting isolates in CF patients is highly stable with no substantial gene acquisition[9;14;74]. It has been reported that also isolates with increased rates of mutation (hypermutators) can emerge, that result in rapid amplification of SNPs, which may facilitate differential mutagenesis and adaptation[10;75]. Recent studies have shown that in one sputum sample of a CF patient a variety of *P. aeruginosa* isolates of the same genotype with variable phenotypes and based on WGS data several SNPs can be detected[7;50;76]. This suggests that in these niches *P. aeruginosa* behaves as a quasispecies, *i.e.* a large group or cloud of related genotypes that exist in an environment of high mutation rate[77]. High mutation rates in changing environments exerting strong and diverse selective pressures, like the CF lung, may therefore lead to adaptive radiation. This can explain that in various longitudinal studies in CF patients, in addition to common parallel evolutionary events in chronically colonizing *P. aeruginosa* isolates, also different unique sets of putative adaptive SNPs have been identified. This study identified a mutation in the *bfmS* gene, one of two genes encoding the BfmR/S two component regulator implicated in biofilm maturation, which occurred during chronic CF colonization that, for the first time, coincided with phenotypic loss of biofilm maturation during chronic colonization and which may represent a novel pathoadaptive mutation. Altered biofilm formation, probable diminished immunogenicity by LPS changes, and altered expression of T3SS, T6SS, and the MexE/F efflux pump are the adaptive evolutionary events in ST406 that in part overlap with adaptations described for other chronic CF isolates. Common adaptive changes that are repeatedly found in chronically infecting isolates may provide novel leads for targeted therapeutic interventions to combat chronic colonization with *P. aeruginosa* in CF patients.

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Supplementary data

Supplemetary Table 1. primers and strains used for S2 specific gene amplification.

Strain code	Primer sequence
S2 1401/1402 lower	GCA GGG AGC ACG ACA CCG ACG AC
S2 1401/1402 upper	CGT CCC ACA AGG TCG GCA AAC ACA
S2 1404 lower	TAC ACC GCC AGC CTT CCT CGT T
S2 1404 upper	TGA CAT CGA AAG CGC CAT TAC AGA
S2 1398/1399 lower	AGC TTC AGC GGG CGG ACC TTT TC
S2 1398/1399 upper	TGA TCG CCA CGC AGA CCT ACG
LES431	kindly provided by Craig Winstanley
LES400	kindly provided by Craig Winstanley
LES58B	kindly provided by Craig Winstanley
Midlands 1	purchased from HPA
PA01	kindly provided by Søren Molin
U-1_10	ICU strain from previous study*
U-1_16	ICU strain from previous study*
U-1_17	ICU strain from previous study*
U-1_23	ICU strain from previous study*
U-1_26	ICU strain from previous study*
U-1_29	ICU strain from previous study*
ZH829	community acquired strain from previous study*
ZH836	community acquired strain from previous study*
ZH843	community acquired strain from previous study*
ZH846	other clinical isolate from previous study*
ZH852	other clinical isolate from previous study*
ZH853	other clinical isolate from previous study*
ZH857	other clinical isolate from previous study*
ZH870	other clinical isolate from previous study*
ZH879	other clinical isolate from previous study*
ZH944	community acquired strain from previous study*
ZH956	community acquired strain from previous study*
ZH979	community acquired strain from previous study*
PA208	CF strain from previous study*
PA212	CF strain from previous study*
PA214	CF strain from previous study*
PA271	CF strain from previous study*
VW186	CF strain from previous study*
VW395	CF strain from previous study*

* REF 25: van Mansfeld *et al*, PLoS One 2010.

Supplementary table 2. Published strains used in SNP-based phylogenetic tree.

strain	origin[a]	Country[a]	year[a]
2192	chronic CF	Boston USA	
39016	cornea, ulcerative keratitis	GB	2003
138244	sputum, pneumonia	Portugal	2011
152504	sputum	Portugal	2011
18A	CF non clonal		
ATCC 14886	soil		
ATCC 700888	biofilm, industrial water system		
B13633	diarrea community acquired, child	China	
C3719	CF Manchester epidemic strain	Gr Britain	
CI27	chronic CF		
CIG1	chronic CF		
DK2	CF chronic	Denmark	2007
E2	tomato plant	Florida, USA	
LCT PA102	ATCC 27853		
LES431	parent of CF patient	Great Br	
LESB58	chronic CF Liverpool epidemic strain	Great Br	
M18	rhizosphere watermelon		
MTB	contaminated soil	India	
NCGM2 S1	urinary tract ingfection MDR outbr	Japan	
NCMG1179	resp tract, MDR outbreak med faci	Japan	2010
PA01	wound, laboratory strain	Australia	1955
PA0579	mutant of PA01	Australia	1975
PA07	clinical, non-respiratory	Argentina	
PA1		military hosp	
PA1R		military hosp	
PA21 ST175	blood, VIM+, outbreak strain	Spain	
PAb1	frostbite clinical sample	USA	
PACS2	CF 6 months	Canada	
PADK2CF510	CF chronic	Denmark	
RP73	CF chronic (16,9 yrs)		
S1	CF first isolate	Netherlands	2004
S2	CF chronic	Netherlands	2007
S3	CF chronic	Netherlands	2007
SCV20265	CF small colony variant, MDR	Italy	2006
SJTD 1	soil	China	
UCBPP PA 14	human burn patient		
XMG	soil	China	2012
VRFPA01	blood	India	
MPAO1_P1	PA01 phenotype 1		
MPAO1_P2	PA01 phenotype 2		

[a] indicated when available as published on NCBI website

Supplementary Table 3. Differential expressed genes between early and late isolate by Affymetrix p<0.05 and >2fold change.

locus-tag	COG function classification	pvalue	ratio S1/S2	gene
PA4489	Adaptation, Protection	0.01	0.43	*magD*
PA2536	Amino acid transport and metabolism	0.02	0.34	
PA2290	Carbohydrate transport and metabolism	0.03	0.48	*gcd*
PA0070	Cell wall/membrane/envelope biogenesis	0.01	0.25	*tagQ1*
PA0045	Cell wall/membrane/envelope biogenesis	0.01	0.29	
PA2684	Cell wall/membrane/envelope biogenesis	0.02	0.30	
PA2457	Cell wall/membrane/envelope biogenesis	0.02	0.46	
PA0072	Cell wall/membrane/envelope biogenesis	0.02	0.48	*tagS1*
PA2454	Coenzyme transport and metabolism	0.03	0.44	
PA0073	Defense mechanisms	0.03	0.34	*tagT1*
PA0046	Function unknown	0.01	0.26	
PA0047	Function unknown	0.02	0.26	
PA0563	Function unknown	0.01	0.27	
PA3021	Function unknown	0.01	0.35	
PA3729	Function unknown	0.01	0.35	
PA0093	Function unknown	0.03	0.39	
PA4490	Function unknown	0.02	0.43	*magC*
PA0094	Function unknown	0.04	0.45	
PA4492	Function unknown	0.04	0.46	*magA*
PA4491	Function unknown	0.03	0.48	*magB*
PA2455	General function prediction only	0.01	0.40	
PA0074	General function prediction only, Signal transduction mechanisms, Transcription, Replication, recombination and repair	0.01	0.33	*ppkA*
PA0087	Hcp secretion island I (HSI-I) T6SS	0.00	0.21	*tssE1*
PA0078	Hcp secretion island I (HSI-I) T6SS	0.00	0.22	*tssL1*
PA0080	Hcp secretion island I (HSI-I) T6SS	0.00	0.22	*tssJ1*
PA0082	Hcp secretion island I (HSI-I) T6SS	0.00	0.23	*ttsA1*
PA0086	Hcp secretion island I (HSI-I) T6SS	0.00	0.23	*tagJ1*
PA0085	Hcp secretion island I (HSI-I) T6SS	0.01	0.24	*hcp1*
PA0076	Hcp secretion island I (HSI-I) T6SS	0.01	0.25	*tagF1*
PA0077	Hcp secretion island I (HSI-I) T6SS	0.00	0.25	*icmF1*
PA0084	Hcp secretion island I (HSI-I) T6SS	0.02	0.26	*ttsC1*
PA0088	Hcp secretion island I (HSI-I) T6SS	0.00	0.27	*tssF1*
PA0089	Hcp secretion island I (HSI-I) T6SS	0.01	0.27	*tssG1*
PA0079	Hcp secretion island I (HSI-I) T6SS	0.01	0.29	*tssK1*
PA0083	Hcp secretion island I (HSI-I) T6SS	0.02	0.30	*ttsB1*
PA1844	Hcp secretion island I (HSI-I) T6SS	0.02	0.30	*tse1*

Supplementary Table 3. Differential expressed genes between early and late isolate by Affymetrix (p<0.05 and >2fold change *(continued)*.

locus-tag	COG function classification	pvalue	ratio S1/S2	gene
PA2685	Hcp secretion island I (HSI-I) T6SS	0.01	0.32	*vgrG4*
PA3484	Hcp secretion island I (HSI-I) T6SS	0.04	0.35	*tse3*
PA0071	Hcp secretion island I (HSI-I) T6SS	0.04	0.45	*tagR1*
PA0126	Hypothetical, unclassified, unknown	0.01	0.24	
PA3716	Hypothetical, unclassified, unknown	0.01	0.34	
PA2781	Hypothetical, unclassified, unknown	0.02	0.37	
PA3661	Hypothetical, unclassified, unknown	0.02	0.40	
PA3850	Hypothetical, unclassified, unknown	0.02	0.42	
PA5441	Hypothetical, unclassified, unknown	0.04	0.42	
PA2456	Hypothetical, unclassified, unknown	0.02	0.44	
PA2792	Hypothetical, unclassified, unknown	0.01	0.45	
PA3483	Hypothetical, unclassified, unknown	0.01	0.45	
PA3485	Hypothetical, unclassified, unknown	0.02	0.47	
PA1639	Hypothetical, unclassified, unknown	0.02	0.48	
PA2540	Lipid transport and metabolism	0.01	0.30	
PA2537	Lipid transport and metabolism	0.02	0.31	
PA2541	Lipid transport and metabolism	0.03	0.46	
PA3727	Lipid transport and metabolism	0.01	0.47	
PA4317	Membrane proteins	0.01	0.27	
PA5113	Membrane proteins	0.01	0.33	
PA2538	Membrane proteins	0.02	0.35	
PA2774	Membrane proteins	0.05	0.39	
PA5114	Membrane proteins	0.05	0.44	
PA3730	Membrane proteins	0.02	0.45	
PA2775	Membrane proteins	0.02	0.47	
PA4318	Membrane proteins	0.03	0.47	
PA0277	Posttranslational modification, protein turnover, chaperones	0.01	0.20	
PA0090	Posttranslational modification, protein turnover, chaperones	0.01	0.24	*clpV1*
PA1791	Posttranslational modification, protein turnover, chaperones	0.02	0.34	
PA1069	Posttranslational modification, protein turnover, chaperones	0.02	0.43	
PA0091	Protein secretion/export apparatus	0.01	0.32	*vgrG1*
PA1202	Secondary metabolites biosynthesis, transport and catabolism	0.01	0.14	
PA2539	Signal transduction mechanisms	0.02	0.27	
PA0075	Signal transduction mechanisms	0.02	0.28	*pppA*

Supplementary Table 3. Differential expressed genes between early and late isolate by Affymetrix (p<0.05 and >2fold change *(continued).*

locus-tag	COG function classification	pvalue	ratio S1/S2	gene
PA0081	Signal transduction mechanisms	0.03	0.36	*fha1*
PA2432	Transcription	0.00	0.19	*bexR*
PA2780	Transcription	0.04	0.36	
PA3267	Transcription	0.03	0.46	
PA0495	Amino acid transport and metabolism	0.04	2.68	
PA0609	Amino acid transport and metabolism, Coenzyme transport and metabolism	0.02	3.06	*trpE*
PA2321	Carbohydrate transport and metabolism	0.04	2.10	
PA2493	Cell wall/membrane/envelope biogenesis	0.00	3.52	*mexE*
PA2494	Defense mechanisms	0.03	2.53	*mexF*
PA4770	Energy production and conversion	0.05	2.71	*lldP*
PA4105	Function unknown	0.00	12.71	
PA4106	Function unknown	0.00	33.81	
PA4104	Function unknown	0.00	45.67	
PA4107	Function unknown	0.00	98.76	
PA0492	General function prediction only	0.04	3.46	
PA3843	Hypothetical, unclassified, unknown	0.02	2.56	
PA3057	Hypothetical, unclassified, unknown	0.01	2.73	
PA4881	Hypothetical, unclassified, unknown	0.02	2.91	
PA3229	Hypothetical, unclassified, unknown	0.00	3.73	
PA4103	Hypothetical, unclassified, unknown	0.00	43.68	
PA4358	Inorganic ion transport and metabolism	0.02	2.18	
PA4359	Inorganic ion transport and metabolism	0.02	2.50	
PA3790	Inorganic ion transport and metabolism	0.00	3.55	*oprC*
PA1707	Intracellular trafficking, secretion, and vesicular transport	0.03	2.59	*pcrH*
PA0494	Lipid transport and metabolism	0.05	3.24	
PA0493	Lipid transport and metabolism	0.03	4.29	
PA1705	Protein secretion/export apparatus, T3SS	0.04	2.14	*pcrG*
PA1709	Protein secretion/export apparatus, T3SS	0.04	2.16	*popD*
PA1706	Protein secretion/export apparatus, T3SS	0.02	2.19	*pcrV*
PA1696	Protein secretion/export apparatus, T3SS	0.02	2.31	
PA1701	Protein secretion/export apparatus, T3SS	0.01	2.64	*pcr3*
PA1708	Protein secretion/export apparatus, T3SS	0.01	3.12	*popB*
PA3842	Protein secretion/export apparatus, T3SS	0.01	4.30	*spcS*
PA3841	Signal transduction mechanisms	0.00	2.66	*exoS*
PA4102	Signal transduction mechanisms	0.00	5.69	*bfmS*
PA4101	Signal transduction mechanisms, Transcription	0.01	7.61	*bfmR*
PA3056	Transcription	0.04	2.01	

Supplementary Table 3. Differential expressed genes between early and late isolate by Affymetrix (p<0.05 and >2fold change *(continued).*

locus-tag	COG function classification	pvalue	ratio S1/S2	gene
PA3055	Transcription	0.02	2.25	

Supplementary Table 4. Antibiotic susceptibility of S1 and S2.

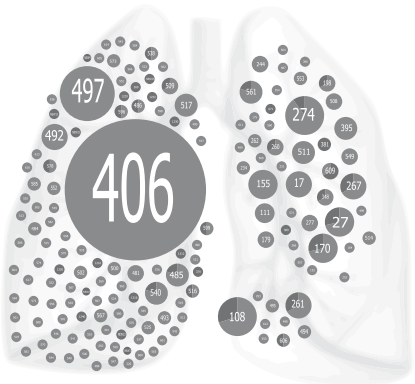
Antibiotics	Diameter agar diffusion in mm[a]	
	S1	S2
ciprofloxacin	19 (R)	19 (R)
levofloxacin	11 (R)	14 (R)
piperacillin	26 (S)	28 (S)
ceftazidime	25 (S)	23 (S)
tobramycin	18 (S)	14 (R)
amikancin	17 (I)	15 (I)
meropenem	27 (S)	35 (S)
piperacillin/tazobactam	32 (S)	35 (S)
colistin*	24	23
trimethoprim*	0	0
trimethoprim/sulfamethoxazole*	0	20
chloramphenicol*	0	17

[a] Resistance breakpoints for R, I, and S were defined according to EUCAST guidelines

* no resistance breakpoint available in EUCAST for disk diffusion

Chapter 8

Summary and general discussion



Cystic fibrosis (CF) is an inherited disorder due to mutations in the CFTR gene affecting the chloride channel in epithelial cells leading to obstruction of good cilliairy clearance of the lungs. CF patients develop acute and later chronic respiratory infection leading to a cycle of further damage and less ability to clear infections, resulting in a significantly reduced life expectancy[1]. However, patients with CF nowadays have an improved life expectancy compared to a few decades ago. More intensive and earlier monitoring and more aggressive antibiotic treatment of pulmonary infections have contributed to this progress. Another intervention designed to improve CF patient's life expectancy that has been implemented in many countries is segregation of CF patients. In follow up of successful segregation policies inhibiting (in)direct contact between patients with and without *Burkholderia cepacia complex* to stop transmission amongst CF patients, broader segregation policies were implemented after detection of epidemic CF related *Pseudomonas aeruginosa* strains in several countries. These strains seemed transmitted between CF patients and were associated with worse clinical outcome[2-4]. Some countries implemented cohort segregation which implies all patients with *P. aeruginosa* in their sputum cultures are separated from patients without *P. aeruginosa* in outpatient clinics and in hospitals and contact between these groups is advised against. Other countries also segregate patients with a certain *P. aeruginosa* clone from other positive patients or segregate all CF patients[5;6]. In the Netherlands a very strict segregation policy, separating all CF patients was implemented in 2006, without extensive knowledge about the population structure of *P. aeruginosa*, prevalence of specific epidemic clones and their potential clinical relevance. For CF patients these measures have had a high impact on their daily lives. Before segregation there were summer camps, information days and other social activities organised for CF patients and their caretakers. After segregation, CF patients are not supposed to meet each other, and are physically separated in the hospital and in outpatient clinics. In this thesis the population structure of *P. aeruginosa* in the Netherlands, properties of the Dutch epidemic clone (ST406) and the effects of the Dutch segregation policy on acquisition of chronic *P. aeruginosa* infection and changes in the population structure were investigated.

In **chapter 2** we describe the population structure of *P. aeruginosa* in Dutch CF patients in 2007, which could serve as a reference point for the future evaluation of the effects of the segregation policy. The detection of *P. aeruginosa* in 57% of all CF patients resembles the prevalence of *P. aeruginosa* in CF patients in other countries[7;8]. However, two highly prevalent clones ST406 and ST497 were identified, that are not detected in other countries and which are genotypically different from other epidemic CF related clones. The finding of high prevalent clones in combination with our observation that only 30% of the CF patients harbour a genetically unique *P. aeruginosa* strain and the

high level of congruence of colonization status and *P. aeruginosa* genotype between tested siblings suggest that in the pre-segregation period *P. aeruginosa* strains were transmitted between CF patients. This is not unique for the Dutch situation since similar findings were reported in other countries like the UK, Belgium and Australia[4;9-11]. Another finding suggestive for patient-to-patient transmission was the observed association of the high prevalent clones with patient's age. ST406 was harboured by approximately 50% of all positive CF patients between 15 and 24 years of age, while ST497 was harboured by older patients (> 25 years). This confirms an earlier study investigating transmission during a Dutch summer camp where an AFLP-based genotype, which appeared to represent ST406, was already present in the younger age groups (in three of the four camps, which were classified by age), while an AFLP-based genotype representing ST497 was present in patients visiting the camp with the oldest CF children[12]. This association of ST406 with age could be due to cohort effects when certain age groups interact more with each other than with other age groups, but could also be attributed to host tropism. The composition (host cell receptors and microbiota) of the ecological niche represented by the CF lung is known to differ with patient's age, and this could be an alternative explanation for the specific link between *P. aeruginosa* genotypes and patient age. Our later longitudinal study described in chapter 6 provided further evidence that the linkage of ST406 with a certain age group is likely to be a cohort effect, since the average age of patients carrying ST406 increased in time, suggesting previous transmission, possibly during an age related event like the summer camps.

In **chapter 3** we demonstrated that *P. aeruginosa* isolates from clinical cultures of different patients groups were highly diverse but also that genotypes were patient population and geographic location specific. For example, the ICU-wards from different hospitals seemed to harbour location specific *P. aeruginosa* clones. Genetic diversity was high and not different between hospitals or between hospital-acquired or community-acquired strains. However, the diversity in one ICU and in CF patients was lower than would be expected on random distribution of types. This indicates that there is no evidence of gross clonal expansion by ongoing patient-to-patient transmission in the wards or via hospital environment in general, but clonal expansion on smaller scale happens within a certain clinical setting. An important finding was that the major clones found in CF patients (ST406 and ST497) are genotypically different from isolates found in non-CF patients suggesting transmission of these clones between CF patients or from a specific CF related source rather than high abundance and frequent transmission from the environment. Although environmental isolates from the Netherlands were not included in this study, environmental isolates that were included in international population studies, using either MLST, MLVA or AT-chip SNP

typing, did not reveal genotypes related to ST406 or ST497 indicating that these genotypes are uniquely associated with Dutch CF patients. Furthermore, absence of these genotypes in non-CF patients strongly suggests that these high prevalent clones are specific for CF patients. Our finding of patient group- and location-specific genotypes with only a limited number of clones distributed across patient population boundaries indicates that transmission of *P. aeruginosa* clones between ICUs, hospital wards and CF patients is rare. This seems to be in contrast to previously published studies that did not demonstrate correlations between genotype and habitat or geographic location[13;14] based on AFLP (amplified fragment length polymorphism) or the existence of a "core genetic lineage" that is predominant in both disease and environment habitats across the world based on AT-chip typing[15]. According to this last study, CF isolates belonging to the so-called "successful core lineage" are ubiquitous in the natural environment and are, therefore, more likely to infect CF patients. Our data, however, suggest no common evolutionary background of *P. aeruginosa* isolates from CF patients nor of *P. aeruginosa* isolates from the other analyzed patient groups.

The discrepancies between these previous studies and our study are most likely due to differences in typing techniques that were used. AT-chip typing, which is based on the presence and absence of 17 SNPs, indexes slowly evolving loci. MLST and MLVA typing used in our studies have, at least in the case of *P. aeruginosa*, a high level of discriminatory power, similar to that of PFGE. This means that MLST and MLVA, in contrast to AT-chip typing, are better able to capture genetic diversity and structure in the *P. aeruginosa* population. Large scale implementation of whole genome sequencing (WGS) to infer the population genomics of *P. aeruginosa* from various sources and geographic locations will shed more light on genetic relatedness and structure in *P. aeruginosa* populations. In chapter 7 we inferred the phylogenetic relatedness of 34 strains based on SNPs in a 3.27 Mb core genome alignment. This analysis revealed a relatively deep phylogenetic split dividing the strains in two major clades. All CF epidemic strains grouped in one clade but are not part of a single monophyletic lineage of CF isolates or combined CF and environmental isolates. This indicates that the major epidemic CF clones have different recent common ancestors, which means that they have emerged independently followed by clonal expansion. Other studies have also shown location or niche specificity of genetically related strains, with horizontal gene transfer dominating evolution in certain niches and clonal expansion in other niches[16]. Possibly, the evolution of *P. aeruginosa* is driven by adaptation to environmental niches with strains that sometimes emerge in clinical settings with successful spread in a certain clinical niche, like CF adapted strains or certain multi-drug resistant genotypes with worldwide prevalence in clinical isolates.

Therefore, we hypothesize that the evolution of *P. aeruginosa* as successful pathogen in CF patients is characterized by the evolutionary development of multiple clones

with different evolutionary trajectories that are well equipped to adapt to and survive in the specific conditions in the CF lung (or other specific niches). Further studies are needed to elucidate the specific adaptations and survival strategies that these strains have adopted to survive in this special niche. In chapter 7 the traits of the Dutch clone are investigated based on genomics, transcriptomics and functional assays.

In **chapter 4** we studied transmission of *P. aeruginosa* during an annual open air Beach Dance event (CFBD) in 2009, which was a unique setting where a small group of CF patients met against medical advice. In this small cohort we identified one possible case of cross-transmission, which involved the Dutch high prevalent clone (ST406). This clone, which was harbored by 32% (=7) of the patients at the beginning of the event, was acquired by one patient at the end of the event. Since we have not found this clone so far in non-CF patients or in other sources (see chapter 2 and 3), we consider this acquisition as a possible transmission event during CFBD 2009. Also a previous study in the Dutch CF summer camps detected three cases of probable transmission and 14/18 cases of possible transmission with genotypes that – in retrospect – appeared the prevalent Dutch clones ST406 and ST497[12]. This indicates that high transmissibility may be at least one of the factors contributing to the prevalence of the Dutch clones (ST406 and ST497).

Additionally, the questionnaires taken during this study revealed that 50% (n=11) of the patients had had contact with other CF patients (beside their household contacts) against medical advice, in the three months after CFBD. There were some patients that were not infected with *P. aeruginosa* in the respiratory tract that still decided to visit this event with a known risk on transmission. Even though segregation was implemented for a few years already, compliance to hygiene rules appeared to be quite low in some of these patients. The need for self-determination and face-to-face contact with fellow CF patients seemed to be considered more important than the risk of chronic *P. aeruginosa* infection, or infection with other putative transmissible pathogens for individual patients. So dependent on policy stringency and impact on daily lives of hygiene instructions there will always be a certain number of patients that will not adhere to medically advised segregation policies.

In **chapter 5** we describe enhanced persistence of the Dutch CF clone (ST406) over a period of nine years in patients that visited CF summer camps in 2001. Next to transmissibility that we investigated in chapter 4, persistence is another possible explanation for the high prevalence of this clone in the Dutch CF patients. Chronically colonized patients often remain colonized with the same strain for many years[17;18]. In this study, persistence of ST406 was significantly higher than of other genotypes in chronically infected patients. However, the longitudinal study described in chapter 6

that included a larger patient cohort but shorter follow-up period, did not demonstrate a statistically significant difference in persistence of ST406 compared to other chronic genotypes. This might be due to differences in patient population. In chapter 5 patients were between 6 and 19 years of age at the start of follow-up while in the study of chapter 6 patients were older (mean age of 18.9 and 25 years in patients with ST406 and other genotypes, respectively). Furthermore, in the first study 49% of patients harbored ST406 versus 19% of patients in the later study. The high persistence of all chronic strains possibly obscures the limited increased persistence when prevalence of ST406 is lower. Although the later study better represents the general CF population, these data do suggest a slightly better persistence for ST406. However, this effect is so small that it cannot explain the high prevalence of ST406.

An important finding in this longitudinal study in chapter 5 is that the clinical relevance of chronic colonization with ST406 compared to other genotypes seems to be limited. A difference in survival or lung function decline between CF patients chronically colonized by the Dutch CF clone or other genotypes could not be demonstrated during nine years of follow-up. This is supported by the larger longitudinal study described in chapter 6 and by a cross-sectional study investigating the chronically infected CF patients that harbored ST406 in 2007 that also failed to demonstrate an association of ST406 with decreased lung function, increased number of hospitalization days in the previous year or nutritional status[19]. This indicates that, although ST406 frequently and successfully infects lungs of CF patients and persists for prolonged periods it does not appear to be more pathogenic compared to more sporadic clones. In chapter 7 the phenotypic and genotypic traits of ST406 are further detailed.

The effect of segregation on acquisition of chronic *P. aeruginosa* infection in Dutch CF patients is not easy to determine. In **chapter 6** the hazard ratio of chronic *P. aeruginosa* acquisition after implementation of segregation was, as compared to before segregation, 0.66. However, given the wide confidence interval this reduced risk on acquisition was not statistically significant. Post hoc analysis did demonstrate a significant effect of segregation on acquisition of chronic infection with *P. aeruginosa* in patients <15 years of age. Previous studies have also reported beneficial effects of segregation, but these data are not easy to interpret because in these studies multiple interventions were implemented at the same time or statistical computations were suboptimal[20-22]. Randomized controlled trials cannot be performed for ethical reasons and patient populations before and after segregation are generally not exactly comparable.

Ongoing transmission despite segregation of ST406 was demonstrated in three already chronically infected patients (all >15 years of age at start of study) that acquired ST406 and could well be caused by lack of adherence to hygiene advises by certain patients, as was demonstrated during CF Beach Dance. Another hypothesis for lack of

effect of segregation in older patients is colonization of the upper respiratory tract with CF adapted strains from before segregation. In newly infected patients after segregation, the prevalent clones ST406 and ST497 were not detected. In total, the prevalence of the dominant *P. aeruginosa* genotypes ST406 and ST497 did not increase, which could be interpreted as a positive effect of segregation on transmission. Also, the level of genetic diversity did not change significantly. However, since we have no genotyping data from before 2007, the prevalence of dominant genotypes and level of genetic diversity could have been stable for many years already before segregation.

So, this observational study suggests that segregation prevented clonal transmission of *P. aeruginosa* strains among CF patients, and decreased chronic acquisitions with *P. aeruginosa* in younger patients. However, acquisition of chronic infection with unique strains is not prevented by segregation and this ultimately limits the effect of segregation on chronic acquisition.

Analysis of clinical data of this large longitudinal cohort of patients did not reveal associations between ST406 and mortality or lung transplantation or with increased lung function decline, confirming the results from the FOK study (chapter 5). However, there is an association of ST406, and other frequently shared genotypes, with antibiotic resistance. This could limit therapeutic options for CF patients with exacerbations and is, therefore, a reason to avoid chronic colonization with ST406 or other shared genotypes. The fact that patients harboring ST406 received inhaled antibiotics more frequently suggests a causal relation with increased resistance in this clone compared to other clones. Yet, what is cause and what is consequence remains speculative. Antibiotic resistance in *P. aeruginosa* ST406 strains isolated in the two cross-sectional genotyping studies did not increase between 2007 and 2011. An alternative hypothesis is that ST406 was already more resistant when introduced into the CF population.

Chapter 7 describes a detailed genomic, transcriptomic and functional comparison of the high prevalent *P. aeruginosa* CF clone ST406, isolated from a single patient at two time points; 2004 and 2007. The aim was to determine the processes that play a role in adaptation of *P. aeruginosa* to the CF lung. CF related *P. aeruginosa* strains are known to lose their virulence factors that are associated with acute infection, like expression of the type three secretion system (T3SS) and motility once strains become adapted. Mucoidity, sustained biofilm formation and slow growth together with metabolic adaptations seem to be main factors of adaptation to the CF lung, in the chronic stage of lung infection[23;24]. However, loss of mucoidity and decreased biofilm formation in late chronic infection have also been described[25;26]. Comparative genomics revealed that the gene content of the 2004 and 2007 isolates was highly stable, and that the main difference between the two isolates was a limited number of 42 high quality SNPs and 10 indels between the two isolates. One of the 24 non-synonymous SNPs was located

in *bmfS*, one of two genes encoding the BfmR/S two-component regulatory system known to control, among others, biofilm maturation in *P. aeruginosa*[27]. This mutation also coincided with decreased expression of this two-component regulatory system in the 2007 isolate and reduced biofilm formation in a semi-static model in this later time-point isolate. It is interesting to note that reduced biofilm formation in strains chronically infecting CF patients have been observed before[26]. During chronic infection of the CF lung clonal diversification occurs, resulting in many different phenotypic (like mucoid and non-mucoid) variants[25;28;29]. It might not be necessary for all clonal variants to sustain biofilm formation even though biofilm protects against stresses from the environment like the host immune system and antibiotics. Some "cooperative traits" like secretion of pyoverdin, elastase and protease and some quorum sensing molecules are down-regulated in chronic CF isolates, indicating loss of social behavior and a role of "cheater strains" profiting from products produced by cooperative strains[30]. Lower-level biofilm formation was not found in that study but may save energy, and more importantly, may increase the opportunity for transmission.

In addition to the changes observed in the two-component regulatory system and biofilm formation other differences were observed between the 2004 and 2007 isolate that include changes in LPS composition, decreased T3SS and increased T6SS expression, and decreased expression of the E/F efflux pump. Both expression of T6SS and the E/F efflux pump are known to be controlled by the MexT regulator. Comparative genomic analysis also revealed a SNP in MexT in the 2007 isolates relative to the 2004 isolate. MexT mutation in longitudinal analysis of adaptation to CF niche has been shown before, as well as LPS modification and loss of T3SS[31-33]. The fact that similar changes in *P. aeruginosa* strains have also been observed in other studies comparing longitudinal isolates from CF lungs indicates that these changes may contribute to adaptation of *P. aeruginosa* to the CF lung.

Conclusion and perspectives

Segregation:

In 2006 "strict segregation", which implies separating all CF patients at all times, was implemented in the Netherlands based on several assumptions without extensive knowledge about local epidemiology. It was generally accepted that chronic infection with *P. aeruginosa* was associated with clinical deterioration. In addition, epidemic clones were reported among CF patients in other countries with presumed associations with more morbidity and hospitalizations. Furthermore, there were reports of failing cohort segregation in Great Britain[34]. So, the main assumptions on which strict segregation was based were that, as in other countries, there were epidemic clones in Dutch

CF patients that were transmitted between CF patients and that were associated with adverse clinical outcome, and that strict segregation would decrease the occurrence of chronic infection by inhibiting transmission of adapted *P. aeruginosa* strains.

This thesis established that the population structure of *P. aeruginosa* in Dutch CF patients is dominated by two clones of which ST406 was mainly found in a cohort of teenagers and young adults in 2007. The epidemic clones from other (surrounding) countries are not present in Dutch CF patients and this Dutch clone is not found outside the Netherlands. Clinical relevance of harboring ST406 is limited to more antibiotic resistance and association with more frequent inhaled antibiotic therapy. Chronically infected patients often keep their own strain for years to lifelong, although 9% of CF patients acquire a super-infection that can result in replacement of the original genotype.

Furthermore, we demonstrated -in post hoc analysis- that segregating all CF patients in the Netherlands seems to decrease acquisition of chronic infection in young CF patients (<15 years of age) and possibly prevents transmission of epidemic or frequently shared strains that are adapted to the CF lung and are resistant to more antibiotic classes. We have observed that also after the implementation of segregation patients will still get chronically infected with sporadic *P. aeruginosa* strains, probably acquired from the environment. However, these strains seem not frequently transmitted between CF patients and are generally less resistant.

The drawbacks of strict segregation have not been investigated extensively. Psychosocial problems linked to loss of social contact and boredom because of isolation measures when hospitalized are reported[34]. Luckily, nowadays a lot of communication can occur via social media and internet access, which provides ample opportunities to talk "face-to-face" without being in the same room. However, being isolated during hospital admission and during the frequent outpatient visits and not being allowed to go on camps with other CF patients or to patient-information events can have a big impact on perceived quality of life. Another possible disadvantage is a possible higher financial burden. Yet, these issues have not been properly studied and might not be as substantial as imagined.

This thesis provides relevant data for the Dutch situation that now can be taken into account for policy making. Abandoning segregation is not recommended since there has been spread of CF related clones among Dutch CF patients, which are associated with more antibiotic resistance and there seems to be a beneficial effect of strict segregation on acquisition of chronic *P. aeruginosa* infection, at least in young patients. However, no association was detected between harboring ST406 and clinical deteriora-

tion compared to other genotypes and the epidemic clones associated with higher morbidity and mortality from abroad are not present in Dutch CF patients.

The question that remains is whether strict segregation is necessary or that cohort segregation (separating chronic infected patients from negative or intermittently infected patients) is also sufficient to prevent spread of resistant strains and/or epidemic clones and reduce acquisition of chronic infection. This question is based on the assumption that cohort segregation will diminish the drawbacks from strict segregation, allowing contact between some groups of CF patients.

An important argument against cohort segregation is more resistance in shared *P. aeruginosa* strains and the 9% of patients that acquire a superinfection with a possible prevalent genotype when already chronically infected. Other arguments against cohort segregation to consider, not described in this thesis, are transmission of other pathogens among CF patients, (like *Mycobacterium avium*) and dependency on availability of culture data with diagnostic delay. Also, practical implications of changing a policy that has been implemented and difficult distinction between chronically and intermittently infected patients favors continuing with the strict segregation policy as implemented in 2006.

Considering all the above, continuing with the policy of strict segregation seems the best way to go, although the effects of cohort and strict segregation have never been compared scientifically. Good communication of research outcome, stimulating and facilitating on-line contact between CF patients and continuous education might improve patients' adherence to segregation policy.

Adaptation to the CF lung

Adaptation to the CF lung of different *P. aeruginosa* strains is driven by changes in the expression of genes that increase protection to a challenging environment due to osmotic and oxidative stress and antibiotic treatment, like genes involved in alginate production, T3SS production, motility and metabolism. Only few mutations in global regulators, with many pleiotropic effects, seem to be needed for optimal adaptation. Since similar changes have been observed in multiple CF strains from different genetic backgrounds, these adaptation represent a clear example of parallel evolution towards an evolutionary peak in the fitness landscape[23]. Although, different evolutionary trajectories have been described for different CF-related *P. aeruginosa* strains to reach an adaptive peak, common altered functions involved in adaptive evolution might provide clues for therapeutic options to combat chronic colonization with *P. aeruginosa* in CF patients.

Investigations on alternative treatment are ongoing. Azithromycin, to reduce quorum sensing and inhalation of compounds that disrupt biofilm formation like recombinant

human DNAse (Pulmozyme) are already being used. Another potential target, also detected in ST406 adaptation, is the loss of O-antigen in the later time-point ST406 isolate on the surface of *P. aeruginosa* in the CF lungs. Loss of the LPS O-antigen, associated with a rough phenotype, reduces immunogenicity but is also associated with better killing by human serum[35]. This might partially explain why bloodstream infections in CF patients with chronic *P. aeruginosa* are rare, even though the lungs are heavily colonized. Insight in the regulatory mechanisms and genes that are important for adaptation to the CF lung may yield ingenious new therapies e.g. involving RNAi or the CRISPR-Cas system. CRISPR-Cas systems are RNA-directed adaptive immune systems, involving gene silencing, in many bacteria and most archaea that recognize nucleic acids of invading plasmids and viruses and these intrinsic systems can be used for the sequence-specific targeting and selective removal of individual strains of bacteria[36]. RNAi is investigated to be used in diverse clinical settings like cancer and neurologic disease and has been shown to effectively inhibit replication of hepatitis C virus in vivo[37;38]. Gene expression can be manipulated by siRNAs, which are 21-23 base pairs in length and are efficacious as exogenous agents in cultured cell- and animal-based systems[39]. *P. aeruginosa* transformed with a vector harbouring siRNA to silence MexB demonstrated reduced MexB mRNA in vitro and reduced viable bacterial load in an animal host model[39]. Adding siRNA, designed to silence coagulase, to *Staphylococcus aureus* culture inhibited coagulase production in vitro and infection with pre-treated bacteria in a mouse model resulted in less viable bacteria. Although not ready for clinical use, siRNA therapy does show possibilities.

The challenge of finding new ways to prevent or eradicate *P. aeruginosa* infections in CF patients is an ongoing process, and thorough knowledge of the population structure of *P. aeruginosa* in CF patients as well as molecular mechanisms involved in *P. aeruginosa* acquisition, persistence and adaptation in CF patients will facilitate this task.

Future investigations should use innovative methods to improve patient care; e.g. targeting essential genes for CF adaptation of *P. aeruginosa* with siRNA as mentioned above. CF lungs are not colonized with only one phenotype of *P. aeruginosa*, but many different clonal variants and contain many other micro-organisms forming a "microbiome". Investigating interactions between these micro-organisms will also provide clues for eradication strategies. Finally, repeatedly monitoring the epidemiology of *P. aeruginosa* in CF patients by cross-sectional genotyping studies (e.g., every five years) will identify emerging clones, and may assist in monitoring the effects of segregation.

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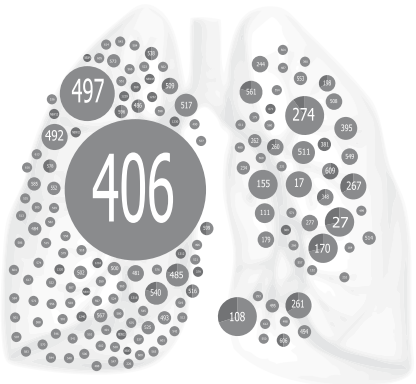
Addendum

- Nederlandse samenvatting
- Dankwoord
- Curriculum vitae
- List of publications



Korte samenvatting voor leken

"Cystische fibrose" (CF) of "taaislijmziekte" is een erfelijke ziekte waarbij patiënten erg dik (taai) slijm hebben dat onder andere tot problemen leidt in de luchtwegen en darmen. Het dikke slijm blokkeert de luchtwegen en wordt minder goed opgehoest. Hierdoor kunnen bacteriën zich veel makkelijker nestelen in de longen. Mensen met CF overlijden daardoor vaak aan de gevolgen van chronische ontsteking van de longen. De prognose van mensen met CF is in de afgelopen jaren enorm verbeterd, maar nog steeds is de gemiddelde levensverwachting slechts 35 tot 40 jaar.

Van de volwassen CF patiënten is meer dan de helft chronisch geïnfecteerd met *Pseudomonas aeruginosa* in zijn longen. *P. aeruginosa* is een bacterie die op vochtige plekken voorkomt en relatief ongevoelig is voor antibiotica. Vroeger werd gedacht dat de patiënten deze bacterie opliepen in hun eigen omgeving en iedereen dus een verschillend type(=stam) had. Zo'n 20 jaar geleden werd duidelijk dat in sommige landen veel CF patiënten dezelfde stam van deze bacterie bij zich droegen. Hierdoor werd het aannemelijk dat patiënten door contact met elkaar te hebben deze stammen aan elkaar doorgeven. Sommige van deze stammen, zoals die uit Engeland en Australië werden geassocieerd met slechtere prognoses en meer ziekenhuisopnames voor de CF patiënten.

Om het verspreiden van eventueel schadelijkere stammen te voorkomen is er in 2005 besloten om CF patiënten te segregeren. Dat wil zeggen dat ze geen lichamelijk contact meer met elkaar mogen hebben, elkaar het beste niet meer kunnen ontmoeten, ze gescheiden van andere CF patiënten op de controles bij de arts komen en ze in aparte kamers verpleegd moeten worden als ze in het ziekenhuis worden opgenomen. CF patiënten hadden tot die tijd zeer intensief contact met lotgenoten op o.a. zomerkampen, voorlichtingsdagen en verschillende uitjes en het segregatiebeleid heeft dus een grote impact op hun leven.

In dit proefschrift wordt onderzocht wat de situatie in Nederland is. Er wordt beschreven dat 15% van de geïnfecteerde Nederlandse CF patiënten dezelfde stam hebben, die vooral bij jongeren gevonden wordt. Deze stam komt alleen bij CF patiënten voor en niet bij patiënten met andere ziekten en ook niet buiten Nederland. Deze stam is niet geassocieerd met een slechtere prognose, maar wel met meer resistentie tegen antibiotica en meer antibiotische behandelingen. Verder wordt het effect van het segregatie beleid onderzocht. Het blijkt dat er weinig verandert in de hoeveelheid en het voorkomen van de verschillende stammen van *P. aeruginosa*, maar er zijn aanwijzingen dat patiënten minder vaak chronisch geïnfecteerd raken. Daarnaast wordt deze Nederlandse stam onderzocht op specifieke eigenschappen die kunnen verklaren waarom deze stam zich onder CF patiënten zo heeft kunnen verspreiden en die mogelijk aanknopingspunten zouden kunnen vormen voor toekomstige therapie.

Nederlandse samenvatting

Ondanks het feit dat de prognose voor patiënten met cystische fibrose (CF) de afgelopen decennia enorm verbeterd is, leidt chronische infectie van de longen nog steeds tot een vroegtijdige dood. Meer dan de helft van de volwassen CF patiënten is chronisch geïnfecteerd met *Pseudomonas aeruginosa. P. aeruginosa* is een bacterie die voorkomt in onze omgeving en in gezonde mensen zelden infecties veroorzaakt. In CF patiënten is kolonisatie met deze bacterie echter geassocieerd met klinische achteruitgang en een slechtere prognose. In 2005 heeft de Gezondheidsraad geadviseerd dat patiënten met CF in Nederland gesegregeerd moeten worden. Dit beleid houdt in dat patiënten apart gezien worden op de poliklinieken en apart verpleegd worden, maar ook dat er wordt afgeraden om fysiek contact te hebben met andere CF patiënten en dat activiteiten zoals de zomerkampen, informatiedagen en andere events afgeschaft werden. Dit besluit is genomen naar aanleiding van onderzoeken in het buitenland die lieten zien dat er transmissie was van *P. aeruginosa* epidemische stammen tussen CF patiënten en dat deze geassocieerd waren met slechtere prognose en meer ziekenhuisopnames dan andere stammen. Daarnaast was er een studie die mogelijke transmissie van *P. aeruginosa* stammen aantoonde op Nederlandse zomerkampen. Echter, men wist niet wat de populatie structuur van *P. aeruginosa* in Nederland was en of er eventuele epidemische stammen (zoals in Engeland) voorkwamen. Dit proefschrift beschrijft de populatie structuur van *P. aeruginosa* in Nederland en het effect van segregatie hierop en op acquisitie van chronische infectie. Daarnaast wordt de Nederlandse prevalente stam ST406 beschreven inclusief klinische relevantie en specifieke eigenschappen.

In hoofdstuk 2 wordt de populatie structuur van *P. aeruginosa* beschreven als zeer divers met een prevalentie onder CF patiënten van 57% wat vergelijkbaar is met de prevalentie in andere landen. Deze studie kan als uitgangspunt dienen om het effect van segregatie later te evalueren. Slechts 30% van de patiënten heeft een uniek genotype *P. aeruginosa* en het blijkt dat broers en zussen met CF veelal met hetzelfde genotype gekoloniseerd zijn. Er worden twee veel voorkomende genotypen beschreven, ST406 en ST497 die niet voorkomen in andere landen. Daarnaast lijkt er een link te zijn tussen leeftijd en de veel voorkomende genotypen. Ongeveer 50% van de patiënten tussen de 15 en 25 jaar oud is gekoloniseerd met ST406, terwijl ST497 juist bij oudere patiënten voorkomt. Deze bevindingen wijzen op mogelijke transmissie van patiënt op patiënt.

In hoofdstuk 3 worden drie typeringsmethoden voor *P. aeruginosa* vergeleken waaronder een nieuw "Multi Locus Variable number of tandem repeats Analysis" (MLVA) typeer schema dat een goede congruentie heeft met "Multi Locus Sequence Analysis" (MLST)

en "Pulsed Field Gel Electrophoresis" (PFGE). Daarnaast blijkt dat de populatiestructuur van *P. aeruginosa* niet alleen heel divers is maar ook specifiek voor de verschillende patiëntengroepen, met weinig onderlinge uitwisseling. Verschillende genotypen worden gevonden in IC patiënten, poliklinische patiënten en CF patiënten maar ook in dezelfde groep (IC patiënten) uit verschillende ziekenhuizen zijn de genotypen verschillend. Er is geen verschil in genetische diversiteit tussen de verschillende patiënten groepen of ziekenhuizen. ST406 en ST497, de veelvoorkomende stammen onder CF patiënten, komen niet voor in niet-CF patiënten. Dit suggereert dat deze stammen niet veel voorkomen in de omgeving en specifiek zijn voor CF. In deze studie zijn geen isolaten uit de directe omgeving of uit het milieu meegenomen, maar in de internationale databases van isolaten getypeerd met MLST en MLVA waarin wel omgevings-isolaten zijn opgenomen komen ST406 en ST497 niet voor. Daarnaast worden deze genotypen ook niet gevonden buiten CF patiënten in een grote typeringsstudie die SNP-typering gebruikt waar ook veel omgevings-isolaten waren geïncludeerd.

Een unieke setting waarin CF patiënten elkaar nog altijd ontmoeten ondanks het segregatie beleid is het jaarlijkse CF Beach Dance evenement. In hoofdstuk 4 wordt het onderzoek naar transmissie tijdens dit evenement in 2009 beschreven. Vanwege het slechte weer was er slechts een kleine groep deelnemers en dit maakt de data lastig te interpreteren. Van de deelnemers was 32% gekoloniseerd met ST406. Er werd één geval van mogelijke transmissie (met ST406) in deze kleine groep gevonden. Omdat ST406 tot nu toe alleen in CF patiënten aangetoond is, lijkt transmissie plausibel. Omdat er slechts een geval van mogelijke transmissie optrad kunnen geen uitspraken gedaan worden over verschil in overdraagbaarheid van de verschillende genotypen. Echter, in een eerder onderzoek naar mogelijke transmissie van *P. aeruginosa* tijdens CF zomerkampen in Nederland betrof het de veelvoorkomende genotypen ST406 en ST497 in alle drie de gevallen van "waarschijnlijke transmissie" en 14 van de 18 gevallen van "mogelijke transmissie". Dit wijst erop dat "makkelijke transmissie" mogelijk bijdraagt aan de prevalentie van deze veelvoorkomende stammen.

Daarnaast bleek tijdens deze studie dat compliance van meerdere CF patiënten betreffende de hygiëne regels laag was en uit de vragenlijsten bleek dat 50% van de CF patiënten contact had gehad met andere CF patiënten (buiten familieleden om) in de drie maanden na het evenement. Ook bezochten patiënten die niet chronisch gekoloniseerd waren met *P. aeruginosa* het evenement wetende dat zij risico liepen om gekoloniseerd te raken.

In hoofdstuk 5 wordt de follow-up studie beschreven van CF patiënten die de zomerkampen bezochten in 2001 waarin de *P. aeruginosa* stammen die destijds waren aangetoond worden vergeleken met de stammen die negen jaar later bij deze patiënten

worden aangetoond. Hieruit blijkt dat genotype ST406 vaker persisteert dan andere genotypen. Deze hogere persistentie kon echter niet bevestigd worden in de grotere longitudinale studie beschreven in hoofdstuk 6. Een tweede conclusie uit hoofdstuk 5 is dat kolonisatie met ST406 niet lijdt tot een slechtere longfunctie of een hogere kans op overlijden of een longtransplantatie na negen jaar. Deze resultaten worden wel geconfirmeerd in de studie van hoofdstuk 6. Blijkbaar kan ST406 makkelijk en langdurig de longen van CF patiënten koloniseren, maar is deze stam niet heel pathogeen, vergeleken met andere genotypen.

In hoofdstuk 6 worden de effecten van het segregatiebeleid geëvalueerd. Omdat de patiëntengroepen voor en na segregatie voor een groot deel uit dezelfde patiënten bestaan, maar ook een deel verschillende patiënten bevatten die qua leeftijdsverdeling niet helemaal vergelijkbaar zijn, is het moeilijk om statistisch betekenisvolle berekeningen uit te voeren. Echter, er lijkt een trend te zijn dat er na segregatie minder risico is op acquisitie van chronische kolonisatie door *P. aeruginosa*. Dit effect lijkt met name op te treden bij jonge CF patiënten. Het aantal chronische acquisities per patiënt per jaar at risk neemt af met de jaren na segregatie. Toch raken elk jaar patiënten chronisch geïnfecteerd met *P. aeruginosa* wat erop wijst dat stammen uit de omgeving ook een belangrijke bijdrage leveren aan chronische acquisitie. Dit is niet uit te bannen met segregatie.

De populatie structuur is niet wezenlijk veranderd na vierjaar segregatie. Er is sprake van hoge diversiteit en er zijn kleine, niet significante dalingen van de prevalentie van *P. aeruginosa* van 57% naar 52% en van het percentage frequent gedeelde stammen van 57% naar 52%. Er wordt geen transmissie van ST406 onder niet-gekoloniseerde patiënten aangetoond maar wel bij drie al chronisch geïnfecteerde patiënten. Mogelijk hebben deze laatste patiënten zich niet voldoende aan de segregatie richtlijnen gehouden of is er toch ergens een onbekende bron van ST406. Ook in deze studie wordt geen associatie gevonden tussen ST406 en slechtere longfunctie of meer risico op overlijden of longtransplantatie. Echter, er werd wel een associatie gevonden tussen ST406 en meer gebruik van inhalatie antibiotica. Daarnaast vertonen ST406 en ook andere frequent gedeelde stammen resistentie tegen meer groepen antibiotica. Het is niet mogelijk oorzaak van gevolg te onderscheiden maar mogelijk leidt infectie met ST406 tot frequentere antibioticakuren waardoor meer resistentie optreedt. Echter, tussen 2007 en 2011 nam de resistentie van ST406 niet significant toe. Een andere mogelijkheid is dat ST406 al resistenter was bij de introductie in de CF patiënten.

In hoofdstuk 7 worden de eigenschappen van ST406 nader onderzocht en wordt gekeken naar evolutie van deze stam in een CF patiënt gedurende drie jaar. Vergeleken met de standaard laboratoriumstam "PA01" die oorspronkelijk uit een wond is geïsoleerd,

groeit ST406 relatief langzaam en brengt weinig virulentie factoren tot expressie. Dit wordt ook voor andere CF gerelateerde stammen beschreven. Vergelijking van de genomen en genexpressie van het vroege en het late isolaat van ST406 toont biofilm maturatie en metabole veranderingen als belangrijke aanpassingen aan de niche in de CF long. Er wordt een puntmutatie gevonden in één van de twee regulator genen *bfmS/R* die essentieel zijn voor maturatie van biofilm in het late isolaat. Daarnaast is expressie van deze genen minder. Adaptaties van ST406 die ook beschreven zijn voor andere CF stammen in de verschillende patiënten zijn aanpassingen in LPS (lipo-polysaccharide) door mutaties in *migA* en *pagL*, een mutatie in *mexT* die de MexEF efflux pomp en het T6SS (type 6 secretie systeem) reguleert, en een mutatie in *exsA* waardoor het T3SS (type 3 secretie systeem) wordt stilgelegd. LPS, de secretie systemen en de efflux pompen beïnvloeden allemaal de virulentie. De overlap in adaptatie tussen de verschillende CF gerelateerde stammen bevestigd dat er sprake is van parallelle evolutie. Genen die belangrijk zijn voor de evolutie in de CF long in de verschillende stammen vormen mogelijk specifieke targets voor therapie. Onderzoek hiernaar zal mogelijk leiden tot betere mogelijkheden om chronische infectie met *P. aeruginosa* te voorkomen of alsnog te eradiceren.

Dankwoord

"It is good to have an end to journey toward; but it is the journey that matters, in the end" (Ernest Hemmingway). Promoveren is een voorrecht. Het is geweldig om onderzoek te mogen doen, je ideeën te testen en antwoorden proberen te vinden en tijdens zo'n traject leer je veel. Een flinke portie frustratietolerantie en doorzettingsvermogen zijn hierbij wel essentiële eigenschappen want onderzoek gaat vaak niet zoals je van te voren had bedacht. En nu ben ik heel blij dat het boekje af is. Tijdens dit promotietraject hebben veel mensen mij bijgestaan, en zonder deze hulp en de medewerking van anderen was dit proefschrift waarschijnlijk niet tot stand gekomen. Veel dank daarvoor. Een aantal van deze mensen wil ik in het bijzonder bedanken.

Ten eerste zijn er natuurlijk de CF patiënten die hun medewerking verlenen aan onderzoek. Dankzij hen is het mogelijk de kennis omtrent deze ziekte te vergroten.

De leden van de leescommissie: Prof. dr. J.W.J. Lammers, Prof. dr. E.A.M. Sanders, Prof. dr. H.A.W.M. Tiddens, Prof. dr. H.J. Grundmann en dr. S.F.T. Thijsen wil ik bedanken voor het beoordelen van het manuscript.

Marc, vanaf het begin mijn promotor, maar ook tijdelijk collega AIOS en snel daarna baas. Bedankt voor de ruimte die je me hebt gegeven om dit promotietraject te kunnen voltooien tijdens mijn opleiding tot arts microbioloog. Heel fijn om een promotor te hebben die via de mail snel bereikbaar is en bij wie je ook met kleine vragen even binnen kunt stappen.

Rob, dank voor je geduldige en betrokken begeleiding al die jaren. Jouw deur stond altijd open en ik heb veel van je geleerd onder andere over typeren en populatiebiologie. Door jouw zorgvuldige feedback op manuscripten en heldere blik op de zaken kon ik steeds weer vooruit.

Kors, dank voor het meedenken met je klinische blik en ervaring als CF behandelaar. Dankzij de samenwerking tussen de afdelingen kinderlongziekten en medische microbiologie werd de link tussen ons onderzoek en de kliniek voor mij een stuk concreter.

Tom, bedankt voor je betrokkenheid en je talent om de essentie van wat gezegd wordt in de meetings (vanuit klinisch, statistisch of biologisch oogpunt) duidelijk te verwoorden en samen te vatten.

Lieke, tijdens mijn maand in Denemarken leerde ik je kennen en ondanks het feit dat we Nederland zagen verliezen met voetbal in een Deens café, was ik meteen geïnspireerd door je enthousiasme voor "ons" onderzoek en je prettige manier van samenwerken. Tijdens onze meetings brainstormden we over onderzoeksplannen, conclusies van stukken of kletsten we even over hoe het nou ging. En inderdaad was het hoogtepunt wel dat we met die grote longfunctiebus naar Bloemendaal reden om kweken af te nemen tijdens de Beach Dance die we midden in de nacht nog even op het lab hebben ingezet. Jij bent al een paar jaar geleden gepromoveerd, en al bijna kinderarts. Veel succes met het afronden van je specialisatie.

Sanne, dank voor de al je MLST werk. Ondanks ontelbare PCRs en te beoordelen DNA sequenties bleef jij enthousiast en vrolijk en je ongecensureerde uitspraken en voorkeur voor roze brachten altijd een glimlach op mijn gezicht. Fijn dat je het zo naar je zin hebt op de derde verdieping.

Bedankt Bertie voor de vele MLVAs, opkweek- en opstuur-acties en allerhande andere klussen die je tussen je andere werkzaamheden door voor me hebt gedaan. Voor jou is het ook bijna klaar. Geniet straks van je pensioen en succes met de projecten waar je dan meer tijd voor zult hebben.

Bedankt, andere mensen van de afdeling medische microbiologie, voor jullie hulp bij experimenten, gezelligheid tijdens borrels, buitendagen en feestjes. In bijzonder de enterokokken groep waar ik de eerste jaren nog soort van bij hoorde. Miranda, dank voor het wegwijs maken in de MLST, Janetta, dank dat je mijn vraagbaak wilde zijn voor het opzetten van de MLVA en andere praktische typeer problemen. Claudia, dank voor het uitvoeren van de MLVAs van de IC monsters. Willem, dank voor het delen van je kennis over de verschillende toepassingen van de genoomdata en je kritische vragen bij presentaties. Fernanda, dank voor het meedenken en doen/begeleiden van de biofilm experimenten. En Mark, bedankt dat je zo goed manieren weet te bedenken om interessante dingen uit een hele berg (genoom)data te halen. In het diagnostiek lab: Alie en Judith, bedankt voor het jarenlang invriezen van pseudomonaden uit de CF sputa, die stammen zijn ons "goud".

Haga ziekenhuis Den Haag: Roland Brimicombe, dankjewel voor het verzamelen van stammen en kweekdata en het meedenken over de verschillende studies en Harry Heijerman, dank voor het kritisch beoordelen van de resultaten van de verschillende studies. Ook de CF verpleegkundigen uit het Haga ziekenhuis wil ik bedanken voor hun medewerking bij het verzamelen van data.

Denmark, DTU: Dear Søren Molin, thanks for giving me the opportunity to do the transcriptome and phenotypic assays in your lab, and for being so nice and hospitable. It was inspiring to work in a group with so much knowledge on *Pseudomonas aeruginosa*. Lei Yang, thanks for your help with the arrays and analysis.

Dank aan mijn opleiders: Professor Verhoef, u nam mij aan in de opleiding tot arts microbioloog in combinatie met een promotietraject en gaf meteen een goede relativatering van de verwachtingen ten aanzien van de impact van promotieonderzoek in het grotere geheel. Daarnaast veel dank voor de gezellige weekenden in Villars. Annemarie Weersink, de eerste jaren van de opleiding tot arts microbioloog was ik onder jouw hoede. Dank voor de ruimte die gecreëerd kon worden om deze promotie te combineren met de opleiding en je luisterend oor en begrip tijdens mindere tijden. En als opleider in de laatste fase Anne Wensing dank voor het vertrouwen en het meedenken over de uiteindelijke realisatie van zowel opleiding als promotie.

Els de Tex, bedankt voor je hulp en het mogelijk maken van zaken afgewisseld met nuttige levenswijsheid of een luisterend oor.

Het voordeel van zo lang in opleiding zijn is dat je veel collega AIOS hebt. (Ex-)AIOS bedankt voor de gezelligheid, de gedeelde biertjes, 'n luisterend oor, even een spiegelmomentje of gekke woordgrappen (ßab@). Naar je werk gaan is een stuk leuker als je leuke collega's hebt!

Dank aan mijn vrienden, van de Spits, het Boni, 8-med en SB (+Kuna) ;-) en anderen (minder goed onder een kopje te plaatsten) voor jullie interesse en support en vooral voor het begrip voor deze drukke periode waarin ik vaak weinig tijd maakte voor jullie. De gezellige etentjes, borrels of bij-klets-koffies die wel plaatsvonden zorgden gelukkig voor de nodige afleiding en plezier. Ik ben weer in voor meer van dat soort genietmomentjes!

Bedankt Laura en Barbara, dat jullie mijn paranimfen willen zijn. Laura, 17 jaar geleden zijn we tegelijk begonnen aan medische biologie en inmiddels een paar levensfases verder is het nog altijd "thuiskomen" bij jou. Barbara, ook al meer dan 15 jaar geleden tegelijk lid geworden, samen bestuur, samen zwemmen en heel vaak eten (inmiddels zeker meer dan 200 x). Fijn dat je zo'n betrokken en geïnteresseerde vriendin bent. Ik ben blij dat jullie naast me staan bij de promotie.

Beste Michiel en Thera, dank voor jullie interesse in mijn progressie en het hosten van de vele logeerpartijtjes de afgelopen tijd.

Lieve familie, broers en schoonzussen, het is fijn dat we het zo gezellig kunnen hebben met elkaar. Familie is belangrijk en het is fijn om te weten dat je er voor elkaar bent.

Lieve pap, dank voor de fijne jeugd die ik dankzij mam en jou heb gehad waarin ik hebt geleerd nieuwsgierig te zijn en de wereld met vertrouwen tegemoet te treden. Heel jammer dat mama dit niet meer mag meemaken.

Lieve Niels, ik ben zo blij dat ik jou ben tegengekomen! Dank dat je er bent, voor je liefde, je enthousiasme en je steun en duizendmaal dank voor de offers die je de afgelopen tijd hebt gebracht om het gezin draaiend te houden met onze lieve kindjes.

Lieve Elisa en Timme, dank voor jullie onvoorwaardelijke liefde die alles steeds weer in het juiste perspectief zet.

Curriculum Vitae

Rosa van Mansfeld was born on February the twelfth in 1978 in Woerden. After graduating from secondary school at the Sint Bonifatius college in Utrecht in 1996 she worked and traveled though Australia and New Zealand for a year. In 1997 she started to study biomedical sciences at the University of Amsterdam and in 2001 she started medical school as well at the University of Amsterdam and did a nursing aid internship in Sumve, Tanzania. In 2004 she performed her research elective at "Harvard Medical School", in Boston, USA, investigating the de regulation of the LPH gene that hydrolyses lactose in the intestine. That same year she received her master's degrees in both medicine and biomedical sciences and started medical rotations. In 2006 she performed an internship Medical Microbiology at the AMC and her final internship was pediatrics at the Tygerberg hospital in Capetown, South Africa. In 2007 she started her residency in Medical Microbiology at the University Medical Centre Utrecht (under supervision of prof. dr. J. Verhoef , dr. A. Weersink and dr. A. Wensing) and started her PhD research project under supervision of prof. dr. M.J.M. Bonten, prof. dr. C.K. van der Ent and dr. R.J.L. Willems that led to this thesis. In 2008 she performed experiments during one month at DTU, Copenhagen under supervision of prof. dr. S. Molin. At the moment she is doing her last year of residency in Medical Microbiology.

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