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PhD-thesis

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Preface

This thesis was written as a partial fulfilment of the requirements to obtain a PhD-degree at the Technical University of Denmark. The work presented here was performed between June 2013 and September 2016 at the Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark (DTU). The work was supervised by Katherine S. Long, Associate Professor at DTU, Søren Molin, Professor at DTU Bioengineering at DTU, and Helle Krogh Johansen, DMSc, Chief Physician at Rigshospitalet, Copenhagen. The work was financed by DTU.

The thesis was evaluated by Professor Lars Jelsbak from DTU, Professor Hanne Ingmer from the University of Copenhagen, and Professor Dr. Susanne Häußler, Head of the Department Molecular Bacteriology at Helmholtz Centre for Infection Research, Germany.

Mikkel Lindegaard
Kgs. Lyngby, September 2016

Abstract

Pseudomonas aeruginosa is a major cause of morbidity and mortality in cystic fibrosis (CF) patients. *P. aeruginosa* infects the CF airways and establishes chronic infections that can last for a lifetime during which *P. aeruginosa* evolves in order to adapt to the environment.

In this PhD thesis, we investigated the evolution of two convergent lineages of *P. aeruginosa* isolated from the early stages of infection in two CF patients using both transcriptomic and proteomic methods. Both lineages harbour sequential mutations in a specific regulatory system, the *retS-gacS-gacA-rsmA-rsmYZ* signalling pathway, which reciprocally regulates the expression of genes attributed to chronic and acute infection states. Additionally, we investigate the effects of the evolution not caused by the mutations in this regulatory system through allelic replacements in the clinical isolates.

We show that the initial stages of infection with *P. aeruginosa* is subject to temporal and differential expression of virulence factors caused by mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signalling pathway. Initially, a mutation in *retS* causes a switch to a chronic infection mode characterised by the expression of the Type VI secretion system (T6SS) and induction of the phenazine biosynthesis operons. The effects of the *retS*-mutation are reversed with a later mutation in either *gacS* or *gacA*, which lowers the expression of the T6SS and the phenazine biosynthesis operons and instead leads to high expression of the Type III secretion system (T3SS). This suggests that the current dogma of this regulatory system does not adequately explain the biological significance of this system, as the opposite mutation pattern would be expected if this dogma were true. Furthermore, we show that

the residual evolution caused by other mutations also has an effect on the expression of virulence factors.

Dansk resumé

Pseudomonas aeruginosa er en stor årsag til morbiditet og dødelighed i cystisk fibrose (CF) patienter. *P. aeruginosa* forårsager infektioner i CF luftvejene og etablerer kroniske infektioner, der kan vare en menneskealder. I denne tid udvikler *P. aeruginosa* sig for at tilpasse sig til miljøet ved at tilegne sig mutationer.

I denne Ph.d.-tese undersøgte vi evolutionen af to konvergerende klontyper af *P. aeruginosa*, der var isoleret fra de tidlige stadier af infektion i to CF patienter ved brug af transkriptom- og proteommetoder. Begge klontyper har sekventielle mutationer i et specifikt regulatorisk system, *retS-gacS-gacA-rsmA-rsmYZ* signalsystemet, der reciprokt regulerer ekspressionen af gener tillagt betydning for enten kroniske infektionstilstande eller akutte infektionstilstande. Endvidere undersøger vi effekterne af evolutionen, der ikke er forårsaget af mutation i dette regulatoriske system ved brug af alleludskiftninger i de kliniske isolater.

Vi viser, at de første stadier af infektion med *P. aeruginosa* er omfattet af temporal og differentielt udtryk af virulensfaktorer forårsaget af mutationer i *retS-gacS-gacA-rsmA-rsmYZ* signalsystemet. Først opstår en mutation i *retS*, hvilket giver et skift til kronisk infektionstilstand karakteriseret ved genudtryk af Type 6 Sekretionssystem (T6SS) og inducering af phenazin-biosynteseoperonerne. Virkningen af mutationen i *retS* bliver omgjort af en mutation i enten *gacS* eller *gacA*, hvilket sænker genudtrykket af T6SS og phenazin-biosynteseoperonerne og i stedet fører til højt genudtryk af Type III sekretionssystemet (T3SS). Dette antyder, at det nuværende dogme om dette regulatoriske system ikke på tilfredsstillende vis beskriver den biologiske signifikans af dette system, da the modsatte mutationsmønster ville være forventet, hvis

dette dogme var sandt. Endvidere viser vi, at den overskydende evolution, forårsaget af andre mutationer, også har en indflydelse på genudtrykket af virulensfaktorer.

Publications

1. **M. Lindegaard**, D. Zühlke, K. Riedel, S. Molin, H. K. Johansen, K. S. Long. (2016). The evolutionary trajectories of *Pseudomonas aeruginosa* isolates from cystic fibrosis airways show temporal expression of virulence genes and lineage specific trends. (in preparation)

2. **M. Lindegaard**, A. Jiménez-Fernández, S. Molin, H. K. Johansen, K. S. Long. (2016). Transcriptomic evolution of two convergent *Pseudomonas aeruginosa* lineages from the cystic fibrosis airways. (in preparation)

Table of Contents

Introduction and thesis outline

Pseudomonas aeruginosa is a major cause of morbidity and mortality in cystic fibrosis (CF) airway infections. It has the ability to establish chronic infections that are difficult to eradicate. This leads to lifelong infections, giving the bacteria ample time to evolve and adapt to the CF airways. The advent of next-generation sequencing (NGS) has given unprecedented insight into how *P. aeruginosa* evolves in the CF airways and has shown that regulatory networks are often the targets of mutations causing major changes in the physiology of the bacterium. Especially the early stages of the infections are characterised by positive selection of mutations, meaning that the mutations that occur are improving the fitness of the bacteria. However, the evolution is a complex process with a multitude of mutations in genes involved in anything from metabolism to virulence. Furthermore, many regulatory systems are interconnected and thus the occurrence and combination of mutations can lead to unexpected results.

The aim of this thesis was to investigate the early adaptation and evolution of clinical *P. aeruginosa* isolates from CF infections. To this end, we investigate two lineages from the CF airways that have mutations in *retS-gacS-gacA-rsmA-rsmYZ* signalling pathway alongside many other mutations. This specific regulatory system serves as a switch between the expression of genes attributed to acute and chronic infection states.

This thesis contains three introductory sections. Section 1 is an introduction to *P. aeruginosa* as a bacterium emphasising its versatility with a special focus on the impressive arsenal of elements involved in virulence, as some of them are regulated by this specific regulatory network. This is followed by an introduction to the evolution of *P. aeruginosa* in the CF airways.

Section 2 gives an introduction to regulation in *P. aeruginosa*. The functions of σ-factors, two-component systems (TCSs), and small RNAs (sRNAs) are explained with select examples that aim to give an idea of the complex regulatory circuits at play. Special focus is given to the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway as it was the subject of study in this thesis.

Section 3 gives the historical background of how this PhD-thesis was conceived and its relevance to the research on the evolution and adaptation of *P. aeruginosa* in CF airway infections. The studies that led to the ideas of this project are presented and the rationale behind the research is explained.

This is followed by section 4, where the conclusions of this thesis are given and future perspectives of what should be investigated next are presented.

Attached are the manuscripts that are the results of the work performed during this PhD.

1. Pseudomonas aeruginosa

Pseudomonas aeruginosa is a motile, Gram-negative, and rod-shaped bacterium, belonging to the genus *Pseudomonas.* The pseudomonads are found in a broad range of environments such as soil and marine environments [1], but also in association with plants and animals. *P. aeruginosa* is the most studied of the genus due to it being an opportunistic pathogen to humans and other mammals, unlike most other members of the *Pseudomonas* genus. Multi-drug resistant *P. aeruginosa* were in 2013 named as a serious threat due to the emergence of strains resistant to the majority of antibiotics, including aminoglycosides, cephalosporins, fluoroquinolones, and carbapenems [2].

P. aeruginosa is a common cause of nosocomial infections in burn wound patients, in mechanically ventilated patients, and the immunosuppressed patients, such as AIDS, cystic fibrosis (CF) and cancer patients [3], due to its ability to create biofilms, its many virulence factors, its innate antibiotic resistance, and its ability to thrive in a vast array of environments.

1.1. Genome characteristics

The first whole-genome sequenced *P. aeruginosa* was PAO1, the most common laboratory strain and originally a wound isolate. The genome was published in 2000 [4] and has a size of 6.3 Mbp, a GC-content of 66.6% and was the largest bacterial genome sequenced at the time. A high proportion (~8%) of its 5570 predicted open reading frames (ORFs) are predicted to encode either transcriptional regulators or two-component systems. Since the release of the first genome, many more strains have been sequenced with the Pseudomonas Genome Database [5] now containing 50 complete genomes and about 1500 unfinished genomes. The genome size ranges from 5.5 to 7 Mbp [6].

The *P. aeruginosa* pan-genome, which represents the entire gene set of all strains of the species, includes at least 9344 genes [7] of which 5233 are shared between all *P. aeruginosa* species (core genome) and the rest represents the genes present only in some strains (accessory genome). Therefore *P. aeruginosa*, as a species, contains considerable genomic diversity between the strains. As part of the accessory genome, some strains contain a variety of pathogenicity islands and genomic islands that can contain genes encoding toxins, adhesins, integrases, transposases, antibiotic and heavy metal resistance genes, making these strains considerably more virulent or capable of surviving in hostile environments than strains lacking these [8], [9].

1.2. Metabolism

P. aeruginosa displays a versatile metabolism like many other members of the *Pseudomonas* genus. Some pseudomonads are capable of growing on more than 100 different simple and complex compounds as carbon and energy sources, owing to their remarkable metabolic diversity [10, pp. 413–415].

The preferred carbon and nitrogen sources of *P. aeruginosa* include short-chain fatty acids, amino acids, carboxylic acids, and polyamines, but the bacterium is also capable of catabolizing sugars through the Entner-Doudoroff pathway [11]. In the presence of multiple substrates, *P. aeruginosa* makes use of carbon catabolite repression control in order to uptake and metabolise the preferred carbon sources first [12]. Furthermore, the ability of *P. aeruginosa* to grow on n-alkanes and halogenated aromatic compounds as sole carbon sources, demonstrates the capability of the bacterium to degrade complex xenobiotics [13], [14].

Energy generation occurs mainly by oxidative phosphorylation, but depending on conditions *P. aeruginosa* will also grow as a facultative anaerobe using alternative electron acceptors such as nitrate through denitrification, or through fermentation of arginine and pyruvate. The genes encoding aerobic respiration, denitrification, and anaerobic fermentation have so far been identified in all strains of *P. aeruginosa,* i.e. as part of the core genome, emphasizing that the metabolic versatility is important to the lifestyle of *P. aeruginosa* in general [7].

1.3. Key elements for virulence

Virulence factors are traits of a bacterium that enable it to establish infection or otherwise be virulent. *P. aeruginosa* has a formidable array of virulence factors available to it, including at least five secretion systems [15], [16] (Figure 1), many iron uptake systems, the ability to form biofilms, secondary metabolites, and intrinsic antibiotic resistance. The combination of these traits enables *P. aeruginosa* to establish infections.

1.3.1. Secretion systems

Secretion systems are proteins or protein-complexes that allow for the secretion of effector molecules, such as toxins, but also proteins that can degrade the environment, such as elastases, lipases, and proteases, in order to release otherwise unavailable nutrients. Some systems work by simply secreting the effector molecules into the environment, while others actively inject the effectors into other cells.

The type I secretion systems (T1SS) (*apr*/*has*-genes) are simple secretion systems that require three components to function; an outer-membrane protein, an inner-membrane ATP-binding cassette (ABC) transporter, and an adaptor connecting the two in the periplasm [17]. At

least three proteins are secreted through these systems [18], AprA, an alkaline protease, AprX, a protein of unknown function, and HasAp, a haem acquisition protein. AprA is capable of degrading collagen, the main structural protein in connective tissues [19]. It has been suggested that HasAp is especially important during the early stages of infection, where iron is scarce as it is capable of acquiring iron through haem from haemoglobin [15].

The type II secretion systems (T2SS) (*xcp*-genes/*hxc*-genes) are very versatile systems. The T2SS Xcp can secrete at least 14 proteins with different functions such as proteases and lipases, but the Hxc secretes only one protein, LapA, an alkaline phosphatase [20]. The two systems seem to be divergent systems that exist in their own clusters consisting of 11 genes in two different loci. A key difference from the T1SS, is that the outer porin is a 12-subunit multimer allowing for even folded exoproteins to pass through [15]. Secreted proteins include LasB, an elastase, which efficiently degrades elastin, a major component of connective tissue [21] of the lungs, suggesting a key function in the infection of the airways. Lipases and phospholipases, such as LipA, LipC, PlcH, and PlcN, have been shown to degrade lung surfactants, but also modify immune function [22], [23]. The exotoxin A, ToxA, inactivates the eukaryotic elongation factor-2 by ADP-ribosylation, thereby halting protein synthesis in the host cell, leading to cell death [24].

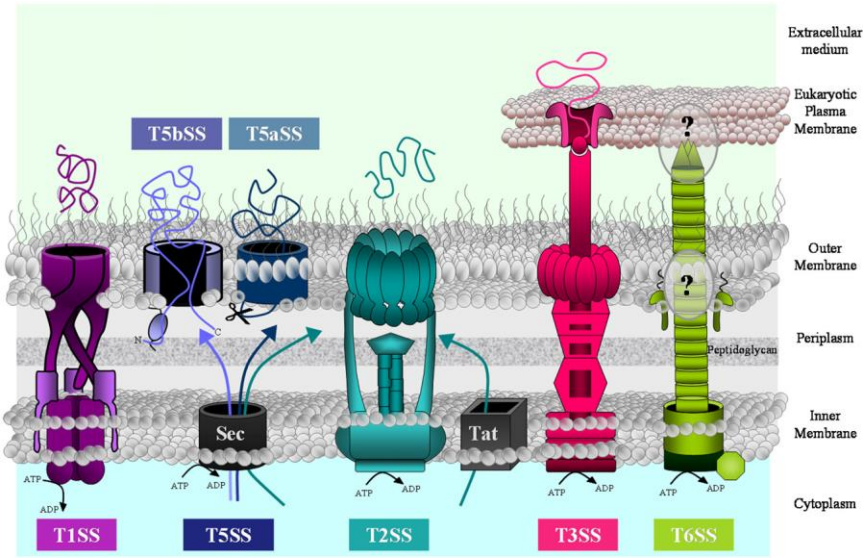

Figure 1. The secretion systems of *P. aeruginosa* showing the different modes of action used. The T1SS secretes effector compounds directly into the extracellular medium. The T2SS and the T5SS make use of the Tat and Sec secretion pathway, respectively, to export effector compounds to the periplasm and then secrete effector compounds through their own machinery. The T3SS and the T6SS secrete effector compounds directly from the cytoplasm to the target through needle-like complexes. Adapted from Bleves, et al., 2010 [15].

The type III secretion system (T3SS) is different from the T1SS and T2SS as it forms a needle-like complex, which helps in injecting effector proteins directly into target cells. This requires a certain degree of complexity and the system consists of 35 clustered genes organized into five operons. The needle-like complex delivers a set of proteins to the target cell membrane that then forms a pore, enabling delivery of effector proteins. At least four effector proteins are injected through this system,

namely ExoS, ExoT, ExoU, and ExoY. ExoS and ExoT are both ADP-ribosyltransferases, like ToxA [25], [26], but unlike ToxA, do not target protein synthesis. Their roles are not fully understood, but they seem to target host signalling pathways, specifically through ADP-ribosylation of Ras, affecting host-cell function, decreasing phagocytosis, and increasing dissemination of *P. aeruginosa* [27]–[30]. ExoY is an adenylate cyclase that impairs the ability of endothelial cell proliferation and vascular repair following lung injury [31]. ExoU is a phospholipase with broad substrate specificity causing tissue destruction and localized immunosuppression [32], [33]. Curiously, all of the four toxins are not present in most strains of *P. aeruginosa*. In fact, the toxins seem to be paired up, where ExoU and ExoT are commonly found together and likewise for ExoS and ExoT [32].

The type V secretion systems (T5SS) are the simplest of them all, consisting of either one protein with two domains, the autotransporters, or two proteins, the two partner secretion systems, where the domains are encoded separately on the genome. The proteins are transported to the outer face of the outer membrane, where they either remain, or are released through proteolytic cleavage [15], [16]. They encode a variety of toxins. EstA is an autotransporter esterase, which sits on the outer face of the outer membrane. It has been shown to be important for rhamnolipid production, which in turn affects cellular motility and biofilm formation [34]. LepA/LepB, a two partner secretion system, secretes a protease that has been suggested to modulate the host response to bacterial infection [35]. CdrA/CdrB, also a two partner secretion system, is responsible for the transport of CdrA, an adhesin, to the outer membrane, which has been found to promote biofilm formation and auto-aggregation in liquid culture [36], [37]. PlpD is a lipolytic enzyme and the function is not well characterised. However, PlpD shows

homology with the ExoU of the T3SS, suggesting immunomodulatory function [38].

The Type VI secretion system (T6SS) is encoded in three loci in the *P. aeruginosa* PAO1 genome, and is the most recently discovered of the secretion systems. Similarly to the T3SS, it injects effector proteins into competing cells. The three T6SSs (HI, HII, and HIII) have distinct evolutionary histories, are regulated by different mechanisms suggesting different functions [39], and are thought to have originated from bacteriophages. At least six effector proteins (Tse1-6) are secreted through the T6SS, and they are encoded next to their cognate immunity proteins (Tsi1-6) that give immunity to the effector proteins. Tse2 has been found to arrest the growth of both prokaryotic and eukaryotic cells lacking the immunity protein, Tsi2 [40]. Tse1 and Tse3 are injected into the periplasm and hydrolyse peptidoglycan leading to cell lysis of bacteria lacking the immunity proteins, Tsi1 and Tsi3 [41]. Tse4-6 also function as antibacterial effectors, but Tse5 and Tse6 were found to inhibit *Escherichia coli* growth even if *E. coli* also expressed the cognate immunity protein, whereas the same was not observed in *P. aeruginosa* [42], suggesting that the immunity proteins are not sufficient to provide immunity to the effector proteins.

1.3.2. Secondary metabolites

P. aeruginosa produces a number of secondary metabolites that give an advantage in the environment and affect both prokaryotic and eukaryotic cells negatively either through inhibition of growth or cell-death. Examples are given below.

Pyocyanin is one of the typical secondary metabolites produced by *P. aeruginosa* and it belongs to the class of phenazines. The genes required for the production of pyocyanin are encoded by two operons,

phzA1B1C1D1E1F1G1 and *phzA2B2C2D2E2F2G2*, and two single genes, *phzM* and *phzS*, which are encoded next to either operon. The *phzM* and *phzS* gene products are responsible for the final conversion into pyocyanin [43]. In laboratory culture, pyocyanin is easily recognisable in high concentrations as it is blue in its oxidised state, usually giving the growth medium a green-blue colour. Pyocyanin is a redox-active compound and is capable of causing intracellular oxidative stress by crossing host cell membranes and generating reactive oxygen species (ROS), superoxide ($O_2 \cdot^-$) and hydrogen peroxide (H_2O_2) [44], [45]. This can result in cellular damage, also increasing inflammation, and cell-death [46]. Furthermore, pyocyanin inhibits the growth of competing bacteria through similar mechanisms [47].

P. aeruginosa is also capable of producing another secondary metabolite, hydrogen cyanide (HCN). The genes encoding the HCN synthase are encoded in an operon, *hcnABC*. It is produced under high cell densities and decreased oxygen availability, but not anoxic conditions. Furthermore, maximum production occurs between 34 °C and 37 °C [48], suggesting that HCN is important in infection scenarios. HCN has been shown to be able to kill competing bacteria both directly [49], and indirectly, through increasing the susceptibility of other bacteria to antibiotics by inhibiting cytochrome oxidase-dependent efflux pumps [50]. Furthermore, HCN shows toxicity towards host cells as it acts as a cellular asphyxiant. CN^- ions are non-competitive inhibitors as they are able to bind to Fe^{3+} in haem, which in turn binds to cytochrome c oxidase, an important component in the respiratory chain of mitochondria, thus preventing oxygen from binding [51], [52]. Interestingly, *P. aeruginosa* can protect itself against this effect by using a cyanide-insensitive oxidase [53].

1.3.3. Iron uptake

In infection settings, most iron will be sequestered by host haem molecules, part of the host aerobic respiration, and is thus not available for uptake. For this reason, *P. aeruginosa* has multiple iron uptake systems suited for different purposes depending on the availability and the oxidation state of the iron [54], [55].

Pyoverdine and pyochelin, also secondary metabolites, are two siderophores capable of chelating Fe^{3+}. *P. aeruginosa* secretes siderophores, which are then taken up by specific receptors. The genes responsible for the production of pyoverdine are encoded by the 14 *pvd* genes [56], whereas the genes for production of pyochelin are encoded in two operons, *pchDBCA* and *pchEFGHI* [57], [58]. Pyoverdines are high-affinity siderophores and are essential for virulence in acute infection models [59]. Pyochelins have lower affinity for iron and seem to be favoured for iron acquisition unless iron limitation is severe [60]. The energy-transducing protein, TonB, is essential as it is required for the reuptake of the siderophores after binding iron by signalling for and mediating transport through other receptor proteins [61], [62].

Additionally, *P. aeruginosa* also has systems (Phu and Hap) for acquiring iron by taking up haem or haem-containing proteins [63]. The Phu system directly extracts haem using a TonB-dependent receptor, whereas the Has-system secretes a haemophore that binds to haem, and the complex is then taken up by another TonB-dependent receptor [54].

In the case of bacterial competition for iron, *P. aeruginosa* is also capable of taking up xenosiderophores, i.e. siderophores from other bacteria and fungi, through a number of TonB-dependent receptors [64].

1.3.4. Biofilm formation capabilities

P. aeruginosa is capable of forming biofilms, which are communities of bacteria embedded in extracellular polymeric substances [65], [66]. Bacteria in biofilms are resistant to antibiotics, phagocytosis, and surfactants and biofilms are difficult to remove once established [67]. *P. aeruginosa* has several systems to produce the extracellular substances composing the biofilm, such as exopolysaccharides and extracellular DNA [68]. The lifestyle of *P. aeruginosa* in biofilms is shown in Figure 2.

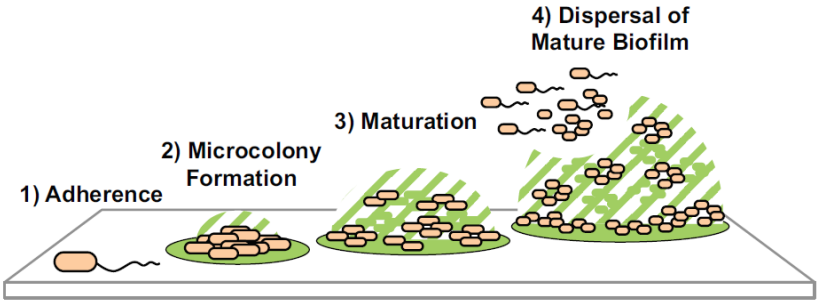

Figure 2. Developmental cycle of *P. aeruginosa* in biofilms. 1) The bacteria attach to a surface. 2) Through cell division, expression of biofilm genes, and adherence of other cells, a microcolony forms. 3) Continued growth of the biofilm. Subpopulations develop due to quorum-sensing and nutrient gradients within the biofilm. 4) Some cells become motile and disperse due to quorum-sensing, external cues, and physical disruption. The dispersing bacteria can then repeat the cycle. Adapted from Taylor, et al., 2014 [69].

The Psl system, encoded by the *psl*-operon, which contains 15 genes from *pslA* to *pslO*, is a major contributor to biofilm formation and leads to enhanced cell-surface and intercellular adhesion in *P. aeruginosa* [70].

When the Psl system is active the biofilm is rich in galactose and mannose [71]. Pel is another biofilm formation system, but its role in biofilm formation is less understood. It is encoded by a six gene operon, *pelABCDEF*, and when active a matrix rich on glucose, sensitive to cellulase, is created [72]. Extracellular DNA is also a key structural component in biofilms and helps in the formation of the characteristic mushroom shapes that are present in mature biofilms. The DNA seems to be random chromosomal DNA [73].

Alginate is another component of biofilms produced by the gene products of the *alg*-genes. The overproduction of alginate leads to the well-known mucoid phenotype, a common hallmark of chronic infections [74]. Alginate has functions in persistence, immunoevasion, and protects bacteria in the matrix from free radicals from the immune system [75].

1.3.5. RND efflux pumps

While not a *de facto* virulence factor, the intrinsic and acquired resistance of *P. aeruginosa* to many antibiotics is important for its ability to establish infections and cause disease in humans and animals as it will often resist treatment by antibiotics [76]. The PAO1 genome encodes multiple efflux pumps of the resistance-nodulation-division (RND) type (Figure 3). However, *P. aeruginosa* is also able to acquire plasmids encoding genes for resistance to antibiotics that it is not intrinsically resistant to, leading to clones resistant to virtually all clinically relevant antibiotics [77]. The four most important RND efflux pumps are MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY [78]–[80]. The pumps consist of three components; an efflux transporter in the inner membrane, an outer membrane channel, and an accessory protein

connecting the two in the periplasm [81]. RND efflux pumps often have broad substrate specificity that is not limited to antibiotics (Table 1).

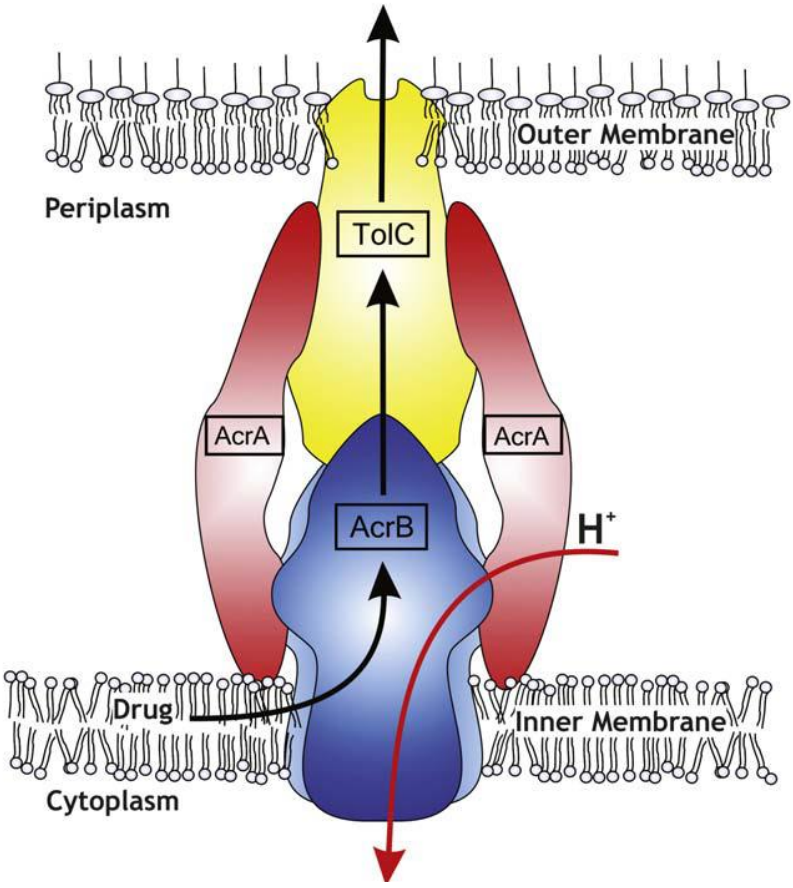

Figure 3. The structure of the AcrAB-TolC RND efflux pump in *E. coli*. It is homologous to the MexAB-OprM in *P. aeruginosa*. AcrB/MexB is inserted into the cytoplasmic membrane and is responsible for substrate recognition. AcrA/MexA is the accessory protein that connects AcrB/MexB to the outer membrane channel. TolC/OprM is the outer membrane channel [82]. Adapted from Blair

and Piddock, 2009 [83].

Table 1. The most important RND efflux pumps of *P. aeruginosa* and their antibiotic substrates. AG: aminoglycosides, BL: β-lactams, CM: chloramphenicol, CP: cephalosporins, FQ: fluoroquinolones, ML: macrolides, NB: novobiocin, TC: tetracycline, TI: tigecycline, TM: trimethoprim, ZBL: zwitterioninc β-lactams. Adapted from Li, et al., 1997 [84].

Efflux pump	**Antibiotic resistance provided**	**References**
MexAB-OprM	AG, BL, CM, ML, NB, TC, TM	[85]–[87]
MexCD-OprJ	CM, CP, FQ, TC	[88]–[90]
MexEF-OprN	CM, FQ	[91], [92]
MexXY	AG, FQ, ML, TC, TI, ZBL	[93], [94]

1.4. *P. aeruginosa* in cystic fibrosis

P. aeruginosa is the major pathogen of CF patients, leading to significant morbidity and mortality for patients by causing chronic lung infections [95].

1.4.1. Cystic fibrosis

CF is a genetically inherited recessive disorder in humans caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene leading to a faulty protein, which results in defective chloride ion transport across epithelial cell surfaces [96]. This causes dehydration of the mucous in the airways, leading to reduced or defective mucociliary clearance and thus chronic infection with bacteria and fungi despite

heavy treatment with antibiotics [97]. The chronic infections result in a state of constant inflammation, permanent remodelling of the airways and decreased lung function [98]. CF patients also suffer from poor food digestion and nutrient absorption, which is treated with pancreatic enzyme replacement therapy [99]. The end result is usually respiratory failure and lung transplantation or death. Before the development of extensive treatment programs, patients would die at a young age due to lung infections [100]. However, a newborn with CF can expect to live upwards of 50 years [101]. CF is most common in people of Northern European descent with an incidence of around 1 in 3000 [102]. In contrast, it occurs in 1 of 350000 people of Japanese descent [103].

The infections of the CF airways are caused by many different species such as *P. aeruginosa*, *Burkholderia cepacia*, *Staphylococcus aureus, Haemophilus influenzae, Achromobacter xylosoxidans, Stenotrophomonos maltophilia* and others [104] (Figure 4). Of special interest are the first three organisms mentioned, due to their high incidence in CF airway infections. At the Copenhagen Cystic Fibrosis Center in Denmark, a large number of clinical isolates of *P. aeruginosa* have been collected and stored longitudinally from CF patients, providing a detailed picture of how these strains evolve and adapt to the CF environment in both early and late stages of infection [105]–[107]. The isolates studied in this thesis are part of this collection.

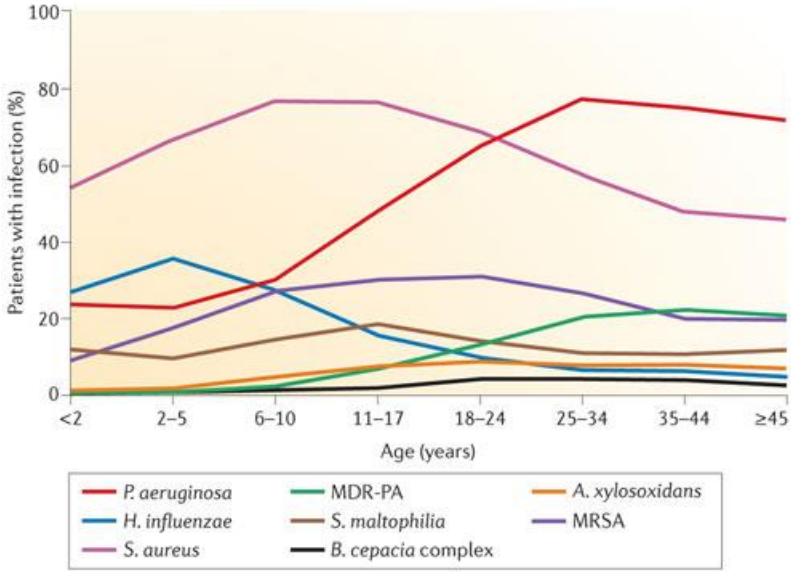

Figure 4. The prevalence of different pathogens in CF patients with the patients age. *P. aeruginosa* becomes the dominant microorganism in the mid-twenties. Adapted from Folkesson, et al., 2012 [95]

1.4.2. Evolution of *P. aeruginosa* in CF

Due to the long term infections of *P. aeruginosa* in the CF airways, the bacteria have ample time to evolve and adapt to the new environment [108]. The CF sputum is a complex medium that allows bacteria to thrive since they are not cleared by the normal mucociliary mechanism.

The selective pressures of the CF airways are not well-understood, but can be presumed to consist of changes in available nutrients, the host defence mechanisms, other microbes, antibiotics, and oxidative and nitrosative stress [108]. The CF sputum is a nutritionally rich growth medium for bacteria and supports bacterial growth to high cell densities ($>10^9$ cells/mL sputum) [109]. The advent of NGS has enabled the

detection of single-point mutations in evolving *P. aeruginosa*. Studies have shown signs of convergent evolution reviewed in Winstanley, et al., 2016 [108]. Mutations have been found in genes related to virulence (attenuation), quorum sensing, motility, iron acquisition, antibiotic resistance (increase), biofilm formation and mucoidy, metabolism (auxotrophy), and transport of small molecules. In particular, mutations are found in regulators, leading to potential large-scale phenotypic changes.

P. aeruginosa shows diversification during infection of the CF airways. However, evolution and adaptation have mostly been studied using single isolates [110]. It has been shown that different parts of the lungs can have different populations of *P. aeruginosa*, but also that the lungs are usually dominated by a single lineage [111]. A single isolate from a sputum sample will accurately represent the population of that sputum sample [110].

1.4.3. Model systems of CF

A major issue in studying the behaviour of *P. aeruginosa* and other bacteria in CF airway infections is the difficulty in recreating the conditions of the infection environment, which can have marked influence on the phenotype of the bacteria [112], [113]. Animal models have proven difficult as they have thus far not been able to accurately depict the long term infection observed in humans due to differences in the manifestation of mutations in the CFTR gene between species [114].

Different versions of media have been composed to mimic the composition of the CF sputum. Two of them are artificial sputum medium (ASM) [112] and synthetic CF sputum medium (SCFM) [113]. They are both based on detailed analyses of the available amino acids, salts, ions, and sugars available in the CF sputum. Furthermore, ASM

also contains mucin and DNA, which creates a viscous mixture to further mimic the CF sputum. Interestingly, in both media formation of microcolonies in the form of small aggregates of bacteria occurs. This is thought to be the growth mode of *P. aeruginosa* in the oxygen-limited CF airways [115].

In conclusion, the metabolic versatility of *P. aeruginosa* combined with its wide array of secretion systems, secondary metabolites, biofilm formation capabilities, iron uptake systems and innate antibiotic resistance make it a formidable opportunistic pathogen. Its ability to form biofilms, to degrade the lung tissue, to modulate the immune defence, and to outcompete other bacteria are key to its persistence and chronicity in lung infections. This leads to a fitter pathogen through evolution and adaptation. In the next section, the regulatory mechanisms of *P. aeruginosa* are explained.

2. Regulation in *Pseudomonas aeruginosa*

P. aeruginosa has one of the highest percentages of genes predicted to be involved in regulation among sequenced bacteria [4], [116] and the regulation occurs on transcriptional, translational and protein levels. Furthermore, many genes are regulated by multiple regulators resulting in an interwoven mesh of regulation. The major regulators are σ-factors (and anti-σ-factors), two component systems (TCSs), and small RNAs (sRNAs). The versatility of *P. aeruginosa* is highly dependent on it being able to respond properly to environmental cues and adapt to the given circumstances, by expressing the appropriate sets of metabolic genes and virulence factors. Regulation in *P. aeruginosa* is complex and many regulatory networks feed into each other as exemplified in Figure 5.

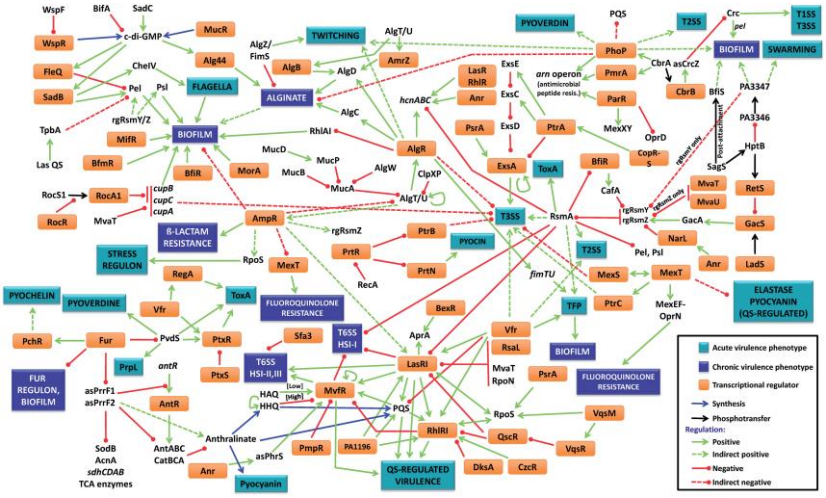

Figure 5. The interconnected regulatory network of virulence in *P. aeruginosa* containing σ-factors, TCSs, and sRNAs. Adapted from Balasubramanian, et al., 2012 [117].

2.1. σ-factors

P. aeruginosa has, as a bacterium, comparatively many σ-factors encoded in its genome with 24 putative σ-factors identified so far [118]. The σ-factors are responsible for the transcription of groups of genes that are necessary under certain circumstances, such as exponential growth (RpoD) or the stationary state (RpoS).

σ-factors function by regulating the transcription of genes by recognising their cognate promoters. The σ-factor binds to the RNA polymerase core enzyme ($\alpha_2\beta\beta'\omega$) forming the holoenzyme ($\alpha_2\beta\beta'\omega\sigma$). In this form, the σ-factor can recognise its cognate promoter sequence and bind to it. This facilitates the melting of the double-stranded DNA, enabling the initiation of transcription by forming the transcription bubble. This results in elongation of the transcript from the template strand leading to the finished messenger RNA that can then be translated into protein or processed further [119]. Defining regulons of σ-factors, i.e. what is regulated by the σ-factor, can be difficult since the effects are often widespread and feed into a host of other regulatory networks.

RpoD (σ^{70}), also called the house-keeping σ-factor, is responsible for the transcription of genes during exponential growth. It has proven difficult to study since it is essential, but a study found that the regulon contains at least 686 genes [120] in the *P. aeruginosa* reference strain PA14.

RpoS (σ^{38}) is generally known as the stationary/stress σ-factor, which comes into play during early-stationary phase, when nutrients are limited. It has a sizeable regulon of upwards of 800 genes and is heavily involved in quorum-sensing, which expands its indirect regulon. It activates the expression of genes involved in chemotaxis and TCSs and represses genes of central intermediary metabolism, chaperones, and

secreted factors. The chemotaxis genes are, in this case, linked to biofilm formation rather than motility [121]. Furthermore, the expression of RpoS is important for tolerance against antibiotics during stationary phase [122].

RpoN was originally named after its connection with nitrogen metabolism. However, it has since been discovered that it has a more versatile role. The regulon is about 600 genes in *P. aeruginosa* PA14 [120]. It is the only σ-factor that does not show homology to RpoD and it functions in a unique way as it requires activator proteins to assert its function. Activator proteins either bind to an upstream enhancer sequence, which then loops back and binds to the RpoN-RNA polymerase holoenzyme, or bind directly to the RpoN-RNA polymerase holoenzyme, enabling transcription [123].

As noted above, there are many more σ-factors in *P. aeruginosa* and a non-exhaustive list has been provided in Table 2.

Table 2. Non-exhaustive list of σ-factors in *P. aeruginosa*. Adapted from Potvin, et al., 2008 [118].

σ-factor	**Function**	**References**
RpoD	House-keeping	[120]
RpoS	Stationary/stress phase	[120], [121], [124], [125]
RpoN	Versatile	[120], [126]–[130]
RpoH	Heatshock response	[120]
RpoF	Flagellin synthesis	[120], [131]
RpoE	Alginate synthesis	[120], [132]
PvdS	Pyoverdine synthesis	[56], [120]

2.2. Two-component systems and GacSA

TCSs are the primary way bacteria sense the environment. *P. aeruginosa* has particularly many of these helping it to adapt to different environments. TCSs are composed of a histidine kinase, which detects environmental signals, and a response regulator that is phosphorylated by the cognate histidine kinase upon activation and thus activates or represses expression of genes necessary for the appropriate response. The genome of *P. aeruginosa* PAO1 encodes 64 putative response regulators and 63 putative histidine kinases [133]. This large number of TCSs allows *P. aeruginosa* to efficiently sense the environment and react accordingly.

An interesting TCS from an infection point-of-view is the GacSA TCS, since it reciprocally regulates gene-expression contributing to either a chronic or an acute infection state. The dogma of this regulatory system is that chronic genes are defined as the *pel*/*psl* biofilm formation operons and the H1-T6SS, whereas the acute infection genes are the T3SS, flagellum and Type IV pili. The GacSA TCS regulatory system is the focus of this thesis because of the occurrence of sequential mutations in clinical isolates of *P. aeruginosa* cultured from patients with CF [134].

The system features at least three other histidine kinases that regulate the system upstream of GacSA in the regulatory chain. The function of GacS, in its dimeric and autophosphorylated state, is to phosphorylate GacA, the response regulator, which then becomes active. In its active state, GacA activates the transcription of two sRNAs, RsmZ and RsmY, the only targets of GacA. These bind to and sequester RsmA, an RNA-binding protein, through characteristic GGA motifs, preventing it from binding to its target mRNAs, relieving suppression of translation and increasing mRNA turnover. The regulators of the GacSA-TCS are LadS, RetS, and PA1611. RetS is a hybrid histidine kinase that binds to GacS,

forming a heterodimer, and prevents the autophosphorylation of GacS. LadS, also a hybrid histidine kinase, has the opposite function. It functions as a phosphorelay mechanism that donates and relays a phosphoryl group to GacS, activating the TCS. Expanding on this is PA1611, another hybrid histidine kinase. Its function is similar to GacS and interacts with RetS, thus preventing RetS from inhibiting GacS (Figure 6). Common for all the histidine kinases in this multicomponent system is that the signals leading to their activation are unknown [134]–[139]. Additionally, this system seems to be a hotspot for mutations in CF airway infections with *P. aeruginosa* [106], [108], [140]. This could indicate that the activating signals are not present in the CF airways or that mutations are a more efficient way of activating/deactivating the signalling cascade. Futhermore, the mutations suggest that this system is important for the adaptation of *P. aeruginosa* to the CF airways.

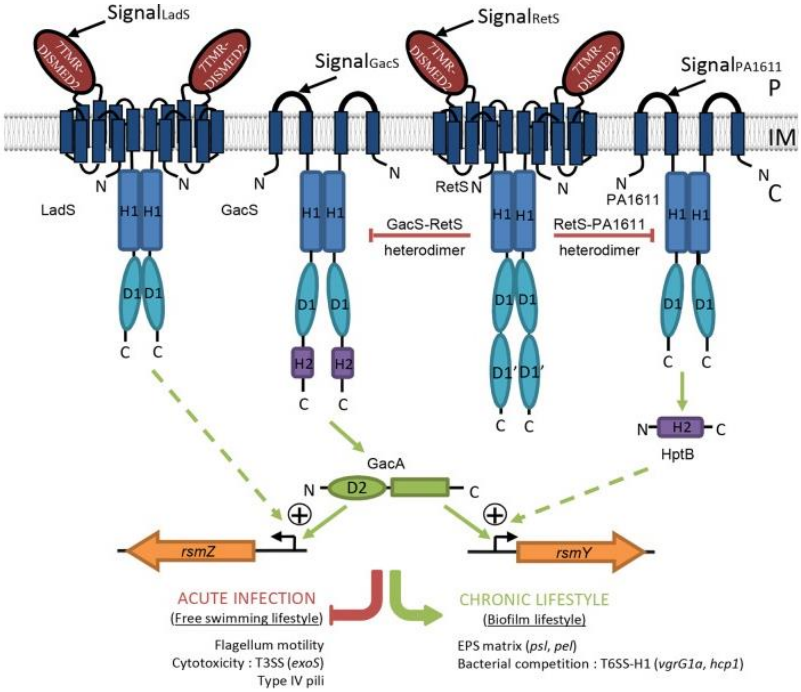

Figure 6. The GacSA TCS and the three histidine kinases (LadS, RetS, PA1611) that modulate the system. Activation of GacS leads to phosphorylation of GacA, which in turn activates transcription of the two sRNAs, RsmZ and RsmY. This leads to expression of chronic lifestyle genes. Adapted from Chambonnier, et al., 2016 [135].

2.3. sRNAs

Small RNAs (sRNAs) add another layer of regulation to bacteria. As the name suggests, they are small RNAs (50-500 bp) that (usually) do not code for a protein. Instead, they act by binding to mRNA or proteins and modulating their function. Two major classes of sRNAs have been defined, *cis*-encoded sRNAs and *trans*-encoded sRNAs. *Cis*-encoded sRNAs are encoded antisense to their targets and thus share extensive

complementarity with their corresponding transcript. *Trans*-encoded sRNAs are not encoded antisense to their targets and have limited complementarity with their targets. These *trans*-encoded sRNAs often require a RNA-chaperone, such as Hfq, to help with base-pairing and asserting their function and can have many different targets, which expands their regulatory function. As noted above in the GacSA TCS, some sRNAs have a different mode of action, where they bind to a protein and sequester it, preventing the binding of mRNAs to the protein [141]–[144]. However, most sRNAs function by either increasing or decreasing translation by binding to mRNAs (Figure 7).

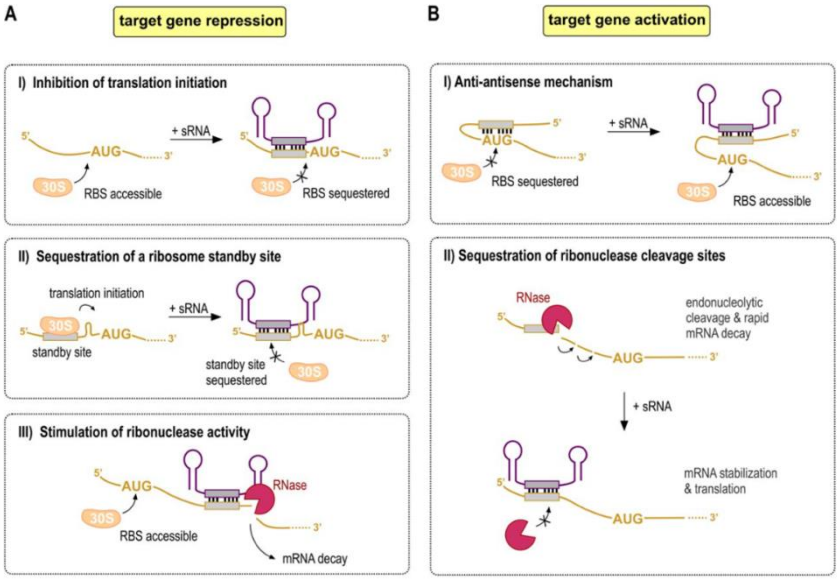

Figure 7. Regulatory mechanisms of sRNAs. A) Target gene repression through (I) inhibition of translation initiation, (II) sequestration of a ribosome standby site, or (III) stimulation of ribonuclease activity increasing mRNA decay. B) Target gene activation through (I) releasing a sequestered ribosome binding site by binding elsewhere on

the mRNA, or (II) sequestration of ribonuclease cleavage sites to decrease mRNA decay thus increasing translation. Adapted from Fröhlich, et al., 2016, [145].

The advent of RNA sequencing (RNA-seq) has facilitated the discovery of sRNAs, and more than 500 sRNAs have been identified in *P. aeruginosa* PAO1 [146], Additionally, 223 intergenic sRNAs were reported in *P. aeruginosa* PA14 [147], and another comparative study identified a 126 sRNA-overlap between PAO1 and PA14 [148]. This suggests that they are integral to regulation, but also that they are involved in strain specific regulation that could have important consequences for the virulence of a specific strain. However, few of them have been characterised. The ones that have been characterised have been shown to have diverse functions and some are involved in the expression of virulence factors. A few examples follow here.

PrrF1 and PrrF2 are two sRNAs involved in iron homeostasis in *P. aeruginosa*. They are >95% identical and are induced under iron limitation. They are encoded in tandem and seem to function in a redundant manner. Curiously, another transcript and sRNA, PrrH, spans both of these. Deletion of the PrrF-locus encoding the sRNAs, leads to loss of virulence in a murine model of acute lung infection [149]–[151].

PhrS is a sRNA that is expressed by ANR, the anaerobic transcriptional regulator, when oxygen is limited. It increases the translation of PqsR, through a structural rearrangement of the mRNA containing the open reading frame of PqsR, which leaves the ribosomal binding site open for binding by the ribosome. PqsR is a positive regulator of the *Pseudomonas* quinolone signal operon, an intercellular signal molecule, and the phenazine biosynthesis operons [152].

CrcZ is a small RNA, functioning much like RsmZ and RsmY. However, it requires the RNA-chaperone, Hfq, to exert its function. The target of CrcZ is the Hfq-Crc complex, which is involved in carbon catabolite repression. The Hfq-Crc complex exerts its regulatory function by binding to mRNA transcripts and preventing their translation. CrcZ thus relieves the post-transcriptional regulation of the Hfq-Crc complex [153]. CrcZ expression is regulated by a TCS, CbrAB, where CbrA is the histidine kinase and CbrB is the response regulator. CbrB is predicted to have a RpoN-binding domain and thus functions as an activator protein [12], [153]–[155]. The carbon catabolite repression mechanism is a clear example of the complexity of regulatory networks and how they feed into each other and cause a cascade of regulatory changes.

In this section, the regulatory systems of *P. aeruginosa* were explained with special focus on the GacSA TCS. It is apparent that regulation in *P. aeruginosa* is a complex matter and that mutations in multiple regulators can lead to results that are not immediately apparent from the single mutations. In the next section, the sequence of events leading to the conception of this PhD-thesis is described.

3. Rationale of the present study

The advent of NGS has enabled massive genome sequencing of clinical isolates of *P. aeruginosa* and the dawn of a new era in the elucidation of how bacteria evolve during long-term infections [105], [106], [156]. This could lead to the development of new treatment strategies and personalised medicine. However, most studies on the evolution of *P. aeruginosa* focus on genomics and phenotypes, and few studies examine the transcriptomic evolution of longitudinal isolates.

A study by Yang, et al., 2011 [157] used genome sequencing, microarray transcriptomic profiling (Affymetrix), and Biolog phenotypic profiling to assess the evolution of longitudinal isolates of the dominant Copenhagen clone types, the DK2 lineage, collected over a period of 40 years. In total, 12 clinical isolates from six patients were examined. They showed that the early and late stages of evolution have different characteristics. The initial stage of evolution was shown to be based on positive selection as measured by the ratio of non-synonymous mutation to synonymous mutations (dN/dS) being more than 1. This tells us that the majority of mutations likely result in altered function of proteins during the first six years of evolution improving the fitness of *P. aeruginosa* in its new environment. However, later in the infection the pattern is the opposite and instead the dN/dS ratio drops below 1, suggesting after the initial stages of infection, adaptation slows down since mutations can no longer improve fitness to the same extent. This was supported by the use of transcriptomic and phenotypic profiling. The majority of changes in the transcriptome and metabolism occurred in the first six years, again suggesting that the initial period is where the major adaptation occurs. Furthermore, they found that mutations in *mucA* (anti-sigma factor of AlgU, alginate biosynthesis), *lasR* (quorum-sensing transcriptional regulator), and *rpoN* accounted for half the differential

gene expression in the first six years, showing the role of mutations in regulatory genes in rapid adaptation.

Closer inspections of mutations in DK2 isolates revealed elaborate rewiring of regulatory networks by Damkiær, et al., 2013 [158]. Here, the wild-type alleles were replaced with the evolved alleles of *mucA*^{DK2} (frame-shift), *algT*^{DK2} (substitution), *rpoN*^{DK2} (substitution), *lasR*^{DK2} (deletion), and *rpoD* ^{DK2} (in-frame deletion) in *P. aeruginosa* PAO1 in a sequential manner and the effects examined. Through a combination of phenotypic assays and gene-expression profiling (microarray), it was discovered that the combination of mutations produced effects that are not obvious from the individual mutations (i.e. epistasis). Four mutations (not including *rpoD*^{DK2}) accounted for 40% of the differential gene expression comparing the clinical isolate and the PAO1 with allelic replacements to PAO1. Hereafter, mutants of PAO1 (*mucA*^{DK2}+*algT* ^{DK2}, *rpoN*^{DK2}, and *lasR*^{DK2}) were constructed and compared their expression patterns to PAO1 and the PAO1 Q-mutant, containing *mucA*^{DK2}+*algT* ^{DK2}, *rpoN*^{DK2}, and *lasR*^{DK2}, showing that while the mutations in some cases act directly or additively on the differential expression of genes, there were also many genes that were only upregulated through the epistasis of all four mutations. Indeed, the combination of all four mutations in the PAO1 Q-mutant led to a significantly increased resistance to ceftazidime and tobramycin, while other combinations of mutations did not. This demonstrates that mutations in regulatory networks can interact in non-obvious manners and result in effects that are only apparent when the mutations can interact with each other. It also serves as a warning of a gene-centric view of evolution and shows that the sum of mutations is greater than the parts. However, the DK2 lineage seems to be a special case as it was highly transmissible and evolved in a

different manner than what is observed in younger patients as described below.

These studies prompted the investigation of clinical *P. aeruginosa* isolates from early CF airway infections as the majority of changes happen in the early stages, leading to the study of Marvig, et al., 2015 [106]. Here, 474 clinical *P. aeruginosa* isolates from early CF airway infections in 34 patients were whole genome sequenced in order to elucidate the evolutionary trajectories and determine if they follow the same pattern, i.e. convergent evolution. 36 lineages of *P. aeruginosa* were discovered as defined by >10,000 single nucleotide polymorphisms between lineages. The study shows that there are 52 genes that are hit more often by mutations than would be expected by chance, called pathoadaptive mutations. Ten of these are predicted to be transcriptional regulators. Another discovery was that mutation in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway and the *mucA/algU* system occur only in a specific order. Mutations would always appear in *retS* before the downstream genes of *gacS*, *gacA*, and *rsmA*. The first mutation in *retS* leads to a chronic infection mode, whereas the second mutation leads to an acute infection mode. In the case of *mucA*/*algU*, there were no cases of *algU* mutating before *mucA*.

The work in this thesis stems from the 474 clinical isolates. The discovery that mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway occur only in a specific order was interesting as it is a system determining the expression of specific virulence factors. We decided to look into the effects of these mutations in two lineages. First by examining the evolutionary trajectories of seven clinical isolates with all the residual evolution included, i.e. mutations in other genes. Secondly, by examining the specific effects of the mutation by replacing the alleles in the non-evolved strains with the mutations in *retS* and *gacS* of the

evolved strains. Furthermore, we also replaced the mutated versions of *retS* and *gacS* in the evolved-strains with the functioning versions from the non-evolved strains, which would show the effects of all the mutations that are not in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway. The current dogma of this system is that it reciprocally regulates chronic and acute infection [134]–[139] modes, but this may not be an accurate nomenclature, as the sequential nature of the mutations are the reverse of what would be expected if this were true.

4. Concluding remarks and perspectives

In this thesis, we show that the initial stages of evolution and adaptation of two lineages of *P. aeruginosa* to the CF airways are complex and characterised by the differential and temporal expression of a multitude of virulence factors. This suggests that the evolution of the initial stages of infection is the result of a changing environment, where the expression of virulence factors is tailored to what is needed at a certain point of time due to external circumstances, such as competing microbes, the host defence, and available nutrients.

In the first paper (in preparation), we investigate two lineages of *P. aeruginosa* isolates from two patients with CF, harbouring their own lineage. Using transcriptomics and proteomics, we investigate how the transcriptome and proteome change over a period of four years of adaptation to the CF airways. The lineages have sequential mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway, which shifts the expression of virulence factors. We show that the mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway are a major source of variation in both the transcriptome and proteome. This leads to differential expression of virulence factors in a reciprocal manner. Additionally, we show that we can correlate mutations in other regulators to their respective transcripts and proteins in complex mutational settings. The reciprocal expression of virulence factors suggests that the selective pressure of the CF airways changes over time and that the expression of virulence factors is tailored to this. The significance of from where the strains were isolated from still needs to be examined, as the DK17 strains were primarily isolated from the sinuses and the DK41 strains were isolated from the lower airways, which could have a role in the evolutionary trajectories.

In the second paper (in preparation), we examine the same strains, but replace the mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway with their wildtype versions. This leads to the discovery that the residual mutations not occurring in this signalling pathway also influence the expression of the virulence factors. This shows that inferring a specific evolutionary trajectory in complex mutational settings does not show the full picture of evolution. Some mutations will hide the effects of other mutations on a transcriptome level, underlining the complex regulatory network of *P. aeruginosa*. This paper still needs a significant amount of work, in particular in examining how similar mutations in two genetic backgrounds behave differently, and a deeper analysis of the proposed differential expression of *rpoD* and *rpoS* is needed.

These results challenge the idea of the *retS-gacS-gacA-rsmA-rsmYZ* being a regulator of chronic and acute infection modes in *P. aeruginosa*. Indeed, if the nomenclature of this system was apt, it would be expected that mutations in *retS* would be predominant in *P. aeruginosa* in the CF airways. However, the effects of the *retS*-mutations are reversed by a later mutation in either *gacS* or *gacA* that should lead to an acute infection mode, the reverse of the dogma in infections of the CF airways. Instead, it seems that the initial *retS*-mutation facilitates the establishment of the infection, whereas the sequential *gacS*/*gacA* mutations could be a response to environmental changes, due to airway remodeling, host response, or changes in the microbiome.

The work in this thesis expands the knowledge on the early stages of *P. aeruginosa* evolution and adaptation in CF infections and shows that a gene-centric view on a genomic level is not enough to accurately describe the evolutionary trajectories, showing the need for more research on the early evolution and adaptation of *P. aeruginosa* in CF airways on a transcriptomic level to properly understand the mechanisms

of adaptation. Additionally, there is an urgent need for model systems that possess the selective pressures found in the CF airways. The behaviour of *P. aeruginosa* in the CF airways needs to be accurately determined for example by *in vivo* transcriptomics. This could give some hints to what the bacteria are experiencing, enabling the simulation of the infection setting. This should be compared with the available model systems to determine how well these systems model the CF airways. Furthermore, the knowledge gained from this would facilitate the development of new model systems that would enable a more exact replication of the stressful environment of the CF airways. It is highly likely that we are missing key aspects of the behavioural mechanisms due to the inadequacy of modelling the CF airways.

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Papers

Manuscript 1

The evolutionary trajectories of *Pseudomonas aeruginosa* isolates from cystic fibrosis airways show temporal expression of virulence genes and lineage specific trends

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The evolutionary trajectories of *Pseudomonas aeruginosa* isolates from cystic fibrosis airways show temporal expression of virulence genes and lineage specific trends

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Summary

Pseudomonas aeruginosa causes long-term infection in the airways of patients with cystic fibrosis (CF) leading to significant morbidity and mortality. During this time, mutations will occur in *P. aeruginosa* as it adapts and evolves to the environment of the CF airways. The evolution is characterized by loss of virulence factors, increased antibiotic resistance and increased biofilm formation or mucoidy. Here, the evolutionary trajectories of two lineages (DK17 and DK41) of *P. aeruginosa* isolated from two young CF patients are investigated at the transcriptomic and proteomic levels. Seven strains were isolated over a period of approximately four years and both lineages have sequential mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway, a key regulatory system for the expression of virulence factors. The data show that the mutations in this system are the major cause of variation in the transcriptomes and proteomes, but that the lineages are also a significant source of variation. Furthermore, the mutations lead to reciprocal expression of the type III and the type VI secretion systems, suggesting that *P. aeruginosa* needs to express different virulence factors at different times during the early stages of infection in response to selection pressures. Additionally, one lineage seems to adapt to microoxic conditions as there is an increased expression of denitrification genes with respect to time of isolation of the isolate. Both lineages also acquire mutations in regulators of resistance-nodulation-division (RND) efflux-pumps, which leads to increased expression of multiple efflux pumps. Furthermore, in line with the similar mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway the transcriptomes and proteomes converge through time, suggesting that the lineages are on similar evolutionary trajectories.

Introduction

Pseudomonas aeruginosa is a major pathogen of cystic fibrosis (CF) infections and leads to significant morbidity and mortality in patients. The majority of cystic fibrosis patients will acquire a *P. aeruginosa* infection at some point in life [1]. These infections may persist for decades giving the bacteria ample time to adapt and evolve to their new niche by accumulation of mutations. Especially the first years of infection are important for the adaptation, when large scale phenotypic changes occur [2]. In line with this, mutations will often occur in regulatory genes and perturb regulatory networks, which can lead to massive phenotypic changes facilitating quick adaptation to the CF airways [3]. The usual evolutionary trajectories concern the loss of virulence factors, increased antibiotic resistance, and increased biofilm formation or mucoidy caused by overproduction of alginate [2], [4], [5]. However, little is known about the selective pressures and the reasons why *P. aeruginosa* evolves in the way it does. Upon entering the body, the bacteria meet the host immune system and a new environment with new energy and carbon sources available. Furthermore, in the attempt to get rid of or curb the infection, patients are treated with numerous antibiotics, which also represent a strong selective pressure for *P. aeruginosa* [6].

The *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway plays a key regulatory role in the reciprocal switching between acute and chronic infection modes. The pathway works through RetS, a hybrid sensor kinase, which inhibits autophosphorylation of GacS, a histidine kinase. GacS, in its active phosphorylated and dimeric state, activates GacA, the cognate response regulator of GacS, which activates transcription of two small RNAs, RsmZ and RsmY. These RNAs sequester RsmA and prevent it from binding to its target mRNAs, thereby relieving the repression of translation [7], [8]. However, RsmA is also capable of promoting the expression of genes [9]. Under acute infection conditions, where RsmA is not sequestered by RsmZ and RsmY, there is expression of the type III secretion system (T3SS), type IV pili, type II secretion, *toxA*, and *lipA* [10]. However, chronic infection conditions (where RsmA is sequestered) are characterized by expression of the *pel* and *psl* operons promoting biofilm formation and the type VI secretion system (T6SS). The RsmA-regulon contains upwards of 500 genes with regulation occurring in both ways either directly at the level of translation or indirectly through regulation of regulatory factors [11].

Here, we investigate the evolution of two lineages of *P. aeruginosa* from two young CF patients comprising a total of seven clinical isolates. Using both transcriptomic and proteomic approaches, we attempt to elucidate the evolutionary trajectories of these lineages that harbor not only sequential mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway, but are also mutated in other genes.

Results

Sequential mutations in the retS-gacS-gacA-rsmA-rsmYZ *signaling pathway*

In a previous study [5], 11 cases of nonsynonymous mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway were recorded. The mutations appeared in a sequential manner, where *retS* always mutated before *gacS*, *gacA*, or *rsmA*, strongly suggesting selection for this sequential mutational pattern (Figure 1).

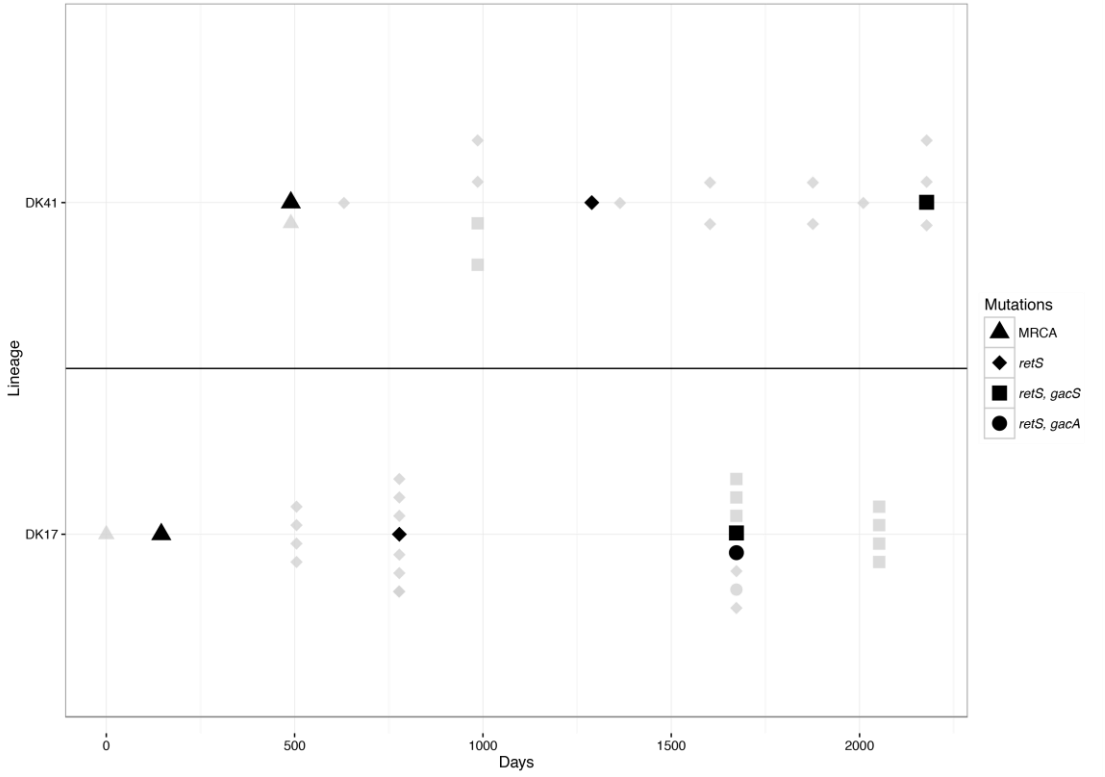


Figure 1. A timeline of the sequence of isolation of strains from the patients. The black symbols represent the isolates used in this study, whereas the light grey were also isolated from the patients, but not used in the study. The mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway are symbolized by the symbol. The

symbols have been scattered along the y-axis for clarity as samples were taken at the same time.

The strains were isolated from two patients (female, born in 1996/male, born in 2001) over approximately a four-year period and belong to two different lineages of *P. aeruginosa*, DK17 and DK41, with each patient being colonized exclusively with one lineage (Table S1). The mutations in *gacS, gacA, and retS* all occur in the first half of the genes and are either indels causing frameshifts or, in one case, a SNP causing a stop codon, suggesting that in every case all gene function is abolished (Figure 2). However, other mutations also occur (Table S2). The first isolate of either lineage will be referred to as the most recent common ancestor (MRCA).

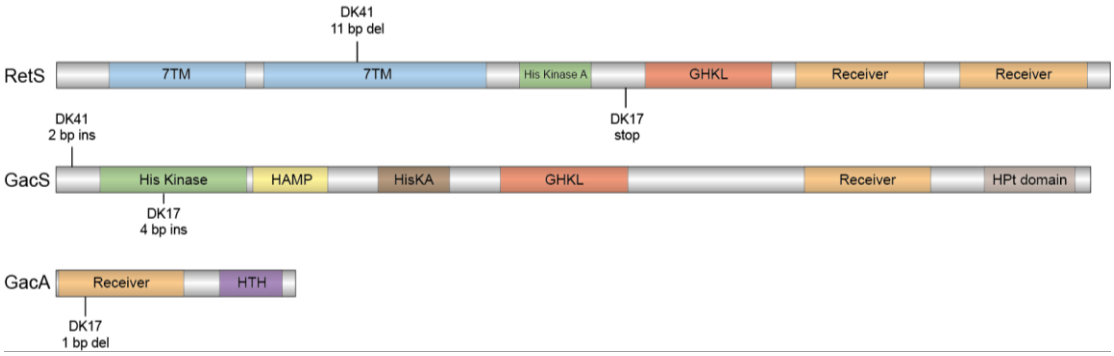


Figure 2. A map showing the proteins with domains, as predicted by pfam [41], of RetS, GacS, and GacA. The location and type of the mutations in the *retS*, *gacS*, and *gacA* genes are shown for both clone types. 7TM = seven-transmembrane domain, His Kinase A/HisKA = histidine kinase phospho-acceptor domain, GHKL = histidine kinase-, DNA gyrase B-, and HSP90-like ATPase domain, Receiver = response regulator receiver domain, His Kinase = uncharacterized signal transduction histidine kinase domain, HAMP = histidine kinase, adenyl cyclase, methyl-accepting proteins, and phosphatase linker domain, HPt domain = histidine-containing phosphotransfer domain, HTH = LuxR-type DNA-binding helix-turn-helix domain.

Proteomics and RNA-seq

The samples taken for proteomic and transcriptomic analysis were harvested from the same cultures at the same time grown at 37 °C in LB medium in late exponential phase. The protein extraction protocol favored cytosolic proteins, which were quantified through LC/IMS^{E} and mapped to *P. aeruginosa* PAO1. In total, 1351 proteins were identified in the DK17 lineage and 1273 in DK41 lineage. The majority

of the proteins are predicted to be cytosolic proteins and are thus overrepresented in the data compared to the database used, as expected (Table 1). The overlap between the two proteomes of the lineages was 1166 proteins. Tables showing all proteins quantified are given as supplemental tables (Tables S4A and S4B). RNA was harvested and converted into cDNA and sequenced on MiSeq and mapped to the genome of *P. aeruginosa* PAO1 with an average of 3.53 million mapped reads (Table S3).

Table 1. Number of detected proteins of the DK17 and DK41 lineages and their localization as predicted by PSORTb [43]–[45].

Localization	**DK17**	**DK41**	**Database (PAO1)**
Cytoplasmic	964 (71.3%)	903 (70.9%)	2591 (46.6%)
Cytoplasmic membrane	88 (6.5%)	89 (7.0%)	1273 (22.9%)
Periplasmic	59 (4.4%)	53 (4.2%)	170 (3.1%)
Outer membrane	24 (1.8%)	27 (2.1%)	172 (3.1%)
Extracellular	13 (1.0%)	14 (1.1%)	69 (1.2%)
Unknown	203 (15.0%)	187 (14.7%)	1285 (23.1%)
Total	1351 (100.0%)	1273 (100%)	5560 (100%)

Regulatory mutations are a major source of variation

With the aim of determining the evolutionary trajectories of two lineages of *P. aeruginosa* from early cystic fibrosis airway infection, and in order to investigate the major sources of variation in the transcriptomes and proteomes, principal component analysis (PCA) was used. The PCA biplot shows the two major factors/components causing variability and interestingly for the transcriptomic data (Figure 3), the first component is represented by mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway, where a negative value corresponds to a mutation in *retS* and a positive value corresponds to a mutation in both *retS* and *gacS/gacA*.

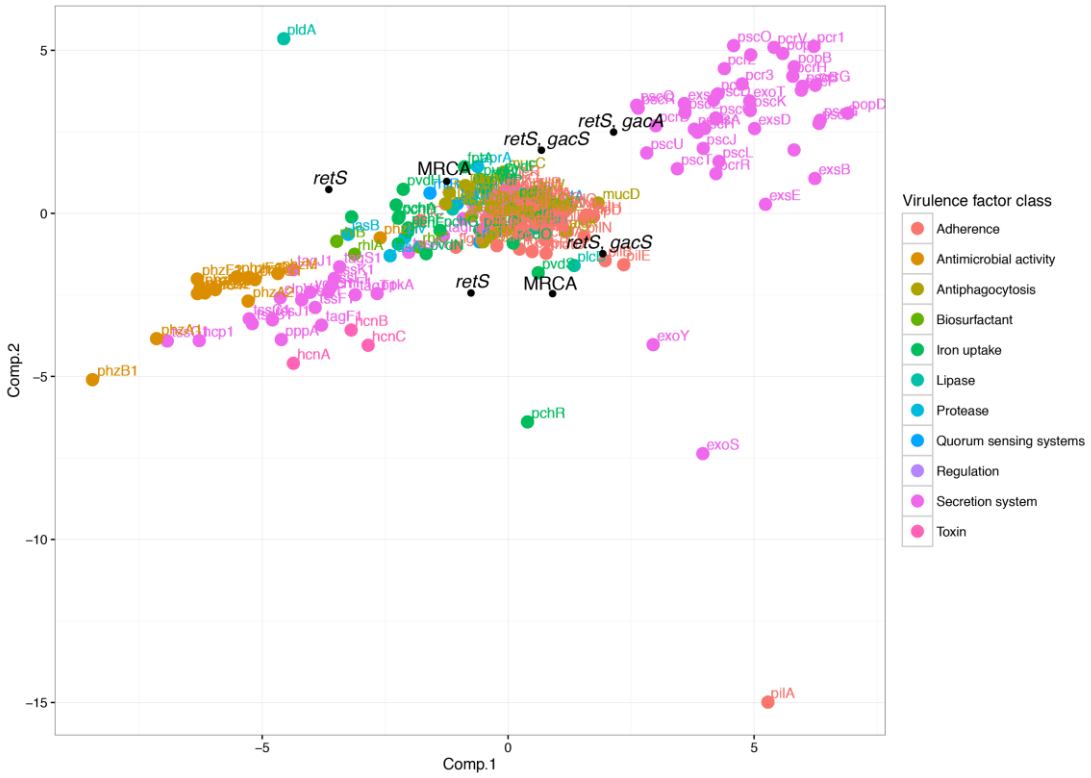


Figure 3. A PCA-biplot of the transcriptome data showing only the virulence factors as obtained from [42]. Component 1 describes 34.3 % of the total variation whereas component 2 describes 29.9 %. Colors describe the virulence factor class. The loadings of the PCA are shown in black and have been amplified for clarity and are thus not to scale.

This indicates that the majority of the transcriptomic changes is indeed caused by mutations in *retS/gacS/gacA* and confirms the reciprocal nature of the regulation. Additionally, the second component (29.9%) divided the clone types with a positive value representing DK17 and a negative value representing DK41 showing that the lineage or genetic background of a strain has a large influence on the transcriptome. Figure 3 clearly shows that the expression of certain virulence factors is associated with mutations in *retS/gacS/gacA*. Indeed, the TT6S, phenazine biosynthesis genes (*phz1/phz2*), the hydrogen cyanide biosynthesis cluster (*hcnABC*), rhamnolipid biosynthesis (*rhlAB*), protease (*lasA*), and elastase (*lasB*) are all associated with mutations in *retS*. Conversely, mutations in *gacS/gacA* are associated with higher expression of the T3SS and lower expression of the aforementioned. Component 3 (11.5%) (data not shown) shows no major trends for DK17. However, for DK41 the strains are distributed along the component with respect to the time of when the

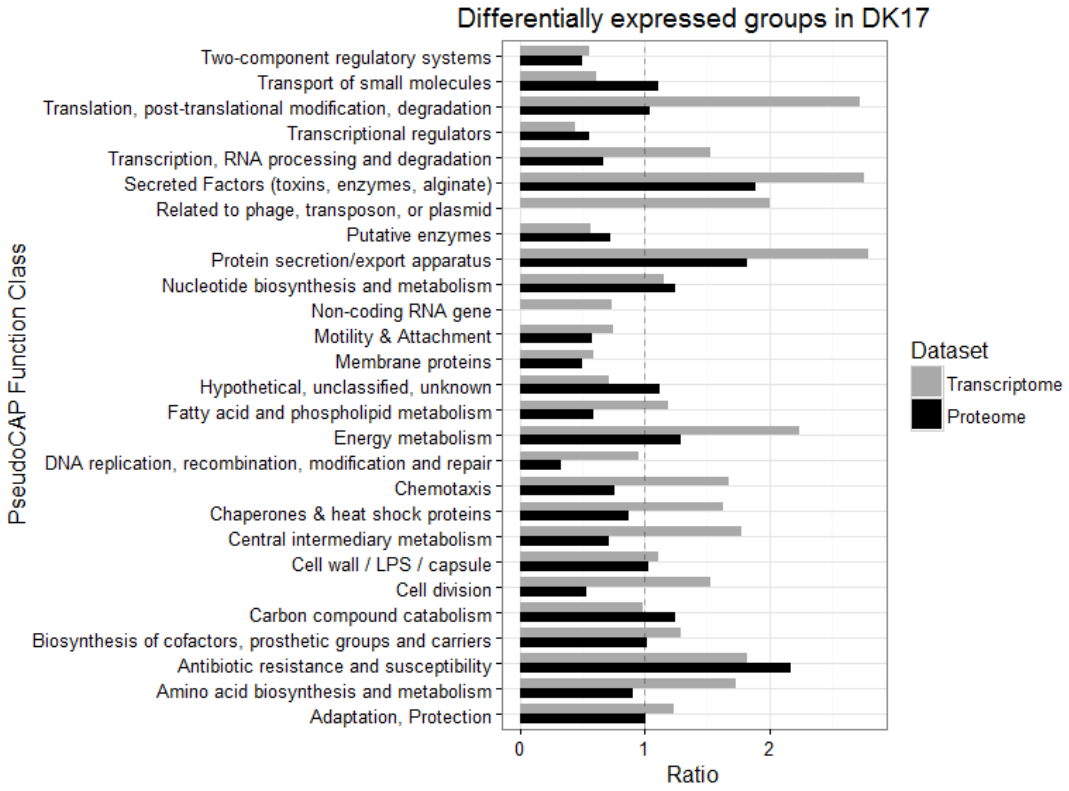
strains were isolated. Interestingly, the later strains are associated with expression of *nir*/*nor*/*nos* genes that are all part of the denitrification pathway.

A similar tendency is seen in the proteomics dataset (Figure S1). Here, the first component (39.1%) seems to describe the lineage specific differences and the second component (33.4%) describes the mutations in *retS/gacS/gacA*. The picture is not as clear as for the transcriptomes. The difference is likely due to the proteomes representing a subset of all proteins, whereas the transcriptomes describes the full mRNA population. The third component is, however, not linked to any denitrification genes. A likely explanation is that only one of the denitrification genes is identified in the dataset (*nosZ*).

Secretion systems and antibiotic resistance genes are differentially expressed

To determine what PseudoCAP groups of genes and proteins that were differentially expressed/abundant in all strains across the lineages, we used ANOVA ($p < 0.05$) on both the transcriptomics and proteomic datasets with respect to lineages and found that in DK17 680 genes were differentially expressed in the transcriptome and 543 proteins were differentially abundant in the proteome with an overlap of 176 genes. In DK41, 146 genes were differentially expressed in the transcriptome and 319 in the proteome with an overlap of only 36 genes. The large difference in differentially expressed genes between lineages can be partially explained by DK17 having four strains and DK41 having three strains.

By comparing the number of differentially expressed genes in a PseudoCAP function class [12] with the total number of genes in the genome of a PseudoCAP function class compared to the overall number of genes in the entire genome, we can by using a binomial distribution determine whether some function classes are overrepresented in the total number of differentially expressed genes. Doing this for both lineages, we see that both the transcriptomic and proteomic datasets agree that some groups are overrepresented (Figure 4).



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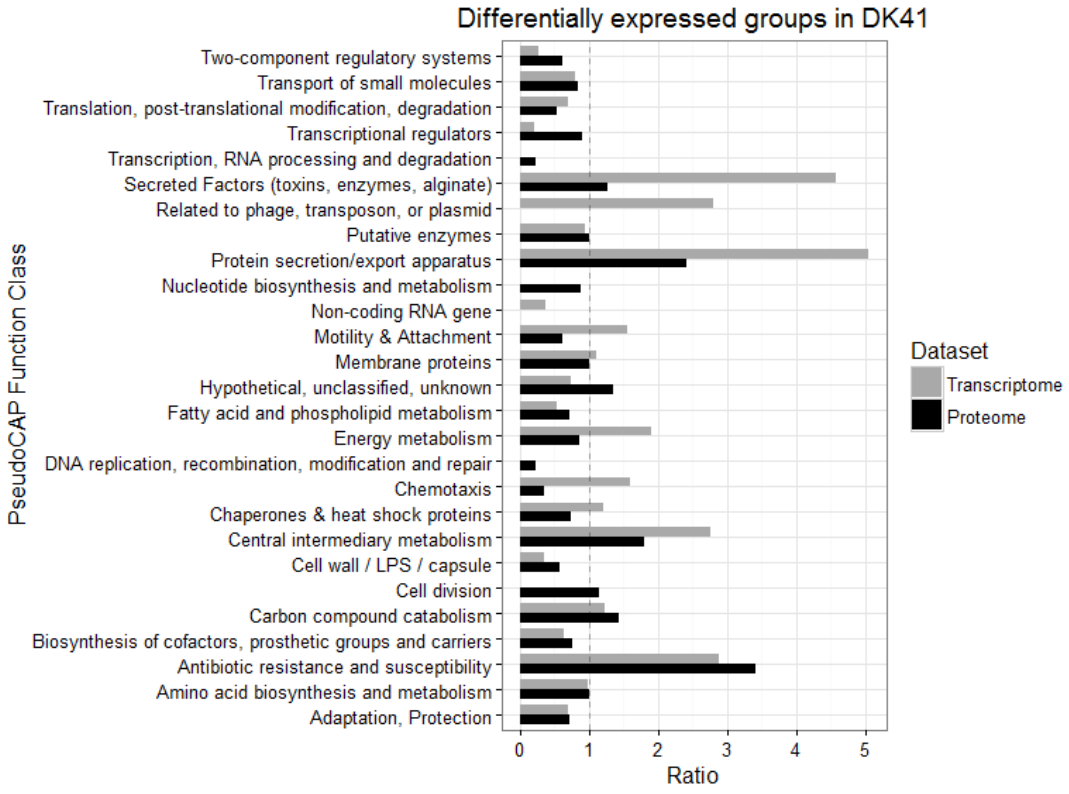

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179 Figure 4A + B. Differential expression of PseudoCAP groups for transcriptome and
180 proteome. The ratios show whether genes are more or less differentially expressed
181 than would be expected if all genes had equal chance of being differentially

expressed. A ratio of more than one means that this group is more differentially expressed than would be expected if differential expression were completely random.

In line with the results from the principal component analysis, the groups called "Protein secretion/export apparatus", "Secreted factors", and "Antibiotic resistance and susceptibility" are overrepresented among differentially expressed genes for both lineages. The first group contains 142 genes that are part of the type I secretion system, type II secretion system, T3SS, and T6SS. Of these, 52 in DK17 and 21 in DK41 are differentially expressed in the transcriptomes. For both lineages, the genes are primarily parts of the Type 3 and Type 6 secretion systems. In DK17 and DK41, the T6SS system shows higher expression (2-fold and 4-fold, respectively) in the isolates with the *retS*-mutation and lower expression (8-fold and 64-fold, respectively) in the isolates that also contain either the *gacS* or *gacA* mutation compared to the *retS*-mutants. For the T3SS, there is virtually no expression in the *retS* mutants of either lineage. However, in the *gacS* mutants, the expression is increased 8-fold and 16-fold in the *gacA* mutant as compared to their respective MRCA. In line with the reciprocal nature of the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway the T3SS is expressed in the opposite manner of the T6SS, meaning that it is downregulated in the *retS*-mutant and upregulated in the *gacS*/*gacA* mutants. In the proteomics data, expression of the T6SS was only observed in the *retS* mutants with the exception of few proteins being quantified in the MRCAs, likely due to their membrane-associated nature.

Interestingly, the mutation in *gacA*, the response regulator, has a larger effect on expression than the *gacS*-mutation, which can also be seen in Figure 3 as the loading for the *retS-gacA* mutant is placed further on the first component than the *retS-gacS* mutant. This can be explained by the fact that a mutation in a response regulator could completely abolish the function of it, whereas some residual function may still be present even if its cognate histidine kinase is not functional or perhaps through crosstalk with other histidine kinases.

The "Secreted factors" group contains 97 genes encoding the products that are secreted and the enzymes necessary for the production of secreted compounds. These include effector proteins of the T3SS and T6SS, but also phenazine biosynthesis genes. The *phz1* and *phz2* operons are 4-fold upregulated in the DK17 *retS* mutant compared to the first isolate. Interestingly, a similar pattern is not observed between the DK41 *retS* mutant and the DK41 MRCA. However, for both DK17 and DK41, the

retS and gacS isolates, show 32- and 8-fold decreases in expression for the *phz1* and *phz2* operons, respectively, compared to the *retS* isolates. The *retS, gacA* isolate of DK17 shows virtually no expression of either the *phz1* or *phz2* operons. The *phz1* and *phz2* operons were only detected in the *retS* mutants in the proteomic data in line with these isolates showing the highest mRNA expression in the same isolates.

Lineage specific trends

Some genes are either only expressed or show a much higher expression in one lineage. Examples include *pilA, exoS, exoY,* and *pchR* that are only expressed in DK41 and *pldA* in DK17 as seen in Figure 3. This is caused by the differences in genetic content as the aforementioned genes are not present in DK17.

As noted above, the expression of denitrification genes is higher in later isolates of DK41 while DK17 has a more erratic pattern. In DK17, the *retS* mutant and the *retS-gacA* mutant show low expression while the MRCA and the *retS-gacS* show a high expression. Moreover, not all genes in the denitrification pathway show higher expression over time. The genes responsible for the first conversion step of nitrate to nitrate are not upregulated, but the remaining steps containing the conversion of nitrite → nitric oxide → nitrous oxide → N_2 (*nir*/*nor*/*nos*) [13] are, suggesting a very specific evolutionary pressure. Only one protein in the denitrification pathway was identified in the proteome data, namely NosZ, which is responsible for the conversion of nitrous oxide into molecular nitrogen. It was only detected in isolates R, M, and 364, which correlates with high mRNA expression. The first isolate in DK17 shows a higher expression of the previously mentioned denitrification genes than the first isolate of DK41. Indeed, it is not until the last isolate of DK41 (~4 years from first isolate) that DK41 has similar expression levels of denitrification genes as the first isolate of DK17. This can explain why differential expression of the denitrification pathway genes is not seen in DK17. Simply put, the level of expression was already high enough from the outset.

Furthermore, in both lineages there are trends of increasing expression concerning multidrug efflux systems of the resistance-nodulation-division family. In DK17, the mRNA levels of *mexXY*[14] and *mexCD-oprJ*[15] increase with the later isolates, where the *retS,* and *retS, gacS,* and *retS, gacA* isolates have up to 16-fold higher expression than the MRCA. None of the respective proteins were detected, likely due to these being membrane-bound proteins that are not favored with the protein

extraction protocol. The expression of the *mexXY*-genes is controlled by the repressor, MexZ [16], which incidentally has a missense mutation in the DNA-binding HTH domain in the later isolates with higher expression of *mexXY*. This strongly suggests that this mutation affects the repression abilities of MexZ. In line with this, a 14-bp deletion in *mexR* is identified in the *retS, gacA*-mutant and is not present in isolates MRCA, *retS*, and *retS, gacS*. *mexR* is a repressor of the *mexAB-oprM* operon and a negative autoregulator of itself [17]. In the *retS, gacA*-mutant, the mRNA levels of *mexAB-oprM* are up to 8-fold higher than in the other isolates of DK17, again suggesting that this mutation affects protein function and leads to faulty repression (Figure 5).

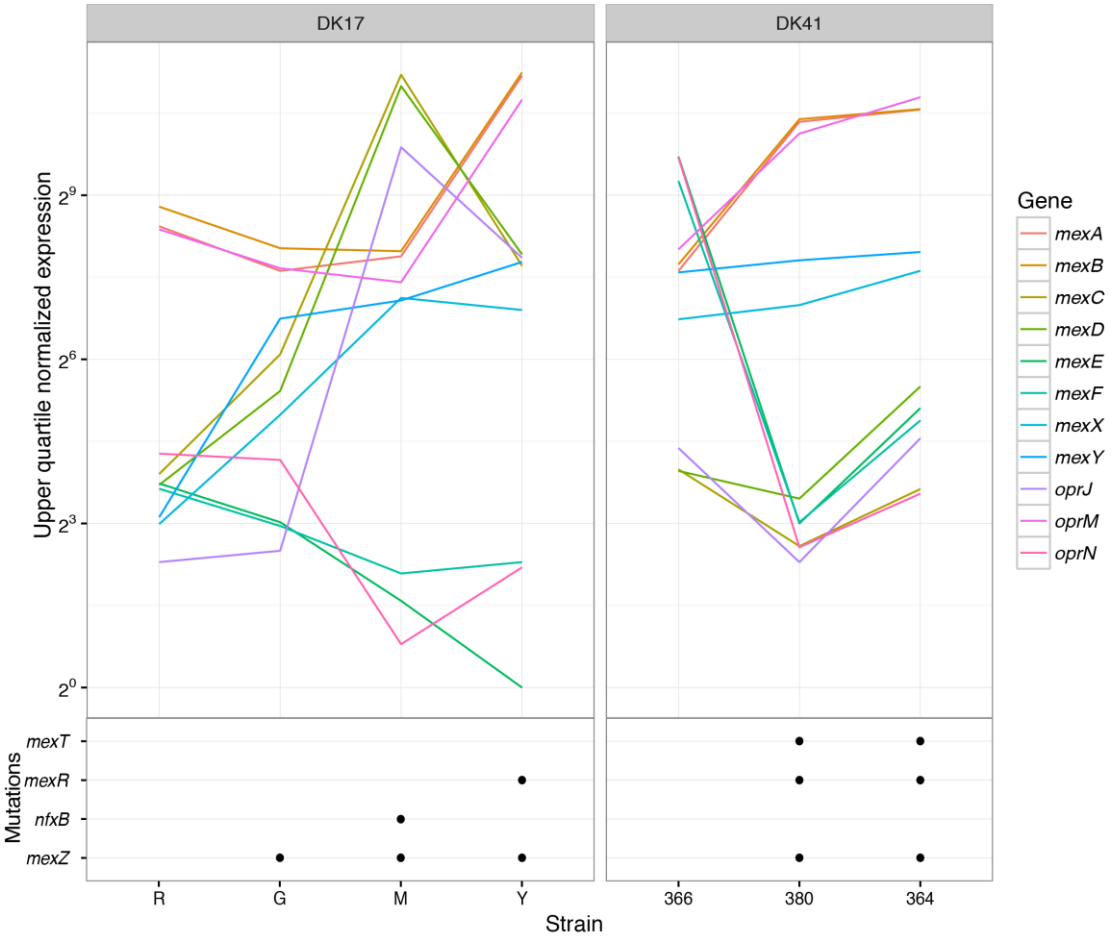

Figure 5. Expression of mRNA of the antibiotic resistance operons *mexAB-oprM*, *mexCD-oprJ*, *mexEF-oprN*, and *mexXY*. The upper part shows the expression of the various antibiotic resistance operons, whereas the bottom part shows whether or not a mutation is present in one of the regulators of the operons. A dot represents a mutation.

In DK41, there is also an upregulation of *mexR* and *mexAB-oprM* in the two later isolates (*retS* and *retS, gacS*-mutants) as compared to the MRCA. Again, this increase in expression coincides with a one bp deletion in the beginning of *mexR* in the later isolates, likely leading to a non-functional protein. As before, this explains the increased expression of the *mexAB-oprM* operon, and shows the negative autoregulation of *mexR*. Also the regulator of the *mexEF*-oprN operon is mutated in this lineage. Interestingly, the regulator, MexT, is a positive regulator [18], [19], meaning that the mutation, a one bp deletion, is likely to lower the expression of the *mexEF-oprN* operon. Indeed, this seems to be the case (Figure 5). However, MexT has also been implicated in regulation of the T3SS through MexS and PtrC [20]. The effects of the mutation in *mexT* on the expression of the T3SS cannot be determined since it is not possible to discern the effects in this mutation from the effects of the mutations the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway.

Another interesting trend is the apparent decreased expression of the *arn*-operon (*arnBCADTEF)* again in the later isolates of DK41 (data not shown). This operon is responsible for a LPS modification system leading to increased resistance towards cationic antimicrobial peptides, polymyxin B/E, and aminoglycosides by addition of positively charged arabinosamine to lipid A [21], [22]. This is surprising and noteworthy as it could lead to a lower resistance against the aforementioned antibiotics that are used as treatment in cystic fibrosis patients.

The transcriptomes and proteomes converge through time

An interesting aspect concerns evolutionary convergence, where lineages will converge towards a similar phenotype, simply because it is the fittest for a given environment. One way to examine whether this is the case for the given strains would be to correlate the transcriptomes and the proteomes of the lineages to each other.

As shown in Figure 6, the Pearson's correlations coefficients for both transcriptomes and proteomes are generally high. The first isolates for both lineages have a correlation of 0.908 and 0.854 for proteomes and transcriptomes, respectively. Moving to the *retS*-mutants, there is a drop in correlation for both datasets (0.886/0.847) suggesting that in the initial stages of adaptation, the lineages are on divergent trajectories. However, by the time the *gacS* mutation is introduced into the lineages, the proteomes and transcriptomes have converged (0.951/0.884) to the highest correlation between any two isolates not belonging to the same lineage. This

suggests that there is indeed an evolutionary trajectory towards a common fitness peak for both lineages.

Another interesting perspective is that, initially, DK17 is more inclined towards the chronic state of the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway. The first isolate of DK17 is most highly correlated with the *retS*-mutant of DK41. In line with this, the expression of the phenazine biosynthesis operons is higher in the first isolate of DK17 than in the first isolate of DK41.

Discussion

The sequential and contingent nature of the mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway, strongly suggests a selection for a specific phenotype during a specific time point during the course of infection. If this is not the case and the end-point of evolution (*retS-gacA/S* double mutation) is the fittest phenotype during any stage of infection, it would be expected that the *gacA/S* mutations would occur more or less as soon as *P. aeruginosa* enters the airways. This suggests that the evolution and adaptation of *P. aeruginosa* is not a trivial process, but instead a process that changes over time possibly due to changes in the environment such as the host airways, host response, or other bacteria/fungi. Numerous studies of *P. aeruginosa* isolates from CF airway infections have shown selection against acute virulence factors [3], [5], [23]. However, this study shows a more nuanced picture where certain virulence factors are expressed during certain periods of infection. Furthermore, some parts of the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway that have been previously reported to be controlled by RsmA [11], were found not to be differentially expressed in this study, e.g. the *pel* and *psl* operons involved in biofilm formation. This could suggest that either other mutations have an effect against this differential expression during infection, or that the regulon can be different from strain to strain as it was reported in *P. aeruginosa* PAK using microarrays and under different conditions [10].

It is possible to speculate as to why these mutations occur in this manner during infection. The initial *retS* mutation, leading to increased expression of the T6SS, phenazines, HCN biosynthetic genes, and more, could be seen as a defense mechanism during the early stages of infection, where competing bacteria, the immune system, and antibiotics cause stress. Here, the most important aspect of

infection for *P. aeruginosa* is survival and the establishment of a niche. Phenazines have been shown to have antimicrobial, antifungal [24], and antimammalian [24] activity by causing oxidative stress. This has obvious advantages during the early stages of infection, when the immune system will react to the infection. However, the later *gacA/S* mutations with downregulation of the aforementioned genes and upregulation of T3SS suggests that after the initial niche-establishment, there is a need for dissemination of the infection. Here, the *P. aeruginosa* spreads from the initial focus of infection and the response of the immune system response is battled by the T3SS, which has been suggested to kill host immune cells[25].

It is surprising that the mutations are necessary to change the phenotype of *P. aeruginosa*. As the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway is a regulatory system, it could be expected that it would be able to perform its function without being rendered inoperable by mutations. However, the signals that this system responds to are not known [7], [10], [11], and this could suggest that the necessary signals for the regulation of the system are simply not present in the human airways. Another possibility is that mutations are more effective at changing the expression of the necessary genes compared to the signals, and that maximum expression is needed in the new environment of the airways. Thus, the only way to regulate the expression of the controlled genes is to mutate the regulators.

The biggest part of the variance of both the transcriptome and the proteome datasets is to be explained by the mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway. A pitfall of this is that both datasets were mapped to the genome of the reference strains, PAO1. As such, genes that are present in either DK17 or DK41, and not in PAO1, are not taken into consideration. If these genes are numerous and different between the two lineages, these would increase the transcriptomic and proteomic diversity, leading to lineages being the greatest source of variance.

The increased expression of the denitrification genes in the DK41 isolates can be linked to two things; (i) the cystic fibrosis mucus has been shown to be microoxic [26], [27] and therefore there is a need for an electron acceptor other than molecular oxygen, and (ii) the immune system can produce nitric oxide (a reactive nitrogen species) as a response to the infection, which can disperse biofilms [28], [29] and damage cells through nitrosative stress [30]. It seems likely that the reason for the increased expression is a combination of both things. Furthermore, some components

of the denitrification pathway have also been linked to functional expression of the T3SS [31], showing that metabolism and virulence can be linked.

Interestingly, the correlation coefficients for the transcriptomes and proteomes are the highest for the last isolates of both lineages with the *gacS* and *retS* mutations. This suggests that the lineages are on a common evolutionary trajectory and concords with the similar mutations observed in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway. The fact that both strains seemingly reach an evolutionary dead-end, seeing as it was not possible to obtain more isolates for years after, suggests that the evolutionary trajectory of sequential mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway is not a viable path of adaptation.

Overall this work shows that the early stages of evolution and adaptation to the CF airways are subject to temporal expression of different virulence factors in *P. aeruginosa*. Specific mutations in regulatory systems confer expression of either the T6SS and the phenazine biosynthesis operons or the T3SS in a sequential manner. Furthermore, mutations in regulators of antibiotic efflux-pump genes increase the transcription of their cognate efflux-pumps. This shows that *P. aeruginosa* is subject to significant adaptation during the early stages of infection and could have impact on future treatment strategies.

Conclusions

This study documents gene expression changes in at least three major groups of genes. The reciprocal nature of the T3SS and the T6SS strongly suggests that the expression of different virulence factors is needed at different stages of infection. The initial *retS* mutation, leading to a chronic infection type, suggests that the first stage of infection requires protection from the host or competing bacteria with expression of T6SS and phenazines. However, the later stage with the *gacS* or *gacA* mutation, promoting acute infection behavior, suggests that at some point during infection the chronic infection behavior is detrimental to the success of the infection. Instead, a phenotype based on motility and the T3SS to combat the immune system and disseminate from the initial focus of infection is needed. Furthermore, the increased expression of denitrification genes in the lineage DK41 suggests that denitrification is important for the survivability of the *P. aeruginosa* infection, at least under some circumstances. Additionally, there is an increase in expression of three operons that

contain efflux pumps across both lineages that is connected to mutations in their respective regulators suggesting that there is a need for increased efflux of antibiotics. However, the fact that no further isolates were culturable from the patients, suggests that the sum of these mutations does not produce a viable phenotype in a CF infection setting.

Materials and methods

Cell handling

All strains were grown in LB-Miller (1% NaCl) at 37 °C with shaking at 200 rpm.

RNA extraction and treatment

O/N cultures were diluted 100 times in a conical flask to a total volume of 100 mL LB. 10 mL of culture was taken per sample at late exponential phase (OD_{600} = 1), transferred to conical tubes with 2 mL of stop solution (95% ethanol, 5% phenol), vortexed thoroughly and left at RT for 5 minutes. The bacteria were pelleted (3500g, 10 min, 4 °C) and the supernatants discarded. The pellets were dissolved in 1 mL of Trizol each and stored at -80 °C until further use. Total RNA extraction and DNA removal by treatment with DNase I were performed as described in [32] and RNA quality was checked on the Agilent Bioanalyzer using the Agilent RNA 6000 Nano Kit. Ribosomal RNA was depleted using MICROBExpress™ Bacterial mRNA Enrichment Kit (Ambion) but with a modification for the removal of 5S RNA as described in [32]. The depletion of rRNA was checked on the Agilent Bioanalyzer using the Agilent RNA 600 Nano Kit.

Library preparation and sequencing

The libraries were prepared using the TruSeq™ RNA Sample Prep Kit v2 (Illumina), the size was checked on the Agilent Bioanalyzer using the DNA high sensitivity assay, the concentration confirmed on Qubit 2.0 Fluorometer, and were sequenced on the Illumina MiSeq in a 2x150 bp paired-end configuration. The reads were mapped to the PAO1 reference genome using Rockhopper [33], [34]. Mapping statistics are found in Table S3.

Protein extraction and sample preparation for MS analysis

Cells were harvested in the late-exponential growth phase (OD_{600} = 1) by centrifugation (10 min, 8500 rpm, 4 °C), resuspended in 1 mL 50 mM TE buffer, pH 7.5, and transferred in a 2 mL screw cap micro tube containing 500 µL glass beads with 0.1 mm diameter. Subsequently, cells were lysed mechanically using the Precellys 24 homogenizator (PeqLab, Erlangen, Germany; 3 × 30 s at 6,800 rpm). Cell debris and glass beads were removed by centrifugation (3 x 20 min at 15,000

rpm, 4 °C). Protein concentration of extracts was determined using a ninhydrin-based assay [35]. Protein extracts (100 µg) were reduced, alkylated and trypsin-digested (Promega, Fitchburg, WI) as previously described [36]. Prior to liquid chromatography/ion-mobility spectrometry (LC/IMS^{E}) analysis, samples were desalted using C18-Stage Tips (Thermo scientific, Waltham, MA) [37] and a complete tryptic digest of alcohol dehydrogenase of yeast (ADH, Waters, Milford, MA) was added to a final concentration of 50 fmol/µL. All experiments were carried out in 3 biological and 3 technical replicates per biological sample.

LC/IMS^{E} data acquisition and analysis

Peptide samples were analyzed with a nanoACQUITY ultraperformance liquid chromatography (UPLC) system (Waters) coupled to a Synapt G2 mass spectrometer (Waters), as previously described [38]. Raw data were processed via the ProteinLynx Global Server (PLGS, Version 2.5.3, Waters) by the Apex3D algorithm including the following parameters: Chromatographic peak width and MS TOF resolution were set to automatic, lock mass charge 2 set to 785.8426 Da/e with a lock mass window of 0.25 Da, low energy threshold 200.0 counts, elevated energy threshold 20.0 counts, intensity threshold 750 counts. The processed data were searched against a *P. aeruginosa* PAO1 database containing 11,226 entries (NCBI, version 2012-11-13) including common laboratory contaminants and the yeast ADH1 sequence. The following search parameters were used: enzyme type trypsin; 1 fragment ion matched per peptide, 5 fragment ions matched per protein, 2 peptide matched per protein; 2 missed cleavages allowed; fixed modification: carbamidomethylation C (+57.0215); variable modifications: deamidation N, Q (+0.9840), oxidation M (+15.9949), pyrrolidonecarboxylacid N-TERM (-27.9949); the false-discovery rate (FDR) was 5%; and the calibration protein was yeast alchohol dehydrogenase 1. For quantitation only proteins were considered that were identified in two out of three biological and technical replicates, respectively (replicate filter). Absolute protein quantification was achieved using the Hi3 approach [39] with yeast ADH as a reference. Differentially expressed proteins were identified using one-way ANOVA (p=0.05) in R and the false discovery rate was controlled by Benjamini-Hochberg procedure.

Transcriptomic data handling

The raw read files were quality checked using FastQC [40]. The expression values provided by Rockhopper were then used for downstream analysis using custom R-scripts. Both lineages were subjected to ANOVA ($p < 0.05$) for differential expression analyses and the false discovery rate was controlled by Benjamini-Hochberg procedure.

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Supplemental material

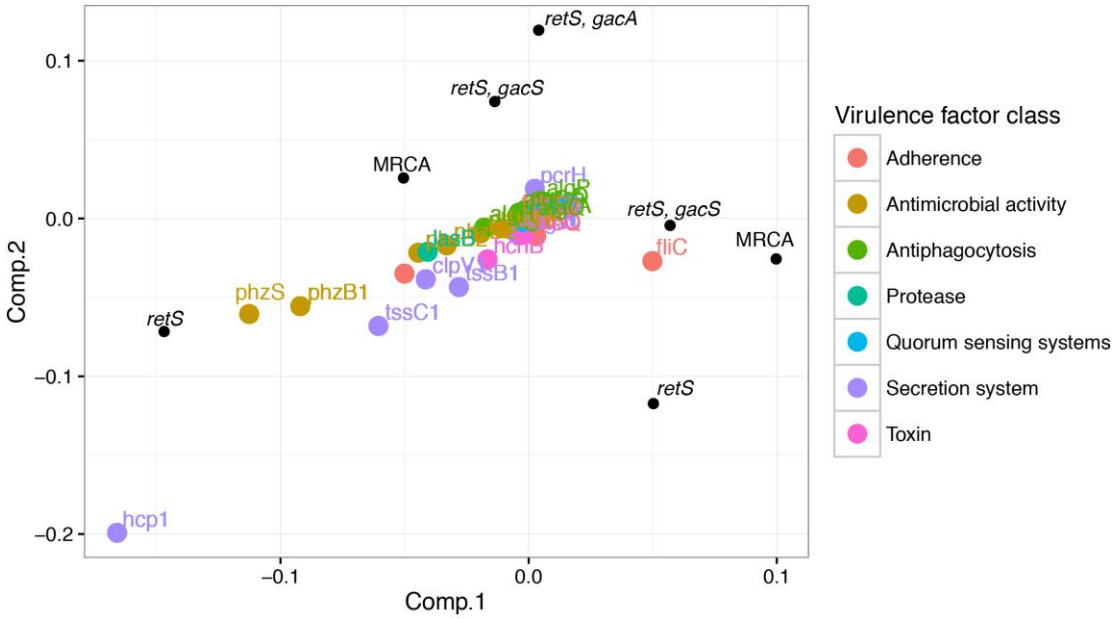

Figure S1. PCA plot of proteomics data. Component 1 explains 39.1% of the variance and component 2 explains 33.4% of the variance.

Table S1. Information about and isolates including the patient, date of isolation, the material of origin of the isolates and the lineage.

Isolate	Patient	Date obtained	Material	Lineage
R (MRCA)	P38F4	1/29/2007	Endolaryngeal suction	DK17
G	P38F4	10/22/2008	Right maxillary sinus	DK17
M	P38F4	04/05/2011	Right maxillary sinus	DK17
Y	P38F4	04/05/2011	Secretion from the ethmoids	DK17
366 (MRCA)	P76M4	01/08/2008	Endolaryngeal suction	DK41
380	P76M4	3/17/2010	Endolaryngeal suction	DK41
364	P76M4	08/22/2012	Sputum sample	DK41

Table S2ABCD. All mutations in the lineages. 1 represents that the mutation specified in ‘qry’ is present in the position specified. Type indicates the nature of the mutation. In the case of single nucleotide polymorphisms, ‘ref’ indicates the base in the reference genome whereas ‘qry’ indicates the mutation.

640 Table S2A. All indels that are different between strains R, G, M, and Y of lineage
641 DK17.

G	M	R	Y	position	ref	qry	type	locus	name	product	pseudocap
0	1	0	0	14445	*	+TGGATAT	Insertion	PA0011		probable 2-OH-lauroyltransferase	Cell wall / LPS / capsule
0	0	0	1	14813	*	-GCGCGTACCGCTG	Deletion	PA0011		probable 2-OH-lauroyltransferase	Cell wall / LPS / capsule
0	1	0	0	370459	*	-A	Intergenic Deletion	PA0328//PA0329	//	55 upstream hypothetical protein//246 downstream conserved hypothetical protein	Hypothetical, unclassified, unknown//Hypothetical, unclassified, unknown
0	0	0	1	451733	*	-ACGAGAACTCGGT	Deletion	PA0411	pilJ	twitching motility protein PilJ	Chemotaxis; Motility & Attachment
0	0	0	1	471602	*	-TTGTTCGTCGATAA	Deletion	PA0424	mexR	multidrug resistance operon repressor MexR	Transcriptional regulators
0	1	1	0	809540	*	-C	Intergenic Deletion	PA0741//PA0742	//	84 upstream conserved hypothetical protein//33 downstream hypothetical protein	Hypothetical, unclassified, unknown//Hypothetical, unclassified, unknown
1	0	1	1	896130	*	+TAAA	Intergenic Insertion	PA0819//PA0820	//	13 downstream hypothetical protein//286 upstream hypothetical protein	Hypothetical, unclassified, unknown//Hypothetical, unclassified, unknown
1	0	1	1	959359	*	-GT	Intergenic Deletion	PA0876//PA0877	//	10 downstream probable transcriptional regulator//124 downstream probable transcriptional regulator	Transcriptional regulators//Transcriptional regulators
1	1	0	1	994775	*	+CGGGAGTGT	Insertion	PA0911		hypothetical protein	Hypothetical, unclassified, unknown
0	1	0	0	1015471	*	+CGTT	Insertion	PA0928	gacS	sensor/response regulator hybrid	Two-component regulatory systems
0	1	0	0	1156537	*	-TGGAGCGCCAG	Deletion	PA1069		hypothetical protein	Hypothetical, unclassified, unknown
0	0	1	0	1233279	*	+GAGCGCC	Intergenic Insertion	PA1141//PA1142	//	148 upstream probable transcriptional regulator//18 downstream probable transcriptional regulator	Transcriptional regulators//Transcriptional regulators
0	1	1	1	1447059	*	+CATTCCCCACA	Intergenic Insertion	PA1334//PA1335	//	141 upstream probable oxidoreductase//167 downstream probable two-component response regulator	Putative enzymes//Transcriptional regulators; Two-component regulatory systems
1	1	1	0	1447060	*	+ATT	Intergenic Insertion	PA1334//PA1335	//	142 upstream probable oxidoreductase//166 downstream probable two-component response regulator	Putative enzymes//Transcriptional regulators; Two-component regulatory systems
0	0	1	1	1495579	*	+CGGAAAAC	Intergenic Insertion	PA1377//PA1378	//	87 downstream conserved hypothetical protein//56 upstream hypothetical protein	Hypothetical, unclassified, unknown//Hypothetical, unclassified, unknown
1	0	1	1	1552312	*	+T	Insertion	PA1425		probable ATP-binding component of ABC transporter	Transport of small molecules
0	0	0	1	1681088	*	-GCCGGCGAG	Deletion	PA1544	anr	transcriptional regulator Anr	Transcriptional regulators
0	0	0	0	2263645	*	-GTCCATGCCGTTCAT	Deletion	PA2065	pcoA	copper resistance protein A precursor	Adaptation, Protection
0	0	0	1	1688689	*	-AGCCA	Deletion	PA1551		probable ferredoxin	Energy metabolism
0	1	1	0	1999485	*	+CGGTTT	Intergenic Insertion	PA1841//PA1842	//	25 downstream hypothetical protein//27 downstream hypothetical protein	Hypothetical, unclassified, unknown//Hypothetical, unclassified, unknown

G	M	R	Y	position	ref	qry	type	locus	name	product	pseudocap
0	1	0	0	2142950	*	+TGGGAAA	Intergenic Insertion	PA1958//PA1959	//bacA	61 upstream probable transporter//223 upstream bacitracin resistance protein	Membrane proteins; Transport of small molecules//Cell wall / LPS / capsule; Adaptation, Protection; Antibiotic resistance and susceptibility
1	0	0	1	2142952	*	+GGAAAAA	Intergenic Insertion	PA1958//PA1959	//bacA	63 upstream probable transporter//221 upstream bacitracin resistance protein	Membrane proteins; Transport of small molecules//Cell wall / LPS / capsule; Adaptation, Protection; Antibiotic resistance and susceptibility
1	1	0	1	2251287	*	+C	Insertion	PA2057		hypothetical protein	Hypothetical, unclassified, unknown
0	0	0	0	2722126	*	-A	Intergenic Deletion	PA2425//PA2426	pvdG//pvdS	596 upstream PvdG//48 upstream sigma factor PvdS	Adaptation, Protection//Transcriptional regulators
1	1	0	1	2379882	*	+AG	Insertion	PA2160		probable glycosyl hydrolase	Putative enzymes
1	1	0	1	2379888	*	+A	Insertion	PA2160		probable glycosyl hydrolase	Putative enzymes
1	1	0	1	2379890	*	+CATTGAGGA	Insertion	PA2160		probable glycosyl hydrolase	Putative enzymes
1	1	0	1	2379890	*	+GA	Insertion	PA2160		probable glycosyl hydrolase	Putative enzymes
1	1	0	1	2379891	*	+T	Insertion	PA2160		probable glycosyl hydrolase	Putative enzymes
1	1	0	1	2400365	*	+GCG	Intergenic Insertion	PA2178//PA2179	//	85 upstream hypothetical protein//290 downstream hypothetical protein	Hypothetical, unclassified, unknown//Hypothetical, unclassified, unknown
0	0	0	1	2400594	*	+TCC	Intergenic Insertion	PA2178//PA2179	//	314 upstream hypothetical protein//61 downstream hypothetical protein	Hypothetical, unclassified, unknown//Hypothetical, unclassified, unknown
0	1	1	0	2571782	*	+G	Insertion	PA2330		hypothetical protein	Hypothetical, unclassified, unknown
1	1	1	0	2623258	*	-GT	Deletion	PA2371		probable ClpA/B-type protease	Translation, post-translational modification, degradation
0	0	0	1	2730153	*	-GCCAGCCCGCCGG	Deletion	PA2434		hypothetical protein	Hypothetical, unclassified, unknown
1	1	0	1	2806290	*	+AACGAAT	Insertion	PA2490		conserved hypothetical protein	Hypothetical, unclassified, unknown
1	0	0	0	2864118	*	-G	Intergenic Deletion	PA2535//PA2536	//	180 downstream probable oxidoreductase//51 downstream probable phosphatidate cytidylyltransferase	Putative enzymes//Fatty acid and phospholipid metabolism
0	0	0	1	2926331	*	-T	Deletion	PA2586	gacA	response regulator GacA	Transcriptional regulators
0	1	1	0	3087585	*	+AATGTAGTGGTC	Intergenic Insertion	PA2729//PA2730	//	94 downstream hypothetical protein//1075 downstream hypothetical protein	Hypothetical, unclassified, unknown//Hypothetical, unclassified, unknown
1	0	0	1	3087587	*	+TGTAGTGGT	Intergenic Insertion	PA2729//PA2730	//	96 downstream hypothetical protein//1073 downstream hypothetical protein	Hypothetical, unclassified, unknown//Hypothetical, unclassified, unknown
1	1	0	0	3249252	*	+C	Insertion	PA2894		hypothetical protein	Hypothetical, unclassified, unknown
1	1	0	1	3496464	*	-GCAGGTCGGT	Deletion	PA3115	fimV	Motility protein FimV	Membrane proteins; Motility & Attachment
1	1	1	0	3526714	*	+TCTG	Intergenic Insertion	PA3141//PA3142	wbpM//	36 upstream nucleotide sugar epimerase/dehydratase WbpM//715 downstream hypothetical protein	Putative enzymes; Membrane proteins; Cell wall / LPS / capsule//Related to phage, transposon, or plasmid

G	M	R	Y	position	ref	qry	type	locus	name	product	pseudocap
0	0	0	1	3557172	*	+CCTCGACTT	Insertion	PA3168	gyrA	DNA gyrase subunit A	DNA replication, recombination, modification and repair
0	1	1	0	3618697	*	+GGCGGA	Intergenic Insertion	PA3230//PA3231	//	230 upstream conserved hypothetical protein//29 downstream hypothetical protein	Hypothetical, unclassified, unknown//Membrane proteins
1	1	0	0	3682825	*	+GGTTTCAGGCGT	Insertion	PA3290		hypothetical protein	Hypothetical, unclassified, unknown
0	1	0	0	3694006	*	+CGCG	Insertion	PA3297		probable ATP-dependent helicase	Transcription, RNA processing and degradation
0	0	1	0	3820916	*	+CCA	Intergenic Insertion	PA3414//PA3415	//	24 downstream hypothetical protein//469 downstream probable dihydrolipoamide acetyltransferase	Hypothetical, unclassified, unknown//Energy metabolism
1	0	1	0	3820918	*	+ATCG	Intergenic Insertion	PA3414//PA3415	//	26 downstream hypothetical protein//467 downstream probable dihydrolipoamide acetyltransferase	Hypothetical, unclassified, unknown//Energy metabolism
1	1	1	0	3974147	*	+GT	Intergenic Insertion	PA3547//PA3548	algL//algI	30 downstream poly(beta-d-mannuronate) lyase precursor AlgL//212 upstream alginate o-acetyltransferase AlgI	Cell wall / LPS / capsule; Adaptation, Protection; Secreted Factors (toxins, enzymes, alginate)//Cell wall / LPS / capsule; Adaptation, Protection; Secreted Factors (toxins, enzymes, alginate)
0	0	1	0	4166949	*	+GTA	Insertion	PA3721	nalC	NalC	Transcriptional regulators; Antibiotic resistance and susceptibility
1	0	1	1	4417080	*	+AA	Insertion	PA3939		hypothetical protein	Hypothetical, unclassified, unknown
1	1	0	1	4432946	*	+CCC	Insertion	PA3952		hypothetical protein	Hypothetical, unclassified, unknown
1	0	0	0	4493463	*	+C	Insertion	PA4013		conserved hypothetical protein	Membrane proteins
1	1	1	0	4714800	*	+GATC	Insertion	PA4211	phzB1	probable phenazine biosynthesis protein	Secreted Factors (toxins, enzymes, alginate)
0	0	0	1	5026059	*	+GG	Insertion	PA4491		conserved hypothetical protein	Hypothetical, unclassified, unknown
0	1	0	0	5155826	*	-CCACCGCGA	Deletion	PA4600	nfxB	transcriptional regulator NfxB	Transcriptional regulators
0	0	1	0	5206144	*	-GGCGATG	Intergenic Deletion	PA4636//PA4637	//	57 downstream hypothetical protein//64 downstream hypothetical protein	Hypothetical, unclassified, unknown//Hypothetical, unclassified, unknown
0	1	0	0	5312996	*	+G	Insertion	PA4730	panC	pantoate--beta-alanine ligase	Biosynthesis of cofactors, prosthetic groups and carriers
1	1	0	1	5384501	*	+AAT	Insertion	PA4798		hypothetical protein	Hypothetical, unclassified, unknown
1	1	0	0	5387754	*	+AGGC	Intergenic Insertion	PA4802//PA4802.1	//	32 downstream tRNA-Sec//12 downstream tRNA-Sec	Hypothetical, unclassified, unknown//Non-coding RNA gene
1	1	0	1	5775356	*	-C	Intergenic Deletion	PA5125//PA5126	ntrC//	550 downstream two-component response regulator NtrC//263 downstream hypothetical protein	Transcriptional regulators; Two-component regulatory systems//Hypothetical, unclassified, unknown
1	0	1	1	5810046	*	+T	Intergenic Insertion	PA5160.1//PA5161	//rmlB	1 downstream tRNA-Thr//235 upstream dTDP-D-glucose 4,6-dehydratase	Non-coding RNA gene//Carbon compound catabolism; Cell wall / LPS / capsule
1	0	1	1	5930755	*	+GCCTGC	Insertion	PA5266		conserved hypothetical protein	Hypothetical, unclassified, unknown
1	0	0	0	6154750	*	+TC	Intergenic Insertion	PA5464//PA5465	//	74 downstream hypothetical protein//34 downstream hypothetical protein	Hypothetical, unclassified, unknown//Hypothetical, unclassified, unknown

642 Table S2B. All single nucleotide polymorphisms that are different between strains R,
643 G, M, and Y of lineage DK17.

G	M	R	Y	position	ref	qry	type	locus	name	product	pseudocap
1	0	1	1	499765	G	A	Intergenic	PA0444//PA0445	//	62 downstream N-carbamoyl-beta-alanine amidohydrolase//339 downstream probable transposase	Nucleotide biosynthesis and metabolism//Related to phage, transposon, or plasmid
0	0	0	1	1665536	C	T	Missense	PA1528	zipA	cell division protein ZipA	Cell division
0	1	0	0	1744763	C	T	Silent	PA1601		probable aldehyde dehydrogenase	Putative enzymes
1	1	0	1	2212705	A	C	Missense	PA2020		probable transcriptional regulator	Transcriptional regulators
1	1	0	1	2455991	A	G	Missense	PA2232	pslB	PslB	Cell wall / LPS / capsule
0	0	0	1	2458028	C	A	Silent	PA2234	pslD	PslD	Cell wall / LPS / capsule; Transport of small molecules
1	1	0	1	2799381	G	A	Missense	PA2480		probable two-component sensor	Two-component regulatory systems
0	0	0	1	4034375	G	A	Silent	PA3598		conserved hypothetical protein	Hypothetical, unclassified, unknown
1	1	0	1	4311423	C	G	Missense	PA3850		hypothetical protein	Hypothetical, unclassified, unknown
0	0	0	1	4768891	A	G	Silent	PA4265	tufA	elongation factor Tu	Translation, post-translational modification, degradation
0	0	0	1	4768975	T	C	Silent	PA4265	tufA	elongation factor Tu	Translation, post-translational modification, degradation
1	1	0	1	5453680	G	T	Nonsense	PA4856	retS	RetS (Regulator of Exopolysaccharide and Type III Secretion)	Two-component regulatory systems

644 Table S2C. All indels that are different between strains 366, 380, and 364 of lineage
645 DK41.

364	366	380	position	ref	qry	type	locus	name	product	pseudocap
1	0	0	340031	*	-AGAAGA	Deletion	PA0301	spuE	polyamine transport protein	Transport of small molecules
1	0	1	398135	*	-A	Intergenic Deletion	PA0353//PA0354	ilvD//	241 upstream dihydroxy-acid dehydratase// 88 downstream conserved hypothetical protein	Biosynthesis of cofactors, prosthetic groups and carriers; Amino acid biosynthesis and metabolism//Hypothetical, unclassified, unknown
0	1	0	453109	*	+A	Insertion	PA0411	pilJ	twitching motility protein PilJ	Chemotaxis; Motility & Attachment
1	0	1	471696	*	-T	Deletion	PA0424	mexR	multidrug resistance operon repressor MexR	Transcriptional regulators
1	0	1	892604	*	-CAG	Deletion	PA0814		conserved hypothetical protein	Hypothetical, unclassified, unknown
1	0	0	949337	*	-T	Deletion	PA0868		conserved hypothetical protein	Hypothetical, unclassified, unknown
1	0	0	1015721	*	+CG	Insertion	PA0928	gacS	sensor/response regulator hybrid	Two-component regulatory systems

364	366	380	position	ref	qry	type	locus	name	product	pseudocap
1	1	0	1249854	*	+A	Intergenic Insertion	PA1154//PA1155	//nrdB	302 upstream conserved hypothetical protein//53 downstream NrdB, tyrosyl radical-harboring component of class Ia ribonucleotide reductase	Hypothetical, unclassified, unknown//Nucleotide biosynthesis and metabolism
1	0	0	1270409	*	-AGGCGAGGGCGA	Deletion	PA1170		conserved hypothetical protein	Membrane proteins
1	0	0	1366984	*	-G	Deletion	PA1259		hypothetical protein	Hypothetical, unclassified, unknown
0	0	1	2235372	*	-TT	Intergenic Deletion	PA2042//PA2043	//	64 downstream probable transporter (membrane subunit)//57 upstream hypothetical protein	Transport of small molecules//Hypothetical, unclassified, unknown
0	1	0	2637061	*	-TG	Deletion	PA2385	pvdQ	3-oxo-C12-homoserine lactone acylase PvdQ	Adaptation, Protection
1	1	0	2756236	*	-GG	Deletion	PA2455		hypothetical protein	Hypothetical, unclassified, unknown
1	0	1	2807546	*	-C	Deletion	PA2492	mexT	transcriptional regulator MexT	Transcriptional regulators
0	1	0	2810971	*	-C	Deletion	PA2494	mexF	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter MexF	Transport of small molecules; Membrane proteins; Antibiotic resistance and susceptibility
1	1	0	3129137	*	+A	Intergenic Insertion	PA2770//PA2771	//	66 upstream hypothetical protein//592 upstream conserved hypothetical protein	Hypothetical, unclassified, unknown//Hypothetical, unclassified, unknown
0	1	1	3486413	*	-A	Intergenic Deletion	PA3105//PA3106	xcpQ//	84 downstream general secretion pathway protein D//3 downstream probable short-chain dehydrogenase	Protein secretion/export apparatus//Putative enzymes
0	1	0	3514799	*	-AAT	Intergenic Deletion	PA3133.1//PA3133.2	//	2 upstream tRNA-Glu//45 downstream tRNA-Ala	Non-coding RNA gene//Non-coding RNA gene
1	0	0	3694826	*	-GCCGCGCCTCG	Deletion	PA3297		probable ATP-dependent helicase	Transcription, RNA processing and degradation
0	1	1	3780318	*	-CCCACAACGC	Intergenic Deletion	PA3371//PA3372	//	6 downstream hypothetical protein//43 downstream conserved hypothetical protein	Hypothetical, unclassified, unknown//Transport of small molecules

364	366	380	position	ref	qry	type	locus	name	product	pseudocap
1	1	0	4009340	*	+CA	Intergenic Insertion	PA3577//PA3578	//	289 upstream hypothetical protein//202 downstream conserved hypothetical protein	Hypothetical, unclassified, unknown//Hypothetical, unclassified, unknown
1	0	0	4607471	*	+GCCG	Intergenic Insertion	PA4118//PA4119	//aph	17 downstream hypothetical protein//107 downstream aminoglycoside 3'-phosphotransferase type IIb	Hypothetical, unclassified, unknown//Antibiotic resistance and susceptibility
1	0	1	4714801	*	+ATCGAA	Insertion	PA4211	phzB1	probable phenazine biosynthesis protein	Secreted Factors (toxins, enzymes, alginate)
1	0	0	4865466	*	-A	Deletion	PA4336		conserved hypothetical protein	Hypothetical, unclassified, unknown
0	1	0	4896208	*	-A	Deletion	PA4367	bifA	BifA	Motility & Attachment; Cell wall / LPS / capsule
1	0	1	5452961	*	-CTTCCGCGGCA	Deletion	PA4856	retS	RetS (Regulator of Exopolysaccharide and Type III Secretion)	Two-component regulatory systems
1	0	1	5677001	*	-AGCGACAGTT	Deletion	PA5040	pilQ	Type 4 fimbrial biogenesis outer membrane protein PilQ precursor	Motility & Attachment
0	1	0	5743831	*	-GCACGTGTA	Deletion	PA5100	hutU	urocanase	Amino acid biosynthesis and metabolism
0	0	1	5807183	*	-CAACAGCGAAA	Deletion	PA5159		multidrug resistance protein	Transport of small molecules; Antibiotic resistance and susceptibility
1	0	1	5942472	*	+C	Insertion	PA5277	lysA	diaminopimelate decarboxylase	Amino acid biosynthesis and metabolism
1	0	1	5986292	*	-G	Intergenic Deletion	PA5316.1//PA5317	//	123 downstream //182 upstream probable binding protein component of ABC dipeptide transporter	Non-coding RNA gene//Transport of small molecules
0	1	0	5986294	*	-C	Intergenic Deletion	PA5316.1//PA5317	//	125 downstream //180 upstream probable binding protein component of ABC dipeptide transporter	Non-coding RNA gene//Transport of small molecules
1	0	0	6120645	*	-GTGAAGGGGTG	Deletion	PA5437		probable transcriptional regulator	Transcriptional regulators

646 Table S2D. All single nucleotide polymorphisms that are different between strains
647 366, 380, and 364 of lineage DK41.

364	366	380	position	ref	qry	type	locus	name	product	pseudocap
1	0	1	2212812	G	T	Missense	PA2020		probable transcriptional	Transcriptional regulators

									regulator	
1	0	1	2808453	G	A	Missense	PA2492	mexT	transcriptional regulator MexT	Transcriptional regulators
1	0	0	4043670	G	A	Missense	PA3609	potC	polyamine transport protein PotC	Membrane proteins; Transport of small molecules
0	1	0	5021956	C	T	Silent	PA4489		conserved hypothetical protein	Hypothetical, unclassified, unknown
1	0	0	5730325	G	A	Silent	PA5090		conserved hypothetical protein	Hypothetical, unclassified, unknown
1	0	0	5730340	T	C	Silent	PA5090		conserved hypothetical protein	Hypothetical, unclassified, unknown

Table S3. Mapping statistics

Sample	**R1**	**R2**	**G1**	**G2**	**M1**	**M2**	**Y1**	**Y2**
Total reads	4101031	3100021	2750399	3060869	3092746	5674693	3966006	4295983
Mapped reads	3909999 (95%)	2967636 (96%)	2583598 (94%)	2924255 (96%)	2943686 (95%)	5351526 (94%)	3799002 (96%)	3873508 (90%)

Sample	**366-1**	**366-2**	**380-1**	**380-2**	**364-1**	**364-2**
Total reads	4560412	3669173	4039980	4013697	3355139	3384675
Mapped reads	4189811 (92%)	3383537 (92%)	3666251 (91%)	3681479 (92%)	3050809 (91%)	3117126 (92%)

Table S4A. Identified and quantified proteins in DK17 with their predicted localization by PSORTb.

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Table S4B. Identified and quantified proteins in DK41 with their predicted localization by pSORTb.

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Table S5A. Differentially expressed transcripts in DK17 as determined by ANOVA ($p < 0.05$)

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Table S5B. Differentially expressed transcripts in DK41as determined by ANOVA ($p < 0.05$)

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Table S5C. Differentially abundant proteins in DK17 as determined by ANOVA ($p < 0.05$)

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Table S5D. Differentially abundant protein in DK41 as determined by ANOVA ($p < 0.05$)

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Manuscript 2

Transcriptomic evolution of two convergent *Pseudomonas aeruginosa* lineages from the cystic fibrosis airways

M. Lindegaard, A. Jiménez-Fernández, S. Molin, H. K. Johansen, K. S. Long

Transcriptomic evolution of *Pseudomonas aeruginosa* of two convergent lineages from the cystic fibrosis airways

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Summary

Pseudomonas aeruginosa is a significant cause of morbidity and mortality in cystic fibrosis (CF) patients due to *P. aeruginosa* establishing chronic infection in the CF airways. During this time, *P. aeruginosa* will evolve and adapt to the new environment through the fixation of mutations in its genome. The common evolutionary trajectories include loss of virulence factors, mucoidy, increased biofilm formation or mucoidy, and increased antibiotic resistance. This evolution is commonly caused by mutations in regulatory genes, which can have large phenotypic consequences. Here the specific effects of mutation in the *retS-gacS-gacA-rsmA-rsmYZ* are investigated, as these occur in a sequential manner where *retS* mutates before *gacS*. Two lineages of *P. aeruginosa* (DK17 and DK41) isolated from the first four years of infection of the CF airways in two patients are examined on a transcriptomic level (RNA-sequencing). Additionally, the mutated alleles of *retS* and *gacS* are moved into the earlier isolated strains not containing these mutations. This enabled the investigation of the effects caused only by *retS* and/or *gacS* mutations without the noise caused by the residual mutations present in the clinical isolates. Furthermore, the non-mutated alleles from the first isolates of DK17 and DK41 were also moved to the later isolates in order to examine the effects of the residual mutations. In the purely clinical isolates, temporal expression of virulence factors such as the Type III secretion system (T3SS), the Type VI secretion system (T6SS), and phenazine biosynthesis operons was observed. However, the mutations not occurring in the *retS-gacS-gacA-rsmA-rsmYZ* signalling pathway also have effects on the expression of virulence factors. In one lineages the residual mutations accentuate the

effects of *retS/gacS* mutations causing increased expression of the phenazines biosynthesis operon and in the other lineage the opposite occurs and the residual mutations counteract the effects leading to lower expression of the phenazines biosynthesis operons.

Introduction

Pseudomonas aeruginosa is a major cause of morbidity and mortality in cystic fibrosis (CF) patients. The bacterial infections of the CF airways may persist for a lifetime with the same *P.* aeruginosa lineage giving ample time to evolve and adapt to the new environment of the CF airways [1]. This environment is not perfectly characterized, but stress factors proposed to exist in the CF airways environment include other microorganisms [2], antibiotics [3], osmotic stress [4], oxidative stress [5], and nitrosative stress [6].

P. aeruginosa has a relatively large genome (PAO1: 6.26 mb) and has 526 genes classified as involved in transcription [7], meaning that at least 9.2% of its 5688 annotated genes probably have some sort of regulatory function. Mutations of regulators can lead to quick adaptation to new environments and it has been shown that regulators are indeed focal points of mutations in sequential *P. aeruginosa* during infection of the CF airways [8]–[10]. Furthermore, the genome contains a large amount of virulence factors including but not limited to the type III secretion system (T3SS), three clusters of type VI secretion system (H1-T6SS, H2-T6SS, and H3-T6SS) [11], [12], pyocyanin and other phenazines [13], [14]. The regulation of these virulence factors is interconnected and complex and relies on various environmental cues [15], e.g. temperature [16] and various stresses.

The *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway reciprocally regulates genes that have been attributed to either chronic or acute infection modes. The regulon encompasses around 500 genes [17] and is regulated through an intricate system of a two-component system (TCS) (GacA/GacS), small RNAs (sRNAs) (RsmYZ), RsmA, and at least three histidine kinases, RetS, LadS, and PA1611 that modulate the activity of the TCS [18]–[20]. RetS, a hybrid sensor kinase, inhibits the autophosphorylation of dimeric GacS, which in its active phosphorylated and dimeric state, will activate GacA, a transcriptional regulator. The sole function of GacA is to activate transcription of the two sRNAs, RsmY and RsmZ. These sRNAs inhibit the function of RsmA by binding directly to it and thereby altering its function. The chronic infection mode is characterized by expression of the type III secretion system, type IV pili biogenesis genes, and iron homeostasis genes [17], whereas the chronic state is characterized by expression of some of the type VI

secretion system clusters, phenazine biosynthesis genes, and the *pel*/*psl* biofilm operons. This regulation occurs through a combination of direct and indirect regulation at both the transcriptional and post-transcriptional levels through RsmA [17]. However, the signal that activates this system is not yet known. Previous studies have found this system to be a hotspot for mutations during infection of the CF airways [9], [21]. Curiously, in the study by Marvig, et al., [9] mutations in this system occurred in a specific order with *retS* always mutating before the *gacS*, *gacA* or *rsmA* genes.

The advent of next generation sequencing has enabled the rapid determination of the entire RNAome [22], [23] with relative ease through RNA-sequencing (RNA-seq), providing a snapshot of the transcriptional landscape of the desired microbe at a certain point in time. This has made it possible to study transcriptomic changes at an unprecedented level of detail, opening up a new world of understanding of cell biology and physiology. The process involves growing the culture, extracting RNA, converting to cDNA, and sequencing on a next-gen platform.

Here the effects of spontaneous mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway in clinical isolates of the *P. aeruginosa* lineages, DK17 and DK41, are investigated using transcriptomics by RNA-seq. Initially, we examine the transcriptomes of six isolates in total from two lineages containing multiple mutations in various genes, but also what appears to be contingent mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway, meaning that mutations appear in a specific order in this system (first *retS* and later *gacS*) during infection of the CF airways. Hereafter, we first moved the mutated version of *retS* into the "wild type" strains of DK17 and DK41 (R and 366, respectively), followed by a second event in which we also inserted the mutated version of *gacS* in order to examine the specific effects caused by the mutations. Afterwards, we moved the "wild-type" versions of these genes into the clinical isolates with the mutated versions of the same genes to determine the effects of the mutations that do not occur in this signaling pathway (in this called 'residual evolution'. Differences in evolution were observed in the DK17 and DK41 lineages. In DK17 there was an increased expression of the phenazine biosynthesis operons *phz1* and *phz2* caused by both the *retS*-mutation in the signaling pathway and the residual mutations. However, in DK41 only the *retS*-mutation caused increased expression of these operons, whereas the residual evolution caused

reduced expression of the operons, effectively cancelling out the effects of each other in the purely clinical isolate.

This work increases the knowledge on the evolution and adaptation of *P. aeruginosa* in the CF airways and shows that single mutation genomic analysis may not be sufficient to accurately describe the possibly convergent evolutionary trajectories found in clinical *P. aeruginosa* isolates.

Results

Strains and allelic replacements

The strains used in this study have been isolated from two young CF patients. The DK17 isolates originate from a female patient born in 1996 with the first isolate (R) being from early 2007, the middle (G) from later 2008, and the last from the middle of 2011. The DK41 isolates originate from a male patient born in 2001 with the first isolate (366) being from the middle of 2008, the middle isolate (380) from early 2010, and the last isolate (364) being from the middle of 2012. Both lineages contain what appear to be contingent mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway, meaning that in both lineages a strain without mutations in this system infects the patients. After approximately two years, a *retS*-mutation appears in the population and subsequently, after approximately two more years, a *gacS*-mutation appears. However, during this period many other residual mutations also appear. In order to examine the effects of the mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway, both the functional and mutated versions of *retS* and *gacS* were moved between these clinical isolates (Figure 1A+B).

Transcriptomic evolution of clinical isolates

All cells were grown to late exponential phase in LB medium and RNA was harvested. cDNA libraries were prepared and sequenced on Illumina NextSeq. The reads were mapped and quantified to the genome of *P. aeruginosa* PAO1 and were checked for differential expression (2-fold, $p < 0.05$). Initially, we examined the transcriptomic evolution of the clinical *P. aeruginosa* isolates, meaning that we were investigating not only the effects of the mutations in *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway, but also the residual mutations. The initial step of evolution occured over a period of about two years for both DK17 and DK41, where both lineages evolved a mutation in *retS*, resulting in strains G and 380, respectively. In DK17, a single-nucleotide polymorphism occured in *retS* causing a premature stop-codon in the middle of the gene, likely leading to loss of function. In DK41, an 11-bp deletion occured about one third into the genes, leading to a frameshift and likely loss-of-function. Comparing the ‘wild-type’ and *retS*-mutant clinical isolates of both lineages, we found that 203 common genes were differentially expressed (2-fold change, $p < 0.05$)

between both lineages. Surprisingly, only 78 of these were differentially expressed in the same direction, meaning that the remaining 135 genes were differentially expressed in opposite direction. Only 25 showed decreased expression in DK17, whereas 102 showed decreased expression in DK41. Some genes of the hcp Secretion Island-I-encoded type VI secretion system (H1-T6SS) (PA0070-PA0091) [12] had increased expression in both *retS* mutants (2-8-fold). The H2-T6SS (PA1656-1671) [24] also had increased expression of 2-10-fold. However, there is no evidence of differential expression of the H3-T6SS (PA2359-PA2373) [25], fitting with reports that this system is not regulated by RetS [25], [26]. Furthermore, the galactophilic lectin, *lecA* [27] (PA2570), show 14-fold and 6-fold increased expression in DK17 and DK41, respectively. Surprisingly, none of the type III secretion system (PA1690-PA1725) genes were found to be differentially expressed in both lineages, even if they have been shown to be regulated by the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway [20]. The *phz1* (PA4210-PA4216) and *phz2* (PA1899-PA1905) phenazine biosynthetic operons have also been reported to be regulated directly by RsmA [28]. However, differential expression was only observed in DK17, where both the *phz1* and *phz2* operons showed a 4-16-fold increased expression in the *retS*-mutant, consistent with observations of green pigmentation during growth (data not shown).

Later in the infection process, we observed that both DK17 and DK41 acquire an additional mutation in *gacS*, the next step in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway, resulting in strains M and 364, respectively. These mutations were again likely loss-of-function mutations, as they were a 4-bp insertion and a 2-bp insertion in DK17 and DK41, respectively. The double mutants had 100 genes in common that were differentially expressed when compared with the previous single *retS*-mutants in the lineages, G and 380. In this case, all 100 genes were differentially expressed in the same direction; 93 showed decreased expression and 7 showed increased expression. Given the reciprocal nature of the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway [29], [30], it would be expected that the H1-T6SS and H2-T6SS operons showed decreased expression in the *retS*, *gacS*-mutants. Indeed, this was the case as the H1-T6SS is under expressed 14-fold in both lineages and the H2-T6SS is under expressed 11-fold. Also the APR-type I secretion system (PA1245-PA1249) [31], [32] was under expressed 4-fold in both lineages. Interestingly, again the *phz1* and

phz2 operons were only found to be differentially expressed in DK17 showing a 32-fold under expression for both operons.

Mutations in *retS* and *gacS* cause large scale transcriptional changes

In order to determine the effects of only the *retS* and *gacS* mutations in the genetic background of both lineages, we replaced first the *retS* gene of the 'wild-type' of both lineages with the mutated versions from the clinical strains, resulting in strains RR^M and $366R^M$, and subsequently also replaced the *gacS* genes with their respective mutated versions, resulting in strains RRG^M and $366RG^M$. Comparing strains R and 366 with the RR^M and $366R^M$, we identified 497 genes that were commonly differentially expressed in both lineages, 294 more than between the clinical isolates. This suggests that the other mutations that occured alongside the *retS*-mutations in the clinical isolates made the effects of the *retS*-mutations less pronounced through epistasis. Furthermore, 387 of these genes were differentially expressed in the same direction with 436 and 376 genes showing increased expression in DK17 and DK41, respectively.

The H1-T6SS operon was overexpressed in both lineages (4-fold) in the *retS*-mutants. Also the H2-T6SS operon showed increased expression; 6-fold in DK17, and 16-fold in DK41. Surprisingly, while the *phz1* and *phz2* operons did not show any differential expression from strain 366 to 380 for DK41, they did in the 'wildtype' with the mutated *retS*, 366 vs $366R^M$. In fact, DK17 showed a 4-fold increased expression of *phz1* operon and 6-fold of the *phz2* operon, whereas DK41 showed a 5-fold and 8-fold increased expression, respectively. This suggests that some of the other mutations in the clinical DK41 *retS*-isolate, 380, had an effect on expression of the *phz1* and *phz2* operons and that differential expression of these was selected against. Another observation that was not observed in the clinical isolates, was in an increase of expression of *rpoS* (PA3622), the sigma factor associated with stationary phase/stress response and regulation of quorum sensing [33], [34]. The differential expression of *rpoS* could be associated with the aggregation seen during growth (not shown), since this could be expected to limit nutrient uptake of the bacteria, and would be expected to have a big influence on the transcriptome of *P. aeruginosa*. In line with this, the expression of *rhlR* (PA3477), the transcriptional regulator of the *rhlAB* operon (PA3478-9) [35], and the *rhlAB* operon itself, the genes responsible for the biosynthesis of rhamnolipids, showed a 20-fold increased expression in

DK17 and 7-fold increased expression in DK41 in strains RR^M and $366R^M$ compared to R and 366, respectively. A 20-fold increase in expression of *lasA* (PA1871) and *lasB* (PA3724) was also observed in both lineages, along with a 2-3-fold increase in expression of *lasR* (PA1430), their transcriptional regulator [36]. No differential expression of the T3SS was observed, suggesting that either this system is not controlled by the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway in these lineages or that the conditions, in which the strains were grown, were not conducive to the expression of the T3SS.

Interestingly, some of the genes that were not differentially expressed in the same direction are the genes of the *atp*-operon (PA5553-PA5561), encoding the subunits of the ATP synthase. In DK17, they showed a 2.5-fold increase in expression whereas they showed a 6-fold decreased expression in DK41.

After introducing the *gacS* mutations into the RR^M and $366R^M$ (resulting in strains RRG^M and $366RG^M$, respectively), no fewer than 1447 genes were differentially expressed commonly between the lineages, comprising just about one third of the genome of *P. aeruginosa* when comparing RR^M with RRG^M and $366R^M$ with $366RG^M$. Surprisingly, 1425 of these were differentially expressed in the same direction. In both lineages, 813 of these showed increased expression whereas 612 showed decreased expression. These numbers seem exceedingly high, however considering the reciprocal nature of the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway regulating around 500 genes [17], the aggregation of *retS*-mutants (not shown), and the increased expression of *rpoS* of RR^M and $366R^M$, regulating 772 genes [34], it could be possible. Furthermore, it turns out that *rpoS* was expressed at a 3-fold and 4-fold lower level in RRG^M and $366RG^M$, respectively. Additionally, *rpoD*, the principal sigma-factor [37], was expressed at a 2-fold and 3-fold higher level for RRG and 366RG, respectively. Taking all this into account, it is not unlikely that this would lead to a complete lifestyle change for *P. aeruginosa* and thus massive transcriptomic changes.

In the double mutants, RRG^M and $366RG^M$, compared to the 'wild-types' with *retS*-mutations, RR^M and $366R^M$, a 14-fold and an 11-fold decrease in expression was observed for the H1-T6SS for DK17 and DK41, respectively. Also the second cluster, H2-T6SS, showed 38-fold and 54-fold decrease in expression for DK17 and DK41, respectively. Interestingly, the T3SS cluster was overexpressed in these strains with a 12-fold higher expression in both lineages. The *phz1* and *phz2* operons were differentially regulated with a much

lower expression in the double mutants compared to the RR^M and $366R^M$. DK17 showed 72-fold lower expression of the *phz2* operon, whereas DK41 showed a 29-fold decrease. A similar pattern was observed for the *phz1* operon, where it was expressed 63-fold lower in DK17 and 20-fold lower in DK41.

Another observation is that the cluster containing the ribosomal proteins and RNA polymerase genes (PA4277-4237) is upregulated 7-fold on average in DK17 and 4-fold on average in DK41, possibly suggesting increased transcriptional activity, which could be linked to the increased expression of *rpoD* and decreased expression of *rpoS*.

Residual mutations also affect the expression of virulence factors

In order to determine the effects of residual evolution, the evolution/transcriptomic changes not caused by mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway, we also moved the 'wild-type' versions of *retS* and *gacS* into the clinical isolates (G, M, 380, and 364) containing the mutated version of the aforementioned genes of the respective lineages. This resulted in GR^{WT} (G with 'wild-type' *retS*), MRG^{WT} (M with 'wild-type' *retS* and *gacS*), $380R^{WT}$ (380 with 'wild-type' *retS*), and $364RG^{WT}$ (364 with 'wild-type' *retS* and *gacS*). Examining only the genes that were differentially expressed in both lineages for the strains R and 366 versus strains GR and 380R, we observed that 413 genes are differentially expressed with 349 being in the same direction. Surprisingly, we observed that some of the genes that were differentially expressed in RR^M and $366R^M$ were also differentially expressed in the clinical isolates, strains GR^{WT} and $380R^{WT}$, without the mutation in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway. For example, nine genes in the H1-T6SS cluster were overexpressed with a 4-fold and 3-fold increase in both GR^{WT} and $380R^{WT}$, respectively. However, a similar trend was not observed for the H2-T6SS. Surprisingly, the T3SS showed decreased expression in DK17 for GR^{WT} when compared to R (8-fold), but the opposite happens for DK41, where $380R^{WT}$ showed a 10-fold overexpression compared to 366. Considering that no differential expression was observed between R and G, and 366 and 380, this suggests that mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway and the mutations outside of this system have an epigenetic effect.

In GR and 380R, we also identified differential expression of some resistance-nodulation-cell division multidrug efflux pump operons. In GR, there was an 18-fold increased expression of *mexXY* (PA2018-2019), and a smaller 3-fold increase in 380R. The *mexEF-oprN* operon was also differentially expressed, but in the other direction. Here we observed a 5-fold decrease in GR and a 60-fold decrease in 380R.

The next steps of residual evolution were contained in the subsequent strains (MRG^{WT} and $364RG^{WT}$), where we have moved the *retS* and *gacS* of R and 366 into strains M and 364, respectively. In this case 95 genes were differentially expressed in both lineages with 68 in the same direction. Most of these genes were scattered through the genome and are either hypothetical proteins or tRNAs. *MexCD* were the only genes of note that are upregulated in both lineages with 65-fold in DK17 and just 3-fold in DK41.

Combination effects of mutation

An example of epigenetic effects was the two phenazine biosynthesis operons, *phz1ABCDEFG* and *phz2ABCDEFG*, that showed markedly different expression patterns in the two lineages when they were subject to *retS*-mutations and the residual evolution (Figure 2A). In RR^{M}, the mutated *retS* gene lead to an increase of expression of 4-fold on average of both operons when compared with R (shown as RR^{M}/R). However, when GR^{WT} is compared with R (shown as GR^{WT}/R) an increase of the phenazine biosynthesis operons WAS still observed with an average of 3-fold. This shows that the residual evolution was also in part responsible of the increased expression of phenazine biosynthesis operons even in absence of the effect related to a *retS*-mutation. Moreover, looking at the clinical isolate G, we observed a combined effect possibly due to both the mutated *retS* and the residual evolution; indeed, we observed an 8-fold over expression on average of both operons (shown as G/R). Therefore, we suggest the possibility of a combination effects of the *retS*-mutation and the residual evolution.

Interestingly, in DK41 regarding the phenazine biosynthesis operons we observed an opposing trend in terms of expression contribution deriving from mutated *retS* and residual evolution (Figure 4B). Here, moving the mutation in *retS* into the 'wild-type' ($366R^{M}$/366) resulted in an increase of expression of 6-fold on average for both *phz* operons, which is higher than what was seen in DK17. However, the genetic background of

380R^{WT} vs. 366 has the opposite effect of G vs. R. For 380R^{WT} compared to 366, we observed an 8-fold decrease in expression of the *phz1* and *phz2* operons. Combining the mutation occurring in *retS* with the residual evolution, the clinical isolate, 380, we observed an expression profile of the *phz1* and *phz2* operons that was very similar to the clinical isolate, 366. In fact, the difference was not sizeable enough to be significantly different. However, as above, the effects of the *retS*-mutation and the residual evolution seem to combine.

Discussion

The adaptation of *P. aeruginosa* to the CF airways is usually recognized as being driven by the loss of virulence factors with the concept being that this would render the bacteria capable of hiding from the immune system [38]. However, here we show that the picture may be more nuanced. Firstly, it appears that different secretion systems are needed at different time points during infection. The overexpression of H1-T6SS and H2-T6SS suggests that these secretion systems are needed during the early stages of infection. However, later the second mutation in this system effectively switches off the expression of the T6SS and instead switches on the T3SS. Furthermore, we also observe that what appears to be signs of convergent evolution on a genomic level, may not necessarily lead to the same effects on a transcriptomic level.

The changes in expression of the *phz1* and *phz2* operons exemplifies that while both lineages show similar mutations in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway from a purely genomic point of view, it is not possible to infer that these mutations will result in similar effects on a transcriptomic or phenotypic level. It appears that while mutating *retS* in the clinical isolates of R and 366 results in an increase of transcription of the *phz1* and *phz2* operons, the mutations that occur alongside the *retS*-mutation, pull the expression in opposite directions in these two lineages. This could mean that the evolution of *P. aeruginosa* in CF patients may show signs of convergence on a genomic level, but, in truth, the larger picture is more refined as an infecting lineage accumulates mutations in many genes that could have pleiotropic effects. The strains come from different lineages that have different genetic background (>10000 SNPs) [9], and it may be optimistic

to expect that they would evolve and behave in the same manner. Furthermore, they were isolated from different patients, which only adds to the noise.

The biological consequences of this differential expression of the phenazine biosynthesis operons for the infection of the CF airways are not known. It has previously been shown that pyocyanin is required for full virulence of *P. aeruginosa* and is thought to have a variety of effects, including defense against the host and functioning as a terminal electron acceptor for respiration [39], meaning that it can help *P. aeruginosa* survive under low-oxygen conditions [14], which can occur in the CF airways [40]. Furthermore, they negatively affect a number of eukaryotic processes including respiration and electron transport.

It also appears that the mutations in *retS*, have a profound effect on the lifestyle of *P. aeruginosa* in general. The observation that transcript levels of *rpoS* increase in strains containing *retS*-mutations confirms this. It has been shown that biofilms of *P. aeruginosa* show increased expression of RpoS-activated transcripts [41] and that nutrient limited *P. aeruginosa* are highly tolerant to antibiotics [42]. This could suggest that this initial *retS*-mutation is a response to the antibiotic treatment pressure that *P. aeruginosa* experiences in the CF airways.

This does not, however, explain why an apparent reversion in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway occurs by mutations in *gacS*. This mutation switches *P. aeruginosa* into acute infection mode, which would seem to be unviable considering the supposed decreased antibiotic resistance caused by the *retS*-mutations mediated through increased expression of *rpoS*-related genes. A previous study by Sall, et al., [43], also observed a mutation in *gacS* in the *P. aeruginosa* strain, CHA. They found that this mutation lowered transcript levels of H1-T6SS and completely abolished protein production of the same. Additionally, they found increased expression of the T3SS and found that it was more virulent in a murine acute model of lung infection. If this is transferrable to *P. aeruginosa* infecting the CF airways, it could have detrimental effects on the patient.

Conclusion

In this study, we have examined 14 transcriptomes of six clinical isolates and eight strains of *P. aeruginosa* that were genetically engineered in the *retS-gacS-gacA-rsmA-rsmYZ* signaling pathway. We show that while similar mutations occur in regulatory systems, this does not mean that we can automatically infer that this will lead to similar transcriptomic or phenotypic effects. It appears that both the genetic background in which these mutations occur and their interplay with other mutations can have surprising effects on genes that may be of clinical importance in CF. This leads to the conclusion that for complex evolutionary patterns and systems, single mutation analysis may not be enough, that mutations have epigenetic effects on each other, and that the genetic background of a given strain plays a large role in differential gene expression.

Materials and methods

Strain handling

Strains (Table 1) were grown at 37 °C in LB. Antibiotic concentrations: *P. aeruginosa*: 50 µg/mL for DK17-derived strains and 200 µg/mL for DK41-derived strains. *E. coli*: 10 µg/mL gentamicin for pEX19Gm constructs and 25 µg/mL kanamycin for *E. coli*/pRK2013.

Genetic constructs

Primers (Table 2) were designed to amplify approximately 800 bp of the desired gene with the desired mutation centered in the fragment using Phusion Hot Start II DNA polymerase with GC buffer. Fragments were gel-purified and cut with the appropriate restriction enzymes according to the manufacturer specifications. The vector, pEX19Gm, was also cut with the appropriate restriction enzymes and gel-purified. Ligation mixtures were set up in a ratio of 5:1 (insert:vector). Ligation occurred for an hour using T4 DNA ligase at room temperature resulting in plasmids in Table 3. Electrocompetent *E. coli* were electrotransformed with the ligation mixtures and incubated at 37 C for 1 hour and plated on LB plates containing 10 µg/mL gentamicin and left O/N at 37 C. The presence of the plasmid was confirmed by colony PCR.

Allelic replacements

Allelic replacements were made using triparental mating. Receptor, helper, donor strains were plated on LB plates containing the appropriate antibiotic and grown for 24-48 h, until an appropriate cell size was obtained. The receptor strains were incubated in 5 mL LB at 42 °C O/N in 50 mL falcon tubes, the helper strain was incubated in 5 mL LB O/N containing 25 µg/mL kanamycin at 30 °C, and donor strains were incubated in 2.5 mL LB containing 10 µg/mL gentamicin at 30 °C O/N. Receptor, help, and donor strains were mixed in a 3:1:1 ratio in a 1.5 mL Eppendorf tube and washed twice with 1 mL of LB with centrifugation at 6500 g for 2 min. The supernatant was discarded and the pellet was resuspended in the remaining supernatant. The pellets were spotted onto LB plates and left at 30 C for at least 6 hours. The mating drops were resuspended in LB and plated on LB plates containing an appropriate amount of gentamicin and incubated at 37 °C for 48 hours. Correct integration of the plasmid was checked using up_fwd/dw_rev and M13fwd/M13rev primers. Strains with integrated plasmids were then cultured O/N and plated on LB plates containing 10% sucrose. Strains were subjected to colony PCR, and correct strains were verified by sanger sequencing (Table 3).

RNA-extraction

Strains were grown in 50 mL LB in baffled shake flasks at 37 °C with shaking at 200 rpm at an initial concentration of OD_{600} = 0.01 and were harvested at OD_{600} = 1. A 10 mL volume of culture was added to 2 mL of ice-cold STOP-solution (95% EtOH, 5% phenol), vortexed vigorously for 15 seconds, incubated at room temperature for 5 min, and vortexed for 5 min (7000 g, 4 °C). The pellet was resuspended in 1 mL of TRIzol® and stored at -80 °C until further use. RNA was extracted using Qiagen© RNeasy mini kits according to manufacturer instructions and the RNA integrity was checked on Agilent Bioanalyzer using the Agilent RNA 6000 Nano Kit. All experiments were conducted with biological duplicate samples.

Data-handling

Read quality was evaluated using FastQC [44]. The reads were mapped using Rockhopper to the genome of *P. aeruginosa* PAO1 and the raw count values were fed to T-REx [45], which uses EdgeR [46] to analyze the

data. Normalization applied was weighted trimmed mean of M-values and genes with a low number of reads were filtered out. Further data handling was conducted in R – statistical computing package [47] and Microsoft Excel.

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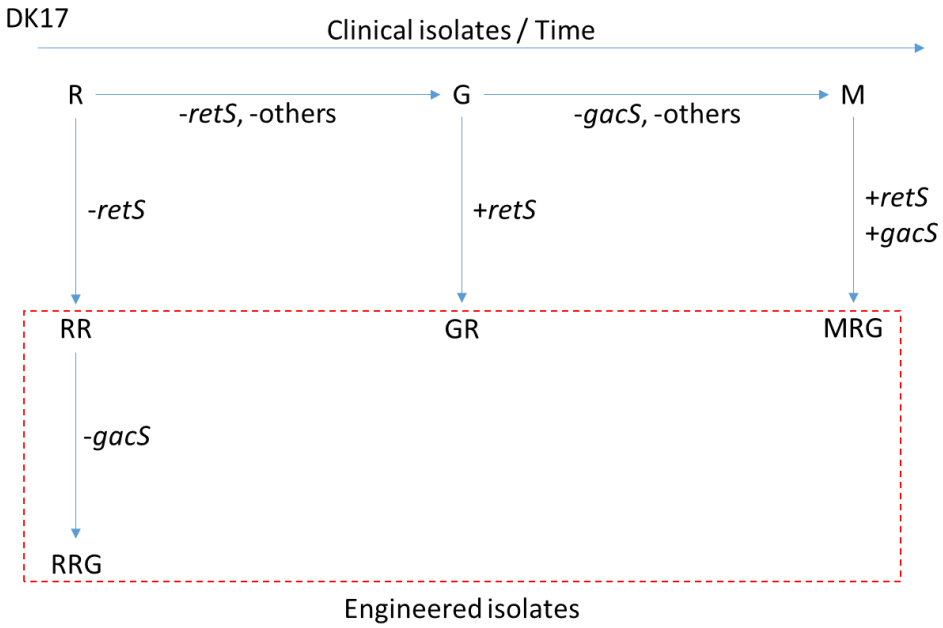
Figures



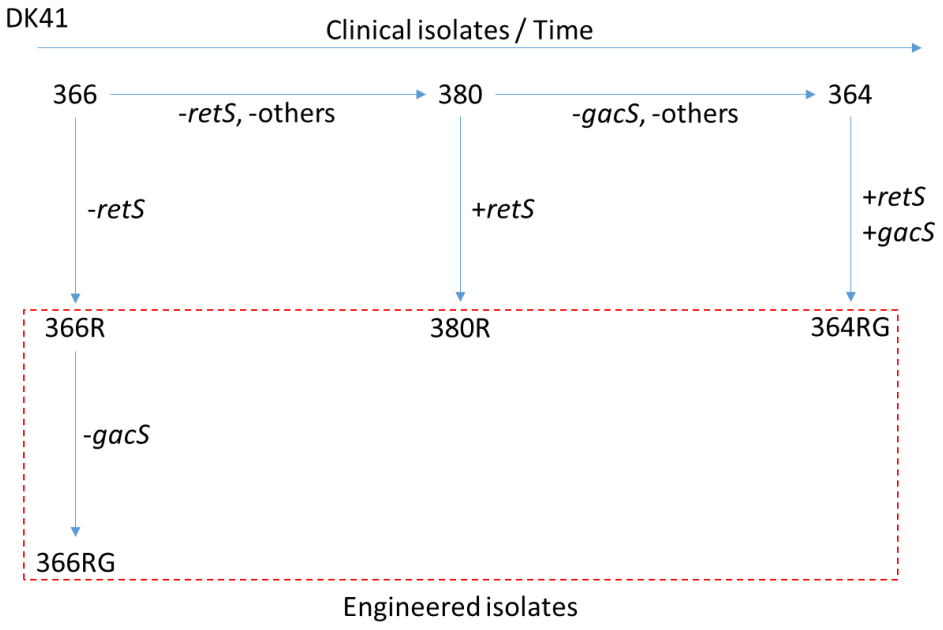
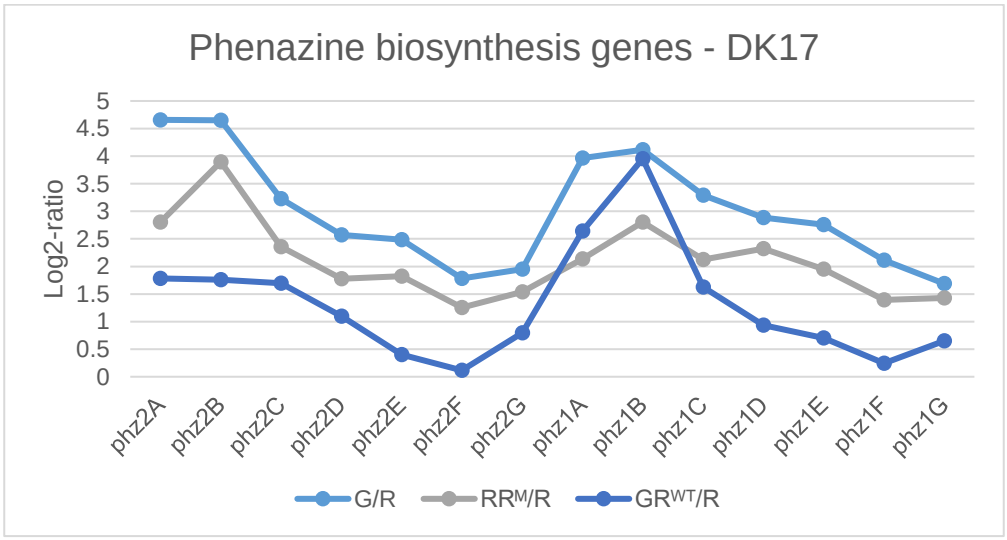


Figure 1A+B. The strategy behind the allelic replacements of *retS* and *gacS*. The timelines are not to scale. For both DK17 and DK41, we moved the mutated versions of *retS* and *gacS* into naïve clinical isolates that have not yet evolved mutations in these genes. This results in strains RR^{M} (R with mutated *retS*), RRG^{M} (R with mutated *retS* and *gacS*), $366R^{M}$ (366 with mutated *retS*), and strain $366RG^{M}$ (366 with mutated *retS* and

gacS). Furthermore, we also moved the presumably functional versions of *retS* and *gacS* into the strains that evolved mutations in the same genes in the patients, resulting in strains GR^{WT}, MRG^{WT}, $380R^{WT}$, and $364RG^{WT}$.



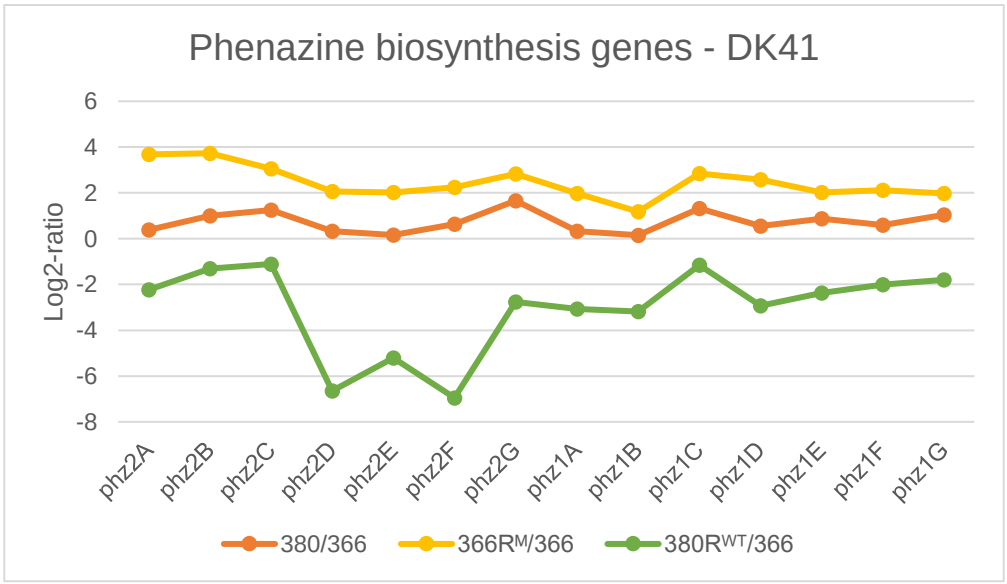


Figure 2A+B. The $\log_2$-ratios of expression the *phz2* (PA1899-1905) and *phz1* (PA4210-4216) operons. All expressions are normalized to the lineages' 'wild-types'.

Tables

Table 1. Strains used in this study.

Strains	**Source**	**Genotype**
R	Clinical isolate	MRCA
G	Clinical isolate	*retS*
M	Clinical isolate	*retS*, *gacS*
366	Clinical isolate	MRCA
380	Clinical isolate	*retS*
364	Clinical isolate	*retS*, *gacS*
RR^{M}	Strain "R" with *retS* mutation from G	*retS*
RRG^{M}	Strain "R" with *retS* mutation from "G/M" and *gacS* mutation from "M"	*retS*, *gacS*
GR^{WT}	Strain "G" with *retS* from strain "R"	*retS*^{WT}
MRG^{WT}	Strain "M" with *retS* and *gacS* from strain "R"	*retS*^{WT}, *gacS*^{WT}
366R^{M}	Strain "366" with *retS* mutation from strain "380"	*retS*
366RG^{M}	Strain "366" with *retS* mutation from "380/364" and *gacS* mutation from "364"	*retS*, *gacS*
380R^{WT}	Strain "380" with *retS* from strain "366"	*retS*^{WT}
364RG^{WT}	Strain "364" with *retS* and *gacS* from strain "366"	*retS*^{WT}, *gacS*^{WT}
E. coli DH5α/pEX19Gm	Cloning / donor	
E. coli/pRK2013	Helper in allelic replacements	

Table 2. Primers used in this study. Bold text signifies restriction enzyme site.

Name	**Sequence (5'-3')**	**Purpose**
HindIII_retS_M_fwd	ATAT**AAGCTT**GGCACCAAGCAACTCGAT	Cloning
EcoRI_retS_M_rev	ATAT**GAATTC**CAGGTTTCGTTGTCGTCCA	Cloning
retS_M_up_fwd	GTGTTCCTGCCGGTACTGTT	Validation
retS_M_dw_rev	ACTGCTGCACCAGCACCTT	Validation

HindIII_gacS_M_fwd	ATAT**AAGCTT**CAGTTCGTCCAGCTCGTTG	Cloning
EcoRI_gacS_M_rev	ATAT**GAATTC**CTTCGTCGCAAGCCGAAT	Cloning
gacS_M_up_fwd	GTTGTGCTGCATTTCCTCCT	Validation
gacS_M_dw_rev	CAATCGTGCCAGTATTCACG	Validation
HindIII_retS_364_fwd	ATAT**AAGCTT**CTCGCGCTCCTACCTGTTCT	Cloning
EcoRI_retS_364_rev	ATAT**GAATTC**AGGAACTCGGCCTTGGTCT	Cloning
retS_364_up_fwd	CGGGTGCAGTACCTGGACTA	Validation
retS_364_dw_rev	GATCTCGTGGCTGATCTTGG	Validation
HindIII_gacS_364_fwd2	ATAT**AAGCTT**GTGGTGCGACAGTTCCAGTT	Cloning
EcoRI_gacS_364_rev2	ATAT**GAATTC**CGGAGTTGGCGAAGAATCTC	Cloning
gacS_364_up_fwd2	ATCAGCAAGAGGCTGGTGAA	Validation
gacS_364_dw_rev2	AGGGCTGACATCAGGATCAC	Validation
M13fwd	GTAAAACGACGGCCAG	Validation
M13rev	CAGGAAACAGCTATGAC	Validation

494

495 Table 3. Plasmids used in this study

Name	**Relevant features**
pEX19Gm	Gm^r, *oriT*, *sacB*, MCS
pEX19Gm::retS^{DK17}	800bp fragment of mutated *retS* from DK17, Gm^r, *oriT*, *sacB*, MCS
pEX19Gm::retS^{DK41}	800bp fragment of mutated *retS* from DK41, Gm^r, *oriT*, *sacB*, MCS
pEX19Gm::retS^{DK17WT}	800bp fragment of wildtype *retS* from DK17, Gm^r, *oriT*, *sacB*, MCS
pEX19Gm::retS^{DK41WT}	800bp fragment of wildtype *retS* from DK17, Gm^r, *oriT*, *sacB*, MCS
pEX19Gm::gacS^{DK17}	800bp fragment of mutated *gacS* from DK17, Gm^r, *oriT*, *sacB*, MCS
pEX19Gm::gacS^{DK41}	800bp fragment of mutated *gacS* from DK41, Gm^r, *oriT*, *sacB*, MCS
pEX19Gm::gacS^{DK17WT}	800bp fragment of wildtype *gacS* from DK17, Gm^r, *oriT*, *sacB*, MCS

pEX19Gm::gacS^{DK41WT}	800bp fragment of wildtype *gacS* from DK41, Gm^{r}, *oriT*, *sacB*, MCS

496