

Investigation of the Competitive Fitness of
Pseudomonas aeruginosa
in Interbacterial Competition

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1.4 Abbreviations used in this study

Abbreviation	Abbreviated term
Approx.	Approximately
AnhMurNAc	1,6-anhydro- <i>N</i> -acetyl muramic acid
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of variance
Bcc	<i>Burkholderia cepacia</i> complex
bp / kbp	base pair / kilo-base pairs
Cb	Carbenicillin
CF	Cystic fibrosis
CFTR	cystic fibrosis Transmembrane Conductance Regulator
CI	Competitive index
Cm	Chloramphenicol
C4-HSL	<i>N</i> -butyryl- <i>L</i> -homoserine lactone
Da / kDa	Dalton / kilo-Dalton
DI-H ₂ O	Deionized water
EC / <i>E. coli</i>	<i>Escherichia coli</i>
EU/EEA	European Union / European Economic Area
FACS	Fluorescence-activated cell sorting
gDNA	Genomic DNA
GlcNAc	<i>N</i> -Acetylglucosamine
Gm	Gentamicin
Irga	Irgasan
Kan	Kanamycin
LPS	Lipopolysaccharide
MM	Master Mix
MurNAc	<i>N</i> -Acetylmuramic acid
NCCT	Next generation sequencing core facility Tübingen
NGS	Next generation sequencing
OD	Optical density
p. / pp.	Page / pages
PA / <i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PBP	Penicillin-binding protein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PQS	<i>Pseudomonas</i> quinolone signal
pwCF	People with cystic fibrosis
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
rpm	Rotations per minute
RT	Room temperature (19-23°C)
sRNA	small RNA
TCS	Two-component-system
Tn	Transposon
TraDIS	Transposon-directed insertion site sequencing
T3SS	Type III Secretion System
T4SS	Type IV Secretion System

Abbreviation	Abbreviated term
T6SS	Type VI Secretion System
UV-Vis	Ultraviolet-visible
WHO	World Health Organization
3OC12-HSL	<i>N</i> -(3-oxododecanoyl)- <i>L</i> -homoserine lactone

2 Summary

2.1 Abstract

Pseudomonas aeruginosa (PA) and members from the *Burkholderia cepacia* complex (Bcc) are closely related bacterial species which are abundantly found in natural environments but are also feared opportunistic pathogens infecting the lungs of people with structural lung diseases like cystic fibrosis (CF). Both PA and Bcc show high rates of intrinsic antibiotic resistance and can easily spread within healthcare facilities due to their genetic flexibility. Both employ their syringe-like Type VI Secretion Systems (T6SS) to deliver toxic effector molecules into bacteria competing for the same resources. While this secretion apparatus has been the subject of extensive research ever since its discovery in 2006 because of its impact on both the virulence and competitive fitness of bacteria, the intricate regulatory network behind its activity is not yet fully understood.

Among the recently described toxic effectors of the T6SS of PA was the amidase AmpDh3. Because our group was examining the role of AmpDh3 in cell-wall recycling of PA and in the induction of the β -lactamase AmpC, we further investigated the secretion of AmpDh3 via the T6SS of PA. We found that AmpDh3, which had been described as a periplasmic cell-wall amidase, is in fact localized in the cytoplasm of PA, and that it is in fact not secreted via the T6SS.

In PA, three distinct T6SS clusters have been identified, all of which fulfill different functions. The H1-T6SS is likely to play a key role in bacterial competition and is known to be regulated by the *gac/rsm* signaling pathway, among others. Therefore, many studies investigating the activity and toxicity of the T6SS used genetic mutations in genes like *retS* or *rsmA* to artificially increase the activity of the T6SS of PA. Since many of the studies investigating the activity and toxicity of the T6SS used genetic mutations in genes like *retS* or *rsmA* to artificially increase the activity of the T6SS of PA via the *gac/rsm* pathway, we established an interbacterial competition assay to investigate the T6SS in competition with Bcc species without over-activating the T6SS. In addition to potentially creating artifacts stemming from T6SS over-activation of the T6SS, manipulating the *gac/rsm* system is known to regulate a multitude of cell responses which may impact the competitive fitness of *P. aeruginosa* in the presence of a competitor bacterium. Using fluorescently labelled PA mutants and unmodified competitor bacteria allowed us to investigate the importance of single effectors or combinations of effectors in the competitive fitness of PA in the presence of Bcc species. We found that the H1-T6SS, but not the H2-T6SS, helps PA to out-compete the Bcc strains, and that multiple effectors act in unison to kill prey cells.

In an attempt to identify further genes that contribute to the competitive fitness of PA in the presence of Bcc species, we employed a transposon-screening competition assay. We identified the histidine kinase AmgS as a previously unrecognized player in the competition of PA. We speculate that it might act as a regulator of GacS, analogously to the previously described sensor kinases *retS* and *ladS*, allowing PA to sense and respond to even more threats from the environment.

2.2 Zusammenfassung

Bei *Pseudomonas aeruginosa* (PA) und Arten des *Burkholderia cepacia*-Komplexes (Bcc) handelt es sich um nahe verwandte Bakterien, welche häufig in diversen natürlichen Habitaten vorkommen, aber ebenso gefürchtete opportunistische Krankheitserreger bei Personen mit strukturellen Lungenerkrankungen wie Cystischer Fibrose (CF) darstellen. Beide Pathogene zeichnen sich durch einen hohen Grad an genetischer Flexibilität und damit verbundener intrinsischer Antibiotikaresistenz sowie leichter Ausbreitung in Gesundheitseinrichtungen aus. Beide Bakterien setzen ihr nadelartiges Typ-VI-Sekretionssystem (T6SS) ein, um toxische Effektoren in konkurrierende Bakterien zu injizieren. Obwohl dieses Sekretionssystem aufgrund seiner Wichtigkeit für Virulenz und Konkurrenz seit seiner Entdeckung im Jahr 2006 Gegenstand umfassender Forschung war, ist das komplexe Netzwerk seiner Regulation noch nicht vollständig verstanden. Unter den kürzlich beschriebenen Effektoren des T6SS von PA findet sich die Amidase AmpDh3. Da unsere Arbeitsgruppe sich mit der Rolle von AmpDh3 im Zellwand-Recycling von PA und der Induktion der β -Lactamase AmpC beschäftigte, untersuchten wir zudem zunächst die Sekretion von AmpDh3 durch das T6SS von PA näher. Dabei fanden wir heraus, dass AmpDh3, welches zunächst als periplasmatische Zellwandamidase beschrieben worden war, wahrscheinlich im Zytoplasma lokalisiert ist und nicht durch das T6SS sekretiert wird. In PA sind drei distinkte T6SS-Cluster mit verschiedenen Funktionen bekannt. Obwohl viele Signalwege parallel das T6SS von PA regulieren, scheint der *gac/rsm*-Signalweg eine zentrale Rolle in der Regulation des antibakteriellen H1-T6SS einzunehmen. Da viele Studien zur Aktivität und Toxizität des T6SS durch genetische Mutationen in Genen wie *retS* und *rsmA* die Aktivität des T6SS artifiziell erhöhen, etablierten wir einen interbakteriellen Wettbewerbsassay, um das T6SS in Konkurrenz mit Bcc-Spezies zu untersuchen, ohne das T6SS selbst zu überaktivieren. Außerhalb des Risikos des Entstehens möglicher Artefakte aufgrund der Überaktivierung des T6SS kann eine Manipulation des *gac/rsm*-Systems eine Vielzahl von Zellantworten beeinflussen, welche die kompetitive Fitness von *P. aeruginosa* in Anwesenheit eines Wettbewerbsbakteriums beeinträchtigen können.

Mittels fluoreszenter PA-Mutanten und nicht-manipulierter Wettbewerbs-Bakterien konnten wir die Bedeutung einzelner Effektoren sowie die von Effektor-Kombinationen auf die kompetitive Fitness von PA in Gegenwart von Bcc-Spezies untersuchen. Wir fanden heraus, dass das H1-T6SS, aber nicht das H2-T6SS PA dabei hilft, sich gegen Bcc-Stämme durchzusetzen, und dass daran mehrere Effektoren in Zusammenarbeit beteiligt sind.

Um weitere Gene zu identifizieren, welche zur kompetitiven Fitness von PA in Gegenwart von Bcc-Spezies beitragen, wendeten wir ein transposon-basiertes Wettbewerbs-Screening an. Wir konnten dadurch die Histidinkinase AmgS als zuvor unbekannten Mitspieler in der

Kompetition von PA identifizieren. Wir gehen davon aus, dass AmgS analog zu den zuvor beschriebenen Histidinkinasen RetS und LadS einen weiteren Regulator von GacS darstellen könnte, welcher es PA erlaubt, weitere externe Stimuli in seine Verteidigungsstrategien zu integrieren.

3 Introduction

3.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an aerobic, Gram-negative bacterium that is abundant in soil and on plants (Green et al., 1974). It has a large genome comprising between 6.3 - 6.5 Mbp in the strains used in this study (D. G. Lee et al., 2006). It is also one of the most frequent pathogens in hospital-acquired pneumonia and ventilator-associated pneumonia (Barbier et al., 2013) as well as in hospital-acquired bacteremia (Magill et al., 2014) and burn wound infections (Azzopardi et al., 2014), but it is also known to cause urinary tract infections in patients with underlying disease like hypertension (Lamas Ferreira et al., 2017). Most bloodstream infections with *P. aeruginosa* arise from lung or urinary tract infections (Thaden et al., 2017).

Severe *P. aeruginosa* infections mainly occur in immunocompromised patients, e.g. those suffering from AIDS or cancer-related neutropenia (Franzetti et al., 1992; Kielhofner et al., 1992; Kim et al., 2017), but notably also patients with cystic fibrosis, where the pathogen enhances inflammation and accelerates damage to the airways (Rosenfeld et al., 2001; Sagel et al., 2009). In healthcare settings, *P. aeruginosa* can be isolated from floors, nurses' hands and bed rails, contaminated medical equipment, but most importantly sinks, faucets and other water-related sources (Blanc et al., 2004; Chitkara & Feierabend, 1981; Hota et al., 2009; Schelenz & French, 2000). Hospital-acquired colonization and infections with *P. aeruginosa* can often be traced back to contaminated hospital water from showers or faucets (Blanc et al., 2004; Rogues et al., 2007).

This thesis was focused on better understanding the interactions of *P. aeruginosa* with competitor species and the interconnection between virulence factors, antibiotic resistance and interspecies competition. As an example, AmpDh3 at the start of this project was described to play a role in β -lactam resistance, in cell wall recycling and as an anti-bacterial effector. In the following sections, I will therefore introduce those aspects, starting with antibiotic resistance mechanisms, the interleaved regulatory pathways involved in the regulation of resistance and virulence with a focus on quorum-sensing mechanisms, and finally the mechanisms of antibacterial competition with focus on the Type VI secretion system.

3.2 Antimicrobial resistance mechanisms

Pseudomonas aeruginosa infections are infamous for being difficult to treat due to a wide variety of antibiotic resistances, which can be intrinsic and due to efflux pumps, or be acquired via gene transfer or mutations (reviewed in Lister et al., 2009). According to the latest annual report from the European Antimicrobial Resistance Surveillance network (EARS-Net) from 2022, 32.4% of all reported *P. aeruginosa* strains in the EU/EEA were

resistant to at least one antimicrobial class, with large geographical variation within the EU in the prevalence of antibiotic resistance. Since 2018, the occurrence of fluoroquinolone-resistant strains has steadily decreased, but the number of reported *P. aeruginosa* strains that are resistant to piperacillin-tazobactam combination therapy has increased since 2018. Approx. 4% of all reported isolates in 2022 were resistant to five different groups of antibiotics, comprising piperacillin-tazobactam, fluoroquinolones, ceftazidime, aminoglycosides, and carbapenems (ECDC, 2023). Due to their very limited treatment options in infections, carbapenem-resistant *P. aeruginosa* strains were ranked second in the priority list of the WHO among pathogens causing severe health burden, which demands discovery, research and development of new antibiotics (World Health WHO, 2017). The following section will give an overview of some of the most notable resistance mechanisms of *P. aeruginosa*.

3.2.1 AmpC β -lactamase

AmpC is a chromosomally encoded cephalosporinase that is found in *P. aeruginosa* and in many *Enterobacteriaceae* (Lister et al., 2009). The *ampC* gene, encoding for a periplasmic penicillinase, was first identified in the genome of *E. coli* K-12 (Burman et al., 1973). Initially found to render bacteria resistant to penicillin G and ampicillin (Burman et al., 1973), AmpC β -lactamases are now known to mediate resistance to most penicillins and cephalosporins, but have low affinities for cefepime and carbapenems (Colom et al., 1995; Jacoby, 2009; Livermore, 1995). In *Pseudomonas aeruginosa*, the carbapenem imipenem even acts as a strong inducer of AmpC (Sanders et al., 1997). Some β -lactamase inhibitors like sulbactam and tazobactam inhibit AmpC-mediated β -lactam resistance, while others like clavulanate can even induce the β -lactamase dose-dependently (Kitzis et al., 1989).

3.2.2 PBP4

PBP4 is a non-essential DD-carboxypeptidase encoded by the *dacB* gene (Iwaya & Strominger, 1977; Matsushashi et al., 1977; Suzuki et al., 1978). Inactivation of PBP4 leads to overproduction of AmpC (Moya et al., 2009). The precise mechanism is still unknown because in *P. aeruginosa*, PBP4 is not the primary carboxypeptidase used in cell wall remodeling. Instead, unlike in other species, PBP5 is used. (Ropy et al., 2015). PBP4 mutants seem to arise under ceftazidime treatment, rendering mutants resistant to the antibiotic (Ramsay et al., 2023). The clinical *P. aeruginosa* isolate ID40 used in this study is known to carry a PBP4-inactivating point mutation in its *dacB* gene (Sonabend et al., 2020).

3.2.3 Efflux pumps: MexAB-OprM, MexXY-OprM and MexEF-OprN

In a large meta-analysis of bacterial genomes, antibiotic efflux mechanisms were recently confirmed as the main mechanism contributing to antibiotic resistance in *P. aeruginosa*

strains isolated from blood and soil (Oliveira et al., 2020). The main drug efflux pumps employed by *P. aeruginosa* belong to the Resistance Nodulation Cell Division (RND) superfamily. These transporters are driven by the proton-motive force and consist of an inner membrane transporter (e.g. MexB), a periplasmic adaptor protein (e.g. MexA), and an exit duct (e.g. OprM) that is anchored to the outer membrane (Daury et al., 2016; Symmons et al., 2009).

MexAB-OprM constitutes an energy-dependent multi-drug efflux system exporting antibiotics like penicillins, fluoroquinolones, tetracycline and chloramphenicol (X. Z. Li et al., 1995; Poole et al., 1993). It is negatively regulated by the repressor proteins NalD and NalC (Cao et al., 2004; Morita et al., 2006).

A different but similar set of proteins, MexX and MexY, also form an outer membrane efflux system together with OprM, conferring resistance to fluoroquinolones, erythromycin and aminoglycosides like gentamicin. Noteworthy, the expression of MexX and MexY is induced by these same antibiotics (Aires et al., 1999; Masuda et al., 2000; Mine et al., 1999). OprA can replace OprM as the outer membrane channel, allowing the transporter to export carbenicillin and sulbenicillin, but it is not found in all strains of *P. aeruginosa* (Morita et al., 2012; Singh et al., 2020).

P. aeruginosa encodes another multidrug efflux system named MexEF-OprN, mediating resistance to a broad range of antibiotics like chloramphenicol, ciprofloxacin, tetracycline, trimethoprim and imipenem (Kohler et al., 1997). Interestingly, this system is also reported to secrete 4-hydroxy-2-heptylquinoline (HAQ) (Lamarche & Deziel, 2011), a precursor of the *Pseudomonas* Quinolone Signal (PQS) quorum sensing molecule 3,4-dihydroxy-2-heptylquinoline (Deziel et al., 2004; Pesci et al., 1999).

3.2.4 Persisters and biofilms

Another strategy of bacteria to evade killing by antibiotics is the formation of less susceptible persister cells. The presence of dormant, non-dividing cells in a bacterial community as a way to evade sterilization by treatment with the antibiotic penicillin was discovered as early as 1944 and these cells were termed “persister cells” (Bigger, 1944). The *hipA* allele (high-persister) *hipA7* was the first recognized gene involved in persister formation and later discovered to induce persister formation via phosphorylation of EF-Tu, leading to inhibition of protein synthesis (Korch et al., 2003; Moyed & Bertrand, 1983; Schumacher et al., 2009). Persister cells were later discovered in biofilms as a small fraction of cells that are highly tolerant to antibiotics (Brooun et al., 2000). It was found that antibiotic tolerance in biofilms is more likely dependent on the presence persister cells than

on other antimicrobial resistance mechanisms (Spoering & Lewis, 2001). In CF patients, strains of *P. aeruginosa* that produce high levels of persister cells, also termed *hip* phenotype, seem to emerge in long-term airway infections. This might be due to long-term high-dose antibiotic therapies selecting for *hip* phenotypes (L. R. Mulcahy et al., 2010).

3.2.5 Mucoid phenotype

Mucoid phenotypes of *P. aeruginosa* in people with cystic fibrosis (pwCF) have been described in chronic infections, being associated with higher alginate production (Govan & Deretic, 1996). Mucoid phenotypes of *P. aeruginosa* are indeed so characteristic for chronic infections that a qPCR screening for mucoidity markers in *P. aeruginosa* has been suggested for discerning intermittent and chronic infections, potentially allowing the therapeutic strategy to be changed early on to better achieve eradication (Boutin et al., 2018). The mucoid phenotype is mainly caused by a heightened production of alginate.

Alginate was first isolated in 1966 from Pseudomonads and found to resemble alginic acid found in seaweeds (Linker & Jones, 1966). *Pseudomonas* strains with high levels of alginate expression quickly revert to their non-mucoid phenotype except under high salt concentrations and addition to glycerol in the medium (Evans & Linker, 1973). High osmolarity leads to higher alginate expression (Berry et al., 1989; Mohr & Deretic, 1990).

3.3 Quorum sensing mechanisms of *P. aeruginosa*

Pseudomonas aeruginosa uses quorum sensing systems for cell-to-cell communication and for the regulation of several virulence genes. This can be exploited to attenuate virulence in vivo and promote immune clearance of *P. aeruginosa* through the use of quorum-sensing blocking agents (Hentzer et al., 2003). It has also been reported that some bacteria have the ability to quench the quorum sensing signals of other species via degradation of signaling molecules, leading to reduced virulence and biofilm formation (Christiaen et al., 2011; Christiaen et al., 2014). The following section including **Figure 1** (on page 10) will give a short overview of the three intertwined quorum-sensing systems that *P. aeruginosa* uses for cell-to-cell communication.

3.3.1 The *Pseudomonas* Quinolone Signal (PQS)

The autoinducer molecule 2-heptyl-3-hydroxy-4-quinolone (PQS) is synthesized by the gene products of *phnAB*, *pqsABCD* and *pqsH* and regulated by PqsR, LasI/R, np20 and PqsE. Upon binding PQS, PqsR activates the transcription of *pqsABCDE* and *phnAB* in a positive feedback loop (Gallagher et al., 2002). PQS also connects the two other known quorum-sensing pathways of *Pseudomonas*, the *las* and the *rhl* systems: activated PqsR directly induces the expression of LasR, which then stimulates both the synthesis of PQS and PqsR

as well as the *rhl* system via RhlI activation and subsequent N-butyryl-L-homoserine lactone (C₄-HSL) production. (McGrath et al., 2004; McKnight et al., 2000; Wade et al., 2005; Xiao et al., 2006). The regulator PqsR is in turn negatively regulated by the RhlR regulator, which is activated via C₄-HSL (Wade et al., 2005).

3.3.2 The *rhl* system and the *las* system

C₄-HSL is synthesized by RhlI and acts as an autoinducer in *Pseudomonas aeruginosa* via binding and subsequent activation of RhlR, acting on virulence genes like *phzB1* (phenazine production) or *hcnA* (cyanide production) and also the expression of LasR (Pearson et al., 1995; Simanek et al., 2022).

N-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL) is synthesized by LasI (Pearson et al., 1994) and acts as a diffusible autoinducer molecule (Passador et al., 1993). LasR, the cognate transcriptional activator of the *las* pathway, regulates LasI expression in a positive feedback loop, as well as *rhlR* and *rhlI*, the proteases LasA, LasB, alkaline protease and toxin A (Gambello & Iglewski, 1991; Gambello et al., 1993; Latifi et al., 1996; Pesci et al., 1997; Toder et al., 1991).

The cyanide producing *hcnABC* gene cluster has also been shown to be regulated by both LasR and RhlR (Pessi & Haas, 2000). Cyanide is known to inhibit the cytochrome C oxidase (Keilin, 1929), leading for example to an inhibition of *Staphylococcus aureus* species (Voggu et al., 2006).

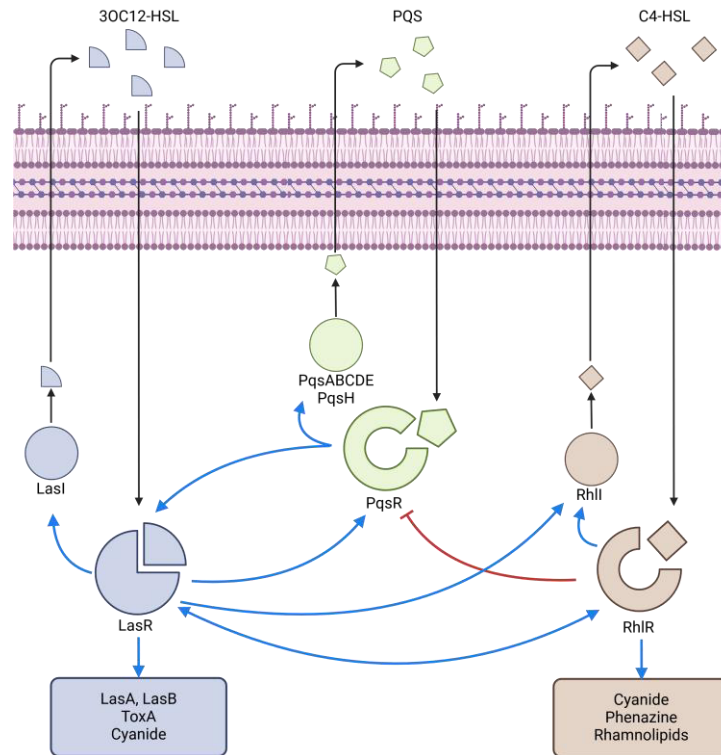


Figure 1: Schematic overview of the three quorum sensing mechanisms of *P. aeruginosa*. Blue arrows indicate positive interactions while red lines with blunt ends represent negative interactions. Dark blue symbols were used for components of the *las* system, green symbols for the *pqs* system and dark red symbols for the *rhl* system. **3OC12-HSL**: *N*-(3-oxododecanoyl)-*L*-homoserine lactone, **PQS** = *Pseudomonas* Quinolone Signal, **C4-HSL** = *N*-butyryl-*L*-homoserine lactone. Created with Biorender.com.

3.4 *Pseudomonas aeruginosa* in the cystic fibrosis lung

3.4.1 The lung microbiome in health and disease

The respiratory tract provides a diverse habitat for microorganisms. The upper airway tract beginning from the nose and mouth is coated by a mucus-covered epithelium, constantly moving air, saliva, microbes and other particles towards the gastrointestinal tract or trachea. The trachea then splits into two bronchi that keep branching off into smaller bronchi and bronchioles and finally, the alveoli. All of this bronchiolar tree is covered by a ciliary epithelium actively transporting the mucus that is produced by specialized cells outwards.

The lower respiratory tract was initially deemed sterile because no other methods than culture-based methods were available. Thanks to next-generation sequencing (NGS) techniques, we now know that this diverse habitat is colonized by microorganisms like bacteria and viruses (reviewed in Aho et al., 2015). The healthy bacterial lung microbiome that most individuals have in common seems to comprise the genera *Pseudomonas*, *Prevotella*, *Streptococcus*, *Fusobacterium*, *Haemophilus*, *Neisseria*, *Veillonella* and *Porphyromonas* in the upper respiratory tract (reviewed in Dickson et al., 2016; Marsland

& Gollwitzer, 2014). The lower parts of the respiratory tract seem to be colonized less densely due to clearance mechanisms of the lung and trachea, like coughing, mucociliary transport and components of the innate immune response (reviewed in Natalini et al., 2023). We now know that the microbiome of the lung develops shortly after birth and is related to the establishment of the gut microbiome (Madan et al., 2012).

In the cystic fibrosis (CF) lung, chloride channels that pump chloride and bicarbonate ions into the airway lumen (CFTR channels) are defective. This leads to a decreased salt concentration in the lung, and by extension, a thickening of the mucus and impaired clearance mechanisms of the lungs, because chlorine ions play a crucial role in maintaining the ion and water equilibrium in the airway mucus (Kerem et al., 1989; D. P. Rich et al., 1990; Welsh, 1986). O₂ gradients seem to be established in this thickened mucus via consumption of oxygen by the underlying epithelia. In the case of *P. aeruginosa* infection, the bacterium rapidly penetrates into the mucus of CF lungs, induces alginate expression, and forms intraluminal microcolonies that additionally consume oxygen and thus contribute to CF-typical steep O₂ gradients in the mucus (Worlitzsch et al., 2002). Additionally, since *P. aeruginosa* can be taken up and cleared by epithelial cells via a mechanism that depends on a functional CFTR, less *P. aeruginosa* cells are taken up in the lungs of people with cystic fibrosis (pwCF), leading to lower clearance rates and a higher bacterial burden in the lung (Pier et al., 1997).

According to the Annual Report of the German cystic fibrosis Registry (2021), the most common chronic infections in pwCF of all age groups were caused by *Staphylococcus aureus* (44 – 83%), followed by *P. aeruginosa*, which seems to establish infections with increasing patient age, ranging from 4.4% in pwCF under the age of 2 to over 44% of pwCF over the age of 18. Other species like *Haemophilus influenzae*, *Burkholderia* sp., *Stenotrophomonas maltophilia*, and *Achromobacter xylosoxidans* are less common in chronic infections, but may cause acute infections with severe impacts on the patients' health. The main cause of death in pwCF in Germany in 2021 were by far cardio-pulmonary diseases (Nährlich L. et al., 2022: German Cystic Fibrosis Registry Annual report 2021). Among the most infamous of the pathogens causing these diseases are members of the *Burkholderia cepacia* complex (Bcc), which were recognized as increasing causes of lung infection in pwCF in the 1980s in Toronto, sometimes causing sudden, rapid and fatal deterioration in previously only mildly affected patients, which became known as “cepacia syndrome”. The symptoms most commonly associated with this syndrome were recurrent or persistent fever, weight loss, and fatal deterioration of lung function, while eradication of the pathogen often turned out to be impossible due to antibiotic resistances (Isles et al., 1984). Later

studies confirmed that colonization with that epidemic *B. cepacia* strain results in excess mortality of pwCF (Ledson et al., 2002). After the discovery that the Bcc comprises many distinct species, the “cepacia syndrome” could mainly be attributed to *B. cenocepacia* and *B. multivorans* (Jones et al., 2004).

3.4.2 Adaptations of *P. aeruginosa* in the cystic fibrosis lung

Pseudomonas aeruginosa accumulates many genetic adaptations during infection in CF patients, often kick-started by selection of hyper-mutator phenotypes with mutations in DNA mismatch-repair-related genes, mostly *mutS* and *mutL* (Mena et al., 2008; E. E. Smith et al., 2006).

Recent findings suggest that the activity of the elastase LasB also decreases in CF patients with chronic *P. aeruginosa* infections, while traits like fast growth, tobramycin susceptibility, green non-mucoid colony phenotypes and the presence of a functional *lasR* gene are associated with early stages of infection (Llanos et al., 2023). Mutations in the *lasR* gene lead to higher expression of a surface-adhesion molecule on epithelial cells, ICAM-1, leading to stronger pulmonary inflammation reactions. This is thought to be related to lower expression of LasR-regulated proteases, which in turn abrogates the degradation of ICAM-1 on epithelial cells (Hennemann et al., 2021). ICAM-1 is part of the immunoglobulin supergene family and is expressed e.g. on vascular endothelial cells, inducing the transendothelial migration of neutrophils into the infected tissue (Hubbard & Rothlein, 2000; C. W. Smith et al., 1989; Springer, 1990). Mutations in *lasR* are also known to confer a selection advantage to *P. aeruginosa* under certain environmental conditions, such as alkaline environment (Heurlier et al., 2005), keratitis (Shanks et al., 2023) or long-term CF infections (Hoffman et al., 2009).

One of the strains used in this study, ID40, carries a transposon insertion in the *lasR* gene starting at F210 and leading to a truncated LasR, with a C-terminus terminating in 212METLKKTS* rather than 212MGNIRRKFGVTSRRVAAIMAVNLGLITL* (own research). Several papers have described point mutations in this region which strongly impair LasR function, like an I215S mutation (Shanks et al., 2023) or the R216L mutation of strain PAO1-D (Heurlier et al., 2005).

3.5 Strains of *P. aeruginosa* used in this study

PAO1 was the first isolate of *P. aeruginosa* to be fully sequenced, revealing the broad range of adaptations to highly diverse ecological niches on a genetic level (Stover et al., 2000). PAO1 is one of the most common reference strains of *P. aeruginosa*, although it is also known to undergo microevolution in research laboratories, leading to diverse phenotypes

and potentially impairing reproducibility of data across research laboratories (Chandler et al., 2019).

Strain UCBPP-PA14 (PA14) was first isolated as a highly virulent strain in a burn patient (Schroth et al., 2018). It carries two pathogenicity islands, PAPI-1 and PAPI-2, of which only part of the latter is present in PAO1 (He et al., 2004). The heightened virulence of PA14 can also in part be attributed to a loss-of-function mutation in the *ladS* gene, which leads to decreased biofilm formation and increased secretion of cytotoxic ExoU via the T3SS (Mikkelsen et al., 2011).

The *P. aeruginosa* strain ID40 is a multidrug-resistant strain of *P. aeruginosa* that was first isolated in a study designed to identify novel prognostic biomarkers in patients with *P. aeruginosa* bloodstream infections (Sonnabend et al., 2020; Willmann et al., 2018). The strain was found to be resistant to piperacillin, cefepime, aztreonam, levofloxacin, ceftazidime, imipenem and ciprofloxacin. The resistance to β -lactam antibiotics can be at least partially explained by the presence of an inactive *dacB* gene leading to *ampC* overexpression (Sonnabend et al., 2020; Torrens et al., 2019). Resistance to fluoroquinolones like ciprofloxacin is at least partially associated with overexpression of the MexEF-OprN efflux pump and mutations in the GyrA subunit (manuscript in preparation). The aforementioned mutation in *lasR* further links it to other pathogenic isolates of *P. aeruginosa*.

3.6 The *Burkholderia cepacia* complex

3.6.1 What is the *Burkholderia cepacia* complex?

The *Burkholderia cepacia* complex (Bcc), as of now, consists of 24 closely related species of pathogens (reviewed in Tavares et al., 2020). While many of them are opportunistic pathogens, the ones most commonly associated with pulmonary infections are *B. cenocepacia* and *B. multivorans*, which are most likely responsible for the so-called “cepacia syndrome” described above (Jones et al., 2004). Despite their role as human and plant pathogens, the genetic diversity and ubiquitous distribution of Bcc species in the environment also provides chances, for example as degraders of the pollutant trichloroethylene (Kilbane et al., 1982), as endophytic nitrogen fixing bacteria that promote plant growth (Reis et al., 2010), or production of compounds that inhibit plant pathogens (Heungens & Parke, 2000, 2001).

Bcc species show a broad spectrum of antibiotic resistances, especially in people with CF (pwCF) (Zhou et al., 2007). Examples are β -lactam resistance mediated by PenB carbapenemase (Trepanier et al., 1997), efflux pumps that confer resistance to

trimethoprim/sulfamethoxazole, levofloxacin, chloramphenicol, ceftazidime and meropenem (Tseng et al., 2014), or resistance to polymyxins which is thought to be related to an altered structure of the lipopolysaccharide (Moore & Hancock, 1986). For Bcc species, non-mucoid phenotypes cause the strongest decline in health of infected pwCF compared to mucoid strains, with common antibiotics used in CF infections like ceftazidime and ciprofloxacin driving the conversion from mucoid to non-mucoid phenotype (Zlosnik et al., 2011). The mucoid phenotype of Bcc may also help evade clearance by the host immune system by inhibiting neutrophil chemotaxis and scavenging reactive oxygen species (ROS) (Bylund et al., 2006; Pedersen et al., 1990).

3.6.2 Strains of the *Burkholderia cepacia* complex (Bcc) prominently used in this study

B. cepacia ATCC 25416 was first isolated in 1948 by Walter H. Burkholder from an onion (first named "*Pseudomonas cepacia*") and later suggested as the *B. cepacia* (or Bcc genomovar I) type strain (Ballard et al., 1970; Yabuuchi et al., 1992).

B. cenocepacia ATCC 17765 (formerly in the Bcc III genomovar group) was initially characterized in a study by Stanier et al. in 1966 (Stanier et al., 1966). It was originally isolated from a urinary tract infection of a child in 1964 in the Bristol Royal Infirmary.

3.7 Interbacterial competition

Interactions between bacteria can both be synergistic and antagonistic. Examples of cooperation in bacterial communities are the secretion of exopolymers which form a stable extracellular matrix to which other species may adhere (reviewed by Joshi et al., 2021), or the secretion of exoenzymes, which make nutrients available to the all community (Fiegna & Velicer, 2003). Examples of antagonism between bacteria are the secretion of soluble antibacterial toxins like hydrogen cyanide (HCN) and pyochelin, leading to ROS-production in *P. aeruginosa* competitors, (Letoffe et al., 2022; Ong et al., 2017), or the deployment of an antibacterial weapon like the T6SS (Pukatzki et al., 2006).

A study by Perault et al. (2020) found that in people with cystic fibrosis, *P. aeruginosa* seems to accumulate T6SS-inactivating mutations during long-term infection, leading to increased predation by Bcc species and rendering the host more susceptible to fatal *B. cenocepacia* lung infections. Those mutations are found for example in *gacS* and *gacA*, *pppA* or *fha1* (Perault et al., 2020), all of which are required for the function of the T6SS in *P. aeruginosa*. Interestingly, mutations were also found in the *retS* gene of those isolates with *gacS* and *gacA* mutations, especially a RetS_{A46V} mutation that occurred in three different isolates, but it is so far unknown if those mutations impact RetS function (Perault et al., 2020).

Certain genera of Proteobacteria like *Enterobacter cloacae* and *Klebsiella* spp. seem to be intrinsically resistant to T6SS attacks without harboring immunity genes against the actual effector toxins, through either their own superior T6SS or group I capsules, (N. Flaughnatti et al., 2021).

3.8 The Type VI Secretion System (T6SS)

3.8.1 Structure and function of the T6SS

The Type VI secretion system (T6SS) was first described in 2006 by two publications in parallel, one describing it as the HIS-I (Hcp1 secretion island I virulence locus of *Pseudomonas aeruginosa* that is, like the Type III secretion system, regulated by RetS and LadS (Mougous et al., 2006), and the other describing it as a conserved bacterial secretion apparatus used for predation of *Dictyostelium* amoeba by *Vibrio cholerae* (Pukatzki et al., 2006). Mougous et al. also found that *icmF1* is needed for correct T6SS assembly, that ATP hydrolysis by ClpV1 at the T6SS foci is essential for secretion of Hcp1 in *P. aeruginosa* and that Hcp1 forms a hexameric ring structure constituting a channel. They also described the existence of two additional IcmF-associated homologous protein (IAHP) clusters, but did not recognize them initially as T6SS loci since they appeared neither involved in virulence nor regulated by RetS or LadS (Mougous et al., 2006). *P. aeruginosa* encodes three distinct T6SS clusters on its genome, termed H1-T6SS, H2-T6SS and H3-T6SS. All three possess a similar structure, but fulfill vastly different functions, which will be described in sections 3.9 - 3.11 (pp. 21 - 26) (Hachani et al., 2011; Mougous et al., 2006; Sana et al., 2012).

Since the T6SS is a relatively newly discovered secretion system and many genes resemble other proteins, there was initially no uniform naming convention for T6SS-related genes in *Pseudomonas* and other species. Consequently, many homologous genes were named differently in different species despite their identical functions. I therefore included **Table 1** which summarizes all interchangeably used denominations for the different genes that I encountered during my research and that are described in the following section.

The Type VI secretion system itself resembles a dynamic, phage tail-like structure, constantly assembling, contracting and disassembling, and most likely shares an evolutionary origin with bacteriophage tails (Basler et al., 2012; Leiman et al., 2009). The central sheath, consisting of an inner sheath formed by Hcp subunits and the outer sheath formed by heterodimers (called VipA/VipB in *V. cholerae* or TssB/TssC in other species), assembles in the cytosol of the bacterium. ClpV is associated with this outer sheath and recycles the outer sheath proteins from the contracted sheath (Basler et al., 2012). Atop the Hcp tube, trimeric VgrG proteins with a needle-tip like structure are located and also translocated as effectors by the T6SS, leading to enhanced virulence e.g. via the actin-crosslinking activity of VgrG-1

(Pukatzki et al., 2007; Pukatzki et al., 2006; Sheahan et al., 2004). The needle tip can be further sharpened and diversified by the presence of cone-shaped proline-alanine-alanine-arginine (PAAR)-repeat effector proteins (Shneider et al., 2013). There are several PAAR effectors that contain an additional effector domain besides the PAAR tip, which bind to the VgrG tip and are then delivered directly to the target cell (Shneider et al., 2013).

Table 1: Summary of interchangeably used names for components of the T6SS. Names usually used in this thesis are written in bold text.

Protein	Localization	Alternative name / homologs
TssA	Distal end of sheath	HsiE
TssB	Outer tail sheath	VipA, HsiB1
TssC	Outer tail sheath	VipB, HsiC1
TssD	Inner tube	Hcp
TssE	Baseplate (wedge complex)	gp25-like protein, HsiF
TssF	Baseplate (wedge complex)	VasA
TssG	Baseplate (wedge complex)	
TssH	Outer sheath after contraction	ClpV
TssI	Needle tip of the Hcp sheath	VgrG
TssJ	Membrane spanning complex (OM lipoprotein “anchor”)	Sci1
TssK	Baseplate (adaptor for wedge complex)	
TssL	Membrane spanning complex (IM „hook-like” structure)	IcmH, DotU
TssM	Membrane spanning complex (Periplasmic arch-like structure)	IcmF , VasK
TagA	Associated with TssA _L	
TagB	Associated with TssA _S	
TagL	Membrane spanning complex	SciZ
TagJ	Associated with TssA _S	

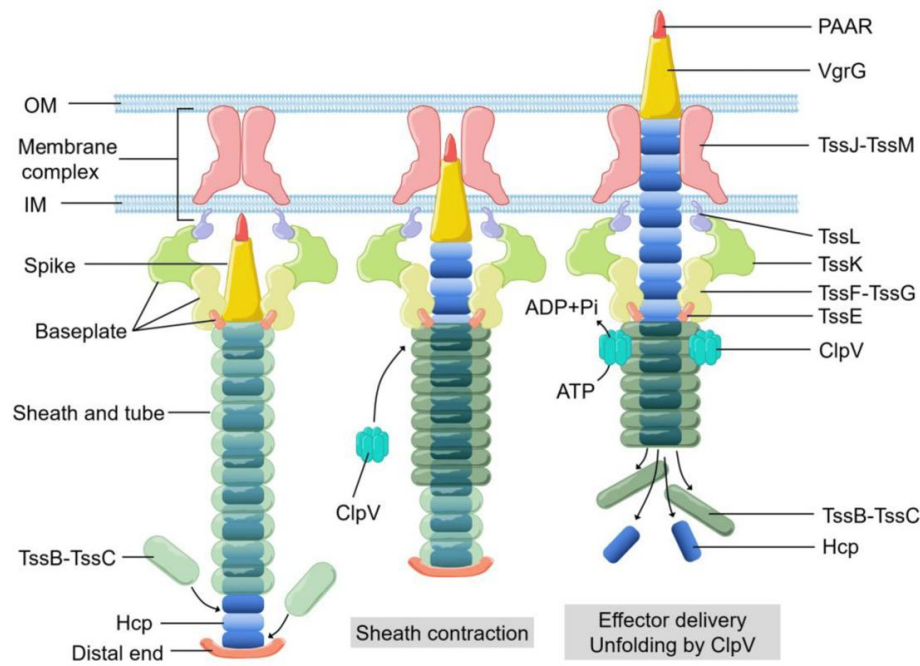


Figure 2: Schematic overview of the structure and function of a T6SS. Figure published under open access Creative Common CC BY license by Yu et al. (2021).

The T6SS membrane complex consists of TssM (called IcmF in this thesis), TssL, TagL, TagP and TssJ (Aschtgen et al., 2010). The membrane core complex assembles by direct interaction of TssJ, an outer membrane anchored protein, with IcmF, a membrane-spanning protein, forming an arch-like structure. Ten of these arches assemble into a cylindrical shape with a 15 Å pore in the middle and recruit TssL in the inner membrane to form a ring-shape with five hooks (shown in **Figure 2**). Although not initially in contact with the outer membrane itself, IcmF (TssM) can change its conformation and contact the outer membrane upon assembly of the complex. This complex resides in the membrane and can be used for multiple T6SS firing events (Durand et al., 2015).

The baseplate of the T6SS is constituted by TssE, TssF, TssG and TssK (Y. J. Park et al., 2018). TssK assembly relies on the TagQRST system and the kinase PpkA and disassembly depends on PppA (Liebl et al., 2019). It acts as an adaptor ring to the membrane complex for the TssFGE “wedge” which contains six copies of TssK, two copies of TssF and one of each TssG and TssE. Six of these wedges come together to form the baseplate around a VgrG trimer (Cherrak et al., 2018; Nazarov et al., 2018; Y. J. Park et al., 2018).

The end of the T6SS sheath facing the bacterial cytoplasm is capped by homo-dodecameric TssA proteins that are essential for the assembly of the T6SS complex (Brunet et al., 2015). TssA is recruited to the T6SS membrane complex early on, then is thought to guide placement of the baseplate and to initiate and coordinate inner and outer sheath assembly (Zoued et al., 2016). It then interacts with TagA, a protein that is associated with the inner

membrane, stops the polymerization of the extending sheath upon contact and stabilizes it in the extended form (Santin et al., 2018). Recent studies suggest that across and within different species, there are two distinct forms of TssA proteins: one long form (TssA_L), which interacts with TagA proteins, and a short form (TssA_S) which instead seems to interact with other proteins like TagB (in *P. putida*) or TagJ (in *P. aeruginosa*), initiating contraction of the sheath immediately after extension, rather than stabilizing TagA (Bernal et al. (2021); Dix et al. (2018); summarized in Figure 3).

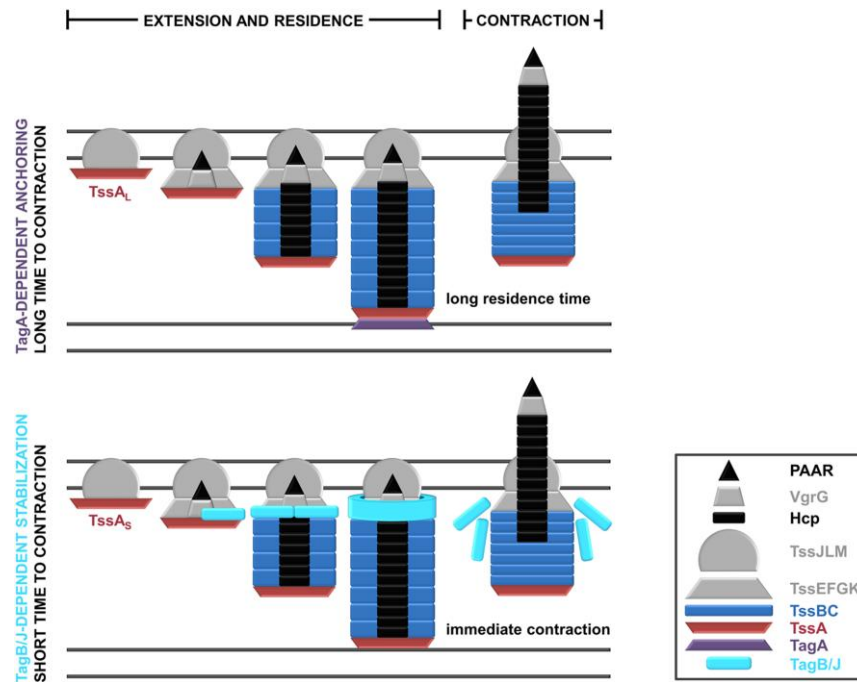


Figure 3: Schematic view of the functions of the two different TssA_L or TssA_S complexes in T6SS assembly. Figure taken from Bernal et al. (2021) (published under CC BY 4.0).

Only the H1-T6SS of *P. aeruginosa* contains the non-essential accessory gene *tagJ/hsiE1*, which interacts with TssB in the outer sheath and, additionally, with ClpV1, possibly recruiting the ATPase to the contracted sheath (Forster et al., 2014; Lossi et al., 2012). Conversely, TagJ was also shown to strongly interact with TssA_S at the distal end of the T6SS tube, potentially stabilizing the extended sheath (Bernal et al., 2021).

3.8.2 Phylogenetic subtypes of the T6SS

Based on phylogenetic analyses of the T6SS of different species, the T6SS was grouped into four distinct subtypes: the T6SS^{i-iv}, of which *P. aeruginosa* (PA) and most Proteobacteria generally possess T6SSⁱ. Based on studies of mainly Proteobacteria genomes, the T6SSⁱ was further clustered into seven subtypes: types i1, i2, i3, i4a, i4b, and i5 (Barret et al., 2011; Barret et al., 2013; J. Li et al., 2015; Russell et al., 2014). For example, *Pseudomonas aeruginosa* harbors the H1-T6SSⁱ³, H2-T6SSⁱ¹ and H3-T6SS^{i4a}, underlining their low phylogenetic relatedness (J. Zhang et al., 2023).

3.8.3 Regulation of the T6SS of *P. aeruginosa*

3.8.3.1 Regulation via the GacS/GacA two-component system

The GacS/GacA two component system, consisting of the GacS sensor kinase and the GacA response regulator, is a regulator of the T6SS, *rhl* and *las* quorum sensing pathways and of the formation of pyocyanin, cyanide, lipase and biofilms (Corbell & Loper, 1995; Hassan et al., 2010; Laville et al., 1992; Parkins et al., 2001; Reimann et al., 1997; J. J. Rich et al., 1994). When GacA becomes phosphorylated by GacS upon sensing a so-far uncharacterized ligand, it activates the expression of the small regulatory RNAs (sRNAs) RsmY and RsmZ (Heeb et al., 2002; Valverde et al., 2003). The signal finally converges on the RsmZ/RsmY/RsmA regulon, where the sRNAs RsmZ and RsmY bind to the post-transcriptional regulator protein RsmA, antagonizing its activity and leading to the repression or de-repression of downstream genes (Heurlier et al., 2004; H. Mulcahy et al., 2008; Valverde et al., 2003). RsmA acts by binding to a recognition sequence on mRNAs, inhibiting their translation (Baker et al., 2002; Liu & Romeo, 1997).

RetS is part of an intricate regulatory network of *P. aeruginosa* and a regulator of the GacS/GacA/Rsm regulatory pathway, which is summarized in **Figure 4**. LadS and RetS have opposite effects on the production of RsmZ. A *retS* mutation leads to overproduction of RsmZ, while a *ladS* mutation results in decreased levels of RsmZ (Ventre et al., 2006). RetS acts not via the phosphorylation cascade of GacA, but by directly binding to the cytoplasmic domain GacS, thereby rendering it incapable of autophosphorylation and preventing it from exhibiting its normal function (Goodman et al., 2009). Additionally, RetS was shown to remove phosphate groups from the phosphotransfer domain of GacS either via phosphatase activity or by accepting those groups itself (Francis et al., 2018). It was found to positively regulate the Type III secretion system (T3SS) and several virulence factors like ToxA or LipA, and negatively regulate the formation of biofilms, type IV pili and the bacterial T6SS (Goodman et al., 2004). PA1611, another histidine kinase, interacts directly with RetS, preventing its antagonizing effect on GacS (Bhagirath et al., 2017; Kong et al., 2013).

LadS, another sensor kinase that also binds directly to GacS, counteracts RetS by delivering additional phosphate groups to GacS on its alternative phosphotransfer domain (Broder et al., 2016; Chambonnier et al., 2016).

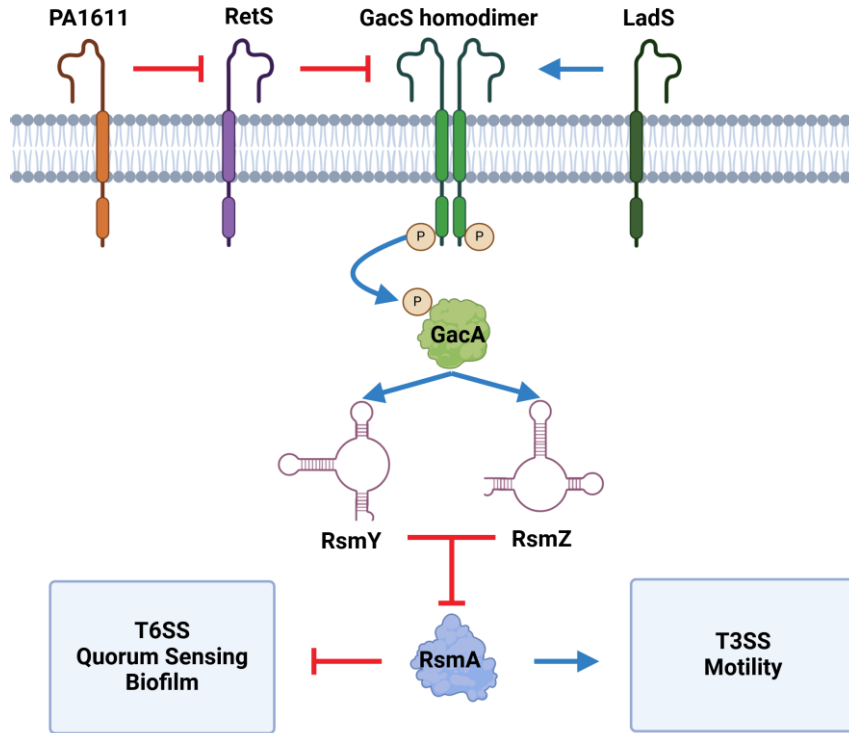


Figure 4: Schematic overview of the GacS/GacA-Rsm regulon. Positive interactions are shown as blue arrows, while negative interactions are shown as red lines with blunt ends. Shapes that represent the sensor-kinases PA1611, RetS, GacS and LadS, as well as those of RsmY, RsmZ and GacA or RsmA are not based on their biological structure and only serve illustrative purposes. This schematic is simplified and focused mainly on those topics that are relevant to this project. Created with Biorender.com.

3.8.3.2 TagQRST-PpkA-PppA-Fha1: Response to cell wall stress

The H1-T6SS of *P. aeruginosa* is activated upon induction of exogenous membrane stresses like polymyxin B, the *Vibrio cholerae* phospholipase effector TseL or the type IV DNA transfer system, but also by endogenous stresses like an inactive *bamA* or *lptD* (Ho et al., 2013; Kamal et al., 2020; Stolle et al., 2021). This activation depends on a second regulatory system, consisting of the TagQRST-PpkA-PppA-Fha1 proteins. TagQ is an outer membrane lipoprotein that anchors TagR to the outer membrane, while TagS and TagT form an inner membrane complex (Casabona et al., 2013). Upon sensing a so far unknown signal associated with membrane stress, TagR mediates the phosphorylation of the membrane-spanning threonine kinase PpkA, in turn leading to the phosphorylation and thereby activation of the cytoplasmic Fha1, which assembles in a stable complex with ClpV1 at the H1-T6SS assembly site (Casabona et al., 2013; Hsu et al., 2009; Mougous et al., 2007). Finally, the signal is quenched via de-phosphorylation of Fha1 by the Ser-Thr phosphatase PppA, which is encoded on the HSI-I island (Mougous et al., 2007). This TagQRST-PpkA-PppA-Fha1 system also seems to be the one responsible for the tit-for-tat dueling behavior of *P. aeruginosa* described in the next chapter (Basler et al., 2013).

3.8.4 Tit-for-tat T6SS dueling behavior in *P. aeruginosa*

While imaging experiments conducted by Basler et al. (2012) revealed several spontaneously assembling T6SS foci in most *Vibrio cholerae* cells, only comparatively few T6SS foci formed in *P. aeruginosa* (Basler & Mekalanos, 2012; Basler et al., 2012). Basler and Mekalanos (2012) found that in *P. aeruginosa*, *retS* mutations lead to the formation of more T6SS foci and that being attacked by a neighboring cell triggers the formation of a T6SS focus at the site of the attack in the neighboring sister cell, and called this behavior “T6SS dueling”. Dueling behavior was not observed in *V. cholerae* (Basler & Mekalanos, 2012). This “tit-for-tat” response in *P. aeruginosa* appears to be a very efficient way to counter-attack “random-firing” T6SS⁺ strains like *V. cholerae*, where *P. aeruginosa* retaliates against the attacker by assembling and firing its own T6SS several times in a row at the site of the attack, killing the attacker (Basler et al., 2013; W. P. J. Smith et al., 2020).

3.9 Effectors of the H1-T6SS

Effector proteins of the T6SS are thought to be delivered to the host cells in several different ways: as C-terminal extensions of the VgrG protein (Pukatzki et al., 2007), bound to VgrG proteins (Dong et al., 2013; Hachani et al., 2011), as extensions of the PAAR tip protein or potentially associated otherwise with the PAAR tip and VgrG proteins (Shneider et al., 2013). The loading process of an effector onto the VgrG protein seems to act as a trigger for T6SS assembly at least in *Agrobacterium tumefaciens* (Wu et al., 2020). The firing of the assembled and loaded T6SS sheath occurs via contraction of the outer sheath by a conformational change in TssBC, but the corresponding trigger remains elusive (Brackmann et al., 2018). Genes encoding bacteriolytic T6SS effectors usually have a cognate immunity gene directly neighboring them on the genome, whose gene product renders the toxin inactive, thereby preventing self-intoxication of the cells or of sibling cells (Hood et al., 2010; Russell et al., 2011). Interestingly, despite expressing immunity proteins for the delivered toxins, in *P. aeruginosa* the attack of a sibling cell via T6SS effectors still triggers the T6SS dueling behavior in the attacked sibling cell (Basler & Mekalanos, 2012). Since the H1-T6SS was one of the main topics of this project, all known effectors will be introduced in the following section and are summarized in **Table 2**.

Table 2: Overview of all known H1-T6SS effectors so far.

Effector	Function	Immunity protein	Described in
Tse1	Peptidoglycan amidase	Tsi1	Hood et al. (2010); Russell et al. (2011)
Tse2	Unknown cytoplasmic target	Tsi2	Hood et al. (2010); Russell et al. (2011)

Effector	Function	Immunity protein	Described in
Tse3	Muramidase	Tsi3	Hood et al. (2010); Russell et al. (2011)
Tse4	Suspected ion pore	Tsi4	LaCourse et al. (2018); Whitney et al. (2014)
Tse5	Pore-forming membrane ion channel	Tsi5	Whitney et al. (2014); Gonzalez-Magana et al. (2022)
Tse6	NAD(P) ⁺ glycohydrolase	Tsi6	Whitney et al. (2014); Whitney et al. (2015)
Tse7	TOX-GHH2 domain-containing DNase	Tsi7	Hachani et al. (2014); Pissaridou et al. (2018)
Tse8	Trandamidosome inhibition	Tsi8	Nolan et al. (2021)

Among the first identified T6SS dependent effector molecules were Tse1-Tse3, with both Tse1 and Tse3 acting through peptidoglycan lysis in target prey cells (Hood et al., 2010; Russell et al., 2011). The precise mode of action of Tse2, to this date, remains unclear.

Although the exact mechanism of action is still unknown, Tse4 seems to act synergistically with the peptidoglycan-targeting effectors Tse1 and Tse3 as well as Tse6. It is thought to act as an ion channel, disrupting the cell's proton motive force and thereby potentially activating autolysins, explaining its synergy with Tse1 or Tse3 (LaCourse et al., 2018).

Tse5, Tse6 and Tse7 all contain PAAR domains and rely on their respective VgrGs, VgrG4, VgrG1, and VgrG1b, for delivery into host cells (Pissaridou et al., 2018; Whitney et al., 2014). While Tse5 forms ion-permissible pores in the cytoplasmic membranes of target cells, leading to depolarization and cell death (Gonzalez-Magana et al., 2022), Tse6 acts as an integral membrane toxin via degradation of NAD⁺ and NADP⁺, and seems to depend on binding to EF-Tu in host cells (Whitney et al., 2015). Tse7 contains a nuclease domain, leading to DNA degradation in target cells, and is secreted via binding of its PAAR domain to VgrG1b (Pissaridou et al., 2018).

Tse8 is the most recently identified and named H1-T6SS effector, targeting the bacterial GatCAB heterotrimer, which is part of the transamidosome, and leading to inhibition of protein synthesis in those host cells that possess such a transamidosome. The secretion of Tse8 seems to depend on VgrG1a (Nolan et al., 2021). The transamidosome is a protein complex exclusively found in prokaryotes lacking an asparagine- or glutamine-aminoacyl-tRNA transferase. Instead, aspartate or glutamate are first transferred to tRNA^{Asn} or tRNA^{Gln}, respectively, and then amidated to asparagine or glutamine by the GatCAB heterotrimer when already attached to the tRNA (Becker et al., 2000; Curnow et al., 1996).

The transposon screening approach that Nolan et al. (2021) used also identified three other potential H1-T6SS effectors, PA0801, PA2778 and PA3272, but there is so far no experimental evidence establishing them as T6SS effectors (Nolan et al., 2021). Since, as of now, all three proposed T6SS effectors of that study are genes of unknown function, except PA3272, which has been identified as a DNA helicase involved in RpoH-dependent stress response (Williamson et al., 2023), they were not included as potential T6SS effectors in this thesis.

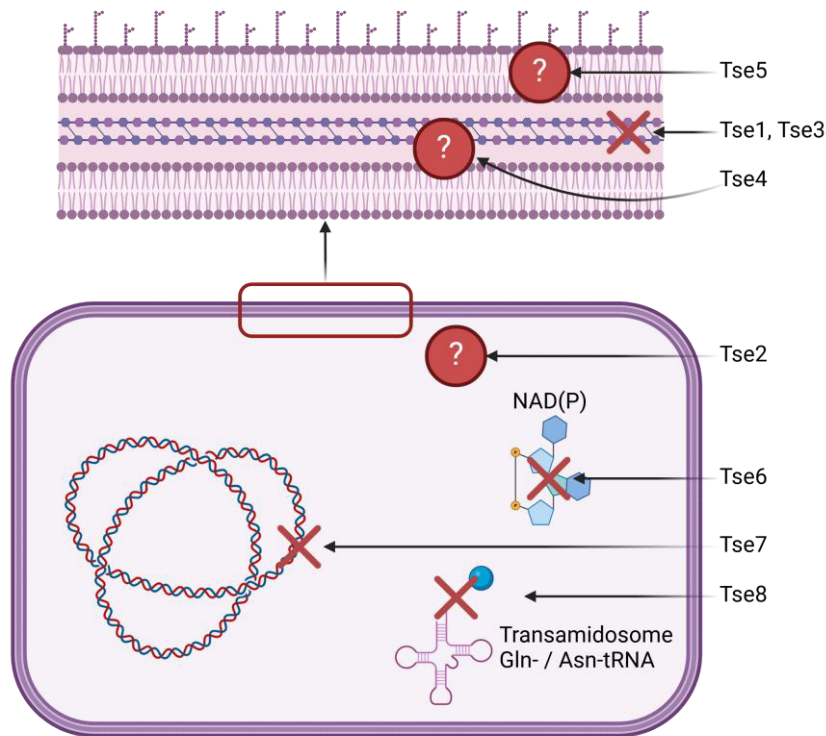


Figure 5: Schematic overview of the known H1-T6SS effectors of *P. aeruginosa* (PA; not depicted) and their targets. Black arrows with text boxes indicate translocation of the effectors Tse1-8 from PA into the host cell. Red crosses indicate disruption of a target structure or process by the effector. Red circles with question marks indicate that the exact target of an effector is unknown, but it is assumed to act in the indicated compartment. Created with Biorender.com.

3.10 The H2-T6SS

The H2-T6SS is regulated via the *las* and *rhl* quorum sensing systems in *P. aeruginosa*, and induced upon iron starvation. It was also found to mediate internalization of *P. aeruginosa* into epithelial cells (Sana et al., 2012). Recent studies suggest that it is activated by RpoN, while H1-T6SS and H3-T6SS are repressed, and that this regulation is mediated by the sigma factor activator 2 (Sfa2) encoded in the H2-T6SS gene cluster (Allsopp et al., 2022). **Table 3** summarizes all known H2-T6SS effectors of *P. aeruginosa* to date.

Table 3: Overview of all known H2-T6SS effectors so far

Effector	Function	Immunity protein	Described in
VgrG2b	Tubulin-dependent uptake into Eukaryotic cells and antibacterial toxic peptidase	Tli3, PA0261	Berni et al. (2019); Sana et al. (2015)
Tle1			Russell et al. (2013)
Tle2			Russell et al. (2013)
Tle3	Periplasmic antibacterial effector	Tli3, PA0261	Barret et al. (2011); Berni et al. (2019); Russell et al. (2013)
PldA / Tle5	Trans-kingdom phospholipase	Tli5	
Tle4 / TplE	Mammalian autophagy inducer and anti-bacterial lipase	Tli4	Jiang et al. (2016); Russell et al. (2013)
ModA	Molybdate acquisition		Wang et al. (2021)
Azurin	Cu ²⁺ uptake		Han et al. (2019)
PA2066	Putative T6SS toxin		Howard et al. (2021)

The H2-T6SS is known to secrete several phospholipases, namely the trans-kingdom effectors PldA and PldB and five other Type VI lipase effectors (*tle1-5*) (Boulant et al., 2018; Jiang et al., 2014; Russell et al., 2013; Wettstadt et al., 2019). It was also reported to secrete ModA, a molybdate binding protein (Wang et al., 2021), TseF, a protein coordinating Fe²⁺ uptake via PQS containing outer membrane vesicles (Lin et al., 2017), and azurin (Han et al., 2019), a copper-containing redox protein that interferes with the p53 signaling pathway and induces apoptosis in host cells (Gao et al., 2017).

VgrG2b has an interesting role as a trans-kingdom effector, mediating tubulin-dependent uptake of *P. aeruginosa* into host cells (Sana et al., 2015), and containing a C-terminal extension that was shown to be toxic to *E. coli* in two independent studies (Berni et al., 2019; Wood et al., 2019).

There is evidence that PA0261, most universally assumed to encode Tli3, (i) is homologous to the known immunity proteins Tsi6 and Tli4, (ii) contains an N-terminal signal peptide localizing it to the periplasm, and (iii) can bind to Tle3 and quench its toxic effect in vivo (Berni et al., 2019). The secretion of the toxic effector Tle3 relies on VgrG2b (Berni et al., 2019). Tle3 itself is loaded onto VgrG2b via an adaptor protein, Tla3, and then acts as an anti-bacterial toxin in the periplasm of the target cell via a yet unknown mechanism (Berni et al., 2019).

Tle5 has a lipase activity, cleaving choline off of phosphatidylcholine and destabilizing or depolarizing the membrane of bacteria as well as mammalian cells, making it another trans-kingdom effector (Jiang et al., 2014; Russell et al., 2013). Tle4 was found to act as a lipolytic

toxin in bacteria and as an autophagy-inducer in mammalian cell lines, disrupting the ER and inducing the unfolded protein response (Jiang et al., 2016).

The H2-T6SS contains no known TagA protein despite having a TssA_L cap, suggesting that TssA2 might act as an anchor itself without requiring an additional partner to keep the T6SS sheath stably extended (Bernal et al., 2021; Schneider et al., 2019).

3.11 The H3-T6SS

The H3-T6SS is the least studied of the three T6SS of *P. aeruginosa*. So far, only four potential effectors of the H3-T6SS have been identified but only two of them, PldB and TseT, experimentally confirmed. There is, however, conflicting data on whether PldB is secreted via the H2- or H3-T6SS system: While one study found that secretion of PldB depends on ClpV3, which is part of the H3-T6SS, but not on ClpV1 or ClpV2 (Jiang et al., 2014), another study found that its secretion depends on VgrG5, which is more likely to be secreted via the H2-T6SS (Wettstadt et al., 2019).

TseT is a TOX-REase-5 (for toxin restriction endonuclease 5) domain containing effector that relies on PAAR4, TecT and co-TecT for killing prey bacteria (Burkinshaw et al., 2018). The postulated effector TseF is thought to be integrated in outer membrane vesicles (OMVs) and to facilitate iron uptake via interaction with the iron-chelating PQS and then engaging with Fe(III)-pyochelin receptor FptA and the porin OprF (Lin et al., 2017). PA0256 is another putative H3-T6SS secreted effector found to directly interact with Hcp3, but remains otherwise uncharacterized (Howard et al., 2021).

3.12 TraDIS based screening assays

Transposon directed insertion-site sequencing (TraDIS) was developed as a high-throughput tool for genome-wide screening of a mutant library of a bacterium of interest under specific conditions. (Langridge et al., 2009). For this purpose, a high-density mutant library is constructed using the pUT-miniTn5pro plasmid, which encodes the cut-and-paste Tn5 transposon, an R6K_{ori} origin of replication, a gentamicin resistance cassette (*aacC1*), an *araC* gene and an outward-facing *araBAD* promoter, while the pUT backbone contains the transposase required for Tn5 mobilization as well as mating functions for conjugation (Goryshin & Reznikoff, 1998; Siehnel et al., 2010).

The plasmid is transferred into *P. aeruginosa* using conjugation and gentamicin-resistant mutants selected until approximately 2 million mutants have been generated (Nolan et al., 2018). After exposing the mutant library to a specific stressor of interest, such as an antibiotic, the surviving cells can be pooled and used to isolate genomic DNA (gDNA). After fragmentation, adapter ligation and a transposon-specific enrichment PCR, the DNA

fragments can be sequenced using next generation sequencing (NGS) to reveal the transposon-flanking regions. This data can then be compared to a reference sample obtained from the same transposon library, but without the stressor added, and processed in exactly the same way. In this way, when comparing the relative abundance of genes found in the sequencing step with the control group, genes involved in resistance to the stressor will be depleted in the stressed group, as these mutants would not have survived under the specific stress (Nolan et al., 2018; Sonnabend et al., 2020).

TraDIS has been used, for example, to identify genes involved in twitching motility in *P. aeruginosa* (Nolan et al., 2018), to identify drug resistance mechanisms in clinical isolate ID40 (Sonnabend et al., 2020), or to identify multidrug efflux systems in *A. baumannii*, in this case coupled with efflux-dependent EtBr staining and cell sorting (Hassan et al., 2016). In the meantime, it has also been used to study the interaction of bacteria, for example by screening a *retS* deficient mutant library of *P. aeruginosa* and a H1-T6SS hyperactive *retS* mutant library to identify H1-T6SS related immunity genes through intraspecific competition (Nolan et al., 2021).

In another study, three so-called interbacterial antagonism resistance clusters (*arc1-3*) were identified in a screening assay using T6SS⁺ and T6SS⁻ *B. thailandensis* as competitors for a *P. aeruginosa* transposon library. They found that these clusters are regulated by Gac/Rsm, that they act mainly as defensive mechanisms, are not part of the general stress response and that they act rather specifically on certain effectors. In particular, Arc3 was found to prevent the accumulation of toxic lysophospholipids (Ting et al., 2022).

3.13 The role of AmpDh3 in cell wall recycling, antimicrobial resistance and interbacterial competition

Gram-negative bacteria generally recover about half of their cell wall peptidoglycan in one generation in a process called “cell wall or peptidoglycan recycling” (reviewed by J. T. Park & Uehara, 2008). The recycling process involves peptidoglycan-cleaving enzymes in the periplasm such as endopeptidases (e.g. PBP4), amidases, and lytic transglycosylases, as well as transporters such as AmpG, which transport degradation products into the cytoplasm of the cell, and cytoplasmic enzymes such as the muramoyl-L-alanine amidase AmpD, which further degrade and recycle those degradation products (Heidrich et al., 2001; Holtje et al., 1975; Jacobs et al., 1994; Jacobs et al., 1995; Korat et al., 1991).

Role of AmpDh3 in cell-wall recycling and antibiotic resistance

AmpD contributes to the β -lactam resistance of *P. aeruginosa* by cleaving the peptide stem off of 1,6-anhydro-*N*-acetyl-muramyl (anhMurNAc) peptides, which bind to and inactivate AmpR. AmpR is a negative regulator of the AmpC β -lactamase, and thereby, a loss of activity

in AmpD leads to inactivation of AmpR and derepression and heightened expression of *ampC* (M. Lee et al., 2009; Torrens et al., 2019). On the contrary, the presence of uridine pyrophosphoryl-N-acetylmuramyl-pentapeptide (UDP-MurNAc-5P), originating from a reaction further downstream the recycling pathway, leads to repression of *ampC* expression by binding to and activating AmpR (Jacobs et al., 1997).

AmpD has two paralogs in *P. aeruginosa*: AmpDh2, which is anchored to the inner leaflet of the outer membrane, and AmpDh3, which was first thought to be localized in the periplasm as well (Juan et al., 2006; W. Zhang et al., 2013). Remarkably, *ampC* becomes derepressed stepwise when *ampD*, *ampDh2* and *ampDh3* are deleted sequentially (Juan et al., 2006). AmpDh3 was shown to catalyze the same reactions as AmpD (W. Zhang et al., 2013), and deletion of *ampDh3* was shown to lead to a cytosolic accumulation of anhydromuramyl-tri- and -pentapeptides (Eggers et al., 2023). The expression of AmpDh3 is positively regulated by AlpA, an antiterminator that, after binding its DNA recognition site, interacts with the RNA-polymerase and lets it bypass intrinsic terminators downstream (Peña et al., 2021). AmpDh3 is negatively regulated by YgfB, which binds to AlpA and is thought to interfere with the binding of AlpA to its regulatory DNA element (Eggers et al., 2023).

Using a TraDIS approach, a previous study by our group aimed to identify genes required to maintain AmpC hyperproduction within the clinical isolate ID40 (Sonnabend et al., 2020). Besides genes that were expected to be involved in the regulation of *ampC* expression, like *ampG*, and genes involved in the peptidoglycan synthesis/remodeling pathway, a so-far uncharacterized gene termed *ygfB* was found to also regulate β -lactam resistance in that study (Sonnabend et al., 2020). Transcriptomic studies at the time had shown that *ygfB* deletion affects the expression of only two co-transcribed neighboring genes: *ampDh3* (PA0807) and PA0808. Further studies revealed that an induction of *ygfB* expression correlates with immediate reduction of *ampDh3* and PA0808 expression, followed by a slightly delayed increase of *ampC* expression. This identified YgfB as a so far unrecognized negative regulator of AmpDh3 and of PA0808 (Eggers et al., 2023).

Role of AmpDh3 in interbacterial competition

Interestingly, while the concept developed that AmpDh3 is negatively regulated by YgfB, Wang et al. (2020) described that AmpDh3 may also function as an antimicrobial effector of a Type VI secretion system (T6SS) of *P. aeruginosa* (Wang et al., 2020). The study suggested that *P. aeruginosa* employs AmpDh3 to kill other *Enterobacteriaceae* such as *E. coli* and *Y. pseudotuberculosis* by cleaving the peptides off of cross-linked peptidoglycan, while PA is protected from self-lysis by expression of the neighboring immunity protein PA0808, which is presumably co-transcribed with *ampDh3* (PA0807) (Wang et al., 2020). They concluded

that the secretion of AmpDh3 relies on Vgr2B secretion via the H2-T6SS, lysing competitor bacteria while protecting themselves from sibling cell attacks through PA0808. Since AmpDh3 and PA0808 are co-expressed, the AmpDh3-mediated impact on *ampC* expression was suspected to be a collateral damage due to imperfect self-protection by the immunity protein PA0808. In either case, beside expression of PA0808, a tight regulation of AmpDh3 by YgfB was supposed to be of importance to prevent self-damage of *P. aeruginosa*. At that time, AmpDh3 was thought to be a periplasmic protein based on its higher affinity for synthetic substrates that mimic cell wall compounds found exclusively in the periplasm compared to those commonly found in the cytoplasm, and was thought to be involved in cell-wall remodeling (M. Lee et al., 2013; W. Zhang et al., 2013). Curiously, the data presented in the study of Wang et al. (Fig. 2(a) and supplementary material, Fig. S3) suggests that AmpDh3, like its cytoplasmic homolog AmpD, is only toxic to *E. coli* when expressed as a fusion peptide with an exogenous *pelB* signal peptide for transport to the periplasm. AmpDh2, which is known to contain a signal peptide for transport to the periplasm, does not require this additional *pelB* tag for showing a toxic effect on *E. coli*. Fig. 2(b) of the same study also provides evidence that AmpDh3 is primarily expressed in the cytoplasm and only gets transported to the periplasm if tagged with an additional *pelB* signal peptide, but this is not discussed further. This piqued our interest because, to the best of our knowledge, this would imply the existence of a so far undiscovered loading mechanism of toxic effectors to the T6SS from the periplasm, and add another layer of complexity to the regulation of AmpDh3, which is known to be involved in cell wall recycling of *P. aeruginosa*.

3.14 Aim of this study

According to the studies of Wang et al. (2020), AmpDh3 was highlighted as a novel H2-T6SS effector complexed with Vgr2B via the H2-T6SS. In the same study, AmpDh3 was reported to be located in the periplasm, and to be involved in peptidoglycan recycling, and, by extension, β -lactam resistance. Own studies indicated that AmpDh3 expression is tightly controlled by YgfB. This raised the following questions to be answered:

- (i) Where is AmpDh3 localized? At the beginning of this study, no other study presenting experimental data had been published that conclusively showed the localization of AmpDh3 in *P. aeruginosa*.
- (ii) Is AmpDh3 secreted via H2-T6SS and does it contribute to lysis of competitors? How does YgfB modulate AmpDh3 secretion and finally, lysis of competitor bacteria? The experiments in the study published by Wang et al. (2020), and most other studies investigating the T6SS of *P. aeruginosa* at the time used a *retS* deficient and, by

extension, T6SS-overexpressing strain. With an *ampDh3*-overexpressing strain, we would potentially be able to investigate the T6SS of *P. aeruginosa* without the need to over-express the T6SS itself, and gain new insights into the functionality and regulation of the T6SS.

(iii) Which of the three T6SS and what effectors are of general importance to kill competitor bacteria? Is there a way to investigate the contribution of single T6SS-effectors to the competitiveness of *P. aeruginosa* without T6SS-overexpression?

(iv) Can we identify novel genes important to compete with other species in an assay that does not rely on the overexpression of T6SS?

To answer such questions a further important aim was to generate tools such as, for instance, generation of deletion mutants, and to establish an assay to measure competition between two species or strains, as well as a transposon-based screening to identify T6SS-related genes contributing to the competitive fitness of *P. aeruginosa*.

4 Materials and Methods

4.1 Materials

4.1.1 Technical equipment

Table 4: Technical equipment used in this study.

Equipment	Designation	Manufacturer
Analytical balance	EG 4200-2NM	Kern & Sohn GmbH, Balingen
Analog Cell Disruptor	250 Sonifier	Emerson Branson, Dietzenbach
Centrifuge	5417R	Eppendorf, Hamburg
Centrifuge	5430 R	Eppendorf, Hamburg
Centrifuge	5810 R	Eppendorf, Hamburg
Centrifuge	Microfuge 20R	Beckman Coulter, Krefeld
Centrifuge	Multifuge 3S-R	Heraeus Instruments, Hanau
Chemiluminescence imaging system	Fusion Solo S	Vilber Lourmat, Eberhardzell
Covaris Focused-Ultrasonicator	M220	Covaris, Brighton (UK)
Electroporation device	Gene Pulser II	Bio-Rad, München
Electrophoresis setup	Mini Protean Tetra Cell	Bio-Rad, München
Flow cytometer	LSRII Fortessa	BD Biosciences, Heidelberg
Gel electrophoresis chamber	Mini-Sub Cell GT	Bio-Rad, München
Gel documentation setup	BioDoc Analyze	Biometra, Göttingen
Gel documentation setup	FastGene® FAS-V	NIPPON Genetics Europe, Düren
Heating block	Thermomixer comfort	Eppendorf, Hamburg
Incubator for bacteria	HeraTherm Incubator	Thermo Fisher, Schwerte
Incubator for bacteria	FunctionLine	Heraeus Instruments, Hanau
Laminar flow safety cabinet	BDK-S 1200, 1300	Weiss, Sonnenbühl
LightCycler	LightCycler 480	Roche, Rotkreuz (CH)
Magnetic stirring hot plate	MR3001 K	Heidolph, Schwabach
Magnetic stirring hot plate	C-MAG HS7	IKA, Staufen
Microvolume UV-Vis Spectrophotometer	NanoDrop™ One	Thermo Fisher, Schwerte
Microwave	MW7775	Severin Elektrogeräte GmbH, Sundern
Mixing block	MB-102	BIOER, Hangzhou (China)
Multistepper	Multipette Plus	Eppendorf, Hamburg
Multi tube holder for vortex	Vortex-Genie 2	Scientific Industries, New York (USA)
Next-generation sequencing system	MiSeqDX	Illumina, San Diego (USA)
pH-electrode	LE438	Mettler Toledo GmbH, Gießen
Photometer	Bio Photometer	Eppendorf, Hamburg

Equipment	Designation	Manufacturer
Pipettes (2.5 µL, 10 µL, 20 µL, 100 µL, 200 µL, 1000 µL)	Reference, Research Plus	Eppendorf, Hamburg
Pipettes (2.5 µL, 10 µL, 100 µL, 200 µL, 1000 µL)	ErgoOne	Starlab GmbH, Hamburg
Pipetting aid	pipetus®	Hirschmann, Eberstadt
Pipetting aid	Accu-jet pro	Brand, Wertheim
Power supply for electrophoresis	PowerPac 200/300	Bio-Rad, München
Qubit Fluorometer	QuBit 2.0	Thermo Fisher, Schwerte
Rocking incubator	Assistant RM5	Karl Hecht, Sondheim
Sequencer	MiSeq	Illumina, San Diego, USA
Shaking incubator for bacteria	Ecotron	INFORS HT, Einsbach
Shaking incubator for bacteria	Multitron	INFORS HT, Einsbach
Shaking incubator for bacteria	Minitron	INFORS HT, Einsbach
Spectral photometer	NanoDrop One	Thermo Fisher, Schwerte
Tabletop centrifuge	miniSpin plus	Eppendorf, Hamburg
Tecan microplate reader	Infinite M200 Pro	Tecan, Crailsheim
Tecan microplate reader	Infinite M Plex	Tecan, Crailsheim
Thermal Printer	DPU-414	Seiko Instruments Inc., Neu-Isenburg
Thermocycler	C1000 Touch	Bio-Rad, München
Thermomixer	comfort	Eppendorf, Hamburg
Ultracentrifuge	Optima MAX-XP	Beckman Coulter, Krefeld
Vacuum pump	CVC 2000	Vacuubrand, Wertheim
Vortex mixer	Vortex Genie 2	Schientific Industries, New York (USA)
Vortex mixer	REAX Top	Heidolph, Schwabach
Water bath	WB 10	Memmert, Schwabach

4.1.2 Expendable materials

Table 5: Expendable materials used in this study.

Product	Manufacturer
AMPure XP Bead-Based Reagent	Beckman Coulter, Krefeld
Blotting paper sheets	Bio-Rad, München
Combitips plus (10 mL)	Eppendorf, Hamburg
Cryotube (1.8 mL)	Thermo Fisher, Schwerte
Cuvette, UV-Vis	Sarstedt, Nümbrecht
Cuvette (electroporation, gene pulser, 0.2 cm electrode cap)	Bio Rad, München
Glass bottles (Duran glass, 50 – 2000 mL)	DWK Life Sciences, Mainz
Glass spatula	WU, Mainz
Gloves (Peha soft nitrile)	Hartmann, Heidenheim
Gloves (Classic nitrile)	Abena, Zörbig
Kimtech Precision wipes	Kimberly-Clark, Dallas (USA)
Inoculation loops (10 µL)	Greiner, Frickenhausen
Lightcycler 480 multiwell plates, 96 wells	Roche, Rotkreuz (CH)
Lightcycler 480 sealing foils	Roche, Rotkreuz (CH)
Microtiter plates (24 well, clear)	Greiner, Frickenhausen
Microtiter plates (96 well, clear, white, black)	Greiner, Frickenhausen

Product	Manufacturer
AMPure XP Bead-Based Reagent	Beckman Coulter, Krefeld
microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm	Covaris, Brighton (UK)
PCR reaction tubes (0.2 ml), 8-tube strip	Biozym Scientific GmbH, Oldendorf
Petri dishes	Greiner, Frickenhausen
Pipette tips (5 mL, 10 mL, 25 mL, 50 mL)	Corning, Amsterdam (NL)
Pipette tips (10 µL)	Brand, Wertheim
Pipette tips (200 µL)	Sarstedt, Nümbrecht
Pipette tips (1000 µL)	Greiner, Frickenhausen
Pipette tips, filter tips, sterile	STARLAB, Hamburg
Qubit assay tubes (0.5 mL)	Thermo Fisher, Schwerte
Reaction tubes safe-seal (1.5 mL, 2 mL)	Eppendorf, Hamburg
Reaction tubes (1.5 mL, 2 mL)	Greiner, Frickenhausen
Reaction tubes (Conical, 15 mL)	Greiner, Frickenhausen
Reaction tubes (Conical, Falcon 50 mL)	Corning, New York (USA)
Reaction tubes (Round bottom, 5 mL, 14 mL)	Greiner, Frickenhausen
Scalpels (surgical, disposable)	Braun, Melsungen
SDS gels (gradient, 4-20%)	Bio-Rad, München
Sealing foil, gas permeable, for microtiter plates	Thermo Fisher, Schwerte
Syringe (10 mL)	Braun, Melsungen
Syringe filters (0.2 µm, 0.45 µm)	Merck, Darmstadt

4.1.3 Chemicals

Table 6: Chemicals used in this study.

Product	Manufacturer
Acetic acid (glacial) (AcOH)	Merck, Darmstadt
Acetone	Merck, Darmstadt
Agar	Roth, Karlsruhe
Agarose for gel electrophoresis (SeaKem)	Lonza, Rockland (USA)
3'-Azido-3'-Desoxythymidin (AZT)	Thermo Fisher, Schwerte
Bactoagar	BD Biosciences, Heidelberg
Bacto tryptone	Thermo Fisher, Schwerte
Bacto yeast extract	Thermo Fisher, Schwerte
β-mercaptoethanol	Applichem, Darmstadt
Boric acid	Applichem, Darmstadt
Calcium chloride dihydrate (CaCl ₂ · 2H ₂ O)	Merck, Darmstadt
Coomassie Brilliant Blue G 250	Applichem, Darmstadt
Descosept	Dr. Schumacher, Malsfeld
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Taufkirchen
Disodium hydrogen phosphate (Na ₂ HPO ₄)	Roth, Karlsruhe
DNase I recombinant, RNase-free	Roche, Rotkreuz (CH)
Ethanol	Merck, Darmstadt
Ethylenediaminetetraacetic acid (EDTA)	Merck, Darmstadt
Para-formaldehyde (PFA; OH(CH ₂ O) _n H)	Merck, Darmstadt
Glucose	Roth, Karlsruhe
Glycerol	Applichem, Darmstadt
Glycine	Applichem, Darmstadt
Hydrochloric acid (HCl)	Merck, Darmstadt
Incidin Plus	Ecolab, Monheim am Rhein

Product	Manufacturer
Isopropanol	Merck, Darmstadt
Isopropyl- β -D-thiogalactopyranosid (IPTG)	VWR, Bruchsal
L-(+)-Arabinose	Sigma-Aldrich, Taufkirchen
L-Rhamnose monohydrate (Rhamnose)	Sigma-Aldrich, Taufkirchen
Lysozyme from chicken egg white	Merck, Darmstadt
Magnesium chloride (MgCl_2)	Applichem, Darmstadt
Methanol	Thermo Fisher, Schwerte
Nicotinamide adenine dinucleotide (NAD^+)	Merck, Darmstadt
Orange G	Sigma-Aldrich, Taufkirchen
Polyethylene glycol 8000 (PEG-8000)	Thermo Fisher, Schwerte
Ponceau S	Applichem, Darmstadt
Potassium acetate	Sigma-Aldrich, Taufkirchen
Sodium chloride (NaCl)	Merck, Darmstadt
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe
Sodium dihydrogen phosphate (NaH_2PO_4)	Sigma-Aldrich, Taufkirchen
Sodium hydroxide (NaOH)	Merck, Darmstadt
Steritop™ Filter Units (0.22 μm pore size)	Merck, Darmstadt
Sucrose	Merck, Darmstadt
SYBR Safe DNA gel stain	Thermo Fisher, Schwerte
SYBR green DNA stain (100 x)	Thermo Fisher, Schwerte
Trichloroacetic acid (TCA)	Sigma-Aldrich, Taufkirchen
Triclosan (Irgasan)	Merck, Darmstadt
Triton X-100	Sigma-Aldrich, Taufkirchen
Tris(hydroxymethyl)aminomethane (Tris)	Applichem, Darmstadt
Trizma base	Sigma-Aldrich, Taufkirchen
Tween 20	Merck, Darmstadt

4.1.4 Buffers and solutions

Table 7: Buffers and solutions used in this study.

Buffer or solution	Ingredients / Manufacturer	Application
5 x SDS running buffer	60.4 g Trizma base 288 g glycine 20 g SDS DI- H_2O ad 2 L	SDS PAGE
5 x TBE electrophoresis buffer	54 g Trizma base 27.5 g boric acid 20 mL 0.5 M EDTA (pH = 8) DI- H_2O ad 2 L	Nucleic acid gel electrophoresis
10 x TBS buffer	24.2 g Trizma base 180 g NaCl adjust pH to 7.4 with HCl DI- H_2O ad 2 L	Western blot
1 x TBS-T buffer	100 mL 10 x TBS buffer 1 mL Tween 20 DI- H_2O	Western blot
10 x Western blot transfer buffer	60.4 g Trizma base 288 g glycine DI- H_2O ad 2 L	Western blot

Buffer or solution	Ingredients / Manufacturer	Application
1 x Western Blot Transfer Buffer with MeOH	100 mL of 10 x Western blot transfer buffer 200 mL methanol DI-H ₂ O ad 1 L	Western blot
Acetic acid solution (5% v/v)	5 mL Acetic acid (glacial) 95 mL DI-H ₂ O	Western blot
Arabinose solution (20% w/v)	2 g L-(+)-Arabinose DI-H ₂ O ad 10 mL	Arabinose stock solution
Bacteria lysis buffer	DNAse 10 µg/mL Lysozyme 20 µg/mL EDTA 1 mM MgCl ₂ 1 mM Triton-X 100 0.5% (v/v) cOmplete protease inhibitor cocktail 1:100 diluted in Buffer K	Chemical lysis of bacteria
BlueBlock PF (10 x)	SERVA Electrophoresis GmbH, Heidelberg	Western Blot
Buffer K	50 mM triethanolamine pH 7.5 250 mM sucrose	
CaCl ₂ solution (1 M)	147 g CaCl ₂ · 2H ₂ O DI-H ₂ O ad 1 L	Dilution to 0.1 M
CaCl ₂ solution (0.1 M)	100 mL CaCl ₂ solution (1 M) 900 mL DI-H ₂ O	Chemically competent cells
CaCl ₂ solution + 15% glycerol (v/v)	100 mL CaCl ₂ -solution (1 M) 300 mL glycerol (50% v/v) 600 mL DI-H ₂ O	Chemically competent cells
cOmplete protease inhibitor cocktail	Sigma-Aldrich, Taufkirchen	
Coomassie Quick Stain Solution (1 x)	800 mg Coomassie Brilliant Blue G-250 100 mL ethanol 900 mL DI-H ₂ O 5 mL 25% HCl (v/v)	Staining gels after SDS-PAGE
EDTA (0.5 M)	9.3 g EDTA adjust pH to 8 with NaOH ddH ₂ O ad 50 mL	Buffer preparation
Gibson isothermal reaction buffer (5 x)	Tris-HCl, pH 7.5 (500 mM) MgCl ₂ (50 mM) dNTPs (1 mM each) PEG-8000 (25%) NAD ⁺ (5 mM) H ₂ O ad 4 mL	Gibson assembly master mix
Gibson reaction master mix (1.33 x)	Taq DNA Ligase (4 U/µL) T5 Exonuclease (0.004 U/µL) Phusion HotStart II DNA Polymerase (0.025 U/µL) in 1 x isothermal reaction buffer	Gibson Assembly
Glycerol solution (50% v/v)	250 mL glycerol	

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Buffer or solution	Ingredients / Manufacturer	Application
	250 mL DI-H ₂ O	
Hybridization Buffer (HT1)	Illumina, San Diego (USA)	TraDIS sample preparation
IPTG (1 M stock solution)	23.83 g IPTG DI-H ₂ O ad 100 mL	Induction of <i>lac</i> -repressor controlled genes
Laemmli-buffer with β -mercaptoethanol (4 x)	900 μ L Laemmli buffer (4 x) 100 μ L β -mercaptoethanol	SDS-PAGE
MgCl ₂ solution (1 M)	9.52 g MgCl ₂ DI-H ₂ O ad 100 mL	PCR reactions and other solutions
Midori Green Xtra DNA stain	NIPPON Genetics Europe, Düren	
NaCl solution (5 M)	14.6 g NaCl DI-H ₂ O ad 50 mL	Preparation of other solutions
Nuclease-free water (Ambion)	Thermo Fisher, Schwerte (Invitrogen)	TraDIS sample preparation
Nuclease-free water (Aqua ad iniection)	Braun, Melsungen	PCR reactions, Protein analysis
Orange G loading buffer	43,7 mL of 87.5% glycerol (v/v) 200 mg Orange G DI-H ₂ O ad 100 mL	
DPBS (Gibco)	Thermo Fisher, Schwerte	Washing of bacterial cultures
Phusion HotStart II DNA Polymerase (2 U/ μ L)	Thermo Fisher, Schwerte	
Ponceau S staining solution (1 x)	100 mg Ponceau S 5% acetic acid solution ad 250 mL	Western blot
Resuspension Buffer (RSB)	Illumina, San Diego (USA)	TraDIS sample preparation
T5 Exonuclease (10,000 U/mL)	New England Biolabs, Frankfurt a.M.	
<i>Taq</i> DNA Ligase (40,000 U/mL)	New England Biolabs, Frankfurt a.M.	
TCA solution (100%)	50 g TCA 350 mL DI-H ₂ O	Protein precipitation
TE-buffer (1 x)	Thermo Fisher, Schwerte (Invitrogen)	TraDIS sample preparation
Sucrose solution (20%)	100 g sucrose DI-H ₂ O ad 500 mL filter through Steritop filter	Generation of electrocompetent bacteria
Sucrose-EDTA solution	500 μ L EDTA solution (0.5 M) 20 g sucrose Adjust pH to 7.3 DPBS ad 100 mL	Spheroblasting of <i>P. aeruginosa</i>

4.1.5 Culture media for bacteria

Table 8: Liquid and solid growth media for bacteria used in this study.

Culture medium	Ingredients / Manufacturer	Application
Liquid media		
Lysogeny Broth (LB; Miller's modification)	10 g tryptone 5 g yeast extract 10 g NaCl DI-H ₂ O ad 1 L	Standard liquid culture medium for all strains
Lysogeny Broth (LB; Lennox' modification)	10 g tryptone 5 g yeast extract 5 g NaCl DI-H ₂ O ad 1 L	Low-salt growth medium
LB-glycerol (20% v/v)	100 mL glycerol 400 mL LB (Miller)	Cryo-conservation of bacteria
LB-sucrose (15% w/v)	10 g tryptone 5 g yeast extract 5 g NaCl 150 g sucrose DI-H ₂ O ad 1 L	Generation of in-frame deletion mutants using pEXG2 derivatives
S.O.C. medium	ThermoFisher, Schwerte	Transformation of competent bacteria
Solid media		
Lysogeny Broth (LB) agar (Miller's modification; 1.5% w/v)	10 g tryptone 5 g yeast extract 10 g NaCl 15 g agar DI-H ₂ O ad 1 L	Standard solid culture medium for all strains
LB agar (3% w/v)	5 g tryptone 2.5 g yeast extract 0/2.5/5 g NaCl (No-salt/Low-salt/Standard LB agar) 15 g agar DI-H ₂ O ad 0.5 L	Inter-bacterial competition assays on solid agar
LB-arabinose (0.1% w/v) plates	2.5 mL arabinose solution (20% w/v) LB agar (Miller) ad 500 mL	Generation of fluorescent <i>P. aeruginosa</i> strains
LB-AZT-IPTG plates	100 mg AZT 500 µL IPTG (1 M) LB agar (Miller) ad 500 mL	Generation of in-frame deletion mutants using pEXG2 derivatives
LB-carbenicillin (200 µg/mL) plates	500 µL carbenicillin solution (200 µg/µL) LB agar (Miller) ad 500 mL	Selection of Gm ^R bacteria
LB-gentamicin (15 µg/mL) plates	750 µL gentamicin solution (10 µg/µL) LB agar (Miller) ad 500 mL	Selection of Gm ^R <i>E. coli</i> strains

Culture medium	Ingredients / Manufacturer	Application
LB-gentamicin (75 µg/mL) plates	3.75 mL gentamicin solution (10 µg/µL) LB agar (Miller) ad 500 mL	Selection of Gm ^R <i>P. aeruginosa</i> strains
LB-gentamicin (75 µg/mL)- irgasan (25 µg/mL) plates	3.75 mL gentamicin solution (10 µg/µL) 500 µL irgasan solution (25 µg/µL) LB agar (Miller) ad 500 mL	Selection of Gm ^R <i>P. aeruginosa</i> strains; negative selection of <i>E. coli</i>
LB-sucrose (15% w/v) plates	10 g tryptone 5 g yeast extract 5 g NaCl 15 g agar 150 g sucrose DI-H ₂ O ad 1 L	Generation of in-frame deletion mutants using pEXG2 derivatives

4.1.6 Kits and reagents

Table 9: Kits and other reagents used in this study.

Product	Manufacturer
BacTiter-Glo Microbial cell viability Assay	Promega, Madison (USA)
Clarity Western ECL Blotting Substrates	Bio-Rad, München
DNeasy UltraClean Microbial Kit	Qiagen, Venlo (NL)
dNTP mix (10 mM each)	Thermo Fisher, Schwerte
DpnI (10 U/µL)	Thermo Fisher, Schwerte
DkBit Peptide 1000x	Promega, Madison (USA)
FACS Clean	BD Biosciences, Heidelberg
FACS Flow	BD Biosciences, Heidelberg
FACS Rinse	BD Biosciences, Heidelberg
Gene Ruler 1 kb plus DNA Ladder	Thermo Fisher, Schwerte
KAPA HiFi HotStart plus dNTPs	Roche, Rotkreuz (CH)
KOD Hot Start DNA Polymerase	Merck, Darmstadt
Laemmli Sample buffer (4 x)	Bio-Rad, München
MangoMix, (2 x MangoTaq DNA Polymerase reaction mix)	Meridian (Bioline), Ohio (USA)
MiSeq Reagent Kit v2 (50-cycles)	Illumina, San Diego (USA)
Monarch Plasmid Miniprep Kit	New England Biolabs, Frankfurt a.M.
Nano-Glo HiBiT Blotting System	Promega, Madison (USA)
Nano-Glo® Live Cell Assay System	Promega, Madison (USA)
Nano-Glo HiBiT Lytic Detection System	Promega, Madison (USA)
PAGE Ruler prestained Protein Ladder (10 – 180 kDa)	Thermo Fisher, Schwerte
PhiX Sequencing Control V3	Illumina, San Diego (USA)
Phusion Phusion Hot Start II DNA Polymerase (2 U/µL)	Thermo Fisher, Schwerte
<i>Pfu</i> Turbo DNA Polymerase	Agilent Technologies, Waldbronn
Qubit dsDNA HS Assay Kit	Thermo Fisher, Schwerte
Wizard SV Gel and PCR Clean-Up System	Promega, Madison (WI, USA)

4.1.7 Antibiotics**Table 10: Antibiotics used in this study with stock concentrations.**

Antibiotic	Abbreviation	Stock concentration	Diluent	Manufacturer
Carbenicillin	Cb	200 mg/mL	DI-H ₂ O	Applichem, Darmstadt
Chloramphenicol	Chlo / Cm	50 mg/mL	Ethanol	Sigma-Aldrich, Taufkirchen
Gentamicin	Gm	10 mg/mL	DI-H ₂ O	Applichem, Darmstadt
Kanamycin	Kan	50 mg/mL	DI-H ₂ O	Applichem, Darmstadt
Triclosan (Irgasan)	Irga	25 mg/mL	Ethanol	Sigma-Aldrich, Taufkirchen

4.1.8 Antibodies**Table 11: Antibodies used in this study with working concentrations**

Antibody	Working dilution	Source / Manufacturer
Goat F(ab') ₂ anti-Rabbit IgG (H+L)-HRPO	1:2000	Dianova, Hamburg
Mouse anti- <i>E. coli</i> RNA Polymerase β	1:1000	BioLegend, San Diego (USA)
Rabbit anti-Mouse IgG (H+L) Secondary Antibody, HRP	1:2000	Thermo Fisher,
Rabbit anti-SurA (PA14)	1:200	Kristina Klein, AG Bohn/Schütz

4.1.9 Oligonucleotides used in this study

Table 12: Oligonucleotides used in this study

Oligo name	Sequence (5' → 3')	Used for	Source
pBBR1MCS-5 derivatives			
1755_pbbRlgbitF	TTAACGCGAATTTTAAACAAAATATTAACGCTT AGCTGTTGATGGTTACTCGGAACA	Generation of pBBR1MCS-5_igBiT	This study
1756_pBBRlgbitR	GATAACAATTTTACACAGGAAACAGCTATGGT CTTCACACTCGAAGATTTTCGTTG	Generation of pBBR1MCS-5_igBiT	This study
1757_pBBRseqIgb itF	TCGCAGTCGGCCTATTGG	Validation of pBBR1MCS-5_igBiT	This study
1758_pBBRseqIgb itR	GCACCCCAGGCTTTACAC	Validation of pBBR1MCS-5_igBiT	This study
pEXG2 and pEXTk derivatives			
1_gib_uni_pEXG2_f	AGGTCGACTCTAGAGGATCC	Linearization of pEXG2 (1 & 2) or pEXTk (1 & 1467) for Gibson assembly	AG Bohn
2_gib_uni_pEXG2_ r	TTCCGGCTCGTATAATGTGT	Linearization of pEXG2 (1 & 2) or pEXTk (1 & 1467) for Gibson assembly	AG Bohn
1467_pexTKuniAR	TTCCGGCTCGTATAATGTGTGGAATTAGCTTA CCTGC	Linearization of pEXG2 (1 & 2) or pEXTk (1 & 1467) for Gibson assembly	This study
4_pEXG2_seq_r	GATCCGGAACATAATGGTG	Sequencing of inserts in pEXG2 after Gibson assembly	AG Bohn
1372_pexAZTlinF	GCAGGTAAGCTAATTCCACAC	Sequencing of inserts in pEXTk after Gibson assembly	This study
1371_pexAZTlinR	AAACGCAAAAGAAAATGCCG	Gibson assembly of pEXTk from pEXG2 by exchange of <i>sacB</i> for IPTG-inducible <i>tdk</i> gene from pVT77	This study
1372_pexAZTlinF	GCAGGTAAGCTAATTCCACAC	Gibson assembly of pEXTk from pEXG2 by exchange of <i>sacB</i> for IPTG-inducible <i>tdk</i> gene from pVT77	This study
1373_AZTcassF	CTTCATAATCGGCATTTTCTTTTGC GTTTTT AATCGTGCGATGCCTTC	Sequencing primers for cloning of pEXTk	This study
1374_AZTcassR	TCGTATAATGTGTGGAATTAGCTTACCTGCCA CACCATCGAATGGTGCAAAAC	Sequencing primers for cloning of pEXTk	This study

Oligo name	Sequence (5' → 3')	Used for	Source
1375_AZTseq1F	CTTATGTCAATTCGAGAATTACG	Sequencing primers for cloning of pEXTk	This study
1376_AZTseq2R	GAGATTCGTGCGGAACATG	Sequencing primers for cloning of pEXTk	This study
1377_AZTseq3F	ACCTATACGCGAACTGAC	Sequencing primers for cloning of pEXTk	This study
1378_AZTseq4F	GTTAACGGCGGGATATAAC	Sequencing primers for cloning of pEXTk	This study
1387_Seq_Vector_pvT77_5_FWD	GCGGATAGTTAATGATCAG	Sequencing primers for cloning of pEXTk	This study
1388_pEXT seqF	ACCCTGAATTGACTCTCTTCC	Sequencing primers for cloning of pEXTk	This study
1072_ampDh3 hibit F	AGCTAATTCACACATTATACGAGCCGAAAT GCTGACCATCGACTACAAC	Gibson assembly of pEXG2_ampDh3-strep-hiBiT; Cloning of pEXTk_ampDh3-strep-hiBiT (insert amplification)	AG Bohn
1073_ampDh3 hibit R	CTTCTCAAATTGAGGATGACTCCACGCGCTGG CCGGGTATTTCTCGTT	Gibson assembly of pEXG2_ampDh3-strep-hiBiT	AG Bohn
1074_strephibit F	CTCTACGCTCTGAACGAGAAATACCCGGCCAG CGCGTGGAGTCATCC	Gibson assembly of pEXG2_ampDh3-strep-hiBiT	AG Bohn
1075_strep hibit R	TCGAGCCCGGGGATCCTCTAGAGTCGACCTTT AGCTAATCTTCTTGAACAGCCG	Gibson assembly of pEXG2_ampDh3-strep-hiBiT	AG Bohn
1076_HiBiT ampdh3linear R	TTAGCTAATCTTCTTGAACAGCCGCCAGCCGC TCACTCCACTCGAACCAC	Gibson assembly of pEXG2_ampDh3-strep-hiBiT	AG Bohn
1077_HiBiT ampdh3linear F	AAATCGGCCAGCAGTTGCAGGTTTCAGGCCGAG GTCGACTCTAGAGGATCCC	Gibson assembly of pEXG2_ampDh3-strep-hiBiT	AG Bohn
1078_HiBiT ampDH3 downF	GGCTGGCGGCTGTTCAAGAAGATTAGCTAAGC CATTAAACCGCGAGCG	Gibson assembly of pEXG2_ampDh3-strep-hiBiT	AG Bohn
963_ampDH3 proof1	ATTCGGCCATTTCGATGAG	Gibson assembly of pEXG2_ampDh3-strep-hiBiT	AG Bohn
1285_ampDh3 S1R	TCGAGCCCGGGGATCCTCTAGAGTCGACCTGC TGGTGACCTGCTCTAC	Gibson assembly of pEXG2_ampDh3-strep-hiBiT ; Cloning of pEXTk_ampDh3-strep-hiBiT (insert amplification)	AG Bohn
1080_hibitampDH 3proof1F	GAAGGAACTCTACGAGGCCG	Gibson assembly of pEXG2_ampDh3-strep-hiBiT	AG Bohn
1081_hibitampDh 3 proof2R	TCTTCTTGAACAGCCGCC	Gibson assembly of pEXG2_ampDh3-strep-hiBiT	AG Bohn

Oligo name	Sequence (5' → 3')	Used for	Source
1270_ <i>icmF1</i> up F	AGCTAATTCACACATTATACGAGCCGGAAGG CGTCTTCGTTGACCTTG	Gibson assembly of pEXTk_Δ <i>icmF1</i>	This study
1271_ <i>icmF1</i> upR	ATGAAAGGCTTCCTCAACTTCTTCGCGCGCGT GTCCGGCTTCAGCCTC	Gibson assembly of pEXTk_Δ <i>icmF1</i>	This study
1272_ <i>icmF1</i> down F	AAGGCGCTCCGGGAGGCTGAAGCCGGACACGC GCGCAAGAAGTTGAG	Gibson assembly of pEXTk_Δ <i>icmF1</i>	This study
1273_ <i>icmF1</i> dn R	CTCGAGCCCGGGGATCCTCTAGAGTCGACCTT GGAGCTGCTGTACCTGTG	Gibson assembly of pEXTk_Δ <i>icmF1</i>	This study
1274_ <i>icmF1</i> seqF	CCGCTGATCAGCGATTGTTC	Validation of pEXTk_Δ <i>icmF1</i>	This study
1275_ <i>icmF1</i> seqR	AACCGCCGGGTGGAAATC	Validation of pEXTk_Δ <i>icmF1</i>	This study
1276_ <i>icmF1</i> inside R	GACCAGTCGGACCTGGAAC	Validation of pEXTk_Δ <i>icmF1</i>	This study
1220_ <i>icmF2</i> _up_F	AGCTAATTCACACATTATACGAGCCGGAAAG CGGTTCGTTTCATTGGC	Gibson assembly of pEXTk_Δ <i>icmF2</i>	This study
1221_ <i>icmF2</i> upR	ATGAAAAACTTCTTCAAGAAAGTCGGCGCCTT CGATCTCGCGGTCCTGC	Gibson assembly of pEXTk_Δ <i>icmF2</i>	This study
1222_ <i>icmF2</i> down F	GCGGAAGCTGCGCAGGACCGCGAGATCGAAGG CGCCGACTTCTTGAAG	Gibson assembly of pEXTk_Δ <i>icmF2</i>	This study
1223_ <i>icmF2</i> downR	TCGAGCCCGGGGATCCTCTAGAGTCGACCTGA CCGTCCTCCTCGATCAC	Gibson assembly of pEXTk_Δ <i>icmF2</i>	This study
1224_ <i>icmF2</i> _seq1 F	CGTACTGCCGATGATCCTGTG	Validation of pEXTk_Δ <i>icmF2</i>	This study
1225_ <i>icmF2</i> _seq2 R	TATTTCCGATGCGCTCTACC	Validation of pEXTk_Δ <i>icmF2</i>	This study
1226_ <i>icmF2</i> _inside3R	CGTGACGTGATGGTGCTC	Validation of pEXTk_Δ <i>icmF2</i>	This study
1263_ <i>retS</i> up F	AGCTAATTCACACATTATACGAGCCGGAAAT CGTCATGGTCGACGGC	Gibson assembly of pEXG2_Δ <i>retS</i>	This study
1264_ <i>retS</i> up R	GCGGCGGCGATCGCGTTCGCCGACCCAGTATA GTCCTATGGCGATCCGAAGC	Gibson assembly of pEXG2_Δ <i>retS</i>	This study

Oligo name	Sequence (5' → 3')	Used for	Source
1265_ <i>retS</i> dn F	CGTGGTACGGCTTCGGATCGCCATAGGACTAT ACTGGGTCGGCGAACGC	Gibson assembly of pEXG2_ <i>ΔretS</i>	This study
1266_ <i>retS</i> dnR	TCGAGCCCGGGGATCCTCTAGAGTCGACCTAT CAGGCGACTGACGATCTG	Gibson assembly of pEXG2_ <i>ΔretS</i>	This study
1665_ <i>retS</i> seqF new2	GAGAAGATCCGCTGGAAC	Validation of pEXG2_ <i>ΔretS</i>	This study
1666_ <i>retS</i> seqR new2	ACCAGCATCTTCAGGTAG	Validation of pEXG2_ <i>ΔretS</i>	This study
1667_ <i>retS</i> inside new2	AGCGAGAACAGGTAGGAG	Validation of pEXG2_ <i>ΔretS</i>	This study
2315_gib_ <i>tse1</i> _up_ for	ATTATACGAGCCGGAAGCATAAATGTAAAGTC TCTACGCCGCACTTTC	Gibson assembly of pEXTk_ <i>Δtse1</i>	This study
2316_gib_ <i>tse1</i> _up_ rev	CAGGCTGCAACTGGCCAGGGAGTAGACGTAGT TGACGATGCATTGATCG	Gibson assembly of pEXTk_ <i>Δtse1</i>	This study
2317_gib_ <i>tse1</i> _dn_ for	ATGGACAGTCTCGATCAATGCATCGTCAACTA CGTCTACTCCCTGGCC	Gibson assembly of pEXTk_ <i>Δtse1</i>	This study
2318_gib_ <i>tse1</i> _dn_ rev	TCGAGCCCGGGGATCCTCTAGAGTCGACCTGC CTCGACGTAGTTCTCC	Gibson assembly of pEXTk_ <i>Δtse1</i>	This study
2319_ <i>tse1</i> _seq_f	CTGGGTCAGAAATGCTCG	Validation of pEXTk_ <i>Δtse1</i>	This study
2320_ <i>tse1</i> _seq_rev	TGGACATGGATGGACCTG	Validation of pEXTk_ <i>Δtse1</i>	This study
2321_ <i>tse1</i> _seq_ins	GACTTCAGGCCCTGGCTC	Validation of pEXTk_ <i>Δtse1</i>	This study
2322_gib_ <i>tse2</i> _up_ for	ATTATACGAGCCGGAAGCATAAATGTAAAGGC GCCTGAACTGTTTCGTC	Gibson assembly of pEXTk_ <i>Δtse2</i>	This study
2323_gib_ <i>tse2</i> _up_ rev	TCCTACGACTACGAGAAAACCAGCCTCACCCG CCAATGGGAAAAAGCC	Gibson assembly of pEXTk_ <i>Δtse2</i>	This study
2324_gib_ <i>tse2</i> _dn_ for	CTAGAGCCCGCGGGCTTTTCCCATTGGCGGG TGAGGCTGGTTTTCTC	Gibson assembly of pEXTk_ <i>Δtse2</i>	This study
2325_gib_ <i>tse2</i> _dn_ rev	TCGAGCCCGGGGATCCTCTAGAGTCGACCTCG CAACCTCTATTTGCTG	Gibson assembly of pEXTk_ <i>Δtse2</i>	This study

Oligo name	Sequence (5' → 3')	Used for	Source
2767 <i>tse2</i> seq for new	CTCTTCGATCAGCCGGTC	Validation of pEXTk_Δ <i>tse2</i>	This study
2768 <i>tse2</i> seq rev new	CTGTGGCTGGAGTTCTTCATC	Validation of pEXTk_Δ <i>tse2</i>	This study
2769 <i>tse2</i> seq ins new	GACGGCGACTTCGTGATAC	Validation of pEXTk_Δ <i>tse2</i>	This study
2329_gib_Δ <i>tse3</i> _up_for	ATTATACGAGCCGGAAGCATAAATGTAAAGAG GATGAAGCCGATGGTG	Gibson assembly of pEXTk_Δ <i>tse3</i>	This study
2330_gib_Δ <i>tse3</i> _up_rev	ATGACCGCCACCAGCGACCTGATCGAGTCATT CCTCGACCCTGGAATG	Gibson assembly of pEXTk_Δ <i>tse3</i>	This study
2331_gib_Δ <i>tse3</i> _down_for	TCATGGGAACCTCATTCCAGGGTCGAGGAATG ACTCGATCAGGTCGCT	Gibson assembly of pEXTk_Δ <i>tse3</i>	This study
2332_gib_Δ <i>tse3</i> _down_rev	TCGAGCCCGGGGATCCTCTAGAGTCGACCTAA AGCGTCTCCGGCAATG	Gibson assembly of pEXTk_Δ <i>tse3</i>	This study
2333_Δ <i>tse3</i> _seq_for	CCTCTACCTTGTAGCGCG	Validation of pEXTk_Δ <i>tse3</i>	This study
2334_Δ <i>tse3</i> _seq_rev	TTGTTCGATGGCACCATCG	Validation of pEXTk_Δ <i>tse3</i>	This study
2335_Δ <i>tse3</i> _seq_ins	CAGGACCTGCGCAAGTTG	Validation of pEXTk_Δ <i>tse3</i>	This study
2336_gib_Δ <i>tse4</i> _up_for	ATTATACGAGCCGGAAGCATAAATGTAAAGGG GGTCTGTAGAATGCGC	Gibson assembly of pEXTk_Δ <i>tse4</i>	This study
2337_gib_Δ <i>tse4</i> _up_rev	CGCGCCGCCGCCGAACGTGTCACCGACGTTGT TGAGGGGCAAGGGAATG	Gibson assembly of pEXTk_Δ <i>tse4</i>	This study
2338_gib_Δ <i>tse4</i> _down_for	TCGCAACTTCCCATTCCCTTGCCCCTCAACAAC GTCGGTGCACAGTTC	Gibson assembly of pEXTk_Δ <i>tse4</i>	This study
2339_gib_Δ <i>tse4</i> _down_rev	TCGAGCCCGGGGATCCTCTAGAGTCGACCTAA CCAGAGCGAAAACACG	Gibson assembly of pEXTk_Δ <i>tse4</i>	This study
2340_Δ <i>tse4</i> _seq_for	TCTTCGGCTACCAGGTGG	Validation of pEXTk_Δ <i>tse4</i>	This study
2341_Δ <i>tse4</i> _seq_rev	CCTCGAACGAGTGGATGG	Validation of pEXTk_Δ <i>tse4</i>	This study
2342_Δ <i>tse4</i> _seq_ins	TCGACGTACATGCAGGGC	Validation of pEXTk_Δ <i>tse4</i>	This study
2393_gib_Δ <i>tse5</i> _up_f_new	ATTATACGAGCCGGAAGCATAAATGTAAAGAG GCGACCCGGACCG	Gibson assembly of pEXTk_Δ <i>tse5</i>	This study

Oligo name	Sequence (5' → 3')	Used for	Source
2394_gib_tse5_up_r_new	CTCCTCTTCTCGTCGCCGACATGGGAAACCGG	Gibson assembly of pEXTk_Δtse5	This study
2395_gib_tse5_dn_f_new	GTTTCCCATGTCTGGCGACGAAGAAGAGGAGTGCCCG	Gibson assembly of pEXTk_Δtse5	This study
2396_gib_tse5_dn_r_new	TCGAGCCCGGGGATCCTCTAGAGTCGACCTATCGTAGGGCGGGATCAGGGTCAG	Gibson assembly of pEXTk_Δtse5	This study
2347_tse5_seq_for	GCTCGAAGATCACCTGG	Validation of pEXTk_Δtse5	This study
2348_tse5_seq_rev	CTCACTCGTCCACGTAGC	Validation of pEXTk_Δtse5	This study
2349_tse5_seq_ins	GCGTCTTCGCTCAGTTCC	Validation of pEXTk_Δtse5	This study
2397_gib_tse6_up_f_new	ATTATACGAGCCGGAAGCATAAATGTAAAGAACGATGCCTCGCTGT	Gibson assembly of pEXTk_Δtse6	This study
2398_gib_tse6_up_r_new	GCCGCCCCGACTGGGCTTCGGTCTGCTTCCCCG	Gibson assembly of pEXTk_Δtse6	This study
2399_gib_tse6_dn_f_new	GGGAAGCAGACCGAAGCCCAGTCGGGCGGCG	Gibson assembly of pEXTk_Δtse6	This study
2400_gib_tse6_dn_r_new	TCGAGCCCGGGGATCCTCTAGAGTCGACCTCGAGCTCTTCGCGACCC	Gibson assembly of pEXTk_Δtse6	This study
2354_tse6_seq_for	TTCCGGGTCGGTTTCGTC	Validation of pEXTk_Δtse6	This study
2355_tse6_seq_rev	GTGCTCCTCGACTACCAG	Validation of pEXTk_Δtse6	This study
2356_tse6_seq_ins	CGGAGCTGGAATCAACC	Validation of pEXTk_Δtse6	This study
2357_gib_tse7_up_for	ATTATACGAGCCGGAAGCATAAATGTAAAGGGGTTGGAGCAGCCTTTG	Gibson assembly of pEXTk_Δtse7	This study
2358_gib_tse7_up_rev	GCCGTTGGCATAGACCTCGTTGGCCATCTAGTTCGCCCTCACTCGCTG	Gibson assembly of pEXTk_Δtse7	This study
2359_gib_tse7_down_for	TAGATGGCCAACGAGGTCTATGCCAACGGCAAAGTCATACCACCCGCG	Gibson assembly of pEXTk_Δtse7	This study
2360_gib_tse7_down_rev	TCGAGCCCGGGGATCCTCTAGAGTCGACCTCGCGGTCACATAGAGCTTG	Gibson assembly of pEXTk_Δtse7	This study
2361_tse7_seq_for	GCCTTGAGTCGAACCCTG	Validation of pEXTk_Δtse7	This study

Oligo name	Sequence (5' → 3')	Used for	Source
2362_tse7_seq_rev	GGCGCTGTAGACGACTTC	Validation of pEXTk_Δtse7	This study
2363_tse7_seq_ins	AGCATCACTTCCTTGCGG	Validation of pEXTk_Δtse7	This study
2401_gib_tse8_up_for	ATTATACGAGCCGGAAGCATAAATGTAAAGCG GGCACAACGCCTCG	Gibson assembly of pEXTk_Δtse8	This study
2402_gib_tse8_up_rev_new	GAGGTTTCCATCGCCCCGCGTACTCCACCGCTG	Gibson assembly of pEXTk_Δtse8	This study
2403_gib_tse8_dn_for_new	CGGTGGAGTACGCGGGGCGATGGAACCTCGG TGACC	Gibson assembly of pEXTk_Δtse8	This study
2404_gib_tse8_dn_rev_new	TCGAGCCCGGGGATCCTCTAGAGTCGACCTGA CCGTGATGCCCAGCGG	Gibson assembly of pEXTk_Δtse8	This study
2368_tse8_seq_for	CAGGTGTTCGTTGGGACG	Validation of pEXTk_Δtse8	This study
2369_tse8_seq_rev	GAAATGGTCCCCCGACG	Validation of pEXTk_Δtse8	This study
2370_tse8_seq_ins	GCGGATCGACCTCGAGGA	Validation of pEXTk_Δtse8	This study
1398_amgS up F	AGCTAATTCCACACATTATACGAGCCGGAAGA CATCACCGCGGTCTGG	Gibson assembly of pEXTk_ΔamgS	This study
1399_amgS up R	AAAACGCCGCTCTGGTTCCCGCAAAGCTTCCC GTTGGGGTTGCTGCTG	Gibson assembly of pEXTk_ΔamgS	This study
1400_amgS dn F	CGCGCCGCGCGGCAGCAGCAACCCCAACGGGA AGCTTTGCGGGAACCAGAG	Gibson assembly of pEXTk_ΔamgS	This study
1401_amgS dn R	TCGAGCCCGGGGATCCTCTAGAGTCGACCTCT GCTCGAACGTTTCCTC	Gibson assembly of pEXTk_ΔamgS	This study
1418_amgS SeqF	AGCCTCTGGCAATTGCTC	Sequencing primers for pEXTk_ΔamgS	This study
1419_amgS SeqR	TCTTACCCGCAACAGGCG	Sequencing primers for pEXTk_ΔamgS	This study
1420_amgS Inside	CGCGAACGCGAATTGATG	Sequencing primers for pEXTk_ΔamgS	This study
2204_pextK gacS upF	ATACGAGCCGGAAGCATAAATGTAAAGGCAGA CATCGGCAGAGAATTG	Gibson assembly of pEXTk_ΔgacS	This study
2205_pextK gacS upR	TCAGAGTTCGCTGGAGTCGAGGCTGGTGGATA CGCGCCCCTTGATGCC	Gibson assembly of pEXTk_ΔgacS	This study

Oligo name	Sequence (5' → 3')	Used for	Source
2206_pextk <i>gacS</i> dnF	TTCAAGGATCTCGGCATCAAGGGGCGCGTATC CACCAGCCTCGACTCC	Gibson assembly of pEXTk_Δ <i>gacS</i>	This study
2207_pextk <i>gacS</i> dnR	TCGAGCCCGGGGATCCTCTAGAGTCGACCTAT CGAGGCCGAGGTAGCC	Gibson assembly of pEXTk_Δ <i>gacS</i>	This study
2208_pextk <i>gacS</i> seq1F	GGATGGGAACATGGCAGC	Validation of pEXTk_Δ <i>gacS</i>	This study
2209_pextk <i>gacS</i> seq2R	GCGAGAGGTCGTCGTTGAC	Validation of pEXTk_Δ <i>gacS</i>	This study
2210_pextK <i>gacS</i> insideR	GTTGGCGATGCGGTTGAG	Validation of pEXTk_Δ <i>gacS</i>	This study
2751_gib_cobT_up_for	ATTATACGAGCCGGAAGCATAAATGTAAAGCA GGCTCAGCAGCAAGGC	Gibson assembly of pEXTk_Δ <i>cobT</i>	This study
2752_gib_cobT_up_rev	GCTATGAGTCTGCAATGGTGGCGCGACACCGA AGCGGCGGTCTCGGAC	Gibson assembly of pEXTk_Δ <i>cobT</i>	This study
2753_gib_cobT_dn_for	TCATGCCGGGCGGTCCGAGACCGCCGCTTCGG TGTCGCGCCACCATTTG	Gibson assembly of pEXTk_Δ <i>cobT</i>	This study
2754_gib_cobT_dn_rev	TCGAGCCCGGGGATCCTCTAGAGTCGACCTCC GCTGTCTGGGCTACGAG	Gibson assembly of pEXTk_Δ <i>cobT</i>	This study
2755_cobT_sec_for	AGCGCCGAAATGCAGTTC	Validation of pEXTk_Δ <i>cobT</i>	This study
2756_cobT_sec_rev	TGCTGCTCTGCGACGATC	Validation of pEXTk_Δ <i>cobT</i>	This study
2757_cobT_sec_inside	TACATCTGCAGCGTCGCG	Validation of pEXTk_Δ <i>cobT</i>	This study
2695_ssub1_gib_up_F_new	CCGGAAGCATAAATGTAAAGGAGTTGAGAGCG GTCTGGAGCG	Gibson assembly of pEXTk_Δ <i>ssub1</i>	This study
2696_ssub1_gib_up_R_new	GGGGTGAAACGGGCGGCGTTGTGGGCAT	Gibson assembly of pEXTk_Δ <i>ssub1</i>	This study
2697_ssub1_gib_down_R_new	AACGCCGCCCGTTTCACCCCTGCCCACG	Gibson assembly of pEXTk_Δ <i>ssub1</i>	This study

Oligo name	Sequence (5' → 3')	Used for	Source
2698_ssub1_gib_dn_R_new	GGATCCTCTAGAGTCGACCTCCTGCCCCAGAC ACCATG	Gibson assembly of pEXTk_Assub1	This study
2620_ssub1_seq_F	GCTTCATGTGGTTGACCC	Validation of pEXTk_Assub1	This study
2621_ssub1_seq_R	ATGGATACCTCGGTGGAC	Validation of pEXTk_Assub1	This study
2622_ssub1_seq_in s	AGTCCTGGAACATCAGCC	Validation of pEXTk_Assub1	This study
2609_gib_pEXTk_02027_up_F	ATTATACGAGCCGGAAGCATAAATGTAAAGAC GCCGTCGAACTCTTCG	Gibson assembly of pEXTk_ΔPROKKA02027	This study
2610_gib_pEXTk_02027_up_R	AGTCATTTCCTGTCGCTGCGTAGCCTCGGCTG	Gibson assembly of pEXTk_ΔPROKKA02027	This study
2611_gib_pEXTk_02027_dn_F	GCCGAGGCTACGCAGCGACAGGAAATGACTCA TGGGG	Gibson assembly of pEXTk_ΔPROKKA02027	This study
2612_gib_pEXTk_02027_dn_R	TCGAGCCCGGGGATCCTCTAGAGTCGACCTTG CTGCCGGCGGC	Gibson assembly of pEXTk_ΔPROKKA02027	This study
2613_02027_seq_F	GTCGTGATAGCGCTCGTAG	Validation of pEXTk_ΔPROKKA02027	This study
2614_02027_seq_R	CAATATCGCTACCCGCAG	Validation of pEXTk_ΔPROKKA02027	This study
2615_02027_seq_in s	GATGTTCTGGCTCGTCGC	Validation of pEXTk_ΔPROKKA02027	This study
2623_gib_05623_up_F	ATTATACGAGCCGGAAGCATAAATGTAAAGCT GGATCGCGCGCG	Gibson assembly of pEXTk_ΔPROKKA05623	This study
2624_gib_05623_up_R	AATCCGCTCGACCGGATTCGCAAGCATGTTTG AGTTCCGCT	Gibson assembly of pEXTk_ΔPROKKA05623	This study
2625_gib_05623_dn_F	AACATGCTTGCGAATCCGGTCGAGCGGATTCA T	Gibson assembly of pEXTk_ΔPROKKA05623	This study
2626_gib_05623_dn_R	TCGAGCCCGGGGATCCTCTAGAGTCGACCTCG ACCAGGTGCGCATCTA	Gibson assembly of pEXTk_ΔPROKKA05623	This study
2627_05623_seq_F	CATGGAACCGGAGACATC	Validation of pEXTk_ΔPROKKA05623	This study

Oligo name	Sequence (5' → 3')	Used for	Source
2628_05623_seq_R	CAGCAACTCAGCCTGATC	Validation of pEXTk_ΔPROKKA05623	This study
2629_05623_seq_ins	ATCAACGGCCTGCTCAAG	Validation of pEXTk_ΔPROKKA05623	This study
2630_gib_05679_up_F	ATTATACGAGCCGGAAGCATAAATGTAAAGCA CGACACCTGTGTCAGCG	Gibson assembly of pEXTk_ΔPROKKA05679	This study
2631_gib_05679_up_R	GGCGCGCTGGCGGAGCACCAGCCAAGTGGTCA TAAGG	Gibson assembly of pEXTk_ΔPROKKA05679	This study
2632_gib_05679_down_F	ACCACTTGGCTGGTGTCTCCGCCAGCGCG	Gibson assembly of pEXTk_ΔPROKKA05679	This study
2633_gib_05679_down_R	TCGAGCCCGGGGATCCTCTAGAGTCGACCTCG TCCGTTCACTCTTTGTCTGG	Gibson assembly of pEXTk_ΔPROKKA05679	This study
2634_05679_seq_F	GATAGGGCATGAACGTCTC	Validation of pEXTk_ΔPROKKA05679	This study
2635_05679_seq_R	CTGTTGAAGCTGCAGCAG	Validation of pEXTk_ΔPROKKA05679	This study
2636_05679_seq_ins	TCCAGCAACTCATCGTCC	Validation of pEXTk_ΔPROKKA05679	This study
2807 <i>gacS</i> sequencing 1 rev	CTGGCTGAAGGCCTTGAAC	Validation of the <i>gacS</i> gene of ID40Δ <i>retS</i> mutants	This study
2808 <i>gacS</i> sequencing 2 up	CAGGACACCGGTATCGGC	Validation of the <i>gacS</i> gene of ID40Δ <i>retS</i> mutants	This study
2809 <i>gacA</i> sequencing 2 for	gtggcggtgtgtctgcta	Validation of the <i>gacA</i> gene of ID40Δ <i>retS</i> mutants	This study
2810 <i>gacA</i> sequencing 2 rev	cgaacatgcggtagacgc	Validation of the <i>gacA</i> gene of ID40Δ <i>retS</i> mutants	This study

Oligo name	Sequence (5' → 3')	Used for	Source
TraDIS			
SplA5_top	G*AGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T	Adapter top strand	AG Bohn/Schütz
SplA5_bottom	/5PHOS/G*ATCGGAAGAGCGGTTCAGCAGGTTTTTTTTTCAAAAAA*A	Adapter bottom strand	AG Bohn/Schütz
SplAP5.1	C*AAGCAGAAGACGGCATACGAGATAACGTGATGAGATCGGTCTCGGCATTC*C	Adapter-specific index primer for Tn amplification and barcoding of sample 1	AG Bohn/Schütz
SplAP5.2	C*AAGCAGAAGACGGCATACGAGATAAACATCGGAGATCGGTCTCGGCATTC*C	Adapter-specific index primer for Tn amplification and barcoding of sample 2	AG Bohn/Schütz
SplAP5.3	C*AAGCAGAAGACGGCATACGAGATATGCCTAAGAGATCGGTCTCGGCATTC*C	Adapter-specific index primer for Tn amplification and barcoding of sample 3	AG Bohn/Schütz
SplAP5.4	C*AAGCAGAAGACGGCATACGAGATAGTGGTCAGAGATCGGTCTCGGCATTC*C	Adapter-specific index primer for Tn amplification and barcoding of sample 4	AG Bohn/Schütz
SplAP5.5	C*AAGCAGAAGACGGCATACGAGATACCACTGTGAGATCGGTCTCGGCATTC*C	Adapter-specific index primer for Tn amplification and barcoding of sample 5	AG Bohn/Schütz
SplAP5.6	C*AAGCAGAAGACGGCATACGAGATACATTGGCGAGATCGGTCTCGGCATTC*C	Adapter-specific index primer for Tn amplification and barcoding of sample 6	AG Bohn/Schütz
SplAP5.7	C*AAGCAGAAGACGGCATACGAGATCAGATCTGGAGATCGGTCTCGGCATTC*C	Adapter-specific index primer for Tn amplification and barcoding of sample 7	AG Bohn/Schütz
SplAP5.8	C*AAGCAGAAGACGGCATACGAGATCATCAAGTGAGATCGGTCTCGGCATTC*C	Adapter-specific index primer for Tn amplification and barcoding of sample 8	AG Bohn/Schütz
SplAP5.9	C*AAGCAGAAGACGGCATACGAGATCGCTGATCGAGATCGGTCTCGGCATTC*C	Adapter-specific index primer for Tn amplification and barcoding of sample 9	AG Bohn/Schütz
qPCR2.1	AATGATACGGCGACCACCGAG	P5 adapter-specific primer for quantification of final amplicates before sequencing	AG Bohn/Schütz
qPCR 2.2	CAAGCAGAAGACGGCATACGA	P7 adapter-specific primer for quantification of final amplicates before sequencing	AG Bohn/Schütz
NTMPseq3	AAGCCTGCTTTCTAGAGACCGGGGACTTATCAG	Transposon-specific primer for transposon amplification, quantification of final amplicates before sequencing and sequencing	AG Bohn/Schütz

Oligo name	Sequence (5' → 3')	Used for	Source
iPCRTagSeq	AAGAGCGGTTTCAGCAGGAATGCCGAGACCGAT CTC	Second sequencing primer; used for barcode sequencing	AG Bohn/Schütz

4.1.10 Plasmids used in this study

Table 13: Plasmids used in this study

Plasmid name	Description	Source
pEXG2	Allelic exchange vector with pBR origin, <i>sacB</i> ⁺ ; Gm ^R	Rietsch et al. (2005)
pEXG2_Δ <i>retS</i>	pEXG2 derivative for in-frame deletion of <i>retS</i> ; Gm ^R	This study
pEXG2_Δ <i>ygfB</i>	pEXG2 derivative for in-frame deletion of <i>ygfB</i> ; Gm ^R	Sonnabend et al. (2020)
pEXG2_Δ <i>ampDh3-strep-hiBiT</i>	pEXG2 derivative for in-frame allelic exchange of <i>ampDh3</i> for <i>ampDh3-strep-hiBiT</i> ; Gm ^R	Eggers et al. (2023)
pEXTk_Δ <i>ampDh3-strep-hiBiT</i>	pEXTk derivate with the <i>ampDh3-strep-hiBiT</i> stretch amplified from pEXG2_Δ <i>ampDh3-strep-hiBiT</i> and integrated into the scar region using Gibson assembly. Gm ^R	Eggers et al. (2023), this study
pEXTk	pEXG2 derivative with in-frame exchange of <i>sacB</i> gene for <i>E. coli</i> thymidine kinase/thymidylate kinase <i>tdk</i> ; Gm ^R	Eggers et al. (2023), this study
pEXTk_Δ <i>ampDh3</i>	pEXTk derivative for in-frame deletion of <i>ampDh3</i> ; Gm ^R	This study
pEXTk_Δ <i>cobT</i>	pEXTk derivative for in-frame deletion of <i>cobT</i> ; Gm ^R	This study
pEXTk_Δ <i>envZ</i>	pEXTk derivative for in-frame deletion of <i>envZ</i> ; Gm ^R	This study
pEXTk_Δ <i>icmF1</i>	pEXTk derivative for in-frame deletion of <i>icmF1</i> ; Gm ^R	This study
pEXTk_Δ <i>icmF2</i>	pEXTk derivative for in-frame deletion of <i>icmF2</i> ; Gm ^R	This study
pEXTk_Δ <i>retS</i>	pEXTk derivative for in-frame deletion of <i>retS</i> ; Gm ^R	This study
pEXTk_Δ <i>ssub1</i>	pEXTk derivative for in-frame deletion of <i>ssub1</i> ; Gm ^R	This study
pEXTk_Δ <i>tse1</i>	pEXTk derivative for in-frame deletion of <i>tse1</i> ; Gm ^R	This study

Plasmid name	Description	Source
pEXTk_Δ <i>tse2</i>	pEXTk derivative for in-frame deletion of <i>tse2</i> ; Gm ^R	This study
pEXTk_Δ <i>tse3</i>	pEXTk derivative for in-frame deletion of <i>tse3</i> ; Gm ^R	This study
pEXTk_Δ <i>tse4</i>	pEXTk derivative for in-frame deletion of <i>tse4</i> ; Gm ^R	This study
pEXTk_Δ <i>tse5</i>	pEXTk derivative for in-frame deletion of <i>tse5</i> ; Gm ^R	This study
pEXTk_Δ <i>tse6</i>	pEXTk derivative for in-frame deletion of <i>tse6</i> ; Gm ^R	This study
pEXTk_Δ <i>tse7</i>	pEXTk derivative for in-frame deletion of <i>tse7</i> ; Gm ^R	This study
pEXTk_Δ <i>tse8</i>	pEXTk derivative for in-frame deletion of <i>tse8</i> ; Gm ^R	This study
pEXTk_ΔPROKKA02027	pEXTk derivative for in-frame deletion of PROKKA02027; Gm ^R	This study
pEXTk_ΔPROKKA05623	pEXTk derivative for in-frame deletion of PROKKA05623; Gm ^R	This study
pEXTk_ΔPROKKA05679	pEXTk derivative for in-frame deletion of PROKKA05679; Gm ^R	This study
pEXTk_Δ <i>ygfB</i>	pEXTk derivative for in-frame deletion of <i>ygfB</i> ; Gm ^R	Eggers et al. (2023), this study
pMRE_Tn7_143 (<i>syfp2</i>)	miniTn7 plasmid for genomic integration of constitutively expressed sYFP2; Gm ^R	pMRE-Tn7-143 was a gift from Mitja Remus-Emsermann (Addgene plasmid # 118559; http://n2t.net/addgene:118559 ; RRID:Addgene_118559)

4.1.11 Bacterial strains used in this study

E. coli strains used in this study

Table 14: E. coli strains used in this study

Strain name	Description	Used for	Obtained from
<i>E. coli</i> DH5 α	Used for maintaining plasmids; F ⁻ ϕ 80/ <i>lacZ</i> Δ M15 Δ (<i>lacZ</i> YA- <i>argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ , m _K ⁺) <i>phoA supE44</i> λ ⁻ <i>thi-1 gyrA96 relA1</i>	Maintenance and propagation of plasmids	Obtained from Thermo Scientific
<i>E. coli</i> SM10 λ <i>pir</i>	Used for mobilization of plasmids; <i>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km</i> λ <i>pir</i>	Conjugal transfer of <i>oriT</i> -containing plasmids from <i>E. coli</i> to <i>P. aeruginosa</i>	Simon et al. (1983)

Other strains used in this study

Table 15: Strains used in this study

Strain name	Description	Obtained from
<i>Burkholderia cenocepacia</i> ATCC 17765	Urinary tract infection isolate of <i>B. cenocepacia</i> ; former genomovar III	American Type Culture Collection (ATCC)
<i>Burkholderia cenocepacia</i> J12315	Epidemic cystic fibrosis isolate of <i>B. cenocepacia</i> ; former genomovar III	American Type Culture Collection (ATCC)
<i>Burkholderia multivorans</i> ATCC 17759	Forest soil isolate and type strain of <i>B. multivorans</i> ; former genomovar II	American Type Culture Collection (ATCC)
<i>Burkholderia cepacia</i> ATCC 25416	<i>B. cepacia</i> type strain; onion isolate; former genomovar I	American Type Culture Collection (ATCC)
<i>Burkholderia multivorans</i> ATCC 17616	Soil isolate of <i>B. multivorans</i> ; former genomovar II	American Type Culture Collection (ATCC)
<i>P. aeruginosa</i> ID40	Strain ID40 of <i>P. aeruginosa</i> ; Human bloodstream isolate, highly antibiotic resistant, <i>lasR</i> mutant, <i>dacB</i> mutant	Willmann et al. (2018)
<i>P. aeruginosa</i> PA14 DSM-19882	Strain PA14 of <i>P. aeruginosa</i> ; Highly virulent human isolate, <i>ladS</i> mutant	DSMZ German Collection of Microorganisms and Cell Cultures GmbH

Strain name	Description	Obtained from
<i>P. aeruginosa</i> PA01 DSM-19880	Strain PA01; Human wound isolate of <i>P. aeruginosa</i>	DSMZ German Collection of Microorganisms and Cell Cultures GmbH
ID40:: <i>ampDh3-strep-hiBiT</i>	In-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i> in strain ID40	Eggers et al. (2023), this study
ID40Δ <i>icmF2</i> :: <i>ampDh3-strep-hiBiT</i>	In-frame <i>icmF2</i> deletion mutant of strain ID40 with in-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i>	This study
ID40Δ <i>icmF2</i> Δ <i>retS</i> :: <i>ampDh3-strep-hiBiT</i>	In-frame <i>icmF2</i> and <i>retS</i> deletion mutant of strain ID40 with in-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i>	This study
ID40Δ <i>retS</i> :: <i>ampDh3-strep-hiBiT</i>	In-frame <i>retS</i> deletion mutant of strain ID40 with in-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i>	This study
ID40Δ <i>ygfB</i> :: <i>ampDh3-strep-hiBiT</i>	In-frame <i>ygfB</i> deletion mutant of strain ID40 with in-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i>	Eggers et al. (2023), this study
ID40Δ <i>ygfB</i> Δ <i>icmF2</i> :: <i>ampDh3-strep-hiBiT</i>	In-frame <i>icmF2</i> and <i>ygfB</i> deletion mutant of strain ID40 with in-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i>	This study
ID40Δ <i>ygfB</i> Δ <i>retS</i> :: <i>ampDh3-strep-hiBiT</i>	In-frame <i>ygfB</i> and <i>retS</i> deletion mutant of strain ID40 with in-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i>	This study
ID40Δ <i>ygfB</i> Δ <i>retS</i> Δ <i>icmF2</i> :: <i>ampDh3-strep-hiBiT</i>	In-frame <i>ygfB</i> , <i>retS</i> and <i>icmF2</i> deletion mutant of strain ID40 with in-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i>	This study
PA14:: <i>ampDh3-strep-hiBiT</i>	In-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i> in strain PA14	This study
PA14Δ <i>icmF2</i> :: <i>ampDh3-strep-hiBiT</i>	In-frame <i>icmF2</i> deletion mutant of strain PA14 with in-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i>	This study
PA14Δ <i>icmF2</i> Δ <i>retS</i> :: <i>ampDh3-strep-hiBiT</i>	In-frame <i>icmF2</i> and <i>retS</i> deletion mutant of strain PA14 with in-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i>	This study
PA14Δ <i>ygfB</i> :: <i>ampDh3-strep-hiBiT</i>	In-frame <i>ygfB</i> deletion mutant of strain PA14 with in-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i>	Bohn/Schütz Lab
PA14Δ <i>ygfB</i> Δ <i>icmF2</i> :: <i>ampDh3-strep-hiBiT</i>	In-frame <i>icmF2</i> and <i>ygfB</i> deletion mutant of strain PA14 with in-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i>	This study
PA14Δ <i>ygfB</i> Δ <i>icmF2</i> Δ <i>retS</i> :: <i>ampDh3-strep-hiBiT</i>	In-frame <i>ygfB</i> , <i>icmF2</i> and <i>retS</i> deletion mutant of strain PA14 with in-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i>	This study
PA14Δ <i>ygfB</i> Δ <i>retS</i> :: <i>ampDh3-strep-hiBiT</i>	In-frame <i>ygfB</i> and <i>retS</i> deletion mutant of strain PA14 with in-frame substitution of <i>ampDh3</i> with <i>ampDh3-strep-hiBiT</i>	This study

Strain name	Description	Obtained from
ID40_ <i>syfp2</i>	ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40Δ <i>amgS</i> _syfp2	In-frame <i>amgS</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40Δ <i>ampDh3</i> _syfp2	In-frame <i>ampDh3</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40Δ <i>cobT</i> _syfp2	In-frame <i>cobT</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40Δ <i>gacS</i> _syfp2	In-frame <i>gacS</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40Δ <i>icmF1</i> _syfp2	In-frame <i>icmF1</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40Δ <i>icmF1</i> Δ <i>icmF2</i> _syfp2	In-frame <i>ygfB</i> and <i>icmF2</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40Δ <i>icmF2</i> _syfp2	In-frame <i>icmF2</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40Δ <i>mepM1</i> _syfp2	In-frame <i>mepM1</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40Δ <i>mepM1</i> Δ <i>mepM2</i> Δ <i>mepM3</i> _syfp2	In-frame <i>mepM1</i> , <i>mepM2</i> and <i>mepM3</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40ΔPROKKA02027_ syfp2	In-frame PROKKA02027 (PA0738) deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40ΔPROKKA05623_ syfp2	In-frame PROKKA05623 (PA3072) deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40ΔPROKKA05679_ syfp2	In-frame PROKKA05679 (PA3016) deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40Δ <i>retS</i> _syfp2	In-frame <i>retS</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40Δ <i>ssub1</i> _syfp2	In-frame <i>ssub1</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study

Strain name	Description	Obtained from
ID40 Δ <i>tse1_syfp2</i>	In-frame <i>tse1</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40 Δ <i>tse1</i> Δ <i>tse5_syfp2</i>	In-frame <i>tse1</i> and <i>tse5</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40 Δ <i>tse1</i> Δ <i>tse5</i> Δ <i>tse2_syfp2</i>	In-frame <i>tse1</i> , <i>tse5</i> and <i>tse2</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40 Δ <i>tse1</i> Δ <i>tse5</i> Δ <i>tse3_syfp2</i>	In-frame <i>tse1</i> , <i>tse5</i> and <i>tse3</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40 Δ <i>tse1</i> Δ <i>tse5</i> Δ <i>tse4_syfp2</i>	In-frame <i>tse1</i> , <i>tse5</i> and <i>tse4</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40 Δ <i>tse1</i> Δ <i>tse5</i> Δ <i>tse6_syfp2</i>	In-frame <i>tse1</i> , <i>tse5</i> and <i>tse6</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40 Δ <i>tse1</i> Δ <i>tse5</i> Δ <i>tse7_syfp2</i>	In-frame <i>tse1</i> , <i>tse5</i> and <i>tse7</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40 Δ <i>tse1</i> Δ <i>tse5</i> Δ <i>tse8_syfp2</i>	In-frame <i>tse1</i> , <i>tse5</i> and <i>tse8</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40 Δ <i>tse2_syfp2</i>	In-frame <i>tse2</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40 Δ <i>tse4_syfp2</i>	In-frame <i>tse4</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40 Δ <i>tse5_syfp2</i>	In-frame <i>tse5</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40 Δ <i>tse5</i> Δ <i>tsi5_syfp2</i>	In-frame deletion mutant of the entire <i>tse5-tsi5</i> region in strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40 Δ <i>tse6_syfp2</i>	In-frame <i>tse6</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40 Δ <i>tse7_syfp2</i>	In-frame <i>tse7</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
ID40 Δ <i>tse8_syfp2</i>	In-frame <i>tse8</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study

Strain name	Description	Obtained from
ID40 Δ <i>ygfB</i> _{<i>syfp2</i>}	In-frame <i>ygfB</i> deletion mutant of strain ID40 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
PA14 _{<i>syfp2</i>}	Strain PA14 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
PA14 Δ <i>ampDh3</i> _{<i>syfp2</i>}	In-frame <i>ampDh3</i> deletion mutant of strain PA14 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
PA14 Δ <i>icmF1</i> _{<i>syfp2</i>}	In-frame <i>icmF1</i> deletion mutant of strain PA14 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
PA14 Δ <i>icmF1</i> Δ <i>icmF2</i> _{<i>syfp2</i>}	In-frame <i>icmF1</i> and <i>icmF2</i> deletion mutant of strain PA14 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
PA14 Δ <i>icmF2</i> _{<i>syfp2</i>}	In-frame <i>icmF2</i> deletion mutant of strain PA14 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study
PA14 Δ <i>ygfB</i> _{<i>syfp2</i>}	In-frame <i>ygfB</i> deletion mutant of strain PA14 carrying <i>syfp2</i> gene from pMRE-Tn7-143 at <i>attTn7</i> site	This study

4.1.12 Computer software and web applications used in this study

Table 16: Computer software and web applications used in this study

Software and version	Manufacturer	Used for
2100 Expert Software	Agilent	BioAnalyzer data evaluation
Basic Local Alignment Search Tool (BLAST)	National Center for Biotechnology Information (NCBI)	Genetic and proteomic alignments
BioRender (web application)	BioRender	Figure assembly
BioTraDIS pipeline v1.4.5	Sanger-pathogens	Next-Generation Sequencing read analysis
Clustal Omega (ClustalX)	Sievers et al. (2011)	Multiple sequence alignments of DNA sequences
CorelDRAW v.24.1.0.360	Corel Corporation	Figure assembly
DeepTMHMM v.1.0.24 (web application)	Hallgren et al. (2022)	Prediction of transmembrane domains in peptides
EndNote21	Clarivate Analytics	Citations and bibliography
BD FACSDiva v6.2	BD Biosciences	FACS data acquisition
FlowJo_10	BD Biosciences	FACS data evaluation

Software and version	Manufacturer	Used for
GraphPad Prism v.10.1.1	Dotmatics	Data evaluation and visualization, statistical analyses
InterPro 98.0 (web application)	Paysan-Lafosse et al. (2023)	Functional analysis of proteins and prediction of domains within peptide sequences
LightCycler 480 software 1.5.0 SP3	Qiagen	qPCR data acquisition and evaluation
Microsoft Office 2016 (Excel, Word, PowerPoint)	Microsoft	Manuscript, Figure assembly, Data evaluation
Picard v2.16.11-1- g9d4b0bc-SNAPSHOT (Java application)	Broad Institute	Extraction of sequencing reads from MiSeq v2 sequencing run (Illumina)
SecReT6 online database (update 2021)	J. Li et al. (2015); J. Zhang et al. (2023)	T6SS literature research
SignalP-6.0 (web application)	Teufel et al. (2022)	Prediction of secretion signal sequences in peptides
SnapGene v.4.2.11	Dotmatics	Genetic analyses, vector and DNA oligonucleotide design, alignment of sequencing data
R v.4.2.3	R Core Team (2023). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/ .	Sequencing data evaluation visualization
VolcanoR (web application)	Goedhart and Luijsterburg (2020)	Creation of volcano plots

4.2 Microbiological methods

4.2.1 Bacteria cultures in liquid medium

All strains used in this study are summarized in **Table 15**. One plastic inoculation loop was used to scrape the top of a bacterial cryo-stock and to inoculate sterile LB in either Erlenmeyer flasks (volumes > 5 mL) covered with aluminum foil or 15 mL tubes (Greiner) with a plastic lid (volumes < 5 mL). If needed, antibiotics were added from stock solutions at the following final concentrations: chloramphenicol (Cm) 25 µg/mL, ampicillin (Amp) 50 µg/mL, gentamicin (Gm) 75 µg/mL for *P. aeruginosa* and 15 µg/mL for *E. coli*. These inocula were incubated over night or for other amounts of time at either 30°C or 37°C, 180-200 rpm with shaking, if not stated otherwise.

4.2.2 Cryo-conservation of bacteria

Cryo-conservation was used to store bacteria indefinitely. Overnight cultures of bacteria were grown at 37°C with selective antibiotics if needed and centrifuged at 4.000 x g or for 10 min. After discarding the supernatant, the pellets were resuspended in 3 mL of LB supplemented with 20% glycerol and evenly distributed to three screw-cap cryo-tubes. Those were immediately placed in a freezer at -80°C.

4.2.3 Photometric determination of cell densities (OD₆₀₀)

For estimation of cell numbers in liquid cultures, the optical density (OD) of that culture was determined using a photometer. For LB cultures, LB was used as a blank, while PBS was used for bacteria suspensions in PBS. 900 µL of the appropriate sterile medium were transferred to a UV-cuvette and thoroughly mixed with 100 µL of bacteria suspension. Measurements of the OD were carried out with light of 600 nm wavelength (OD₆₀₀). We approximated that an OD₆₀₀ of 1 equals 10⁹ *P. aeruginosa* cells.

4.2.4 Determination of colony forming units (CFU) in a bacteria suspension

To count the number of viable bacteria in a given culture, the OD₆₀₀ of the culture was determined. The suspension was diluted in 10-fold diluting steps in sterile PBS in a total volume of 1 mL in 1.5 mL reaction cups. Each dilution step was mixed using a vortex mixer for at least 5 seconds before continuing the dilution series. The theoretical number of bacteria in each dilution step was calculated based on the OD₆₀₀ and those dilutions with expected 10¹ to 10⁴ CFU/mL were chosen for plating. 100 µL of those dilutions were spread on either non-selective LB-agar plates or selective agar plates for total or species-specific CFU determination, respectively, and incubated over night at 30°C or 37°C. The colonies on each plate with 2-200 visible colonies were counted the next day and used to calculate the CFU/mL in the original sample.

4.2.5 Growth curves of bacteria in liquid medium

Overnight cultures of each strain were prepared in 5 mL LB medium. Their OD was adjusted to $OD_{600} = 0.01$ in 5 mL of fresh growth medium and 1 mL was transferred to each of three wells of a clear Greiner flat-bottom 24 well plate. A negative growth control of sterile medium was included as a blank for each measurement. The plates were covered with a lid, incubated at 30°C or 37°C with shaking in a Tecan microplate reader and the OD_{600} was measured every 10 min. After 18-20 h, the measurement was stopped.

4.2.6 Generation of electrocompetent bacteria

Overnight cultures of the desired strain were grown in 5 mL LB. Each strain was then washed twice with sterile filtered, cold 300 mM sucrose solution (w/v). Each wash step comprised centrifugation at 4.000 x g for 10 min at 4°C, discarding the supernatant and resuspending in 5 mL of cold 300 mM sucrose solution. After one more centrifugation step, the pellet was resuspended in 100 µL ice-cold 20% sucrose solution and stored on ice until further use.

4.2.7 Transformation of electrocompetent bacteria with plasmids

100 µL of electrocompetent bacteria cells were mixed with 20-100 ng plasmid DNA and transferred into an electroporation cuvette. An electric pulse at 2.5 kV voltage and a capacity setting of 25 µF (low range 200 Ω, high range 600 Ω) were used to transform the bacteria with a plasmid. Immediately after the pulse, 200 µL SOC-medium were added to the cuvette and then transferred to a 1.5 mL reaction cup filled with 800 µL SOC-medium. The suspension was incubated at 37°C with shaking for 1 h. After centrifugation at 11.000 x g for 1 min, the supernatant was discarded, the pellet was resuspended in roughly 100 µL of left over SOC medium in the cup and then spread onto a selective LB-agar-plate containing an appropriate antibiotic. The plates were incubated over night at 37°C to obtain transformant colonies.

4.2.8 Generation of chemically competent (Ca^{2+} -competent) bacteria

Bacterial cells were streaked onto LB-agar plates, which were supplemented with a selective antibiotic if needed, and incubated over night at 37°C. Single colonies were used to inoculate 4 mL LB medium and incubated over night at 37°C with 200 rpm shaking. The overnight cultures were transferred into 400 mL LB-media and grown at 37°C with shaking until they reached an OD_{600} of 0.4 - 0.6, usually after 2 - 3 hours. The suspensions were transferred to 50 mL Falcon tubes and placed on ice for 20 min. After centrifugation at 3,000 x g for 10 min at 4°C, the supernatant was discarded, and the cells resuspended in a total of 24 mL of cold 0.1 M $CaCl_2$ solution. After incubation on ice for 30 min, the suspensions were centrifuged at 3,000 x g for 10 min at 4°C, the supernatant was discarded,

and the pellets resuspended in a total of 6.4 mL cold 0.1 M CaCl₂ solution supplemented with 15% glycerol. The suspensions were used to prepare 100 µL aliquots of the cells that were flash frozen in liquid nitrogen and stored at -80°C for up to 6 months.

4.2.9 Transformation of chemically competent (Ca²⁺) bacteria with plasmids

One frozen aliquot of chemically competent bacteria was thawed on ice and mixed with 20-100 ng of plasmid DNA or Gibson Assembly mix. After incubation on ice for 20 min, a heat shock was performed in a heating block at 42°C for 30-40 s. After 2 min incubation on ice, 1 mL of room temperature SOC medium was added, and the suspension incubated at 37°C for 1 h on a horizontal shaker with 180-200 rpm shaking. The suspension was centrifuged at 11.000 x g for 2 min, the supernatant was discarded, and the pellet resuspended in 100 µL sterile LB and then spread out onto a selective LB-agar plate containing an appropriate antibiotic. The plates were incubated over night at 37°C or until transformant colonies appeared.

4.2.10 Transformation of *Pseudomonas aeruginosa* using conjugation

Overnight cultures from *P. aeruginosa* (PA) strains and *Escherichia coli* SM10λpir (EC) containing a conjugative plasmid were grown in 5 mL LB. 400 µL PA suspension was mixed with 200 µL EC suspension. 200 µL from that mix were transferred as spots onto either LB-agar plates and incubated over night at 37°C or LB-agar plates supplemented with 0.1% arabinose for pMRE-Tn7 plasmids, and incubated at 30°C overnight. Transformants were selected by scraping the grown bacteria lawn from the plate using an inoculation loop and resuspending it in 2 mL LB medium. 100 µL of this suspension were spread out on a LB-agar plate supplemented with 200 µg/mL triclosan (Irgasan), and an additional selective antibiotic if needed, before incubation at 37°C or 30°C overnight or until colonies appeared.

4.2.11 Interbacterial competition assays on solid agar

Competition assays were used to investigate the competitiveness of a strain of interest against a competitor. In short, two competing strains were mixed, of which one expressed a yellow fluorescent protein (sYFP2), similarly to the method described in a pre-print publication by Habich et al. (2022). The ratio of fluorescent to non-fluorescent cells before and after co-incubation on solid agar for 21 h was determined using a flow cytometer and used to calculate a competitive index for the fluorescent strain, usually *P. aeruginosa*. Single-strain, unmixed samples were used as controls for the potential loss of fluorescence in the labelled strains in each biological replicate.

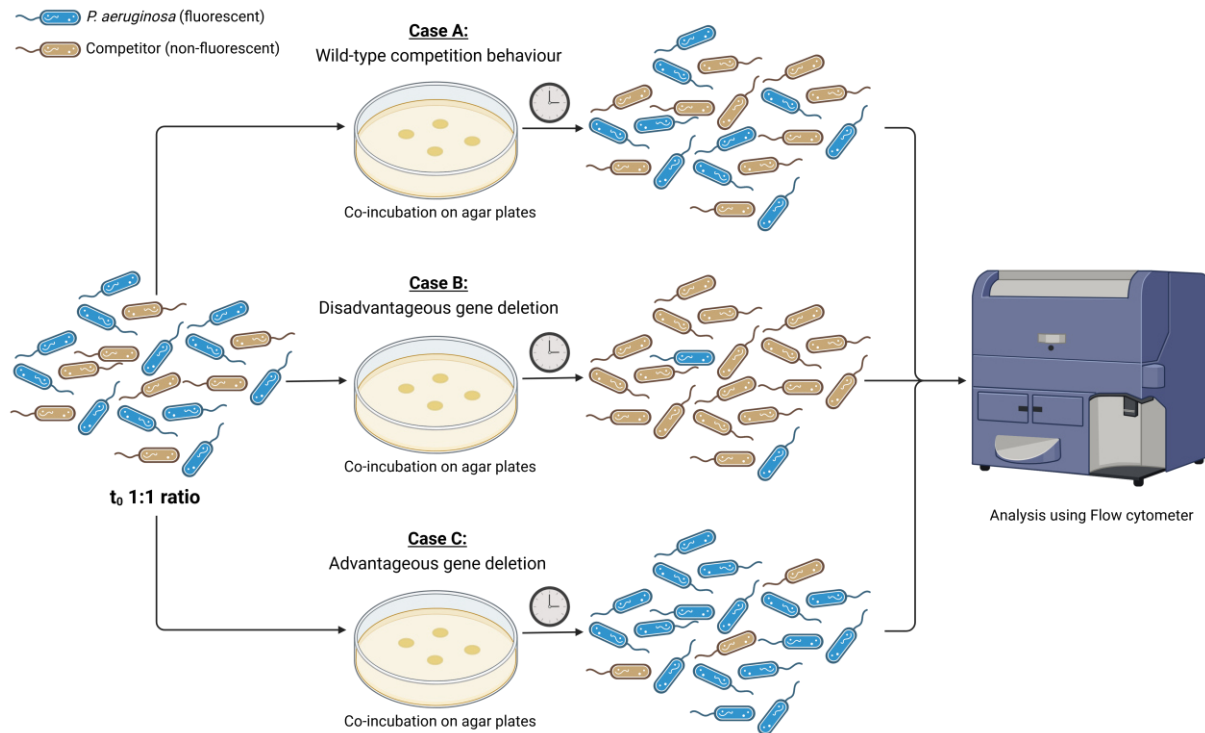


Figure 6: Schematic overview of the flow cytometry-based interbacterial competition assay. Blue cells represent fluorescently-labeled *P. aeruginosa* (PA) cells, and brown cells represent non-fluorescent competitor bacteria cells. Clock symbols indicate co-incubation for 21 h. Three scenarios and their expected outcome are shown. **Case A** shows the hypothetical behavior of a wild-type strain with neither growth advantage nor disadvantage, **Case B** shows the outcome of competition if PA carries a mutation that decreases its competitive fitness, and **Case C** shows the expected outcome if PA carries a mutation that increases its competitive fitness against the tested competitor. The ratios of PA to competitor after co-incubation are determined using flow cytometry to differentiate fluorescent from non-fluorescent cells and used to calculate the competitive index for each strain. Figure created with Biorender.com

Co-incubation on solid agar and FACS sample preparation

Overnight cultures of all strains of interest were grown at either 30°C or 37°C in 5 - 10 mL LB medium as indicated. Competition assays between a strain of interest and *B. cepacia* or *B. cenocepacia* were performed at 30°C unless indicated otherwise. Thorough mixing of bacterial suspensions at each step using a vortex mixer proved to be essential to obtain reproducible results, since especially in PBS, the bacteria sank quickly to the bottom of their reaction cups, and sometimes formed visible clumps.

The overnight cultures were harvested by centrifugation for 15 min at 4.000 x g at room temperature (RT). Supernatants were discarded and bacterial pellets were resuspended in an equal amount of PBS. The centrifugation and resuspension steps were repeated to wash the pellets. After determination of the OD₆₀₀, an equivalent of 0.3 or 0.5 OD units (equivalent to 3 x 10⁸ or 5 x 10⁸ cells) were harvested for each strain, with an extra 10⁹ bacteria added to account for losses in further washing steps. After centrifugation, the supernatant was discarded and the pellets resuspended in an equal amount of PBS, usually 1 mL. If the

calculated OD₆₀₀/mL in a given culture was expected to be greater than 2, the sample was further diluted to an OD₆₀₀/mL $\leq$ 2.

The OD₆₀₀ of each suspension was determined again. To obtain each competition mix, an equal amount by OD₆₀₀ of both competing strains were mixed in a new reaction cup, usually OD₆₀₀ = 0.3 or 0.5 of both strains (equivalent to 3×10^8 or 5×10^8 cells). The mixed suspension was centrifuged at 11.000 x g for 3 min and the supernatant removed before resuspending the pellet in PBS to obtain a final density of OD₆₀₀ = 1 / mL (approx. 10^9 bacteria / mL). 10 μ L of the mixed suspensions and of all single-strain suspensions (approx. 10^7 bacteria) were spotted onto a LB-plate containing 3% agarose and incubated for 21 h at 30°C or 37°C. The left-over suspension of the mixed samples as well as an equivalent of OD₆₀₀ = 0.2 of the non-mixed samples was centrifuged at 11.000 rpm for 3 min, the supernatant was removed, and the pellet resuspended in 200 μ L of 4% *p*-formaldehyde solution in PBS (w/v; 4% PFA). It is important that no methanol is contained in the PFA solution as a stabilizing agent, to prevent degradation of fluorophores. These samples were termed “t₀ samples”.

After incubation for 21 h, the grown bacterial spots were punched out using the tip of a 5 mL Falcon tube (“FACS tube”) and transferred into sterile 14 mL Falcon tubes containing 1 mL sterile PBS using a 10 μ L pipet tip. The bacteria lawns were resuspended with a pipet until the agar snips were completely clear and no clumps were visible. After determining the OD₆₀₀, an equivalent of OD₆₀₀ = 0.2 (approx. 2×10^8 cells) was harvested into a fresh reaction cup. The samples were centrifuged, the supernatant was removed, and the pellet resuspended in 200 μ L of 4% PFA solution. These samples were termed “t₂₁ samples”.

Both the t₀ and the t₂₁ samples were shaken for at least 1 h on a horizontal shaker at room temperature (23°C) with vigorous shaking > 1000 rpm to prevent clumping of the cells and stored at 4°C for up to two days.

Sample evaluation using a flow cytometer

The ratios of fluorescent to non-fluorescent cells in the t₀ and t₂₁ samples were determined using a flow cytometer (LSRFortessa™ Cell Analyzer; Preconfigured BD LSR II [typical setup]). All samples were diluted 1:50 – 1:200, depending on the optical density of each sample. For detection of all particles, FSC voltage was set to 604 and SSC voltage was set to 275. sYFP2 fluorescence was detected by excitation at 488 nm with a voltage of 549 and a 530/30 nm emission filter.

Since the detection of bacteria in a flow cytometer is sensitive to impurities in the flow cytometer itself, the cytometer was cleaned before each run using the following protocol:

The instrument was switched to a flow rate of 60 $\mu\text{L}/\text{min}$ and washed for 5 min with FACS clean solution, then FACS rinse solution, then distilled water. To account for background events resulting from FACS Flow reagent or the PBS used to dilute all samples, a compensation line was created with PBS to blank each sample. The compensation line was measured in triplicates for each time point by recording all events detected with pure PBS in 10 second increments between 10 and 70 seconds at a constant flow rate of 60 $\mu\text{L}/\text{min}$. This compensation line was measured in triplicates for each time point. We hypothesized that the rate of false-positive detection, which mostly consists of non-fluorescent particles in the flow cytometer under our conditions would remain constant over time. We used this rate to subtract false-positive events from each sample, resulting in more precise calculations of the ratios of fluorescent to non-fluorescent cells.

Each sample was then measured using the BD FACSDiva™ v6.2 software (BD Biosciences) and the particle density in each sample was adjusted so that the instrument detected < 1000 events/s. This event density was found to minimize the occurrence of multiple particles passing the light source at the same time in an FSC-A – FSC-H plot. In the FACSDiva software, a P1 gate was drawn in an FSC-A – SSC-A plot around the densest cell population. For each sample, all events were recorded until 10,000 events within the P1 gate were detected. The data was exported as .fcs files and further processed in FlowJo v.10 (BD Biosciences).

Data processing of FACS data: Gating in FlowJo

Raw .fcs files containing all events from the FACS measurements were processed in FlowJo v.10. An FSC-A – SSC-A-plot was generated of a t_0 sample containing both fluorescent and non-fluorescent cells. A polygonal P1-gate was then drawn around the densest population of events, called the “bacteria gate”. This P1 gate was applied to all samples, manually tested for accuracy on each sample and adjusted if necessary.

Subsequently, a histogram displaying the fluorescence intensity (corresponding to sYFP2 expression) of the same sample used for generating the P1 gate was generated. A gate dividing the two prominent FITC-A⁻ and FITC-A⁺ populations was generated and applied to all samples’ “bacteria gate”. The accuracy of the gate was confirmed for each sample and adjusted if necessary. Subsequently, the count of all events in the FITC⁻ and FITC⁺ events as well as the time that each sample took to measure were exported in an Excel-file for further data processing in GraphPad Prism and Microsoft Excel. The data evaluation process is schematically shown in **Figure 7**.

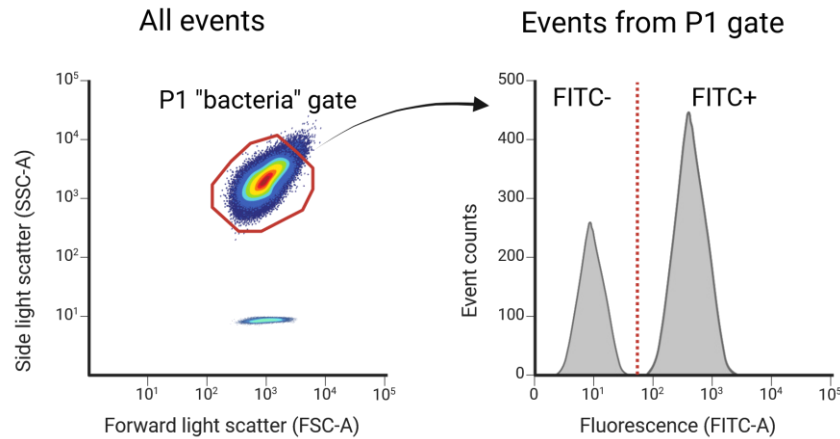


Figure 7: Schematic depiction of the gating strategy used in flow cytometry-based competition assays. All steps shown were performed using the FlowJo v.10.1.1 software. All recorded events (single dots in the left graph) were displayed as an FSC-A – SSC-A plot and a two-dimensional P1 gate (dark red polygon) was drawn around the densest population of events, represented by the dark red color of the population. All events from the P1 gate were then displayed in a histogram of the FITC-A fluorescence intensity, corresponding to the levels of sYFP2 expression in the fluorescence-labelled *P. aeruginosa* cells. A one-dimensional gate (dashed line) was applied to the histogram to separate the fluorescent (FITC+) from the non-fluorescent (FITC-) cells. The ratios of fluorescent to non-fluorescent cells before and after co-incubation of competitor strains was used to calculate a competitive index for each tested strain of *P. aeruginosa*. Figure created with Biorender.com.

Compensation line and blanking of all sample data in GraphPad Prism and Microsoft Excel

First, a compensation line for both the FITC- and FITC+ events was calculated from the measurements with pure PBS via linear regression in GraphPad Prism v.10.1.1, using the smallest sum of squares for fitting the curve. The Y-intercept of the regression curves was manually set to 0 since no events can be detected when measuring for 0 seconds. The best-fit-values for both slopes, corresponding to the rate of false-positive events per second in the FITC- or FITC+ gate, was then multiplied by the measuring time length of each sample to obtain the hypothetical number of false-positive events in each gate of each sample. This number was then subtracted from the total number of events in the respective gate to obtain blanked counts before calculating the percentage of fluorescent cells in each sample.

Calculation of the competitive index of *P. aeruginosa* in competition assays

The blanked ratios of each sample were used to calculate the competitive index of *P. aeruginosa* in competition with another strain, as suggested by previous publications (LaCourse et al., 2018; Perault et al., 2020). The calculation was slightly adjusted to fit our assay better where the blanking step using a PBS compensation line leads to a potential skew of the absolute bacteria detected in each sample. Instead of using absolute counts to calculate the competitive index, we used the percentages of *P. aeruginosa* in each sample and at each time point. We used the following formula:

Equation 1: Calculation of the competitive index (CI) of *P. aeruginosa* (PA) in co-incubation with a competitor (Comp). The percentage of PA after co-incubation (t_{21}) was divided by the percentage of the corresponding competitor at t_{21} and subsequently by the same ratio before co-incubation (t_0). If $\log_{10}(\text{CI}) > 0$, the ratio of PA increased from t_0 to t_{21} ; If $\log_{10}(\text{CI}) < 0$, the ratio of PA decreased from t_0 to t_{21} ; If $\log_{10}(\text{CI}) = 0$, the ratio of PA to competitor did not change between t_0 and t_{21} .

$$\text{CI}_{\text{PA}} = \frac{\% \text{PA}_{t_{21}}}{\% \text{Comp}_{t_{21}}} \bigg/ \frac{\% \text{PA}_{t_0}}{\% \text{Comp}_{t_0}}$$

This equation yields the same results as if inserting the absolute count of PA and competitor in the respective conditions instead of percentages, as suggested by Perault et al. (2020), but is more robust towards slightly different sample sizes at t_{21} and t_0 . In either way, the obtained values are calculated through a ratio of ratios, resulting in log-normally distributed data, which was $\log_{10}$ -transformed to obtain normally distributed data. The $\log_{10}(\text{CI}_{\text{PA}})$ is a measure for how much the co-culture of the two species was shifted towards PA (resulting in a $\log(\text{CI}_{\text{PA}}) > 0$) or towards the competitor ($\log(\text{CI}_{\text{PA}}) < 0$) after co-incubation.

A problem arises when in two biological replicates, the percentage of PA in the mixed culture is not identical, but the final mix after co-incubation still reaches a similar ratio of PA to competitor in each replicate. This leads to overall lower CIs when the ratio at t_0 is systematically skewed towards the competitor in one replicate, which can easily happen due to human error during sample preparation. This would make it difficult to compare data between replicates, and would only allow for the comparison between samples with the same percentage of PA in the t_0 sample.

We found that this error mostly results from the systematic miscalculation of the amount of competitor that was added to each tested PA strain, meaning that within one replicate, the ratios of PA to competitor at t_0 were mostly consistent between different PA strains. This allows for the normalization of all PA strains in co-culture with one competitor to the corresponding fluorescent PA wild-type (wt) by subtracting the $\log(\text{CI})$ of the wt sample from each other tested PA strain as shown in **Equation 2**.

Equation 2: Calculation of the relative competitive fitness ($\Delta \log_{10}[\text{CI}]$) against a certain competitor of a PA gene deletion mutant (PAi) compared to the PA wild-type (PAwt).

$$\Delta \log_{10}(\text{CI})_{\text{PAi}} = \log_{10}(\text{CI})_{\text{PAi}} - \log_{10}(\text{CI})_{\text{PAwt}}$$

This $\Delta \log(\text{CI})$ value then is an indicator for the competitive strength of each PA mutant when compared to the PA wt, where $\Delta \log(\text{CI}) < 0$ means that the mutant has lower “competitive fitness” than its wt, and $\Delta \log(\text{CI}) > 0$ means that the mutant has a higher competitive strength. We reasoned that when aiming to compare the impact of a certain gene deletion on the competitiveness of PA under certain conditions, this $\Delta \log(\text{CI})$ is a better indicator

than the log(CI), which only indicates how the ratio of PA to competitor changed during co-incubation.

4.3 Molecular biological methods

4.3.1 Isolation of genomic DNA (gDNA) from bacteria

For isolation of bacterial genomic DNA, the DNeasy UltraClean Microbial Kit (QiaGen) was used according to the manufacturer's instructions. 1.8 mL of bacteria suspension were used as the input material and the final samples were eluted in 50 μ L DNase free water. The concentration of extracted DNA was determined using a NanoDrop One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific) and the DNA was subsequently stored at -20°C.

4.3.2 Isolation of plasmid DNA from bacteria

For isolation of plasmid DNA from bacteria, the Monarch® Plasmid Miniprep Kit (New England BioLabs) was used. 5 mL (high-copy plasmids) or 20 mL (low-copy plasmids) of a given bacterium were grown over night in LB medium supplemented with adequate antibiotics for the plasmid of interest. After centrifugation at 4.000 x g for 10 minutes, the supernatant was discarded and the pellet resuspended in 200 μ L resuspension buffer. The protocol was adjusted for low-copy plasmids by using twice the amount of resuspension buffer, lysis buffer and neutralization buffer. The obtained sample was then loaded onto one collection column in several loading steps and subsequently treated as the protocol suggests. The purified plasmid DNA was eluted in 50 μ L of DNase-free water. The concentration of extracted DNA was determined using a NanoDrop One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific) and is was subsequently stored at -20°C.

4.3.3 Polymerase chain reaction (PCR)

PCR was used for amplification of DNA sequences of interest. DNA double strands are first denatured at high heat, then oligonucleotide primers bind to specific regions of the DNA at a certain annealing temperature, and finally, the single strands are complemented by a DNA polymerase starting from the primers in 5' $\rightarrow$ 3' orientation before the cycle is repeated. Theoretically, the amount of DNA is doubled in each reaction cycle.

Different DNA polymerases were used for different applications:

- (i) A relatively fast to set up, error prone MangoMix polymerase was used for amplification of fragments with a certain expected length, e.g. to control if a specific sequence is present or absent and
- (ii) An error-proof polymerase was used to amplify fragments for downstream applications like sequencing or cloning experiments. Different polymerases give

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different results for certain amplicates and were used interchangeably in this study, whenever one reaction failed to deliver results of sufficient quality.

The reaction protocols for each polymerase will be listed below:

Detailed running protocol for PCR reactions using MangoMix

Table 17: Reaction components for a PCR using MangoMix

Component	Amount per reaction	Final concentration
2 x MangoMix	7.5 μ L	1 x
Primers (10 μ M)	0.5 μ L each	0.33 μ M each
Template DNA	variable	2 ng (plasmid) or 20 ng (gDNA) or Very little bacterial pellet
DMSO ¹	0 - 0.75 μ L	0-5% (v/v)
DNase-free water	Ad 15 μ L	
Total volume	15 μ L	

1: DMSO was added in cases where MangoMix PCR did not yield PCR products of sufficient quality

PCR reactions were performed in a thermal cycler (Bio-Rad) with the specific cycling conditions for the listed in **Table 18**.

Table 18: Cycling conditions for PCR reactions using MangoMix.

Step	Temperature [°C]	Time [min:s]	Repeats
Initial denaturation	95	2:00 - 5:00 min ¹	1
Melting	95	0:30	25 - 30
Primer annealing	Lowest T _M of primers -3°C	0:30	
Strand extension	72	30 s/kbp	
Final extension	72	5:00	1
Hold	4	∞	

1: 5 min extension time were used if bacterial pellets were used as template DNA

Detailed running protocol for PCR reactions using KAPA HiFi DNA polymerase

Table 19: Reaction components for a PCR using KAPA HiFi DNA polymerase.

Component	Amount per reaction	Final concentration
5 x KAPA HiFi Buffer (Fidelity or GC) ¹	5 μ L	1 x
Primers (10 μ M)	0.75 μ L each	0.33 μ M each
dNTPs (2 mM each)	2.5 μ L each	0.2 mM each
Template DNA	variable	20 ng gDNA
KAPA HiFi DNA polymerase (1 U/ μ L)	0.25 μ L	0.01 U/ μ L
DMSO ¹	0 - 1.25 μ L	0 - 5% (v/v)
DNase-free water	Ad 25 μ L	

1: Fidelity buffer was used for non-high GC DNA and for cases in which GC buffer failed to deliver satisfying results, supplemented with 5% DMSO or without DMSO, as suggested by the manufacturer.

PCR reactions were performed in a thermal cycler (Bio-Rad) with the specific cycling conditions for the listed in **Table 20**.

Table 20: Cycling protocol for PCR reactions using KAPA HiFi DNA polymerase.

Step	Temperature [°C]	Time [min:s]	Repeats
Initial denaturation	95	2:00 - 5:00 min	1
Melting	98	0:20	25 - 30
Primer annealing	Lowest T _M of primers -3°C	0:15	
Strand extension	72	30 s/kbp	
Final extension	72	5:00	1
Hold	4	∞	

Detailed running protocol for PCR reactions using KOD Hot Start DNA polymerase

Table 21: Reaction components for a PCR using KOD Hot Start DNA polymerase.

Component	Amount per reaction	Final concentration
10 x buffer for KOD polymerase	2.5 µL	1 x
25 mM MgSO ₄	1.5 µL	1.5 mM
Primers (10 µM)	0.75 µL each	0.33 µM each
dNTPs (2 mM each)	2.5 µL each	0.2 mM each
Template DNA	20 ng gDNA	
KOD Hot Start DNA polymerase (1 U/µL)	0.25 µL	0.01 U/µL
DNase-free water	Ad 25 µL	

PCR reactions were performed in a thermal cycler (Bio-Rad) with the specific cycling conditions for the listed in **Table 22**.

Table 22: Cycling protocol for PCR reactions using KOD Hot Start DNA polymerase.

Step	Temperature [°C]	Time [min:s]	Repeats
Initial denaturation	95	2:00 min	1
Melting	95	0:20	25 - 30
Primer annealing	Lowest T _M of primers -3°C	0:15	
Strand extension	70	15 s/kbp	
Final extension	70	5:00	1
Hold	4	∞	

Detailed running protocol for PCR reactions using *PfuTurbo* DNA polymerase

Table 23: Reaction components for a PCR using *PfuTurbo* DNA polymerase.

Component	Amount per reaction	Final concentration
10 × Cloned Pfu reaction buffer	2.5 µL	1 x
Primers (10 µM)	0.75 µL each	0.33 µM each
dNTPs (10 mM each)	0.75 µL	0.3 µM each
Template DNA	50-100 ng gDNA	
<i>PfuTurbo</i> DNA polymerase (2.5 U/µL)	1.0 µL	0.1 U/µL
DNase-free water	Ad 25 µL	

PCR reactions were performed in a thermal cycler (Bio-Rad) with the specific cycling conditions for the listed in **Table 24**.

Table 24: Cycling protocol for PCR reactions using *PfuTurbo* DNA polymerase.

Step	Temperature [°C]	Time [min:s]	Repeats
Initial denaturation	95	2:00 min	1
Melting	95	0:30	25 - 30
Primer annealing	Lowest T _M of primers -3°C	0:30	
Strand extension	72	1 min/kbp	
Final extension	72	10:00	1
Hold	4	∞	

Detailed running protocol for PCR reactions using Phusion High Fidelity DNA polymerase

Table 25: Reaction components for a PCR using Phusion High Fidelity DNA polymerase.

Component	Amount per reaction	Final concentration
5X Phusion GC buffer	5 µL	1X
Primers (10 µM)	1.25 µL each	0.5 µM each
dNTPs (10 mM)	0.5 µL	200 µM
Template DNA	variable	< 250 ng
DMSO	0.75 µL	3%
Phusion DNA polymerase	0.25 µL	1 U

Total volume ad 25 µL with nuclease-free water

PCR reactions were performed in a thermal cycler (Bio-Rad) with the specific cycling conditions for the listed in **Table 27**.

Table 26: Cycling protocol for PCR reactions using Phusion High Fidelity DNA polymerase.

Step	Temperature [°C]	Time [min:s]	Repeats
Initial denaturation	98	0:30	1
Melting	98	0:30	25 - 30
Primer annealing	Lowest T _M of primers -3°C	0:30	
Strand extension	72	1 min/kbp	
Final extension	72	10:00	1
Hold	4	∞	

4.3.4 Agarose gel electrophoresis of DNA samples

Agarose gel electrophoresis was used to separate and detect DNA fragments of different sizes. For gel electrophoresis after a PCR reaction using MangoMix, no loading buffer was added. Otherwise, 5 µL of DNA solution were mixed with 2 µL of Orange G loading buffer. 1% agarose gels (w/v in 0.5 x TBE buffer supplemented with Midori Green Xtra DNA stain) were prepared by boiling the gel, filling a gel chamber with the molten gel and then allowing it to cool to room temperature.

5 – 7 μ L DNA sample with loading dye were loaded into the gel pockets and the gel was submerged in 0.5 x TBE buffer in a Mini-Sub Cell GT electrophoresis chamber (Bio-Rad). An electric tension of 120 V was applied to the gel for 45 min for separation of DNA fragments of different sizes. The fluorescently stained DNA lanes were imaged using the FastGene FAS-V gel documentation system (NIPPON Genetics).

4.3.5 Generation of in-frame deletion mutants of *P. aeruginosa* using allelic exchange

Genomic in-frame deletion mutants of *P. aeruginosa* were generated using the protocol described by Klein et al. (2019). In a first step, mutagenesis plasmids using pEXG2 or pEXTk as backbones were generated containing up- and downstream flanking regions of the gene of interest with a truncated *goi* (30 bp of the 3' end and 30 bp of the 5' end of the gene flanked by ~800 bp flanking region on either side), following the method described by Gibson et al. (2009) (referred to as Gibson Assembly below). After transfer of the mutagenesis plasmid into the *P. aeruginosa* strain of interest via conjugation from *E. coli* SM10 λ pir, the plasmid is integrated into the genome via strand cross-over in one of the homologous regions. After selection of merodiploid colonies (containing both the wt allele and the integrated plasmid with the mutant allele), the counter-selection cassettes of the respective plasmids (*sacB* in pEXG2 and *tdk* in pEXTk) were used to select colonies in which a second cross-over event has taken place, leading to the loss of the integrated plasmid and a haploid strain, containing either the wt or mutant allele. MangoMix PCR was used to screen colonies for the presence of a mutant allele as well as the absence of a wt allele.

Primer design for Gibson Assembly

The process of creating plasmids for in-frame deletion of a gene of interest (*goi*) is schematically shown in **Figure 8**.

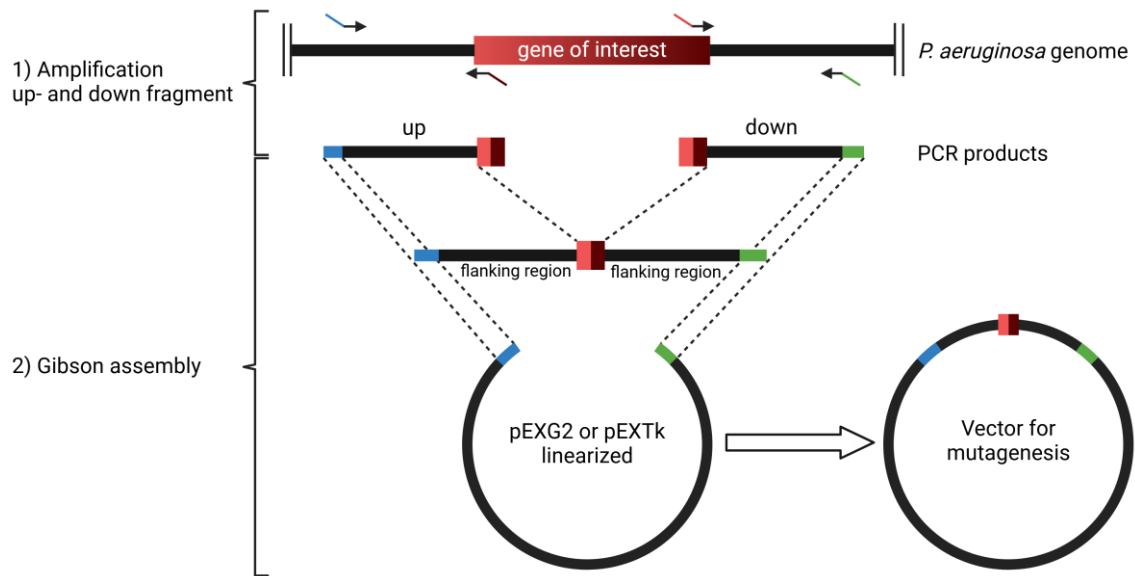


Figure 8: Schematic overview of the Gibson assembly process. In a first step, using PCR an up- and down-fragment are created, containing 30 bp of the gene of interest (*goi*) flanked by an 800 bp genomic flanking region. The primers used in this amplification consist of an 18 bp annealing sequence (black arrows) with a 30 bp overhang (angled, colored lines) complementing the regions for annealing using Gibson cloning (colors representing matching genetic sequences). In the second step (Gibson assembly), homologous regions of the up- and down-fragment and the linearized vector are annealed to form the final vector containing the fragmented *goi* (red) flanked by 800 bps up- and downstream of the *goi*, which are homologous to the native gene locus of the *goi*. Figure created with Biorender.com.

PCR reactions using one of the proofreading DNA polymerases KAPA HiFi DNA polymerase, KOD Hot Start DNA polymerase or *PfuTurbo* DNA polymerase were used to generate all DNA fragments for Gibson Assembly. Two pairs of primers were used: one amplifying the first 30 bps of the 5' coding region of the gene of interest (*goi*) with ~800 bps of the upstream flanking region ("up fragment"), and the other pair amplifying 30 bps of the 3' end of the *goi* and ~800 bps of the downstream flanking region ("down fragment"). Those primers amplifying the fragmented *goi* sequences in both the up- and down fragment were designed so that they contain an overlap of ~30 bp with the other fragment. Those primers of the up- or down fragment that bind in the flanking regions were designed to contain a 30 bp overlap with the cloning vector backbone after linearization. The vector was linearized by PCR reaction with a set of primers flanking the multiple cloning site and facing outwards from it. Successful amplification of all PCR reactions was verified using agarose gel electrophoresis. All primers were ordered from Sigma Aldrich or Integrated DNA Technologies.

Gibson Assembly reaction

Gibson Assembly was used to incorporate DNA fragments into the suicide vectors pEXG2 or pEXTk (description in Table 13). Gibson assembly was performed using a self-made master mix, containing a Phusion HotStart DNA polymerase, Taq DNA ligase and T5 exonuclease.

100 ng of linearized vector DNA (20-30 fmol) were added to the master mix and mixed with the 5-fold amount of each insert (100-150 fmol). The amount of insert needed was approximated from the lengths of each DNA fragment, assuming the same positive linear correlation between the number of base pairs and molecular weight in each, e.g. 650 Da per base pair. The mix was incubated at 50°C for 30 min, then cooled to room temperature before adding 1 µL (10 U) of DpnI (Thermo Fisher). The mix was incubated at 37°C for 1 h and subsequently transformed into chemically competent *E. coli* DH5α cells as described on p. 59. Colonies with the correct inserts were identified using MangoMix PCR amplification and subsequently sequenced.

Allelic exchange

Plasmids used for mutagenesis of *P. aeruginosa* were first transferred into *E. coli* SM10λpir. Then, *P. aeruginosa* was transformed with the plasmid using conjugation as described in section 4.2.10 (p. 60). Since neither pEXG2 nor pEXTk can be replicated by *P. aeruginosa*, merodiploid colonies with the plasmid integrated after a first cross-over event were selected for their antibiotic resistance cassette. Different methods were used for the selection of haploid colonies after a second cross-over event:

- (i) If pEXG2 was used, single gentamicin-resistant (merodiploid) colonies were streaked onto a non-selective LB-agar plate and incubated over night. The next day, those colonies were grown in 3 mL LB medium supplemented with 20% sucrose for 3 h and subsequently plated onto an LB-agar plate supplemented with 20% sucrose. The pEXG2 plasmid contains a *sacB* gene encoding levansucrase, which synthesizes levan polymers from sucrose that have a toxic effect in Gram-negative bacteria (Gay et al., 1983). Those colonies that can grow in sucrose have undergone a second cross-over event, leading to an allelic exchange between genomic and plasmid DNA. The outcome can be a haploid colony containing either the wt or mutant allele, or a merodiploid colony in which *sacB* contains an inactivating mutation as shown in **Figure 9**.
- (ii) If pEXTk was used, single gentamicin-resistant (merodiploid) colonies were streaked onto non-selective LB-agar plates and incubated over night at 37°C. The resulting colonies were then grown in 3 mL LB supplemented with 1 mM IPTG for 3 h. In pEXTk, *sacB* was exchanged for an IPTG-inducible *E. coli*-derived thymidine kinase/thymidylate kinase fusion gene (*tdk*) from the pVT77 cloning vector. This gene converts 3'-azido-2'-dideoxythymidine (AZT) to 3'-azido-2'-dideoxythymidine 5'-triphosphate (AZT-TP), a chain-terminating thymidine analogue during DNA replication, thus leading to cell intoxication (Holzer et al., 2005). Haploid double cross-over colonies were selected by plating out the bacteria on LB-agar plates supplemented

with 200 mg/L AZT and 1 mM IPTG and incubation at 37°C over night or until colonies appeared.

In both cases, haploid and mutant allele-containing colonies were screened for using MangoMix colony PCR. Two PCR reactions were performed for each mutant: (i) A primer pair binding upstream and downstream of the gene of interest (*goi*), amplifying a long DNA stretch, if the wt allele is present, or a short stretch (~500 bp), if the mutant allele is present; and (ii) a primer pair binding outside the *goi* and inside the excised sequence, amplifying ~500 bp if the wt gene is present, but failing to amplify any DNA if the mutant allele is present.

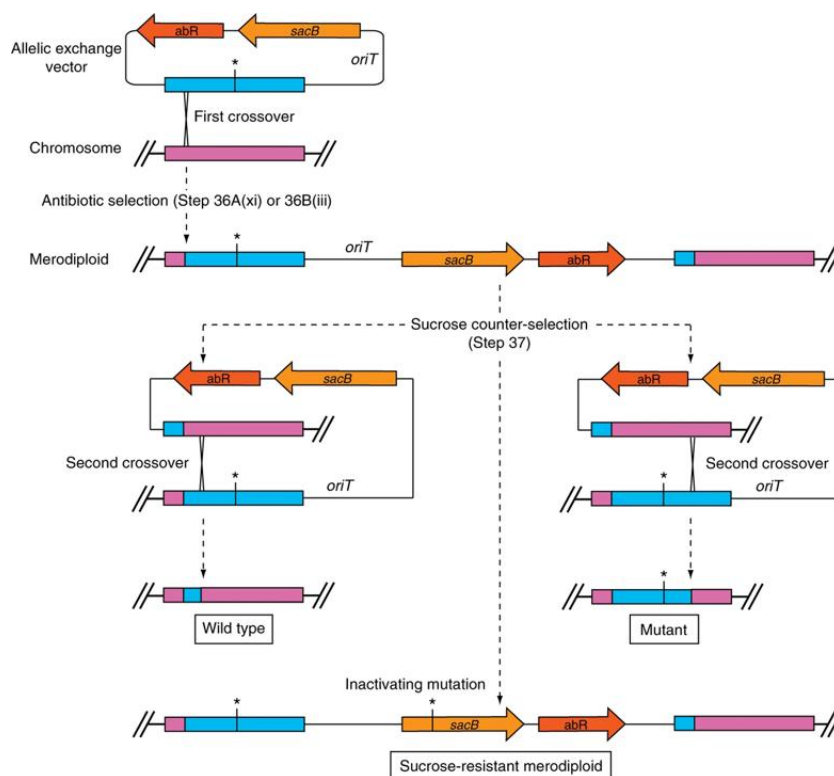


Figure 9: Schematic overview over the generation of in-frame deletion mutants in *P. aeruginosa* using allelic exchange vector. Asterisks indicate any insertion, deletion or base substitution. Blue and pink boxes indicate homologous regions on the plasmid and genome, respectively. The method depicted here shows the use of the *sacB* counter-selection cassette encoded e.g. on pEXG2. For pEXTk, *sacB* was substituted by *tdk*, which requires induction via IPTG and counter-selection via AZT. Figure was taken from Hmelo et al. (2015) (Figure 1) and licensed for reuse in this thesis by Springer Nature.

4.3.6 Generation of AmpDh3-HiBiT knock-in mutants

For generation of *P. aeruginosa* strains expressing HiBiT-tagged AmpDh3, the pEXG2_ampDh3-strep-hiBiT vector published in Eggers et al. (2023) was used. In short, the vector contains the *ampDh3* gene of *P. aeruginosa* ID40 with an additional Strep-tag-spacer followed by a HiBiT-tag immediately downstream and in frame with the gene, which is flanked by 800 bps up- and downstream regions, which are homologous to the flanking

regions of *ampDh3* in the ID40 genome. Transfer of the vector followed by allelic exchange is performed as described in section 4.3.5 (p. 70). PCR reactions to confirm allelic exchange were performed with (i) a primer-pair that binds within the *hiBiT*-tag (Primer # 1081) and 500 bp upstream within the *ampDh3* gene (Primer # 1080), or (ii) a primer pair that binds upstream (Primer # 1080) and downstream (Primer # 963) of the *strep-hiBiT*-tag, amplifying a stretch of DNA that is 60 bp larger if the *strep-hiBiT*-tag is present.

4.3.7 Sanger sequencing of plasmids

Purified plasmids were sequenced after cloning and validated via PCR by Eurofins Genomics (EU) after preparation of the samples according to the instructions for TubeSeq Supreme service. Each plasmid was sequenced with primers indicated in **Table 12**.

4.3.8 Generation of sYFP2-expressing *P. aeruginosa* strains

pMRE-Tn7-143 was used to transform *P. aeruginosa* via conjugation. Conjugation was carried out on LB-agar plates supplemented with 0.1% arabinose and at 30°C to induce the transcription of transposase encoded on the plasmid. The transposase mobilizes the Tn7 insertion element containing a Gm resistance gene (Gm^R), a Cm resistance gene, and a *syfp2* gene under the control of a constitutive, strong *nptII* promoter into the *attTn7* site of *P. aeruginosa*. Until this point, all strains must be grown at 30°C because pMRE-Tn7-143 has the thermo-unstable pSC101 origin of replication and is a conditional suicide plasmid (Schlechter et al., 2018). After conjugation, mutants carrying the insertion element are selected on LB-agar plates supplemented with Gm (75 µg/mL) and Irgasan (25 µg/mL). After incubation at 37°C over night, the colonies have lost the plasmid and carry the insertion sequence. Single colonies were picked to inoculate 5 mL of sterile LB-medium, grown over night at 37°C with 200 rpm shaking and checked for fluorophore expression with a flow cytometer as described in section 4.2.11. In short, 200 µL of bacteria suspension were fixed in 4% PFA-solution (w/v), analyzed using the flow cytometer to observe fluorescence of all single cells and compared with a fixed, non-fluorescent reference strain. Strains with > 95% fluorescent cells were used to create cryo-stocks.

4.3.9 Protein tagging using the NanoLuc HiBiT-LgBiT-system

The HiBiT tag used prominently in this work is part of the NanoLuc reporter system manufactured by Promega. The NanoLuc luciferase is based on the luciferase of the deep-sea shrimp *Oplophorus gracilirostris* and was engineered to produce a strong glow-type luminescence signal in the presence of furimazine, an imidazopyrazinone. The reaction is ATP-independent, but requires oxygen. NanoLuc was designed for use in mammalian cells and shows higher luminescence and better physical stability towards temperature and pH than firefly luciferase, as well as a wide dynamic range (Hall et al., 2012).

This NanoLuc luciferase was then used to design the complementation reporter NanoBit, consisting of a large 18 kDa subunit (called LgBiT) and a smaller, 1.3 kDa subunit (called SmBiT) that weakly interacts with LgBiT to form an active luciferase. This process can be exploited in protein-interaction studies where LgBiT is fused to one peptide of interest and SmBiT to the other (Dixon et al., 2016). In this study, a second interaction partner of LgBiT was used that, like SmBiT, consists of 11 amino acids, but binds LgBiT with a much higher affinity. This subunit is termed HiBiT and used as an intracellular reporter tag for the presence of certain proteins and their abundance in the cell (Schwinn et al., 2018).

More recently, this system has been employed to investigate the translocation of HiBiT-tagged T3S substrates from *Salmonella* Typhimurium into LgBiT-expressing HeLa cells (Pais et al., 2022; Westerhausen et al., 2020).

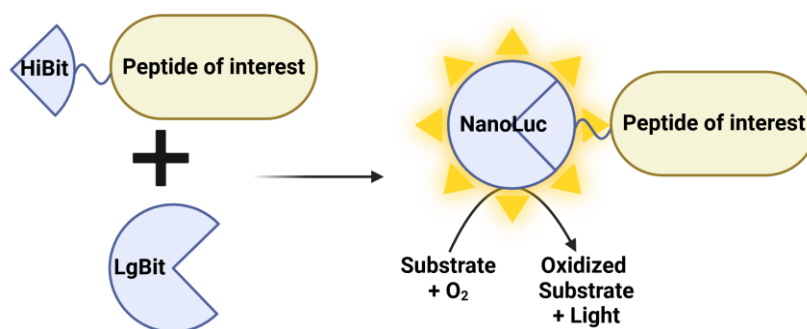


Figure 10: Schematic of the NanoLuc HiBiT system (Promega). A peptide of interest is tagged with an 11 kDa HiBiT-tag that forms an active luciferase when LgBiT is added. The formation of luciferase can be detected by adding the substrate furimazine, which is oxidized ATP-independently by NanoLuc luciferase. Created with Biorender.com.

In this study, strains were used in which the *ampDh3* gene was replaced by a sequence encoding *ampDh3* fused to sequences encoding first a Strep-tag and subsequently the HiBiT-tag via allelic exchange. This allows the specific detection of the AmpDh3-HiBiT fusion protein in the culture supernatant of a growing bacterial culture by first adding LgBiT and then a substrate called furimazine. LgBiT and HiBiT form an active luciferase that gives off a luminescence signal when processing furimazine.

4.3.10 Protein expression and secretion assays of HiBiT-tagged peptides

Overnight cultures of *P. aeruginosa* strains encoding a genomically HiBiT-tagged peptide of interest were grown at 37°C with 200 rpm shaking. Then, day cultures were prepared by diluting the suspensions 1:20 in fresh LB medium. The day cultures were grown at 37°C with 200 rpm shaking for 3 hours and then centrifuged for 10 min at 4,000 x g. The supernatant was carefully removed, filtered through a 0.2 µm pore size syringe filter into a sterile reaction cup and stored on ice. For Western blot detection, samples of this supernatant were used to precipitate proteins using TCA as described in section 4.3.12.

The bacterial pellets were resuspended in 1 mL of PBS and centrifuged at 10,000 x g for 1 min. The supernatant was discarded and the pellets resuspended in 1 mL of PBS. The OD₆₀₀ of the suspensions was determined and an equivalent of OD = 1 (approx. 10⁹ cells) were harvested by centrifugation at 10,000 x g for 1 min. The supernatant was discarded and the pellet resuspended in 500 µL of bacteria lysis buffer (see **Table 7** on p. 33).

After incubation on ice for 30 min, 50 µL of the lysates were transferred into a white F-bottom 96-well plate (Greiner). 50 µL of the non-precipitated supernatant containing all secreted peptides were also added to the same plate. Then, 50 µL of the HiBiT Lytic Detection system buffer supplemented with LgBiT peptide and furimazine were added to each well and mixed briefly. The luminescence intensity in each well was measured using a Tecan microplate reader.

4.3.11 Subcellular fractionation of *P. aeruginosa* via spheroblasting

To determine the subcellular localization of peptides in *P. aeruginosa*, a method adapted from Wang et al. (2020) was used, involving spheroblasting of *P. aeruginosa* cells. Overnight cultures were prepared and diluted in 20 mL of sterile LB to inoculate day cultures to a final OD₆₀₀ of 0.05. The day cultures were incubated at 37°C with 200 rpm shaking to a final OD₆₀₀ of 1.5 before harvesting 10 mL of each culture by centrifugation at 4,000 x g for 10 min. The supernatants were discarded and the pellets resuspended in 500 µL of PBS supplemented with 2.5 mM EDTA and 20% sucrose (w/v). The suspensions were incubated 20 min at room temperature before adding 500 µL of ice-cold DI-H₂O. After 5 min of incubation at 4°C with gentle shaking (550 rpm), the samples were centrifuged at 7,000 x g for 20 min at 4°C. This step yields soluble, periplasmic proteins in the supernatants and insoluble, cytoplasmic and membrane-bound proteins in spheroblasts in the pellet. The supernatant was removed and filtered through a 0.22 µm pore size filter, while the pellets were resuspended in 500 µL of 1 x Laemmli buffer and boiled for 10 min at 95°C. The sterile filtered supernatants were used for TCA precipitation of the contained periplasmic peptides as described below.

4.3.12 Precipitation of proteins from culture supernatants

Proteins were precipitated from solutions using trichloroacetic acid (TCA) and resuspended in Laemmli-buffer for analysis via SDS-PAGE and Western blot.

The supernatants were filtered using a 0.22 µm pore size filter. Then, 250 µL of TCA solution (100%) were added per 1 mL of supernatant. The samples were incubated on ice for 30 minutes and then centrifuged at >14,000 x g for 5 minutes at 4°C. The supernatant was discarded, and the pellets were washed twice with ice-cold 90% acetone (v/v) by adding 400 µL of 90% acetone, centrifuging at 14,000 x g for 5 min at 4°C, and discarding the

supernatant. The dry pellets were completely dried by incubating them at 95°C for 1 min in a heating block. The pellets were resuspended in 36 µL of nuclease-free water and 12 µL of 4 x Laemmli-buffer supplemented with β-mercaptoethanol. The samples were boiled at 95°C for 10 min and stored at -20°C for future use in SDS-PAGE. In SDS-PAGE, 20 µL of these samples were loaded per gel pocket.

4.3.13 Sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For SDS-PAGE, Mini-PROTEAN® TGX precast gels (BioRad) were used. 10 µL of sample were loaded in each pocket if not stated otherwise, with one lane loaded with 5 µL of Prestained Protein Marker (10 – 180 kDa, Thermo Fisher). Gel electrophoresis was performed using an SDS-PAGE electrophoresis chamber (Bio-Rad). 1 x SDS buffer was used as a running buffer and a voltage of 110 V was applied for 60 min. Gels were carefully removed from the running chamber for either Coomassie staining or Western blot.

4.3.14 Coomassie staining

SDS-gels after gel electrophoresis were transferred into a plastic container and washed with warm, deionized water (DI-H₂O) three times. The gels were covered with DI-H₂O and heated in a microwave oven until boiling, then placed on a rocking incubator for 5 min. The gels were washed three times with warm DI-H₂O and then covered with Coomassie Quick Stain solution. The gels were heated in the microwave oven until boiling and placed on the rocking incubator for 5 min. Then, they were rinsed once with DI-H₂O and placed into a closed plastic container and covered with DI-H₂O. A paper towel was placed in the container and the container was placed on the rocking incubator with gentle rocking over night to allow de-staining of the gels. For imaging, the gels were placed on the white-light conversion screen of the Fusion Solo S imaging system.

4.3.15 Western Blot

SDS-gels after gel electrophoresis were removed from the SDS-PAGE running buffer and transferred to nitrocellulose membranes using a wet-blot system and application of an electric field, allowing the charged proteins to migrate through the gel onto the membrane. From the anode-facing side to the cathode-facing side, a “blotting-sandwich” was built using a blotting filter sponge, a blotting paper filter sheet, the nitrocellulose membrane, the SDS-gel, another filter paper and another sponge, all of which were drenched and submerged in 1 x Western Blot Transfer Buffer. For the transfer, a BioRad Mini-Protean blotting chamber was filled with 1 x Western Blot Transfer Buffer, an ice block, a magnetic stirring rod and the blotting sandwich. Blotting was performed by applying an electric current of 350 mA to the blotting chamber for 60 min at 4°C with stirring.

The blotting sandwich was disassembled and rinsed once by covering the membrane with DI-H₂O. The successful transfer of proteins onto the membrane was verified by covering the membrane with 1 x Ponceau S staining solution and placing it on a rocking incubator for 10 min. The membrane was then carefully rinsed with DI-H₂O. Since the Ponceau S staining solution unspecifically stains all proteins bound to the membrane, but not the membrane itself, protein bands become visible after 1-3 washing steps with water. The stain can be fully removed by washing the membrane multiple times with DI-H₂O.

4.3.16 Immunostaining of peptides on nitrocellulose membranes

After Western blotting, nitrocellulose membranes were rinsed once with DI-H₂O. If multiple proteins were detected from the same membrane, the membranes were carefully cut between the protein lanes of interest and treated separately. 5 mL of 1 x BlueBlock PF blocking reagent were added to the membranes and incubated at room temperature for 1 h or over night at 4°C on a rocking incubator. Primary antibodies were then added to the membranes in appropriate dilutions as indicated in section 4.1.8, **Table 11** (p. 38). The membranes were incubated for 1 h at room temperature or over night at 4°C on a rocking incubator. All liquid was discarded and the membrane was incubated three times with TBS-T for 5 min on a rocking incubator to rinse off the primary antibodies. The membrane was covered with 5 mL BlueBlock solution containing the appropriately diluted secondary antibody as indicated in section 4.1.8, **Table 11**. After 1 h incubation at room temperature or over night at 4°C, the solution was discarded and the membrane rinsed again three times with TBS-T solution. The membrane was then covered with PBS.

Since the secondary antibodies we used were fused to a horse-radish peroxidase, we used an ECL substrate (Bio-Rad) to detect secondary antibodies bound to peptides of interest on the nitrocellulose membrane. The membrane was transferred into a cut-open plastic bag and covered with 200 - 400 µL of the mixed ECL substrate. Air bubbles were removed and the luminescence signal detected using the Fusion Solo S imaging system (Vilber).

4.3.17 Detection of HiBiT-tagged peptides on nitrocellulose membranes

HiBiT-tagged proteins on a nitrocellulose membrane can be detected without immune-staining, but instead by adding LgBiT peptide and furimazine to the membrane. LgBiT forms an active luciferase with HiBiT and enzymatically sequesters furimazine, releasing photons that can be measured as luminescence. LgBiT and HiBiT on their own have no catalytic activity. Blocking of the membrane is not required if using the Nano-Glo HiBiT Blotting System (Promega).

After Western blotting, nitrocellulose membranes were rinsed once with DI-H₂O. The membrane was washed once with TBS-T solution on a rocking incubator for 5 min. The TBS-T solution was discarded, the membrane was covered with 5 mL HiBiT Blotting system detection buffer (containing LgBiT, but no substrate) and incubated for 1 hour at room temperature or at 4°C over night on a rocking incubator. Then, 10 µL of substrate (furimazine) were added to the buffer and incubated for 5 min on a rocking incubator. The membrane was transferred into a cut-open plastic bag and the luminescence signal detected using the Fusion Solo S imaging system (Vilber).

4.3.18 Transposon-Directed Insertion Site Sequencing (TraDIS)

A TraDIS screening assay was performed to identify genes of *P. aeruginosa* (PA) that are involved in interbacterial competition against members of the *Burkholderia cepacia* complex (Bcc). In short, a transposon (Tn) library of PA ID40 was co-incubated on LB-agar with two Bcc strains: once with *B. cepacia* ATCC 25416 and once with *B. cenocepacia* ATCC 17765. For reference, PA ID40 Tn library was incubated on plate without a competitor. Generally, Tn mutants that are deficient in essential cell functions should not be able to survive in either treatment condition. Mutants in which the Tn insertion disrupts a gene that is essential for PA to compete with the Bcc strains should have a growth disadvantage only in those conditions where they are competing with Bcc strains, but not in the reference group. Tn mutants in which the Tn disrupts a gene that is detrimental for the survival of PA in co-incubation with Bcc strains should have a growth advantage over other mutants in co-incubation with Bcc strains. DNA isolation, sequencing and comparing the relative abundance of gene insertions across the whole genome of PA in all three conditions allows for the screening of those genes that are either beneficial or detrimental for PA when co-incubated with a Bcc strain on solid agar. **Figure 11** is a graphical abstract of all steps involved in the TraDIS assay that are described in detail below.

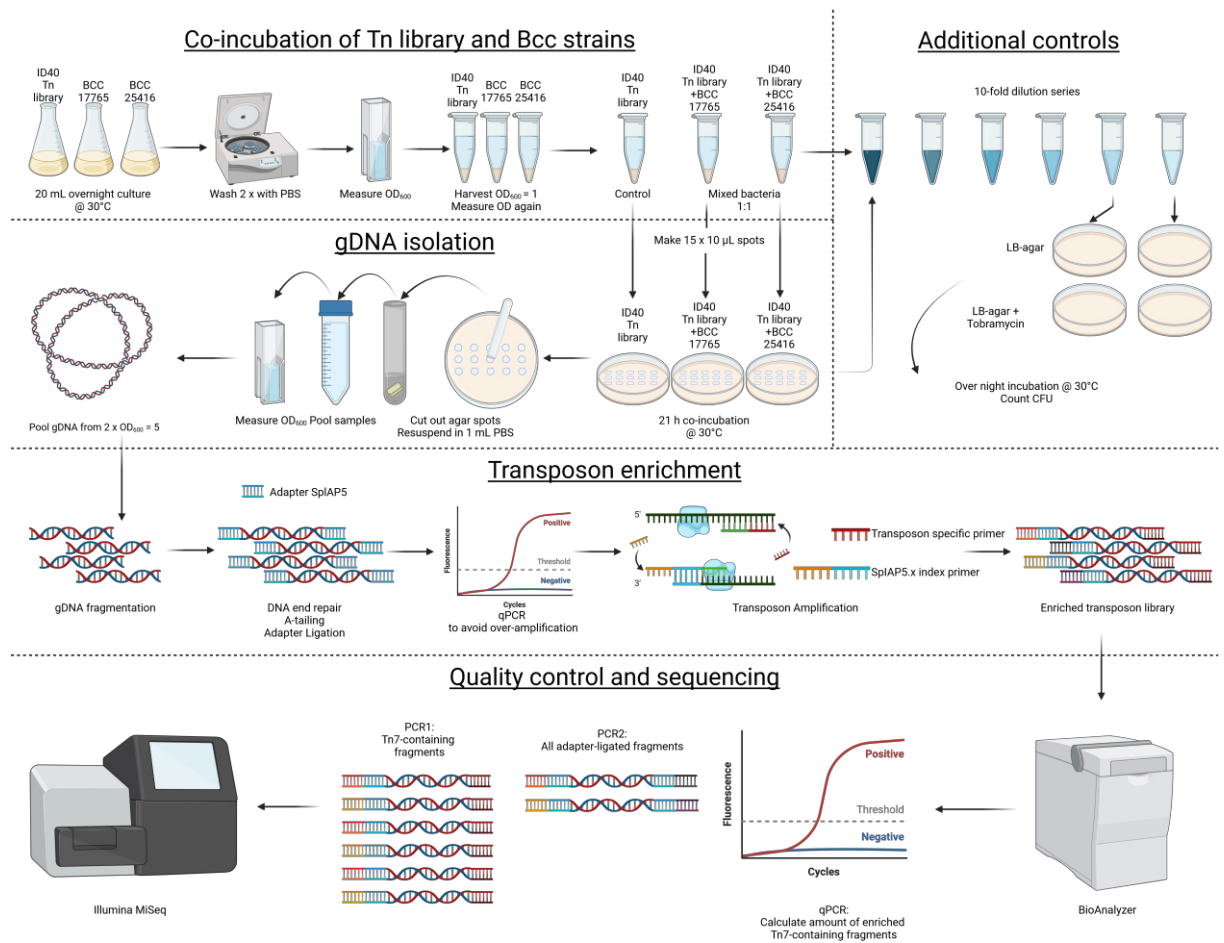


Figure 11: Graphical abstract summarizing all steps involved in the TraDIS screening experiment. In short, an ID40 transposon (Tn) library was co-incubated with either *B. cepacia* ATCC 25416 or *B. cenocepacia* ATCC 17765 on solid LB-agar for 21 h in triplicates. As a control, ID40 Tn library without competitor was treated the same way. After co-incubation (t_{21}), the bacteria were resuspended in PBS and genomic DNA (gDNA) was isolated. To confirm equal ratios of both bacteria in the mix, and to control the competition behavior of both strains during co-incubation, dilution series of all samples were prepared before and after co-incubation, and dilutions were plated out on LB-agar without antibiotics or supplemented with 5 μ g/mL of tobramycin. The previously isolated gDNA was sheared using a sonicator. After end repair, A-tailing and adapter-ligation, the transposon-containing fragments were amplified in a PCR reaction. Finally, the enriched libraries were confirmed to contain sufficient ratios of Tn-containing fragments of adequate, equal length, then pooled and sequenced using Illumina MiSeq sequencing technology. Figure created with Biorender.com.

Co-incubation of *P. aeruginosa* transposon library and *B. cepacia* or *B. cenocepacia*

Cultures of *P. aeruginosa* ID40 (PA) transposon (Tn) library and *B. cepacia* or *B. cenocepacia* (Bcc strains) were grown. The transposon library that was used was generated by Sonnabend et al. (2020). One aliquot of 1 mL volume of the frozen ID40 Tn library was thawed on ice and centrifuged for 2 min at 10,000 $\times$ g at 4°C. The supernatant was discarded and resuspended in 1 mL fresh LB medium. This suspension was added to 100 mL sterile LB broth in an Erlenmeyer shaking flask. Overnight cultures of the Bcc strains were prepared as described in section 4.2.1. All cultures were incubated at 30°C over night on a horizontal shaking incubator with 180 rpm stirring for 16 h. The overnight cultures were

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centrifuged at 4,000 x g for 30 min. The pellets were resuspended in PBS and washed again with PBS. After determination of the OD₆₀₀, an equivalent of OD₆₀₀ = 10 (approx. 10¹⁰ bacteria) were harvested in triplicates for each strain (samples A-C) and centrifuged for 10 min at 10,000 x g. The supernatant was discarded and the pellets were resuspended in PBS to a final density of OD₆₀₀ = 1/mL. The OD₆₀₀ was confirmed again and the strains were mixed 1:1 by OD (with OD = 2 or approx. 2 x 10⁹ cells of each strain) in the following way to yield nine samples:

Table 27: Description of the nine samples used in the TraDIS assay.

Sample number	Mixed strains	Description
1	ID40 Tn library (A)	ID40 reference control condition A
2	ID40 Tn library (A) + <i>B. cepacia</i> (A)	ID40 + <i>B. cepacia</i> co-incubation sample A
3	ID40 Tn library (A) + <i>B. cenocepacia</i> (A)	ID40 + <i>B. cenocepacia</i> co-incubation sample A
4	ID40 Tn library (B)	ID40 reference control condition B
5	ID40 Tn library (B) + <i>B. cepacia</i> (B)	ID40 + <i>B. cepacia</i> co-incubation sample B
6	ID40 Tn library (B) + <i>B. cenocepacia</i> (B)	ID40 + <i>B. cenocepacia</i> co-incubation sample B
7	ID40 Tn library (C)	ID40 reference control condition C
8	ID40 Tn library (C) + <i>B. cepacia</i> (C)	ID40 + <i>B. cepacia</i> co-incubation sample C
9	ID40 Tn library (C) + <i>B. cenocepacia</i> (C)	ID40 + <i>B. cenocepacia</i> co-incubation sample C

For each suspension (containing approx. 2 x 10⁷ cells each), 10 µL were spotted 15 times on a fresh LB-agar plate (3% agar [w/v]), using a separate plate for each sample. After incubation for 21 h, the grown bacterial spots were punched out using the tip of a 5 mL Falcon tube ("FACS tube") and transferred into sterile 14 mL Falcon tubes containing 1 mL sterile PBS using a 10 µL pipet tip. The bacteria lawns were resuspended with a pipet until the agar snips were completely clear and no clumps were visible. All spots belonging to the same sample were pooled in a fresh 14 mL Falcon tube and the OD₆₀₀ was determined. An equivalent of OD₆₀₀ = 10 was harvested and used for isolation of genomic DNA as described in section 4.3.1 (p. 66). The DNA was stored at -20°C until further use in downstream applications.

To confirm that all cultures were mixed in the proper ratios, a 10-fold dilution series was made from the samples used to make co-incubation spots (t₀ samples) as well as the samples after co-incubation (t₂₁) as described in section 4.2.4 (p. 58). LB-agar plates were used to allow growth of PA and Bcc species, and LB-agar plates supplemented with tobramycin (5 µg/mL) were used to select for Bcc strains only. We reasoned that, since we used a Tn

library of PA ID40, selection of ID40 strains with an antibiotic would always fail to allow all colonies to grow. The ratios of PA to Bcc in the t_0 samples was calculated by dividing the calculated CFU/mL from the tobramycin-containing plates (Bcc CFU) by the one from the LB-agar plates (total CFU). We aimed for a 1:1 ratio of PA to Bcc in all conditions.

Fragmentation of gDNA samples via ultrasonication

The concentrations of the gDNA samples from the previous step were determined using QuBit™, since it is less sensitive to contaminants in the samples than NanoDrop technologies. We used QuBit dsDNA broad range buffer (Thermo Fisher Scientific) to dilute our samples 1:100 and measure their DNA concentration.

5 µg of DNA were diluted in 130 µL nuclease-free water and transferred into a micro tube for the Covaris M220 Ultrasonicator. The running protocol used to fragment the DNA samples is shown in **Table 28**:

Table 28: Running protocol for the Covaris M220 Ultrasonicator to obtain 300 bp long gDNA fragments.

Feature	Value
Duty factor	20 %
Peak Incident Power	50 W
Cycles per burst	200
Treatment time	75 s
Temperature	20 °C

This step should yield mainly 300 bp long fragments of gDNA. Each sample was split into 2 separate tubes for downstream applications, each containing 65 µL of DNA fragments. The fragments were purified using AMPure XP Bead-Based Reagent (Beckman Coulter GmbH) with 1.5 x beads per volume of sample. The beads were washed twice with 80% ethanol solution and the DNA was eluted in 50 µL RSB solution (Illumina). The samples were stored at -20°C until further use.

End repair and A-tailing of the fragments

The NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs) was used for end repair and A-tailing of the sheared gDNA samples, to allow adapter ligation in the next step.

7 µL NEBNext Ultra II DNA End Prep Reaction Buffer and 3 µL NEBNext Ultra II DNA End Prep Enzyme Mix were added to each DNA sample from the previous step. The samples were incubated for 30 min at 20 °C and then 65 °C for 30 min in a thermocycler with a constant lid temperature of 75°C.

Adapter ligation

The SplA5 adapter was annealed by first mixing the SplA5_top and SplA5_bottom fragments 1:1 to a total concentration of 15 μM in TE-buffer. The following running protocol was used to anneal the adapter in a thermocycler:

Table 29: Running protocol for annealing SplA5 adapter in a thermocycler. Lid temperature was kept at 105°C.

Temperature	Time [min:s]
95°C	2:00
Ramp down to 25°C (0.1 °C /s)	45:00
10°C	Hold

2.5 μL of the annealed adapter was added to each end-prepped sample (60 μL volume) to a final concentration of 0.4 μM . To each, 1 μL of NEBNext Ligation Enhancer and NEBNext ultra II Ligation MM were added and the sample was incubated for 15 min at 20°C in a thermocycler, with the lid heating turned off. The samples were cleaned up using the AMPure XP Bead-Based Reagent (Beckman Coulter GmbH) in two steps: First with 1 x beads per volume of sample to bind small DNA fragments, but not the larger, adapter-ligated fragments. Then in a second step, these supernatants containing the ligated fragments were split again into two tubes: one containing 100 μL and one containing 80 μL volume. Each was mixed with 0.8 x AMPure XP beads per volume. The final samples were washed twice with 80% ethanol and eluted in 7 μL RSB solution before re-uniting all samples that stem from the same original DNA sample, resulting in a total elution volume of 28 μL for each of the 9 DNA samples.

Optimization of the cycle number for Tn amplification using quantitative PCR (qPCR)

A quantitative PCR (qPCR) mimicking the PCR conditions for transposon amplification was used to determine at which cycle count the amplification reaction should occur to prevent over-amplification of fragments. We decided to use the same PCR conditions as for transposon enrichment (see below). The only difference was the addition of SYBR safe DNA stain in the qPCR reaction mixes. The following reactions were set up in a 96-well PCR plate:

Table 30: Reaction setup for qPCR to determine the optimal cycle number for transposon amplification.

Reagent	Amount
End-prepped, adapter-ligated gDNA	10 μL
2 x KAPA HiFi HotStart ReadyMix	25 μL
Primer 1 (NTMPseq3) 10 μM	5 μL
Primer 2 (SPIAP5.X)* 10 μM	5 μL
100 x SYBR Safe DNA stain	0.25 μL

Reagent	Amount
H ₂ O	4.75 µL

*: The primers SPIA5.X are listed in **Table 12** (p. 39). SPIA5.1 was used for sample 1, SPIA5.2 for sample 2 etc.

The following running protocol was used in the LightCycler:

Table 31: Running protocol for the determination of optimal transposon amplification conditions in the LightCycler.

Step	Temperature [°C]	Duration [min:s]	Repeats
Initial denaturation	95	2:00	x 1
Amplification cycles	98	0:20	x 30
	65	0:30	
	72	0:30	
Final Extension	72	2:00	x 1
Hold	40	-	x 1
Lid temperature	100		

Data was processed using the LightCycler 480 Software v.1.5. We settled on the amplification cycle that showed a beginning exponential amplification of all samples per cycle to prevent over-amplification.

Transposon enrichment PCR

For enrichment of the transposon-containing, adapter-ligated gDNA fragments, the same qPCR protocol was used as described in the previous section. The only changes were the omission of the SYBR safe stain, which was replaced by an equal volume of water, and the use of PCR reaction cups to perform the reaction in a thermocycler. The fragments were cleaned up using AMPure XP beads as described above, with the addition of 0.8 x beads per volume of PCR reaction and elution in 30 µL RSB solution. The DNA concentration was measured using QuBit dsDNA broad range reagents, as described above.

Final quality control using qPCR and capillary gel electrophoresis

A qPCR was performed to determine the total amount of adapter-ligated fragments in the samples as compared to the number of transposon-containing fragments. Two separate qPCR reactions were performed at the same time: one containing two adapter-specific primers and one using one adapter-specific and one transposon-specific primer. For both reactions, the KAPA SYBR® FAST qPCR Kit (Roche Diagnostics) was used. After the last amplification cycle, a melting curve was recorded for all samples, revealing if fragments of varying sizes were present in one of the samples. The following reactions were set up in a white, 96-well PCR plate:

Table 32: Reactions set up for quality control qPCR reaction using the KAPA SYBR FAST qPCR Kit.

*: Template DNA was added 1:10,000 or 1:100,000 diluted in TE-buffer. For the standard curve, 4 µL of standards 1-6 included in the kit were used. Water was used as a negative control. All measurements were performed in duplicates.

Reagent	Volume for PCR1 (adapter specific)	Volume for PCR2 (transposon specific)
Nuclease-free water		5.2 µL
KAPA SYBR fast 2 x buffer		10 µL
Primer qPCR2.1 (10 µM)	0.4 µL	0
Primer qPCR2.2 (10 µM)	0.4 µL	0.4 µL
Primer NTMPseq3 (10 µM)	0	0.4 µL
Template DNA*		4 µL

Each DNA sample was diluted 1:10,000 and 1:100,000 in 10 mM Tris-buffer at pH = 8. The following running protocol was used for the LightCycler:

Table 33: Running protocol used for quality control qPCR reactions.

Step	Time [min]	Temperature [°C]	Repetitions
Denaturation	5:00	95	x 1
Cycling	0:30	95	x 35
	0:45	60	x 35
Melting curve	0:15	95 (4.4°C/s ramp rate)	x 1
	0:15	65 (2.2°C/s ramp rate)	x 1
	Continuously	95 (0.11°C/s ramp rate)	x 1
Cooling	∞	4	

Using the LightCycler 480 Software v.1.5, the crossing-point (CP) values of all amplification curves were used to interpolate a compensation line using the reference standards 1-6. Considering the dilution steps, the concentration of DNA in each sample was then interpolated using the slope of the compensation line and the CP of each respective sample. The ratio of Tn-carrying amplicates to all amplicates was calculated to validate sufficient Tn enrichment in the amplification step.

All samples were furthermore analyzed via capillary gel electrophoresis using the 2100 BioAnalyzer system (Agilent). 1.5 µL of each sample were loaded onto the Bioanalyzer chip together with gel dye, the marker and the DNA ladder following the manufacturers descriptions. The length of all fragments in each sample was analyzed using the 2100 Expert Software (Agilent) and used to verify that all fragments were of equal size and no over-amplification of the fragments had occurred. These results complement the results obtained from the melting curve during the LightCycler run.

Sequencing of the TraDIS libraries

Sequencing was performed by the Next Generation Sequencing Center Core Facility of the Universitätsklinikum Tübingen (NCCT) using the MiSeq Reagent Kit v2 (Illumina®). The samples were first distributed into two pools as described in **Table 34**:

Table 34: Pooling step of all samples for transposon Illumina sequencing.

Sample number	United in
1 (ID40 Tn library A control)	Pool 1
2 (ID40 Tn library A + <i>B. cepacia</i> A)	Pool 2
3 (ID40 Tn library A + <i>B. cenocepacia</i> A)	Pool 1
4 (ID40 Tn library B control)	Pool 1
5 (ID40 Tn library B + <i>B. cepacia</i> B)	Left out
6 (ID40 Tn library B + <i>B. cenocepacia</i> B)	Pool 1
7 (ID40 Tn library C control)	Pool 2
8 (ID40 Tn library C + <i>B. cepacia</i> C)	Pool 2
9 (ID40 Tn library C + <i>B. cenocepacia</i> C)	Pool 2

For pooling, all samples were united as indicated into two pools and the concentration of each sample was adjusted to 4 nM in the final pools. Since pooling more than 4 samples bears the risk of a low amplification of each single fragment, we decided to leave out sample 5 from the sequencing step.

To determine the final concentration of all fragments in each pool, another qPCR was performed as described in the previous section, with the same protocol as described in **Table 32** and **Table 33**. The sample pool was then diluted to 8 pM. The pools were denatured with 0.2 M NaOH and mixed with 10% of PhiX to optimize the results in a high-density transposon library as described by Barquist et al. (2016). The flow cell was then loaded according to manufacturer descriptions of the MiSeq Reagent Kit v2 (Illumina®). 4 µL of both 100 µM primer NTMPseq3 (transposon-specific) and 100 µM Illumina Read Sequencing Primer 1 in 600 µL HT1 buffer were loaded into port 18 of the sequencing kit cartridge and 4 µL of 100 µM index primer iPCRtagseq in 600 µM HT1 buffer were loaded into slot 19 of the cartridge.

Sequencing and Data processing of TraDIS sequencing data

Sequencing, data analysis and visualization were performed by the NCCT. The samples were sequenced in 2 batches on a MiSeq v2 (50 cycles) with a run mode of 52,10,10,0.

For the extraction of the reads, the ExtractIlluminaBarcodes and IlluminaBasecallsToSam commands of picard (v2.16.11-1-g9d4b0bc-SNAPSHOT) were used. For both tools, the read structure was defined as 42T10M10B. The analysis of the reads was performed with the pipelines included in the Bio-Tradis (v1.4.5) package of sanger-pathogens. The

bacteria_tradis pipeline was executed with the transposon tag CCAACCTGTT and the flags `-n 2 -v -r`. For the insert side plots, the trim3 parameter was set to 0,1.

For the visualizations, the included tradiscomparison.R script was adapted. The data were visualized with VolcaNoseR (Goedhart & Luijsterburg, 2020), ggplot2 (v3.4.4) and plotly (v4.10.3).

The resulting list of reads was mapped to the genome of *P. aeruginosa* ID40. The log₂ of the fold change (log₂FC) of the relative abundance of Tn-containing sequences with and without *B. cepacia* or *B. cenocepacia* was compared in all replicates, and tested for significant changes using t-tests ($\alpha = 0.05$).

4.4 Statistical analysis and hypothesis testing

4.4.1 Tests used in AmpDh3 production and secretion assays

Statistical analyses and hypothesis testing were performed in Microsoft Excel and Graphpad Prism v.10.1.1. Before further evaluation, all data sets were tested for normal or log-normal distribution in Graphpad Prism using the D'Agostino-Pearson omnibus normality test and the Anderson-Darling test. A false discovery rate (FDR; α) of 5% was set as cut-off for the rejection of the null hypothesis in all statistical tests performed. Generally, data from luminescence measurements using a Tecan reader was found to follow a log-normal distribution and was subsequently log-transformed using GraphPad Prism function $Y = \log_{10}(Y)$ before further data analysis.

Hypothesis testing using one-way analysis of variance (ANOVA) was performed using GraphPad Prism to investigate the effect of a single factor, for example a gene deletion, on the dependent variable, for example fluorescence. Normality of the data was assumed after testing as described above, and a mixed effects model was fit. Homoscedasticity of data was confirmed using the Brown-Forsythe test.

ANOVA testing was followed up with multiple comparisons using Tukey's range test to identify groups that are different from one another. The test was set to report multiplicity-adjusted P values. The FDR was set to 5%.

For descriptive statistics, the built-in function of GraphPad Prism was used to calculate means, medians, standard deviations and confidence intervals of data sets, which were then used to calculate approximate differences between single data sets.

4.4.2 Hypothesis testing in competition assays

In competition assays, up to three variables were changed at the same time: (i) the genotype of the ID40 competitor, (ii) the competitor species and (iii) the growth conditions, for

example different temperatures or salinities. For simplicity, only two variables were changed at the same time. To compare the effects of these variables on the competitive fitness of *P. aeruginosa*, two-way ANOVA was performed, and followed up by Šidák's multiple comparisons test to compare the means of different groups under different conditions. Normality of the data and homoscedasticity were tested using the D'Agostino-Pearson omnibus normality test and Spearman's test for heteroscedasticity, respectively. The FDR was set to 5%.

To assess whether a certain gene deletion mutant has a different competitive fitness in co-incubation with a certain competitor as compared to the ID40 wt, the $\Delta\log_{10}(\text{CI})$ value was calculated as described in section 4.2.11 (pp. 60 - 66). Using the GraphPad Prism software, one-sample t-tests were performed to assess whether the data set was significantly different from 0, indicating that the respective tested strain has a growth advantage or disadvantage when compared to the ID40 wt.

5 Results I: Investigation of AmpDh3 as an H2-T6SS effector

5.1 AmpDh3 is a cytosolic protein

Parts of the results in the following section were previously published in Eggers et al. (2023). In pp. 3 and 6 of the PDF file, the conclusion that AmpDh3 is located in the periplasm is outlined. That section was written by Ole Eggers based on experiments performed by me, as shown in Supplementary Figure 4 of the document. This figure shows a Western blot performed and formatted by me. Description of the method on p. 15 of the publication (“Subcellular fractionation of *Pseudomonas aeruginosa* by spheroblasting”) was written by me, but it is also described in different words in the methods of this thesis (section 4.3.11, p. 76). The Western blot shown in **Figure 12** of this thesis is a different replicate of the assay shown in the published work of Eggers et al. (2023). However, the data shown in **Figure 12** also present results of the localization of AmpDh3 in strain PA14 of *P. aeruginosa*. To comprehensively include the results into the framework of this thesis, the following section was re-worded and put into a different context than in the aforementioned publication.

AmpDh3 is a zinc protease that was first hypothesized to be located in the periplasm of *P. aeruginosa* based on the high affinity of AmpDh3 to a synthetic substrate mimicking peptidoglycan fragments that are found mainly in the periplasm (W. Zhang et al., 2013). This hypothesis was adopted by other publications and also cited in a publication reporting that AmpDh3 is secreted via the H2-T6SS, despite raising questions about the loading mechanism to the T6SS and the biological function of AmpDh3 in *P. aeruginosa* (Wang et al., 2020). To clarify in which compartment of the bacterial cell AmpDh3 is located, the peptide was first investigated in silico. This was also done to confirm that the strains used in this study, PA14 and ID40, did not contain alterations in their AmpDh3 gene, like alternative start sites, that would lead to periplasmic expression of AmpDh3. For studying the localization and secretion of AmpDh3 in this study, *P. aeruginosa* strains that had a *hiBiT*-tag fused to the *ampDh3* gene at its native locus were generated. The gene sequence of AmpDh3-HiBiT of strains ID40 and PA14 was translated in silico using a DNA translation tool by ExPASy. The peptide sequence was used to predict possible periplasm-targeting signal peptide sequences with the prediction tool SignalP-5.0. No signal peptide sequences were predicted in silico.

To further investigate the localization of AmpDh3 in *P. aeruginosa* experimentally, a subcellular fractionation assay via spheroblasting adapted from a protocol by Wang et al. (2020) was used as described in section 4.3.11, p. 76. To achieve higher expression levels of AmpDh3-HiBiT, *ygfB* gene deletion mutants of strains ID40 and PA14 were also tested in this assay, since they express higher amounts of AmpDh3. The results are shown in **Figure 12**. PA14 was chosen as a model organism for its heightened virulence compared to other

strains (Mikkelsen et al., 2011) and because it was known from previous assays that it generally produces higher levels of AmpDh3 than other strains. ID40 was chosen because it is a highly β -lactam resistant clinical isolate that over-produces the AmpC β -lactamase at least in part in an AmpDh3-dependent manner (Eggers et al., 2023; Sonnabend et al., 2020).

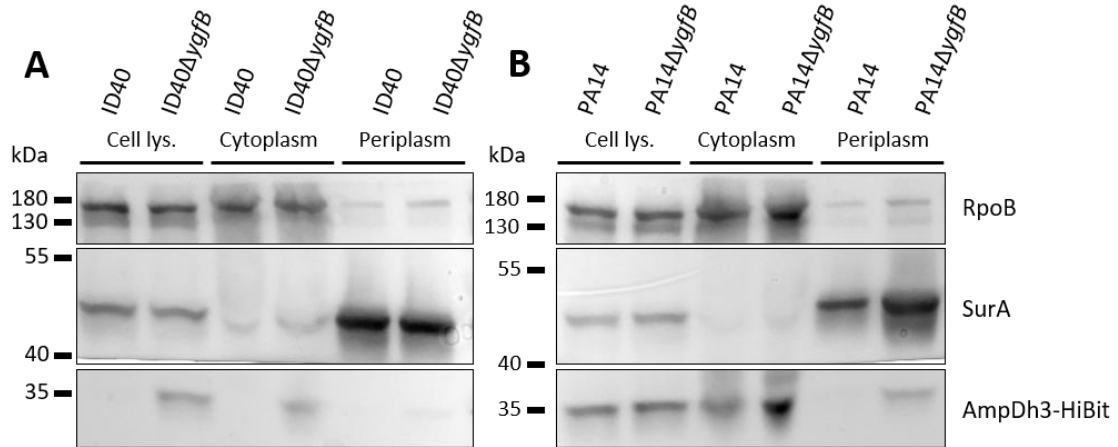


Figure 12: Localization of AmpDh3-HiBiT in the cytosol of *P. aeruginosa* via subcellular fractionation. Fractions containing the periplasmic proteins (**Periplasm**) were sterile filtered and proteins precipitated with TCA. Proteins from the cytoplasm and the plasma membranes are contained in the (**Cytoplasm**) fractions. Whole cell lysates (**Cell lys.**) were used as expression controls. All strains depicted carry a *hiBiT*-tag fused to their *ampDh3* gene. RNA polymerase subunit β (**RpoB**) as a cytosolic housekeeping protein was used as a loading control and as a marker for cytoplasmic proteins. **SurA** was used as a marker for periplasmic proteins. RpoB and SurA were detected using immunostaining on the membrane while AmpDh3-HiBiT was detected using the NanoGlo HiBiT blotting system (Promega). **Primary antibodies / their dilutions:** Mouse anti-*E. coli* RNA Polymerase β / 1:1000 (**RpoB**), Rabbit anti-SurA (PA14) / 1:200 (**SurA**). **Secondary antibodies / their dilutions:** Rabbit anti-Mouse IgG (H+L) Secondary Antibody, HRP / 1:2000 (**RpoB**); Goat F(ab')₂ anti-Rabbit IgG (H+L)-HRPO / 1:2000 (**SurA**). A protein marker (PageRuler Prestained Protein Ladder, Thermo Fisher) was used in the SDS-PAGE to confirm the correct sizes of all detected proteins on the membrane (marker bands not shown). All shown samples were blotted on the same membrane. Before immuno-staining and detection of the protein lanes, the Western blot membrane was sliced apart between the RpoB, SurA and AmpDh3 band and the different peptides were detected separately. Exposure times during detection in all shown blots were adjusted to allow visual detection of lanes on the membrane without over-exposure of single bands. The shown blot shows representative results from $n = 2$ biological replicates.

Figure 12 shows the localization of the reference proteins RNA-polymerase subunit β (RpoB; cytosolic) and SurA (periplasmic) compared to the localization of AmpDh3-HiBiT in *P. aeruginosa* strains ID40 (**A**) and PA14 (**B**) after before and after spheroblasting. Whole cell lysates were compared to both periplasmic and cytosolic fractions to visualize which protein is enriched in which fraction when compared to the whole cell lysates. Comparison of the *ygfB* deletion mutants of both strains with the respective wt background confirmed stronger expression of AmpDh3 in the *ygfB* null background, as seen in the darker AmpDh3-HiBiT bands in both *ygfB* deletion mutants when compared to the wt. The deletion of *ygfB* did not impact the levels of RpoB or SurA in either strain's cell lysate.

As expected, RpoB was enriched in the cytosolic fraction of the spheroblasts, while SurA was enriched in the fraction containing soluble periplasmic proteins. In both ID40 and PA14, AmpDh3-HiBiT was enriched in the fraction containing cytosolic proteins and membrane-bound proteins (**Figure 12**, “**Cytoplasm**”). Since neither the signal peptide prediction tool SignalP-6.0 nor the transmembrane-domain prediction tool DeepTMHMM v.1.0.24 predicted periplasmic signal peptides or transmembrane domains in AmpDh3-HiBiT from ID40 or PA14, it is highly unlikely that the peptide is directly membrane-bound and instead indicates that it is found soluble in the cytosol of the strains. The fainter periplasmic band of AmpDh3-HiBiT could likely be attributed to partial cell lysis during the spheroblasting, together with the faint RpoB band in the periplasmic fractions.

5.2 Secretion of AmpDh3 does not rely on *icmF2*

5.2.1 Secretion and production assays

At the start of this study, we aimed at investigating the H2-T6SS-dependent secretion of AmpDh3 in *P. aeruginosa* that was reported by Wang et al. (2020). We therefore performed a Nanoluciferase assay as described by Westerhausen et al. (2020) using *P. aeruginosa* strains ID40 and PA14 expressing AmpDh3-HiBiT, as well as several in-frame gene deletion mutants of those strains.

Day cultures of all tested strains were grown for 3 h and the supernatants were harvested and filtered, while the pellets were chemically lysed as described in section 4.3.10 (p. 75). LgBiT peptide was then added to the supernatants and lysates, interacting with the HiBiT tag to form an active NanoLuc luciferase. After addition of its substrate, furimazine, a luminescence signal can then be detected using a Tecan reader.

To investigate whether AmpDh3 is secreted in a H2-T6SS-dependent manner, the intracellular production of AmpDh3-HiBiT was compared to the amount detected in the supernatant. Additionally, the AmpDh3 levels in the cytosol and supernatant of the wild type (wt) background was compared to the respective levels in an *icmF2* deletion mutant, which is expected to be H2-T6SS deficient, a *ygfB* mutant background, which is expected to express higher intercellular levels of AmpDh3, and a *ygfB* and *icmF2* double deletion mutant, which should produce high intercellular levels of AmpDh3 but not be able to secrete it via the H2-T6SS. The results are shown in **Figure 13**, where graphs **A** and **B** show the luminescence intensity of the cell pellet lysates and supernatants of ID40 strains, respectively, and graphs **C** and **D** show the luminescence intensity of the cell pellet lysates and supernatants of PA14 strains.

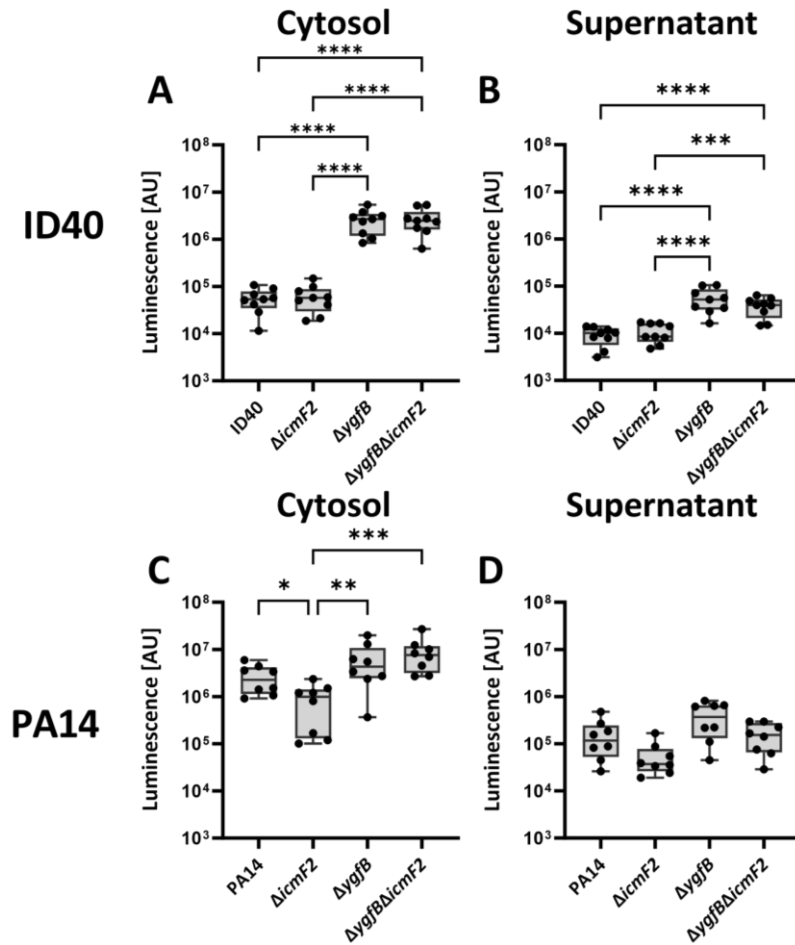


Figure 13: Cytosolic expression and secretion of HiBiT-tagged AmpDh3 in *P. aeruginosa* strains ID40 (A, B) and PA14 (C, D). A and C show luminescence signals from lysed cell pellets, B and D show luminescence signals from culture supernatants. All strains depicted carried a *hiBiT*-tag fused to their *ampDh3* gene. Luminescence stems from a HiBiT-tag fused to AmpDh3 that interacts with LgBiT in the reaction buffer to form an active luciferase that metabolizes furimazine in the buffer. Luminescence was measured using a Tecan plate reader. Boxes represent the mean (center line) and 25% to 75% confidence intervals (upper and lower borders) of $n = 9$ (A, B) or $n = 8$ (C, D) biological replicates. Each dot represents one biological replicate as the mean of three technical replicates. Whiskers range from the minimum to the maximum value. Hypothesis testing was performed on log-normally distributed, log-transformed data using one-way ANOVA. Multiple comparisons between all groups were performed using Tukey post-hoc testing. Asterisks indicate significant differences between groups: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. P values ≥ 0.05 are not shown.

As expected, **Figure 13 A** shows an approx. 45-fold increase in luminescence in the cell pellet of the mutant of ID40 $\Delta ygfB$ and ID40 $\Delta ygfB\Delta icmF2$ compared to ID40 wt or ID40 $\Delta icmF2$. Deletion of *icmF2* alone showed no impact on the luminescence signal in the wt background or the *ygfB* deletion background. In PA14 cell pellet lysates (**Figure 13 C**), the luminescence signal in the PA14 wt strain was approx. 50 times higher compared to the ID40 wt (**Figure 13 A**), but the PA14 $\Delta ygfB$ mutant showed only an approx. 2.5-fold increase in luminescence compared to ID40 $\Delta ygfB$. Interestingly, the deletion of *icmF2* led to a significant reduction in the luminescence signal of the PA14 wt background. The deletion of *ygfB* in the PA14 wt background caused no significant difference in luminescence levels of

the pellet lysates, but caused a 10-fold decrease of luciferase activity in the PA14 Δ *ygfB* Δ *icmF2* background compared to PA14 Δ *ygfB*. In the *ygfB* deletion background, *icmF2* deletion caused no significant change in the luminescence signal of the cell lysates.

Comparison of the luminescence signals derived from AmpDh3-HiBiT production in the cytosol (**Figure 13 A and C**) with the data derived from culture supernatants (**Figure 13B and D**) provides insight into the possible pathways of AmpDh3 secretion in *P. aeruginosa*. Notably, luminescence signals were detected in all tested and sterile filtered culture supernatants. In **Figure 13 B**, the luminescence signal intensities followed the same pattern as in **Figure 13 A**. Deletion of *icmF2* in the ID40 wt or ID40 Δ *ygfB* background did not impact the luminescence signal in the culture supernatant compared to the *icmF2*⁺ strain, while the deletion of *ygfB* in the ID40 wt or ID40 Δ *icmF2* background increased the luminescence signal 3 - 6-fold. Taken together, these results suggest that the higher levels of AmpDh3 in the cytoplasm contributed to higher levels of secretion of AmpDh3 into the culture supernatant. On the other hand, the secretion of AmpDh3 did not seem to rely on the presence of an intact *icmF2* gene, and by extension, an intact H2-T6SS, in strain ID40.

In the PA14 background (**Figure 13 D**), neither deletion of *icmF2* nor of *ygfB* significantly changed the luminescence signal in the supernatants of any of the tested PA14 mutants. Purely qualitative assessment of the measured values, however, revealed an approx. 2.5-fold increase of luminescence signal upon *ygfB* deletion in both the wt background and the *icmF2* deletion background. Deletion of *icmF2* led to a 0.65-fold decrease of AmpDh3 secretion in the wt and *ygfB* deletion backgrounds of PA14. The decrease of luminescence signal in the supernatant of the *icmF2* deletion mutant compared to the wt background was paralleled in the decrease of luminescence in the cell lysate depicted in **Figure 13 C**, but the decrease upon *icmF2* deletion in the *ygfB* mutant background was not. This might indicate that the secretion of AmpDh3 is indeed dependent on an intact *icmF2* gene in PA14 if high levels of AmpDh3 are present in the cytosol. However, since none of the differences in luminescence detected in the supernatants of the PA14 strains showed statistical significance in any of the tested groups, this observation is most likely attributable to unknown confounders. As in strain ID40, the deletion of *icmF2* in PA14 strains did not lead to lower secretion levels of AmpDh3-HiBiT.

Taken together, (i) the secretion of AmpDh3-HiBiT did not seem to rely on an intact *icmF2* gene in the two tested strains of *P. aeruginosa* and (ii) higher levels of AmpDh3 production in the cytosol in the tested strains seemed to correlate with higher AmpDh3 secretion rates into the supernatants. To validate the results of this secretion assay, and to see whether other factors play a role in the higher secretion rates of AmpDh3 in ID40 than in PA14, an

SDS-PAGE followed by a Western blot was performed with samples obtained from cell lysates and TCA-precipitated peptides from culture supernatants.

5.2.2 Confirmation of the findings using Western blot analysis

To confirm the results from the AmpDh3-HiBiT secretion assay performed with the NanoGlo Live Cell Assay kit (Promega), SDS-PAGE and Western blot detection of AmpDh3-HiBiT from cell pellet lysates and culture supernatants was performed. Proteins from TCA-precipitated supernatant were obtained as described in section 4.3.12 (p. 76). The left-over cell pellets were resuspended in 25 μ L water before 25 μ L of 4 x Laemmli buffer with 10% β -mercaptoethanol (v/v) were added, and the samples boiled for 10 min at 95°C. The RNA-polymerase subunit β (RpoB) was used as a loading control and to confirm that proteins in the supernatant stem from secretion and not cell lysis, since RpoB is localized in the cytosol of bacteria.

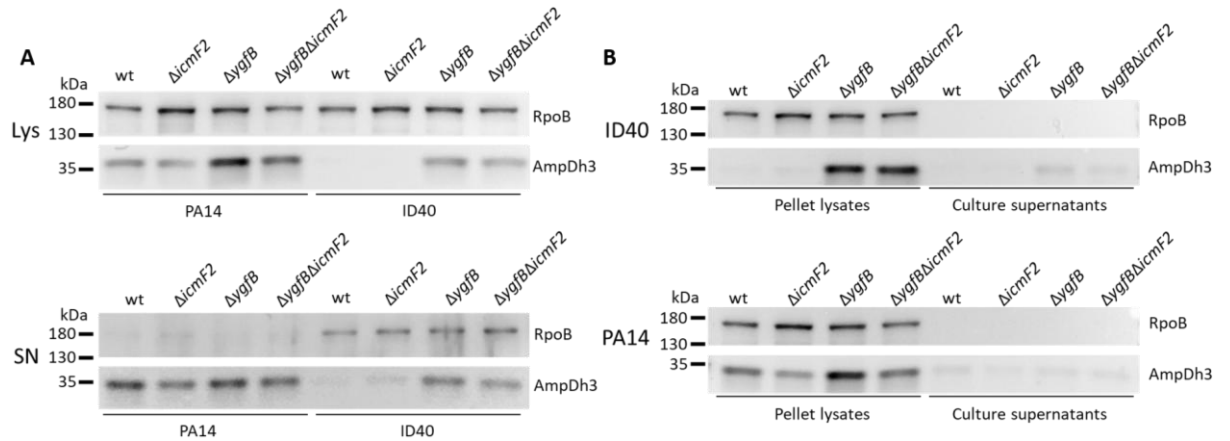


Figure 14: Western blot after SDS-PAGE of cell lysates (Lys) and TCA-precipitated peptides from culture supernatants (SN) from ID40 and PA14 strains. The same lysate and supernatant samples were used for SDS-PAGE and Western blot depicted in A and B. (A) allows comparison of AmpDh3-HiBiT production and secretion in strains PA14 and ID40 while (B) shows the relative abundance of RpoB and AmpDh3-HiBiT in the cytosol and culture supernatant of each strain. All strains depicted carried a *hiBiT*-tag fused to their *ampDh3* gene. RNA polymerase subunit β (RpoB) is a cytosolic housekeeping protein that was used as control for loading equal amounts of protein into every gel pocket to allow comparison of all lanes (loading control). RpoB was detected using immunostaining on the membrane, while AmpDh3-HiBiT was detected using the NanoGlo HiBiT blotting system (Promega). **Primary and secondary antibody / their dilutions:** Mouse anti-*E. coli* RNA Polymerase β / 1:1000 and Rabbit anti-Mouse IgG (H+L) Secondary Antibody, HRP / 1:2000. A protein marker (PageRuler Prestained Protein Ladder, Thermo Fisher) was used in the SDS-PAGE to confirm the correct sizes of both proteins detected on the membrane. Before immuno-staining and detection of the protein lanes, the Western blot membranes were sliced apart between the RpoB and AmpDh3 band and detected separately. Exposure times during detection in all shown blots were adjusted to allow visual detection of lanes on the membrane without over-exposure of single bands. Therefore, intensities of lanes cannot be compared directly between different membranes due to different methods of detection and different exposure times. The shown blot shows representative results from $n = 3$ blots.

Figure 14 shows the results from the SDS-PAGE followed by Western blot, with cell lysates and culture supernatants from ID40 and PA14 strains. Like in section 5.2.1, Figure 13, all

strains contained a *hiBiT*-tagged *ampDh3* gene, either in a wt background, an *icmF2* deletion background, a *ygfB* deletion background or an *icmF2* and *ygfB* double deletion background. For the detection of RpoB and AmpDh3-HiBiT on the Western blot membrane, the membranes were separated and the two proteins detected separately on their membrane snips. PA14 samples and ID40 samples after SDS-PAGE were blotted on separate membranes.

In **Figure 14 A**, the comparison of band intensities between PA14 and ID40 cell lysates showed that all PA14 strains that were tested produced higher levels of AmpDh3-HiBiT in the cytosol than the respective ID40 strains. This difference was most prominent when comparing both the PA14 wt and PA14 Δ *icmF2* mutant to the respective ID40 strains. The intensity of AmpDh3-HiBiT bands increased in both PA14 and ID40 strains in the Δ *ygfB* and Δ *ygfB* Δ *icmF2* mutant backgrounds when compared to the strains carrying an intact *ygfB* gene. In both PA14 and ID40, the AmpDh3-HiBiT band appeared fainter in the Δ *ygfB* Δ *icmF2* strain than in the Δ *ygfB* strain, but so did the respective RpoB band, indicating that the observed difference may stem from different amounts of protein loaded to the SDS-gel. Overall, *ygfB* deletion appeared to result in higher production of AmpDh3-HiBiT in both the PA14 and ID40 strains, while *icmF2* deletion may have resulted in lower AmpDh3 production levels, as observed in **Figure 13**.

When comparing the abundance of RpoB and AmpDh3-HiBiT in the supernatant (SN) of PA14 and ID40 in **Figure 14 A**, the intensity of the RpoB bands in the ID40 background appeared stronger than in the PA14 background, while the intensity of AmpDh3-HiBiT bands appeared stronger in all PA14 strains compared to the respective ID40 strains. Since RpoB is a cytosolic protein, its presence in the culture supernatant can only originate from cell rupture or unspecific secretion via unspecific mechanisms like outer membrane vesicles. Since RpoB bands appeared more prominent in ID40 than in PA14, this may indicate that during growth in the day cultures, more ID40 cells were lysed than PA14 cells. However, even in PA14, AmpDh3-HiBiT was detectable in the supernatant, suggesting an active mode of secretion that does not rely on cell lysis. The intensity of AmpDh3-HiBiT lanes of the PA14 wt and Δ *ygfB* mutant appeared equal, while both the Δ *icmF2* and Δ *ygfB* Δ *icmF2* mutant showed less prominent, but similarly intense bands. In ID40, both Δ *ygfB* mutant backgrounds showed more prominent AmpDh3-HiBiT bands in the supernatant than the respective *ygfB*⁺ strains, and the band in the ID40 Δ *ygfB* Δ *icmF2* mutant appeared less intense than the one of the Δ *ygfB* mutant.

When comparing the levels of RpoB detected in the cell lysates of both PA14 and ID40 strains to the levels detected in culture supernatant precipitates in **Figure 14 B**, it became

apparent that the RpoB levels detected in the supernatant were much lower than those in the cell lysates when detected at the same exposure time as the cell lysate samples. The apparently higher rate of cell lysis of ID40 compared to PA14 observed in **Figure 14 A** could therefore be assumed to have a negligible effect on the secretion rates of AmpDh3-HiBiT.

In PA14, the deletion of *icmF2* in the wt and $\Delta ygfB$ mutant seemed to lead to reduced production of AmpDh3-HiBiT in the cytoplasm in **Figure 14 A**, which may explain the reduced levels of secretion into the supernatant in the same strains in **Figure 14 B**. In both the secretion assay using the NanoGlo Live Cell Assay (**Figure 13**) and using SDS-PAGE and Western blot (**Figure 14**), all PA14 strains seemed to produce higher levels of AmpDh3 in the cytosol than the respective ID40 strains. The increase of AmpDh3 production upon deletion of *ygfB* was more pronounced in ID40 backgrounds than in PA14 strains. The data from the Western blot (**Figure 14**) closely matches the data obtained from using the NanoGlo Live Cell Assay system (**Figure 13**). Overall, both in PA14 and ID40 strains, AmpDh3-HiBiT was secreted into the culture supernatant. Since the deletion of *icmF2* prevents the correct assembly of the H2-T6SS, no secretion of AmpDh3-HiBiT was expected in *icmF2* deficient strains if it relied on the H2-T6SS. Since AmpDh3 was detectable in the supernatant of both ID40 and PA14 *icmF2* deletion mutants (**Figure 13** and **Figure 14**), we conclude that the secretion of AmpDh3 does not or at least not exclusively rely on the H2-T6SS.

5.3 The production and secretion of AmpDh3 does not rely on the *gac/rsm* regulon

Since we could not replicate the findings of the study published by Wang et al (2020) which came to the conclusion that AmpDh3 is secreted via the H2-T6SS, we next wanted to investigate whether the expression of AmpDh3, like other T6SS substrates, is regulated via the *gac/rsm* regulon (Allsopp et al., 2017), and whether over-activated T6SS activity enhances AmpDh3 secretion into the culture supernatant. The secretion of AmpDh3-HiBiT was therefore tested in a *retS* deletion background of *P. aeruginosa* using the NanoGlo Live Cell Assay system (Promega). RetS is a negative regulator of the *gac/rsm* signaling pathway. Samples were obtained in the same way as described in section 5.2.1. Both *ygfB* deletion mutants, leading to higher production of AmpDh3 in the cytosol, and *icmF2* deletion mutants that are unable to assemble a functional H2-T6SS, were included in the experiment.

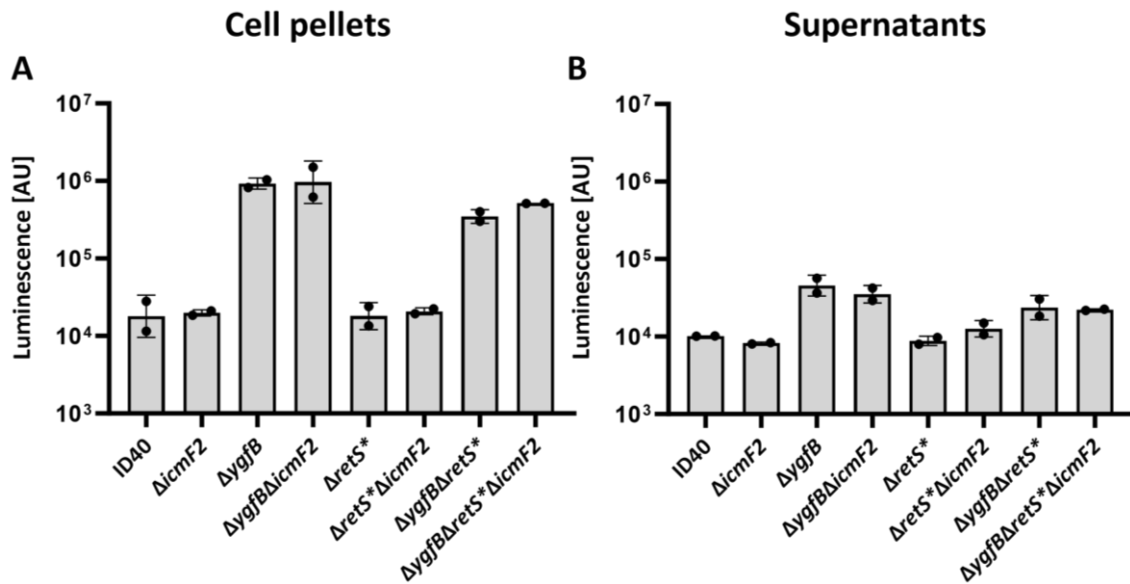


Figure 15: Production and secretion of HiBiT-tagged AmpDh3 in *P. aeruginosa* strain ID40 does not depend on the H2-T6SS or the *gac/rsm* regulon. (A) shows luminescence signals from lysed cell pellets, (B) shows luminescence signals from culture supernatants. Asterisks in the single and multiple *retS* deletion mutants indicate that the strains additionally carried a mutated *gacS* gene that encodes a most likely dysfunctional GacS. This mutation was only recognized in later assays. All strains depicted carry a *hiBiT*-tag fused to their *ampDh3* gene. Luminescence stems from a HiBiT-tag fused to AmpDh3 that interacts with LgBiT to form an active luciferase that metabolizes furimazine in the buffer. Luminescence was measured using a Tecan plate reader. Bars and whiskers show the mean and standard deviation of $n = 2$ biological replicates. Each dot represents one biological replicate as the mean of three technical replicates.

Figure 15 shows the production in the cytosol (A) and secretion into the culture supernatant (B) of AmpDh3-HiBiT in strain ID40. As previously described in **Figure 13** and **Figure 14**, the deletion of *icmF2* did not reduce the production of cytosolic AmpDh3 in this strain, but deletion of *ygfB* led to higher production of AmpDh3 (**Figure 15 A**). Deletion of *retS* did not alter the production of AmpDh3 compared to either the $\Delta icmF2$ and $\Delta ygfB$ single mutant background nor to the $\Delta ygfB\Delta icmF2$ double mutant background. The luminescence intensities correlating with of AmpDh3-HiBiT levels that were detected in the cell pellet lysates in strains ID40 wt, $\Delta icmF2$, $\Delta ygfB$ and $\Delta ygfB\Delta icmF2$ match the luminescence values shown in **Figure 13**, p. 92.

The bars in **Figure 15 B** represent the amount of AmpDh3-HiBiT detected in culture supernatants of the ID40 strains. Only the deletion of *ygfB* in ID40 wt, $\Delta icmF2$, $\Delta retS$ or $\Delta retS\Delta icmF2$ backgrounds led to higher secreted AmpDh3-levels in the supernatant. The deletion of *retS* did not increase AmpDh3-HiBiT secretion levels, nor did the deletion of *icmF2*. The deletion of *ygfB* in the ID40 wt, $\Delta icmF2$, $\Delta retS$ and $\Delta retS\Delta icmF2$ backgrounds led to an approx. 10-fold higher increase in production than in secretion of AmpDh3-HiBiT, suggesting that the secretion rate of AmpDh3 may not strictly be proportional to AmpDh3 levels in the cytosol, and may actually be limited by some unknown factor. Since the deletion

of *icmF2* did not cause a difference in the AmpDh3-HiBiT levels detected in the supernatant compared to the respective *icmF2*⁺ strain, that limiting factor was most likely not the capacity of the H2-T6SS, since deletion of *icmF2* would abrogate the H2-T6SS's function. Interestingly, the *retS* mutant background of ID40 was later found to harbor a 178 bp long deletion in its *gacS* gene, most likely leading to the expression of a non-functional GacS signal kinase. Since (i) all three T6SS of *P. aeruginosa* are regulated by the *gac/rsm* regulatory pathway, (ii) the deletion of GacS usually results in reduced secretion of gene products via the T6SS, and (iii) RetS acts upstream of GacS in the regulation pathway, the *retS* mutants used in this study should show much lower secretion of AmpDh3 compared to their respective *retS*⁺ backgrounds. That is, however, not the case, and instead, *retS* and *gacS*-deficient mutants showed no altered secretion of AmpDh3 whatsoever. The *gacS* mutation in these strains is described in more detail in section 6.4.7 on p. 128, when it was first discovered. Either way, the secretion of AmpDh3 in the tested strains did not appear to rely on the *gac/rsm* pathway.

6 Results II: Investigation of competitive fitness of PA ID40

6.1 Assay parameters: Temperature and co-incubation time

To investigate the competitive fitness of single gene deletion mutations in *P. aeruginosa* (PA) in co-incubation with a competitor strain, a constitutively expressed *syfp2* gene was integrated into the genome of different PA strains. The fluorescent strains were then co-incubated with a competitor bacterium on LB-agar plates for extended periods of time to assess whether the gene deletion affects the competitive fitness of the PA mutant strain compared to the PA wt. Flow cytometry was used to discern fluorescent from non-fluorescent bacteria and calculate a ratio of fluorescent bacteria before and after co-incubation. Those ratios were then used to calculate a competitive index as a ratio of the ratios after and before co-incubation.

To ensure that the temperatures and co-incubation parameters (i) allow the PA strains to continuously express sYFP2 (YFP) and (ii) do not favor the selection of *syfp2* inactivating mutants, fluorescence-labelled ID40 and PA14 strains were grown in liquid LB medium with or without shaking or on LB agar plates containing 3% agarose, both at 30°C and 37°C for 21 h and 42 h. The ratios of fluorescent to non-fluorescent cells of overnight cultures before and after the respective incubation times at the different incubation parameters was measured using a flow cytometer. While the Tn7 insertion element encoding *yfp* carries a gentamicin resistance cassette that would allow for selection of *yfp*-encoding PA strains, addition of an antibiotic would not be feasible in co-incubation experiments with other strains. The aim of this assay was to determine parameters that allow growth of YFP expressing PA strains in the absence of a selective antibiotic.

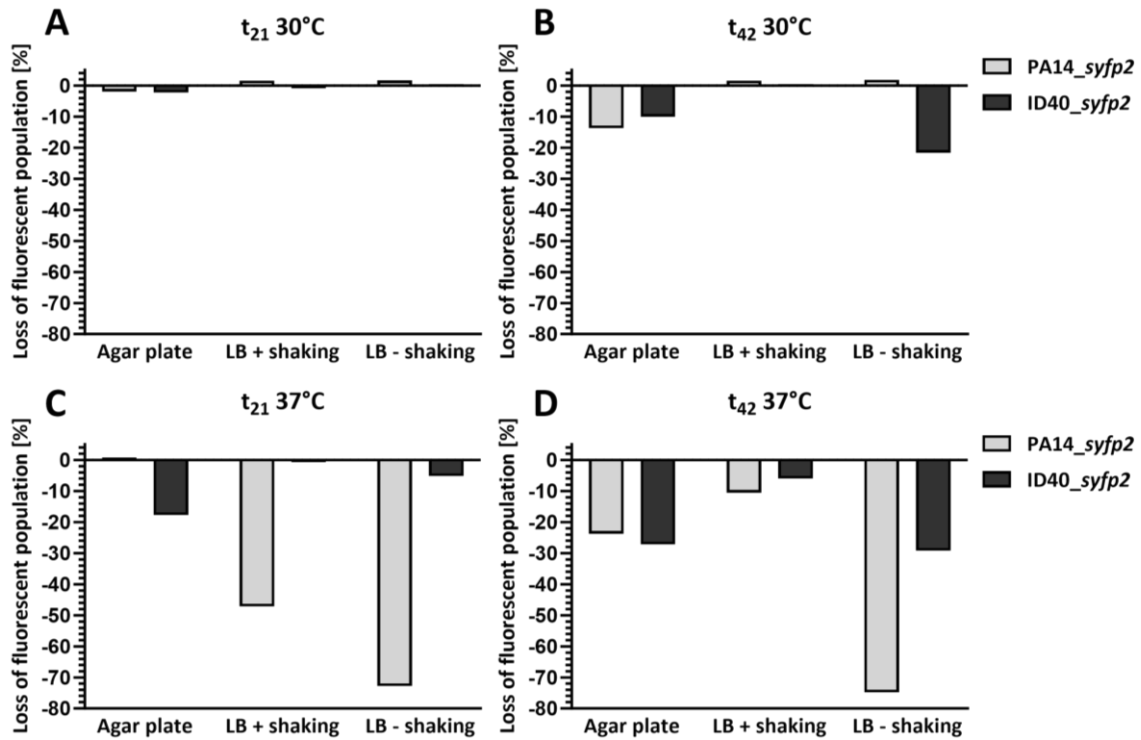


Figure 16: Loss of fluorescent populations [%] in single cultures of *P. aeruginosa* strains PA14 and ID40 with a genomically integrated *syfp2*-encoding Tn7 insertion element under different growth conditions. Approx. 10^7 cells of each strain were either grown on LB-agar plates containing 3% agarose, in 5 mL LB medium with 200 rpm shaking or in 5 mL LB medium without shaking for 21 h (t_{21} , A and C) or 42 h (t_{42} , B and D) at 30°C (A and B) or 37°C (C and D). Ratios of fluorescent to non-fluorescent subpopulations were determined using flow cytometry. The ratio of fluorescent to non-fluorescent populations in each sample before co-incubation was subtracted from the respective ratios at t_{21} or t_{42} to obtain the shown loss of fluorescent populations in [%].

Figure 16 shows the loss of fluorescent populations within a pure culture of genomically fluorescence-labelled *P. aeruginosa* strains at 21 h or 42 h of co-incubation relative to the ratio before incubation under various growth conditions. When grown for 21 h (t_{21}) at 30°C, as shown in **Figure 16 A**, the percentage of fluorescent cells within the cultures changed by less than 5% compared to the ratio before incubation (t_0). Incubation for 42 h (t_{42}) at 30°C (**Figure 16 B**) led to an approx. 10% decrease in the abundance of fluorescent cells in the cultures that were incubated on agar plates, and an approx. 20% decrease in strain PA14 when incubated in liquid LB medium without shaking.

When grown for 21 h at 37°C (**Figure 16 C**), ID40 showed a 16% decrease of the fluorescent population when incubated on an LB-agar plate, but no decrease if incubated in shaken LB-medium. Interestingly, PA14 showed a decrease of fluorescent population of approx. 46% when incubated in liquid LB at 37°C with shaking and a decrease by 72% when incubated without shaking.

When incubated for 42 h at 37°C (**Figure 16 D**), both PA14 and ID40 population showed an approx. 25% decrease in the abundance of fluorescent cells when incubated on LB-agar.

When incubated in liquid LB, ID40 showed a decrease of approx. 5% of the fluorescent population, while PA14 showed a decrease by approx. 10%. Both strains showed a strong decrease in the abundance of fluorescent cells within those cultures that were incubated in liquid LB medium without shaking, where the decrease in PA14 is approx. 74% and approx. 30% in ID40.

The aim of this experiment was to find growth conditions in which the fluorescence-labelled PA14 and ID40 strains show no loss of their fluorescent population when incubated on LB-agar plates for extended periods of time. The only condition where neither PA14 nor ID40 showed a decrease in fluorescent population compared to the time point before incubation was an incubation time of 21 h at 30°C. The loss of fluorescent populations was lower in all conditions that involve incubation at 30°C compared to incubation at 37°C, and larger when liquid cultures were grown without shaking than with shaking, indicating that the loss of fluorescence in a subpopulation of cells might correlate with faster growth and decreased oxygenation or conditions where strains may form biofilms. Why those non-fluorescent cells lost their fluorescent properties remains unknown, but they appeared to be either a metabolically inactive subpopulation, dead cells that did not lyse upon death or a subpopulation of cells that lost or inactivated the *syfp2* gene or promoter region and had a selective advantage over those cells that constitutively express sYFP2.

The flow cytometry-based competition assay that was employed to investigate the competitive fitness of *P. aeruginosa* (PA) over competitor strains relies on the distinction of fluorescent PA cells from non-fluorescent competitor cells. A loss of fluorescence in subpopulations of PA would lead to skewed data, causing an under-estimation of the PA population in all co-incubation experiments. Because incubation at 37°C and at incubation times longer than 42 h seemed to favor the loss of fluorescence in some PA cells, co-incubation experiments like competition assays were performed at 30°C, and for 21 h over night.

6.2 Screening for competitors that show T6SS-dependent competition with *P. aeruginosa*

To assess the competitive fitness of *P. aeruginosa* (PA) in co-incubation with a competitor strain, different criteria of that competitor must be fulfilled. To allow for assessment of the competitive fitness of PA gene deletion mutants towards the competitor over extended periods of time, an ideal competitor should (i) be found in similar ecological niches as *P. aeruginosa*, (ii) grow at a similar rate as PA and (iii) should neither completely suppress the growth of PA nor be completely suppressed by PA. Additionally, the H1-T6SS of PA, which is most commonly associated with interbacterial competition of PA, is repressed

under standard growth conditions and is activated by an attacking T6SS⁺ competitor strain or other membrane-damaging stresses (Basler et al., 2013; Basler & Mekalanos, 2012). Therefore, to assess the contribution of the H1-T6SS and single H1-T6SS effector gene deletions on the competitive fitness of PA, the competitor strain should ideally be a Gram-negative bacterium that also harbors an antibacterial T6SS. The clinical bloodstream isolate ID40 was chosen for this experiment because PAO1, which is used as a common PA laboratory strain, is known to undergo microevolution in laboratories and might have acquired specific adaptations to this environment in the past (Chandler et al., 2019). The strain is itself a derivative of strain PAO (DSM-1707) carrying a genomic inversion of 1.7 Mbp (Stover et al., 2000). PA14, on the other hand, is known to harbor an inactivating insertion mutation in its *ladS* gene, which is a positive regulator of the H1-T6SS, and is not an ideal candidate to study the function of the H1-T6SS (Mikkelsen et al., 2011). To our knowledge, ID40 does not carry any gene mutations in known regulators of the T6SS.

Like *P. aeruginosa*, members of the *Burkholderia cepacia* complex (Bcc) are found abundantly in natural environments like soil and on plants, but are also associated with severe respiratory tract infections in people with cystic fibrosis (Isles et al., 1984; Kilbane et al., 1982; Nährlich L. et al., 2022). *B. cepacia* ATCC 25416 contains two T6SS loci in its genome: one T6SS^{i4b} and one T6SSⁱ³.

6.2.1 Competition between ID40 strains and members of the Bcc

Four strains of the Bcc were tested in competition with *P. aeruginosa* ID40, of which one belongs to the species *B. cepacia* (ATCC 25416), one belongs to the species *B. cenocepacia* (ATCC 1776) and two belong to the species *B. multivorans* (ATCC 17616 and ATCC 17759). To investigate the role that the H1-T6SS and the H2-T6SS of *P. aeruginosa* play in competitive fitness, ID40 Δ *icmF1* (with a defect H1-T6SS system) and ID40 Δ *icmF2* (with a defect H2-T6SS) were compared to the ID40 wt.

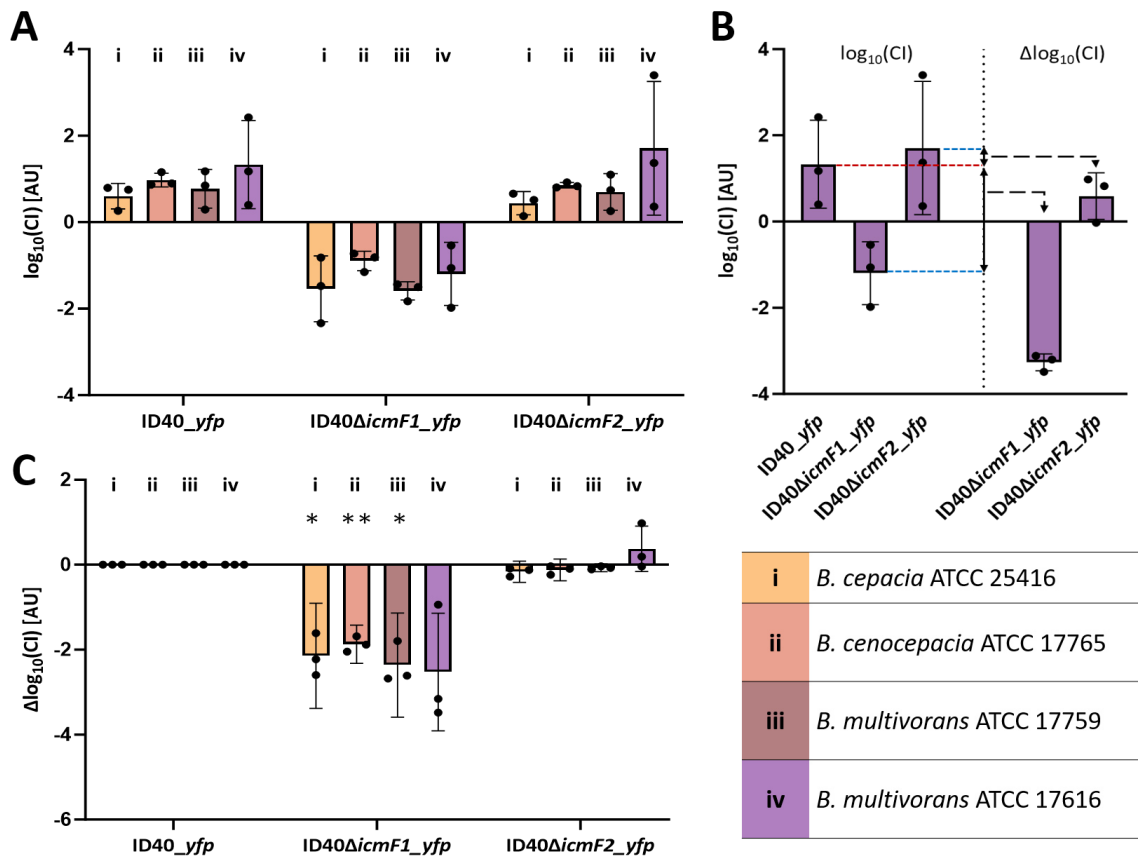


Figure 17: Investigation of the competitive fitness of *P. aeruginosa* (PA) ID40 strains against members of the *Burkholderia cepacia* complex (Bcc). ID40 wt, ID40 Δ icmF1 and ID40 Δ icmF2 carrying a constitutively expressed *yfp2* gene were co-incubated with (i) *B. cepacia* ATCC 25416, (ii) *B. cenocepacia* ATCC 17765, (iii) *B. multivorans* ATCC 17759 and (iv) *B. multivorans* ATCC 17616 for 21 h at 30°C on LB-agar plates containing 3% agar. The ratio of fluorescent cells (PA) to non-fluorescent cells (Bcc species) was determined using a flow cytometer. The ratios of the bacteria before co-incubation (t_0) and after co-incubation (t_{21}) were used to calculate the competitive index (CI) in arbitrary units (AU) for each PA strain in co-incubation with each Bcc strain. (A) depicts the $\log_{10}$ -transformed CI values for each PA strain in co-incubation with each Bcc strain. (B) exemplifies the normalization procedure of $\log_{10}$ -transformed CI values to the wildtype by subtracting the $\log_{10}(\text{CI})$ value of the *yfp*-labelled ID40 wt strain (red dotted line) from each $\log_{10}(\text{CI})$ value of each ID40 gene deletion mutant (blue dotted lines) in each of the three replicates (indicated by alternating arrows). As a result of this normalization step, the $\Delta\log_{10}(\text{CI})$ values of the ID40 wt equals 0 in all replicates. For simplicity, only the values derived from co-incubation with *B. cenocepacia* ATCC 17616 are shown, because they illustrate that $\Delta\log_{10}(\text{CI}) < 0$ indicates a loss of competitive fitness of the ID40 mutant strain compared to the wt and $\Delta\log_{10}(\text{CI}) > 0$ indicates a gain of competitive fitness compared to the wt. (C) depicts the $\Delta\log_{10}(\text{CI})$ values of the three ID40 strains in co-incubation with the four Bcc species (i-iv) from (A). Bars and whiskers depict the mean and standard deviation of $n = 3$ biological replicates. One-sample t-tests were performed to determine if the samples are different from 0, indicating that the respective gene deletion impacts the competitive fitness of *P. aeruginosa* ID40: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. P values ≥ 0.05 are not shown.

Figure 17 depicts the results of a competition assay between fluorescence-labelled ID40 wt, ID40 Δ icmF1 and ID40 Δ icmF2 and four members of the Bcc: *B. cepacia* ATCC 25416, *B. cenocepacia* ATCC 17765, *B. multivorans* ATCC 17759 and *B. multivorans* ATCC 17616. The strains were mixed in a 1:1 ratio (by OD₆₀₀) and co-incubated for 21 h on LB-agar plates containing 3% agar. The ratio of fluorescent to non-fluorescent cells, corresponding to the

ratio of PA to Bcc cells, before co-incubation and after co-incubation was determined using a flow cytometer and used to calculate a competitive index for each PA strain, which was $\log_{10}$ -transformed and is depicted in **Figure 17 A**. As exemplified in **Figure 17 B**, those $\log_{10}(\text{CI})$ values were blanked by subtracting the $\log_{10}(\text{CI})$ of the ID40 wt strain from the $\log_{10}(\text{CI})$ of each PA mutant strain in each replicate. This calculated $\Delta\log_{10}(\text{CI})$ value for each PA - Bcc co-incubation sample is depicted in **Figure 17 C**. A $\Delta\log_{10}(\text{CI})$ value smaller than 0 indicates that the ID40 mutant strain shows a loss of competitive fitness against the tested competitor strain compared to the ID40 wt. A $\Delta\log_{10}(\text{CI})$ value greater than 0 indicates that the tested ID40 mutant has a gain of competitive fitness compared to the ID40 wt.

As shown in **Figure 17 C**, the deletion of *icmF1* in a *yfp*-expressing ID40 background led to a decrease of competitive fitness in co-incubation with all four tested Bcc strains, indicated by a $\Delta\log_{10}(\text{CI}) < 0$. The deletion of *icmF2* did not impact the competitive fitness of ID40 against any of the tested Bcc strains compared to the ID40 wt. On first glance, the deletion of *icmF2* seemed to result in improved competitive fitness of ID40 in co-incubation with *B. multivorans* ATCC 17616. However, descriptive statistics revealed that the 95% confidence intervals of the mean of the three biological replicates includes 0 as a value. In other words, there is a chance of < 5% that the deletion did have an effect on the competitive fitness of *P. aeruginosa* under the tested conditions, meaning that the observed difference can likely be attributed to stochastic scatter.

Taken together, the deletion of *icmF1*, but not *icmF2* in sYFP2-expressing ID40 strains led to reduced competitive fitness of PA ID40 in co-incubation with all tested Bcc strains.

6.2.2 Growth curves of ID40 and Bcc strains

To confirm that the effects observed in section 6.2.1 do not result from growth differences between the tested *P. aeruginosa* (PA) ID40 and *Burkholderia cepacia* complex (Bcc) strains, all tested strains, as well as non-*yfp* expressing ID40 wt were individually grown for 21 h in 1 mL LB medium in a clear 24-well microplate at 30°C with shaking. An additional well was filled with 1 mL of sterile LB for blanking. The OD₆₀₀ within the wells was measured every 10 minutes using a microplate reader (Tecan) and the OD₆₀₀ of each sample was measured at five different points per well in a cross shape. This was done to reduce data scatter due to potential biofilm formation in the center of the wells during growth. Both incubation and measurements were done continually in the microplate reader. The mean of all measurement points was blanked by subtracting the mean of the LB blank at each time point, and growth curves were generated using GraphPad Prism and displayed in **Figure 18**.

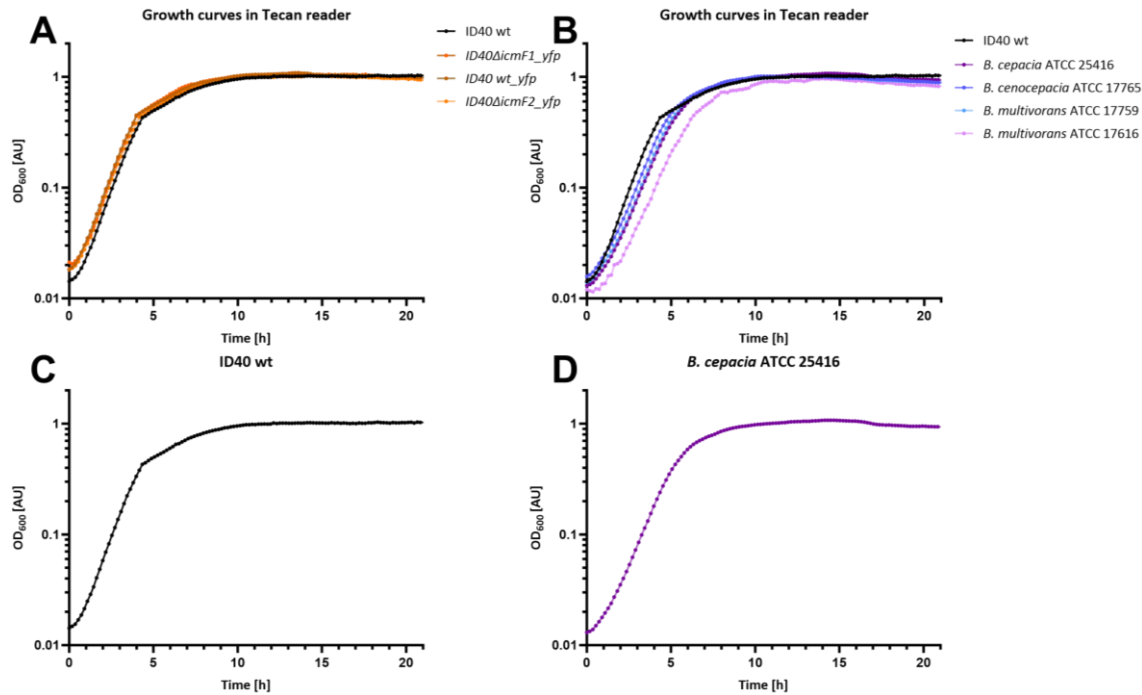


Figure 18: Growth curves of ID40 wt, *yfp*-expressing ID40 strains (A and C) and members of the *B. cepacia* complex (Bcc) (B and D). Bacteria were grown for 21 h at 30°C in 1 mL of LB-medium in a microplate reader (Tecan) in a 24-well plate (Greiner). The OD₆₀₀ of all cultures was measured in 10 min intervals. The plate was shaken in between measurements. In each well, the OD₆₀₀ was measured at five points at each time point to avoid data scatter due to potential biofilm formation during growth. Sterile LB was treated like the samples and was used as sterility control and blank. Colored dots represent the mean of all five measurements after subtracting the OD₆₀₀ value from the sterile LB blank, connected with a line. The data is shown on a log-linear scale to account for the exponential nature of bacterial growth. All growth curves were detected at the same time in the same 24-well plate in the Tecan reader, and were separated into two figures (A and B) for better visibility of the data. The growth curve of ID40 wt (black dots and line) is the same curve in both graphs and serves as reference for both. C and D serve as examples for the different growth behavior of *P. aeruginosa* ID40 and *B. cepacia* strains, exemplified by ID40 wt (C) and *B. cepacia* ATCC 25416 (D).

Figure 18 shows the blanked growth curves of all strains used in the competition assay shown section 6.2.1, as well as the ID40 wt for comparison. Since the plot shows a log-linear depiction of exponential growth, exponential growth appears as a linear slope in the graph, and low OD₆₀₀ values appear to scatter more than in a linear-linear depiction of the same data. It is important to note that the OD₆₀₀ values measured with the microplate reader do not necessarily correspond to the same values measured in a UV/Vis spectrometer, and that the OD₆₀₀ is dimensionless data.

The growth curves of all ID40 strains showed a lag-phase during the first hour of growth, then entered the log-linear growth phase before abruptly switching to a slower log-linear growth after 4 h of growth and an OD₆₀₀ of approx. 0.43 (Figure 18 C). This biphasic growth was commonly observed for ID40 strains growing in LB in our lab (unpublished data). The second log-linear growth phase of the ID40 strains lasted until 6.7 h of growth or an OD₆₀₀ of 0.78 before starting to decline. The ID40 strains reached a plateau at an OD of approx. 1

after 11 h of growth. Neither the introduction of the constitutively expressed *syfp2* gene nor the deletion of *icmF1* or *icmF2* seemed to have an impact on the growth behavior of ID40 under the tested conditions.

The tested members of the Bcc did not show the same biphasic growth as the ID40 strains, but seemed to show a comparatively longer lag-phase than the ID40 strains (**Figure 18 D**). Notably, *B. multivorans* ATCC 17616 only entered the log-linear growth phase after 2 h of growth, and the log-linear section of the growth curve appeared less steep than the one of the other Bcc species. The other Bcc species entered the log-linear phase after approx. 1.5 h of growth before the slope of the curve started to decline again after approx. 5 h. They reached a plateau at approximately the same time as the ID40 strains after 11 h of growth at an OD of approx. 1, whereas the *B. multivorans* strain ATCC 17616 seemed to reach its plateau phase after 13 h of growth. All tested strains except the ID40 wt strain then showed a decrease of their OD₆₀₀ in the well over time, starting after approx. 14.5 h of growth.

All in all, neither the expression of sYFP2 nor the deletion of *icmF1* or *icmF2* had a negative effect on the growth of ID40 strains under standard growth conditions, although it can not be excluded that it does negatively influence their fitness in the presence of a stressor, e.g. a competitor. PA ID40 and Bcc species, except *B. multivorans* ATCC 17616, showed a similar growth behavior, with the Bcc species reaching the log-linear growth phase approx. 30 min later than the ID40 strains, and their plateau phase around the same time as the ID40 strains, maybe due to the biphasic growth of ID40. Since all Bcc strains except *B. multivorans* ATCC 17616 grew at similar rates to sYFP2-expressing PA ID40 at 30°C, they appear to be suitable competitors for PA.

To maintain feasibility of co-incubation assays within a reasonable amount of time, and to ensure that all of the steps required could be performed at approximately the same time for all co-incubation pairs, two out of the three Bcc members were chosen for further investigation of the competitive fitness of *P. aeruginosa*. *B. cepacia* ATCC 25416 was chosen as the type strain for *B. cepacia*, since it represents a more environmental, plant-pathogenic member of the Bcc. *B. cenocepacia* ATCC 17765 was chosen as a representative of human pathogenic Bcc strains, since *B. cenocepacia* is commonly associated with the fatal “cepacia syndrome” in people with cystic fibrosis. Both strains represent different ecological niches and potential competitors of *P. aeruginosa* in natural environments and in human disease.

6.2.3 Impact of high- and low-salt conditions on the growth and competition of ID40 and Bcc species

Both *P. aeruginosa* and some *B. cepacia* species have a broad tolerance to changing salt concentrations in the environment (Elabed et al., 2019; Paul et al., 2021). To investigate

whether different NaCl concentrations impact the competitive fitness of *P. aeruginosa* (PA) ID40 in co-incubation with *B. cepacia*, competition assays on LB Miller (10 g/L NaCl) and NSLB (0 g/L) agar plates were performed. To investigate whether potential differences in competitive fitness of ID40 or *B. cepacia* strains in the two conditions stems from growth-related phenotypes, growth curves of the tested strains were measured in LB and NSLB medium.

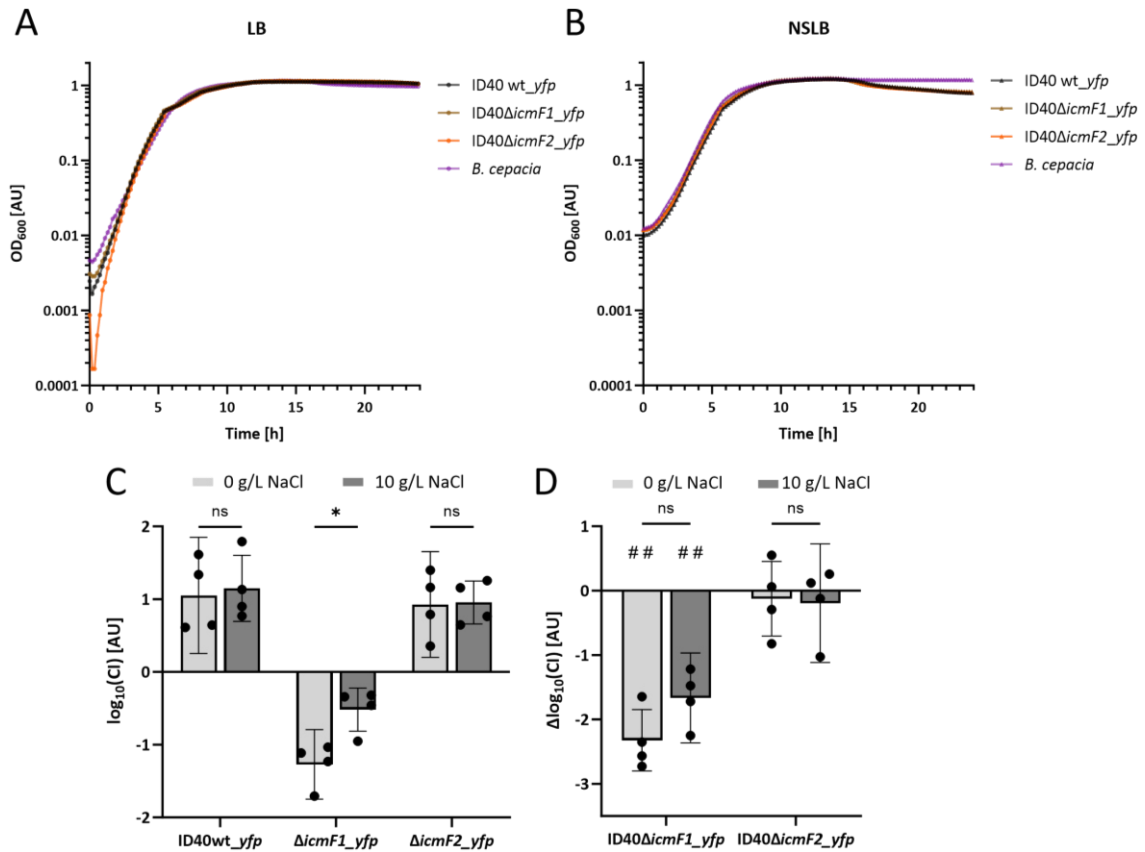


Figure 19: Competition and growth of *P. aeruginosa* ID40 strains and *B. cepacia* ATCC 25416 in LB medium supplemented with 10 g/L NaCl (LB) or 0 g/L NaCl (NSLB). All ID40 strains shown in this figure constitutively express *syfp2* (*yfp*). (A, B) Growth curves were measured in a microplate reader (Tecan) by continuously measuring the OD₆₀₀ in 10 min intervals, with incubation at 30°C and shaking in between measuring points. Colored dots represent the mean of all five measurements after subtracting the OD₆₀₀ value from the sterile LB blank, connected with a line. Growth curves in LB (A) and NSLB (B) were measured in the same run, but split into two graphs for better visibility. Competition assays (C, D) were carried out either on LB-agar or NSLB-agar plates with 3% agar at 30°C with 21 h of co-incubation. Bars and whiskers represent the mean and standard deviation of either the log₁₀-transformed competitive index (log₁₀[CI]) of ID40 strains (C) or the log₁₀(CI) of the *icmF1* and *icmF2* deletion mutants after subtracting the log(CI) of the ID40 wt (Δlog₁₀[CI], D), of *n* = 4 biological replicates (black dots). The means of log₁₀(CI) values of the ID40 strains in competition with *B. cepacia* on LB plates were compared to the respective means of log₁₀(CI) values on NSLB plates using two-way ANOVA followed up by Šidák's multiple comparisons test. The same tests were used for comparing the means of Δlog₁₀(CI) values of ID40 mutants in competition with *B. cepacia* on LB plates to the respective Δlog₁₀(CI) in competition on NSLB plates. Multiplicity-adjusted P-values are shown as follows: ns *P* > 0.05; * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001; **** *P* < 0.0001. Homoscedasticity of data was confirmed using the Spearman rank correlation test (recommended by GraphPad Prism), while normality was confirmed using the D'Agostino-Pearson omnibus (K2) test (done in GraphPad Prism). Additionally, one-sample t-tests were performed to determine if the samples are different from 0, indicating that the respective gene deletion impacts the competitive fitness of *P. aeruginosa* ID40: # *P* < 0.05; ## *P* < 0.01; ### *P* < 0.001; #### *P* < 0.0001. P values ≥ 0.05 are not shown.

Figure 19 shows growth curves of strains ID40 wt, ID40Δ*icmF1*, ID40Δ*icmF2* and *B. cepacia* ATCC 25416 in LB medium containing either 10 g/L NaCl (LB) (**Figure 19 A**) or 0 g/L NaCl (NSLB) (**Figure 19 B**), as well as competition assays between the ID40 strains and *B. cepacia* (**Figure 19 C and D**).

The growth curves shown in **Figure 19 A** demonstrated at first glance that all tested strains grow similarly to each other in LB medium, as observed in section 6.2.2. The log-linear growth phase started after approx. 1 h of growth in all strains. All ID40 strains showed the same biphasic growth as was observed in the previous section, this time after approx. 5.4 h of growth around an OD₆₀₀ of 0.46. The second phase of slower, log-linear growth lasted until approx. 8 h of growth, before the growth rate declined and reached a plateau at OD₆₀₀ of 1.1 after approx. 13.5 h of growth. *B. cepacia* ATCC 25416 did not show a biphasic growth. Log-linear growth of this strain in LB lasted until reaching an OD₆₀₀ of 0.48 after approx. 6 h of growth before declining and also reaching a plateau at the same density and approximately at the same time as the ID40 strains. The OD₆₀₀ of all tested strains started to decline slightly after approx. 16.5 h of growth, with the OD₆₀₀ of all ID40 strains reaching 1.05 and the OD of *B. cepacia* reaching 0.97 after 24 h of growth.

When growing in NSLB (**Figure 19 B**), the exponential growth phase of all strains started after approx. 1.5 h of growth and lasted until the strains had grown for 5.4 h (*B. cepacia*) or 5.8 h (*P. aeruginosa*) to an OD of approx. 0.48. All ID40 strains again showed an abrupt shift to slower log-linear growth until reaching an OD₆₀₀ of approx. 0.83 after 7.5 h of growth. After the end of log-linear growth, the growth rate in all strains slowly decreased until the OD₆₀₀ reached a plateau at approx. 1.2 after 13 h of growth. The OD₆₀₀ then started to decrease in all ID40 strains until reaching 0.8, while the OD of *B. cepacia* remained constant.

Taken together, the growth curves demonstrated that the NaCl concentration in the growth medium had no particular effect on the growth behavior of ID40 or *B. cepacia* during exponential (log-linear) growth, since all strains reached the exponential and stationary phases at approximately the same time and at the same cell densities. In all tested conditions, ID40 showed a biphasic growth curve with no lag-phase in between log-linear growth phases, independent of the salt concentration in the medium. However, the ID40 strains showed a decline of their OD₆₀₀ when grown in NSLB after reaching the plateau, which was not observed in LB supplemented with 10 g/L NaCl.

Figure 19 C and **D** shows the competitive fitness of PA ID40 strains in competition with *B. cepacia* ATCC 25416. The three tested PA strains were ID40 wt, an *icmF1* deletion mutant and an *icmF2* deletion mutant, which are deficient in the assembly of the H1-T6SS or H2-T6SS, respectively, and all constitutively expressed sYFP2. As observed in section 6.2.1, **Figure 17** (p. 61), only the deletion of *icmF1*, but not the deletion of *icmF2* reduced the competitive fitness of ID40 in competition with *B. cepacia*. Comparison of the log₁₀(CI) values of each ID40 strain competing on LB agar with those of the same strain competing on NSLB agar (**Figure 19 C**) via 2-way ANOVA and multiple comparison testing revealed a

significant difference in competitive fitness on NSLB agar only in the *icmF1* deletion mutant. ID40 Δ *icmF1* seems to have a higher competitive fitness when co-incubated with *B. cepacia* on LB-agar with 10 g/L NaCl than on NSLB agar, represented by a lower $\log_{10}(\text{CI})$ value when competing on NSLB agar. When normalizing the data in each biological replicate to the competition behavior of the ID40 wt background ($\Delta\log_{10}(\text{CI})$, **Figure 19 D**), the difference in the competitive fitness of ID40 Δ *icmF1* when competing on LB and NSLB agar was not statistically significant. This could mean that the difference observed when comparing the $\log_{10}(\text{CI})$ values before blanking against the ID40 wt could originate from confounding factors like day-to-day variation between replicates.

All tested ID40 strains, but not *B. cepacia* also showed a decreasing OD₆₀₀ after reaching the maximum cell density when growing in liquid NSLB medium, but not in LB medium (**Figure 19 A and B**). If the growth of ID40 and *B. cepacia* in late stages of co-incubation differs in one condition, but not the other, this can lead to skewed $\log_{10}(\text{CI})$ values in that condition, and direct comparison between the same strains in those conditions would not be feasible. However, when comparing the difference in competitive fitness of each mutant to the ID40 wt of that data set, the resulting $\Delta\log_{10}(\text{CI})$ becomes normalized to the growth of the ID40 in each condition, making it possible to compare the competitive fitness of mutant strains between different growth conditions. Since this experiment aimed at investigating the effects of gene deletion mutants on the competitive fitness of the ID40 wt when competing with *B. cepacia* on LB-agar with or without salt, the “blanked against the wt” $\Delta\log_{10}(\text{CI})$ value is a better point of reference than the $\log_{10}(\text{CI})$ values.

6.3 Contribution of AmpDh3 to the competitive fitness of *P. aeruginosa* in competition with *B. cepacia*

Having established an assay for the investigation of T6SS-related competitive fitness of *P. aeruginosa* ID40, this was used to investigate the effects of *ampDh3* deletion or -overexpression in *P. aeruginosa* in competition with *B. cepacia*. A study by Wang et al. (2020) had previously shown that AmpDh3 secretion into competitor bacteria leads to cell death, using a *retS* deficient strain of *P. aeruginosa*, which over-expresses the T6SS, and *E. coli* or *Y. pseudotuberculosis* as competitor strains. Using *ampDh3*-over-expressing *ygfB* deletion strains of PA ID40 and PA14, we aimed at recreating these results without introducing a mutation into the PA strains that would dysregulate the T6SS itself. In previous assays we had observed that in ID40, the deletion of *ygfB* leads to a higher production and secretion of AmpDh3, which should supposedly lead to higher toxicity towards competitor cells if AmpDh3 is delivered to target bacteria via the H2-T6SS.

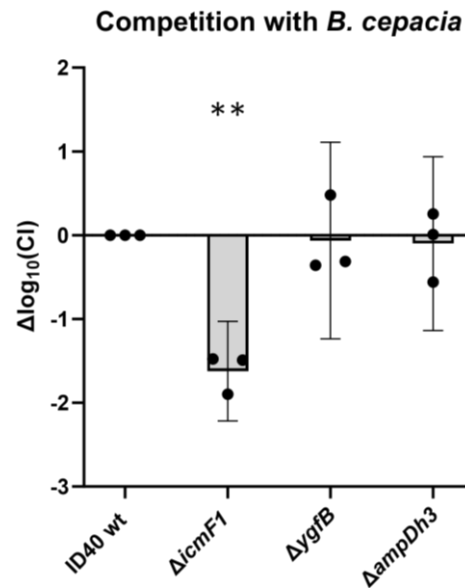


Figure 20: Competition between ID40 strains and *B. cepacia* to test the impact of *ygfB* and *ampDh3* deletion on the competitive fitness of ID40. All ID40 strains shown express *yfp* from a constitutive promoter. An *icmF1* deletion mutant was used as positive control. Competition assays were carried out either on LB-agar plates with 3% agar at 30°C for 21 h of co-incubation. The log-transformed competitive index ($\log_{10}[\text{CI}]$) of the ID40 strains is shown after subtracting the $\log(\text{CI})$ of the ID40 wt ($\Delta\log_{10}[\text{CI}]$), of $n = 3$ biological replicates (black dots). Bars and whiskers represent the mean and 95%-confidence interval of the mean. One-sample t-tests were performed to determine if the samples are different from 0, indicating that the respective gene deletion impacts the competitive fitness of *P. aeruginosa* ID40: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. P values ≥ 0.05 are not shown.

Figure 20 shows the outcome of the competition assay between *B. cepacia* ATCC 25416 and ID40 strains expressing chromosomally integrated *yfp* from a constitutively active promoter. The competitive indices (CI) of the ID40 strains were calculated for each replicate from the ratios of PA:BC after and before co-incubation at 30°C for 21 h. The CI values were log-transformed and the $\log(\text{CI})$ of the wt was subtracted from the $\log(\text{CI})$ of the individual strains to show the competitive fitness of the mutants relative to the wildtype. An *icmF1* deletion mutant was used as positive control for a gene that would negatively impact the competitive fitness upon deletion, as shown in **Figure 17** (p. 103).

Neither the deletion of *ygfB*, leading to AmpDh3 overexpression and -secretion as shown in **Figure 15** (p. 97), nor the genomic deletion of *ampDh3* led to a change in the competitive fitness of the mutants compared to the ID40 wildtype. At least in competition with *B. cepacia*, AmpDh3 did not seem to play a role in competition of ID40. This observation is in line with the finding that the H2-T6SS did not play a role in the competition of PA ID40 in competition with *B. cepacia*, as shown above in section 6.2.1 (pp. 102 - 104).

6.4 Contribution of H1-T6SS effectors to the competitive fitness of *P. aeruginosa* ID40 in competition with *B. cepacia* complex strains

Since the H1-T6SS, but not the H2-T6SS of *P. aeruginosa* (PA) greatly contributes to the competitive fitness of PA against members of the *B. cepacia* complex (Bcc), gene deletion mutants of all known H1-T6SS effectors, *tse1-8*, were created. Despite our best efforts, we were unable to successfully generate a PA ID40 Δ *tse3* mutant. The functions and targets of all known and tested H1-T6SS effectors are specified in **Table 2** (p. 21).

6.4.1 Tse7 is a PAAR-effector with low levels of conservation in *P. aeruginosa*

During the construction of plasmids for the generation of an ID40 Δ *tse7* mutant, *in silico* alignment of the gene sequence of *tse7* of strain PA01 (PA0099) against the genome of ID40, and curiously, also against the genome of PA14, failed. Basic Local Alignment Search Tool (BLAST) analysis of the translated peptide returned no homologous genes in other *P. aeruginosa* strains. The gene sequences of the assumed Tse7 homologs in strains PA01, PA14 and ID40, based on their location on the genome, were translated using the ExPASy translate tool and aligned using Clustal Omega multiple sequence alignment, as shown in **Figure 21**.

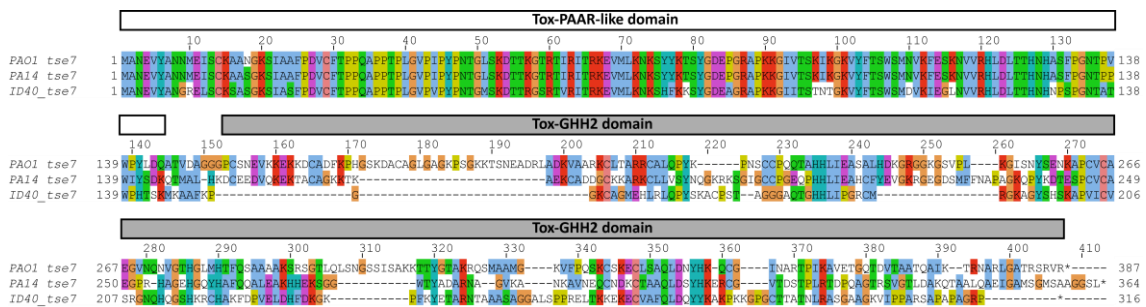


Figure 21: Multiple sequence alignment of predicted Tse7 peptides in *P. aeruginosa* PA01, PA14 and ID40. The sequence of PA01 was used for determining the predicted PAAR- (white box) and Tox-GHH2 domains (grey box), as suggested by InterPro (PA0099). Translated amino acid sequences of the gene sequences were predicted using the ExPASy translation tool, and multiple sequence alignments were performed with Clustal Omega (Sievers et al., 2011), using the built-in function of SnapGene, and displayed in Jalview. Colors represent conserved amino acids based on their biochemical properties (Clustal X). Dashes indicate missing amino acids compared to the other aligned sequences.

The alignments of all three sequences revealed a high level of conservation in the PAAR domain of the PAAR effectors, but a very low level of conservation in the Tox-GHH2 domains, where the amino acid sequence of ID40 was more dissimilar to PA01 than the sequence of PA14. At this point, it is impossible to say whether Tse7 of PA14 or ID40 have the same function as Tse7 of PA01. Importantly, the assumed Tsi7 immunity peptides of the three strains were also highly dissimilar when examined the same way as their cognate Tse7 effectors (not shown), which might indicate that the three effectors co-evolved differently

in all three tested strains. Interestingly, Tse7 and Tsi7 appear to be the only known H1-T6SS effector-immunity protein pair that shows such a high level of divergence between the three PA strains. Despite the low level of conservation between the strains, the gene in ID40 will be referred to as *tse7*. This finding fits a study currently published as a pre-print by Habich et al. (2022), which analyzed and compared the T6SS effectors of 52 diverse clones of *P. aeruginosa*. They found that *tse7* is among those effectors with high variation between isolates, and that the gene is therefore part of the so-termed accessory effectors of *P. aeruginosa* (Habich et al., 2022).

6.4.2 Competitive fitness of H1-T6SS effector deletion mutants against Bcc strains

The competitive fitness of PA ID40 H1-T6SS effector deletion mutants were tested against *B. cepacia* ATCC 25416 and *B. cenocepacia* ATCC 17765, and compared to fitness of the ID40 wt (see **Figure 22**). After some initial experiments using only the ID40 strains and *B. cepacia* (**Figure 22 A**, white circles), it became clear that *tse1*, *tse5* and *tse8* deletion caused the greatest loss of competitive fitness in PA ID40 wt against *B. cepacia* ATCC 25416. To assess whether there exists a specific set of H1-T6SS effectors (“*tse* effectors”) that is sufficient for PA ID40 to outcompete Bcc species, *tse* effectors were sequentially knocked out in ID40 *tse1* and *tse5* double deletion mutant strains and their competitive fitness against a Bcc competitor was compared to the *icmF1* (H1-T6SS-) deletion mutant. Interestingly, a *tse3* deletion mutant was successfully created in an ID40 *tse1* and *tse5* double deletion background, but not in the ID40 wt background.

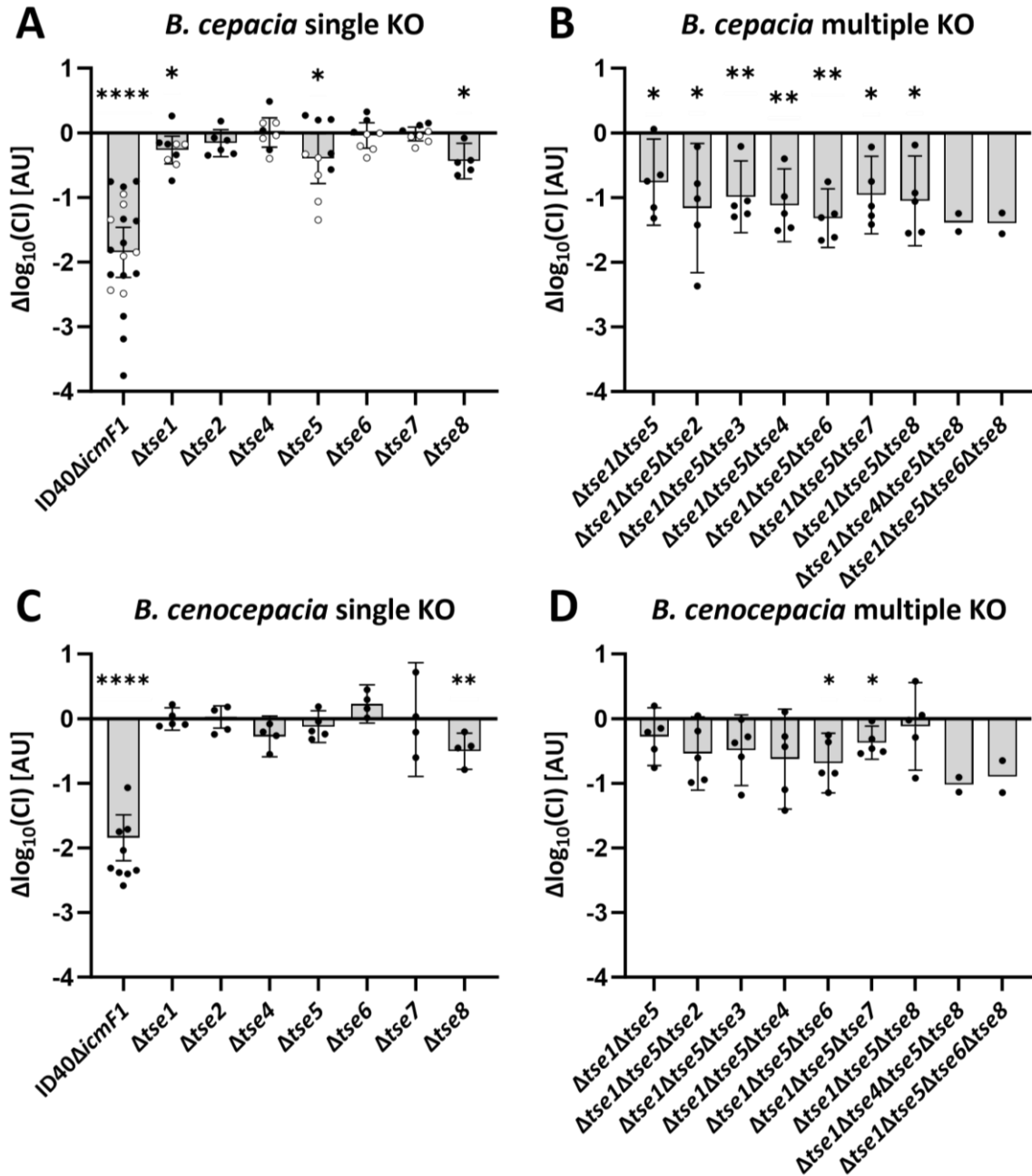


Figure 22: Effect of the deletion of one or multiple H1-T6SS effectors (*tse1-8*) on the competitive fitness of PA ID40 in competition with Bcc species compared to the fitness of the ID40 wt. ID40 Δ *icmF1* was used as H1-T6SS deficient control strain. All ID40 strains used in this assay constitutively express sYFP2. Bars and whiskers represent the mean and 95%-confidence interval of the $\log_{10}(\text{CI})$ of the *icmF1* and *tse1-8* deletion mutants after subtracting the $\log(\text{CI})$ of the ID40 wt ($\Delta\log_{10}[\text{CI}]$, wt not shown), of biological replicates (black and white dots). White circles represent initial replicates that were used to determine *tse1* and *tse5* double deletion mutants as the starting point for sequential deletion of more *tse* effectors to find a set of effectors that are sufficient for ID40 to outcompete Bcc strains. One-sample t-tests were performed to determine if the samples are different from 0, indicating that the respective gene deletion impacts the competitive fitness of *P. aeruginosa* ID40: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. P values ≥ 0.05 are not shown.

Figure 22 illustrates summarized competition assays performed between ID40 wt, ID40 Δ *icmF1* and ID40 Δ *tse1-8* deletion mutants, and the Bcc strains *B. cepacia* ATCC 25416 (A and B) or *B. cenocepacia* ATCC 17765 (C and D). Since the data was normalized to the

$\log_{10}(\text{CI})$ of ID40 wt within every replicate by subtracting it from the respective $\log_{10}(\text{CI})$ of the tested ID40 deletion mutants, the ID40 wt itself is not shown. The resulting $\Delta\log_{10}(\text{CI})$ was compared between *tse1-8* deletion mutants and the H1-T6SS deficient *icmF1* deletion mutant. The deletion of a *tse* effector or set of effectors that is sufficient for outcompeting the Bcc competitor strains should result in a competitive disadvantage that is comparable to the ID40 Δ *icmF1* mutant.

In a first set of competition assays, only ID40 Δ *icmF1*, ID40 Δ *tse1*, ID40 Δ *tse4*, ID40 Δ *tse5*, ID40 Δ *tse6* and ID40 Δ *tse7* had been successfully obtained and tested only against the *B. cepacia* ATCC 25614 strain as shown in **Figure 22 A**, represented by the white circles. None of the tested effector deletions resulted in a loss of competitiveness that was comparable to ID40 Δ *icmF1*, but the deletion of *tse1* and *tse5* seemed to have the stronger impact on the competitiveness of ID40 than the deletion of the other tested effectors. To identify a potential set of effectors that are sufficient for ID40 to outcompete Bcc species ("core effector set"), a *tse1* and *tse5* double deletion mutant was created, followed by the sequential deletion of further *tse* effectors and competition assays with *B. cepacia* and *B. cenocepacia*, as shown in **Figure 22 B and D**. In *B. cepacia*, the cumulative deletion of *tse1* and *tse5* had a slightly stronger negative impact on the competitive fitness of ID40 than the single effector deletion mutants. The subsequent deletion of further *tse* effectors, especially *tse4* and *tse6*, resulted in an even lower $\Delta\log_{10}(\text{CI})$, but none of the tested deletion mutants had a competitive disadvantage against *B. cepacia* that was comparable to the ID40 Δ *icmF1* control strain, indicating that even more than those effectors are needed for ID40 to successfully outcompete *B. cepacia*. In summary, *tse1*, *tse5* and *tse8* provide a competitive advantage to ID40 when competing with *B. cepacia*, but no single effector deletion can explain the loss of fitness observed when deleting the *icmF1* gene. Cumulative deletion of *tse1*, *tse5* and other *tse* effectors resulted in a stronger loss of competitiveness in ID40 than the single deletion of those genes, but that cumulative effect appeared to be unspecific for any effector or combination of effectors under the tested conditions.

In competition with *B. cenocepacia* (**Figure 22 C and D**), the deletion of *tse1* did not result in a loss of competitiveness of ID40 compared to the ID40 wt, and the deletion of *tse5* only showed a minor negative impact on the competitiveness of ID40. Interestingly, the deletion of *tse4* negatively affected the competitive fitness of ID40 in competition with *B. cenocepacia*, but not with *B. cepacia*. The deletion of *tse8* negatively impacted the competitive fitness of ID40 compared to the ID40 wt both in competition with *B. cepacia* and *B. cenocepacia*, but not as strongly as the deletion of *icmF1* did. Surprisingly, the simultaneous deletion of *tse1* and *tse5* did not negatively impact the competitive fitness of

ID40 compared to the wildtype when competing with *B. cenocepacia*, although the single deletion of *tse5* did. The additional deletion of *tse2-7* in the ID40 Δ *tse1* Δ *tse5* background led to decreased fitness of ID40 in competition with *B. cenocepacia* when compared to the fitness of the ID40 wt, except for the deletion of *tse4*, in which the effect on the competitiveness of the triple deletion mutant was comparable to the effect on the single deletion of *tse4* in ID40. Interestingly, the Δ *tse1* Δ *tse5* Δ *tse8* triple deletion showed no negative effect on the competitiveness of ID40 compared to the wt when competing with *B. cenocepacia*, although the single deletion of *tse8* had the strongest negative effect out of all tested *tse1-8* single deletion mutants.

In summary, it appears that different sets of H1-T6SS effectors provide a competitive advantage to ID40 when competing with *B. cepacia* and with *B. cenocepacia*. While in *B. cepacia*, the deletion of *tse1*, *tse5* and *tse8* resulted in a loss of competitiveness in ID40, *tse4* and *tse8* deletions were the only ones resulting in a competitive disadvantage in competition with *B. cenocepacia*. While the cumulative deletion of *tse1* and *tse5* resulted in a disadvantage for ID40 in competition with *B. cepacia*, no such disadvantage was observed in competition with *B. cenocepacia*. Deletion of further *tse* effectors in an ID40 Δ *tse1* Δ *tse5* background resulted in a loss of competitiveness against both *B. cepacia* and *B. cenocepacia*, except for *tse8* deletion. The ID40 Δ *tse1* Δ *tse5* Δ *tse8* triple knock-out mutant showed a loss of competitive fitness compared to the ID40 wt when competing with *B. cepacia*, but not when competing with *B. cenocepacia*. This was especially curious because the single deletion of *tse8* had a relatively large negative impact on the competitiveness of ID40 against both Bcc strains.

It is remarkable that, despite targeting different sites and structures in the competitor cell, none of the tested H1-T6SS effector single, double or triple deletion mutants showed an impact on the competitive fitness of ID40 that was comparable to the ID40 Δ *icmF1* mutant. This may also imply the presence of effectors other than those already identified, which are used by ID40 to simultaneously attack Bcc strains at various target sites to outcompete Bcc species. Furthermore, *B. cepacia* appeared to be able to better take advantage of inactivating mutations in ID40 effectors than *B. cenocepacia*, despite *B. cenocepacia* being able to outcompete the T6SS-deficient ID40 Δ *icmF1* strain approximately as efficiently as *B. cepacia*. The deletion of multiple *tse* effectors gave *B. cepacia* the opportunity to better outcompete the ID40 mutant when compared to *B. cenocepacia*, represented by overall smaller negative changes in the competitiveness of ID40 with multiple *tse* gene deletions when competing with *B. cenocepacia* rather than *B. cepacia*. This capability of *B. cepacia* to better take advantage of a loss of competitive fitness in ID40 strains can not be explained by a different

growth phenotype of *B. cepacia* and *B. cenocepacia*, since both strains grew at equal rates as shown in **Figure 18**.

6.4.3 Screening for tobramycin concentrations for selection of *B. cepacia* complex species

Transposon-directed insertion site sequencing was used in this study to identify genes that are needed for *P. aeruginosa* (PA) ID40 to compete with *Burkholderia cepacia* complex (Bcc) strains. The bacteria were mixed in a 1:1 ratio with either *B. cepacia* ATCC 25416 or *B. cenocepacia* ATCC 17765 in triplicates. To verify that the initial inoculum of all replicates contained equal amounts of ID40 transposon (Tn) library and the Bcc strains, the CFU of at least one of the strains in the inoculum had to be counted and compared to the total CFU in the inoculum. Selection of ID40 in this case was not a viable option, since the Tn library might contain an unknown number of mutants that are susceptible to the chosen antibiotic, which may lead to underestimation of the CFU of ID40 in the original inocula. Instead, the more homogeneous Bcc populations were selected on LB-agar plates with a suitable antibiotic.

From previous tests, it was known that *B. cepacia* and *B. cenocepacia* were resistant to tobramycin, while *P. aeruginosa* ID40 was sensitive. To determine at which concentration the ID40 Tn library was killed, but neither of the Bcc strains, LB-agar plates without antibiotics, or supplemented with 5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL or 80 µg/mL of tobramycin were prepared, and all three tested strains were streaked onto each plate using an inoculation loop. The plates were incubated over night at 30°C. No colonies of the ID40 Tn library were visible on either of the plates containing tobramycin, while the number of colonies of both Bcc strains decreased with increasing tobramycin concentrations, but even at 80 µg/mL tobramycin, there were still colonies visible on the plates. Based on this observation, LB-agar plates supplemented with 5 µg/mL of tobramycin were used to allow for selective growth of both Bcc species, but not of the ID40 Tn library, with minimal negative impact on the growth of the Bcc strains, if at all.

6.4.4 Sequencing run analysis

After mixing the ID40 Tn library with Bcc species, and after co-incubation on plates, samples from all conditions were used to count CFU either on non-selective LB-plates or on LB-agar containing 5 µg/mL of tobramycin to allow for selective growth of only the *Burkholderia* species. The abundance of the Bcc species in the mix was then calculated as percentage from the counted CFU on both plate types. In the initial inocula, between 48.44% and 66.7% of the total CFU could be attributed to *B. cepacia* or *B. cenocepacia* species. After co-incubation over night, the abundance of Bcc species was between 1.5% and 4.1%, indicating that the

Bcc strains had mostly been outcompeted by *P. aeruginosa*, and that their genomic DNA did likely not interfere with the further sequencing process. For one of the three biological replicates containing ID40 and *B. cepacia* after co-incubation, no colonies were visible on the LB-plates supplemented with tobramycin. Since due to read limitations, the flow cells that were used for sequencing (MiSeq reagent kit v2) yield the best results when pooling no more than 4 samples per sequencing run, and only two flow cells were used for this screening, this sample was excluded from pooling and sequencing. The samples were pooled as shown in **Table 34**, p. 86.

The performance of the sequencing run on the MiSeq chip can be determined by comparing the total number of reads to the maximum possible number of reads, and by assessing the read output of each single sample within each of the two pools, as shown in **Figure 23**.

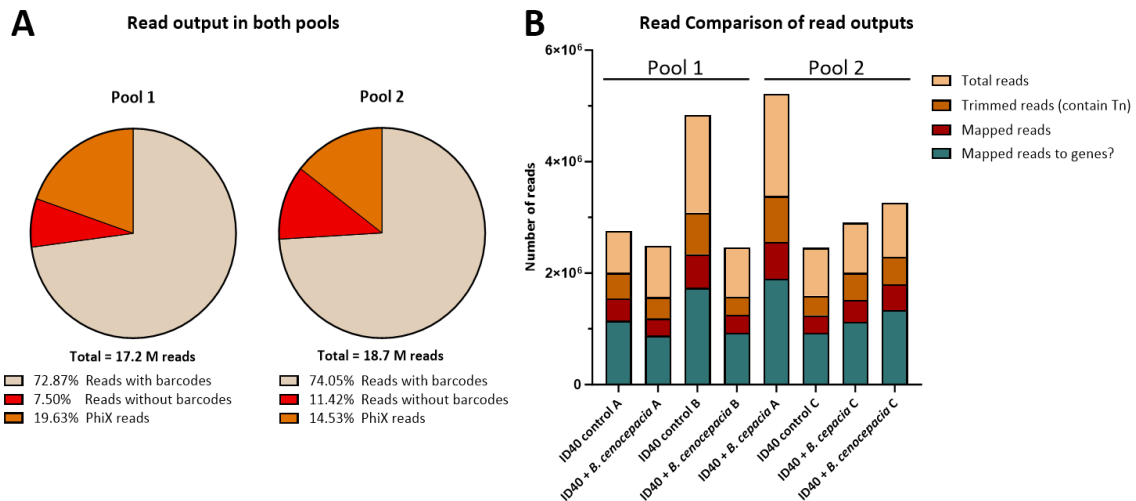


Figure 23: Visual comparison of all samples sequenced using the MiSeq Reagent Kit v2. (A) Total read output of flow cell 1 (Pool 1) and flow cell 2 (Pool 2), split into reads containing barcode sequences (beige) or containing no barcodes, further split into reads originating from PhiX (orange) and reads that could not be attributed to any sequence (red). PhiX, an adapter-ligated control DNA library for Illumina sequencing runs, was added to each pool to optimize clustering on the flow cells and overall read quality of reads with low base variability. (B) Per-sample analysis of the barcode-containing reads in pools 1 and 2. The superimposed bars represent the total number of reads per barcoded sample (beige), the trimmed reads containing a transposon marker sequence (orange), the number of those reads that were successfully mapped to the ID40 genome (red), and reads mapped to coding sequences (teal).

Figure 23 A shows the overall performance of the two sequencing runs with sample pool 1 and sample pool 2. While the MiSeq Reagent Kit v2 can sequence up to 15 million reads passing filter per run according to the manufacturer, the two flow cells used for this study yielded in total 17.2 million reads (flow cell 1 with pool 1) and 18.7 million total reads (flow cell 2 with pool 2), respectively. While the calculated amount of PhiX added to each pool was 10%, flow cell 1 yielded 19.63% PhiX reads and flow cell 2 yielded 14.53% PhiX reads. The amount of reads that could be attributed neither to barcodes nor to PhiX was 7.5% in flow

cell 1 and 11.42% in flow cell 2. To assess whether all samples were pooled evenly, and the overall quality of the reads, the reads were further analyzed per sample.

The read distribution of the single barcoded samples in both pools appeared to be mostly homogenous, as seen in **Figure 23 B**. One sample per pool, the ID40 control of replicate B in pool 1 and the ID40 sample mixed with *B. cepacia* from replicate A in pool 2, yielded higher read counts than all other samples in the respective pool. Those two samples make up approx. 38% of all reads in their pool, while all other samples range from 17% to 23% of total reads. Of those reads, between 63% and 72% contained a transposon sequence and of those trimmed reads, 76% to 79% were mapped to the genome, with a total of 1,200,000 to 2,500,000 mapped reads per sample. Between approx. 880,000 and approx. 1,900,000 of those mapped reads per sample were located in predicted coding sequences on the ID40 genome, which comprises 6485 predicted coding sequences in total. Of those mapped sequences, between 911 and 1108 genes were found with an abundance ≤ 10 reads and 662 – 738 genes with an abundance of 0 reads, which most likely comprise essential genes of ID40. Other studies using a similar approach have previously identified ~ 400 - 800 essential genes of single *P. aeruginosa* strains, with a shared essential genome of 321 genes (Poulsen et al., 2019).

The mapped reads were further analyzed by assessing the clustering of reads between replicates through a multidimensional scaling plot (MDS plot) of a principal component analysis of the log₂-fold change (logFC) between samples. The plots revealed sufficient clustering of conditions and controls, respectively, and separation of controls from conditions, with no batch effects of the separate sequencing runs on the logFC detectable.

6.4.5 Data evaluation

For further data evaluation, the relative changes in the abundance of Tn-containing sequences were compared between the three ID40 controls and the competition conditions with either *B. cepacia* (two replicates) or *B. cenocepacia* (three replicates). For visualization of the data, volcano plots were generated of either condition, as shown in **Figure 24**.

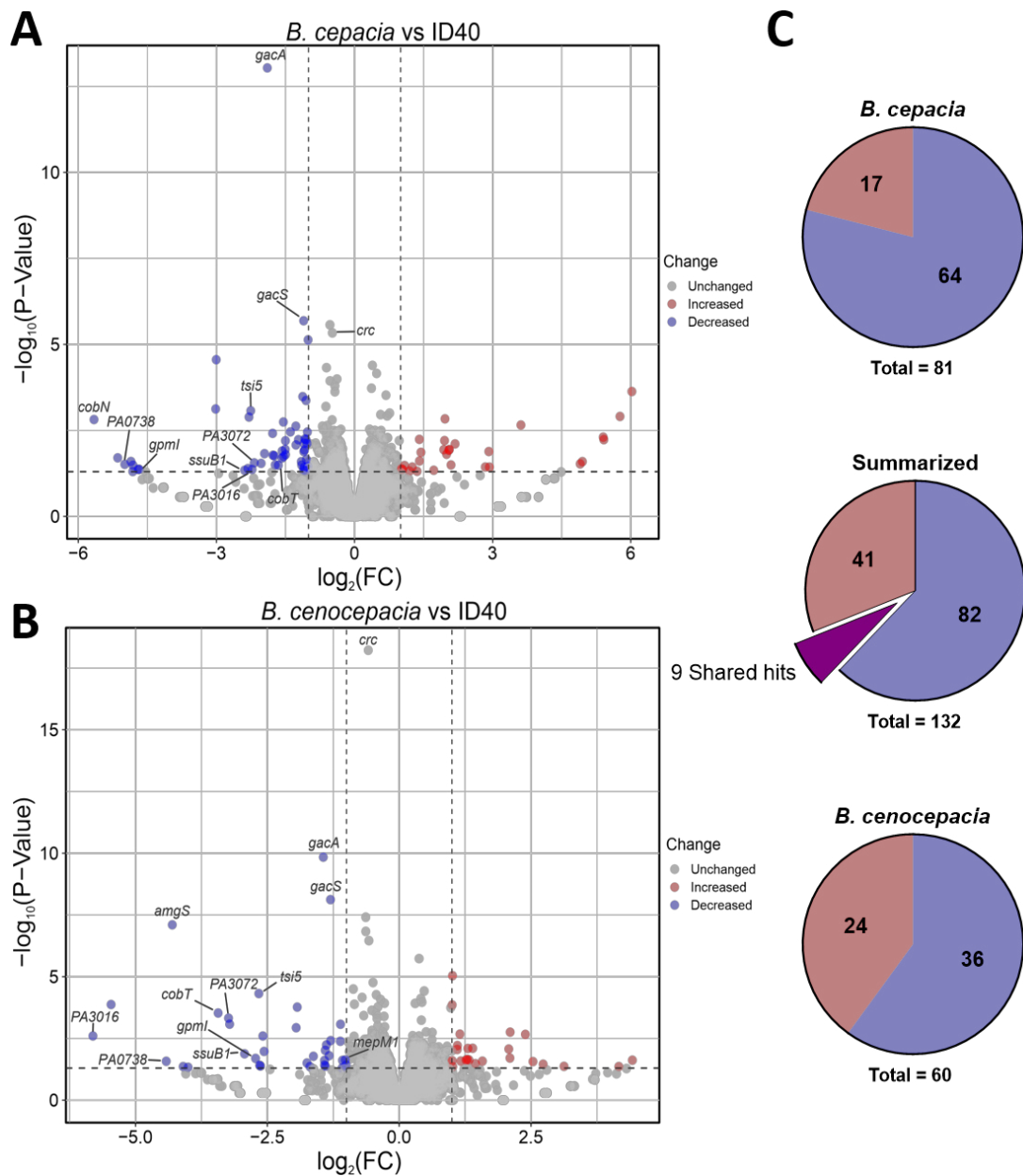


Figure 24: Visual representation of the relative change of transposon-containing sequences in an ID40 transposon library when incubated with *B. cepacia* (A) or *B. cenocepacia* (B), compared to a control condition without competitors. Each dot shown in the plots represents a gene in which a transposon insertion was identified. An ID40 Tn library was either incubated on LB-agar over night (control) or together with *B. cepacia* (A) or *B. cenocepacia* (B). After sequencing of the samples after co-incubation and mapping the Tn-containing reads to the ID40 genome, the relative abundance of each read in each condition was compared to the relative abundance of that read in the control without *B. cepacia* or *B. cenocepacia* strains present. The volcano plots show the $\log_2$ fold change ($\log_2[\text{FC}]$) of each mapped read compared to the control condition (X-axis) and the statistical significance (as $-\log_{10}(\text{P-Value})$) of that change for each read. Reads that are significantly less abundant ($\log_2[\text{FC}] < -1$) in the test condition compared to the control conditions are shown in blue, while reads that are significantly more abundant ($\log_2[\text{FC}] > 1$) are shown in red. (C) depicts the number of increased (red) and decreased reads (blue) in both single conditions, as well as a summary of unique increased or decreased reads and reads that were significantly altered in both conditions (purple). (A) summarizes two biological replicates, while (B) shows three biological replicates. An FDR (P-Value) = 0.05 was chosen as cutoff for significant changes. Visualized using VolcanoR (Goedhart & Luijsterburg, 2020).

The single dots shown in **Figure 24** represent single genes in which transposon insertions were found when sequencing fragmented genomic DNA of the shown samples. The relative abundance of each gene was calculated in the condition in which the ID40 transposon (Tn) library was incubated with either *B. cepacia* or *B. cenocepacia* and compared to the control condition in which the ID40 Tn library was incubated alone. Since during the sequencing data evaluation process, all reads that did not contain a Tn sequence were filtered out, essential genes of ID40 will likely not be detected in either condition, because those mutants would not survive in any of the tested conditions. Genes that bear a fitness advantage for ID40 in co-incubation, but are not essential for ID40, would be detected more abundantly in the ID40 control than in the co-incubation condition, since a transposon insertion most likely renders those genes inactive, giving ID40 with Tn insertions in those genes a growth disadvantage over the Bcc strains. Those genes are depicted as blue dots in **Figure 24**. On the contrary, transposon insertions in genes that gave ID40 a growth disadvantage against *B. cepacia* or *B. cenocepacia*, but are not essential otherwise, would lead to a growth advantage for those Tn mutants in co-incubation with the competitors. Those genes are depicted as red dots in **Figure 24**. The data gating strategy used in this assay was based on a sensitive first gating step that allowed more false-positive hits to pass the chosen filter step, and further filtering those broad hits by comparing the genes that provided *P. aeruginosa* ID40 a growth advantage in competition with to closely related *Burkholderia* species. In a first filtering step, we therefore chose a relatively low fold-change of 2 ($\log_2(\text{FC}) \geq 1$) and a false discovery rate (P-value) ≤ 0.05 as a gate for reads that were relevant to the survival of ID40 in competition with the respective Bcc species, as shown in **Figure 24**. Then, genes that were relevant in both of the tested conditions for ID40 to out-compete Bcc were further evaluated.

In co-incubation with *B. cepacia*, a total of 64 genes were identified in which less Tn-insertions were found than in the controls, and 17 genes in which more Tn insertions were found. After co-incubation with *B. cenocepacia*, 36 genes were identified in which less Tn insertions occurred compared to the control conditions, and 24 genes in which more Tn insertions occurred. Remarkably, the *crc* gene showed a highly significant (P-value $< 10^{-15}$), but not very big change ($\text{FC} < 2$) between the condition in co-incubation of the ID40 Tn library with *B. cenocepacia* compared to the control conditions (**Figure 24 B**). Investigation of the sequencing data of the annotated Tn-containing genes revealed that *crc* was by far the most detected read in all tested conditions and controls, with read counts between 21,000 and 45,000 in every sequenced sample. The second most abundant read in most samples was TUEID40_ *hxaA_1*, but the read count for that gene ranges between 2,700 and 5,800. It is annotated as a large, 12 kbp genomic region, which is more likely to have a

random transposon insertion occur than a smaller gene, contributing to the higher read count compared to other genes. In comparison, *crc* is only annotated as a 780 bp long genomic sequence, so the high read counts can not be attributed to a high chance of a transposon insertion occurring. To date, we were unable to locate the source of the extraordinarily high read counts of *crc* in all samples, but suspect a growth advantage of *crc* mutants under the tested conditions, as outlined in Appendix, **Figure 28** (p. 177) and further discussed in section 7.2 (pp. 136 - 138).

In an attempt to identify conserved genes that bear a general fitness advantage towards either *B. cepacia* or *B. cenocepacia*, nine genes that were significantly more or less abundant in both co-incubation conditions compared to the control conditions were identified (**Figure 24 C**). Out of those genes, none were found with increased abundance in both conditions, and one essential gene of *P. aeruginosa* according to Poulsen et al. (2019), *gpml*, was also identified. This might be an artifact where the transposon insertion did not result in a complete loss of function of the translated peptide inactive, but only impaired the function of Gpml. **Table 35** shows a list of nine genes that were identified in this way which were candidate genes for providing ID40 with a fitness advantage in competition with *B. cepacia* complex species. The gene names shown in the table represent the most likely homologs in PAO1 based on similarity in their amino acid sequence. Two genes that were identified only in competition with *B. cenocepacia*, but for which in-frame genomic knock-out mutants were readily available in our strain collection, were also included in further analyses.

Table 35: List of genes identified in the TraDIS screening assay that may contribute to the fitness of ID40 in co-incubation with *B. cepacia* complex (Bcc) species. The listed genes showed a significantly lower rate of transposon insertions when ID40 was co-incubated with both Bcc species compared to the control condition. Two additional genes, *mepM1* and *amgS*, that were found in only one of the two tested conditions, but for which in-frame deletion mutants were readily available in our lab from previous studies, were also included in further investigation. One gene, *gpmI*, was identified in the screening, but was previously described to be essential for *P. aeruginosa* strains (highlighted in red). The values shown are the log₂-fold change of the relative frequency of Tn-insertions and the respective P-value compared to the ID40 wt control condition.

Condition	Locus tag	PA01 ortholog	Function	log ₂ FC	P-value
<i>B. cepacia</i>	TUEID40_01324	<i>cobT</i>	Nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase	-1,65	0,0326
	TUEID40_06236	<i>gacA</i>	Response regulator GacA	-1,89	< 0,0001
	TUEID40_01819	<i>gacS</i>	Signal transduction histidine-protein kinase GacS	-1,11	< 0,0001
	TUEID40_03140	<i>gpmI</i>	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	-4,70	0,0429
	TUEID40_02027	PA0738	Hypothetical protein	-4,99	0,0306
	TUEID40_05679	PA3016	Hypothetical protein; associated with aminoglycoside resistance	-2,32	0,0406
	TUEID40_05623	PA3072	Hypothetical protein	-2,18	0,0277
	TUEID40_05234	<i>ssuB1</i>	Aliphatic sulfonates import ATP-binding protein SsuB	-2,39	0,0456
	TUEID40_06059	<i>tsi5</i>	Hypothetical protein	-2,26	0,0008
<i>B. cenocepacia</i>	TUEID40_01324	<i>cobT</i>	Nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase	-3,43	0,0003
	TUEID40_06236	<i>gacA</i>	Response regulator UvrY	-1,44	< 0,0001
	TUEID40_01819	<i>gacS</i>	Signal transduction histidine-protein kinase BarA	-1,30	< 0,0001
	TUEID40_03140	<i>gpmI</i>	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	-2,72	0,0204
	TUEID40_02027	PA0738	Hypothetical protein	-4,42	0,0266
	TUEID40_05679	PA3016	Hypothetical protein	-5,81	0,0025
	TUEID40_05623	PA3072	Hypothetical protein	-3,24	0,0005
	TUEID40_05234	<i>ssuB1</i>	Aliphatic sulfonates import ATP-binding protein SsuB	-2,93	0,0132
	TUEID40_06059	<i>tsi5</i>	Hypothetical protein	-2,66	< 0,0001
<i>B. cenocepacia</i> only	TUEID40_03219	<i>amgS</i>	Histidine kinase of AmgSR two-component system	-4,30	< 0,0001
	TUEID40_04290	<i>mepM1</i>	Murein endopeptidase MepM1	-1,02	0,0235

6 Results II: Investigation of competitive fitness of PA ID40

The list of genes shown in **Table 35** fulfill a diverse set of functions in *P. aeruginosa*. GacS and GacA constitute a two-component system that regulates the T6SS and its effectors. One of the H1-T6SS effectors is Tse5 with its cognate immunity protein Tsi5, which was also identified as contributing to the competitiveness of ID40 in previous assays described in section 6.4.2 (p. 113). *gpmI* was marked in red because it is an essential gene in *P. aeruginosa* (Poulsen et al., 2019).

The *cobT* gene (TUEID40_01324) shown in **Table 35** was annotated as *cobT_1* in the automatically annotated genome of ID40. BLAST alignment of the gene sequence of the *cobT_1* against the *cobU* gene of PAO1 and the *cobT* gene of PA14 revealed that (i) *cobU* of PAO1 and *cobT* of PA14 are homologous to each other and to the *cobT* genes, but not the *cobU* genes of other *P. aeruginosa* strains and that (ii) a supposed loss of three non-consecutive base pairs in the homologous region in the ID40 genome led to the fragmentation of *cobT* into the supposed *cobT_1-3* genes. Further analysis revealed that the predicted active center of CobT in PA14 and PAO1 lies within the region of the automatically annotated *cobT_1* gene. Since out of all automatically annotated *cobT_1-3* genes, only *cobT_1* showed a significant change in abundance of Tn-insertions in competition with the competitor strains, this also suggests that in this region, a transposon insertion had the biggest impact on the competitiveness of ID40. We assumed that the supposed base pair deletions stem from inaccuracies in the initial sequencing of the ID40 genome, and *cobT_1* will be referred to as *cobT* in the following. For the further validation process, the entire *cobT_1-3* region was deleted (ID40 Δ *cobT*). CobT plays a role in the synthesis of vitamin B12 in *P. aeruginosa* and encodes the nicotinate mononucleotide:5,6-dimethyl benzimidazole phosphoribosyltransferase (Cameron et al., 1991; B12 biosynthesis reviewed by Warren et al., 2002).

The *ssub1* gene encodes SsuB, which is part of the ATP binding cassette (ABC)-type transporter SsuABC. In *E. coli*, this transporter is part of the *ssuEADCB* system that is required for the utilization of aliphatic sulfonates as sulfur source. SsuB constitutes the ATP-binding domain of the ABC-transporter (van Der Ploeg et al., 1999).

Out of the three uncharacterized genes PA0738, PA3016 and PA3072, only PA3016 has a suggested known function. It has previously been associated with resistance towards aminoglycosides like tobramycin (S. Lee et al., 2009; Sanz-Garcia et al., 2019), but mutants have also been shown to display a hyperresistant phenotype towards β -lactam antibiotics (Dotsch et al., 2009).

The two-component system (TCS) AmgRS is also correlated with aminoglycoside resistance in *P. aeruginosa* (S. Lee et al., 2009). S. Lee et al. (2009) found that a deletion of this TCS

results in reduced aminoglycoside resistance as well as reduced virulence, possibly due to a decreased resistance against host stresses. This resistance is mediated by induction of AmgRS by mistranslated proteins in the outer membrane, leading to the direct induction of the expression of proteases HtpX and PA5528 and the resulting, indirect heightened expression of MexXY-OprM efflux pumps (Lau et al., 2015). AmgRS was also found to play a role in zinc-resistance of *P. aeruginosa* (Poole et al., 2019).

MepM1 is a murein endopeptidase (EP) that is thought to be involved in peptidoglycan cross-linking. MepM2 is also an EP but, unlike MepM1, is not regulated by the protease CtpA (Srivastava et al., 2018). Both of these genes were found to play a role in β -lactam resistance of *P. aeruginosa* (Sonnabend et al., 2020). PA4404 was also found to belong to the same family of peptidases as MepM1 and MepM2 and to be regulated by CtpA (Srivastava et al., 2018), and will be referred to as MepM3 as suggested by Klein et al. (2019).

6.4.6 Validation of the hits found in the TraDIS screening assay

Competition assays were performed to validate those genes identified in the TraDIS screening assay that may contribute to the fitness of *P. aeruginosa* ID40 in competition with members of the *B. cepacia* complex. In-frame single gene deletion mutants of these genes in ID40 were tested for their fitness in competition against *B. cepacia* and *B. cenocepacia*. To identify those mutants that have a general growth disadvantage or non-specific competitive disadvantage, competition assays of the same mutants against the ID40 wt strain were performed. Those mutants that had a general growth defect should also be out-competed or outgrown by their own wildtype strain. The log-transformed competitive index (log[CI]) of the fluorescent “attacker” mutants was calculated for each non-fluorescent competitor and then normalized to the log(CI) of the fluorescent ID40 wt. In competition of the fluorescent ID40 wt with the non-fluorescent ID40 wt, potential growth defects of the fluorescently labelled strain stemming from a higher metabolic burden or YFP expression should become apparent. Normalization of the log(CI) values to the log(CI) of the fluorescent ID40 wt served as a blanking step for all non-specific growth effects, and gave information about the relative competitive fitness of each mutant compared to the ID40 wt ($\Delta\log[CI]$). An ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ triple deletion mutant was also included in the assay, although only *mepM1* was identified as a candidate gene in the TraDIS screening assay. Since MepM1, MepM2 and MepM3 are thought to act in tandem as murein endopeptidases, a more pronounced phenotype was expected in the triple deletion mutant than in the *mepM1* single knock-out mutant. The ID40 $\Delta tsi5$ mutant was not included in the study, since Tsi5 constitutes the cognate immunity gene of Tse5, and deletion of the *tsi5* gene would result in the mutants being killed by sibling cells. A $\Delta tse5/tsi5$ double mutant,

however, would most likely have the same effect in competition with *B. cepacia* complex species as observed in section 6.4.2 (p. 113), but be killed by ID40 wt cells. The results of the competition assays are shown in **Figure 25**.

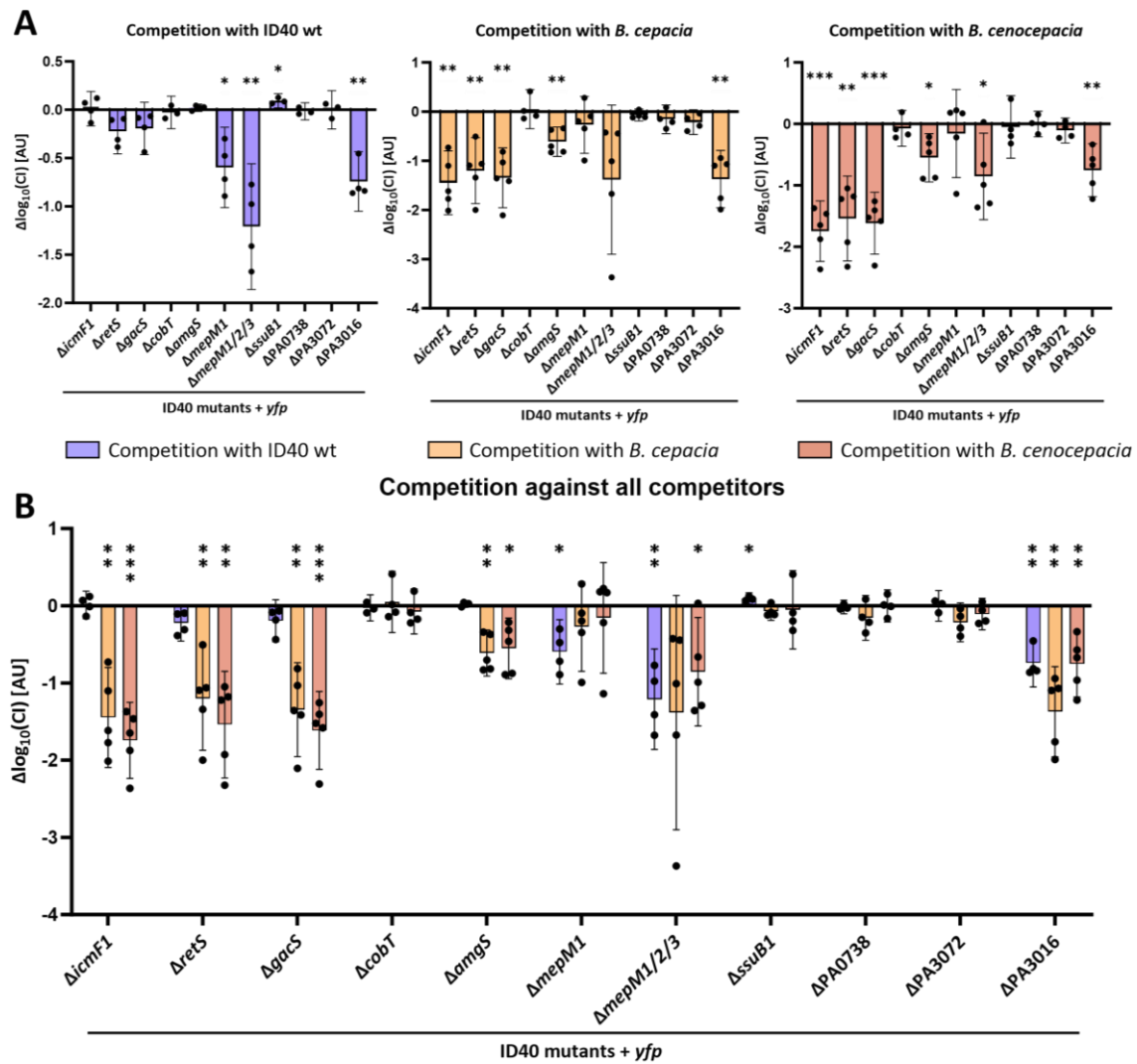


Figure 25: Validation of candidate genes contributing to the competitive fitness of *P. aeruginosa* in competition with *B. cepacia* complex species. The genes identified in the previously described TraDIS assay were deleted in ID40 wt, and those gene deletion mutants were labelled through a chromosomally integrated, constitutively *syfp2* gene, and used in interspecies competition assays with *B. cepacia* or *B. cenocepacia*. Competition assays with the ID40 wt were conducted in parallel to test whether the observed effects stem from general growth defects or generalized competitive disadvantages. An ID40 ΔretS *yfp* strain was tested since it was thought to out-compete the competitor strains due to T6SS-hyperactivity. Bars and whiskers depict the mean and 95%-confidence interval of the mean of *n* biological replicates of the difference between the log-transformed competitive index ($\log(CI)$) of the mutants to the $\log(CI)$ of the ID40 wt *yfp* strain ($\Delta\log(CI)$). Black dots represent the individual biological replicates. *n* = 4 for competition against ID40; *n* = 5 in assays with competition against *B. cepacia* and *B. cenocepacia*. **A** and **B** depict the same data set, where **B** is a summary of all singular tested conditions shown in **A**, allowing direct comparison of the competitive fitness of the tested strains in all three conditions. One-sample t-tests were performed to determine if the samples are different from 0, indicating that the respective gene deletion impacts the competitive fitness of *P. aeruginosa* ID40: * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001; **** *P* < 0.0001. *P* values $\geq$ 0.05 are not shown.

Figure 25 shows the results of competition assays performed with deletion mutants in genes identified in the TraDIS screening assay to validate if those genes are important in the competition between ID40 and *B. cepacia* complex (Bcc) species. The data shown in the figure are the competitive indices of each mutant in competition with their respective

competitor relative to the competitive index of the ID40 wt ($\Delta\log[\text{CI}]$), which is not shown in the figure. A $\Delta\log(\text{CI})$ value above 0 means that the mutant had a fitness advantage compared to the ID40 wt in the tested condition, while a $\log(\text{CI})$ below 0 means that the mutant has a fitness disadvantage compared to the ID40 wt. An ID40 ΔicmF1 mutant was used as a positive control, since we had seen in previous assays that the deletion of *icmF1* led to a severe fitness disadvantage of ID40 compared to the wildtype strain in competition with Bcc species, as shown in **Figure 17** (p. 103). Additionally, a *retS* deletion mutant of ID40 was included as a positive control for genes that may provide ID40 a competitive advantage upon deletion, since RetS acts as a negative regulator of H1-T6SS activity. As shown in **Figure 25**, the opposite effect was observed, and the deletion of *retS* led to a severe growth defect, comparable to that of *icmF1* deletion of the mutant in competition with both Bcc species, but only a minute disadvantage in competition with the ID40 wt. Interestingly, this effect closely matched the effect of the deletion of *gacS*, even though GacS is antagonized by RetS. The deletion of all three genes - *icmF1*, *retS*, and *gacS* - led to a strong loss of competitive fitness of the mutants in co-incubation with Bcc species, but not in co-incubation with ID40 wt.

Four of the tested gene deletion mutants showed no impact on the competitive fitness against either of the three tested competitor strains, those genes being *cobT*, *ssuB1*, PA0738 and PA3072. Furthermore, three of the tested gene deletion strains - ID40 ΔmepM1 , $\Delta\text{mepM1}/2/3$, and ΔPA3016 - did show a competitive disadvantage in competition with Bcc species, but also in competition with the ID40 wt, implying that the gene deletions conveyed a general growth or competition disadvantage for the mutants that was not specific to the competition with Bcc species. The simultaneous deletion of *mepM1*, *mepM2* and *mepM3* resulted in a stronger negative impact on the fitness of ID40 compared to the *mepM1* single gene deletion, implying that the three genes work in unison and cumulatively constitute an important factor for the fitness of ID40.

The only tested gene deletion mutant besides ID40 ΔgacS that showed a relative fitness disadvantage only in competition with the two Bcc species, but not in competition with the ID40 wt, was the ID40 ΔamgS mutant. While the fitness disadvantage in competition with the Bcc strains was not as pronounced as the one resulting from *icmF1* or *gacS* deletion, it was highly reproducible and comparable in both tested Bcc strains.

6.4.7 Follow-up investigation of the ID40 ΔretS mutant used in the competition assays

Because the phenotype of the ID40 ΔretS mutant used in the assays of section 6.4.6 was so drastically different from what had previously been described in other publications, and so

intriguingly similar to the phenotype of the ID40 Δ *gacS* mutant, the *gacS* and *gacA* genomic regions of ID40 Δ *retS* mutant strains were sequenced. Those genomic regions were amplified from the genome using KAPA DNA polymerase using primers 2809 and 2810 for amplification of *gacA* and Phusion DNA polymerase with primers 2208 and 2209 for the much longer *gacS* amplified (**Table 12:** Oligonucleotides used in this study, p. 39). While the *gacA* sequence was identical to that of the ID40 wt, the *gacS* sequence of the ID40 Δ *retS* mutants also contains a 179 bp long deletion from bp 1996 to bp 2174, resulting in a frame-shift and the fusion of the reading frame of *gacS* with that of the neighboring *ldhA* gene. According to the InterPro database, this region contains the REC domain of GacS in PAO1, which is needed for the interaction between GacS and GacA (Workentine et al., 2009). The phenotype of the *retS* mutant observed in competition with species of the *Burkholderia cepacia* complex (Bcc) is therefore likely related to the additional mutation of ID40 that was most likely acquired after or during the mutagenesis process. The results from section 5.3 (p. 96), showing the investigation of the secretion of AmpDh3 via the H2-T6SS, were also re-evaluated in this light.

Interestingly, the only strains with genomic knock-out mutations of *retS* that we were able to construct in this study were ID40 Δ *retS* and PA14 Δ *retS*. The ID40 Δ *retS* mutant turned out to also contain a probably non-functional *gacS* gene, while PA14 is known to contain an inactivating mutation in *ladS*, which is a positive regulator of GacS, and an antagonist of RetS. One might speculate that the deletion of *retS* has a deleterious effect on the ID40 mutant in question, which may be reversed by an additional deletion in *gacS*, thereby rendering the entire *gac/rsm* pathway inactive. Since the deletion of *retS* leads to a strong de-repression of the regulatory pathway, this mutation would lead to heightened T6SS activity, stimulation of quorum sensing mechanisms via over-production of 4-HSL, and biofilm formation, while T3SS activity and motility genes would be down-regulated.

6.5 Investigation of the biological function of *amgS* on the competitiveness of *P. aeruginosa*

To investigate whether AmgS is involved in the regulation of the H1-T6SS and thereby impacts the competitiveness of ID40 in co-incubation with *B. cepacia* complex strains described in section 6.4.6 (p. 125), competition assays between ID40 strains expressing YFP and a non-fluorescent ID40 Δ *tse5tsi5* mutant were performed. The ID40 Δ *tse5tsi5* mutant lacks the Tse5/Tsi5 effector-immunity pair, and is therefore susceptible to attacks from ID40 wt cells expressing a functional H1-T6SS and the Tse5 toxin. However, if the competitor either does not express Tse5 or has an inactive T6SS, e.g. through deletion of *icmF1*, the *tse5tsi5* deletion mutant is not lysed by the attacker strain. Therefore, a genomic

mutation that impacts the activity of the T6SS was expected to show a phenotype similar to the ID40 Δ *icmF1* mutant in co-incubation with the ID40 Δ *tse5tsi5* mutant. The Tse5/Tsi5 toxin-immunity gene pair was chosen because *tsi5* was identified as the only toxin or immunity gene in the TraDIS screening assay for genes contributing to the competitive fitness of ID40, and was therefore expected to show the strongest phenotype of the H1-T6SS immunity genes. **Figure 26** depicts a schematic overview explaining the outcomes of the competition assay between ID40 mutant strains and the ID40 Δ *tse5tsi5* mutant.

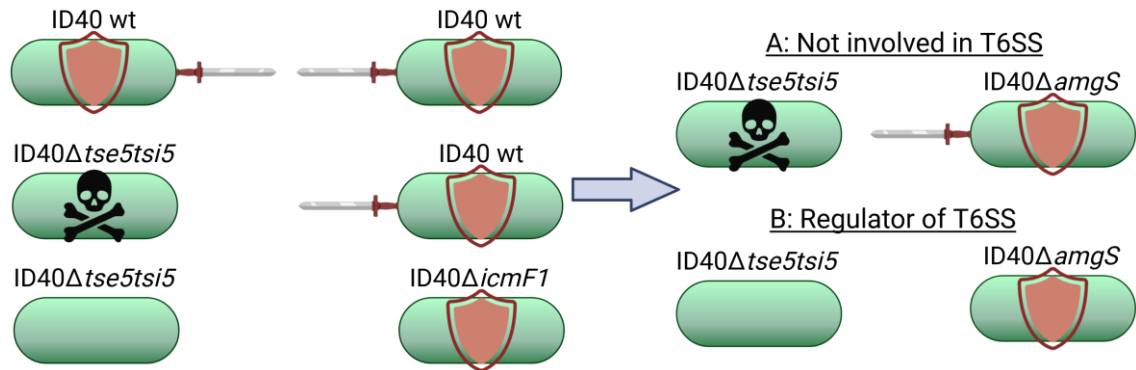


Figure 26: Conceptual visualization of the setup for investigating if AmgS is involved in the regulation of the H1-T6SS. Sword symbols represent the active H1-T6SS loaded with Tse5, while shield symbols represent the immunity protein Tsi5. Skull and bones represent the respective strain being lysed by the attacker strain. A Δ *tse5tsi5* gene deletion mutant of *P. aeruginosa* ID40 is susceptible to attacks from ID40 strains with an active H1-T6SS (ID40 wt), but not to a strain with an inactive H1-T6SS (ID40 Δ *icmF1*). In competition with ID40 Δ *amgS*, there are two possible outcomes: If AmgS is not a regulator of the H1-T6SS, the mutant should show the same phenotype as the wildtype in competition with ID40 Δ *tse5tsi5* (A). If AmgS contributes to the activity of the H1-T6SS, the mutant should show a similar phenotype to the ID40 Δ *icmF1* mutant in competition with ID40 Δ *tse5tsi5* (B). Created with Biorender.com.

The outcome of the competition assays between ID40 Δ *tse5tsi5* with ID40 wt, ID40 Δ *icmF1* and ID40 Δ *amgS* is shown in **Figure 27**.

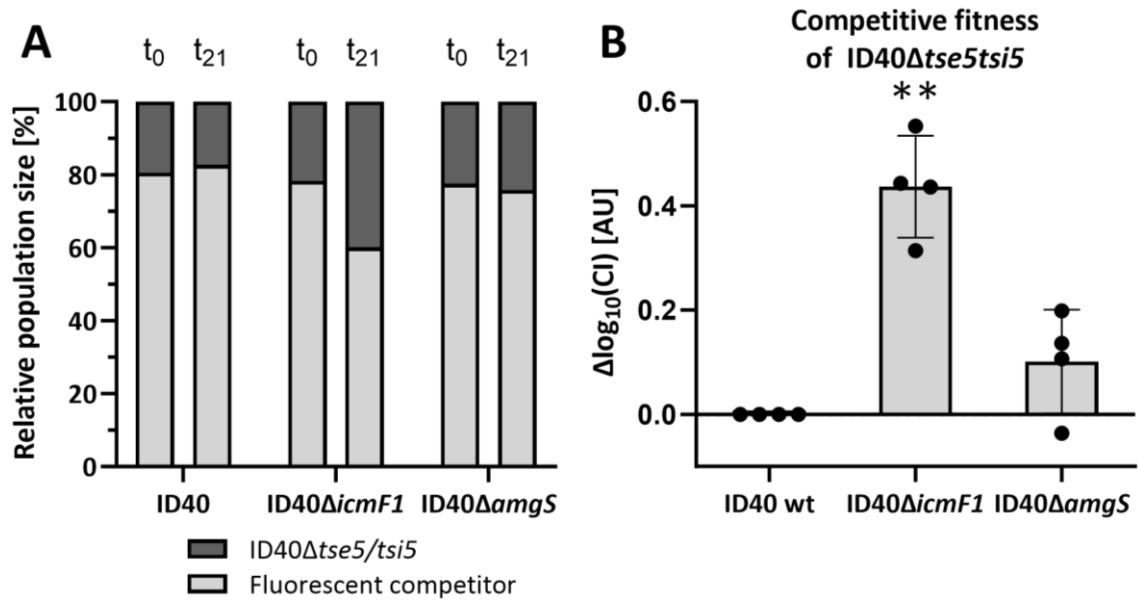


Figure 27: Competition assays between ID40Δtse5tsi5 and ID40 gene deletion mutants. (A) depicts the outcome of one out of four competition assays that were performed as the relative abundance in [%] of fluorescent competitors and non-fluorescent ID40Δtse5tsi5 cells before (t₀) and after co-incubation for 21 h (t₂₁) on LB-agar plates. The names of the respective competitors are shown on the X-Axis. (A) is representative of four biological replicates. (B) The competitive index (CI) was calculated for ID40Δtse5tsi5 in competition with each tested mutant. The log(CI) of ID40Δtse5tsi5 mutant in competition with ID40 wt_yfp was then subtracted from the log(CI) in competition with each mutant in each biological replicate to assess whether the mutant shows a fitness advantage or disadvantage against the three competitors (Δlog[CI]). Bars and whiskers depict the mean and 95%-confidence interval of the Δlog(CI) of n = 4 biological replicates. One-sample t-tests were performed to determine if the samples are different from 0, indicating that the respective gene deletion impacts the competitive fitness of *P. aeruginosa* ID40: * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001. P values ≥ 0.05 are not shown.

Figure 27 shows the outcome of competition assays that were performed between ID40Δtse5tsi5 and fluorescent competitor strains derived from ID40. **Figure 27 A** shows representative results for the changes in the relative abundance of each strain before competition (t₀) and after co-incubation (t₂₁). On first glance, it became clear that (i) the initial inoculum did not contain a 1:1 ratio of the competitors and (ii) that the deletion of the Tse5/Tsi5 effector-immunity pair alone did not affect the ability of the strain to grow in the presence of T6SS⁺ sibling cells (ID40 wt). However, in co-incubation with T6SS deficient cells (ID40ΔicmF1), the ID40Δtse5tsi5 mutant was able to slightly outgrow the competitor, implying that the ID40ΔicmF1 cells had a growth disadvantage against that strain. Since the ID40ΔicmF1 mutant should withstand all attacks from T6SS⁺ sibling cells – in this case ID40Δtse5tsi5 –, because of the presence of immunity genes, this growth disadvantage was not due to a competitive disadvantage, and more likely implied a general growth disadvantage. Although this effect did not appear particularly strong at first, with the ratio of ID40Δtse5tsi5 to ID40 wt not changing during co-incubation and the relative amount of ID40ΔicmF1 falling from approx. 80% to approx. 60% during co-incubation with ID40Δtse5tsi5, it was highly reproducible. This is reflected in **Figure 27 B**, which shows the

difference between the competitive fitness ($\log[CI]$) of the ID40 $\Delta tse5tsi5$ strain in co-incubation with each competitor and its fitness in competition with the ID40 wt ($\Delta\log[CI]$). Data from four independent biological replicates of the competition assay are depicted. The assay confirmed that the competitive fitness of ID40 $\Delta tse5tsi5$ was significantly higher in competition with ID40 $\Delta icmF1$ than in competition with the ID40 wt. In competition with ID40 $\Delta amgS$, the data were inconclusive, with ID40 $\Delta tse5tsi5$ showing higher competitiveness than in competition with the wt in three of the replicates, and lowered competitiveness in one of the replicates. Notably, the overall effect size is very small in this assay so that it remains unclear if ID40 $\Delta amgS$ affects the function of the T6SS of ID40. However, the effect size may be increased through additional deletion of further effector-immunity gene pairs, for example of the Tse8/Tsi8 pair. As shown in **Figure 22** (p. 114), the deletion of *tse8* impaired the competitive fitness of ID40 at least in competition with Bcc strains, making a ID40 $\Delta tse5tsi5\Delta tse8tsi8$ deletion mutant a potential candidate to investigate the effect of gene deletions such as *amgS* on the activity of the H1-T6SS of *P. aeruginosa*.

7 Discussion

7.1 AmpDh3 is a cytosolic protein that is secreted into the supernatant of *P. aeruginosa*, but not via the H2-T6SS

Prior to the beginning of this project, AmpDh3 was assumed to be a periplasmic protein which is secreted by *P. aeruginosa* via the H2-T6SS (Wang et al., 2020; W. Zhang et al., 2013). The suggested localization of AmpDh3 in the periplasm was at first purely derived from the fact that AmpDh3 has a higher affinity to synthetic substrates mimicking those cell wall building blocks that are more abundantly found in the periplasm in a fascinating study by W. Zhang et al. (2013). Wang et al. (2020) therefore assumed that AmpDh3 was localized in the periplasm of *P. aeruginosa*, but did not present experimental data for this. However, they presented a strikingly simple method for sub-cellular fractionation of *P. aeruginosa* cells in their methods section, which allows distinguishing the localization of periplasmic proteins and those that are either attached to cytoplasmic membranes or localized in the cytosol. Since to the best of our knowledge, all other known T6SS-dependent toxic effectors are loaded to the T6SS from the cytosol, we decided to investigate the localization of AmpDh3 experimentally in the strains PA14 and ID40, which we used in this study, using that same method. As shown in section 5.1 (pp. 89 - 91), we concluded that AmpDh3 is in fact localized in the cytosol and not in the periplasm of *P. aeruginosa*, and also found no signal sequence in the primary structure of AmpDh3 that would be required for the transport into the periplasm *in silico*. These findings were also published in a study of our group (Eggers et al., 2023). At that time, another study had been published which came to the same conclusion, namely that AmpDh3 is located in the cytosol (Colautti et al., 2023).

The secretion of AmpDh3 via the H2-T6SS in the study by Wang et al. (2020) was shown with Western blots of culture supernatants and cell pellets of *P. aeruginosa* strain PAO1 with a genomic deletion of *retS*. We first aimed at replicating these findings to further investigate the secretion and translocation of AmpDh3 into the culture supernatant or into competitor species using two strains expressing HiBiT-tagged AmpDh3. Despite our best efforts, and using the highly sensitive NanoLuc system to detect AmpDh3 in the supernatant of our strains, we were unable to replicate these findings. However, we did make some changes to the assays as they were performed by Wang et al. (2020).

Firstly, we did not use strain PAO1 as reference strain, but another commonly used reference strain, PA14. PA14 is a highly pathogenic strain which, as we knew from previous experiments, expresses relatively high levels of AmpDh3 compared to strain ID40. ID40 is a highly antibiotic-resistant strain that is known to over-express the β -lactamase AmpC (Eggers et al., 2023; Sonnabend et al., 2020). Instead of using a *retS* deficient background,

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we aimed to investigate the secretion of AmpDh3 in wildtype strains, which only harbor the *hiBiT*-tag downstream and in frame of the *ampDh3* gene. For inactivation of the H2-T6SS, Wang et al. (2020) deleted *clpV2*, which is responsible for disassembly of the contracted T6SS after firing (Basler et al., 2012). However, we reasoned that without *clpV2*, the assembly and firing of the T6SS could still occur, and instead decided to delete *icmF2*, which should prevent the assembly of the H2-T6SS, leading to a tighter shut-down of the T6SS (Durand et al., 2015; Mougous et al., 2006). To stimulate the expression of AmpDh3 in the cytosol of our strains, especially in ID40, we also used *ygfB* deletion mutants, which are known to over-express AmpDh3 compared to the wild-type strains (Eggers et al., 2023).

Neither by detection of AmpDh3-HiBiT in the culture supernatant directly by using the NanoGlo Live Cell Assay (Promega) nor by SDS-PAGE and Western blot analysis did we observe decreased secretion of AmpDh3 in *icmF2*-deficient strains (section 5.2, pp. 91 - 96). Only in the *ygfB* deficient ID40 strain, which over-expresses AmpDh3 compared to the wild-type, the secretion levels of AmpDh3 into the supernatant also increased, but additional deletion of *icmF2* had no impact on the secretion levels of AmpDh3. While we also aimed at creating a *retS* deficient ID40 strain to see if secretion of AmpDh3 can be stimulated as shown by Wang et al. (2020), we later recognized that the *retS* deletion strain had also acquired an inactivating mutation in its *gacS* gene. Since RetS acts by inhibiting GacS, this explains why we did not observe heightened secretion of AmpDh3 in the *retS* deletion mutant (see **Figure 15**, p. 97). However, the inactivation of GacS should lead to a T6SS-inactivating phenotype, so that the supposed *retS* deletion mutant would be expected to show lower secretion of AmpDh3, especially in an AmpDh3-overexpressing background. Since the ID40 Δ *retS* mutant showed no change in either the expression nor in the secretion of AmpDh3, we concluded that the secretion of AmpDh3 does not rely on the H2-T6SS, which is regulated via the *gac/rsm* regulon (Allsopp et al., 2017).

At the end of 2022, a pre-print version of the study done by Colautti et al. (2023) was published, presenting data that closely match my own observations and that lead to the conclusion that AmpDh3 and its suggested immunity gene, PA0808, most likely do not constitute a H2-T6SS-dependent effector-immunity pair. As with my own data presented in this thesis, Colautti and colleagues were unable to replicate the findings of Wang et al. and concluded that (i) AmpDh3 is in fact not secreted via the H2-T6SS and that (ii) AmpDh3 and PA0808 do not constitute an effector-immunity pair (Colautti et al., 2023). Intriguingly, the study performed by Colautti and colleagues showed no secretion of AmpDh3 into the culture supernatant of *P. aeruginosa* at all. Since both the VSV-G tag used in that study and the HiBiT tag, which I used to tag AmpDh3, consist of 11 amino acids, the different tags

should not negatively impact the secretion of AmpDh3 itself in either study. It is however possible that the NanoLuc system used in this study is more sensitive than immunostaining of proteins on the Western blot membrane, and can detect lower quantities of protein on the membrane, which would explain why we did detect some relatively low quantities of AmpDh3 in the supernatant of all tested strains. Previous studies have reported detection limits of HiBiT-tagged proteins of downwards of 10^{-18} moles (1 amol) (Schwinn et al., 2018).

Interestingly, **Figure 12** (p. 90) also shows that in ID40 culture supernatants, more RNA polymerase β (RpoB) was found than in culture supernatants of PA14 strains, although still in negligible amounts compared to the cell lysates. Since RpoB is a strictly cytosolic protein that is not actively secreted, the presence of RpoB in the supernatant likely stems from cell lysis during the three-hour incubation of our strains before harvesting the supernatants and cells. It is unlikely that the cells were lysing because of a lack of nutrients, especially because our growth curves indicate that ID40 is still growing exponentially after that amount of time (**Figure 18**, p. 105). Rather, some sort of self-lysis pathway that is more active in ID40 than in PA14 may be responsible for the higher abundance of RpoB in culture supernatants of ID40. Programmed cell death, for example via the *alpBCDE* lysis cassette, is known to enhance virulence of *P. aeruginosa* during infection, and induces the formation of outer membrane vesicles (OMVs) (McFarland et al., 2015; L. Turnbull et al., 2016). However, the secretion of AmpDh3 does not appear to rely solely on these self-lysis mechanisms of *P. aeruginosa*, because secretion was still detected in strain PA14 despite lower RpoB levels in the supernatant compared to strain ID40.

Previous studies have also described that AmpDh3 is expressed in PAO1 at higher levels after X-ray irradiation, and have detected the presence of AmpDh3 in outer membrane vesicles (OMVs) (Maredia et al., 2012; L. Zhang et al., 2020). A special kind of OMV formation in Gram-negative bacteria is the so-called explosive cell lysis, which leads to the encapsulation of DNA and cytosolic proteins into outer membrane vesicles that form from membrane fragments (Lynne Turnbull et al., 2016). Explosive cell lysis, which can be induced via ciprofloxacin or mitomycin C treatment of *P. aeruginosa* cells, was found to induce the formation of AmpDh3-containing OMVs, as well as a generally higher AmpDh3 expression. The bacteriolytic properties of those OMVs derived from explosive cell lysis towards Gram-positive bacteria is, however, not attributable to AmpDh3, and AmpDh3 is in fact thought to counter-act cell lysis and OMV formation (Chen et al., 2022). The presence of AmpDh3 in the culture supernatants of *P. aeruginosa* strains in our experiments may therefore be derived from OMVs that are released into the culture supernatant constantly, and also upon cell lysis of bacteria during growth. This relatively unspecific mode of

secretion of AmpDh3 via OMVs would also explain why more AmpDh3 is detected in the supernatant of AmpDh3-overexpressing strains.

Overall, the question remains why our observations differ so drastically from the study of Wang et al. (2020), but overall match the conclusions drawn by Colautti et al. (2023). While Wang et al. did not present immediate experimental evidence for the localization of AmpDh3 in the cell and used a foreign signal sequence to export AmpDh3 to the periplasm of *E. coli*, my own results and those presented in the study of Colautti et al., who also performed subcellular fractionation experiments, present strong evidence that AmpDh3 is localized in the cytosol instead of the periplasm. Furthermore, Colautti et al. also present evidence that the suggested cognate immunity protein to AmpDh3, PA0808, is also localized in the cytosol and does not interact with AmpDh3, using three orthogonal experimental approaches to investigate this interaction (Colautti et al., 2023). Concerning possible explanations for discrepancies between their own findings and those presented by Wang et al. (2020), Colautti et al. (2023) argue that (i) Wang et al. might have investigated a lab-adapted sub-strain of PA01 that presents a unique, but not conserved phenotype that does secrete AmpDh3 in a H2-T6SS-dependent manner, or (ii) expression of AmpDh3 and PA0808 from a non-native locus might have influenced their results, and that (iii) the observed interaction of AmpDh3 with PA0808 may be an artifact of unspecific interactions of the tagged proteins (Colautti et al., 2023).

7.2 Impact of Crc on the growth of *P. aeruginosa* ID40 in LB medium

When grown in liquid LB medium, the ID40 wt shows a biphasic growth, resembling diauxic growth (**Figure 18**, p. 105). This growth behavior usually occurs when a medium contains different carbon sources, of which some are preferred by the bacterium, and is defined by the occurrence of two exponential growth phases with a lag-phase in between (Narang & Pilyugin, 2007). *Pseudomonas* species are known to use a different catabolite-repression mechanism than *Enterobacteriaceae* or *Firmicutes*, which utilize mainly DNA-binding regulators for carbon catabolite repression (reviewed by Görke & Stülke, 2008). Instead, the post-transcriptional global regulator Crc in *Pseudomonas* species binds to the mRNAs in concert with another regulator, Hfq, blocking the translation of regulator proteins of e.g. catabolic benzoate and alkane degradation pathways (Moreno & Rojo, 2008; Moreno et al., 2007; Sonnleitner et al., 2018). Alignment of the sequences of a regulator of Crc activity, the regulatory RNA *crcZ* (Sonnleitner et al., 2009), revealed that in ID40, *crcZ* contains one nucleotide deletion within the last 23 bp of the small RNA (C385) and one point mutation (G145A). However, it is unclear if these mutations are responsible for the altered growth curves of ID40. Additionally, the upstream region of *crcZ* in strain ID40 contains a point

mutation inside the transcription regulator IHF's consensus sequence 48 bp – 60 bp upstream of the transcriptional start (C51A), but since that mutation lies within the variable region of the consensus sequence, it will most likely not impact the transcription of *CrcZ* (Mangan et al., 2006). In strain PAO1, the deletion of *crcZ* leads to the inability to use some carbon sources, like alanine (both stereoisomers), L-Leucine, L-Ornithine, D-Fructose, D-Gluconic acid, glycerol, D-mannitol, and less common molecules as carbon sources, while the deletion of *crc* did not impact any of these (Sonnleitner et al., 2018). *CrcZ* levels usually increase upon exhaustion of preferable carbon sources like succinate in the medium, and sequesters mRNA-binding post-translational regulators (Sonnleitner et al., 2009; Sonnleitner & Blasi, 2014). On the proteome level, *Crc* was found to negatively regulate proteins involved in uptake and catabolism of certain amino acids and peptides, and to positively regulate *hcp1* among other genes. Additionally, *crc* mutants over-produce pyocyanin, and show reduced expression of quorum-sensing regulated genes, impaired motility, and increased biofilm, exopolysaccharide and aggregate formation (Linares et al., 2010).

In the TraDIS screening assay described in section 6.4.6 (p. 125), *crc* was the gene sequence in all conditions in which a transposon insertion was most commonly detected out of all genes of *P. aeruginosa* ID40. This could indicate that a transposon insertion in this gene could provide a growth advantage compared to the ID40 wt, so that those cells in the transposon library that contain a mutation in this gene are selected during growth and are maybe already over-represented in the transposon library pre-culture. One might speculate that specifically strain ID40 has a so far undefined defect in the use of a certain carbon source that limits growth in LB medium. LB medium has previously been described to contain relatively low concentrations of sugars, which force the cells to adapt their metabolism as soon as those sugars are depleted, and use amino acids as carbon sources (Sezonov et al., 2007). In that study, *E. coli* strain K-12 showed a diauxic-like growth, switching from one exponential growth phase to a following one early on, at around OD = 0.3. Although those studies have been carried out using *E. coli*, ID40 showed a very similar phenotype in this study, switching from its first exponential growth phase to the second one starting at OD = 0.3. Strains PAO1 and PA14 did not show such a diauxic growth-like behavior under the same conditions, indicating that as a bloodstream isolate, ID40 may not be well adapted to growth under laboratory conditions, and may have some unique metabolic properties compared to the other two strains, which have been used as model organisms in research laboratories for many years. In fact, when comparing the growth of ID40 wt to the growth of an ID40 Δ *crc*, the *crc* deletion mutant behaves like the wild-type and grows at a very similar rate at first, but keeps growing for longer than the wt, reaching

a higher optical density before the OD starts to decline again (Appendix, **Figure 28**, p. 177). This might be the reason why mutants containing an inactivating Tn insertion in their *crc* gene within an ID40 transposon library are enriched compared to all other mutants, because the ID40 Tn library has been grown in liquid LB overnight multiple times.

7.3 Establishment of a FACS-based interbacterial competition assay for *P. aeruginosa* and members of the *B. cepacia* complex

The assay established in this study presents an easily modifiable, relatively high-throughput assay that allows for the investigation of the competitive fitness of certain bacterial strains in co-incubation with a competitor of choice. While competition assays using fluorescence-labelled bacteria have been described in previous studies (Habich et al., 2022; Stannek et al., 2014), the assay was adapted to our specific needs. To investigate contact-dependent competition between *P. aeruginosa* (PA) and a competitor species, the two species need to be in close contact during co-incubation. Therefore, co-incubation was performed on solid agar and the cells were resuspended from cut-out agar spots of the plates after extended amounts of time (21 h). This resuspension technique of transferring a cut-out agar snip into a tube filled with PBS and then resuspending the bacteria using a pipet tip was chosen because it allows complete removal of the bacterial pellet without also damaging the agar, both of which may be difficult to achieve when using inoculation loops as described by Hachani et al. (2013). Furthermore, it is easy to see if there is a thin layer of bacteria still adhering to the agar snip, because it becomes fully clear and see-through when all bacteria are resuspended from it.

To monitor whether all YFP-expressing PA strains maintained stable expression of YFP under the chosen assay conditions, reference samples of non-mixed strains were used. Using the pMRE-Tn7 family of plasmids encoding fluorescent proteins that can integrate into the genome of *P. aeruginosa* at the very specific *attTn7* site also circumvents problems arising from spontaneous loss of the YFP-encoding gene due to random dilution effects during growth. The constitutive expression of *syfp2* is not toxic to the bacteria itself, and also does not present a metabolic burden to the cells, as evidenced by the growth curves in section 6.2.2, **Figure 18** (p. 105).

We were therefore at first surprised to observe a severe loss of fluorescent populations in YFP-expressing PA strains that were incubated for more than 24 h, and even earlier when incubating those strains at 37°C instead of 30°C, as shown in **Figure 16**, p. 100. Interestingly, this loss of fluorescent populations also occurred when PA strains were grown in liquid medium, and faster if the medium was not shaken during incubation. This raises the question what causes the loss of fluorescent cells. The fact that the loss of

fluorescence in a portion of the population appears to be temperature- and time-dependent suggests that this effect is related to the growth of the PA strains. More specifically, the non-fluorescent cells may be metabolically inactive or dormant cells that are known to arise in PA upon biofilm formation, especially in deeper layers of biofilms that are less oxygenated than the upper layers (Williamson et al., 2012). This would also explain that more non-fluorescent cells are found when the strains were incubated in unshaken medium compared to medium that was shaken, since shaking the cultures should lead to higher oxygenation of the culture medium, and cells can more easily aggregate into biofilms when not shaken. Additionally, the YFP molecule requires oxygen to mature, which would demand major adjustments to this assay for investigation of competition between fluorescent and non-fluorescent strains in anoxic conditions, and would also explain why some cells may be unable to express active YFP in low-oxygen biofilms (Heim et al., 1994). In a thin, spread-out bacterial lawn on an LB-agar plate, enough oxygen should be present to penetrate the entire biofilm, as evidenced by the fact that even after incubation for 21 h on LB-agar, no major loss of a fluorescent sub-population of cells was detected in either of the tested strains at 30°C. Only after longer periods of time, as the bacteria start to deplete nutrients in the agar and adapt their metabolism (42 h), an increase of non-fluorescent cells was observed.

The biggest advantage of the fluorescence-based competition assay used in this study is that it is a semi-quantitative competition assay that may be easily applied to investigate the interaction of *P. aeruginosa* with a broad range of possible competitors in the future. In a more traditional approach, the mixed cultures of the bacteria would usually be harvested before and after co-incubation, and serial dilutions of the bacteria would be plated out on separate selective agar plates to count colony forming units (CFU) of either competitor the next day. While this is feasible to investigate the competitive fitness of relatively antibiotic-susceptible strains of bacteria, since a broad range of selective agar plates would be available for selection of either strain, it may be difficult to find adequate antibiotic combinations when investigating two highly resistant isolates. In our case, the *P. aeruginosa* strain ID40 used in this study is highly antibiotic-resistant, and pairing this strain with another famously antibiotic resistant genus of bacteria, *Burkholderia*, presented a challenge in finding antibiotics that would be selective for one strain, but not the other, and vice versa. Ideally, the growth of the positively selected strain should also not be impaired by the chosen antibiotic at the chosen concentration. When investigating the influence of the deletion of certain T6SS-related genes in *P. aeruginosa* in competition with more than one genus of bacteria, it would become nearly impossible to find a combination of antibiotics that would positively select ID40 on one hand, and the respective competitor on the other. For our TraDIS experiment, we found that tobramycin presented the only viable option for

selecting either *B. cepacia* or *B. cenocepacia*, but not ID40. On the other hand, when using *yfp*-expressing *P. aeruginosa* strains, that same set of strains can easily be challenged with a wide variety of non-fluorescent competitor strains, even highly resistant ones, without much effort and without the need to know the antibiotic resistance profile of each tested strain. To this end, the FACS-based competition assay should be preferred over more traditional approaches to competition assays which are based on counting the CFU of both competitors on separate selective agar plates.

7.4 *retS* deletion may negatively impact the fitness of ID40

While *retS* deletion mutants have been reported in several strains such as PA103, PA01, PAK and PA14, we were unable to obtain a *retS* single deletion mutant in strain ID40 (Goodman et al., 2004; Laskowski et al., 2004; Moscoso et al., 2011). While the genomic deletion of *retS* in strain ID40 was successful after many attempts, we later recognized that the tested colony in question contained an additional deletion mutation in *gacS* gene, rendering the deletion of *retS* ineffective. While it is tempting to attribute this specific mutation to bad luck during the mutagenesis process, it is also possible that an over-activation of the *gac/rsm* pathway by *retS* deletion is disadvantageous for the mutants.

The first step to obtain a *retS* knock-out mutant - the conjugative transfer and recombinational integration of the mutator plasmid into the genome of ID40 - was successful in most attempts. However, the resulting merodiploid mutants either acquired an inactivating mutation in the counter-selection cassette, yielding merodiploid colonies, or the second cross-over occurred in a region that led to a wild-type genotype of the mutants. It is surprising that the first successful *retS* deletion mutant of strain ID40 that we obtained after many attempts just so happened to harbor an additional *gacS*-inactivating mutation.

The *gac/rsm* signaling pathway is thought to act as a switch from chronic to acute infection, with chronic infections being associated with T3SS activity, flagella and type IV pili, while chronic infections are associated with increased biofilm production and T6SS activity (Goodman et al., 2004; Laskowski et al., 2004; Moscoso et al., 2011).

A study investigating the longitudinal adaptations of clonal *P. aeruginosa* lineages within people with cystic fibrosis (pwCF) observed that 11 isolates from a hospital in Copenhagen that were obtained from young patients seemed to have acquired *retS* mutations followed by *gacS*- or *gacA*-inactivating mutations over time (Marvig et al., 2015). The same may have occurred in our ID40 Δ *retS* deletion mutant, since the described deletion within the *gacS* gene was not present in the ID40 wt genome, but only in the *retS* deletion mutant. The fact that this mutation occurs naturally in vivo as well as, apparently, in vitro, may hint that *retS*

deletion alone negatively impacts the fitness of *P. aeruginosa* and may increase the selective pressure for reverting mutations through the inactivation of downstream regulatory genes like *gacA* and *gacS*. This underlines the importance of investigating the T6SS machinery of *P. aeruginosa* in strains that do not contain a mutation that would over-activate the *gac/rsm* signaling cascade. Instead, other methods of stimulating the expression of the T6SS of PA should be investigated. For example, LeRoux et al. (2015) demonstrated that treating the cells with cell lysates obtained from sibling cells stimulate the expression and activity of the H1-T6SS via a hypothetical diffusible stress signal, in a response that they termed the *P. aeruginosa* response to antagonism (PARA) (LeRoux et al., 2015). It is also known that envelope stress-inducing agents like polymyxin B, EDTA or even extracellular DNA can induce the T6SS via activation of the TagQRST-PpkA-PppA-Fha1 pathway (Ho et al., 2013; Wilton et al., 2016). In theory, these factors provide more physiological activation states of the T6SS than deletion of integral components of the *gac/rsm* regulation pathways, and might allow a more detailed investigation of the components and the regulation of the T6SS of *P. aeruginosa*.

7.5 The impact of *tse* effectors on the competitive fitness of *P. aeruginosa*

In our competition assays between *P. aeruginosa* (PA) and members of the *B. cepacia* complex (Bcc; *B. cepacia* or *B. cenocepacia*), we found that none of the single gene deletion mutants of *tse1-8* (*tse* effectors) alone impaired the competitive fitness of PA under the tested conditions to the same extent as the T6SS-inactivating *icmF1* mutation (**Figure 21**, p. 112). The strongest consistent effect that was also observable in competition with both *B. cepacia* and *B. cenocepacia* was observed upon deletion of *tse8*, which acts by inhibiting the bacterial transamidosome, leading to impaired protein synthesis (Nolan et al., 2021). Out of the *tse* effectors with known modes of action, *tse8* is the only one acting on the protein synthesis of target cells. Importantly, *tse8* would be expected to only act as a toxin in those recipient cells that use transamidosomes for the synthesis of glutamine or asparagine (Nolan et al., 2021).

Effectors of the H1-T6SS of *P. aeruginosa* were previously described to show different levels of activity in certain environmental conditions. For example, both Tse4 and Tse5, which both probably constitute ion pores leading to cell depolarization, were reported to be more active in high salinity environments, while Tse5 also increases its activity with increasing pH and with decreasing temperatures (LaCourse et al., 2018). It is therefore possible that the “environmental” conditions in our study do not allow all *tse* effectors to demonstrate maximum toxicity. The same study of LaCourse et al. (2018) found that Tse1, Tse4 and Tse6 tend to act synergistically with other *tse* effectors more often than other effectors. Strongly

synergistic effector pairs were Tse1 and Tse4, Tse3 and Tse4, Tse1 and Tse5 and Tse4 and Tse6. Antagonistic effects were observed when combining Tse2 and Tse4, Tse2 and Tse6, and Tse4 and Tse5. The study was conducted at a time when Tse7-8 were not known as T6SS effectors yet (LaCourse et al., 2018). Importantly, competition experiments in that study were performed between PA *retS* deletion mutants and PA strains that were made susceptible to certain *tse* effectors by deletion of the effector-immunity pairs (LaCourse et al., 2018). All of these synergistically acting Tse effectors were also found to be conserved “core effectors” among clinical isolates of *P. aeruginosa* in the study by Habich et al. (2022).

While we did not test these combinations specifically, we did observe that the effect of combined deletion of *tse* effectors is generally larger than the effect of the single *tse1-8* gene deletions. However, none of the triple deletion mutants of *tse* effectors in ID40 stood out to impair the fitness of ID40 more strongly than any other combination of effector deletion mutations. It is important to keep in mind that the ID40 triple and quadruple *tse* deletion mutants shown in **Figure 22** (p. 114) still encode five other *tse* effectors, which are likely sufficient to allow ID40 to out-compete Bcc competitors, albeit less effectively than the wild-type. Strikingly, in competition with *B. cenocepacia*, the additional deletion of *tse8* in the $\Delta tse1\Delta tse5$ double deletion background of ID40 led to increased competitive fitness of the strain in comparison to the double deletion mutant. Such an antagonistic effect is surprising in the sense that Tse1 (cell wall amidase), Tse5 (pore-forming toxin of the inner membrane) and Tse8 (inhibitor of the transamidosome) (i) all have different targets inside the cell and (ii) at least *tse8* deletion was shown to impair the competitive fitness of ID40. Upon additional deletion of *tse4* or *tse6*, which alone did not show negative impact on competition with either Bcc strain upon deletion, the mutants showed a decreased competitive fitness compared to the triple and double deletion mutant, which might indicate that the observed effect upon *tse1tse5tse8* triple deletion depends on either Tse4 or Tse6. More replicates may however be required to be able to exclude random scatter of the data as a source for the observed effect, since the overall effect size is still rather small in competition assays between ID40 and *B. cenocepacia*, compared to the scatter of the data.

Importantly, in competition with Bcc strains, the deletion of *retS* does not seem to be necessary to investigate the effects of single gene deletions on the competitive fitness of *P. aeruginosa* strains. As far as we are aware, these findings allow for the first time the assessment of H1-T6SS effectors on the fitness of a competitor strain without manipulating the regulation of the T6SS or the expression of its effectors itself.

At least strain *B. cenocepacia* J2315 was also reported to encode two homologs of *tse4*, BCAL1292 and BCAL2505, making it probable that the strain also encodes a cognate

immunity gene that might be sufficient to render the strain immune to the *tse4* toxin of attacking *P. aeruginosa* cells (Spiewak et al., 2019). The T6SS-1 system of *Burkholderia* species is known to play a role in bacterial competition (Angus et al., 2014; Schwarz et al., 2010). Furthermore, bacteria from densely populated habitats like the human gut are also known to acquire immunity towards competitor attacks, for example via acquisition of immunity proteins (Ross et al., 2019). This study by Ross et al (2019) found that several species of the genus *Bacillus* isolated from the human gut encode genes that share homology with other known T6S immunity genes but lack a cognate effector gene in their genome. These genes tend to cluster together on the genome and provide protection against T6SS attacks from strains encoding a cognate effector of the immunity gene (Ross et al., 2019). It is possible that besides *B. cenocepacia*, other members of the *B. cepacia* complex (Bcc) also encode either effector-immunity genes that resemble those of *P. aeruginosa*, or orphan immunity genes, and are therefore resistant to attacks from certain H1-T6SS effectors. This might explain why the single gene deletion of some of the tested *tse* effectors (Tse2, Tse4, Tse6, Tse7) did not impact the competitive fitness of PA ID40 in competition with the tested Bcc strains, but others (Tse1, Tse5, Tse8) did. Additionally, physical barriers like exopolysaccharides and capsule production can also potentially protect bacteria from T6SS-mediated predation (Nicolas Flaugnatti et al., 2021; Toska et al., 2018). Although Bcc strains are known to be able to form biofilms (Conway et al., 2004), this is unlikely to be the reason for the Bcc strains evading attacks by some *tse* effectors, since that defensive strategy should allow the Bcc strains to resist T6SS-attacks by all effectors. However, different levels of biofilm production between the Bcc isolates might explain why *B. cepacia* seems to be more resistant than *B. cenocepacia* towards T6SS-mediated attacks by *P. aeruginosa* ID40, and is able to better take advantage of defects in the T6SS activity of PA.

7.6 TraDIS discussion

7.6.1 Method evaluation

Our approach taken to investigate the presence of factors influencing the competitive fitness of *Pseudomonas aeruginosa* (PA) using a TraDIS assay was unconventional in several ways. Firstly, using a non-laboratory-adapted patient isolate of PA, ID40, as basis for the generation of a transposon library may at first seem unusual, and to our knowledge, has not been done by other groups, but only by our lab (Sonnabend et al., 2020). While also adding a certain risk of an unknown factor leading to a unique, niche phenotype in the strain's competitive behavior, we argue that investigating a non-lab-adapted strain may provide the chance to discover genetic factors that do contribute to competition with other species, and which might have undergone negative selection in decade-long single cultures in laboratory strains like PA01 and PA14. Within the scope of this study however, it is important to

highlight that we did not aim at recreating the environmental conditions of e.g. the lung of a person with CF, but rather saw this as a proof-of-concept approach to identifying potential non-T6SS related factors in competition behavior of PA. As a trade-off for not being able to detect the essential genes of PA, this TraDIS-based assay offers the opportunity to uncover the involvement of already described or even unknown genes in competition. For example, we showed for the first time that *amgS* influences the competitive fitness of PA. The large amount of data generated from the TraDIS experiment of course also has to be filtered for relevant genes. One might think of several options for filtering this data set:

- (i) Through post-hoc functional filtering of the hits, e.g. for genes that are likely to be related to competition behavior. While effectively reducing the size of the data set, and filtering for likely relevant genes, the risk of this approach would be to overlook those genes that may have an unexpected, previously undescribed relation to the research question. Because the aim of this study was to identify genes that were previously not linked to the competitive fitness of *P. aeruginosa*, functional post-hoc filtering was not an option for evaluation of the data set.
- (ii) Through diligent statistical analysis of the raw data, setting stringent cut-off values for significance and fold-change of relevant genes. It may however be challenging to find appropriate cut-off-values for the desired application. For example, when treating a transposon library evenly with an antibiotic, as described for example by Sonnabend et al. (2020), every cell is equally likely to encounter the same concentration of antibiotic, and the resulting data set may be easy to evaluate statistically, since the outcome is not influenced by confounders like varying selective pressure. However, when working with a growing bacterial culture of mixed competitors on a solid surface, one can expect that not every mutant within the culture is equally likely to encounter the same number of competitor cells. Imagining the transposon library as a sum of sub-populations of bacteria makes it clear that in this case, some mutants that would have a growth disadvantage in competition with Bcc strains may profit from other sub-populations that can fend off competitors, while some competition-competent mutants may encounter over-proportionally high numbers of competitors and be overwhelmed and eradicated. This will lead to more false-positive and false-negative hits compared to a TraDIS setup where every cell encounters the same selection pressure. To account for this intrinsic confounding factor, leading to higher “background noise” of hits, one may instead opt to choose less stringent criteria for statistical analysis, with the risk of letting more false-positive hits, but also more false-negative hits pass this filtering step. In our case, because the impact of single gene deletions on the competitive fitness may

be minute as seen with the *tse* effector deletion mutants, we decided to choose this option and add a second filtering step for those false-positive hits thereafter.

- (iii) Because we were interested in conserved ways of *P. aeruginosa* to compete with strains of the *Burkholderia cepacia* complex, we compared genes that played a role in competition with both *B. cepacia* and *B. cenocepacia*. This allowed us to narrow down the relatively large set of genes that were potentially involved in the competitive fitness of *P. aeruginosa* from 132 genes to only nine genes, which formed the intersection between those two competition conditions. This step allowed us to filter out most false-positive hits in favor of those genes that are relevant to the competition behavior of *P. aeruginosa* and made proper evaluation of those hits through in-frame gene deletion and competition assays viable.

As described in the methods section, we performed the TraDIS assay in biological triplicates for competition of ID40 with *B. cenocepacia*, but in duplicates with *B. cepacia*. Since a higher number of biological replicates tends to reduce the number of false-positive hits, and give the results more statistical power, we would expect to see more hits (including more false-positive hits) in the competition between ID40 and *B. cepacia* compared to the competition with ID40 and *B. cenocepacia*. Indeed, 81 genes were identified as candidates involved in competition with *B. cepacia*, while only 60 were identified in competition with *B. cenocepacia*. We therefore opted to investigate the condition with more statistical power more closely, and identified two genes that were of particular interest to our group: *mepM1*, which was found to play a role in β -lactam resistance by Sonnabend et al. (2020), and *amgS*. For these reasons, we decided to also include *amgS* and *mepM1* in our validation process, and to investigate their potential involvement in the competitive fitness of *P. aeruginosa*.

7.6.2 Results evaluation

Most genes that were identified in the TraDIS screening assay as possible candidates impacting the competitive fitness of *P. aeruginosa* (PA) ID40 were found in competition with either *B. cepacia* or *B. cenocepacia* only, and not in both conditions. Out of a total of 132 candidate genes, only 9 out of them were identified in both conditions.

For all these genes with the exception of *gpml*, *tsi5* and *gacA*, single gene deletion mutants were created and used for further validation through competition assays. Both *mepM1* and *amgS* were also included in the validation process because gene deletion mutants of those genes were readily available in the lab's strain collection. A *retS* deletion mutant, which later turned out to also harbor an inactivating mutation in *gacS*, was initially intended as a control for the effect of an over-active T6SS on the competitiveness. Additionally, a triple knock-out

mutant of *mepM1*, *mepM2* and *mepM3* was included for analyzing the potential cumulative effect of those endopeptidases on the competitiveness of PA.

A competition disadvantage of PA against both Bcc species was found upon deletion of the genes *gacS*, *amgS*, *retS* (with additional *gacS* inactivation), *mepM1*, *mepM1/2/3*, and PA3016. Closer investigation showed that the latter three genes or combination of gene deletions also lead to a general competitive disadvantage of the mutants against the ID40 wt. These disadvantages could therefore be attributed to a general loss of fitness and are not specific for interbacterial competition. The genomic deletion of *cobT*, *ssuB1*, PA0738 and PA3072 had no impact on the fitness of these mutants.

Interestingly, both *gacA* and *gacS*, which are part of the central regulatory pathway of the T6SS in PA but no structural components of the T6SS (e.g. *icmF1*) were identified in this screening assay, even though we saw a strong negative effect of *icmF1* deletion on the competitive fitness of the ID40 strain. A possible explanation for this is that the deletion of the *gacS/gacA* two-component system has negative impacts on the competitive fitness of *P. aeruginosa* beyond the sole loss of T6SS activity. For example, biofilm formation has been shown to increase resistance of bacteria towards T6SS-mediated attacks (Toska et al., 2018), and the *gac/rsm* signaling pathway is also known to regulate biofilm formation (Irie et al., 2010). Additionally, deletion of *gacS* leads to increased motility of *P. aeruginosa* via Type IV pili, and motility is known as a factor that increases intermixing of strains and, by extension, the likelihood of falling victim of T6SS-mediated attacks by competitors (Rudzite et al., 2023). Because the inactivation of *gacS/gacA* affects more than one mechanism of T6SS-attack evasion, these cumulative effects may be more readily detected than those of mutations in structural components of the T6SS alone.

Interestingly, one more T6SS-related gene that was found in our TraDIS assay was the immunity gene *tsi5*. It is curious that no other immunity gene appeared to sufficiently impact the competitive fitness of *P. aeruginosa*, especially because (i) deletion of an immunity gene without additional deletion of the cognate toxin is expected to be toxic to *P. aeruginosa* and (ii) we observed no negative impact of *tse5tsi5* deletion on the competitive fitness of *P. aeruginosa* ID40 in competition with the ID40 wt, as shown in **Figure 27** (p. 131). It is however possible that the *B. cepacia* and *B. cenocepacia* strains used in the TraDIS assay also encode a toxin that resembles Tse5 of *P. aeruginosa* and is normally inactivated by Tsi5. In that case, while the *tsi5* mutants would be killed by T6SS predation from sibling, Tse5-expressing cells in the *P. aeruginosa* control conditions, the additional predation by *B. cepacia* and *B. cenocepacia* would inflict additional predation stress and lead to an even stronger reduction of *tsi5*-inactive mutants in the assay. BLAST alignment of the

Tse5 peptide sequence of *P. aeruginosa* against the genome of *B. cepacia* strains revealed a certain, albeit low, similarity to the DNA deaminase DddA of *B. cenocepacia* (Identity = 28.7%), although the respective immunity genes, *tsi5* and *dddI*, share no similarity. As an additional caveat, both Tse5 and DddA are large peptides (1317 aa and 1427 aa, respectively), and might be bound to share a certain level of identity just by coincidence (de Moraes et al., 2021). The DNA deaminase DddA is a T6SS-dependent toxic effector of *B. cenocepacia*, and was recently shown to mutagenize target bacteria by deamination of cytosine, leading to an arrested replication complex and DNA degradation or, at lower levels, to the accumulation of mutations (de Moraes et al., 2021).

Out of all genes detected with the TraDIS screening assay that may play an important role in competition of *P. aeruginosa* with Bcc strains, *gacS* and *amgS* were the only ones that did not also result in a general growth defect against the own wild-type strain, as shown in **Figure 25** (p. 127). While the deletion of *gacS* is known to result in lower T6SS activity in *P. aeruginosa*, giving the strain a competitive disadvantage over T6SS-sensitive competitor strains (Perault et al., 2020), *amgS* has so far not been linked to competition behavior of *P. aeruginosa*. It encodes for the sensor kinase AmgS, which is part of the AmgRS two-component-system (S. Lee et al., 2009). The AmgRS system has previously been described to contribute to increased aminoglycoside resistance in PA by sensing misfolded peptides in the periplasm, and activating the membrane protease HtpX, a membrane protein of unknown function, PA5582, and a modulator of the FtsH protease called YccA, which help to relieve membrane stress caused by misfolded proteins that can originate from either aminoglycosides or Zn²⁺ ions in the supernatant (Hinz et al., 2011; Poole et al., 2019). There are several ways in which AmgS might provide a competitive advantage to PA in competition with Bcc strains:

- (i) AmgS directly or indirectly senses an attack by the Bcc strains, for example through one of their T6SS effectors, and activates its target genes, which then protect PA from the effects of the attack.
- (ii) AmgS is another, so far unrecognized positive regulator of the *gac/rsm* pathway in PA, allowing the cells to either retaliate against the attackers or to over-activate their own T6SS. Since there are already at least three sensor kinases known to regulate the activity of GacS – LadS, RetS, and PA1611 – upon deletion of *amgS*, PA loses some, but not all competitive fitness through the T6SS.

Additionally, other TCS of *P. aeruginosa* like the AlgZ-AlgR-TCS were also suggested to play a role in regulating the *gac/rsm* pathway through increasing the expression of *rsmZ* and

rsmY (Intile et al., 2014). The AmgR-AmgS TCS might act in a similar way, but not necessarily on *rsmY* or *rsmZ*, further fine-tuning this central regulatory switch of different lifestyles of *P. aeruginosa*.

A relatively recent publication by Ting et al. (2022) has employed a TraDIS approach to identify genes that are specifically involved in the resistance of PA towards T6SS attacks by *B. thailandensis* (*B. thai*). To this end, a mutant library of PA was co-incubated with either T6SS⁺ or T6SS⁻ strain of *B. thai* (Ting et al., 2022). That study identified three gene clusters (Arc1-3) that help PA fend off attacks by the T6SS of *B. thai*, but those clusters only became relevant when PA was greatly outnumbered in the initial inoculum by *B. thai*. In our screening assay, none of these genes were identified. This might indicate that the defensive strategies of PA played a subordinate role in our screening assay, where we used a 1:1 ratio inoculum of both PA and Bcc strains. Rather, it seems that in competition with Bcc strains under the chosen conditions, attack is the best defensive strategy for PA, since those gene mutations that heavily impacted the competitive fitness of PA in competition with Bcc strains were either deletion of *icmF1*, *gacS*, or multiple *tse* effectors, which all are related to the antibacterial H1-T6SS, as seen in **Figure 22** (p. 114) or **Figure 25** (p. 127). Of course, the aim of our screening assay was also a different one from the one that Ting et al. (2022) had. While Ting and colleagues aimed specifically at identifying those genes that help PA defend against attacks from *B. thai*, our screening assay aimed at identifying all factors that contribute to the survival of PA in the presence of Bcc strains, be they offensive or defensive strategies. It appears that in the conditions chosen for our competition assays, offensive strategies outweighed the importance of defensive strategies of PA. However, it would be interesting to look into the role of defensive strategies as well, because that is a possible mode of action of AmgS, which we found to play a role in interbacterial competition of *P. aeruginosa*. A possible approach to investigating this would be to change the ratio of PA:Bcc in the initial inoculum in favor of the Bcc strains, so that the importance of defensive strategies of PA may increase, and then investigating if *amgS* deletion shows a stronger impact on the competitiveness of *P. aeruginosa*. This would then help to discern whether AmgS acts via the *gac/rsm* regulatory pathway, through its target genes, or through another, unknown mechanism.

7.7 Next steps in uncovering the mode of action of AmgS in competition

To investigate the way in which *amgS* regulates the competition behavior of *P. aeruginosa*, several approaches are being taken at the time of writing this thesis.

- (i) The promoters of *rsmZ* and *rsmY* were fused to a reporter gene, in this case the NanoLuc luciferase, and those constructs will be integrated into the genome of *P. aeruginosa*

(PA). If AmgS is a regulator of the T6SS of PA via the *gac/rsm* signaling pathway upstream of *rsmZ/Y*, the levels of expression of NanoLuc luciferase should differ between an *amgS* deletion mutant and the PA wt.

- (ii) As with the *rsmZ/Y* promoters, a construct for NanoLuc expression under the control of the *exsA* promoter will be employed to investigate the possible effects of *amgS* deletion on the downstream regulatory cascade of *rsmZ/Y*. ExsA is a central regulator of the T3SS of PA (Brutinel et al., 2008) and is itself positively regulated by RsmA (H. Mulcahy et al., 2006). Since the T6SS of *P. aeruginosa* is repressed under non-inducing conditions (Basler et al., 2013), investigating the inversely regulated site of the *gac/rsm* signaling pathway may serve as a better readout for said pathway. For example, the expression levels of NanoLuc under the control of the *exsA* promoter could be compared between a PA wt strain, a *gacS* deletion mutant (expected to show higher *exsA* promoter activity than the wt) and an *amgS* deletion mutant. This, together with the *rsmZ/Y* promoter activity assays, would allow us to assess whether the AmgS/R TCS regulates the competitive fitness of PA through the *gac/rsm* signaling pathway, which finally affects the T6SS activity of PA.
- (iii) Because the AmgS/R TCS plays a role in aminoglycoside resistance of *P. aeruginosa* through activation of genes that help relieve stress of misfolded periplasmatic peptides (Hinz et al., 2011; Poole et al., 2019), it is possible that a similar mode of action helps PA withstand attacks from *B. cepacia* complex (Bcc) strains causing similar stresses. We therefore will generate in-frame gene deletion mutants of the genes encoding HtpX and PA5528, alone or in combination with *amgS* deletion, in *P. aeruginosa*. Testing the fitness of those gene deletion mutants in competition with Bcc strains and comparing their fitness to the PA wt will allow us to assess whether the AmgS/R-dependent activation of HtpX and PA5528 helps PA withstand attacks from Bcc strains.

Bibliography

- Aho, V. T. E., Pereira, P. A. B., Haahtela, T., Pawankar, R., Auvinen, P., & Koskinen, K. (2015).** The microbiome of the human lower airways: a next generation sequencing perspective. *World Allergy Organ J*, 8(1), 23. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/26140078>. doi:10.1186/s40413-015-0074-z
- Aires, J. R., Kohler, T., Nikaido, H., & Plesiat, P. (1999).** Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother*, 43(11), 2624-2628. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/10543738>. doi:10.1128/AAC.43.11.2624
- Allsopp, L. P., Collins, A. C. Z., Hawkins, E., Wood, T. E., & Filloux, A. (2022).** RpoN/Sfa2-dependent activation of the *Pseudomonas aeruginosa* H2-T6SS and its cognate arsenal of antibacterial toxins. *Nucleic Acids Res*, 50(1), 227-243. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/34928327>. doi:10.1093/nar/gkab1254
- Allsopp, L. P., Wood, T. E., Howard, S. A., Maggiorelli, F., Nolan, L. M., Wettstadt, S., & Filloux, A. (2017).** RsmA and AmrZ orchestrate the assembly of all three type VI secretion systems in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A*, 114(29), 7707-7712. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/28673999>. doi:10.1073/pnas.1700286114
- Angus, A. A., Agapakis, C. M., Fong, S., Yerrapragada, S., Estrada-de los Santos, P., Yang, P., Song, N., Kano, S., Caballero-Mellado, J., de Faria, S. M., Dakora, F. D., Weinstock, G., & Hirsch, A. M. (2014).** Plant-associated symbiotic *Burkholderia* species lack hallmark strategies required in mammalian pathogenesis. *PLoS One*, 9(1), e83779. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/24416172>. doi:10.1371/journal.pone.0083779
- Aschtgen, M. S., Thomas, M. S., & Cascales, E. (2010).** Anchoring the type VI secretion system to the peptidoglycan: TssL, TagL, TagP... what else? *Virulence*, 1(6), 535-540. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/21178498>. doi:10.4161/viru.1.6.13732
- Azzopardi, E. A., Azzopardi, E., Camilleri, L., Villapalos, J., Boyce, D. E., Dziewulski, P., Dickson, W. A., & Whitaker, I. S. (2014).** Gram negative wound infection in hospitalised adult burn patients--systematic review and metanalysis. *PLoS One*, 9(4), e95042. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/24751699>. doi:10.1371/journal.pone.0095042
- Baker, C. S., Morozov, I., Suzuki, K., Romeo, T., & Babitzke, P. (2002).** CsrA regulates glycogen biosynthesis by preventing translation of *glgC* in *Escherichia coli*. *Mol Microbiol*, 44(6), 1599-1610. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/12067347>. doi:10.1046/j.1365-2958.2002.02982.x
- Ballard, R. W., Palleroni, N. J., Doudoroff, M., Stanier, R. Y., & Mandel, M. (1970).** Taxonomy of the aerobic pseudomonads: *Pseudomonas cepacia*, *P. marginata*, *P. alliiicola* and *P. caryophylli*. *J Gen Microbiol*, 60(2), 199-214. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/5488054>. doi:10.1099/00221287-60-2-199
- Barbier, F., Andremont, A., Wolff, M., & Bouadma, L. (2013).** Hospital-acquired pneumonia and ventilator-associated pneumonia: recent advances in epidemiology and management. *Curr Opin Pulm Med*, 19(3), 216-228. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/23524477>. doi:10.1097/MCP.0b013e32835f27be
- Barquist, L., Mayho, M., Cummins, C., Cain, A. K., Boinett, C. J., Page, A. J., Langridge, G. C., Quail, M. A., Keane, J. A., & Parkhill, J. (2016).** The TraDIS toolkit: sequencing and analysis for dense transposon mutant libraries. *Bioinformatics*, 32(7), 1109-1111. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/26794317>. doi:10.1093/bioinformatics/btw022
- Barret, M., Egan, F., Fargier, E., Morrissey, J. P., & O'Gara, F. (2011).** Genomic analysis of the type VI secretion systems in *Pseudomonas* spp.: novel clusters and putative effectors

- uncovered. *Microbiology (Reading)*, 157(Pt 6), 1726-1739. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/21474537>. doi:10.1099/mic.0.048645-0
- Barret, M., Egan, F., & O'Gara, F. (2013).** Distribution and diversity of bacterial secretion systems across metagenomic datasets. *Environ Microbiol Rep*, 5(1), 117-126. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/23757140>. doi:10.1111/j.1758-2229.2012.00394.x
- Basler, M., Ho, B. T., & Mekalanos, J. J. (2013).** Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. *Cell*, 152(4), 884-894. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/23415234>. doi:10.1016/j.cell.2013.01.042
- Basler, M., & Mekalanos, J. J. (2012).** Type 6 secretion dynamics within and between bacterial cells. *Science*, 337(6096), 815. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/22767897>. doi:10.1126/science.1222901
- Basler, M., Pilhofer, M., Henderson, G. P., Jensen, G. J., & Mekalanos, J. J. (2012).** Type VI secretion requires a dynamic contractile phage tail-like structure. *Nature*, 483(7388), 182-186. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/22367545>. doi:10.1038/nature10846
- Becker, H. D., Min, B., Jacobi, C., Raczniak, G., Pelaschier, J., Roy, H., Klein, S., Kern, D., & Söll, D. (2000).** The heterotrimeric *Thermus thermophilus* Asp-tRNAAsn amidotransferase can also generate Gln-tRNA^{Gln}. *FEBS Letters*, 476(3), 140-144. Retrieved from <https://febs.onlinelibrary.wiley.com/doi/abs/10.1016/S0014-5793%2800%2901697-5>. doi:https://doi.org/10.1016/S0014-5793(00)01697-5
- Bernal, P., Furniss, R. C. D., Fecht, S., Leung, R. C. Y., Spiga, L., Mavridou, D. A. I., & Filloux, A. (2021).** A novel stabilization mechanism for the type VI secretion system sheath. *Proc Natl Acad Sci U S A*, 118(7). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/33558227>. doi:10.1073/pnas.2008500118
- Berni, B., Soscia, C., Djermoun, S., Ize, B., & Bleves, S. (2019).** A Type VI Secretion System Trans-Kingdom Effector Is Required for the Delivery of a Novel Antibacterial Toxin in *Pseudomonas aeruginosa*. *Front Microbiol*, 10, 1218. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/31231326>. doi:10.3389/fmicb.2019.01218
- Berry, A., DeVault, J. D., & Chakrabarty, A. M. (1989).** High osmolarity is a signal for enhanced *algD* transcription in mucoid and nonmucoid *Pseudomonas aeruginosa* strains. *J Bacteriol*, 171(5), 2312-2317. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/2496102>. doi:10.1128/jb.171.5.2312-2317.1989
- Bhagirath, A. Y., Pydi, S. P., Li, Y., Lin, C., Kong, W., Chelikani, P., & Duan, K. (2017).** Characterization of the Direct Interaction between Hybrid Sensor Kinases PA1611 and RetS That Controls Biofilm Formation and the Type III Secretion System in *Pseudomonas aeruginosa*. *ACS Infect Dis*, 3(2), 162-175. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/27957853>. doi:10.1021/acsinfecdis.6b00153
- Bigger, J. W. (1944).** Treatment of Staphylococcal Infections With Penicillin by Intermittent Sterilisation. *The Lancet*, 244(6320), 497-500. Retrieved from [https://doi.org/10.1016/S0140-6736\(00\)74210-3](https://doi.org/10.1016/S0140-6736(00)74210-3).
- Blanc, D. S., Nahimana, I., Petignat, C., Wenger, A., Bille, J., & Francioli, P. (2004).** Faucets as a reservoir of endemic *Pseudomonas aeruginosa* colonization/infections in intensive care units. *Intensive Care Med*, 30(10), 1964-1968. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/15257431>. doi:10.1007/s00134-004-2389-z
- Boulant, T., Boudehen, Y. M., Filloux, A., Plesiat, P., Naas, T., & Dortet, L. (2018).** Higher Prevalence of PldA, a *Pseudomonas aeruginosa* Trans-Kingdom H2-Type VI Secretion System Effector, in Clinical Isolates Responsible for Acute Infections and in Multidrug Resistant Strains. *Front Microbiol*, 9, 2578. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30420847>. doi:10.3389/fmicb.2018.02578
- Boutin, S., Weitnauer, M., Hassel, S., Graeber, S. Y., Stahl, M., Dittrich, A. S., Mall, M. A., & Dalpke, A. H. (2018).** One time quantitative PCR detection of *Pseudomonas aeruginosa* to discriminate intermittent from chronic infection in cystic

- fibrosis. *J Cyst Fibros*, 17(3), 348-355. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/29336943>. doi:10.1016/j.jcf.2017.12.007
- Brackmann, M., Wang, J., & Basler, M. (2018).** Type VI secretion system sheath inter-subunit interactions modulate its contraction. *EMBO Rep*, 19(2), 225-233. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/29222345>. doi:10.15252/embr.201744416
- Broder, U. N., Jaeger, T., & Jenal, U. (2016).** LadS is a calcium-responsive kinase that induces acute-to-chronic virulence switch in *Pseudomonas aeruginosa*. *Nat Microbiol*, 2, 16184. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/27775685>. doi:10.1038/nmicrobiol.2016.184
- Brooun, A., Liu, S., & Lewis, K. (2000).** A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother*, 44(3), 640-646. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/10681331>. doi:10.1128/AAC.44.3.640-646.2000
- Brunet, Y. R., Zoued, A., Boyer, F., Douzi, B., & Cascales, E. (2015).** The Type VI Secretion TssEFGK-VgrG Phage-Like Baseplate Is Recruited to the TssJLM Membrane Complex via Multiple Contacts and Serves As Assembly Platform for Tail Tube/Sheath Polymerization. *PLoS Genet*, 11(10), e1005545. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/26460929>. doi:10.1371/journal.pgen.1005545
- Brutinel, E. D., Vakulskas, C. A., Brady, K. M., & Yahr, T. L. (2008).** Characterization of ExsA and of ExsA-dependent promoters required for expression of the *Pseudomonas aeruginosa* type III secretion system. *Mol Microbiol*, 68(3), 657-671. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/18373522>. doi:10.1111/j.1365-2958.2008.06179.x
- Burkinshaw, B. J., Liang, X., Wong, M., Le, A. N. H., Lam, L., & Dong, T. G. (2018).** A type VI secretion system effector delivery mechanism dependent on PAAR and a chaperone-co-chaperone complex. *Nat Microbiol*, 3(5), 632-640. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/29632369>. doi:10.1038/s41564-018-0144-4
- Burman, L. G., Park, J. T., Lindstrom, E. B., & Boman, H. G. (1973).** Resistance of *Escherichia coli* to penicillins: identification of the structural gene for the chromosomal penicillinase. *J Bacteriol*, 116(1), 123-130. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/4200837>. doi:10.1128/jb.116.1.123-130.1973
- Bylund, J., Burgess, L. A., Cescutti, P., Ernst, R. K., & Speert, D. P. (2006).** Exopolysaccharides from *Burkholderia cenocepacia* inhibit neutrophil chemotaxis and scavenge reactive oxygen species. *J Biol Chem*, 281(5), 2526-2532. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/16316987>. doi:10.1074/jbc.M510692200
- Cameron, B., Blanche, F., Rouyez, M. C., Bisch, D., Famechon, A., Couder, M., Cauchois, L., Thibaut, D., Debussche, L., & Crouzet, J. (1991).** Genetic analysis, nucleotide sequence, and products of two *Pseudomonas denitrificans* *cob* genes encoding nicotinate-nucleotide: dimethylbenzimidazole phosphoribosyltransferase and cobalamin (5'-phosphate) synthase. *J Bacteriol*, 173(19), 6066-6073. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/1917841>. doi:10.1128/jb.173.19.6066-6073.1991
- Cao, L., Srikumar, R., & Poole, K. (2004).** MexAB-OprM hyperexpression in NalC-type multidrug-resistant *Pseudomonas aeruginosa*: identification and characterization of the *nalC* gene encoding a repressor of PA3720-PA3719. *Mol Microbiol*, 53(5), 1423-1436. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/15387820>. doi:10.1111/j.1365-2958.2004.04210.x
- Casabona, M. G., Silverman, J. M., Sall, K. M., Boyer, F., Coute, Y., Poirol, J., Grunwald, D., Mougous, J. D., Elsen, S., & Attree, I. (2013).** An ABC transporter and an outer membrane lipoprotein participate in posttranslational activation of type VI secretion in *Pseudomonas aeruginosa*. *Environ Microbiol*, 15(2), 471-486. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/22765374>. doi:10.1111/j.1462-2920.2012.02816.x

- Chambonniere, G., Roux, L., Redelberger, D., Fadel, F., Filloux, A., Sivaneson, M., de Bentzmann, S., & Bordi, C. (2016).** The Hybrid Histidine Kinase LadS Forms a Multicomponent Signal Transduction System with the GacS/GacA Two-Component System in *Pseudomonas aeruginosa*. *PLoS Genet*, 12(5), e1006032. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/27176226>. doi:10.1371/journal.pgen.1006032
- Chandler, C. E., Horspool, A. M., Hill, P. J., Wozniak, D. J., Schertzer, J. W., Rasko, D. A., & Ernst, R. K. (2019).** Genomic and Phenotypic Diversity among Ten Laboratory Isolates of *Pseudomonas aeruginosa* PAO1. *J Bacteriol*, 201(5). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30530517>. doi:10.1128/JB.00595-18
- Chen, Y. C., Kalawong, R., Toyofuku, M., & Eberl, L. (2022).** The role of peptidoglycan hydrolases in the formation and toxicity of *Pseudomonas aeruginosa* membrane vesicles. *MicroLife*, 3, uqac009. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/37229443>. doi:10.1093/femsml/uqac009
- Cherrak, Y., Rapisarda, C., Pellarin, R., Bouvier, G., Bardiaux, B., Allain, F., Malosse, C., Rey, M., Chamot-Rooke, J., Cascales, E., Fronzes, R., & Durand, E. (2018).** Biogenesis and structure of a type VI secretion baseplate. *Nat Microbiol*, 3(12), 1404-1416. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30323254>. doi:10.1038/s41564-018-0260-1
- Chitkara, Y. K., & Feierabend, T. C. (1981).** Endogenous and exogenous infection with *Pseudomonas aeruginosa* in a burns unit. *Int Surg*, 66(3), 237-240. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/6797982>.
- Christiaen, S. E., Brackman, G., Nelis, H. J., & Coenye, T. (2011).** Isolation and identification of quorum quenching bacteria from environmental samples. *J Microbiol Methods*, 87(2), 213-219. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/21856341>. doi:10.1016/j.mimet.2011.08.002
- Christiaen, S. E., Matthijs, N., Zhang, X. H., Nelis, H. J., Bossier, P., & Coenye, T. (2014).** Bacteria that inhibit quorum sensing decrease biofilm formation and virulence in *Pseudomonas aeruginosa* PAO1. *Pathog Dis*, 70(3), 271-279. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/24415453>. doi:10.1111/2049-632X.12124
- Colautti, J., Bullen, N. P., & Whitney, J. C. (2023).** Lack of evidence that *Pseudomonas aeruginosa* AmpDh3-PA0808 constitute a type VI secretion system effector-immunity pair. *Mol Microbiol*, 119(2), 262-274. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/36577706>. doi:10.1111/mmi.15021
- Colom, K., Fdz-Aranguiz, A., Suinaga, E., & Cisterna, R. (1995).** Emergence of resistance to beta-lactam agents in *Pseudomonas aeruginosa* with group I beta-lactamases in Spain. *Eur J Clin Microbiol Infect Dis*, 14(11), 964-971. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/8654447>. doi:10.1007/BF01691378
- Conway, B.-A. D., Chu, K. K., Bylund, J., Altman, E., & Speert, D. P. (2004).** Production of Exopolysaccharide by *Burkholderia cenocepacia* Results in Altered Cell-Surface Interactions and Altered Bacterial Clearance in Mice. *The Journal of Infectious Diseases*, 190(5), 957-966. Retrieved from <https://doi.org/10.1086/423141>. doi:10.1086/423141
- Corbell, N., & Loper, J. E. (1995).** A global regulator of secondary metabolite production in *Pseudomonas fluorescens* Pf-5. *J Bacteriol*, 177(21), 6230-6236. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/7592389>. doi:10.1128/jb.177.21.6230-6236.1995
- Curnow, A. W., Ibba, M., & Soll, D. (1996).** tRNA-dependent asparagine formation. *Nature*, 382(6592), 589-590. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/8757127>. doi:10.1038/382589b0
- Daury, L., Orange, F., Taveau, J. C., Verchere, A., Monlezun, L., Gounou, C., Marreddy, R. K., Picard, M., Broutin, I., Pos, K. M., & Lambert, O. (2016).** Tripartite assembly of RND multidrug efflux pumps. *Nat Commun*, 7, 10731. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/26867482>. doi:10.1038/ncomms10731

- de Moraes, M. H., Hsu, F., Huang, D., Bosch, D. E., Zeng, J., Radey, M. C., Simon, N., Ledvina, H. E., Frick, J. P., Wiggins, P. A., Peterson, S. B., & Mougous, J. D. (2021). An interbacterial DNA deaminase toxin directly mutagenizes surviving target populations. *Elife*, 10, e62967. Retrieved from <https://doi.org/10.7554/eLife.62967>. doi:10.7554/eLife.62967
- Deziel, E., Lepine, F., Milot, S., He, J., Mindrinos, M. N., Tompkins, R. G., & Rahme, L. G. (2004). Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. *Proc Natl Acad Sci U S A*, 101(5), 1339-1344. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/14739337>. doi:10.1073/pnas.0307694100
- Dickson, R. P., Erb-Downward, J. R., Martinez, F. J., & Huffnagle, G. B. (2016). The Microbiome and the Respiratory Tract. *Annu Rev Physiol*, 78, 481-504. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/26527186>. doi:10.1146/annurev-physiol-021115-105238
- Dix, S. R., Owen, H. J., Sun, R., Ahmad, A., Shastri, S., Spiewak, H. L., Mosby, D. J., Harris, M. J., Batters, S. L., Brooker, T. A., Tzokov, S. B., Sedelnikova, S. E., Baker, P. J., Bullough, P. A., Rice, D. W., & Thomas, M. S. (2018). Structural insights into the function of type VI secretion system TssA subunits. *Nat Commun*, 9(1), 4765. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30420757>. doi:10.1038/s41467-018-07247-1
- Dixon, A. S., Schwinn, M. K., Hall, M. P., Zimmerman, K., Otto, P., Lubben, T. H., Butler, B. L., Binkowski, B. F., Machleidt, T., Kirkland, T. A., Wood, M. G., Eggers, C. T., Encell, L. P., & Wood, K. V. (2016). NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chem Biol*, 11(2), 400-408. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/26569370>. doi:10.1021/acscchembio.5b00753
- Dong, T. G., Ho, B. T., Yoder-Himes, D. R., & Mekalanos, J. J. (2013). Identification of T6SS-dependent effector and immunity proteins by Tn-seq in *Vibrio cholerae*. *Proc Natl Acad Sci U S A*, 110(7), 2623-2628. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/23362380>. doi:10.1073/pnas.1222783110
- Dotsch, A., Becker, T., Pommerenke, C., Magnowska, Z., Jansch, L., & Haussler, S. (2009). Genomewide identification of genetic determinants of antimicrobial drug resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*, 53(6), 2522-2531. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19332674>. doi:10.1128/AAC.00035-09
- Durand, E., Nguyen, V. S., Zoued, A., Logger, L., Pehau-Arnaudet, G., Aschtgen, M. S., Spinelli, S., Desmyter, A., Bardiaux, B., Dujeancourt, A., Roussel, A., Cambillau, C., Cascales, E., & Fronzes, R. (2015). Biogenesis and structure of a type VI secretion membrane core complex. *Nature*, 523(7562), 555-560. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/26200339>. doi:10.1038/nature14667
- ECDC. (2023). *Antimicrobial resistance in the EU/EEA (EARS-Net) - Annual epidemiological report for 2022*. Retrieved from Stockholm:
- Eggers, O., Renschler, F. A., Michalek, L. A., Wackler, N., Walter, E., Smollich, F., Klein, K., Sonnabend, M. S., Egle, V., Angelov, A., Engesser, C., Borisova, M., Mayer, C., Schutz, M., & Bohn, E. (2023). YgfB increases beta-lactam resistance in *Pseudomonas aeruginosa* by counteracting AlpA-mediated *ampDh3* expression. *Commun Biol*, 6(1), 254. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/36894667>. doi:10.1038/s42003-023-04609-4
- Elabed, H., Gonzalez-Tortuero, E., Ibacache-Quiroga, C., Bakhrouf, A., Johnston, P., Gaddour, K., Blazquez, J., & Rodriguez-Rojas, A. (2019). Seawater salt-trapped *Pseudomonas aeruginosa* survives for years and gets primed for salinity tolerance. *BMC Microbiol*, 19(1), 142. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/31234794>. doi:10.1186/s12866-019-1499-2
- Evans, L. R., & Linker, A. (1973). Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. *J Bacteriol*, 116(2), 915-924. Retrieved

- from <https://www.ncbi.nlm.nih.gov/pubmed/4200860>. doi:10.1128/jb.116.2.915-924.1973
- Fiegna, F., & Velicer, G. J. (2003).** Competitive fates of bacterial social parasites: persistence and self-induced extinction of *Myxococcus xanthus* cheaters. *Proc Biol Sci*, 270(1523), 1527-1534. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/12965020>. doi:10.1098/rspb.2003.2387
- Flaughnatti, N., Isaac, S., Lemos Rocha, L. F., Stutzmann, S., Rendueles, O., Stoudmann, C., Vesel, N., Garcia-Garcera, M., Buffet, A., Sana, T. G., Rocha, E. P. C., & Blokesch, M. (2021).** Human commensal gut Proteobacteria withstand type VI secretion attacks through immunity protein-independent mechanisms. *Nat Commun*, 12(1), 5751. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/34599171>. doi:10.1038/s41467-021-26041-0
- Flaughnatti, N., Isaac, S., Lemos Rocha, L. F., Stutzmann, S., Rendueles, O., Stoudmann, C., Vesel, N., Garcia-Garcera, M., Buffet, A., Sana, T. G., Rocha, E. P. C., & Blokesch, M. (2021).** Human commensal gut Proteobacteria withstand type VI secretion attacks through immunity protein-independent mechanisms. *Nature Communications*, 12(1), 5751. Retrieved from <https://doi.org/10.1038/s41467-021-26041-0>. doi:10.1038/s41467-021-26041-0
- Forster, A., Planamente, S., Manoli, E., Lossi, N. S., Freemont, P. S., & Filloux, A. (2014).** Coevolution of the ATPase ClpV, the sheath proteins TssB and TssC, and the accessory protein TagJ/HsiE1 distinguishes type VI secretion classes. *J Biol Chem*, 289(47), 33032-33043. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/25305017>. doi:10.1074/jbc.M114.600510
- Francis, V. I., Waters, E. M., Finton-James, S. E., Gori, A., Kadioglu, A., Brown, A. R., & Porter, S. L. (2018).** Multiple communication mechanisms between sensor kinases are crucial for virulence in *Pseudomonas aeruginosa*. *Nat Commun*, 9(1), 2219. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/29880803>. doi:10.1038/s41467-018-04640-8
- Franzetti, F., Cernuschi, M., Esposito, R., & Moroni, M. (1992).** *Pseudomonas* infections in patients with AIDS and AIDS-related complex. *J Intern Med*, 231(4), 437-443. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/1588272>. doi:10.1111/j.1365-2796.1992.tb00957.x
- Gallagher, L. A., McKnight, S. L., Kuznetsova, M. S., Pesci, E. C., & Manoil, C. (2002).** Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. *J Bacteriol*, 184(23), 6472-6480. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/12426334>. doi:10.1128/JB.184.23.6472-6480.2002
- Gambello, M. J., & Iglewski, B. H. (1991).** Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. *J Bacteriol*, 173(9), 3000-3009. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/1902216>. doi:10.1128/jb.173.9.3000-3009.1991
- Gambello, M. J., Kaye, S., & Iglewski, B. H. (1993).** LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (*apr*) and an enhancer of exotoxin A expression. *Infect Immun*, 61(4), 1180-1184. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/8454322>. doi:10.1128/iai.61.4.1180-1184.1993
- Gao, M., Zhou, J., Su, Z., & Huang, Y. (2017).** Bacterial cupredoxin azurin hijacks cellular signaling networks: Protein-protein interactions and cancer therapy. *Protein Sci*, 26(12), 2334-2341. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/28960574>. doi:10.1002/pro.3310
- Gay, P., Le Coq, D., Steinmetz, M., Ferrari, E., & Hoch, J. A. (1983).** Cloning structural gene *sacB*, which codes for exoenzyme levansucrase of *Bacillus subtilis*: expression of the gene in *Escherichia coli*. *J Bacteriol*, 153(3), 1424-1431. Retrieved from

<https://www.ncbi.nlm.nih.gov/pubmed/6402497>. doi:10.1128/jb.153.3.1424-1431.1983

- Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., 3rd, & Smith, H. O. (2009).** Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods*, 6(5), 343-345. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19363495>. doi:10.1038/nmeth.1318
- Goedhart, J., & Luijsterburg, M. S. (2020).** VolcanoR is a web app for creating, exploring, labeling and sharing volcano plots. *Sci Rep*, 10(1), 20560. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/33239692>. doi:10.1038/s41598-020-76603-3
- Gonzalez-Magana, A., Altuna, J., Queralt-Martin, M., Largo, E., Velazquez, C., Montanhez, I., Bernal, P., Alcaraz, A., & Albesa-Jove, D. (2022).** The *P. aeruginosa* effector Tse5 forms membrane pores disrupting the membrane potential of intoxicated bacteria. *Commun Biol*, 5(1), 1189. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/36335275>. doi:10.1038/s42003-022-04140-y
- Goodman, A. L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R. S., & Lory, S. (2004).** A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev Cell*, 7(5), 745-754. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/15525535>. doi:10.1016/j.devcel.2004.08.020
- Goodman, A. L., Merighi, M., Hyodo, M., Ventre, I., Filloux, A., & Lory, S. (2009).** Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. *Genes Dev*, 23(2), 249-259. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19171785>. doi:10.1101/gad.1739009
- Görke, B., & Stülke, J. (2008).** Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat Rev Microbiol*, 6(8), 613-624. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/18628769>. doi:10.1038/nrmicro1932
- Goryshin, I. Y., & Reznikoff, W. S. (1998).** Tn5 in vitro transposition. *J Biol Chem*, 273(13), 7367-7374. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/9516433>. doi:10.1074/jbc.273.13.7367
- Govan, J. R., & Deretic, V. (1996).** Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev*, 60(3), 539-574. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/8840786>. doi:10.1128/mr.60.3.539-574.1996
- Green, S. K., Schroth, M. N., Cho, J. J., Kominos, S. K., & Vitanza-jack, V. B. (1974).** Agricultural plants and soil as a reservoir for *Pseudomonas aeruginosa*. *Appl Microbiol*, 28(6), 987-991. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/4217591>. doi:10.1128/am.28.6.987-991.1974
- Habich, A., Galeev, A., Vargas, V. C., Vogler, O., Ghoul, M., Andersen, S. B., Johansen, H. K., Molin, S., Griffin, A. S., & Unterweger, D. (2022).** Core and accessory effectors of type VI secretion systems contribute differently to the intraspecific diversity of *Pseudomonas aeruginosa*. *bioRxiv*, 2022.2004.2011.487527. Retrieved from <https://www.biorxiv.org/content/biorxiv/early/2022/04/11/2022.04.11.487527.full.pdf>. doi:10.1101/2022.04.11.487527
- Hachani, A., Allsopp, L. P., Oduko, Y., & Filloux, A. (2014).** The VgrG proteins are "a la carte" delivery systems for bacterial type VI effectors. *J Biol Chem*, 289(25), 17872-17884. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/24794869>. doi:10.1074/jbc.M114.563429
- Hachani, A., Lossi, N. S., & Filloux, A. (2013).** A visual assay to monitor T6SS-mediated bacterial competition. *J Vis Exp*(73), e50103. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/23542679>. doi:10.3791/50103
- Hachani, A., Lossi, N. S., Hamilton, A., Jones, C., Bleves, S., Albesa-Jove, D., & Filloux, A. (2011).** Type VI secretion system in *Pseudomonas aeruginosa*: secretion and multimerization of VgrG proteins. *J Biol Chem*, 286(14), 12317-12327. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/21325275>. doi:10.1074/jbc.M110.193045

- Hall, M. P., Unch, J., Binkowski, B. F., Valley, M. P., Butler, B. L., Wood, M. G., Otto, P., Zimmerman, K., Vidugiris, G., Machleidt, T., Robers, M. B., Benink, H. A., Eggers, C. T., Slater, M. R., Meisenheimer, P. L., Klaubert, D. H., Fan, F., Encell, L. P., & Wood, K. V. (2012). Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chem Biol*, 7(11), 1848-1857. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/22894855>. doi:10.1021/cb3002478
- Hallgren, J., Tsirigos, K. D., Pedersen, M. D., Armenteros, J. J. A., Marcatili, P., Nielsen, H., Krogh, A., & Winther, O. (2022). DeepTMHMM predicts alpha and beta transmembrane proteins using deep neural networks. *bioRxiv*, 2022.2004.2008.487609. Retrieved from <https://www.biorxiv.org/content/biorxiv/early/2022/04/10/2022.04.08.487609.full.pdf>. doi:10.1101/2022.04.08.487609
- Han, Y., Wang, T., Chen, G., Pu, Q., Liu, Q., Zhang, Y., Xu, L., Wu, M., & Liang, H. (2019). A *Pseudomonas aeruginosa* type VI secretion system regulated by CueR facilitates copper acquisition. *PLoS Pathog*, 15(12), e1008198. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/31790504>. doi:10.1371/journal.ppat.1008198
- Hassan, K. A., Cain, A. K., Huang, T., Liu, Q., Elbourne, L. D., Boinett, C. J., Brzoska, A. J., Li, L., Ostrowski, M., Nhu, N. T., Nhu Tdo, H., Baker, S., Parkhill, J., & Paulsen, I. T. (2016). Fluorescence-Based Flow Sorting in Parallel with Transposon Insertion Site Sequencing Identifies Multidrug Efflux Systems in *Acinetobacter baumannii*. *mBio*, 7(5). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/27601573>. doi:10.1128/mBio.01200-16
- Hassan, K. A., Johnson, A., Shaffer, B. T., Ren, Q., Kidarsa, T. A., Elbourne, L. D., Hartney, S., Duboy, R., Goebel, N. C., Zabriskie, T. M., Paulsen, I. T., & Loper, J. E. (2010). Inactivation of the GacA response regulator in *Pseudomonas fluorescens* Pf-5 has far-reaching transcriptomic consequences. *Environ Microbiol*, 12(4), 899-915. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/20089046>. doi:10.1111/j.1462-2920.2009.02134.x
- He, J., Baldini, R. L., Deziel, E., Saucier, M., Zhang, Q., Liberati, N. T., Lee, D., Urbach, J., Goodman, H. M., & Rahme, L. G. (2004). The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proc Natl Acad Sci U S A*, 101(8), 2530-2535. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/14983043>. doi:10.1073/pnas.0304622101
- Heeb, S., Blumer, C., & Haas, D. (2002). Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J Bacteriol*, 184(4), 1046-1056. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/11807065>. doi:10.1128/jb.184.4.1046-1056.2002
- Heidrich, C., Templin, M. F., Ursinus, A., Merdanovic, M., Berger, J., Schwarz, H., de Pedro, M. A., & Holtje, J. V. (2001). Involvement of N-acetylmuramyl-L-alanine amidases in cell separation and antibiotic-induced autolysis of *Escherichia coli*. *Mol Microbiol*, 41(1), 167-178. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/11454209>. doi:10.1046/j.1365-2958.2001.02499.x
- Heim, R., Prasher, D. C., & Tsien, R. Y. (1994). Wavelength mutations and posttranslational autooxidation of green fluorescent protein. *Proc Natl Acad Sci U S A*, 91(26), 12501-12504. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/7809066>. doi:10.1073/pnas.91.26.12501
- Hennemann, L. C., LaFayette, S. L., Malet, J. K., Bortolotti, P., Yang, T., McKay, G. A., Houle, D., Radzioch, D., Rousseau, S., & Nguyen, D. (2021). LasR-deficient *Pseudomonas aeruginosa* variants increase airway epithelial mICAM-1 expression and enhance neutrophilic lung inflammation. *PLoS Pathog*, 17(3), e1009375. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/33690714>. doi:10.1371/journal.ppat.1009375

- Hentzer, M., Wu, H., Andersen, J. B., Riedel, K., Rasmussen, T. B., Bagge, N., Kumar, N., Schembri, M. A., Song, Z., Kristoffersen, P., Manefield, M., Costerton, J. W., Molin, S., Eberl, L., Steinberg, P., Kjelleberg, S., Hoiby, N., & Givskov, M. (2003). Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J*, 22(15), 3803-3815. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/12881415>. doi:10.1093/emboj/cdg366
- Heungens, K., & Parke, J. L. (2000). Zoospore homing and infection events: effects of the biocontrol bacterium *Burkholderia cepacia* AMMDR1 on two oomycete pathogens of pea (*Pisum sativum* L.). *Appl Environ Microbiol*, 66(12), 5192-5200. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/11097889>. doi:10.1128/AEM.66.12.5192-5200.2000
- Heungens, K., & Parke, J. L. (2001). Postinfection Biological Control of Oomycete Pathogens of Pea by *Burkholderia cepacia* AMMDR1. *Phytopathology*, 91(4), 383-391. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/18943851>. doi:10.1094/PHYTO.2001.91.4.383
- Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., Camara, M., Williams, P., & Haas, D. (2004). Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol*, 186(10), 2936-2945. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/15126453>. doi:10.1128/JB.186.10.2936-2945.2004
- Hinz, A., Lee, S., Jacoby, K., & Manoil, C. (2011). Membrane proteases and aminoglycoside antibiotic resistance. *J Bacteriol*, 193(18), 4790-4797. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/21764915>. doi:10.1128/JB.05133-11
- Hmelo, L. R., Borlee, B. R., Almblad, H., Love, M. E., Randall, T. E., Tseng, B. S., Lin, C., Irie, Y., Storek, K. M., Yang, J. J., Siehnell, R. J., Howell, P. L., Singh, P. K., Tolker-Nielsen, T., Parsek, M. R., Schweizer, H. P., & Harrison, J. J. (2015). Precision-engineering the *Pseudomonas aeruginosa* genome with two-step allelic exchange. *Nat Protoc*, 10(11), 1820-1841. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/26492139>. doi:10.1038/nprot.2015.115
- Ho, B. T., Basler, M., & Mekalanos, J. J. (2013). Type 6 secretion system-mediated immunity to type 4 secretion system-mediated gene transfer. *Science*, 342(6155), 250-253. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/24115441>. doi:10.1126/science.1243745
- Holtje, J. V., Mirelman, D., Sharon, N., & Schwarz, U. (1975). Novel type of murein transglycosylase in *Escherichia coli*. *J Bacteriol*, 124(3), 1067-1076. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/357>. doi:10.1128/jb.124.3.1067-1076.1975
- Holzer, G. W., Mayrhofer, J., Gritschenberger, W., & Falkner, F. G. (2005). Dominant negative selection of vaccinia virus using a thymidine kinase/thymidylate kinase fusion gene and the prodrug azidothymidine. *Virology*, 337(2), 235-241. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/15882885>. doi:10.1016/j.virol.2005.04.009
- Hood, R. D., Singh, P., Hsu, F., Guvener, T., Carl, M. A., Trinidad, R. R., Silverman, J. M., Ohlson, B. B., Hicks, K. G., Plemel, R. L., Li, M., Schwarz, S., Wang, W. Y., Merz, A. J., Goodlett, D. R., & Mougous, J. D. (2010). A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell Host Microbe*, 7(1), 25-37. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/20114026>. doi:10.1016/j.chom.2009.12.007
- Hota, S., Hirji, Z., Stockton, K., Lemieux, C., Dedier, H., Wolfaardt, G., & Gardam, M. A. (2009). Outbreak of multidrug-resistant *Pseudomonas aeruginosa* colonization and infection secondary to imperfect intensive care unit room design. *Infect Control Hosp Epidemiol*, 30(1), 25-33. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19046054>. doi:10.1086/592700
- Howard, S. A., Furniss, R. C. D., Bonini, D., Amin, H., Paracuellos, P., Zlotkin, D., Costa, T. R. D., Levy, A., Mavridou, D. A. I., & Filloux, A. (2021). The Breadth and Molecular Basis of Hcp-Driven Type VI Secretion System Effector Delivery. *mBio*, 12(3), e0026221.

- Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/34061601>. doi:10.1128/mBio.00262-21
- Hsu, F., Schwarz, S., & Mougous, J. D. (2009).** TagR promotes PpkA-catalysed type VI secretion activation in *Pseudomonas aeruginosa*. *Mol Microbiol*, 72(5), 1111-1125. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19400797>. doi:10.1111/j.1365-2958.2009.06701.x
- Hubbard, A. K., & Rothlein, R. (2000).** Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. *Free Radic Biol Med*, 28(9), 1379-1386. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/10924857>. doi:10.1016/s0891-5849(00)00223-9
- Intile, P. J., Diaz, M. R., Urbanowski, M. L., Wolfgang, M. C., & Yahr, T. L. (2014).** The AlgZR two-component system recalibrates the RsmAYZ posttranscriptional regulatory system to inhibit expression of the *Pseudomonas aeruginosa* type III secretion system. *J Bacteriol*, 196(2), 357-366. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/24187093>. doi:10.1128/JB.01199-13
- Irie, Y., Starkey, M., Edwards, A. N., Wozniak, D. J., Romeo, T., & Parsek, M. R. (2010).** *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. *Mol Microbiol*, 78(1), 158-172. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/20735777>. doi:10.1111/j.1365-2958.2010.07320.x
- Isles, A., Maclusky, I., Corey, M., Gold, R., Prober, C., Fleming, P., & Levison, H. (1984).** *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. *J Pediatr*, 104(2), 206-210. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/6420530>. doi:10.1016/s0022-3476(84)80993-2
- Iwaya, M., & Strominger, J. L. (1977).** Simultaneous deletion of D-alanine carboxypeptidase IB-C and penicillin-binding component IV in a mutant of *Escherichia coli* K12. *Proc Natl Acad Sci U S A*, 74(7), 2980-2984. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/331323>. doi:10.1073/pnas.74.7.2980
- Jacobs, C., Frere, J. M., & Normark, S. (1997).** Cytosolic intermediates for cell wall biosynthesis and degradation control inducible beta-lactam resistance in gram-negative bacteria. *Cell*, 88(6), 823-832. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/9118225>. doi:10.1016/s0092-8674(00)81928-5
- Jacobs, C., Huang, L. J., Bartowsky, E., Normark, S., & Park, J. T. (1994).** Bacterial cell wall recycling provides cytosolic muropeptides as effectors for beta-lactamase induction. *EMBO J*, 13(19), 4684-4694. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/7925310>. doi:10.1002/j.1460-2075.1994.tb06792.x
- Jacobs, C., Joris, B., Jamin, M., Klarsov, K., Van Beeumen, J., Mengin-Lecreulx, D., van Heijenoort, J., Park, J. T., Normark, S., & Frere, J. M. (1995).** AmpD, essential for both beta-lactamase regulation and cell wall recycling, is a novel cytosolic N-acetylmuramyl-L-alanine amidase. *Mol Microbiol*, 15(3), 553-559. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/7783625>. doi:10.1111/j.1365-2958.1995.tb02268.x
- Jacoby, G. A. (2009).** AmpC beta-lactamases. *Clin Microbiol Rev*, 22(1), 161-182, Table of Contents. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19136439>. doi:10.1128/CMR.00036-08
- Jiang, F., Wang, X., Wang, B., Chen, L., Zhao, Z., Waterfield, N. R., Yang, G., & Jin, Q. (2016).** The *Pseudomonas aeruginosa* Type VI Secretion PGAP1-like Effector Induces Host Autophagy by Activating Endoplasmic Reticulum Stress. *Cell Rep*, 16(6), 1502-1509. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/27477276>. doi:10.1016/j.celrep.2016.07.012
- Jiang, F., Waterfield, N. R., Yang, J., Yang, G., & Jin, Q. (2014).** A *Pseudomonas aeruginosa* type VI secretion phospholipase D effector targets both prokaryotic and eukaryotic

- cells. *Cell Host Microbe*, 15(5), 600-610. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/24832454>. doi:10.1016/j.chom.2014.04.010
- Jones, A. M., Dodd, M. E., Govan, J. R., Barcus, V., Doherty, C. J., Morris, J., & Webb, A. K. (2004).** *Burkholderia cenocepacia* and *Burkholderia multivorans*: influence on survival in cystic fibrosis. *Thorax*, 59(11), 948-951. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/15516469>. doi:10.1136/thx.2003.017210
- Joshi, R. V., Gunawan, C., & Mann, R. (2021).** We Are One: Multispecies Metabolism of a Biofilm Consortium and Their Treatment Strategies. *Front Microbiol*, 12, 635432. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/33584635>. doi:10.3389/fmicb.2021.635432
- Juan, C., Moya, B., Perez, J. L., & Oliver, A. (2006).** Stepwise upregulation of the *Pseudomonas aeruginosa* chromosomal cephalosporinase conferring high-level beta-lactam resistance involves three AmpD homologues. *Antimicrob Agents Chemother*, 50(5), 1780-1787. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/16641450>. doi:10.1128/AAC.50.5.1780-1787.2006
- Kamal, F., Liang, X., Manera, K., Pei, T. T., Kim, H., Lam, L. G., Pun, A., Hersch, S. J., & Dong, T. G. (2020).** Differential Cellular Response to Translocated Toxic Effectors and Physical Penetration by the Type VI Secretion System. *Cell Rep*, 31(11), 107766. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/32553162>. doi:10.1016/j.celrep.2020.107766
- Keilin, D. (1929).** Cytochrome and respiratory enzymes. *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character*, 104(730), 206-252. Retrieved from <https://royalsocietypublishing.org/doi/abs/10.1098/rspb.1929.0009>. doi:doi:10.1098/rspb.1929.0009
- Kerem, B., Rommens, J. M., Buchanan, J. A., Markiewicz, D., Cox, T. K., Chakravarti, A., Buchwald, M., & Tsui, L. C. (1989).** Identification of the cystic fibrosis gene: genetic analysis. *Science*, 245(4922), 1073-1080. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/2570460>. doi:10.1126/science.2570460
- Kielhofner, M., Atmar, R. L., Hamill, R. J., & Musher, D. M. (1992).** Life-threatening *Pseudomonas aeruginosa* infections in patients with human immunodeficiency virus infection. *Clin Infect Dis*, 14(2), 403-411. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/1554824>. doi:10.1093/clinids/14.2.403
- Kilbane, J. J., Chatterjee, D. K., Karns, J. S., Kellogg, S. T., & Chakrabarty, A. M. (1982).** Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of *Pseudomonas cepacia*. *Appl Environ Microbiol*, 44(1), 72-78. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/7125648>. doi:10.1128/aem.44.1.72-78.1982
- Kim, H. S., Park, B. K., Kim, S. K., Han, S. B., Lee, J. W., Lee, D. G., Chung, N. G., Cho, B., Jeong, D. C., & Kang, J. H. (2017).** Clinical characteristics and outcomes of *Pseudomonas aeruginosa* bacteremia in febrile neutropenic children and adolescents with the impact of antibiotic resistance: a retrospective study. *BMC Infect Dis*, 17(1), 500. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/28716109>. doi:10.1186/s12879-017-2597-0
- Kitzis, M. D., Ferre, B., Coutrot, A., Acar, J. F., & Gutmann, L. (1989).** In vitro activity of combinations of beta-lactam antibiotics with beta-lactamase inhibitors against cephalosporinase-producing bacteria. *Eur J Clin Microbiol Infect Dis*, 8(9), 783-788. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/2556277>. doi:10.1007/BF02185845
- Klein, K., Sonnabend, M. S., Frank, L., Leibiger, K., Franz-Wachtel, M., Macek, B., Trunk, T., Leo, J. C., Autenrieth, I. B., Schutz, M., & Bohn, E. (2019).** Deprivation of the Periplasmic Chaperone SurA Reduces Virulence and Restores Antibiotic Susceptibility of Multidrug-Resistant *Pseudomonas aeruginosa*. *Front Microbiol*, 10, 100. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30846971>. doi:10.3389/fmicb.2019.00100
- Kohler, T., Michea-Hamzehpour, M., Henze, U., Gotoh, N., Curty, L. K., & Pechere, J. C. (1997).** Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. *Mol Microbiol*, 23(2), 345-354. Retrieved from

- <https://www.ncbi.nlm.nih.gov/pubmed/9044268>. doi:10.1046/j.1365-2958.1997.2281594.x
- Kong, W., Chen, L., Zhao, J., Shen, T., Surette, M. G., Shen, L., & Duan, K. (2013).** Hybrid sensor kinase PA1611 in *Pseudomonas aeruginosa* regulates transitions between acute and chronic infection through direct interaction with RetS. *Mol Microbiol*, 88(4), 784-797. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/23560772>. doi:10.1111/mmi.12223
- Korat, B., Mottl, H., & Keck, W. (1991).** Penicillin-binding protein 4 of *Escherichia coli*: molecular cloning of the *dacB* gene, controlled overexpression, and alterations in murein composition. *Mol Microbiol*, 5(3), 675-684. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/2046551>. doi:10.1111/j.1365-2958.1991.tb00739.x
- Korch, S. B., Henderson, T. A., & Hill, T. M. (2003).** Characterization of the *hipA7* allele of *Escherichia coli* and evidence that high persistence is governed by (p)ppGpp synthesis. *Mol Microbiol*, 50(4), 1199-1213. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/14622409>. doi:10.1046/j.1365-2958.2003.03779.x
- LaCourse, K. D., Peterson, S. B., Kulasekara, H. D., Radey, M. C., Kim, J., & Mougous, J. D. (2018).** Conditional toxicity and synergy drive diversity among antibacterial effectors. *Nat Microbiol*, 3(4), 440-446. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/29459733>. doi:10.1038/s41564-018-0113-y
- Lamarche, M. G., & Deziel, E. (2011).** MexEF-OprN efflux pump exports the *Pseudomonas* quinolone signal (PQS) precursor HHQ (4-hydroxy-2-heptylquinoline). *PLoS One*, 6(9), e24310. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/21957445>. doi:10.1371/journal.pone.0024310
- Lamas Ferreiro, J. L., Alvarez Otero, J., Gonzalez Gonzalez, L., Novoa Lamazares, L., Arca Blanco, A., Bermudez Sanjurjo, J. R., Rodriguez Conde, I., Fernandez Soneira, M., & de la Fuente Aguado, J. (2017).** *Pseudomonas aeruginosa* urinary tract infections in hospitalized patients: Mortality and prognostic factors. *PLoS One*, 12(5), e0178178. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/28552972>. doi:10.1371/journal.pone.0178178
- Langridge, G. C., Phan, M. D., Turner, D. J., Perkins, T. T., Parts, L., Haase, J., Charles, I., Maskell, D. J., Peters, S. E., Dougan, G., Wain, J., Parkhill, J., & Turner, A. K. (2009).** Simultaneous assay of every *Salmonella* Typhi gene using one million transposon mutants. *Genome Res*, 19(12), 2308-2316. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19826075>. doi:10.1101/gr.097097.109
- Laskowski, M. A., Osborn, E., & Kazmierczak, B. I. (2004).** A novel sensor kinase-response regulator hybrid regulates type III secretion and is required for virulence in *Pseudomonas aeruginosa*. *Molecular Microbiology*, 54(4), 1090-1103. Retrieved from <https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2958.2004.04331.x>. doi:<https://doi.org/10.1111/j.1365-2958.2004.04331.x>
- Latifi, A., Foglino, M., Tanaka, K., Williams, P., & Lazdunski, A. (1996).** A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol Microbiol*, 21(6), 1137-1146. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/8898383>. doi:10.1046/j.1365-2958.1996.00063.x
- Lau, C. H., Krahn, T., Gilmour, C., Mullen, E., & Poole, K. (2015).** AmgRS-mediated envelope stress-inducible expression of the *mexXY* multidrug efflux operon of *Pseudomonas aeruginosa*. *Microbiologyopen*, 4(1), 121-135. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/25450797>. doi:10.1002/mbo3.226
- Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago, G., & Haas, D. (1992).** Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proc Natl Acad Sci U S A*, 89(5), 1562-1566. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/1311842>. doi:10.1073/pnas.89.5.1562

- Ledson, M. J., Gallagher, M. J., Jackson, M., Hart, C. A., & Walshaw, M. J. (2002).** Outcome of *Burkholderia cepacia* colonisation in an adult cystic fibrosis centre. *Thorax*, 57(2), 142-145. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/11828044>. doi:10.1136/thorax.57.2.142
- Lee, D. G., Urbach, J. M., Wu, G., Liberati, N. T., Feinbaum, R. L., Miyata, S., Diggins, L. T., He, J., Saucier, M., Deziel, E., Friedman, L., Li, L., Grills, G., Montgomery, K., Kucherlapati, R., Rahme, L. G., & Ausubel, F. M. (2006).** Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. *Genome Biol*, 7(10), R90. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/17038190>. doi:10.1186/gb-2006-7-10-r90
- Lee, M., Artola-Recolons, C., Carrasco-Lopez, C., Martinez-Caballero, S., Heseck, D., Spink, E., Lastochkin, E., Zhang, W., Hellman, L. M., Boggess, B., Hermoso, J. A., & Mobashery, S. (2013).** Cell-wall remodeling by the zinc-protease AmpDh3 from *Pseudomonas aeruginosa*. *J Am Chem Soc*, 135(34), 12604-12607. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/23931161>. doi:10.1021/ja407445x
- Lee, M., Zhang, W., Heseck, D., Noll, B. C., Boggess, B., & Mobashery, S. (2009).** Bacterial AmpD at the crossroads of peptidoglycan recycling and manifestation of antibiotic resistance. *J Am Chem Soc*, 131(25), 8742-8743. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19496566>. doi:10.1021/ja9025566
- Lee, S., Hinz, A., Bauerle, E., Angermeyer, A., Juhaszova, K., Kaneko, Y., Singh, P. K., & Manoil, C. (2009).** Targeting a bacterial stress response to enhance antibiotic action. *Proc Natl Acad Sci U S A*, 106(34), 14570-14575. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19706543>. doi:10.1073/pnas.0903619106
- Leiman, P. G., Basler, M., Ramagopal, U. A., Bonanno, J. B., Sauder, J. M., Pukatzki, S., Burley, S. K., Almo, S. C., & Mekalanos, J. J. (2009).** Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. *Proc Natl Acad Sci U S A*, 106(11), 4154-4159. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19251641>. doi:10.1073/pnas.0813360106
- LeRoux, M., Kirkpatrick, R. L., Montauti, E. I., Tran, B. Q., Peterson, S. B., Harding, B. N., Whitney, J. C., Russell, A. B., Traxler, B., Goo, Y. A., Goodlett, D. R., Wiggins, P. A., & Mougous, J. D. (2015).** Kin cell lysis is a danger signal that activates antibacterial pathways of *Pseudomonas aeruginosa*. *Elife*, 4. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/25643398>. doi:10.7554/eLife.05701
- Letoffe, S., Wu, Y., Darch, S. E., Beloin, C., Whiteley, M., Touqui, L., & Ghigo, J. M. (2022).** *Pseudomonas aeruginosa* Production of Hydrogen Cyanide Leads to Airborne Control of *Staphylococcus aureus* Growth in Biofilm and In Vivo Lung Environments. *mBio*, 13(5), e0215422. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/36129311>. doi:10.1128/mbio.02154-22
- Li, J., Yao, Y., Xu, H. H., Hao, L., Deng, Z., Rajakumar, K., & Ou, H. Y. (2015).** SecReT6: a web-based resource for type VI secretion systems found in bacteria. *Environ Microbiol*, 17(7), 2196-2202. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/25640659>. doi:10.1111/1462-2920.12794
- Li, X. Z., Nikaido, H., & Poole, K. (1995).** Role of *mexA-mexB-oprM* in antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*, 39(9), 1948-1953. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/8540696>. doi:10.1128/AAC.39.9.1948
- Liebl, D., Robert-Genthon, M., Job, V., Cogoni, V., & Attree, I. (2019).** Baseplate Component TssK and Spatio-Temporal Assembly of T6SS in *Pseudomonas aeruginosa*. *Front Microbiol*, 10, 1615. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/31379775>. doi:10.3389/fmicb.2019.01615
- Lin, J., Zhang, W., Cheng, J., Yang, X., Zhu, K., Wang, Y., Wei, G., Qian, P. Y., Luo, Z. Q., & Shen, X. (2017).** A *Pseudomonas* T6SS effector recruits PQS-containing outer membrane vesicles for iron acquisition. *Nat Commun*, 8, 14888. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/28348410>. doi:10.1038/ncomms14888
- Linares, J. F., Moreno, R., Fajardo, A., Martinez-Solano, L., Escalante, R., Rojo, F., & Martinez, J. L. (2010).** The global regulator Crc modulates metabolism, susceptibility to antibiotics and virulence in *Pseudomonas aeruginosa*. *Environ Microbiol*, 12(12),

- 3196-3212. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/20626455>. doi:10.1111/j.1462-2920.2010.02292.x
- Linker, A., & Jones, R. S. (1966).** A new polysaccharide resembling alginic acid isolated from pseudomonads. *J Biol Chem*, 241(16), 3845-3851. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/5916397>.
- Lister, P. D., Wolter, D. J., & Hanson, N. D. (2009).** Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin Microbiol Rev*, 22(4), 582-610. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19822890>. doi:10.1128/CMR.00040-09
- Liu, M. Y., & Romeo, T. (1997).** The global regulator CsrA of *Escherichia coli* is a specific mRNA-binding protein. *J Bacteriol*, 179(14), 4639-4642. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/9226279>. doi:10.1128/jb.179.14.4639-4642.1997
- Livermore, D. M. (1995).** beta-Lactamases in laboratory and clinical resistance. *Clin Microbiol Rev*, 8(4), 557-584. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/8665470>. doi:10.1128/CMR.8.4.557
- Llanos, A., Achard, P., Bousquet, J., Lozano, C., Zalacain, M., Sable, C., Revillet, H., Murris, M., Mittaine, M., Lemonnier, M., & Everett, M. (2023).** Higher levels of *Pseudomonas aeruginosa* LasB elastase expression are associated with early-stage infection in cystic fibrosis patients. *Sci Rep*, 13(1), 14208. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/37648735>. doi:10.1038/s41598-023-41333-9
- Lossi, N. S., Manoli, E., Simpson, P., Jones, C., Hui, K., Dajani, R., Coulthurst, S. J., Freemont, P., & Filloux, A. (2012).** The archetype *Pseudomonas aeruginosa* proteins TssB and TagJ form a novel subcomplex in the bacterial type VI secretion system. *Mol Microbiol*, 86(2), 437-456. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/22906320>. doi:10.1111/j.1365-2958.2012.08204.x
- Madan, J. C., Koestler, D. C., Stanton, B. A., Davidson, L., Moulton, L. A., Housman, M. L., Moore, J. H., Guill, M. F., Morrison, H. G., Sogin, M. L., Hampton, T. H., Karagas, M. R., Palumbo, P. E., Foster, J. A., Hibberd, P. L., & O'Toole, G. A. (2012).** Serial analysis of the gut and respiratory microbiome in cystic fibrosis in infancy: interaction between intestinal and respiratory tracts and impact of nutritional exposures. *mBio*, 3(4). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/22911969>. doi:10.1128/mBio.00251-12
- Magill, S. S., Edwards, J. R., Bamberg, W., Beldavs, Z. G., Dumyati, G., Kainer, M. A., Lynfield, R., Maloney, M., McAllister-Hollod, L., Nadle, J., Ray, S. M., Thompson, D. L., Wilson, L. E., Fridkin, S. K., Emerging Infections Program Healthcare-Associated, I., & Antimicrobial Use Prevalence Survey, T. (2014).** Multistate point-prevalence survey of health care-associated infections. *N Engl J Med*, 370(13), 1198-1208. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/24670166>. doi:10.1056/NEJMoa1306801
- Mangan, M. W., Lucchini, S., Danino, V., Croinin, T. O., Hinton, J. C., & Dorman, C. J. (2006).** The integration host factor (IHF) integrates stationary-phase and virulence gene expression in *Salmonella enterica* serovar Typhimurium. *Mol Microbiol*, 59(6), 1831-1847. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/16553887>. doi:10.1111/j.1365-2958.2006.05062.x
- Maredia, R., Devineni, N., Lentz, P., Dallo, S. F., Yu, J., Guentzel, N., Chambers, J., Arulanandam, B., Haskins, W. E., & Weitao, T. (2012).** Vesiculation from *Pseudomonas aeruginosa* under SOS. *ScientificWorldJournal*, 2012, 402919. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/22448133>. doi:10.1100/2012/402919
- Marsland, B. J., & Gollwitzer, E. S. (2014).** Host-microorganism interactions in lung diseases. *Nat Rev Immunol*, 14(12), 827-835. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/25421702>. doi:10.1038/nri3769

- Marvig, R. L., Sommer, L. M., Molin, S., & Johansen, H. K. (2015).** Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. *Nature Genetics*, 47(1), 57-64. Retrieved from <https://doi.org/10.1038/ng.3148>. doi:10.1038/ng.3148
- Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H., & Nishino, T. (2000).** Contribution of the MexX-MexY-oprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*, 44(9), 2242-2246. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/10952562>. doi:10.1128/AAC.44.9.2242-2246.2000
- Matsubashi, M., Takagaki, Y., Maruyama, I. N., Tamaki, S., Nishimura, Y., Suzuki, H., Ogino, U., & Hirota, Y. (1977).** Mutants of *Escherichia coli* lacking in highly penicillin-sensitive D-alanine carboxypeptidase activity. *Proc Natl Acad Sci U S A*, 74(7), 2976-2979. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/331322>. doi:10.1073/pnas.74.7.2976
- McFarland, K. A., Dolben, E. L., LeRoux, M., Kambara, T. K., Ramsey, K. M., Kirkpatrick, R. L., Mougous, J. D., Hogan, D. A., & Dove, S. L. (2015).** A self-lysis pathway that enhances the virulence of a pathogenic bacterium. *Proc Natl Acad Sci U S A*, 112(27), 8433-8438. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/26100878>. doi:10.1073/pnas.1506299112
- McGrath, S., Wade, D. S., & Pesci, E. C. (2004).** Dueling quorum sensing systems in *Pseudomonas aeruginosa* control the production of the *Pseudomonas* quinolone signal (PQS). *FEMS Microbiol Lett*, 230(1), 27-34. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/14734162>. doi:10.1016/S0378-1097(03)00849-8
- McKnight, S. L., Iglewski, B. H., & Pesci, E. C. (2000).** The *Pseudomonas* quinolone signal regulates *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol*, 182(10), 2702-2708. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/10781536>. doi:10.1128/JB.182.10.2702-2708.2000
- Mena, A., Smith, E. E., Burns, J. L., Speert, D. P., Moskowitz, S. M., Perez, J. L., & Oliver, A. (2008).** Genetic adaptation of *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients is catalyzed by hypermutation. *J Bacteriol*, 190(24), 7910-7917. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/18849421>. doi:10.1128/JB.01147-08
- Mikkelsen, H., McMullan, R., & Filloux, A. (2011).** The *Pseudomonas aeruginosa* reference strain PA14 displays increased virulence due to a mutation in *ladS*. *PLoS One*, 6(12), e29113. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/22216178>. doi:10.1371/journal.pone.0029113
- Mine, T., Morita, Y., Kataoka, A., Mizushima, T., & Tsuchiya, T. (1999).** Expression in *Escherichia coli* of a new multidrug efflux pump, MexXY, from *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*, 43(2), 415-417. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/9925549>. doi:10.1128/AAC.43.2.415
- Mohr, C. D., & Deretic, V. (1990).** Gene-scrambling mutagenesis: generation and analysis of insertional mutations in the alginate regulatory region of *Pseudomonas aeruginosa*. *J Bacteriol*, 172(11), 6252-6260. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/2121708>. doi:10.1128/jb.172.11.6252-6260.1990
- Moore, R. A., & Hancock, R. E. (1986).** Involvement of outer membrane of *Pseudomonas cepacia* in aminoglycoside and polymyxin resistance. *Antimicrob Agents Chemother*, 30(6), 923-926. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/3028253>. doi:10.1128/AAC.30.6.923
- Moreno, R., & Rojo, F. (2008).** The target for the *Pseudomonas putida* Crc global regulator in the benzoate degradation pathway is the BenR transcriptional regulator. *J Bacteriol*, 190(5), 1539-1545. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/18156252>. doi:10.1128/JB.01604-07
- Moreno, R., Ruiz-Manzano, A., Yuste, L., & Rojo, F. (2007).** The *Pseudomonas putida* Crc global regulator is an RNA binding protein that inhibits translation of the AlkS

- transcriptional regulator. *Mol Microbiol*, 64(3), 665-675. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/17462015>. doi:10.1111/j.1365-2958.2007.05685.x
- Morita, Y., Cao, L., Gould, V. C., Avison, M. B., & Poole, K. (2006).** *nalD* encodes a second repressor of the *mexAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*. *J Bacteriol*, 188(24), 8649-8654. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/17028276>. doi:10.1128/JB.01342-06
- Morita, Y., Tomida, J., & Kawamura, Y. (2012).** Primary mechanisms mediating aminoglycoside resistance in the multidrug-resistant *Pseudomonas aeruginosa* clinical isolate PA7. *Microbiology (Reading)*, 158(Pt 4), 1071-1083. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/22282519>. doi:10.1099/mic.0.054320-0
- Moscato, J. A., Mikkelsen, H., Heeb, S., Williams, P., & Filloux, A. (2011).** The *Pseudomonas aeruginosa* sensor RetS switches Type III and Type VI secretion via c-di-GMP signalling. *Environmental Microbiology*, 13(12), 3128-3138. Retrieved from <https://enviromicro-journals.onlinelibrary.wiley.com/doi/abs/10.1111/j.1462-2920.2011.02595.x>. doi:https://doi.org/10.1111/j.1462-2920.2011.02595.x
- Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., Goodman, A. L., Joachimiak, G., Ordóñez, C. L., Lory, S., Walz, T., Joachimiak, A., & Mekalanos, J. J. (2006).** A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science*, 312(5779), 1526-1530. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/16763151>. doi:10.1126/science.1128393
- Mougous, J. D., Gifford, C. A., Ramsdell, T. L., & Mekalanos, J. J. (2007).** Threonine phosphorylation post-translationally regulates protein secretion in *Pseudomonas aeruginosa*. *Nat Cell Biol*, 9(7), 797-803. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/17558395>. doi:10.1038/ncb1605
- Moya, B., Dotsch, A., Juan, C., Blázquez, J., Zamorano, L., Haussler, S., & Oliver, A. (2009).** Beta-lactam resistance response triggered by inactivation of a nonessential penicillin-binding protein. *PLoS Pathog*, 5(3), e1000353. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19325877>. doi:10.1371/journal.ppat.1000353
- Moyed, H. S., & Bertrand, K. P. (1983).** *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *J Bacteriol*, 155(2), 768-775. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/6348026>. doi:10.1128/jb.155.2.768-775.1983
- Mulcahy, H., O'Callaghan, J., O'Grady, E. P., Adams, C., & O'Gara, F. (2006).** The posttranscriptional regulator RsmA plays a role in the interaction between *Pseudomonas aeruginosa* and human airway epithelial cells by positively regulating the type III secretion system. *Infect Immun*, 74(5), 3012-3015. doi:10.1128/iai.74.5.3012-3015.2006
- Mulcahy, H., O'Callaghan, J., O'Grady, E. P., Macia, M. D., Borrell, N., Gomez, C., Casey, P. G., Hill, C., Adams, C., Gahan, C. G., Oliver, A., & O'Gara, F. (2008).** *Pseudomonas aeruginosa* RsmA plays an important role during murine infection by influencing colonization, virulence, persistence, and pulmonary inflammation. *Infect Immun*, 76(2), 632-638. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/18025099>. doi:10.1128/IAI.01132-07
- Mulcahy, L. R., Burns, J. L., Lory, S., & Lewis, K. (2010).** Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. *J Bacteriol*, 192(23), 6191-6199. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/20935098>. doi:10.1128/JB.01651-09
- Nährlich L., e., M., B., & J., W. (2022).** *German Cystic Fibrosis Registry – Annual Report 2021*. Retrieved from https://www.muko.info/fileadmin/user_upload/was_wir_tun/register/berichtsbaende/annual_report_2021.pdf

- Narang, A., & Pilyugin, S. S. (2007).** Bacterial gene regulation in diauxic and non-diauxic growth. *J Theor Biol*, 244(2), 326-348. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/16989865>. doi:10.1016/j.jtbi.2006.08.007
- Natalini, J. G., Singh, S., & Segal, L. N. (2023).** The dynamic lung microbiome in health and disease. *Nat Rev Microbiol*, 21(4), 222-235. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/36385637>. doi:10.1038/s41579-022-00821-x
- Nazarov, S., Schneider, J. P., Brackmann, M., Goldie, K. N., Stahlberg, H., & Basler, M. (2018).** Cryo-EM reconstruction of Type VI secretion system baseplate and sheath distal end. *EMBO J*, 37(4). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/29255010>. doi:10.15252/embj.201797103
- Nolan, L. M., Cain, A. K., Clamens, T., Furniss, R. C. D., Manoli, E., Sainz-Polo, M. A., Dougan, G., Albesa-Jove, D., Parkhill, J., Mavridou, D. A. I., & Filloux, A. (2021).** Identification of Tse8 as a Type VI secretion system toxin from *Pseudomonas aeruginosa* that targets the bacterial transamidosome to inhibit protein synthesis in prey cells. *Nat Microbiol*, 6(9), 1199-1210. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/34413503>. doi:10.1038/s41564-021-00950-8
- Nolan, L. M., Whitchurch, C. B., Barquist, L., Katrib, M., Boinett, C. J., Mayho, M., Goulding, D., Charles, I. G., Filloux, A., Parkhill, J., & Cain, A. K. (2018).** A global genomic approach uncovers novel components for twitching motility-mediated biofilm expansion in *Pseudomonas aeruginosa*. *Microb Genom*, 4(11). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30383525>. doi:10.1099/mgen.0.000229
- Oliveira, W. K., Ferrarini, M., Morello, L. G., & Faoro, H. (2020).** Resistome analysis of bloodstream infection bacterial genomes reveals a specific set of proteins involved in antibiotic resistance and drug efflux. *NAR Genom Bioinform*, 2(3), lqaa055. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/33575606>. doi:10.1093/nargab/lqaa055
- Ong, K. S., Cheow, Y. L., & Lee, S. M. (2017).** The role of reactive oxygen species in the antimicrobial activity of pyochelin. *J Adv Res*, 8(4), 393-398. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/28580180>. doi:10.1016/j.jare.2017.05.007
- Pais, S. V., Westerhausen, S., Bohn, E., & Wagner, S. (2022).** Analysis of SPI-1 Dependent Type III Secretion and Injection Using a NanoLuc Luciferase-Based Assay. *Methods Mol Biol*, 2427, 57-71. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/35619025>. doi:10.1007/978-1-0716-1971-1_6
- Park, J. T., & Uehara, T. (2008).** How bacteria consume their own exoskeletons (turnover and recycling of cell wall peptidoglycan). *Microbiol Mol Biol Rev*, 72(2), 211-227, table of contents. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/18535144>. doi:10.1128/MMBR.00027-07
- Park, Y. J., Lacourse, K. D., Cambillau, C., DiMaio, F., Mougous, J. D., & Veasler, D. (2018).** Structure of the type VI secretion system TssK-TssF-TssG baseplate subcomplex revealed by cryo-electron microscopy. *Nat Commun*, 9(1), 5385. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30568167>. doi:10.1038/s41467-018-07796-5
- Parkins, M. D., Ceri, H., & Storey, D. G. (2001).** *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. *Mol Microbiol*, 40(5), 1215-1226. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/11401724>. doi:10.1046/j.1365-2958.2001.02469.x
- Passador, L., Cook, J. M., Gambello, M. J., Rust, L., & Iglewski, B. H. (1993).** Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science*, 260(5111), 1127-1130. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/8493556>. doi:10.1126/science.8493556
- Paul, S. I., Rahman, M. M., Salam, M. A., Khan, M. A. R., & Islam, M. T. (2021).** Identification of marine sponge-associated bacteria of the Saint Martin's island of the Bay of Bengal emphasizing on the prevention of motile *Aeromonas septicemia* in *Labeo rohita*. *Aquaculture*, 545, 737156. Retrieved from

- <https://www.sciencedirect.com/science/article/pii/S004484862100819X>.
doi:<https://doi.org/10.1016/j.aquaculture.2021.737156>
- Paysan-Lafosse, T., Blum, M., Chuguransky, S., Grego, T., Pinto, B. L., Salazar, G. A., Bileschi, M. L., Bork, P., Bridge, A., Colwell, L., Gough, J., Haft, D. H., Letunic, I., Marchler-Bauer, A., Mi, H., Natale, D. A., Orengo, C. A., Pandurangan, A. P., Rivoire, C., Sigrist, C. J. A., Sillitoe, I., Thanki, N., Thomas, P. D., Tosatto, S. C. E., Wu, C. H., & Bateman, A. (2023).** InterPro in 2022. *Nucleic Acids Res*, 51(D1), D418-D427. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/36350672>. doi:10.1093/nar/gkac993
- Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H., & Greenberg, E. P. (1994).** Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci U S A*, 91(1), 197-201. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/8278364>. doi:10.1073/pnas.91.1.197
- Pearson, J. P., Passador, L., Iglewski, B. H., & Greenberg, E. P. (1995).** A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A*, 92(5), 1490-1494. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/7878006>. doi:10.1073/pnas.92.5.1490
- Pedersen, S. S., Kharazmi, A., Espersen, F., & Hoiby, N. (1990).** *Pseudomonas aeruginosa* alginate in cystic fibrosis sputum and the inflammatory response. *Infect Immun*, 58(10), 3363-3368. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/2401567>. doi:10.1128/iai.58.10.3363-3368.1990
- Peña, J. M., Prezioso, S. M., McFarland, K. A., Kambara, T. K., Ramsey, K. M., Deighan, P., & Dove, S. L. (2021).** Control of a programmed cell death pathway in *Pseudomonas aeruginosa* by an antiterminator. *Nat Commun*, 12(1), 1702. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/33731715>. doi:10.1038/s41467-021-21941-7
- Perault, A. I., Chandler, C. E., Rasko, D. A., Ernst, R. K., Wolfgang, M. C., & Cotter, P. A. (2020).** Host Adaptation Predisposes *Pseudomonas aeruginosa* to Type VI Secretion System-Mediated Predation by the *Burkholderia cepacia* Complex. *Cell Host Microbe*, 28(4), 534-547. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/32755549>. doi:10.1016/j.chom.2020.06.019
- Pesci, E. C., Milbank, J. B., Pearson, J. P., McKnight, S., Kende, A. S., Greenberg, E. P., & Iglewski, B. H. (1999).** Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A*, 96(20), 11229-11234. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/10500159>. doi:10.1073/pnas.96.20.11229
- Pesci, E. C., Pearson, J. P., Seed, P. C., & Iglewski, B. H. (1997).** Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol*, 179(10), 3127-3132. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/9150205>. doi:10.1128/jb.179.10.3127-3132.1997
- Pessi, G., & Haas, D. (2000).** Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*. *J Bacteriol*, 182(24), 6940-6949. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/11092854>. doi:10.1128/JB.182.24.6940-6949.2000
- Pier, G. B., Grout, M., & Zaidi, T. S. (1997).** Cystic fibrosis transmembrane conductance regulator is an epithelial cell receptor for clearance of *Pseudomonas aeruginosa* from the lung. *Proc Natl Acad Sci U S A*, 94(22), 12088-12093. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/9342367>. doi:10.1073/pnas.94.22.12088
- Pissaridou, P., Allsopp, L. P., Wettstadt, S., Howard, S. A., Mavridou, D. A. I., & Filloux, A. (2018).** The *Pseudomonas aeruginosa* T6SS-VgrG1b spike is topped by a PAAR protein eliciting DNA damage to bacterial competitors. *Proc Natl Acad Sci U S A*, 115(49), 12519-12524. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30455305>. doi:10.1073/pnas.1814181115

- Poole, K., Hay, T., Gilmour, C., & Fruci, M. (2019). The aminoglycoside resistance-promoting AmgRS envelope stress-responsive two-component system in *Pseudomonas aeruginosa* is zinc-activated and protects cells from zinc-promoted membrane damage. *Microbiology (Reading)*, 165(5), 563-571. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30835196>. doi:10.1099/mic.0.000787
- Poole, K., Krebes, K., McNally, C., & Neshat, S. (1993). Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *J Bacteriol*, 175(22), 7363-7372. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/8226684>. doi:10.1128/jb.175.22.7363-7372.1993
- Poulsen, B. E., Yang, R., Clatworthy, A. E., White, T., Osmulski, S. J., Li, L., Penaranda, C., Lander, E. S., Shores, N., & Hung, D. T. (2019). Defining the core essential genome of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A*, 116(20), 10072-10080. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/31036669>. doi:10.1073/pnas.1900570116
- Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D., & Mekalanos, J. J. (2007). Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proc Natl Acad Sci U S A*, 104(39), 15508-15513. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/17873062>. doi:10.1073/pnas.0706532104
- Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., Heidelberg, J. F., & Mekalanos, J. J. (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proc Natl Acad Sci U S A*, 103(5), 1528-1533. doi:10.1073/pnas.0510322103
- Ramsay, K. A., Rehman, A., Wardell, S. T., Martin, L. W., Bell, S. C., Patrick, W. M., Winstanley, C., & Lamont, I. L. (2023). Ceftazidime resistance in *Pseudomonas aeruginosa* is multigenic and complex. *PLoS One*, 18(5), e0285856. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/37192202>. doi:10.1371/journal.pone.0285856
- Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A., & Haas, D. (1997). The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol Microbiol*, 24(2), 309-319. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/9159518>. doi:10.1046/j.1365-2958.1997.3291701.x
- Reis, V. M., Baldani, J. I., Baldani, V. L. D., & Dobereiner, J. (2010). Biological Nitrogen Fixation in Gramineae and Palm Trees. *Critical Reviews in Plant Sciences*, 19(3), 171-266. doi:10.1080/07352680091139213
- Rich, D. P., Anderson, M. P., Gregory, R. J., Cheng, S. H., Paul, S., Jefferson, D. M., McCann, J. D., Klinger, K. W., Smith, A. E., & Welsh, M. J. (1990). Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. *Nature*, 347(6291), 358-363. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/1699126>. doi:10.1038/347358a0
- Rich, J. J., Kinscherf, T. G., Kitten, T., & Willis, D. K. (1994). Genetic evidence that the gacA gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. *J Bacteriol*, 176(24), 7468-7475. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/8002569>. doi:10.1128/jb.176.24.7468-7475.1994
- Rietsch, A., Vallet-Gely, I., Dove, S. L., & Mekalanos, J. J. (2005). ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A*, 102(22), 8006-8011. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/15911752>. doi:10.1073/pnas.0503005102
- Rogues, A. M., Boulestreau, H., Lasheras, A., Boyer, A., Gruson, D., Merle, C., Castaing, Y., Bebear, C. M., & Gachie, J. P. (2007). Contribution of tap water to patient colonisation with *Pseudomonas aeruginosa* in a medical intensive care unit. *J Hosp Infect*, 67(1), 72-78. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/17728016>. doi:10.1016/j.jhin.2007.06.019

- Ropy, A., Cabot, G., Sanchez-Diener, I., Aguilera, C., Moya, B., Ayala, J. A., & Oliver, A. (2015).** Role of *Pseudomonas aeruginosa* low-molecular-mass penicillin-binding proteins in AmpC expression, beta-lactam resistance, and peptidoglycan structure. *Antimicrob Agents Chemother*, 59(7), 3925-3934. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/25896695>. doi:10.1128/AAC.05150-14
- Rosenfeld, M., Gibson, R. L., McNamara, S., Emerson, J., Burns, J. L., Castile, R., Hiatt, P., McCoy, K., Wilson, C. B., Inglis, A., Smith, A., Martin, T. R., & Ramsey, B. W. (2001).** Early pulmonary infection, inflammation, and clinical outcomes in infants with cystic fibrosis. *Pediatr Pulmonol*, 32(5), 356-366. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/11596160>. doi:10.1002/ppul.1144
- Ross, B. D., Verster, A. J., Radey, M. C., Schmidtke, D. T., Pope, C. E., Hoffman, L. R., Hajjar, A. M., Peterson, S. B., Borenstein, E., & Mougous, J. D. (2019).** Human gut bacteria contain acquired interbacterial defence systems. *Nature*, 575(7781), 224-228. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/31666699>. doi:10.1038/s41586-019-1708-z
- Rudzite, M., Subramoni, S., Endres, R. G., & Filloux, A. (2023).** Effectiveness of *Pseudomonas aeruginosa* type VI secretion system relies on toxin potency and type IV pili-dependent interaction. *PLoS Pathog*, 19(5), e1011428. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/37253075>. doi:10.1371/journal.ppat.1011428
- Russell, A. B., Hood, R. D., Bui, N. K., LeRoux, M., Vollmer, W., & Mougous, J. D. (2011).** Type VI secretion delivers bacteriolytic effectors to target cells. *Nature*, 475(7356), 343-347. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/21776080>. doi:10.1038/nature10244
- Russell, A. B., LeRoux, M., Hathazi, K., Agnello, D. M., Ishikawa, T., Wiggins, P. A., Wai, S. N., & Mougous, J. D. (2013).** Diverse type VI secretion phospholipases are functionally plastic antibacterial effectors. *Nature*, 496(7446), 508-512. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/23552891>. doi:10.1038/nature12074
- Russell, A. B., Wexler, A. G., Harding, B. N., Whitney, J. C., Bohn, A. J., Goo, Y. A., Tran, B. Q., Barry, N. A., Zheng, H., Peterson, S. B., Chou, S., Gonen, T., Goodlett, D. R., Goodman, A. L., & Mougous, J. D. (2014).** A type VI secretion-related pathway in Bacteroidetes mediates interbacterial antagonism. *Cell Host Microbe*, 16(2), 227-236. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/25070807>. doi:10.1016/j.chom.2014.07.007
- Sagel, S. D., Gibson, R. L., Emerson, J., McNamara, S., Burns, J. L., Wagener, J. S., Ramsey, B. W., Inhaled Tobramycin in Young Children Study, G., & Cystic Fibrosis Foundation Therapeutics Development, N. (2009).** Impact of *Pseudomonas* and *Staphylococcus* infection on inflammation and clinical status in young children with cystic fibrosis. *J Pediatr*, 154(2), 183-188. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/18822427>. doi:10.1016/j.jpeds.2008.08.001
- Sana, T. G., Baumann, C., Merdes, A., Soscia, C., Rattei, T., Hachani, A., Jones, C., Bennett, K. L., Filloux, A., Superti-Furga, G., Voulhoux, R., & Bleves, S. (2015).** Internalization of *Pseudomonas aeruginosa* Strain PAO1 into Epithelial Cells Is Promoted by Interaction of a T6SS Effector with the Microtubule Network. *mBio*, 6(3), e00712. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/26037124>. doi:10.1128/mBio.00712-15
- Sana, T. G., Hachani, A., Bucior, I., Soscia, C., Garvis, S., Termine, E., Engel, J., Filloux, A., & Bleves, S. (2012).** The second type VI secretion system of *Pseudomonas aeruginosa* strain PAO1 is regulated by quorum sensing and Fur and modulates internalization in epithelial cells. *J Biol Chem*, 287(32), 27095-27105. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/22665491>. doi:10.1074/jbc.M112.376368
- Sanders, C. C., Bradford, P. A., Ehrhardt, A. F., Bush, K., Young, K. D., Henderson, T. A., & Sanders, W. E., Jr. (1997).** Penicillin-binding proteins and induction of AmpC beta-lactamase. *Antimicrob Agents Chemother*, 41(9), 2013-2015. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/9303404>. doi:10.1128/AAC.41.9.2013

- Santin, Y. G., Doan, T., Lebrun, R., Espinosa, L., Journet, L., & Cascales, E. (2018).** In vivo TssA proximity labelling during type VI secretion biogenesis reveals TagA as a protein that stops and holds the sheath. *Nat Microbiol*, 3(11), 1304-1313. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30275513>. doi:10.1038/s41564-018-0234-3
- Sanz-Garcia, F., Alvarez-Ortega, C., Olivares-Pacheco, J., Blanco, P., Martinez, J. L., & Hernando-Amado, S. (2019).** Analysis of the *Pseudomonas aeruginosa* Aminoglycoside Differential Resistomes Allows Defining Genes Simultaneously Involved in Intrinsic Antibiotic Resistance and Virulence. *Antimicrob Agents Chemother*, 63(5). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30858210>. doi:10.1128/AAC.00185-19
- Schelenz, S., & French, G. (2000).** An outbreak of multidrug-resistant *Pseudomonas aeruginosa* infection associated with contamination of bronchoscopes and an endoscope washer-disinfector. *J Hosp Infect*, 46(1), 23-30. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/11023719>. doi:10.1053/jhin.2000.0800
- Schlechter, R. O., Jun, H., Bernach, M., Oso, S., Boyd, E., Munoz-Lintz, D. A., Dobson, R. C. J., Remus, D. M., & Remus-Emsermann, M. N. P. (2018).** Chromatic Bacteria - A Broad Host-Range Plasmid and Chromosomal Insertion Toolbox for Fluorescent Protein Expression in Bacteria. *Front Microbiol*, 9, 3052. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30631309>. doi:10.3389/fmicb.2018.03052
- Schneider, J. P., Nazarov, S., Adaixo, R., Liuzzo, M., Ringel, P. D., Stahlberg, H., & Basler, M. (2019).** Diverse roles of TssA-like proteins in the assembly of bacterial type VI secretion systems. *EMBO J*, 38(18), e100825. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/31403721>. doi:10.15252/embj.2018100825
- Schroth, M. N., Cho, J. J., Green, S. K., Kominos, S. D., & Microbiology Society, P. (2018).** Epidemiology of *Pseudomonas aeruginosa* in agricultural areas(). *J Med Microbiol*, 67(8), 1191-1201. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30067169>. doi:10.1099/jmm.0.000758
- Schumacher, M. A., Piro, K. M., Xu, W., Hansen, S., Lewis, K., & Brennan, R. G. (2009).** Molecular mechanisms of HipA-mediated multidrug tolerance and its neutralization by HipB. *Science*, 323(5912), 396-401. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19150849>. doi:10.1126/science.1163806
- Schwarz, S., West, T. E., Boyer, F., Chiang, W. C., Carl, M. A., Hood, R. D., Rohmer, L., Tolker-Nielsen, T., Skerrett, S. J., & Mougous, J. D. (2010).** *Burkholderia* type VI secretion systems have distinct roles in eukaryotic and bacterial cell interactions. *PLoS Pathog*, 6(8), e1001068. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/20865170>. doi:10.1371/journal.ppat.1001068
- Schwinn, M. K., Machleidt, T., Zimmerman, K., Eggers, C. T., Dixon, A. S., Hurst, R., Hall, M. P., Encell, L. P., Binkowski, B. F., & Wood, K. V. (2018).** CRISPR-Mediated Tagging of Endogenous Proteins with a Luminescent Peptide. *ACS Chem Biol*, 13(2), 467-474. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/28892606>. doi:10.1021/acscchembio.7b00549
- Sezonov, G., Joseleau-Petit, D., & D'Ari, R. (2007).** *Escherichia coli* physiology in Luria-Bertani broth. *J Bacteriol*, 189(23), 8746-8749. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/17905994>. doi:10.1128/JB.01368-07
- Sheahan, K. L., Cordero, C. L., & Satchell, K. J. (2004).** Identification of a domain within the multifunctional *Vibrio cholerae* RTX toxin that covalently cross-links actin. *Proc Natl Acad Sci U S A*, 101(26), 9798-9803. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/15199181>. doi:10.1073/pnas.0401104101
- Shneider, M. M., Buth, S. A., Ho, B. T., Basler, M., Mekalanos, J. J., & Leiman, P. G. (2013).** PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. *Nature*, 500(7462), 350-353. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/23925114>. doi:10.1038/nature12453
- Siehn, R., Traxler, B., An, D. D., Parsek, M. R., Schaefer, A. L., & Singh, P. K. (2010).** A unique regulator controls the activation threshold of quorum-regulated genes in

- Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A*, 107(17), 7916-7921. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/20378835>. doi:10.1073/pnas.0908511107
- Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J. D., & Higgins, D. G. (2011).** Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol*, 7, 539. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/21988835>. doi:10.1038/msb.2011.75
- Simanek, K. A., Taylor, I. R., Richael, E. K., Lasek-Nesselquist, E., Bassler, B. L., & Paczkowski, J. E. (2022).** The PqsE-RhlR Interaction Regulates RhlR DNA Binding to Control Virulence Factor Production in *Pseudomonas aeruginosa*. *Microbiol Spectr*, 10(1), e0210821. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/35019777>. doi:10.1128/spectrum.02108-21
- Simon, R., Priefer, U., & Pühler, A. (1983).** A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. *Bio/Technology*, 1(9), 784-791. Retrieved from <https://doi.org/10.1038/nbt1183-784>. doi:10.1038/nbt1183-784
- Singh, M., Sykes, E. M. E., Li, Y., & Kumar, A. (2020).** MexXY RND pump of *Pseudomonas aeruginosa* PA7 effluxes bi-anionic beta-lactams carbenicillin and sulbenicillin when it partners with the outer membrane factor OprA but not with OprM. *Microbiology (Reading)*, 166(11), 1095-1106. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/32909933>. doi:10.1099/mic.0.000971
- Smith, C. W., Marlin, S. D., Rothlein, R., Toman, C., & Anderson, D. C. (1989).** Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. *J Clin Invest*, 83(6), 2008-2017. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/2566624>. doi:10.1172/JCI114111
- Smith, E. E., Buckley, D. G., Wu, Z., Saenphimmachak, C., Hoffman, L. R., D'Argenio, D. A., Miller, S. I., Ramsey, B. W., Speert, D. P., Moskowitz, S. M., Burns, J. L., Kaul, R., & Olson, M. V. (2006).** Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci U S A*, 103(22), 8487-8492. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/16687478>. doi:10.1073/pnas.0602138103
- Smith, W. P. J., Brodmann, M., Unterweger, D., Davit, Y., Comstock, L. E., Basler, M., & Foster, K. R. (2020).** The evolution of tit-for-tat in bacteria via the type VI secretion system. *Nat Commun*, 11(1), 5395. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/33106492>. doi:10.1038/s41467-020-19017-z
- Sonnabend, M. S., Klein, K., Beier, S., Angelov, A., Kluj, R., Mayer, C., Gross, C., Hofmeister, K., Beuttner, A., Willmann, M., Peter, S., Oberhettinger, P., Schmidt, A., Autenrieth, I. B., Schutz, M., & Bohn, E. (2020).** Identification of Drug Resistance Determinants in a Clinical Isolate of *Pseudomonas aeruginosa* by High-Density Transposon Mutagenesis. *Antimicrob Agents Chemother*, 64(3). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/31818817>. doi:10.1128/AAC.01771-19
- Sonnleitner, E., Abdou, L., & Haas, D. (2009).** Small RNA as global regulator of carbon catabolite repression in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A*, 106(51), 21866-21871. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/20080802>. doi:10.1073/pnas.0910308106
- Sonnleitner, E., & Blasi, U. (2014).** Regulation of Hfq by the RNA CrcZ in *Pseudomonas aeruginosa* carbon catabolite repression. *PLoS Genet*, 10(6), e1004440. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/24945892>. doi:10.1371/journal.pgen.1004440
- Sonnleitner, E., Wulf, A., Campagne, S., Pei, X. Y., Wolfinger, M. T., Forlani, G., Prindl, K., Abdou, L., Resch, A., Allain, F. H., Luisi, B. F., Urlaub, H., & Blasi, U. (2018).** Interplay between the catabolite repression control protein Crc, Hfq and RNA in Hfq-dependent translational regulation in *Pseudomonas aeruginosa*. *Nucleic Acids Res*, 46(3), 1470-

1485. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/29244160>. doi:10.1093/nar/gkx1245
- Spiewak, H. L., Shastri, S., Zhang, L., Schwager, S., Eberl, L., Vergunst, A. C., & Thomas, M. S. (2019).** *Burkholderia cenocepacia* utilizes a type VI secretion system for bacterial competition. *Microbiologyopen*, 8(7), e00774. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30628184>. doi:10.1002/mbo3.774
- Spoering, A. L., & Lewis, K. (2001).** Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J Bacteriol*, 183(23), 6746-6751. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/11698361>. doi:10.1128/JB.183.23.6746-6751.2001
- Springer, T. A. (1990).** Adhesion receptors of the immune system. *Nature*, 346(6283), 425-434. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/1974032>. doi:10.1038/346425a0
- Srivastava, D., Seo, J., Rimal, B., Kim, S. J., Zhen, S., & Darwin, A. J. (2018).** A Proteolytic Complex Targets Multiple Cell Wall Hydrolases in *Pseudomonas aeruginosa*. *mBio*, 9(4). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30018106>. doi:10.1128/mBio.00972-18
- Stanier, R. Y., Palleroni, N. J., & Doudoroff, M. (1966).** The aerobic pseudomonads: a taxonomic study. *J Gen Microbiol*, 43(2), 159-271. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/5963505>. doi:10.1099/00221287-43-2-159
- Stannek, L., Egelkamp, R., Gunka, K., & Commichau, F. M. (2014).** Monitoring intraspecies competition in a bacterial cell population by cocultivation of fluorescently labelled strains. *J Vis Exp*(83), e51196. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/24473333>. doi:10.3791/51196
- Stolle, A. S., Meader, B. T., Toska, J., & Mekalanos, J. J. (2021).** Endogenous membrane stress induces T6SS activity in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A*, 118(1). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/33443205>. doi:10.1073/pnas.2018365118
- Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrenner, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S., & Olson, M. V. (2000).** Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, 406(6799), 959-964. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/10984043>. doi:10.1038/35023079
- Suzuki, H., Nishimura, Y., & Hirota, Y. (1978).** On the process of cellular division in *Escherichia coli*: a series of mutants of *E. coli* altered in the penicillin-binding proteins. *Proc Natl Acad Sci U S A*, 75(2), 664-668. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/345275>. doi:10.1073/pnas.75.2.664
- Symmons, M. F., Bokma, E., Koronakis, E., Hughes, C., & Koronakis, V. (2009).** The assembled structure of a complete tripartite bacterial multidrug efflux pump. *Proc Natl Acad Sci U S A*, 106(17), 7173-7178. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19342493>. doi:10.1073/pnas.0900693106
- Tavares, M., Kozak, M., Balola, A., & Sa-Correia, I. (2020).** *Burkholderia cepacia* Complex Bacteria: a Feared Contamination Risk in Water-Based Pharmaceutical Products. *Clin Microbiol Rev*, 33(3). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/32295766>. doi:10.1128/CMR.00139-19
- Teufel, F., Almagro Armenteros, J. J., Johansen, A. R., Gislason, M. H., Pihl, S. I., Tsirigos, K. D., Winther, O., Brunak, S., von Heijne, G., & Nielsen, H. (2022).** SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nat Biotechnol*, 40(7), 1023-1025. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/34980915>. doi:10.1038/s41587-021-01156-3
- Thaden, J. T., Park, L. P., Maskarinec, S. A., Ruffin, F., Fowler, V. G., Jr., & van Duin, D. (2017).** Results from a 13-Year Prospective Cohort Study Show Increased Mortality

- Associated with Bloodstream Infections Caused by *Pseudomonas aeruginosa* Compared to Other Bacteria. *Antimicrob Agents Chemother*, 61(6). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/28373189>. doi:10.1128/AAC.02671-16
- Ting, S. Y., LaCourse, K. D., Ledvina, H. E., Zhang, R., Radey, M. C., Kulasekara, H. D., Somavanshi, R., Bertolli, S. K., Gallagher, L. A., Kim, J., Penewit, K. M., Salipante, S. J., Xu, L., Peterson, S. B., & Mougous, J. D. (2022).** Discovery of coordinately regulated pathways that provide innate protection against interbacterial antagonism. *Elife*, 11. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/35175195>. doi:10.7554/eLife.74658
- Toder, D. S., Gambello, M. J., & Iglewski, B. H. (1991).** *Pseudomonas aeruginosa* LasA: a second elastase under the transcriptional control of lasR. *Mol Microbiol*, 5(8), 2003-2010. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/1766376>. doi:10.1111/j.1365-2958.1991.tb00822.x
- Torrens, G., Hernandez, S. B., Ayala, J. A., Moya, B., Juan, C., Cava, F., & Oliver, A. (2019).** Regulation of AmpC-Driven beta-Lactam Resistance in *Pseudomonas aeruginosa*: Different Pathways, Different Signaling. *mSystems*, 4(6). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/31796566>. doi:10.1128/mSystems.00524-19
- Toska, J., Ho, B. T., & Mekalanos, J. J. (2018).** Exopolysaccharide protects *Vibrio cholerae* from exogenous attacks by the type 6 secretion system. *Proc Natl Acad Sci U S A*, 115(31), 7997-8002. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/30021850>. doi:10.1073/pnas.1808469115
- Trepanier, S., Prince, A., & Huletsky, A. (1997).** Characterization of the *penA* and *penR* genes of *Burkholderia cepacia* 249 which encode the chromosomal class A penicillinase and its LysR-type transcriptional regulator. *Antimicrob Agents Chemother*, 41(11), 2399-2405. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/9371340>. doi:10.1128/AAC.41.11.2399
- Tseng, S. P., Tsai, W. C., Liang, C. Y., Lin, Y. S., Huang, J. W., Chang, C. Y., Tyan, Y. C., & Lu, P. L. (2014).** The contribution of antibiotic resistance mechanisms in clinical *Burkholderia cepacia* complex isolates: an emphasis on efflux pump activity. *PLoS One*, 9(8), e104986. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/25153194>. doi:10.1371/journal.pone.0104986
- Turnbull, L., Toyofuku, M., Hynen, A. L., Kurosawa, M., Pessi, G., Petty, N. K., Osvath, S. R., Cárcamo-Oyarce, G., Gloag, E. S., Shimoni, R., Omasits, U., Ito, S., Yap, X., Monahan, L. G., Cavaliere, R., Ahrens, C. H., Charles, I. G., Nomura, N., Eberl, L., & Whitchurch, C. B. (2016).** Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nature Communications*, 7(1), 11220. Retrieved from <https://doi.org/10.1038/ncomms11220>. doi:10.1038/ncomms11220
- Valverde, C., Heeb, S., Keel, C., & Haas, D. (2003).** RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHA0. *Mol Microbiol*, 50(4), 1361-1379. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/14622422>. doi:10.1046/j.1365-2958.2003.03774.x
- van Der Ploeg, J. R., Iwanicka-Nowicka, R., Bykowski, T., Hryniewicz, M. M., & Leisinger, T. (1999).** The *Escherichia coli* *ssuEADCB* gene cluster is required for the utilization of sulfur from aliphatic sulfonates and is regulated by the transcriptional activator Cbl. *J Biol Chem*, 274(41), 29358-29365. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/10506196>. doi:10.1074/jbc.274.41.29358
- Ventre, I., Goodman, A. L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., Bleves, S., Lazdunski, A., Lory, S., & Filloux, A. (2006).** Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci U S A*, 103(1), 171-176. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/16373506>. doi:10.1073/pnas.0507407103
- Voggu, L., Schlag, S., Biswas, R., Rosenstein, R., Rausch, C., & Gotz, F. (2006).** Microevolution of cytochrome bd oxidase in *Staphylococci* and its implication in resistance to respiratory toxins released by *Pseudomonas*. *J Bacteriol*, 188(23), 8079-

8086. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/17108291>. doi:10.1128/JB.00858-06
- Wade, D. S., Calfee, M. W., Rocha, E. R., Ling, E. A., Engstrom, E., Coleman, J. P., & Pesci, E. C. (2005).** Regulation of *Pseudomonas* quinolone signal synthesis in *Pseudomonas aeruginosa*. *J Bacteriol*, 187(13), 4372-4380. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/15968046>. doi:10.1128/JB.187.13.4372-4380.2005
- Wang, T., Du, X., Ji, L., Han, Y., Dang, J., Wen, J., Wang, Y., Pu, Q., Wu, M., & Liang, H. (2021).** *Pseudomonas aeruginosa* T6SS-mediated molybdate transport contributes to bacterial competition during anaerobiosis. *Cell Rep*, 35(2), 108957. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/33852869>. doi:10.1016/j.celrep.2021.108957
- Wang, T., Hu, Z., Du, X., Shi, Y., Dang, J., Lee, M., Hesek, D., Mobashery, S., Wu, M., & Liang, H. (2020).** A type VI secretion system delivers a cell wall amidase to target bacterial competitors. *Mol Microbiol*, 114(2), 308-321. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/32279364>. doi:10.1111/mmi.14513
- Warren, M. J., Raux, E., Schubert, H. L., & Escalante-Semerena, J. C. (2002).** The biosynthesis of adenosylcobalamin (vitamin B12). *Nat Prod Rep*, 19(4), 390-412. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/12195810>. doi:10.1039/b108967f
- Welsh, M. J. (1986).** An apical-membrane chloride channel in human tracheal epithelium. *Science*, 232(4758), 1648-1650. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/2424085>. doi:10.1126/science.2424085
- Westerhausen, S., Nowak, M., Torres-Vargas, C. E., Bilitewski, U., Bohn, E., Grin, I., & Wagner, S. (2020).** A NanoLuc luciferase-based assay enabling the real-time analysis of protein secretion and injection by bacterial type III secretion systems. *Mol Microbiol*, 113(6), 1240-1254. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/32068313>. doi:10.1111/mmi.14490
- Wettstadt, S., Wood, T. E., Fecht, S., & Filloux, A. (2019).** Delivery of the *Pseudomonas aeruginosa* Phospholipase Effectors PldA and PldB in a VgrG- and H2-T6SS-Dependent Manner. *Front Microbiol*, 10, 1718. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/31417515>. doi:10.3389/fmicb.2019.01718
- Whitney, J. C., Beck, C. M., Goo, Y. A., Russell, A. B., Harding, B. N., De Leon, J. A., Cunningham, D. A., Tran, B. Q., Low, D. A., Goodlett, D. R., Hayes, C. S., & Mougous, J. D. (2014).** Genetically distinct pathways guide effector export through the type VI secretion system. *Mol Microbiol*, 92(3), 529-542. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/24589350>. doi:10.1111/mmi.12571
- Whitney, J. C., Quentin, D., Sawai, S., LeRoux, M., Harding, B. N., Ledvina, H. E., Tran, B. Q., Robinson, H., Goo, Y. A., Goodlett, D. R., Raunser, S., & Mougous, J. D. (2015).** An interbacterial NAD(P)(+) glycohydrolase toxin requires elongation factor Tu for delivery to target cells. *Cell*, 163(3), 607-619. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/26456113>. doi:10.1016/j.cell.2015.09.027
- WHO. (2017).** *Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis*. Retrieved from Geneva:
- Williamson, K. S., Dlakic, M., Akiyama, T., & Franklin, M. J. (2023).** The *Pseudomonas aeruginosa* RpoH (sigma(32)) Regulon and Its Role in Essential Cellular Functions, Starvation Survival, and Antibiotic Tolerance. *Int J Mol Sci*, 24(2). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/36675051>. doi:10.3390/ijms24021513
- Williamson, K. S., Richards, L. A., Perez-Osorio, A. C., Pitts, B., McInnerney, K., Stewart, P. S., & Franklin, M. J. (2012).** Heterogeneity in *Pseudomonas aeruginosa* biofilms includes expression of ribosome hibernation factors in the antibiotic-tolerant subpopulation and hypoxia-induced stress response in the metabolically active population. *J Bacteriol*, 194(8), 2062-2073. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/22343293>. doi:10.1128/JB.00022-12

- Willmann, M., Götting, S., Bezdan, D., Maček, B., Velic, Marschal, M., Vogel, W., Flesch, I., Markert, U., Schmidt, A., Kübler, P., Haug, M., Javed, M., Jentzsch, B., Oberhettinger, P., Schütz, M., Bohn, E., Sonnabend, M., Klein, K., & Peter, S. (2018). Multi-omics approach identifies novel pathogen-derived prognostic biomarkers in patients with *Pseudomonas aeruginosa* bloodstream infection.
- Wilton, M., Wong, M. J. Q., Tang, L., Liang, X., Moore, R., Parkins, M. D., Lewenza, S., & Dong, T. G. (2016). Chelation of Membrane-Bound Cations by Extracellular DNA Activates the Type VI Secretion System in *Pseudomonas aeruginosa*. *Infect Immun*, 84(8), 2355-2361. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/27271742>. doi:10.1128/IAI.00233-16
- Wood, T. E., Howard, S. A., Forster, A., Nolan, L. M., Manoli, E., Bullen, N. P., Yau, H. C. L., Hachani, A., Hayward, R. D., Whitney, J. C., Vollmer, W., Freemont, P. S., & Filloux, A. (2019). The *Pseudomonas aeruginosa* T6SS Delivers a Periplasmic Toxin that Disrupts Bacterial Cell Morphology. *Cell Rep*, 29(1), 187-201 e187. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/31577948>. doi:10.1016/j.celrep.2019.08.094
- Workentine, M. L., Chang, L., Ceri, H., & Turner, R. J. (2009). The GacS-GacA two-component regulatory system of *Pseudomonas fluorescens*: a bacterial two-hybrid analysis. *FEMS Microbiol Lett*, 292(1), 50-56. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/19191877>. doi:10.1111/j.1574-6968.2008.01445.x
- Worlitzsch, D., Tarran, R., Ulrich, M., Schwab, U., Cekici, A., Meyer, K. C., Birrer, P., Bellon, G., Berger, J., Weiss, T., Botzenhart, K., Yankaskas, J. R., Randell, S., Boucher, R. C., & Döring, G. (2002). Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J Clin Invest*, 109(3), 317-325. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/11827991>. doi:10.1172/JCI13870
- Wu, C. F., Lien, Y. W., Bondage, D., Lin, J. S., Pilhofer, M., Shih, Y. L., Chang, J. H., & Lai, E. M. (2020). Effector loading onto the VgrG carrier activates type VI secretion system assembly. *EMBO Rep*, 21(1), e47961. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/31808291>. doi:10.15252/embr.201947961
- Xiao, G., He, J., & Rahme, L. G. (2006). Mutation analysis of the *Pseudomonas aeruginosa* *mvfR* and *pqsABCDE* gene promoters demonstrates complex quorum-sensing circuitry. *Microbiology (Reading)*, 152(Pt 6), 1679-1686. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/16735731>. doi:10.1099/mic.0.28605-0
- Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T., & Arakawa, M. (1992). Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol Immunol*, 36(12), 1251-1275. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/1283774>. doi:10.1111/j.1348-0421.1992.tb02129.x
- Yu, K. W., Xue, P., Fu, Y., & Yang, L. (2021). T6SS Mediated Stress Responses for Bacterial Environmental Survival and Host Adaptation. *Int J Mol Sci*, 22(2). Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/33418898>. doi:10.3390/ijms22020478
- Zhang, J., Guan, J., Wang, M., Li, G., Djordjevic, M., Tai, C., Wang, H., Deng, Z., Chen, Z., & Ou, H. Y. (2023). SecReT6 update: a comprehensive resource of bacterial Type VI Secretion Systems. *Sci China Life Sci*, 66(3), 626-634. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/36346548>. doi:10.1007/s11427-022-2172-x
- Zhang, L., Zhao, S. Q., Zhang, J., Sun, Y., Xie, Y. L., Liu, Y. B., Ma, C. C., Jiang, B. G., Liao, X. Y., Li, W. F., Cheng, X. J., & Wang, Z. L. (2020). Proteomic Analysis of Vesicle-Producing *Pseudomonas aeruginosa* PAO1 Exposed to X-Ray Irradiation. *Front Microbiol*, 11, 558233. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/33384665>. doi:10.3389/fmicb.2020.558233
- Zhang, W., Lee, M., Hesek, D., Lastochkin, E., Boggess, B., & Mobashery, S. (2013). Reactions of the three AmpD enzymes of *Pseudomonas aeruginosa*. *J Am Chem Soc*,

- 135(13), 4950-4953. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/23510438>. doi:10.1021/ja400970n
- Zhou, J., Chen, Y., Tabibi, S., Alba, L., Garber, E., & Saiman, L. (2007).** Antimicrobial susceptibility and synergy studies of *Burkholderia cepacia* complex isolated from patients with cystic fibrosis. *Antimicrob Agents Chemother*, 51(3), 1085-1088. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/17158942>. doi:10.1128/AAC.00954-06
- Zlosnik, J. E., Costa, P. S., Brant, R., Mori, P. Y., Hird, T. J., Fraenkel, M. C., Wilcox, P. G., Davidson, A. G., & Speert, D. P. (2011).** Mucoid and nonmucoid *Burkholderia cepacia* complex bacteria in cystic fibrosis infections. *Am J Respir Crit Care Med*, 183(1), 67-72. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/20709823>. doi:10.1164/rccm.201002-0203OC
- Zoued, A., Durand, E., Brunet, Y. R., Spinelli, S., Douzi, B., Guzzo, M., Flaugnatti, N., Legrand, P., Journet, L., Fronzes, R., Mignot, T., Cambillau, C., & Cascales, E. (2016).** Priming and polymerization of a bacterial contractile tail structure. *Nature*, 531(7592), 59-63. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/26909579>. doi:10.1038/nature17182

8 Appendix

8.1 Growth curve of the ID40 Δ crc mutant compared to the wt

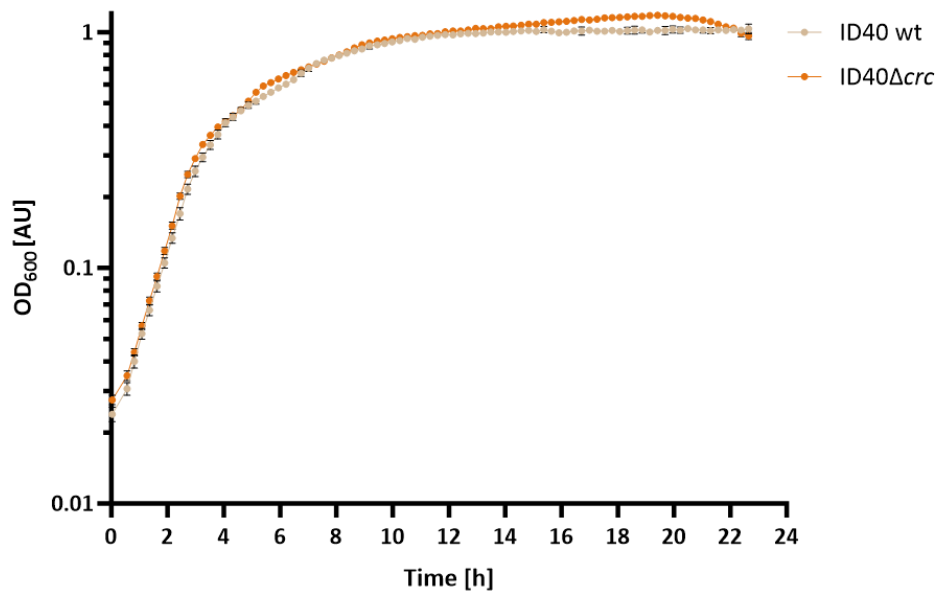


Figure 28: Comparison of the growth curves of ID40wt and the ID40 Δ crc mutant. Both strains were grown from day cultures in a 24-well clear flat-bottom plate (Greiner) at 30°C with shaking (amplitude 4) in a Tecan reader. Initial inoculum: OD₆₀₀ = 0.1 mL⁻¹; measurement every 15 min for 23 h. Dots and whiskers represent the mean and standard deviation of n = 3 technical replicates. The OD was blanked by subtracting the mean of the OD of three LB control wells at each time point.

9 Eidesstattliche Erklärung

Ich erkläre hiermit, dass ich die zur Promotion eingereichte Arbeit mit dem Titel:

Investigation of the Competitive Fitness of *Pseudomonas aeruginosa* in Interbacterial Competition

selbständig verfasst, nur die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen als solche gekennzeichnet habe. Ich erkläre, dass die Richtlinien zur Sicherung guter wissenschaftlicher Praxis der Universität Tübingen (Beschluss des Senats vom 25.5.2000) beachtet wurden. Ich versichere an Eides statt, dass diese Angaben wahr sind und dass ich nichts verschwiegen habe. Mir ist bekannt, dass die falsche Abgabe einer Versicherung an Eides statt mit Freiheitsstrafe bis zu drei Jahren oder mit Geldstrafe bestraft wird.

Tübingen, den 05.07.2025

10 Danksagung

10.1 Vorwort

Ich bin gescheitert. Ich habe versucht, diese Danksagung auf eine Seite... na gut, zwei Seiten... Ach, was soll's: drei Seiten... jedenfalls habe ich versucht, nur die wichtigsten Menschen zu erwähnen, und bin daran gescheitert, irgendeine Reihenfolge festzulegen. Ich sehe diese ganze Dissertation als ein einziges langes Gruppenprojekt, das ohne die Beiträge jedes und jeder einzelnen niemals hätte vervollständigt werden können. Ihr mögt mir verzeihen, dass ich euch einfach allen ohne irgendeine Priorisierung danken werde.

10.2 Danksagung

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An dieser Stelle sind natürlich direkt und unausweichlich alle weiteren aktuellen und ehemaligen Mitglieder der AG Bohn/Schütz zu nennen, die mich durch alle Phasen meiner Reise begleitet haben, und bei unseren wöchentlichen Meetings gleichermaßen kräftig geholfen haben, die gesammelte kreative Energie in ein rundes Forschungsprojekt zu bündeln. Auch wenn ich gerne möchte, so würde es jeden Rahmen sprengen, alle einzeln aufzuzählen, aber es sei gesagt, dass ich euch alle schrecklich vermissen werde und dass ohne euch das Ganze einfach nur eine Doktorarbeit gewesen wäre. PD Dr. Monika Schütz gilt dabei der größte Dank. Obwohl du nicht als offizielle Betreuerin gelistet bist, hatte ich den unfassbar wertvollen Luxus einer ständigen Doppelbetreuung zu jeder Zeit, bei jeder

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