Studies on the role of protein secretion systems in *Pseudomonas fluorescens* biofilm formation, and development of a tunable gene expression system for such analyses

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Abstract

Plant growth-promoting rhizobacteria (PGPRs), such as *Pseudomonas fluorescens*, support plants via different mechanisms such as biocontrol (suppressing pathogenic organisms), phytostimulation (modulation of plant-hormone levels) or biofertilization (by improving nutrient supply) and thus are important for growth yields. Therefore, it is important to understand interactions of the bacteria and plant roots. Many PGPRs are able to form a biofilm on plant roots, so they live in an adherent cell community on the root surface. Different bacterial surface structures (e.g. pili or fimbriae) and secreted proteins (e.g. adhesins or enzymes) are involved in biofilm formation or plant growth promotion.

Therefore, the first part of this study investigated the influence of selected secreted proteins or protein secretion systems on biofilm formation of *P. fluorescens*. Genes encoding translocated substrates or secretion system components were deleted to abolish their function. Two genes encoding type 1 secretion system (T1SS) components (*lapA*, encoding an adhesin and *aprE*, encoding a membrane fusion protein) were identified, whose deletion lead to weaker biofilm formation. Further analyses of the Apr T1SS revealed that its substrate AprA (an extracellular protease) was not directly involved in biofilm formation. The deletion of *aprE* most likely resulted in mistargeting AprA into the periplasm, which could influence biofilm formation.

In the second part of the study, a new tightly controlled sugar-independent expression system was established for *P. fluorescens* A506. The gene *PflA506_4486* was predicted to encode a putative anthranilic acid inducible regulator for the expression of an adjacent *antABC* operon for anthranilic acid degradation to catechol. *PflA506_4486* and the putative promoter region for *antABC* expression were cloned into a shuttle vector system for *Escherichia coli* and *Pseudomonas*. *gfp* was put under control of this anthranilate-inducible promoter (P_{antA}) system as reporter for the examination of promoter activity.

In this study it was shown that the anthranilate-inducible promoter system is tightly controlled by inducer concentration and can be tuned to expression levels suitable for physiological analyses. Compared with a rhamnose-inducible promoter system, the P_{antA}-system was advantageous in *P. fluorescens* A506. Moreover, this new system is working in *P. fluorescens* as well as in *Pseudomonas putida* and *Pseudomonas aeruginosa*, which are the most intensively studies pseudomonads.

Keywords: T1SS, Biofilm formation, Anthranilate, Recombinant gene expression

Zusammenfassung

Pflanzenwachstumsfördernde Rhizobakterien (PGPRs), wie *Pseudomonas fluorescens*, unterstützen Pflanzen über verschiedene Mechanismen, wie Biokontrolle (Unterdrückung pathogener Organismen), Phytostimulation (Anpassung des Pflanzen-Hormonlevels) oder Biofertilisation (Nährstoffversorgung) und sind somit relevant für deren Erträge. Daher ist es wichtig, die Wechselwirkungen von Pflanze und Bakterien zu verstehen. Viele PGPRs bilden Biofilme auf Wurzeloberflächenund leben somit in einer Zellgemeinschaft. Sekretierte Proteine (z.B. Adhesine, Enzyme) und verschiedene Oberflächenstrukturen der Bakterien (z.B. Pili, Fimbrien) wirken bei der Biofilmbildung oder in der Förderung des Pflanzenwachstums mit.

Im ersten Teil dieser Arbeit wurde daher der Einfluss sekretierter Proteine bzw. von Protein-Sekretionssystemen auf die Biofilmbildung von *P. fluorescens* untersucht. Gene wurden deletiert, die für ein zu transportierendes Substrat oder für Komponenten des Sekretionssystems codieren und somit deren Funktion aufgehoben. Zwei Gene, die für T1SS-Komponenten codieren (*lapA* für ein Adhesin und *aprE* für ein Membranfusionsprotein), konnten identifiziert werden, deren Deletion zu einer schwächeren Biofilmbildung führte. Weitere Untersuchungen des Apr T1SS zeigten, dass dessen Substrat AprA (eine extrazelluläre Protease) nicht direkt in der Biofilmbildung involviert war. Die Deletion von *aprE* führte sehr wahrscheinlich zu einer Fehlleitung von AprA in das Periplasma, was die Biofilmbildung beeinflussen könnte.

Im zweiten Teil dieser Arbeit wurde ein neues, eng kontrollierbares, zuckerunabhängiges Expressionssystem in *P. fluorescens* A506 etabliert. Das Gen *PflA506_4486* codiert für einen vorhergesagten Anthranilsäure-induzierbaren Regulator, der das direkt benachbarte *antABC* Operon reguliert (zuständig für den Abbau von Anthranilsäure zu Catechol). *PflA506_4486* und die vermutete Promotorregion für die Expression von *antABC* wurden in ein *Shuttle*-Vektorsystem für *Escherichia coli* und *Pseudomonas* kloniert. Als Reporter der Promotoraktivität wurde *gfp* unter die Kontrolle dieses Anthranilsäure-induzierbaren Promotors gesetzt.

In dieser Arbeit wurde gezeigt, dass das Anthranilsäure-induzierbare Promotorsystem eng regulierbar ist und somit auf das jeweils gewünschte Expressionslevel abgestimmt werden kann (wie für physiologische Untersuchungen). In *P. fluorescens* A506 ist es einem Rhamnose-induzierbaren Promotorsystem überlegen, und funktioniert darüberhinaus genauso gut in den meist untersuchten Pseudomonaden *Pseudomonas putida* und *Pseudomonas aeruginosa*.

Schlagwörter: T1SS, Biofilmbildung, Anthranilsäure, Recombinante Genexpression

List of abbreviations

Abbreviation	Meaning
ABC	ATP-binding cassette
AHL	Acyl homoserine lactones
Amp/Amp^R	Ampicillin / ampicillin resistance
ATP	Adenosine triphosphate
BAM	β-barrel assembly machinery
BCCP	Biotin carboxyl carrier protein
bp	Base pairs
BSA	Bovine albumin serum
bzw.	*Beziehungsweise* (German)
Carb/CarbR	Carbenicillin/ carbenicillin resistance
CV	Crystal violet
Da	Dalton
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
E. coli	*Escherichia coli*
e.g.	*Exempli gratia* (Latin)
ECL	Enhanced chemiluminescence
eDNA	Environmental DNA
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substance
et al.	*Et alii* (Latin)
GFP	Green fluorescence protein
glc	Glucose
GMP	Guanosine monophosphate
h	Hour(s)
His	Hexahistidine
HRP	Horseradish peroxidase
HHQ	4-hydroxy-2-heptylquinoline
i.e.	*Id est* (Latin)
Kan/Kan^R	Kanamycin/kanamycin resistance
LapA	Large adhesion protein A

LapD	Large adhesion protein D
LapG	Large adhesion protein G
LB	Luria-Bertani complex medium
MCS	Multiple cloning site
MW	Molecular weight
OD_{600}	Optical density measured at 600 nm
Ori	Origin of replication
Orf	Open reading frame
P. aeruginosa	*Pseudomonas aeruginosa*
P. brassicacearum	*Pseudomonas brassicacearum*
P. cichorii	*Pseudomonas cichorii*
P. fluorescens	*Pseudomonas fluorescens*
P. putida	*Pseudomonas putida*
P. syringae	*Pseudomonas syringae*
PAGE	Polyacylamide gel elektrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGPR	Plant growth-promoting rhizobacteria
pH	Potentia Hydrogenii
PMSF	Phenylmethylsulfonyl fluoride
psi	Pounds per square inch
PVDF	Polyvinylidenefluoride
QS	Quorum sensing
RBS	Ribosomale binding site
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
SD	Shine Dalgarno sequence
SDS	Sodium dodecyl sulfate
Sec	General secretion pathway
SOC	Super optimal broth with catabolite repression
T1SS	Type 1 secretion system
T2SS	Type 2 secretion system
T3SS	Type 3 secretion system

T4SS	Type 4 secretion system
T5SS	Type 5 secretion system
T6SS	Type 6 secretion system
T7SS	Type 7 secretion system
T8SS	Type 8 secretion system
T9SS	Type 9 secretion system
Tat	Twin-arginine translocation
TCA cycle	Tricarboxylic acid cycle
TEMED	N,N,N′,N′-Tetramethylethane-1,2-diamine
TES	Tris-EDTA-Sucrose
TR	Transcription regulator
Tris	Tris(hydroxymethyl)aminomethane
TSS	Transformation and storage solution
UTR	Untranslated region
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
WT	Wildtype
z.B.	*Zum Beispiel* (German)
× g	Folds of gravity (g = 9.81 m/s^2)

Nucleic acids

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

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1 Introduction

Within the phylum proteobacteria, in the class of γ-proteobacteria and therein in the order of pseudomonadales, the family of pseudomonadaceae with its genus *Pseudomonas* can be found. This genus was first introduced by Migula in 1894 (Migula, 1894). Pseudomonads describe a large number of motile rod-shaped Gram-negative non spore-forming bacteria with a polar flagellum. They are able to use many organic substrates for their growth and they inhabit a wide range of terristric, marine and limnic habitats also in relation to plants or humans or animals (Palleroni, 1981). Some of the pseudomonads are pathogenic. Whereas some pseudomonads (e.g. *Pseudomonas aeruginosa* and *Pseudomonas putida*) are opportunistic human pathogens, some other pseudomonads are plant pathogens (e.g. *Pseudomonas syringae* and *Pseudomonas cichorii*) (Lyczak *et al.*, 2000, Kerr and Snelling, 2009, Kim *et al.*, 2012, Fernández *et al.*, 2015), and other pseudomonads are mutualistic, including those that belong to the plant growth-promoting rhizobacteria (PGPRs), such as *Pseudomonas fluorescens* or *Pseudomonas chlororaphis* (Dowling and O'Gara, 1994, Lugtenberg and Kamilova, 2009, Santoyo *et al.*, 2012, Shen *et al.*, 2013).

Many species of the pseudomonads can produce a green fluorescent siderophore (pyoverdine) and show under iron-limiting conditions a fluorescent colour, such as e.g. *Pseudomonas aeruginosa*, *Pseudomonas fluorescens, Pseudomonas putida* or *Pseudomonas syringae*.

1.1 Plant growth-promoting rhizobacteria

Pseudomonads play important roles in many ecosystems. They often occur in mutualistic or pathogenic life styles associated with plants, animals or humans (Peix *et al*., 2018). Because bacteria such as *P. fluorescens* are beneficial for plants, they are termed plant growth-promoting rhizobacteria (PGPRs), first described by Kloepper and Schroth in 1978 (Kloepper and Schroth, 1978).

They support plant growth via different mechanisms, such as biocontrol (the out-competition of pathogens in the rhizosphere, secretion of pathogen-inhibiting compounds such as antibiotics, siderophores (Van Loon, 2007), hydrogen cyanide (Ramette *et al.*, 2003)), modulation of plant-hormone levels (phytostimulation), or biofertilization by improvement of nutrient supply (Lugtenberg *et al.*, 2002) (Figure 1).

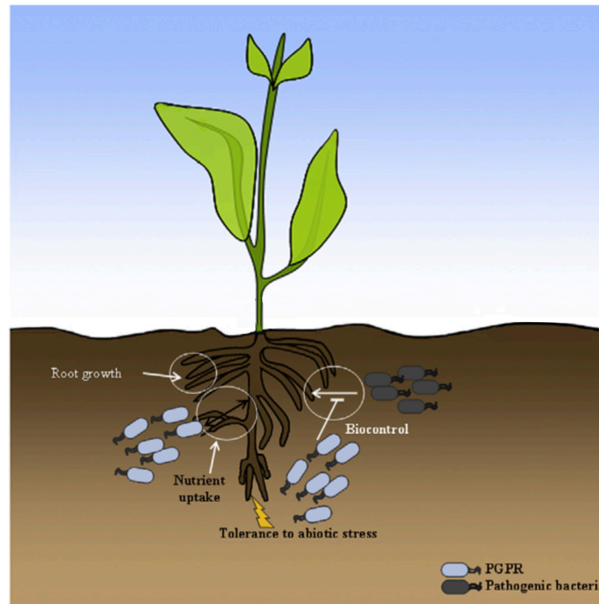

Figure 1: Benefits to plants from interaction with plant growth-promoting bacteria (Pérez-Montaño *et al.*, 2014, modified). By mechanisms such as biocontrol, PGPRs can improve the tolerance to abiotic stress and nutrient uptake of plants. This leads to better growing plants that are more tolerant against pathogenic bacteria.

1.1.1 Biofilm formation

Many PGPRs are able to form biofilms on surfaces, e.g. plant roots. In these biofilms, they are able to live in a community of microorganisms that belong either to the same species or to multiple species. A biofilm describes microorganisms living in a community adherent on a biotic or abiotic surface. The bacteria are positioned in a biofilm matrix that forms a scaffold. The bacterial cells pass different stages during biofilm formation. Starting with the planktonic stage, freely swimming cells attach to a surface. During the attachment stage, cells stick reversibly, then irreversibly to the surface. By clonal growth of the cells the microcolony formation stage is reached, followed by formation of a macrocolony that eventually disperses to release planktonic cells (Figure 2). While the shape of the microcolonies varies, they have in common that the bacteria are encased in a matrix.

Protein structures on cell surfaces, such as pili (Klausen *et al.*, 2003a, b), flagella (O'Toole and Kolter, 1998, Klausen *et al.*, 2003a, b), proteins (Monds *et al.*, 2007, Newell *et al.*, 2009, Borlee *et al.*, 2010) and extracellular polysaccharides (Nivens *et al.*, 2001, Wozniak *et al.*, 2003, Friedman and Kolter, 2004a, b, Jackson *et al.*, 2004, Matsukawa and Greenberg, 2004, Ma *et al.*, 2006, Ryder *et al.*, 2007, Starkey *et al.*, 2009, Byrd *et al.*, 2010) are involved in biofilm formation by mediating the surface interaction (Mann and Wozniak, 2012). It is unclear, whether biofilm formation is a prerequisite for plant growth promotion by these bacteria.

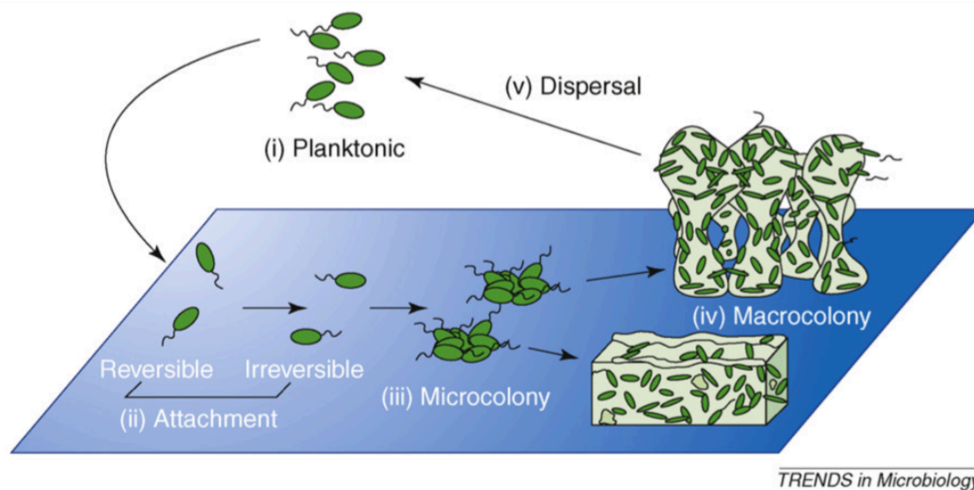

Figure 2: Different stages of biofilm formation (Monds and O'Toole, 2009). The bacterial cells pass different stages during biofilm formation, such as the planktonic stage, the reversible and irreversible attachment stage, microcolony and macrocolony formation and the dispersal stage.

Different molecules contribute to *Pseudomonas* biofilms, such as proteins, polysaccharides and eDNA. Proteins are important for surface attachment and most likely also for cell-cell interaction. Two specific classes of polysaccharides of biofilm-forming *Pseudomonas* strains have been shown to have an influence on biofilm formation: capsular polysaccharides (forming a coat around the microorganism) and aggregative polysaccharides (offering structural integrity). All these polysaccharides improve the surface tolerance, support adherence and aggregation of the bacteria (Osman *et al.*, 1986, Penaloza-Vazquez *et al.*, 1997, Keith *et al.*, 2003, Laue *et al.*, 2006). The extracellular matrix of the biofilm is described as extracellular polymeric substance (EPS). It is important for biofilm formation, and the largest part of total biomass in biofilms is represented by the EPS (Sutherland, 2001a, b, Flemming *et al.*, 2007, Rasamiravaka *et al.*, 2015). Besides proteins and polysaccharides, nucleic acid is part of the EPS and plays an important role in stabilization of the biofilm matrix (Whitchurch *et al.*, 2002, Allesen-Holm *et al.*, 2006, Yang *et al.*, 2007). During different biofilm formation stages, different components of the matrix are more predominant or necessary than others. For *P. aeruginosa* it is shown that polysaccharides are present during all developmental stages of biofilm formation, while nucleic acids become more present in the matrix during later maturation stages (Klausen *et al.*, 2003a, b, Webb *et al.*, 2003, Allesen-Holm *et al.*, 2006, Yang *et al.*, 2007, Ma *et al.*, 2009).

Proteins support the surface attachment of the bacteria, they most likely also support cell-cell-interactions, and are part of the biofilm matrix.

Biofilm formation protects the microorganism from harmful outer impacts such as antibiotics (Hoyle and Costerton, 1991), pH gradients (Davey and O'Toole, 2000), UV irradiation (Elasri and Miller, 1999), desiccation (Chang *et al.*, 2007) and predation (Matz *et al.*, 2005). The biofilm formation of pseudomonads is not sufficiently investigated yet and, besides human pathogenic *P. aeruginosa*, the plant growth-promoting *P. fluorescens* became a model organism for biofilm research.

1.1.2 Regulation of biofilm formation by quorum sensing

Biofilm formation is suggested to be influenced or partly controlled by quorum sensing (QS) (Parsek and Greenberg, 2005). QS is a regulatory mechanism of microorganisms. Small molecules are secreted by the cells that can be recognized by the cells of the population. The concentration of these QS molecules depends upon cell density. The more cells there are in the surrounding of a cell, the more quorum sensing signals are sensed. These signals are used to repress or induce specific QS controlled genes in response to cell density.

Different classes of QS signals are described so far (Irie and Parsek, 2008). One of them is the class of acyl homoserine lactone signals that has been found in different Gram-negative proteobacteria, including the genus *Pseudomonas*. The acyl side chain can vary between different species and quorum sensing systems. The structure and length of the side chain can influence the permeability of the molecule across the cell membrane (Pearson *et al*., 1999, Irie and Parsek, 2008).

The synthesis of the signal molecules of the acyl homoserine lactones (AHL) is mostly catalyzed by an enzyme of the LuxI family, and a LuxR family protein is involved in signal recognition (Figure 3) (Parsek and Greenberg, 2000).

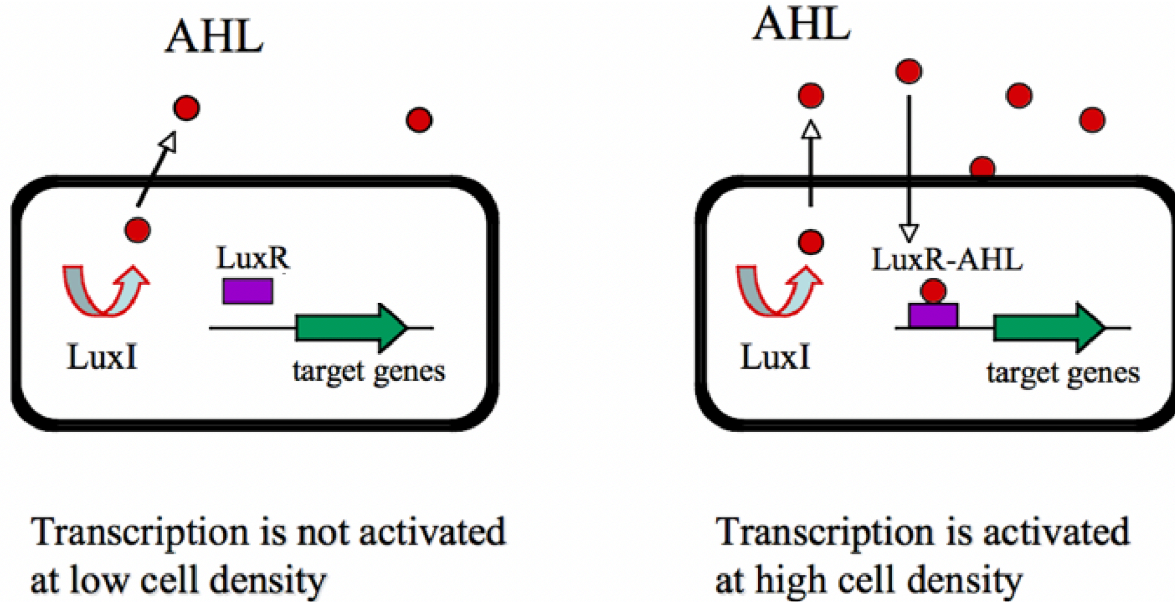

Figure 3: High level of AHL (respectively high cell density) activates gene expression of target genes (Li and Tian, 2012, modified). The acyl homoserine lactone level can work as an indicator for cell density and influence the gene expression of target genes. The bacterial cell membrane is permeable for AHL, so the produced AHL can pass the cell membrane. When many AHL releasing bacteria are close to each other, the concentration of AHL in the extracellular matrix increases. Because of membrane permeability the concentration of AHL inside the bacteria also increases. AHL can activate gene expression of target genes when it is inside the bacteria.

In pathogenic *P. aeruginosa*, experiments with a disabled *las* acyl homoserine lactone system have shown that the biofilm formation is affected and the microorganisms form a flat unstructured biofilm (Davies *et al*., 1998). A disabled acyl homoserine lactone system (*ppu*) in *P. putida* leads to a differently structured biofilm that has distinct microcolonies and water channels (Steidle *et al*., 2002).

The effect of a disabled quorum sensing system on biofilm formation has also been investigated with other bacteria, such as *Aeromonas hydrophila* (Lynch *et al*., 2002), *Klebsiella pneumoniae* (Balestrino *et al*., 2005), *Listeria monocytogenes* (Belval *et al*., 2006), *Serratia liquefaciens* (Labbate *et al*., 2004), *Serratia marcescens* (Rice *et al*., 2005). It was shown that the biofilm formation is influenced by the disabled quorum sensing system, leading to different phenomena: a thinner biofilm (*Serratia liquefaciens*), more biofilm formation (*Listeria monocytogenes*), an altered biofilm maturation (*Aeromonas hydrophila*) or an impaired biofilm dispersal or development (*Serratia marcescens* and *Klebsiella pneumoniae*).

1.1.3 Two-component system GacS-GacA

In many γ-proteobacteria a two-component regulatory system was found, whose corresponding target genes and consequently the corresponding gene products are involved in different processes, including biofilm formation, quorum sensing, the production of extracellular products and motility (Lapouge *et al.*, 2008). This GacS-GacA-two-component regulatory system consists of a sensor kinase (GacS) that can be autophosphorilated and that transfers the phosphate to its response regulator (GacA) (Lapouge *et al.*, 2008). The activated response regulator is responsible for regulation of the production of small regulatory RNAs. These RNAs bind to repressor proteins which relieve the repression of target genes (Lapouge *et al.*, 2008).
The two-component regulatory system can be activated by extracellular signals or molecules, that were released from e.g. *Pseudomonas* populations that are grown to high population densities (Lapouge *et al.*, 2008). Dubuis and Haas (2007) and Dubuis *et al.* (2007) have shown that intraspecies and interspecies signalling is possible (Dubuis and Haas, 2007, Dubuis *et al.*, 2007).
The quorum sensing system of different *Pseudomonas* is positively controlled by the GacS-GacA-two-component system via different acyl homoserine lactones (Lapouge *et al.*, 2008, Kay *et al.*, 2006, Lalouna *et al.*, 2012).

1.1.4 Regulation of biofilm formation in *Pseudomonas fluorescens*

In non-pathogenic *P. fluorescens* (and pathogenic *P. aeruginosa*) cyclic-di-GMP is part of a sensor system that is involved in biofilm formation.
In *P. fluorescens* Pf0-1 the secretion and surface localisation of the large adhesion protein A (LapA) is necessary for biofilm formation (for initial attachment) on every abiotic surface tested (including hydrophilic and hydrophobic surfaces) (O'Toole and Kolter, 1998, Hinsa *et al.*, 2003). LapA is an about 520 kDa sized adhesin (containing a type 1 secretion signal and characteristic amino acid repeats) that is conserved in *P. fluorescens* and *P. putida* (Hinsa *et al.*, 2003, Satchell, 2011). In *P. fluorescens* the secretion and surface localisation of LapA is regulated by cyclic-di-GMP and inorganic phosphate. For biofilm formation, a high level of cyclic-di-GMP is required as well as a sufficient concentration of inorganic phosphate (Monds *et al.*, 2007, Navarro *et al.*, 2011, Newell *et al.*, 2011, Newell *et al.*, 2009).
A low level of inorganic phosphate is sensed by a two-component system (PhoBR) which activates *rapA* (among other genes) (Jenal and Malone, 2006, Wanner, 1996). *rapA* encodes a

phosphodiesterase that reduces cyclic-di-GMP, thus the cyclic-di-GMP level decreases (Jenal and Malone, 2006). A low inorganic phosphate level does also lead to the cleavage of LapA from the cell surface by the periplasmic cysteine protease LapG, which targets the N-terminus of the adhesin LapA (Newell *et al*., 2011).

Cytoplasmic cyclic-di-GMP binds to LapD, an inner membrane cyclic-di-GMP effector protein that undergoes conformational change upon cyclic-di-GMP binding. This conformational change enables LapD to bind LapG (periplasmic cysteine protease), preventing the cleavage of LapA (Navarro *et al*., 2011, Newell *et al*., 2009).

These mechanisms prevent the cells from forming a biofilm under unfavorable conditions (Newell *et al*., 2011).

1.1.5 Roles of protein secretion systems in biofilm formation

As all proteins on bacterial surfaces are synthesized in the cytoplasm, protein secretion systems are expected to contribute to plant growth promotion. Secreted proteins allow bacteria to interact, influence or communicate with their environment. For example, they provide nutrients for uptake by secretion of mainly hydrolytic enzymes or they can transfer effector proteins into hosts.

Translocation systems can influence biofilm formation. Borlee *et al*. (2010) showed that the T5SS translocates a putative c-di-GMP regulated adhesin (CdrA) that promotes the biofilm formation of *P. aeruginosa* (Borlee *et al*., 2010). Another adhesin protein the large adhesion protein A (LapA) is involved in biofilm formation and therefore it has to be transported to the surface of the bacteria. The large LapA (523 kDa) is translocated via a specific T1SS.

As described in chapter 1.1.4, it is shown that a regulatory mechanism, resulting in the cleavage of LapA from the cell surface, prevents *P. fluorescens* Pf0-1 from biofilm formation under unfavorable conditions (Newell *et al*., 2011).

With regard to their cell envelope structure, bacteria can be divided into two groups: Gram-positive bacteria and Gram-negative bacteria (Figure 4). Gram-positive bacteria usually have only one compartment, the cytoplasm, surrounded by a cytoplasmic membrane. The structure of Gram-negative bacteria such as *P. fluorescens* or *Escherichia coli* is more complex. They usually have two compartments, the cytoplasm and the periplasm that are separated by an inner membrane. An outer membrane surrounds the periplasm and separates the cell from the extracellular space.

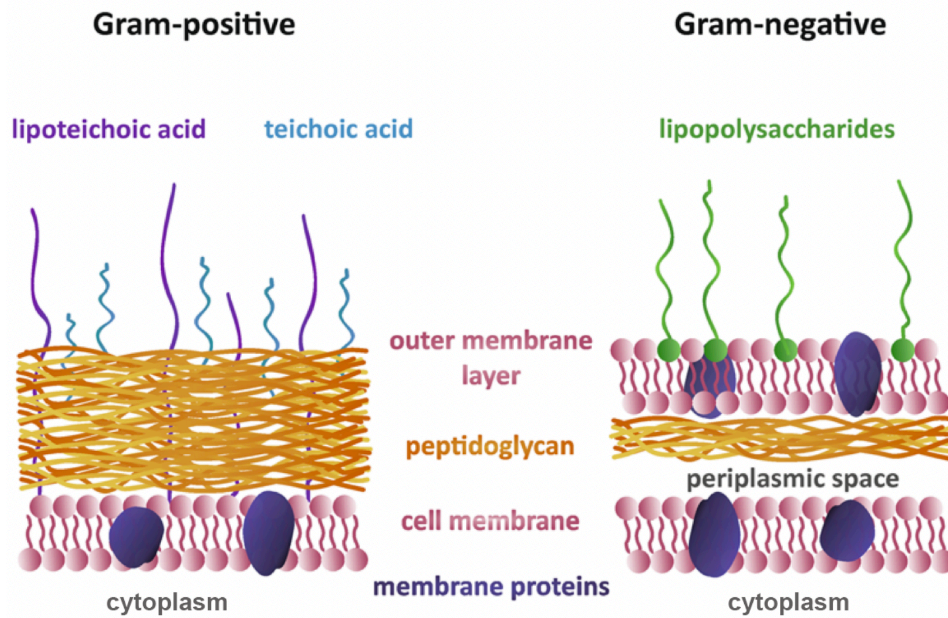

Figure 4: Cell wall structure for Gram-positive and Gram-negative bacteria (Lovering *et al.*, 2010, Clifton *et al.*, 2013, Pajerski *et al.*, 2019, modified). The cell wall structure of bacteria can be split into two classes, the Gram-positive and the Gram-negative bacteria. The structure of Gram-positive bacteria is simpler as it is built up of a cytoplasmic membrane that surrounds the cytoplasm. Outside the membrane there is a thick peptidoglycan layer. The Gram-negative bacteria have two membranes, the cytoplasmic membrane that surrounds the cytoplasm and an outer membrane. Between these two membranes, there is the periplasmic space that contains a thin peptidoglycan layer.

Proteins are synthesized in the cytoplasm. As some of the proteins have their destination and function in the periplasm or in the extracellular space, they have to be translocated across the inner membrane or both membranes. Other proteins are located in the inner or outer membrane and have to be inserted. Different protein secretion and insertion systems are found in bacteria that allow the proteins to get to their destinations.

As in all Gram-negative bacteria, proteins of *P. fluorescens* have to be translocated across two membranes to reach the surface or to be released into the extracellular space. This can happen in one or in two steps, i.e. secretion can be achieved either by a single secretion system that crosses two membranes or by a combination of separate secretion systems in the inner and outer membranes. A growing number of bacterial secretion systems have been described so far, nine of which being classified as T1SS to T9SS (for **T**ype **X** **S**ecretion **S**ystem), some of them are shown in figure 5.

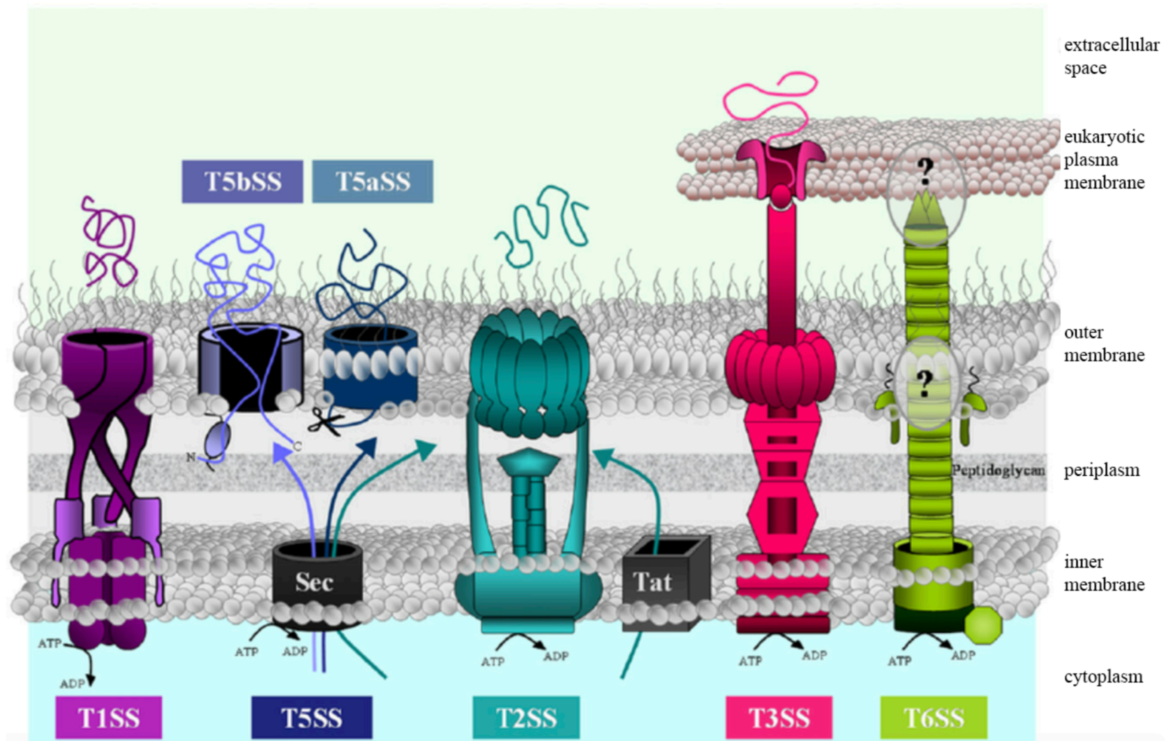

Figure 5: Schematic overview of different translocation systems (Blevesa *et al*., 2010, modified). As bacteria have different characteristics (such as the cell wall structure or the properties such as virulence), different translocation systems with different structures are found in bacteria. Some translocation systems can translocate substrates from the cytoplasmic matrix directly into a host cell across three membranes (T3SS, T6SS), while others only translocate the substrate across the inner membrane (Sec- and Tat-pathway). Translocation systems such as T1SS allow the Gram-negative cell to release substrates directly into the extracellular matrix by a one-step mechanism, so that the substrate does not get in contact with the periplasm.

T1SS, T3SS, T4SS and T6SS belong to the one-step-mechanisms. The translocated protein is not resting in the periplasm at any time during translocation. T3SS allow for the secretion of a protein across the inner and outer membrane and the membrane of a host cell and releases the substrate directly into the cytoplasm of a host cell.
The two-step-mechanism relies on the Tat- or Sec-pathway that translocate the proteins in a first step across the inner membrane.
The Sec-pathway is responsible for the translocation of many periplasmic proteins. While this pathway only transports proteins in an unfolded state, proteins that require the secretion in a folded state are translocated via the Tat-system. In a second step, translocation systems such as T5SS translocate the previously translocated proteins from the periplasm across the outer membrane. Mostly these substrates are exotoxins (Dalbey and Kuhn, 2012).

Many membrane proteins are integrated into the inner membrane via the SRP dependant Sec-pathway. The Sec translocase forms a transmembrane channel and can laterally release

hydrophobic regions of the proteins into the membrane. A protein family of membrane insertases found in archaea, mitochondria, thylakoids and bacteria is involved in membrane insertion, while the YidC invertase of Gram-negative *E. coli* is well investigated (Kuhn and Kiefer, 2017, Kuhn *et al.*, 2003, Kiefer and Kuhn, 2007, Zhang *et al.*, 2009).
Two mechanisms are known for the integration of proteins that are located in the outer membrane. First these proteins have to be translocated into the periplasm via the Sec- or the Tat-pathway. Lipoproteins are then integrated into the outer membrane via the Lol-system (Tokuda and Matsuyama, 2004) and β-barrel proteins are integrated via the BAM-system (Knowles *et al.*, 2009).

1.1.5.1 Type 1 secretion systems

The type 1 secretion pathway is widespread in Gram-negative bacteria and many of them are pathogenic. The first identified T1SS in bacteria was the hemolysin A (hlyA) T1SS, that was found in *E. coli*. The transportation machinery is built up of three proteins, the ATP-binding cassette (ABC) transporter, the membrane fusion protein and an outer membrane protein (Wagner *et al.*, 1983, Wandersman and Delepelaire, 1990). The size of the transported substrate varies from small (e.g. 20 kDa sized HasA from *Serratia marcescens*) (Létoffé *et al.*, 1994) up to large (e.g. 900 kDa sized LapA from *P. fluorescens*) (Hinsa *et al.*, 2003). The substrates have a C-terminal signal sequence required for transport. The best investigated T1SS is the HlyA T1SS that became a model for T1SS.
The HlyA T1SS consists of HlyB (ABC transporter), HlyD (membrane fusion protein) and TolC (outer membrane protein). The genes encoding the ABC transporter and the membrane fusion protein are located next to each other on the genome in one operon. Besides *hlyB* and *hlyD*, the substrate HlyA and a HlyC are encoded in this operon. HlyC is not necessary for translocation of HlyA but it acylates HlyA and thus activates it (Stanley *et al.*, 1994, Stanley *et al.*, 1998). The outer membrane protein TolC is not encoded within this operon (Wandersman and Delepelaire, 1990). As Thomas *et al.* (2014) describe, genes of proteins that promote the function of the T1SS substrate are sometimes found within the operon although they are not required for transport (Thomas *et al.*, 2014).
In 2000 Koronakis *et al.* were able to solve the structure of the outer membrane protein TolC and it was investigated that TolC is able to form homotrimers to build a channel that reaches deeply into the periplasm (Koronakis *et al.,* 2000). The part that is located in the outer

membrane has a β-barrel structure and the part that reaches into the periplasm shows a long α-helical structure (Koronakis *et al.,* 2000, Thanabalu *et al.*, 1998, Koronakis *et al.*, 1997).
The membrane fusion protein HlyD is connecting the outer membrane protein with the ABC transporter and is located in the cytoplasmic membrane (Higgins *et al.*, 2004, Schulein *et al.*, 1992, Wang *et al.*, 1991). It is assumed that HlyD forms oligomers, Thanabalu *et al.* (1998) are suggesting trimeric structures (Thanabalu *et al.*, 1998), whereas Lee *et al.* (2012) are suggesting hexametric oligomers (Lee *et al.*, 2012). For other membrane fusion proteins such hexametric structures could be shown (Trepout *et al.*, 2010, Xu *et al.*, 2011, Janganan *et al.*, 2011). The ABC transporters are energized by ATP and can translocate substrates across membranes independent from concentration gradients (Higgins, 1992). Usually ABC transporters are consisting of two transmembrane domains (Kerr, 2002) and two nucleotide binding domains and these domains are either in one polypeptide or on multiple peptides that are assembling (Kerr, 2002, Davidson *et al.*, 2008, Zolnerciks *et al.*, 2011). For translocation, the ABC transporter HlyB is forming dimers. A single HlyD protein is only consisting of one transmembrane domain and one ATP binding site (Schmitt *et al.*, 2003, Zaitseva *et al.*, 2005, Zaitseva *et al.*, 2006).
For being secreted by the T1SS the corresponding substrates need a C-terminal secretion signal although there is no conserved signal shown for all T1SS.

1.1.5.2 Type 5 secretion systems

As already mentioned, adhesins may influence surface interaction of bacteria and thus may be involved in biofilm formation. In 2010 it was shown for *P. aeruginosa* that the T5SS substrate CdrA adhesin is promoting biofilm formation (Borlee *et al*., 2010). T5SS are simple translocation systems. T5SS can be split into subgroups. The T5aSS group describes autotransporters that have all required information for translocation in a single protein (Dautin and Bernstein, 2007, Yen *et al*., 2008). The T5SS is based on Sec translocation, so the protein requires an N-terminal Sec-signal peptide for transport across the cytoplasmic membrane (see 1.1.5.3). This signal peptide can be cleaved after transport. The C-terminal region of the T5SS protein contains a domain that forms a β-barrel structure into the outer membrane of Gram-negative bacteria. Another domain of the same protein is the passenger domain that passes the outer membrane through the β-barrel structure of its C-terminal region. When the passenger domain is exposed to the outside of the cell, it can be cleaved from the β-barrel structure to be released from the cell surface or it may remain anchored to the outer membrane (e.g. this is suggested for EstA in *P. aeruginosa*). Slightly more complex are the T5bSS, in

which the β-barrel domain and the passenger domain are located on two polypeptides. Examples of T5bSS in *P. aeruginosa* are the LepA/LepB system (Kida *et al.*, 2008), that is suggested to be involved in eukaryotic cell attachment, and CdrA/CdrB (Borlee *et al.*, 2010), that was shown to promote cell aggregation respectively biofilm formation.

1.1.5.3 Sec- and Tat-pathways

For the translocation of proteins across the cytoplasmic membrane, two widely spread translocation systems are known. These systems are found in bacteria, archaea and in eukarya. The general secretion system (Sec-system) and the twin-arginine-translocation system (Tat-system) are mainly responsible for this translocation.

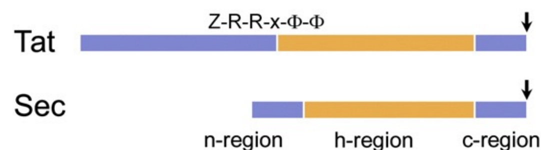

Figure 6: N-terminal signal sequence of Tat- and Sec-substrates (Natale *et al.*, 2008). The Tat- and Sec-substrates require an N-terminal signal sequence for transport. The signal peptides of both pathways show a similar structure, they are built up of a positively charged N-terminal n-region, a hydrophobic core in the h-region and a hydrophilic C-terminal c-region with a cleavage site. The Tat-signal peptide requires a conserved amino acid sequence between the n- and h-region. Arrow indicates cleavage site.

Substrates for these translocation systems require an N-terminal signal sequence. The structure of the signal sequence for both pathways is similar. It is built up of a positively charged N-terminal n-region, a hydrophobic core (h-region) and a hydrophilic C-terminal c-region. To cleave the signal peptide from the protein after transport, a conserved cleavage site for the membrane bound type 1 signal peptidase is encoded in the c-region (Figure 6). During or shortly after transport across the membrane, this type 1 signal peptidase cleaves the signal peptide from the mature protein (Paetzel *et al.*, 2002). The Tat-signal peptide is more complex than the Sec-signal peptide. While the Sec-signal sequence does not require a specific amino acid sequence, a highly conserved amino acid sequence is needed for translocation via the Tat-system. In between of the n- and h-region, the twin arginine motive must be contained for transport: S/T-R-R-x-F-L-K (Berks *et al.*, 1996).

1.2 Expression systems for pseudomonads

For homologous or heterologous gene expressions in *Pseudomonas*, a vector system is required. In 1994 West *et al.*, improved *Escherichia-Pseudomonas* shuttle vectors deriving from pUC18/19 vectors. One of the resulting vectors is the pUCP20 shuttle vector that is widely used in experimental studies. West *et al.* (1994) started with pUCP18/19 vectors that were established by Schweizer (1991) by combining parts of the plasmid pUC18 (Yanisch-Perron *et al.*, 1985) (Figure 7 black part of the vector map) and pRO1614 (Olsen *et al.*, 1982) (Figure 7, stippled part of the vector map) (Schweizer, 1991, West *et al.*, 1994). By deleting a fragment of pUCP18 West *et al.* (1994) tried to enhance gene cloning in *P. aeruginosa* and its related organisms. Nowadays, the pUCP20 vector is widely used for studies in different pseudomonads.

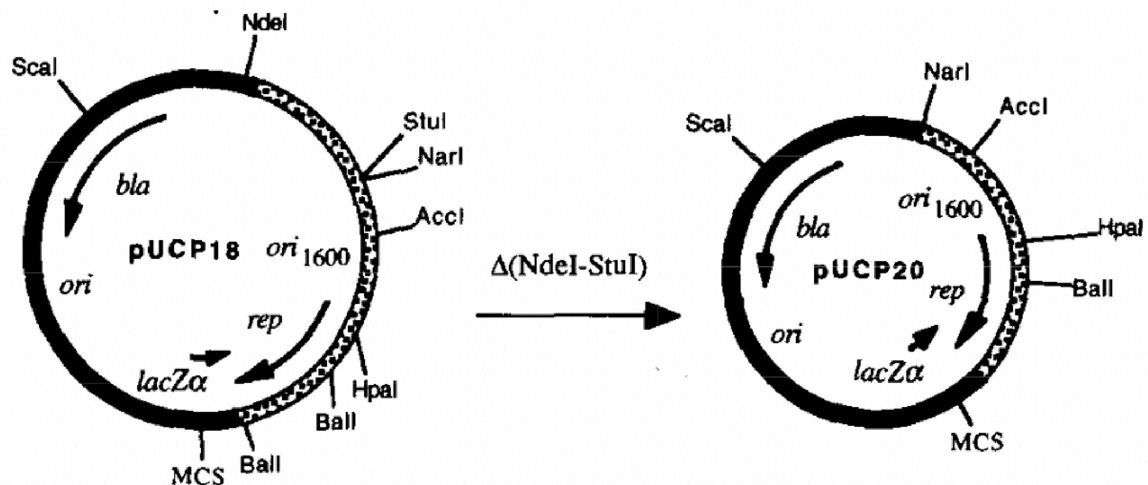

Figure 7: Construction of the pUCP20 *Escherichia-Pseudomonas* shuttle vector, starting from pUCP18 (West *et al.*, 1994, modified). By deleting a part of pUCP18 that is not required for plasmid replication in Pseudomonas or Escherichia the small *Escherichia-Pseudomonas* shuttle vector pUCP20 is generated.

For some experiments (especially for physiological analyses) a tightly controlled gene expression is needed. Not only a specific expression level but also the growth phase or time point in which the gene expression is induced can be important. Some expressed gene products might disturb cell growth respectively cellular physiology and therefore, gene expression during early growth phases might cause a strong selection pressure (Mermod *et al.*, 1986). In such situations inducible gene expressions are necessary.

For different purposes it can become important to regulate the quantity of proteins that are produced by controlling the expression of a specific gene. In some cases, small amounts of

protein production are desired while in others protein overproduction is aimed. Especially when gene products cause selection pressure on the cells, it may become important to induce the gene expression at a specific time point or growth phase during cell cultivation. To study complementation, localization, interaction and transport of specific functional proteins sometimes a tightly controlled expression system becomes necessary. Some inducible promoter systems for the often-investigated model organisms *P. fluorescens*, the human opportunistic pathogen *P. aeruginosa* and *P. putida* are already described.

Mermod *et al.* (1986) showed a benzoate/toluate-inducible system for a high-level gene expression (Mermod *et al.*, 1986). *P. putida* switches from uninduced to fully induced by addition of 1 µM inducer. But the promoter shows a leakage of about 5 % in *P. putida* and *P. aeruginosa* (Mermod *et al.*, 1986) so that there is always some background gene expression. Later on, Smits *et al.* (2001) introduced another system for high-level expression, the dicyclopropylketone-inducible system (Smits *et al.*, 2001).

Three sugar induced systems $LacI^q/P_{tac}$, $AraC/P_{araB}$ and $RhaSR/P_{rhaB}$ were compared in *P. aeruginosa* and Meisner and Goldberg (2016) could only observe a tightly controlled gene expression with the $RhaSR/P_{rhaB}$ promoter system (Meisner and Goldberg, 2016). This system does also function in *P. putida*, as Jeske and Altenbuchner (2010) showed (Jeske and Altenbuchner, 2010).

Earlier, de Lorenzo *et al.* (1993) showed that the $LacI^q/P_{tac}$-based expression system is very leaky under non-induced conditions (Lorenzo *et al.*, 1993).

Transcription regulation in bacteria involves proteins (named transcription regulators, TRs) that activate or repress specific promoters inside the cell. In 1966 Sheppard and Englesberg described as one of the first a transcriptional activator found in *E. coli*, AraC (Sheppard and Englesberg, 1966). Later on, more than 100 proteins showing a sequence homology to a 99 amino acid segment of AraC were detected (Gallegos *et al.*, 1997). AraC family regulators describe a large group of transcriptional regulators in bacteria to modulate and control cellular metabolism to adapt to environmental conditions. Mostly the AraC family TRs act as activators for gene expression (Martin and Rosner, 2001).

1.3 Aim of the study

The aim of this study was to investigate the role of protein secretion systems and their secreted protein substrates on biofilm formation in the plant growth-promoting bacterium *P. fluorescens* A506. As a first approach, candidate genes encoding components of

translocation systems had to be chosen and deleted by the scar-less in-frame gene deletion method that has been previously established in our lab for *P. fluorescens* (Ringel *et al.*, 2016). The influence of these deletions on biofilm formation had to be examined.

For physiological studies, systems for tightly controlled heterogenous and homogenous gene expression in *P. fluorescens* had to be developed.

2 Material and Methods

All materials (e.g. flasks, pipette tips) used for experiments were sterilized by autoclaving (at a temperature of 121 °C and a pressure of 1 bar) before use. Heat sensitive substances (e.g. antibiotics, L-rhamnose) were sterilized by filtration with polyvinylidenefluoride (PVDF) membrane syringe filters (0.22 µm). For all growth media, buffers and solutions desalinated water from a PURELAB flex type I water purification system was used containing a LC197 biofilter (Elga LabWater/Veolia Water Technologies, Celle).

2.1 Strains, plasmids and oligonucleotides used in this study

All strains, plasmids and oligonucleotides that were used in this study are listed in 2.1.1, 2.1.2 and 2.1.3 in tables 1, 2 and 3.

2.1.1 Strains

Table 1: Strains used for this study.

Strain	Characteristics	Reference
Escherichia coli DH5α λ *pir*+ (DSM 06897)	Derivative of strain MM294. *F*- phi80*d lacZ*Delta*M*15 *end*A1 *rec*A1 *hsd*R17 *(rk⁻mk⁻)* *sup*E44 *thi*-l *lambda⁻* *gyr*A96 *rel*A1? Delta*(lac*ZYA-*arg*F*)*U169	Skerman *et al.*, 1980
Pseudomonas aeruginosa PAO1 (DSM 22644)	Wildtype strain, isolated from an infected wound	Skerman *et al.*, 1980
Pseudomonas brassicacearum DBK11 (DSM 13227)	type strain, isolated from rhizoplane of *Brassica napus*	Achouak *et al.*, 2000
Pseudomonas fluorescens A506	Wildtype strain, isolated from pear leaf surface	Wilson and Lindow, 1993
Pseudomonas fluorescens A506 Δ*aprA*	Wildtype strain *P. fluorescens* A506 with deleted *aprA*	This study

Pseudomonas fluorescens A506 Δ*aprAIDE*	Wildtype strain *P. fluorescens* A506 with deleted *aprAIDE*	This study
Pseudomonas fluorescens A506 Δ*aprAIDEF*	Wildtype strain *P. fluorescens* A506 with deleted *aprAIDEF*	This study
Pseudomonas fluorescens A506 Δ*aprD*	Wildtype strain *P. fluorescens* A506 with deleted *aprD*	This study
Pseudomonas fluorescens A506 Δ*aprE*	Wildtype strain *P. fluorescens* A506 with deleted *aprE*	This study
Pseudomonas fluorescens A506 Δ*aprF*	Wildtype strain *P. fluorescens* A506 with deleted *aprF*	This study
Pseudomonas fluorescens A506 Δ*aprI*	Wildtype strain *P. fluorescens* A506 with deleted *aprI*	This study
Pseudomonas fluorescens A506 Δ*fppA*	Wildtype strain *P. fluorescens* A506 with deleted *fppA*	This study
Pseudomonas fluorescens A506 Δ*hlyD*	Wildtype strain *P. fluorescens* A506 with deleted *hlyD*	This study
Pseudomonas fluorescens A506 Δ*lapA*	Wildtype strain *P. fluorescens* A506 with deleted *lapA*	This study
Pseudomonas fluorescens A506 Δ*PflA506_0159*	Wildtype strain *P. fluorescens* A506 with deleted *PflA506_0159*	This study
Pseudomonas fluorescens A506 Δ*PflA506_2789*	Wildtype strain *P. fluorescens* A506 with deleted *PflA506_2789*	This study

Pseudomonas fluorescens A506 Δ*PflA506_2974*	Wildtype strain *P. fluorescens* A506 with deleted *PflA506_2974*	This study
Pseudomonas putida DSM291^{T}	Type strain	Skerman *et al.*, 1980

2.1.2 Plasmids

Table 2: Plasmids used for this study.

Plasmid	**Characteristics**	**Reference**
pABS-*aprD*	cm^{R}, pABS derivate for AprD production	This study
pABS-*aprDEF*	cm^{R}, pABS derivate for AprDEF production	This study
pABS-*tatABC*$_{his}$	cm^{R}, pABS derivate for TatABC$_{his}$ production	Dr. Denise Mehner-Breitfeld
pBW-*tatABC*$_{his}$	amp^{R}, pBW derivate for TatABC$_{his}$ production	Dr. Hendrik Geise
pBW-*aprA*	amp^{R}, pBW derivate for AprA production	This study
pBW-*aprAI*	amp^{R}, pBW derivate for AprAI production	This study
pBW-$_{his}$*aprA*	amp^{R}, pBW derivate for $_{his}$AprA production	This study
pBW-$_{his}$*aprAI*$_{Strep}$	amp^{R}, pBW derivate for $_{his}$AprAI$_{Strep}$ production	This study
pBW-*mat-hip*$_{Strep}$	amp^{R}, pBW derivate	Graubner *et al.*, 2007
pBW-$_{Strep}$*aprA*	amp^{R}, pBW derivate for $_{Strep}$AprA production	This study
pBW-$_{Strep}$*aprAI*$_{his}$	amp^{R}, pBW derivate for $_{Strep}$AprAI$_{his}$ production	This study
pHT01-*gfp*	Expression vector for *B. subtilis*, amp^{R}, cam^{R}, ColE1 ori, *repA*, *lacI*, *gfp*	Mulvenna *et al.*, 2019
pK18	pMB1 (ColEI) ori, oriV, kan^{R}, *lacZα*	Pridmore, 1987
pK18*mobSacB*	Derivative of pK18 with *mob* region of RP4 and a modified *sacB* of *Bacillus subtilis*	Schäfer *et al.*, 1994

pK18*mobSacB*-Δ*aprA*	pK18*mobSacB* plasmid with flanking regions of *aprA* for scar-less *aprA* deletion in *P. fluorescens* A506	This study
pK18*mobSacB*-Δ*aprAIDE*	pK18*mobSacB* plasmid with flanking regions of *aprAIDE* for scar-less *aprAIDE* deletion in *P. fluorescens* A506	This study
pK18*mobSacB*-Δ*aprAIDEF*	pK18*mobSacB* plasmid with flanking regions of *aprAIDEF* for scar-less *aprE* deletion in *P. fluorescens* A506	This study
pK18*mobSacB*-Δ*aprD*	pK18*mobSacB* plasmid with flanking regions of *aprD* for scar-less *aprD* deletion in *P. fluorescens* A506	This study
pK18*mobSacB*-Δ*aprE*	pK18*mobSacB* plasmid with flanking regions of *aprE* for scar-less *aprE* deletion in *P. fluorescens* A506	This study
pK18*mobSacB*-Δ*aprF*	pK18*mobSacB* plasmid with flanking regions of *aprF* for scar-less *aprF* deletion in *P. fluorescens* A506	This study
pK18*mobSacB*-Δ*aprI*	pK18*mobSacB* plasmid with flanking regions of *aprI* for scar-less *aprI* deletion in *P. fluorescens* A506	This study
pK18*mobSacB*-Δ*fppA*	pK18*mobSacB* plasmid with flanking regions of *fppA* for scar-less *fppA* deletion in *P. fluorescens* A506	This study
pK18*mobSacB*-Δ*hlyD*	pK18*mobSacB* plasmid with flanking regions of *hlyD* for scar-less *hlyD* deletion in *P. fluorescens* A506	This study
pK18*mobSacB*-Δ*lapA*	pK18*mobSacB* plasmid with flanking regions of *lapA* for scar-less *lapA* deletion in *P. fluorescens* A506	This study
pK18*mobSacB*-Δ*PflA506_0159*	pK18*mobSacB* plasmid with flanking regions of *PflA506_0159* for scar-less *PflA506_0159* deletion in *P. fluorescens* A506	This study

pK18*mobSacB*-Δ*PflA506_2789*	pK18*mobSacB* plasmid with flanking regions of *PflA506_2789* for scar-less *PflA506_2789* deletion in *P. fluorescens* A506	This study
pK18*mobSacB*-Δ*PflA506_2974*	pK18*mobSacB* plasmid with flanking regions of *PflA506_297 4*for scar-less *PflA506_2974* deletion in *P. fluorescens* A506	This study
pUCP20	*Escherichia-Pseudomonas* shuttle vector, amp^R, *lacZ alpha*, pMB1 (ColE1) ori (for *E. coli*), contains pRO1614 region required for replication of pMB1ori in pseudomonads.	West *et al*., 1994
pUCP20-ANT1	pUCP20-based *Escherichia-Pseudomonas* shuttle vector for anthranilate-regulated gene expression, amp^R, *lacZ alpha*, ColE1 ori (*E. coli*), *antR*, *antA* promoter	This study
pUCP20-ANT1-*gfp*	pUCP20-ANT1 with *gfp* as reporter gene for the anthranilate-regulated *antA* promoter	This study
pUCP20-ANT1-MCS	pUCP20-ANT1 with multiple cloning site of pUCP20	This study
pUCP20-ANT2	As pUCP20-ANT1, but amp^R exchanged by kan^R	This study
pUCP20-ANT2-*gfp*	pUCP20-ANT2 with *gfp* as reporter gene for the anthranilate-regulated *antA* promoter	This study
pUCP20-ANT2-MCS	pUCP20-ANT2 with MCS from pUCP20	This study
pUCP20-RHA1	pUCP20-based *Escherichia-Pseudomonas* shuttle vector for rhamnose-regulated gene expression, amp^R, *lacZ alpha*, ColE1 ori (*E. coli*), *rhaSR*, *rhaB* promoter	This study
pUCP20-RHA2	As pUCP20-RHA1, but amp^R exchanged by kan^R	This study
pUCP20-RHA2-*gfp*	pUCP20-RHA2 with *gfp* as reporter gene for the rhamnose-regulated *rhaB* promoter	This study

2.1.3 Oligonucleotides

Table 3: Oligonucleotides used for this study.

Primer name	Sequence (5‘ → 3‘)	Usage
Primers for deletion experiments and T1SS analysis		
PfA506_*aprA*-DF-LH	CGGTGTACTGGTGCATGCCG GTGGTCATGGAAAC	*aprA* deletion region (*P. fluorescens* A506)
PfA506_*aprA*-DR-LH	TGCAAATGGCTGGTGGCCAG TTGGGTGGAC	*aprA* deletion region (*P. fluorescens* A506)
PfA506_*aprA*-F1-LH	CTAGCCGTCGACAGGCGCGA TAGGCGGTGGCACCGGCATG ATTTG	*aprA* left flanking region (*P. fluorescens* A506)
PfA506_*aprA*-F2-LH	GTAGCCTGATGTAAAGCGCG CGGTGCTTCGG	*aprA* right flanking region (*P. fluorescens* A506)
PfA506_*aprA*-R1-LH	CACCGCGCGCTTTACATCAG GCTACTTTTGACATAAACGT ACTTCCTTGTTTGC	*aprA* left flanking region (*P. fluorescens* A506)
PfA506_*aprA*-R2-LH	CGCCTAAAGCTTAACAGGTA GATCGGGAACCACGGCGCAT CG	*aprA* right flanking region (*P. fluorescens* A506)
PfA506_*aprAIDE*-F1-LH	CTAGATCCCGGGAGGCGCGA TAGGCGGTGGCACCGGCAT GATTTG	*aprAIDE* left flanking region (*P. fluorescens* A506)
PfA506_*aprAIDE*-F2-LH	GAGGAATGAGCATGAAGCCA GTG	*aprAIDE* right flanking region (*P. fluorescens* A506)
PfA506_*aprAIDE*-R1-LH	AACACTGGCTTCATGCTCAT TCCTCTTTTGACATAAACGT ACTTCCTTGTTTGC	*aprAIDE* left flanking region (*P. fluorescens* A506)
PfA506_*aprAIDE*-R2-LH	CGCCTAGTCGACGGGTCTTG CCTTCCAGCTCGTACTC	*aprAIDE* right flanking region (*P. fluorescens* A506)

PfA506_*aprAIDEF*-F1-LH	CTAGATCCCGGGAGGCGCGATAGGCGGTGGCACCGGCATGATTTG	*aprAIDEF* left flanking region (*P. fluorescens* A506)
PfA506_*aprAIDEF*-F2-LH	AAACTGTAGGAGCGAGCTTGCTCGCGAAAAACGTCAACGATGACGTG	*aprAIDEF* right flanking region (*P. fluorescens* A506)
PfA506_*aprAIDEF*-R1-LH	GCGAGCAAGCTCGCTCCTACAGTTTTTTTGACATAAACGTACTTCCTTGTTTGC	*aprAIDEF* left flanking region (*P. fluorescens* A506)
PfA506_*aprAIDEF*-R2-LH	CGCCTAGTCGACATACACCAGTACTTGGCGACGCCGCATTTG	*aprAIDEF* right flanking region (*P. fluorescens* A506)
PfA506_*aprD*-DF-LH	GATTTCCTCAGCGCCAGTTCCAATGCCGAC	*aprD* deletion region (*P. fluorescens* A506)
PfA506_*aprD*-DR-LH	ATCGAGCATCTGGTCGGCGGAGATCAGG	*aprD* deletion region (*P. fluorescens* A506)
PfA506_*aprD*-F1-LH	CTAGCCGTCGACGTTCTCTGATGTGGGCGGCCTGGTGGGCAATGTATCC	*aprD* left flanking region (*P. fluorescens* A506)
PfA506_*aprD*-F2-LH	CAGGCCGGAAGGAATCCAGGCGCATGAG	*aprD* right flanking region (*P. fluorescens* A506)
PfA506_*aprD*-R1-LH	ATGCGCCTGGATTCCTTCCGGCCTGCTTCGCCATATTAATTGTTTTTCCTTGCAG	*aprD* left flanking region (*P. fluorescens* A506)
PfA506_*aprD*-R2-LH	CGCCTAAAGCTTAAATGCACCGGCAGGTGCCCTTCCACTTCC	*aprD* right flanking region (*P. fluorescens* A506)
PfA506_*aprE*-DF-LH	GCCAACGCTGGTTTGGCGTGCATTC	*aprE* deletion region (*P. fluorescens* A506)
PfA506_*aprE*-DR-LH	CCTCGGCAGATGCCAGCGCTTTCTGATAGG	*aprE* deletion region (*P. fluorescens* A506)

PfA506_*aprE*-F1-LH	CTAGCCGGATCCAATCCTTG CGCCTGTGCCTGCAATCC	*aprE* left flanking region (*P. fluorescens* A506)
PfA506_*aprE*-F2-LH	GAGGAATGAGCATGAAGCCA GTG	*aprE* right flanking region (*P. fluorescens* A506)
PfA506_*aprE*-R1-LH	AACACTGGCTTCATGCTCAT TCCTCGCTGCTCATGCGCCT GGATTCCTTCCG	*aprE* left flanking region (*P. fluorescens* A506)
PfA506_*aprE*-R2-LH	CGCCTACTGCAGGGGTCTTG CCTTCCAGCTCGTACTC	*aprE* right flanking region (*P. fluorescens* A506)
PfA506_*aprF*-DF-LH	AGCCGCATCCTGGTGAAAGA GGGCGAGTTG	*aprF* deletion region (*P. fluorescens* A506)
PfA506_*aprF*-DR-LH	AGCAGCACCTGCAAGGCTTGC TCGTTGG	*aprF* deletion region (*P. fluorescens* A506)
PfA506_*aprF*-F1-LH	CTAGCCGAATTCCAGAGCGA GCGTGACAACCTCTCCAGCA TC	*aprF* left flanking region (*P. fluorescens* A506)
PfA506_*aprF*-F2-LH	AAACTGTAGGAGCGAGCTTG CTCGCGAAAAACGTCAACGA TGACGTG	*aprF* right flanking region (*P. fluorescens* A506)
PfA506_*aprF*-R1-LH	GCGAGCAAGCTCGCTCCTAC AGTTTTGGCTTCATGCTCAT TCCTCAGTCAACGC	*aprF* left flanking region (*P. fluorescens* A506)
PfA506_*aprF*-R2-LH	CGCCTATCTAGAATACACCA GTACTTGGCGACGCCGCATT TG	*aprF* right flanking region (*P. fluorescens* A506)
PfA506_*aprI*-DF-LH	CCTTCGGCAACTACAGCAGCG GCCAGGACG	*aprI* deletion region (*P. fluorescens* A506)
PfA506_*aprI*-DR-LH	TGACCTGCTCGAAACTGACT TGGCCGTTGG	*aprI* deletion region (*P. fluorescens* A506)

PfA506_*aprI*-F1-LH	CTAGCCGTCGACCCTGAACAACTATGGCCGGCAGACCCTGAC	*aprI* left flanking region (*P. fluorescens* A506)
PfA506_*aprI*-F2-LH	AAACTTTAATTAAAAGTTATAAGCATATAACTCG	*aprI* right flanking region (*P. fluorescens* A506)
PfA506_*aprI*-R1-LH	TGCTTATAACTTTTAATTAAAGTTTACGTGGCATATCCAACTCCGGCCTGGAGC	*aprI* left flanking region (*P. fluorescens* A506)
PfA506_*aprI*-R2-LH	CGCCTAAAGCTTGCCGAACTCCATTGCTTCCACACTGCAATC	*aprI* right flanking region (*P. fluorescens* A506)
PfA506_*fppA*-DF-LH	CAGCCCTGGTCATCTTCGTCGAG	*fppA* deletion region (*P. fluorescens* A506)
PfA506_*fppA*-DR-LH	AACTGGATGGCGCTGCAACTGAG	*fppA* deletion region (*P. fluorescens* A506)
PfA506_*fppA*-F1-LH	CTAGAGGAATTCGGTAAGCGGTTGAGGCTGCACAC	*fppA* left flanking region (*P. fluorescens* A506)
PfA506_*fppA*-F2-LH	TACGTTTAATCAGGAGTTGTGTAGG	*fppA* right flanking region (*P. fluorescens* A506)
PfA506_*fppA*-R1-LH	CCTACACAACTCCTGATTAAACGTACTGGATCACGTAAAAAACATCCC	*fppA* left flanking region (*P. fluorescens* A506)
PfA506_*fppA*-R2-LH	CGCCTACCTGCAGGAGCGCTGGCTGGACAGCAACAAGCATC	*fppA* right flanking region (*P. fluorescens* A506)
PfA506_*hlyD*-DF-LH	TGCCGAAAGCGAGCGCCAGTATG	*hlyD* deletion region (*P. fluorescens* A506)
PfA506_*hlyD*-DR-LH	GGCTTTATCGGGTTAGAAAGGCGCATC	*hlyD* deletion region (*P. fluorescens* A506)

PfA506_*hlyD*-F1-LH	CTAGCCGGATCCTCGGGCCT GTCGATGAACATGACC	*hlyD* left flanking region (*P. fluorescens* A506)
PfA506_*hlyD*-F2-LH	GAGCGTTGATCTTCAGCGCC TGTACC	*hlyD* right flanking region (*P. fluorescens* A506)
PfA506_*hlyD*-R1-LH	GTACAGGCGCTGAAGATCAA CGCTCAAGCAACACTGATCT GCCCCTTC	*hlyD* left flanking region (*P. fluorescens* A506)
PfA506_*hlyD*-R2-LH	CGCCTACTGCAGAGCGGTTA TCCCAGAACACCAGGTC	*hlyD* right flanking region (*P. fluorescens* A506)
PfA506_*lapA*-DF-LH	ATTTTAATGAGCAGTGTTGT TG	*lapA* deletion region (*P. fluorescens* A506)
PfA506_*lapA*-DR-LH	AGGTTCAGCGCCAGGTTCTC GG	*lapA* deletion region (*P. fluorescens* A506)
PfA506_*lapA*-F1-LH	CTAGCCGGATCCTGTTTGCC GGCGACCAGATCG	*lapA* left flanking region (*P. fluorescens* A506)
PfA506_*lapA*-F2-LH	CACACTTAAGACACGATTAA TCGG	*lapA* right flanking region (*P. fluorescens* A506)
PfA506_*lapA*-R1-LH	GCCGATTAATCGTGTCTTAA GTGTGACTGCTCATTAAAAT TTTCTCCGG	*lapA* left flanking region (*P. fluorescens* A506)
PfA506_*lapA*-R2-LH	CGCCTACTGCAGTTGCCTTC CAGCTTGATCGTC	*lapA* right flanking region (*P. fluorescens* A506)
PfA506_*PflA506_0159*-DF-LH	GCCAGCCTGTCGCGCAACAA C	*PflA506_0159* deletion region (*P. fluorescens* A506)
PfA506_*PflA506_0159*-DR-LH	CATATGCTGTATCGCTGGCA ACC	*PflA506_0159* deletion region (*P. fluorescens* A506)

PfA506_*PflA506_0159*-F1-LH	CTAGCCGGATCCACTCGGCCTGGGTGACCAACTGATG	*PflA506_0159* left flanking region (*P. fluorescens* A506)
PfA506_*PflA506_0159*-F2-LH	AGAAATTAATGCCTACCCTTTTAAATGACTGG	*PflA506_0159* right flanking region (*P. fluorescens* A506)
PfA506_*PflA506_0159*-R1-LH	TTTAAAAGGGTAGGCATTAATTTCTAACATCCATTGTCAGGCTCTCATGC	*PflA506_0159* left flanking region (*P. fluorescens* A506)
PfA506_*PflA506_0159*-R2-LH	CGCCTAAAGCTTCCGTGCCACGGAGCAGTGCTATTC	*PflA506_0159* right flanking region (*P. fluorescens* A506)
PfA506_*PflA506_2789*-DF-LH	GCGCCAGCCTGGATTGGGACAATCCG	*PflA506_2789* deletion region (*P. fluorescens* A506)
PfA506_*PflA506_2789*-DR-LH	CCCTGACAATCCGGCTGTACACC	*PflA506_2789* deletion region (*P. fluorescens* A506)
PfA506_*PflA506_2789*-F1-LH	CTAGCCGGATCCCCTCGCGCAATTCGATGCTCAG	*PflA506_2789* left flanking region (*P. fluorescens* A506)
PfA506_*PflA506_2789*-F2-LH	GTTAATTAGAGAAATTTTATATGCTGTGTCC	*PflA506_2789* right flanking region (*P. fluorescens* A506)
PfA506_*PflA506_2789*-R1-LH	AGCATATAAAATTTCTCTAATTAACAACGTCCATGTCATGTCTCGAAGC	*PflA506_2789* left flanking region (*P. fluorescens* A506)
PfA506_*PflA506_2789*-R2-LH	CGCCTACTGCAGGAACTCGTCAACCCTAATAAATCTGTCCAC	*PflA506_2789* right flanking region (*P. fluorescens* A506)
PfA506_*PflA506_2974*-DF-LH	GATCTGGATCCGGTGGTGATCAAG	*PflA506_2974* deletion region (*P. fluorescens* A506)

PfA506_*PflA506_2974*-DR-LH	GCATGCGCTACCGCGATGACCC	*PflA506_2974* deletion region (*P. fluorescens* A506)
PfA506_*PflA506_2974*-F1-LH	CTAGCCGGATCCCCCGCCAATGCCCATATCAC	*PflA506_2974* left flanking region (*P. fluorescens* A506)
PfA506_*PflA506_2974*-F2-LH	GACTTTTAATTCATCCATCCACCCGAACAG	*PflA506_2974* right flanking region (*P. fluorescens* A506)
PfA506_*PflA506_2974*-R1-LH	CGGGTGGATGGATGAATTAAAAGTCTGACGACATCGAAAAACCCTCTGG	*PflA506_2974* left flanking region (*P. fluorescens* A506)
PfA506_*PflA506_2974*-R2-LH	CGCCTAAAGCTTCTGCATCAGGACGTCGGCATGGATGAACTC	*PflA506_2974* right flanking region (*P. fluorescens* A506)
Primers used for heterologous gene expression in *E. coli*		
PfA506_*aprA*-BamHI-R-LH	CCGACTGGATCCTTAGGCTACGATGTCACTGG	Amplification of *aprA* for insertion in pBW-plasmid
PfA506_*aprA*-NdeI-F-LH	CCGACTCATATGATGTCAAAAGTAAAAGACAAAGC	Amplification of *aprA* for insertion in pBW-plasmid
PfA506_*aprD*-BamHI-R-LH	CCGACTGGATCCTTATGCGCCTGGATTCCTTC	Amplification of *aprD* for insertion in pBW-plasmid
PfA506_*aprD*-NdeI-F-LH	CCGACTCATATGATGGCGAAGTCCCATGGGG	Amplification of *aprD* for insertion in pBW-plasmid
PfA506_*aprE*-BglII-R-LH	CCGACTAGATCTTCATTCCTCAGTCAACGCAGAGC	Amplification of *aprE* for insertion in pBW-plasmid
PfA506_*aprF*-BglII-R-LH	CCGACTAGATCTTTACAGTTTTCCATCCGGGC	Amplification of *aprF* for insertion in pBW-plasmid

PfA506_*aprI*-BamHI-R-LH	CCGACTGGATCCTTAAAGTT TTCGTTGCAGCC	Amplification of *aprI* for insertion in pBW-plasmid
PfA506_*aprI*$_{tag}$-BamHI-R-LH	CCGACTGGATCCAAGTTTTC GTTGCAGCC	Amplification of *aprI*$_{tag}$ for insertion in pBW-plasmid
PfA506_$_{his}$*aprA*-NdeI-F-LH	CCGACTCATATGATGCATCA CCACCACCATCACTCAAAAG TAAAAGACAAAGC	Amplification of $_{his}$*aprA* for insertion in pBW-plasmid
PfA506_$_{Strep}$*aprA*-NdeI-F-LH	CCGACTCATATGATGTGGAG CCACCCGCAGTTCGAAAAGT CAAAAGTAAAAGACAAAGC	Amplification of $_{Strep}$*aprA* for insertion in pBW-plasmid
Primers used for promoter studies		
Pant-fus-F1-LH	CACTCATTAGGCACCCCAGG CTGAGATCCTCCAGGCACCC CATAC	Amplification of *antA* from *P. fluorescens* A506
PantA-SpeI-SbfI-R-LH	CGCTCCTGCAGGCAGGTCAC ACTAGTTTGATCATGGCTAA ACGGTGAGCC	Amplification of *antA* from *P. fluorescens* A506
pUCP20-SapI-F-LH	AGGAAGCGGAAGAGCGCCCA ATAC	Amplification of a fragment from pUCP20
Pant-fus-R1-LH	GTATGGGGTGCCTGGAGGAT CTCAGCCTGGGGTGCCTAAT GAGTG	Amplification of a fragment from pUCP20
Ter-SbfI-F-LH	GCTTCCTGCAGGGTTTTCGT TCCACTGAGCGTCAGAC	Amplification of the terminator from pK18
Ter-HindIII-R-LH	GCTCAAGCTTCAGATTACGC GCAGAAAAAAAGG	Amplification of the terminator from pK18
KanR-SspI-SD-F-LH	GCATAATATTACAGGATGAG GATCGTTTCGC	Amplification of the kan^{R} resistance cassette from pK18*mobSacB*

KanR-BsaI-R-LH	GCTAACCGCGAGACCTCAGAAGAACTCGTCAAGAAG	Amplification of the kan^{R} resistance cassette from pK18*mobSacB*
SD-GFP-SpeI-F-LH	GCAGACTAGTAAAGGAGGAAGGATCCATGA	Amplification of *gfp* with the SD sequence from pHT01-*gfp*
GFP-SbfI-R-LH	GCAGCCTGCAGGTTATTTGTATAGTTCATCCATGCC	Amplification of *gfp* with the SD sequence from pHT01-*gfp*
MCS-F-LH	GCCGACTAGTAGGAGATATACATATGGAATTCGAGCTCGGTACCCGGGGATCCTC	Amplification of the MCS of pUCP20
MCS-R-LH	TGCCAAGCTTGCATGCCTGC	Amplification of the MCS of pUCP20
Prha-fus-F-LH	CACTCATTAGGCACCCCAGGCAAAGAGTGGAACAATGCAGG	Amplification of the *rhaSR* operon and the P_{rhaB}-promoter of *E. coli*
Prha-SpeI-SbfI-R-LH	CGCTCCTGCAGGCAGGTCACACTAGTTGAATTTCATTACGACCAGTC	Amplification of the *rhaSR* operon and the P_{rhaB}-promoter of *E. coli*
Prha-Fus-R-LH	CCTGCATTGTTCCACTCTTTGCCTGGGGTGCCTAATGAGTG	Amplification of a part of pUCP20
Kan^{R}-AccI-F-LH	ACGTGGCCTGTAGACGTCCTAAAAG	Fusion of the resistance cassette and part of the pUCP20 vector backbone
Kan^{R}-fus-R-LH	GCGAAACGATCCTCATCCTGTAATATTATTGAAGCATTTATCAGGG	Fusion of the resistance cassette and part of the pUCP20 vector backbone

2.2 Cultivation conditions

Different bacteria were cultivated in this study for different purposes, thus different media and growth conditions and cultivation techniques were used as described below.

2.2.1 Media and growth conditions

All microorganisms used in this study (*P. fluorescens*, *P. putida*, *P. aeruginosa* and *E. coli*) were either cultivated in liquid Luria-Bertani (LB) medium, King's B medium, M63 or M9 minimal medium (Table 5) or grown on agar plates consisting of LB medium and
1.5 % (w/v) agar or King's medium and 1.5 % (w/v) agar (Table 6). All media used for experiments with *Pseudomonas* were supplemented with 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0) For selection, different antibiotics were added to the medium (Table 7).

For the transformation of *E. coli*, an additional medium was required. SOC (Super Optimal broth with Catabolite repression) medium (Table 5) was added to the cells after the heat shock.

Some of the components for the media were not heat-resistant. These components could not be sterilized by autoclavation and therefore they had to be sterile filtered instead.

All the microorganisms were cultivated under aerobic conditions but at different temperatures as shown in table 4. Liquid cell cultures were incubated with additional shaking (160 rpm) to ensure a sufficient supply of oxygen.

Table 4: Cultivation temperatures for different bacteria.

Microorganism	Cultivation temperature
P. fluorescens	30 °C
P. brassicacearum	30 °C
P. putida	30 °C
P. aeruginosa	37 °C
E. coli	37 °C

Table 5: Composition of the media used for the study.

Medium	Component	Concentration
LB medium	Tryptone	1 % (w/v)
	NaCl	1 % (w/v)
	Yeast extract	0.5 % (w/v)
King's B medium	Peptone	2 % (w/v)
	K_2HPO_4	0.15 % (w/v)
	$MgSO_4$ x 7 H_2O	0.15 % (w/v)
	Glycerol	1 % (w/v)
M63 minimal medium (supplemented with magnesium sulfate, glucose and casamino acids)	KH_2PO_4	0.3 % (w/v)
	K_2HPO_4	0.7 % (w/v)
	$(NH_4)_2SO_4$	0.2 % (w/v)
	$MgSO_4$	1 mM
	Glucose	0.1 % (w/v)
	Casamino acids	0.5 % (w/v)
M9 mineral salt medium (supplemented with 0.4 % (w/v) glucose and 100 µM $FeCl_3$)	KH_2PO_4 x 7 H_2O	1.28 % (w/v)
	KH_2PO_4	0.3 % (w/v)
	NaCl	0.05 % (w/v)
	NH_4Cl	0.1 % (w/v)
	$MgSO_4$	2 mM
	$CaCl_2$	0.1 mM
	Glucose	0.4 % (w/v)
	$FeCl_3$	100 µM
SOC medium	Tryptone	2 % (w/v)
	Yeast extract	0.5% (w/v)
	NaCl	10 mM
	KCl	2.5 mM
	$MgCl_2$	10 mM
	$MgSO_4$	10 mM
	Glucose	10 mM

Table 6: Components of agar plates used in this study.

Medium	Component	Concentration
LB plates	Tryptone NaCl Yeast extract Agar	1 % (w/v) 1 % (w/v) 0.5 % (w/v) 1.5 % (w/v)
LB plates	Tryptone NaCl Yeast extract Sucrose Agar	1 % (w/v) 1 % (w/v) 0.5 % (w/v) 10 % (w/v) 1.5 % (w/v)
Skim milk LB plates	Tryptone NaCl Yeast extract Skim milk Agar	1 % (w/v) 1 % (w/v) 0.5 % (w/v) 1 % (w/v) 1.5 % (w/v)
King's B plates	Peptone K_2HPO_4 $MgSO_4$ x 7 H_2O Glycerol Agar	2 % (w/v) 0.15 % (w/v) 0.15 % (w/v) 1 % (w/v) 1.5 % (w/v)

Table 7: Concentration of antibiotics used in this study.

Antibiotic	Stock solution	Pseudomonads	*E. coli*
Ampicillin	100 mg/ml	100 µg/ml	100 µg/ml
Kanamycin	50 mg/ml	50 µg/ml	50 µg/ml
Carbenicillin	100 mg/ml	200 µg/ml	-
Chloramphenicol	25 mg/ml (solved in ethanol)	-	25 µg/ml

2.2.2 Preparation of cryo cultures

To store the strains at -80 °C, cryo cultures were created. They consisted of 500 µl overnight culture and 500 µl of LB medium with 30 % (w/v) glycerol. For *P. putida* strains King's B

medium with 30 % (w/v) glycerol was used. The cryo culture was stored at a temperature of -80 °C.

2.2.3 Overnight cultures

For overnight cultures 5 ml up to 20 ml liquid medium were inoculated with either a single colony picked from agar plate or cells from a cryo culture. Cultures were incubated over night at the respective incubation temperature with constant shaking (160 rpm). When minimal medium was used, overnight cultures were incubated for up to 30 h.

2.2.4 Optical density determination

Cell growth was controlled by optical density measurement with a photometer (Libra S11, Biochrom) using light of 600 nm (OD_{600}). The pure growth medium was used as a reference. If the OD_{600} exceeded 0.3, the cells were diluted with fresh medium.

2.2.5 Growth curves

For comparing the growth behavior of different strains growth curves were measured in a 96 well microtiter dish plate. Fresh medium was inoculated with so much overnight culture that the cultures had a starting OD_{600} of 0.015. 100 µl of these cultures and fresh medium as a reference were pipetted into each well of the microtiter dish plate. Due to possible evaporation effects, the outer wells of the plate were filled with fresh medium but not used for the measurements. Growth curves were based on measurements of the OD_{600}. To investigate possible autoinduction of a promoter during cultivation fluorescence (excitation at 488 nm, emission at 509 nm) was additionally measured. The cultures were grown at 30 °C with shaking. The Infinite 200 PRO microplate reader (TECAN, Switzerland) and the Synergy Mx Multi-Mode Microplate Reader (BioTek Instruments, Winooski, USA) were used and the OD_{600} and the fluorescence were measured every 15 minutes.

2.2.6 Biofilm formation assay

To compare biofilm formation ability of different *P. fluorescens* strains, the microtiter dish biofilm formation assay that was published by O'Toole in 2011 for *P. aeruginosa* was adapted to the needs of *P. fluorescens* (O'Toole, 2011). M63 minimal medium supplemented with

magnesium sulfate, glucose and casamino acids (Table 5) was used for cultivation. 5 ml of overnight culture were taken and the cells washed three times by centrifugation (16,000 × g for 2 minutes at RT) and resuspension in fresh medium. The OD_{600} of the washed culture was measured and the culture was diluted to an OD_{600} of 2. This culture was diluted by a factor of 100. 100 µl of this diluted culture was used for one well of a 96 well dish. In this study, 6 replicate wells were used and sterile M63 minimal medium (supplemented with magnesium sulfate, glucose and casamino acids) was used as a reference. The 96 well dish was incubated at 20 °C for 20 h.

After incubation, the liquid culture was carefully removed from each well. Each well was washed carefully twice by adding and removing 250 ml saline (0.9 % (w/v) NaCl). 125 µl staining solution (0.1 % (w/v) crystal violet in water) was pipetted into each well. After 15 minutes of incubation at RT the staining solution is carefully removed. Each well is washed three times by carefully adding and removing 250 ml saline (0.9 % (w/v) NaCl). 125 µl of 30 % (w/v) acetic acid were added to each well to solubilize the crystal violet. The dish was incubated for 15 minutes at RT. The solubilized crystal violet was pipetted into a fresh 96 well plate. The absorbance of each well was quantified in a plate reader at 550 nm (Synergy Mx Multi-Mode Microplate Reader (BioTek Instruments, Winooski, USA)).

2.2.7 Cultivation of pseudomonads for promoter studies

2 ml overnight culture were taken. The cells were washed three times by centrifugation (16,000 × g for 2 minutes at RT) and resuspension in fresh medium. The OD_{600} of the washed culture was measured and fresh 100 ml Erlenmeyer flasks with four bottom baffles and 20 ml fresh medium were inoculated to a final OD_{600} of 0.1. The cultures were inoculated at the respective temperature (*P. fluorescens* and *P. putida* at 30 °C, *P. aeruginosa* at 37 °C) with additional shaking. Gene expression was induced by addition of anthranilic acid (1 M stock solution in DMSO) or rhamnose (1 M stock solution in distilled water) to different final concentrations (10 mM, 1 mM, 0.1 mM, 0.01 mM, 0.001 mM and 0 mM). A negative control was not induced and for repression of the rhamnose-system, 1 % (w/v) glucose was added instead of rhamnose. Inoculation of the culture was continued for 1, 2 or 3 hours.

2.2.8 Cultivation of *Escherichia coli* for localization studies of heterogeneously expressed *aprAIDEF*

For localization studies with *E. coli*, the genes encoding heterogenous T1SS translocation machinery AprDEF were encoded under the control of a constitutive promoter on a pABS-vector system. The soluble proteins AprAI were encoded on a pBW-vector and the gene expression was under the control of a L-rhamnose-inducible promoter. The cells of an overnight culture grown on LB were washed by centrifugation (12,000 × g for 2 minutes at TR) and resuspension in fresh LB medium. The OD_{600} was measured and 100 ml flasks with 50 ml LB were inoculated with an OD_{600} of 0.05. To one of these cultures, glucose was added to a final concentration of 1 % (w/v) to inhibit gene expression of *aprAI*. The cultures were incubated at 37 °C with additional shaking (160 rpm). When the OD_{600} of the cultures that had no additional glucose reached 0.3, gene expression of *aprAI* was induced by addition of L-rhamnose to a final concentration of 0.1 % (w/v). The cultivation of the cell cultures was continued until the OD_{600} reached 1.0.
The cells were harvested by centrifugation (4,500 × g for 10 minutes at a temperature of 4 °C). After discarding the supernatant, the cell pellet was immediately used for periplasmic fractionation.

2.3 Molecular genetic methods

The different molecular genetic methods that were used in this study are described below.

2.3.1 Preparation of genomic DNA

For using the genomic DNA of *E. coli* or *P. fluorescens* as a PCR template two different methods were used for preparation. Genomic DNA was either prepared with the “Gentra Puregene Yeast/Bact. Kit” (Qiagen) or by phenol/chloroform precipitation. The preparation with the kit was done as described by the manufacturer and 500 µl overnight culture were used for it.
For the phenol/chloroform precipitation 4 ml of an overnight culture of *E. coli* were used. The cells were harvested by centrifugation (11,300 × g for 5 minutes at RT) and the supernatant was discarded. The cells were washed by resuspending them in 1 ml of TE buffer followed by centrifugation at 11,300 × g for 5 minutes at RT. The cell pellet was resuspended in TE buffer (700 µl). 25 µl of lysozyme solution (10 mg/ml) was added and the suspension was inverted

several times and incubated for one hour at 37 °C. Next, 50 µl of Proteinase K (10 mg/ml) and 150 µl of a 6 % (w/v) laurylsarcosin solution were added and the suspension was incubated at 37 °C for one hour. Afterwards, 500 µl of a 1:1 phenol/chloroform mix (pH 8.0) were added, the reaction tube was inverted several times, followed by a centrifugation step (18,000 × g for 15 minutes at a temperature of 4 °C). The aqueous phase was transferred into a fresh reaction tube. 500 µl of chloroform were added and the tube was inverted several times. After another centrifugation step (18,000 × g for 15 minutes at 4 °C) 600 µl of the aqueous phase were transferred into a fresh reaction tube and 1.2 ml of absolute ethanol (100 %) were added. The solution was incubated at a temperature of -20 °C for 30 minutes. The DNA precipitated and was subsequently pelleted by centrifugation (18,000 × g for 15 minutes at a temperature of 4 °C). The pellet was resuspended with 1 ml of ethanol (70 %). After a final centrifugation step (18,000 × g for 15 minutes at a temperature of 4 °C) the DNA pellet was dried and resuspended in 200 µl of nuclease-free water. The concentration was measured at the NanoDrop 2000 (ThermoFisher Scientific) with a wavelength of 260 nm. The genomic DNA was stored at a temperature of -20 °C.

2.3.2 Preparation of plasmids

The peqGOLD Plasmid Miniprep Kit I (peqlab) was used for plasmid preparation. 4 ml of overnight culture were used. The preparation was done according to the manufacturer's protocol. The plasmid was eluted in 50 µl of nuclease-free water and stored at a temperature of -20 °C.

2.3.3 Polymerase chain reactions

DNA was amplified from different sources such as genomic DNA or plasmids by the polymerase chain reaction (PCR). For DNA amplification two cyclers were used: the FlexCycler2 and the Tpersonal 48 ThermoCycler (Biometra/Analytik Jena AG, Jena).

2.3.3.1 Standard PCR

A 50 µl standard reaction was prepared as described in table 8, using primers listed in table 3. For the standard PCRs the Phusion® High-Fidelity-DNA-Polymerase (New England Biolabs, Frankfurt am Main) was used. The annealing temperature for the primers was 60 °C, only when no DNA could be amplified, or unspecific amplification products appeared, the temperature

was adjusted. The annealing time depended on the product length, the used polymerase amplified 1 kbp within 15 to 30 seconds. DMSO was always used for amplification from genomic DNA, for the other templates it was replaced by distilled water. The standard PCR program is shown in table 9.
The size of PCR products was controlled with agarose gel electrophoresis.

Table 8: Components of a routine PCR.

Component	50 µl reaction
5X Phusion GC Buffer	10 µl
10 mM dNTPs (10 pmol/µl)	1 µl
10 µM forward primer (25 pmol/µl)	1 µl
10 µM reverse primer (25 pmol/µl)	1 µl
template DNA	20-30 ng
DMSO	5 µl
Phusion® High- Fidelity-DNA-Polymerase	0.5 µl
Nuclease-free water	Add to 50 µl

Table 9: Thermocycling conditions for a standard PCR.

Step	Temperature	Time
Initial denaturation	98 °C	30 seconds
35 cycles	98 °C 45-72 °C 72 °C	10 seconds 30 seconds 30 seconds per kilobase
Final Extension Hold	72 °C 16 °C	10 minutes

2.3.3.2 Fusion PCR

The fusion PCR describes a variation of the standard PCR to fuse two DNA fragments. The first step of the fusion PCR was a standard PCR that amplified the two DNA fragments that should be fused. One primer for one of these fragments had a 5`-end that was complementary to the 5`-end of one primer for the other fragment. The resulting two fragments should have an overlapping region of 25 bp. In the second step, the actual fusion PCR was done. The reaction

for fusion PCR was set up similarly to a standard PCR reaction. Here the two fragments with the homologue regions were used as template DNA and no primers were added to the reaction. The two fragments were used in a 1:1 ratio, using 10 fmol of each fragment as template. The PCR was started as a standard PCR for 10 cycles. During these cycles the homologue regions of the two fragments should anneal and the fusion product should be produced. After the 10 cycles, 1 µl of the outer primers (25 pmol/µl) for amplification of the fusion product were added to the reaction. The PCR was continued for 25 further cycles. The PCR product was cleaned up by agarose gel electrophoresis and gel extraction.

2.3.3.3 Colony PCR

For colony PCR, 1 ml overnight culture was harvested by centrifugation at 16,000 × g for 2 minutes at RT. The pellet was resuspended in 1 ml distilled water and heated to 98 °C for 10 minutes. The cells were sedimented by centrifugation at 16,000 × g for 1 minute at RT and chilled on ice. For colony PCR 2 µl supernatant were used as template. The colony PCR was prepared as described in table 10 and a colony PCR program is shown in table 11.

Table 10: Components of a colony PCR.

Component	25 µl reaction
5X Phusion GC Buffer	5 µl
10 mM dNTPs (10 pmol/µl)	0.5 µl
10 µM forward primer (25 pmol/µl)	0.5 µl
10 µM reverse primer (25 pmol/µl)	0.5 µl
template DNA	2 ml
DMSO	2.5 µl
Phusion® High- Fidelity-DNA-Polymerase	0.25 µl
Nuclease-free water	Add to 25 µl

Table 11: Thermocycling conditions for a colony PCR.

Step	Temperature	Time
Initial denaturation	98 °C	30 seconds
30 cycles	98 °C 45-72 °C 72 °C	10 seconds 15 seconds 15 seconds per kilobase
Final Extension Hold	72 °C 16 °C	5 minutes

2.3.4 Scar-less gene deletion

This method was established by Ringel *et al*. (2016) (Ringel *et al*., 2016). First, the pK18*mobsacB* deletion plasmid was produced containing the approximately 1 kb sized left and right flanking regions of the target gene. Next, the deletion plasmid was transferred into *P. fluorescens* by electroporation and incubated overnight on agar plates (with the respective antibiotic). On the agar plate single colonies should appear that were picked and incubated in a 5 ml liquid overnight culture (with the respective antibiotic). The next day, the overnight culture is diluted two times 1:1000 and 50 µl of the final dilution is put on a LB agarose plate with 10 % (w/v) sucrose and without the previously used antibiotic. The next day, a few clones were picked from the agar plate and tested for growth ability on two LB agar plates with either the previously used antibiotic or with 10 % (w/v) sucrose. Those clones, that only grew on the sucrose containing agar plate, were picked and 5 ml liquid cultures were inoculated (without antibiotic). Theses cultures were used as templates for colony PCR the next day. When colony PCR confirmed the gene deletion, a cryo culture of the respective cell culture was created. To confirm that the left and right flanking regions of the target gene were not influenced by the deletion method, genomic DNA was extracted. The deletion area was amplified from the genomic DNA and used for DNA sequencing.

2.3.5 Agarose gel electrophoresis

To separate DNA fragments according to their length, agarose gel electrophoresis was performed. The “Perfect BlueTM Gelsystem” Mini S, Mini M and Mini L from peqlab/VWR (Erlangen) were used. The agarose gel was prepared with 1 x TAE buffer. The concentration of the agarose in the gel varied from 1 % (w/v) up to 2 % (w/v), depending on the size of the

DNA fragments. To separate small DNA-fragments (smaller than 500 bp) agarose gels containing 2 % (w/v) agarose were used. For larger DNA fragments, gels containing 1 % (w/v) agarose were used for gel electrophoresis. To stain the DNA in the agarose gel, HDGreenTM Plus (Intas, Göttingen) was added to the gel to a final concentration of 0.05 µl/ml.
Sample volumes of up to 50 µl were loaded onto the pockets of the gel. Before loading the samples on the gel, 6-fold sample buffer (50 % (w/v) glycerin, 200 mM EDTA, 0.2 % (w/v) xylencyanol or bromophenol blue) was added to the sample. The final concentration of the sample buffer in the sample was 1-fold.
To determine the size of the DNA fragments on the gel, 5 µl of specific DNA ladders (FastLoad 1 kb DNA ladder and FastLoad 100 bp DNA ladder by SERVA, Heidelberg) were used.
The agarose gel electrophoresis was run at 120 V. To detect the DNA, of UV-light (360 nm) was used at the Intas-UV-System Gel Stick (Intas, Göttingen).

2.3.6 Gel extraction

Digested or amplified DNA fragments that were used for gel electrophoresis were extracted from the agarose gel. After gel electrophoresis DNA fragments of the desired length were cut out of the gel with a razor blade. Next, the DNA was extracted with the NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Düren). The extraction was done according to the manufacturer's protocol. Finally, the DNA was eluted with 20 µl of warm nuclease-free water and stored at a temperature of -20 °C.

2.3.7 Determination of DNA concentration

To determine the concentration of DNA in samples such as plasmids or gel extraction, the Nanodrop2000 spectral photometer (Thermo Scientific, Dreieich) was used.

2.3.8 Restriction digest

DNA was digested by restriction enzymes for different purposes such as linearization of plasmids, preparation of PCR products for ligation or for testing a plasmid for correctness. The DNA was digested with one (single digest) or two (double digest) restriction enzymes. Depending on the amount of DNA the reaction volume was between 20 µl (for a test digestion of a plasmid) and 50 µl (for plasmids and for DNA fragments that should serve as inserts). The components for a restriction digest are shown in table 12. Whenever possible the High-Fidelity

(HF®) version of the restriction enzymes was used. The restriction digest was incubated at a temperature of 37 °C for two hours. PCR products and single digested plasmids were subsequently cleaned up by NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren) (eluting in 20 µl of nuclease-free water), whereas a double digested plasmid was run on an agarose gel. Afterwards, the DNA was extracted from the gel.

Table 12: Components of a restriction digest.

Component	**20 µl reaction (test digestion)**	**50 µl reaction (plasmid and DNA fragment for later ligation)**
DNA	10 µl	40 µl
10 x *CutSmart*® buffer (NEB)	2 µl	5 µl
Restriction Enzyme(s)	1 µl (each)	2.5 µl (each)
Nuclease-free water	Add to 20 µl	Add to 40 µl

2.3.9 Ligation

After the clean-up of the digested PCR products and vector backbones, the DNA fragments were ligated by the T4 DNA Ligase (New England Biolabs, Frankfurt am Main). For each vector backbone - insert combination two reactions were set up, varying in the ratio of vector backbone to insert. The reaction was set up according to the protocol shown in table 13. Vector backbone and insert were used in a 1:3 and 1:5 ratio. The ligation was prepared on ice and the reaction was incubated overnight at a temperature of 16 °C.

Table 13: Components of a ligation.

Component	**20 µl reaction**
T4-DNA-Ligase Buffer (NEB)	2 µl
Vector	~ 12.5 ng per kbp
Insert	~ 37.5 ng per kbp (1:3 ratio) or ~ 62.5 ng per kbp (1:5 ratio)
T4-DNA-Ligase (NEB)	1 µl
Nuclease-free water	Add to 20 µl

2.3.10 Transformation of *Escherichia coli* (DH5α)

For transformation of *E. coli* DH5α, ultracompetent cells had to be prepared. 500 µl of an LB overnight culture were used to inoculate 25 ml LB. This culture was incubated until the OD_{600} reached 0.6. Then, the cells were stored on ice for 10 minutes. The cells were harvested by centrifugation at 6,800 × g for 10 minutes at 4 °C. The supernatant was discarded and the cells were resuspended in 7.5 ml of transformation buffer (10 mM PIPES (pH 6.7), 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl). Next, the culture was incubated on ice for 45 minutes. The cells were sedimented by centrifugation (6,800 × g for 10 minutes at 4 °C) and the supernatant was discarded. After resuspending the cells in 2.5 ml transformation buffer, they were incubated for 45 minutes on ice. Afterwards, 160 µl of DMSO were slowly added to the cell suspension. Cells were incubated on ice for 10 minutes. Finally, the culture was aliquoted, liquid nitrogen was used to freeze the aliquots and afterwards they were stored at -80 °C.
For transformation of the ultracompetent *E. coli*, 100 µl of the cells were used. Cells were carefully defreezed on ice. 10 µl of a ligation reaction (2.3.9) respectively plasmid DNA were added to the cells and carefully mixed. The cells were incubated on ice for 30 minutes. Next, the cells were heat shocked for 1 minute at 42 °C, followed by a 2 minute incubation step on ice. 1 ml SOC medium (2 % (w/v) tryptone, 0,5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to the cells and the cells were incubated for 1 hour at 37 °C with gentle shaking. Cells were harvested by centrifugation (3.800 × g for 5 minutes at RT), resuspended in 100 µl LB and put on an agar plate containing the respective antibiotic. The agar plate was incubated at 37 °C.

2.3.11 Transformation of *Pseudomonas*

In this study, non-pathogenic and pathogenic *Pseudomonas* were transformed with electroporation.

2.3.11.1 *Pseudomonas fluorescens*

For transformation of *P. fluorescens*, 6 ml overnight culture grown in LB was used. The culture was aliquoted into four 1.5 ml cultures. The cultures were harvested by centrifugation (16,000 × g for 2 minutes at RT) and the cell pellets were washed two times with 1 ml of 300 mM sucrose. Finally, all four cell pellets were resuspended in 100 ml of 100 mM sucrose. 100 µl of these cells were used for electroporation and put into a 2 mm electroporation cuvette. 10 – 50 ng

of a replicable plasmid or 300 – 500 ng of non-replicable plasmid or chromosomal DNA was added and the electroporation was performed with the Gene Pulser Xcell System electroporator from Biorad (Hercules, California, United States) at 25 µF, 200 Ω and 2.5 kV. Afterwards, 1 ml LB medium was added to the culture and the cells were incubated at 30 °C for 2 h with gentle shaking. Next, the cells were harvested (3,800 × g for 5 minutes at RT) and the supernatant was discarded. The cells were resuspended in 100 µl LB and put to an agar plate containing the respective antibiotic for selection. The plate was incubated at 30 °C overnight.

2.3.11.2 *Pseudomonas aeruginosa* and *Pseudomonas putida*

For transformation of *P. aeruginosa* and *P. putida* a 5 ml overnight culture in LB (*P. aeruginosa*) respectively in King's B (*P. putida*) was needed. Cells were harvested by centrifugation (16,000 × g for 2 minutes at RT) and washed three times by resuspending the pellet in 1 ml distilled water followed by centrifugation (16,000 × g for 2 minutes at RT). Depending on the amount of the bacteria, the cell pellet was resuspended in 100 µl – 1000 µl of distilled water. 100 µl of cell suspension were pipetted into a 2 mm electroporation cuvette and 100 ng plasmid was added. Electroporation was performed with the Gene Pulser Xcell System electroporator from Biorad (Hercules, California, United States) at 25 µF, 200 Ω and 2.5 kV. Immediately, 900 µl LB medium (*P. aeruginosa*) respectively King's B medium (*P. putida*) were added into the electroporation cuvette. Afterwards, the cell suspension was pipetted into a fresh 1.5 ml reaction tube and incubated for 1 hour at 37 °C (*P. aeruginosa*) respectively 30 °C (*P. putida*). Finally, 50 µl of the cell suspension were plated on an agar plate (LB respectively King's B) containing the respective antibiotic for selection. The remaining 950 µl cell suspension were harvested by centrifugation (16,000 × g for 2 minutes at RT), resuspended in 10 µl medium and also plated on an agar plate containing the respective antibiotic for selection. The agar plates were incubated overnight at 37 °C (*P. aeruginosa*) respectively 30 °C (*P. putida*).

2.3.12 DNA sequencing

To prove the correctness of generated plasmids, the regions of interests on these plasmids were sequenced. Regions of interest were ligation sites where vector backbone and insert were ligated and the middle of a fusion PCR product, where the two fragments were fused together. Seqlab/Microsynth (Göttingen) offers a sequencing service with the Sanger sequencing method. 12 µl of plasmid solution (40-100 ng/µl) and 3 µl of primer solution (20µM) were mixed in a 1.5 ml reaction tube and sent for sequencing. Primers that bind 50 bp to 100 bp next to the region of interest were chosen.

2.4 Biochemical methods

All biochemical methods that were used in this study are described below.

2.4.1 Subcellular fractionation of *Escherichia coli*

For periplasmic fractionation of *E. coli* the cell pellet (2.2.8) was resuspended in 20 ml of TES buffer and incubated for 10 minutes at RT. The cells were sedimented by centrifugation (3,800 × g for 10 minutes at a temperature of 4 °C) and the supernatant discarded. 1 ml of ice cold 5 mM $MgSO_4$-solution was used for resuspending the cells and the cultures were incubated on ice for 20 minutes. Next, the cells were sedimented by centrifugation (20,800 × g for 10 minutes at a temperature of 4 °C). The periplasmic fraction (the supernatant) was carefully transferred into a fresh reaction tube and stored on ice. The pellet was resuspended in 1 ml of ice cold 5 mM $MgSO_4$-solution and used for cell disruption with sonification (2.4.2).

2.4.2 Cell disruption with sonification

The cells prepared in 2.4.1 were used for cell disruption as a first step to separate the cytoplasmic fraction from the membrane fraction. The samples were kept on ice during the whole cell disruption process. DNaseI and protease inhibitor PMSF (final concentration of 1 mM) were added to the samples. The cell disruption was performed with the Sonifier 250 (by Branson). The cells were treated three times for 30 s with sonification (Duty Cycle 40 %, Output control 3.5), with a break of 30 seconds between each treatment. Next, the cell debris was separated by centrifugation (4,500 × g for 15 minutes at a temperature of 4 °C).

The supernatant was directly used for fractionation (2.4.3). The cell debris pellet was resuspended in 0.9 ml of 5 mM $MgSO_4$-solution and prepared for SDS-PAGE. 100 µl of the resuspended cell debris sample were prepared for SDS-PAGE by adding 100 µl SDS sample buffer (100 mM Tris-HCl (pH 6.8), 20 % (v/v) glycerin, 4 % (w/v) SDS, 0.2 % (w/v) bromophenol blue, 200 mM DTT). The SDS-PAGE samples were incubated at 95 °C for 5 minutes and stored at -20 °C.

2.4.3 Fractionation of disrupted *Escherichia coli*

The supernatant of the previously disrupted cell samples after centrifugation from 2.4.2 was used for ultracentrifugation at 130,000 × g for 30 minutes at a temperature of 4 °C to separate the cytoplasm from the membrane fraction. The upper 75 % of the resulting supernatant was carefully pipetted into a fresh reaction tube, the lower 25 % of the supernatant was discarded. The membrane pellet was resuspended in 0.9 ml of 5 mM $MgSO_4$-solution.
100 µl of the cytoplasmic and the membrane fraction were prepared for SDS-PAGE by adding 100 µl SDS sample buffer (100 mM Tris-HCl (pH 6.8), 20 % (v/v) glycerin, 4 % (w/v) SDS, 0.2 % (w/v) bromophenol blue, 200 mM DTT) each. The SDS-PAGE samples were incubated at 95 °C for 5 minutes and stored at -20 °C.

2.4.4 SDS-PAGE

To detect specific proteins in cell samples, proteins within the sample were separated according to their length by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The composition of the SDS gels varied depending on the size of the proteins that should be analyzed. For small proteins a 15 % SDS gel was prepared, for larger proteins a 12.5 % or 10 % SDS gel was used (Table 14). Gels were 1 mm thick.

Table 14: Composition of SDS-PAGE gels.

Component	Stacking gel (5 ml)	Separating gel (10 ml)		
		10 % SDS gel	12 % SDS gel	15 % SDS gel
Acryl/Bisacryl amide (30% w/v)	0.67 ml	3.4 ml	4 ml	5 ml
Distilled water	2.975 ml	3.8 ml	3.2 ml	2.2 ml
1.5 M Tris-HCl, pH 8.8	-	2.6 ml	2.6 ml	2.6 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml	-	-	-
10 % (w/v) SDS	50 µl	100 µl	100 µl	100 µl
10 % (w/v) APS	50 µl	100 µl	100 µl	100 µl
TEMED	5 µl	10 µl	10 µl	10 µl

The SDS-PAGE was performed in *Minigel-Twin* chambers (Biometra/Analytik Jena, Jena). To estimate the size of proteins within the cell samples, 3 µl of PageRuler^{TM} Prestained Protein Ladder (Thermo Scientific, Dreieich) were used as reference (Figure 8).

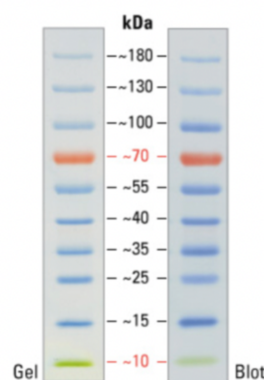

Figure 8: PageRuler^{TM} Prestained Protein Ladder.

The running buffer for electrophoresis was composed of 200 mM glycin, 20 mM Tris and 1 % (w/v) SDS. The volume of the loaded samples was 10 µl and the gel was run at 10 mA. When the running had passed the stacking gel, the gel was run at 20 mA.

2.4.5 Coomassie stain

For an unspecific staining of the proteins on the SDS-PAGE gel, the Coomassie stain was used. The SDS gel was carefully removed from the electrophoresis chamber and incubated overnight in Coomassie staining solution (50 % (v/v) ethanol, 7.5 % (v/v) acetic acid and 0.25 % (w/v) Coomassie Brillant Blue G250). Afterwards, the staining solution was removed and the stained gel was incubated in decolorizing solution (5 % (v/v) ethanol and 7.1 % (v/v) acetic acid). The solution was exchanged every few hours until the gel became clear enough to see the stained proteins.

2.4.6 Semi-dry Western blot

To detect specific proteins in the samples used for SDS-PAGE, specific antibodies were used. Before protein detection, the proteins had to be transferred from the SDS gel onto a nitrocellulose membrane (Amersham™ Protran® 0.2µm NC western blotting membranes, nitrocellulose (GE Healthcare, Freiburg)) by semi-dry Western blot in *Fastblot B44* chambers (Biometra/Analytik Jena, Jena).
The acrylamide gels were carefully removed from the gel electrophoresis chamber (from 2.4.4). Whatman® cellulose chromatography filter papers (GE Healthcare, Freiburg), the acrylamide gel and the nitrocellulose membrane were equilibrated in Towbin buffer (25 mM Tris, 192 mM glycin, 20 % (v/v) methanol).
Directly on the anode of the blotting chamber, a wet filter paper was placed. On top of that, the membrane and then the acrylamide gel were placed. The acrylamide gel was covered by a second filter paper. Excess buffer next to the filter paper and air bubbles were carefully removed. The cathode of the blotting chamber was put on top. The blotting was performed at 0.7 mA per square cm for 60 minutes. Afterwards, the membrane was blocked by incubating it at 4 °C overnight in 20 ml of blocking solution (5 % (w/v) skim milk powder in PBS buffer (4 mM Na_2HPO_4, 2 mM KH_2PO_4, 13,7 mM NaCl, 3 mM KCl)). Next, the membranes were used for antibody detection (2.4.7).

2.4.7 Protein detection with antibodies

The nitrocellulose membrane from 2.4.6 was washed by incubating it three times for 5 minutes with PBS buffer (4 mM Na_2HPO_4, 2 mM KH_2PO_4, 13.7 mM NaCl, 3 mM KCl)) with gentle shaking.

Different antibodies were used in this study (Table 15). First, the membrane was incubated with the primary antibody for 2 h at RT with gentle shaking. The primary antibody was diluted in PBS buffer (4 mM Na_2HPO_4, 2 mM KH_2PO_4, 13.7 mM NaCl, 3 mM KCl) with 0.5 % (w/v) BSA and 0.02 % (w/v) NaN_3. Next, the antibody solution was removed and the membrane was washed by incubating it three times for 5 minutes with PBS buffer (4 mM Na_2HPO_4, 2 mM KH_2PO_4, 13.7 mM NaCl, 3 mM KCl) with gentle shaking. The secondary antibody and the recombinant antibody were both incubated together in PBS buffer (4 mM Na_2HPO_4, 2 mM KH_2PO_4, 13.7 mM NaCl, 3 mM KCl) with 0.5 % (w/v) BSA for 1 h.

After antibody incubation, the membrane was washed by incubating it three times for 5 minutes with PBS buffer (4 mM Na_2HPO_4, 2 mM KH_2PO_4, 13.7 mM NaCl, 3 mM KCl) with gentle shaking.

Table 15: Antibodies used in this study.

Antibody	Type of antibody	Antigen	Dilution	Reference
Anti-His-tag	Mouse-IgG (Sera) monoclonal	Tetra-/Penta-His	1:5000	QUIAGEN (Hilden)
Goat-anti-mouse-HRP-conjugate	Goat-IgG polyclonal	Mouse-IgG	1:5000	Carl Roth (Karlsruhe)
Strep-Tactin-AP-conjugate	recombinant	Strep-Tag® II	1:5000	IBA (Goettingen)

After the antigen incubation and the washing steps, the immune stained membrane was incubated with 100 mM Tris HCl (pH 8.0), 1.25 mM luminol, 0.225 mM p-coumaric acid. Directly before development 6 µl 30 % (v/v) H_2O_2 was added to the solution. The chemiluminescence signals were detected by ChemoStar 6.0 ECL (Intas, Göttingen).
The recombinant Strep-Tactin-AP conjugate was detected via an alkaline phosphatase reaction. The immune stained membrane was washed and incubated in AP buffer for 10 minutes. Afterwards, the membrane was incubated with 10 ml of AP buffer and 100 µl of 2.5 % (w/v) BCIP (in 100 % DMF) and 100 µl of 5 % (w/v) NBT (in 70 % (v/v) DMF). The membrane was incubated at a temperature of 37 °C for about 20 minutes. The AP reaction was stopped by washing the membrane with distilled water.

2.4.8 Fixation of cells

For cell fixation, formaldehyde was freshly prepared from paraformaldehyde. Cells that were used for flow cytometry had to be fixed. 1 ml cell culture was harvested by centrifugation (16,000 × g for 2 minutes at RT) and resuspended in 1 ml PBS containing 2 % (w/v) formaldehyde. Cells were incubated at RT for 10 minutes. Afterwards, the cells were sedimented by centrifugation (16,000 × g for 2 minutes at RT). Next, the fixed cells were washed one time resuspending them in 1 ml PBS supplemented with 20 mM Tris-HCl (pH 7.5) followed by centrifugation (16,000 × g for 2 minutes at RT). Finally, they were resuspended in 1 ml PBS supplemented with 20 mM Tris-HCl (pH 7.5). The OD_{600} was determined and the samples were diluted to a final OD_{600} of 0.01. Samples were stored on ice and used for flow cytometry.

2.4.9 Flow cytometry

For flow cytometry the Guava EasyCyte Flow Cytometer and the software GuavaSoft 2.7 (Merck, Darmstadt) was used. The measurement was taken according to the manufacturer's protocol.

2.5 Microscopy

Microscopy of GFP-fluorescence inside cells should give first information about promoter activity respectively promoter inducibility. To detect GFP-fluorescence, living bacteria were investigated by epifluorescence microscopy on agarose slides to avoid bacterial movement.

2.5.1 Preparation of agarose slides

To fix the cells on a surface for microscopy, agarose slides were used. 500 µl of a previously boiled agarose solution (1 % (w/v) agarose in distilled water) were pipetted on a slide and a second slide was directly laid on top helping to achieve a smooth and even surface of the agarose. After polymerization the upper slide was carefully removed and the agarose remained on the bottom slide. 2 µl of cell culture was put on the agarose slide. Finally, a coverslip was put on top and the sample was used for microscopy.

2.5.2 Epifluorescence microscopy

For epifluorescence microscopy a Zeiss Axio Imager.M2 (Zeiss, Jena) was used with 498 nm light for excitation and 516 nm for emission and filter set 13 for GFP fluorescence detection. Photos were taken with an AxioCam MRm camera (Zeiss, Jena) at 1000 x magnification (Plan-Neofluar 100x N.A. 1.3 objective). For comparable results the illumination time was set to 30 s. The softwares AxioRel 4.8.2 and Zen 2.3 (blue edition) (Carl Zeiss, Jena) were used for taking and process the images.

2.6 Buffers

All buffers used in this study are listed in table 16.

Table 16: Buffers used in this study.

Buffer	Component	Concentration
Agarose sample buffer (6-fold)	Glycerin	50 % (w/v)
	EDTA	200 mM
	xylencyanol or bromophenol blue	0.2 % (w/v)
AP buffer	Tris-HCl, pH 9.5	100 mM
	NaCl	100 mM
	$MgCl_2$	50 mM
PBS (pH 7.4)	NaCl	13.7 mM
	KCl	3 mM
	Na_2HPO_4	4 mM
	KH_2PO_4	2 mM
SDS running buffer	Tris	20 mM
	Glycin	200 mM
	SDS	1 % (w/v)
SDS sample buffer (2-fold)	Glycin	20 % (w/v)
	Tris-HCl, pH 6.8	100 mM
	SDS	4 % (w/v)
	Bromophenol blue	0.2 % (w/v)
	DTT	200 mM
TAE buffer	Tris	40 mM
	Acetic acid	20 mM
	EDTA	1 mM
TE buffer	Tris-HCl, pH 8.0	10 mM
	EDTA	1 mM
TES buffer	Tris-HCl, pH 8.0	10 mM
	EDTA	0.1 mM
	Sucrose	20 % (w/v)

Towbin buffer	Tris Glycin Methanol	25 mM 192 mM 20 % (v/v)
Transformation buffer	PIPES (pH 6.7) $MnCl_2$ $CaCl_2$ KCl	10 mM 55 mM 15 mM 250 mM
TSS buffer (pH 6.5)	Tryptone Yeast extract NaCl PEG 6000 DMSO $MgSO_4$	10 % (w/v) 5 % (w/v) 10 % (w/v) 10 % (w/v) 5 % (w/v) 33 mM

2.7 Centrifuges and rotors

All centrifuges and rotors used in this study are listed in table 17.

Table 17: Centrifuges and rotors used in this study.

Centrifuge	Rotor	sample volume	Speed	Temperature
Eppendorf *MiniSpin Plus*	Blackline Rotor	Up to 2 ml	Up to 13,000 × g	RT
Eppendorf *Centrifuge 5804R*	F-45-30-11	Up to 2 ml	3,800 × g up to 20,800 × g	4 °C
	A-4-44	Up to 50 ml	4,500 × g	4 °C
Thermo Scientific *Sorvall Discovery M120SE*	S55-A2	1.5 ml	130,000 x g	4 °C

2.8 Chemicals

The chemicals used in this study were purchased from suppliers listed in table 18.

Table 18: Suppliers of chemicals used in this study.

Suppliers
Alfa Aesar (Kandel)
AppliChem (Darmstadt)
Becton Dickinson GmbH (Heidelberg)
Carl Roth (Karlsruhe)
IBA (Göttingen)
Merck KGaA (Darmstadt)
New England Biolabs (Frankfurt am Main)
Oxoid Limited (Basingstoke)
SERVA (Heidelberg)
Sigma-Aldrich (Taufkirchen)
VWR (Erlangen)

3 Results

3.1 Deletion of a component of the T1SS for the transport of the protease AprA affects biofilm formation

To investigate the relevance of protein translocation systems for biofilm formation, genes encoding key components of translocation systems were deleted and the biofilm formation of different deletion strains was compared. Two different strategies were used to choose candidate genes for deletion experiments. First, the genome of our lab strain *P. fluorescens* A506 was scanned by bioinformatic tools (established by Michael Ringel in our lab) that detect possible components of secretion systems. Thereafter, the presence of genes encoding homologous proteins that are known to influence biofilm formation in other related bacteria was examined in the genome of our lab strain. Seven candidate genes were chosen: *lapA, hlyD, aprE, PflA506_2974, fppA, PflA506_2789* and *PflA506_0159.*

lapA encodes the T1SS substrate LapA, the large adhesion protein A (LapA, 523 kDa). As already described in chapter 1.1.4, the secretion and surface localisation of LapA is necessary for biofilm formation of *P. fluorescens* Pf0-1. At conditions unfavorable for biofilm formation, LapA is cleaved from the cell surface, preventing the bacterium from biofilm formation (Newell *et al*., 2011).

hlyD encodes HlyD, a 51 kDa sized membrane fusion protein that connects the ATPase subunit of the inner membrane with an outer membrane pore to form a T1SS. This specific T1SS translocates LapA. Its name is derived from the homologous T1SS component of the *E. coli* hemolysine secretion system.

aprE encodes AprE, a 48 kDa membrane fusion protein of another T1SS. This system may be involved in transport of a protease. The function of this transport system has not been shown yet.

PflA506_2974 encodes a predicted T5SS autotransporter with unknown function that may be involved in formation of surface protein structures.

fppA encodes a 18 kDa prepilin peptidase A. This peptidase is responsible for maturation of the pilin precursor Flp, which is essential for the biogenesis of pili. De Bentzmann *et al.* (2006)

showed that overproduction of FppA and Flp enhances surface attachment and promotes biofilm formation in *P. aeruginosa* (de Bentzmann *et al.*, 2006).

In *Erwinia chrysanthemi* it was shown that a HecA protein (3850 aa) is important for surface attachment and aggregate formation and virulence (Rojas *et al.*, 2002, Park *et al.*, 2014). In our lab strain *P. fluorescens* A506 there are two predicted unknown adhesin/hemagglutinines, that both belong to the HecA family: PflA506_2789 (312 kDa; 3013 aa) and PflA506_0159 (587 kDa; 5695 aa).

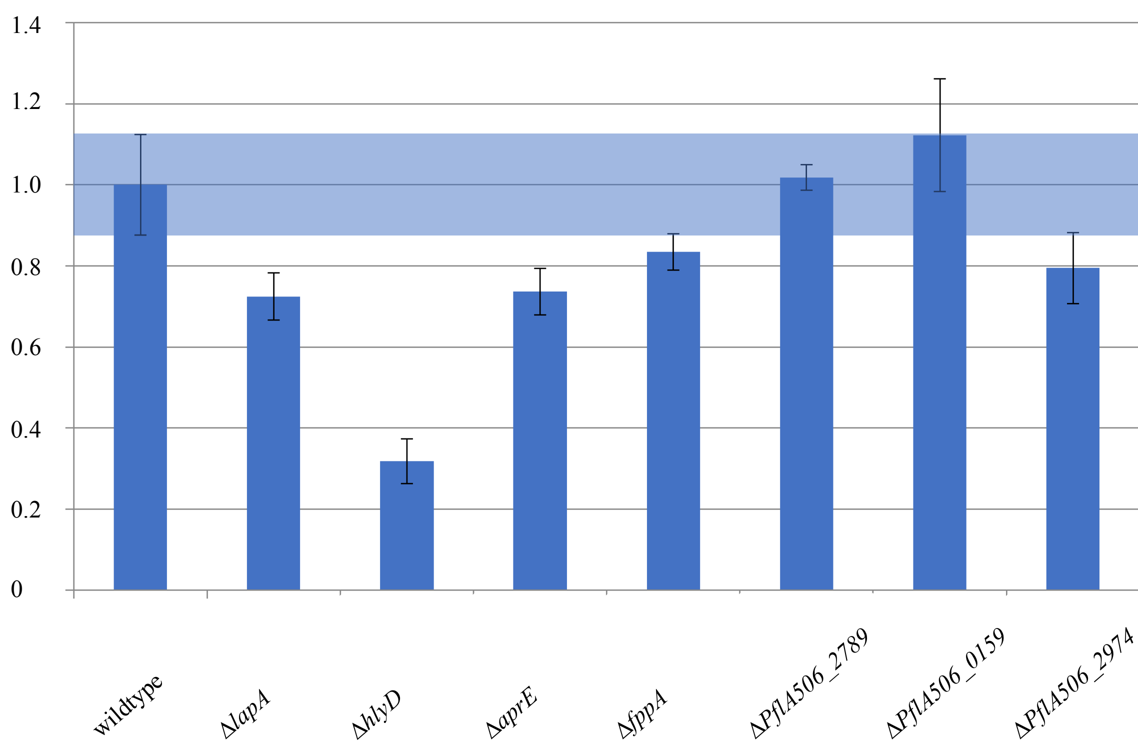

Figure 9: **Biofilm formation assay of wildtype strain *P. fluorescens* A506 and different deletion strains of *P. fluorescens* A506.** The deletion strains were lacking the single genes encoding LapA, HlyD, AprE, FppA or the unnamed proteins PflA506_2789, PflA506_0159 or PflA506_2974. The amount of biofilm is normalized to the amount of biofilm formed by the wildtype strain. The blue rectangle indicates the amount of biofilm formed by the wildtype strain. Deletion of *lapA*, *hlyD* and *aprE* negatively influenced biofilm formation.

Some of the deletion strains were forming less biofilm than the wild type strain (Figure 9). The deletion of *hlyD* had the strongest impact on biofilm formation, encoding the T1SS membrane fusion protein that was necessary for the export of LapA. The impact of the deleted exporter (HlyD) was even stronger than the impact of the deleted substrate (LapA). This might indicate the existence of so far not identified further HlyD substrates with relevance for biofilm

formation that are also transported by this exporter. *lapA* was (in contrast to the findings of O'Toole and Kolter (1998) and Hinsa *et al*. (2003) in *P. fluorescens* Pf0-1) not absolutely necessary for biofilm formation in the lab strain *P. fluorescens* A506 (Figure 9) (O'Toole and Kolter, 1998, Hinsa *et al*., 2003). The candidate gene *aprE*, also encoding a T1SS component, showed also the same impact on biofilm formation as did *lapA*. FppA and PflA506_2974 might also slightly influence biofilm formation. The two predicted unknown adhesin/hemagglutinines, that both belong to the HecA family (PflA506_2789 and PflA506_0159) seemed to have no influence on biofilm formation.
The results showed the importance but no strict requirement of at least three candidate genes (respectively their corresponding proteins) for biofilm formation.

3.2 A mistargeting of AprA to the periplasm likely causes the reduced biofilm formation

The T1SS that belongs to AprE is expected to export a potential protease into the environment. Five proteins belong to this T1SS and their corresponding genes are encoded on the genome of *P. fluorescens* A506 directly next to each other (Figure 10).

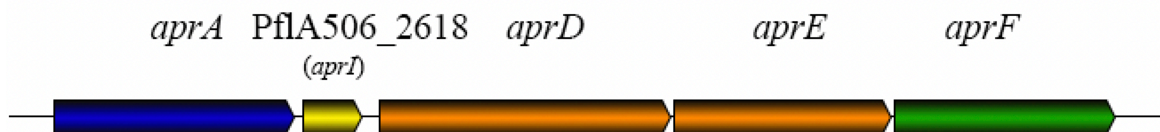

Figure 10: Genes encoding the T1SS components in *P. fluorescens* A506. Gene color indicates the localization of the corresponding gene product as annotated on pseudomonas.com (Winsor et al., 2016). The blue gene encodes an extracellular protein, the yellow gene encodes a periplasmic protein, the two orange genes are described as encoding membrane proteins and the green gene is encoding an outer membrane protein.

Four of the T1SS component genes are named: *aprA, aprD, aprE* and *aprF*. *aprA* encodes the T1SS substrate, that is supposed to be a protease. The T1SS translocation machinery is encoded by *aprDEF*, with *aprD* encoding the ABC transporter, *aprE* encoding the membrane fusion protein and *aprF* encoding the outer membrane protein. The small unnamed gene *PflA506_2618* is located on the genome of *P. fluorescens* A506 between *aprA* and the genes encoding the translocation machinery. In the pseudomonas.com database (Winsor *et al.*, 2016) the gene product of the unnamed gene annotated as a protease inhibitor.

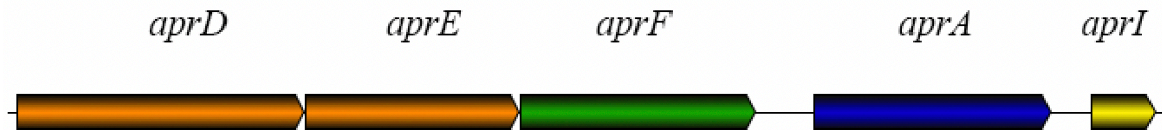

Figure 11: Genes encoding the T1SS components in *P. aeruginosa* PAO1. Gene color indicates the localization of the corresponding gene product as annotated on pseudomonas.com (Winsor *et al.*, 2016). The blue gene encodes an extracellular protein, the yellow gene encodes a periplasmic protein, the two orange genes are described as encoding membrane proteins and the green gene is encoding an outer membrane protein.

This translocation system can also be found in *P. aeruginosa* PAO1. As in *P. fluorescens*, the T1SS components are encoded by five genes: *aprAIDEF*, although the order of the genes is different on the genomes (Figure 11). By aligning the amino acid sequence of the components of the T1SS of *P. aeruginosa* PAO1 with the corresponding sequences from *P. fluorescence* A506 an identity of approximately 50 % for each protein is observed. The small protein AprI from *P. aeruginosa* PAO1 has an identity of 41 % with PflA506_2618 from *P. fluorescens* A506 and conserved regions can be seen (Figure 12). Because PflA506_2618 is related to the AprI from *P. aeruginosa* PAO1, PflA506_2618 is named AprI in this study.

```
PA01 aprI  17   FFSTGI-SMASSLILLSASDLAGQWTLQQDEAPAICHLELRDSEVAEASGYDLGGDTACL  75
                F S G  +MASSL+L +++ LAGQW L+Q +   +C L+L +   A       L GD AC
A506 aprI  16   FVSAGAHAMASSLVLPTSAQLAGQWELKQQDQ--VCALKLVEQANA------LEGDIACA  67

PA01 aprI  76   TRWLPSEPRAWRPTPAGIALLERGGLTLMLLGRQGEGDYRVQKGDGGQLVLRR  128
                 +WL   +P   W PTP GI L     G  +  L RQ EGDY+ +   G  + L+R
A506 aprI  68   EQWLGDKPLTWSPTPDGIWLFNAEGSGITHLNRQKEGDYQARTKAGEVVRLQR  120
```

Figure 12: Alignment of the amino acid sequence of AprI from *P. aeruginosa* PAO1 with the amino acid sequence of AprI from *P. fluorescens* A506. The alignment was performed with BlastP (Altschul *et al.*, 1997).

Different deletion strains were created with the scar-less deletion method (Ringel *et al.*, 2016), lacking genes encoding only one component of the T1SS (Δ*aprA*, Δ*aprI*, Δ*aprD*, Δ*aprE*, Δ*aprF*) or multiple genes (Δ*aprAIDEF*).

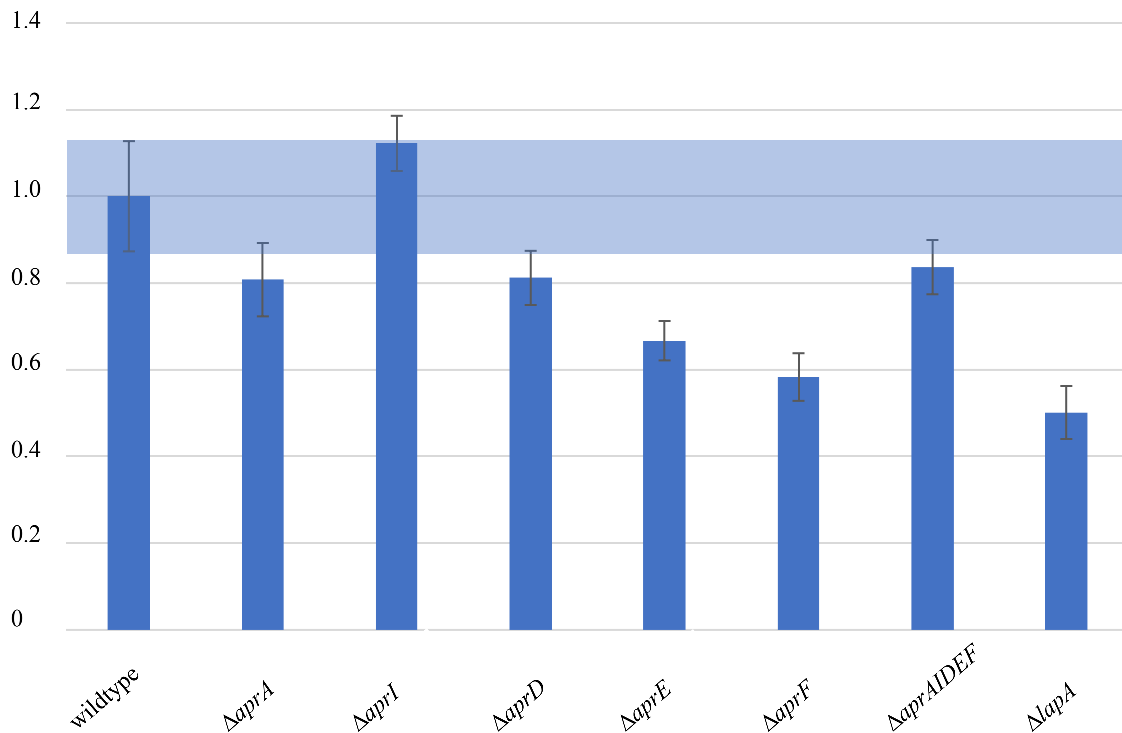

Figure 13: Biofilm formation assay of the wildtype strain *P. fluorescens* A506 in comparison to indicated deletion strains. The deletion strains were lacking the single genes *aprA*, *aprI*, *aprD*, *aprE*, *aprF*, *lapA* or the genes *aprAIDEF*. The amount of biofilm is normalized to the amount of biofilm formed by the wildtype strain. The blue rectangle indicates the amount of biofilm formed by the wildtype strain. Deletion of *aprE*, *aprF* and *lapA* negatively influenced biofilm formation.

Biofilm formation of wildtype *P. fluorescens* A506 and *P. fluorescens* A506 Δ*lapA* served as controls for comparison (Figure 13). The deletion of *aprA*, *aprI*, *aprD* and of the whole T1SS genes (*aprAIDEF*) hardly influenced biofilm formation (Figure 13). Only the deletion of *aprE* and *aprF* lead to a reduced amount of biofilm. As *aprA* could be deleted without influencing biofilm formation, the substrate of this T1SS AprA was not necessary for biofilm formation. A weaker biofilm was observed when the transport of AprA across the inner membrane was still possible but transport across the outer membrane was disabled by deleting *aprE* encoding the membrane fusion protein or *aprF* encoding the outer membrane protein.

A growth curve of the deletion strains showed that the deletions did not influence growth behavior of *P. fluorescens* A506 (Figure 14). Thus, a weaker biofilm was not caused by weaker cell growth.

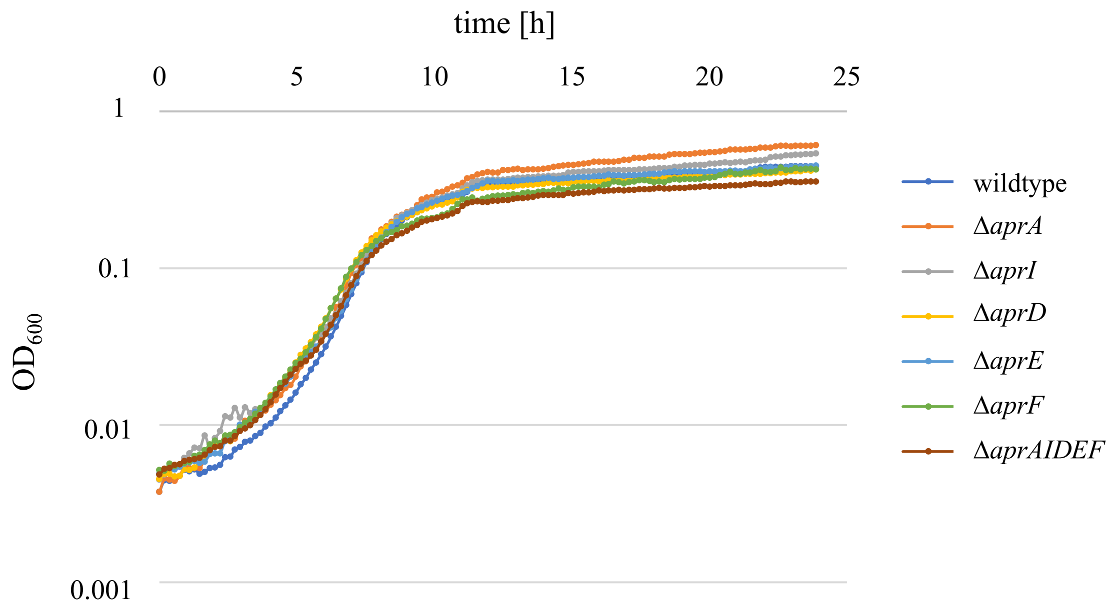

Figure 14: Growth curves of wildtype *P. fluorescens* A506 and different deletion strains. Deletion strains were lacking the single genes *aprA*, *aprI*, *aprD*, *aprE*, *aprF*, *lapA* or the genes *aprAIDEF*. All strains tested showed similar growth behavior.

To conclude, the results indicate that the phenotype of *P. fluorescens* A506 is not caused by not secreted T1SS but it may be caused by a mistargeted AprA into the periplasm.

3.3 The inhibitor AprI inhibits protease activity in the environment of *Pseudomonas fluorescens* A506 colonies

As AprA is described as a protease in *P. aeruginosa*, the protease activity in the environment of colonies grown on skim milk agar was tested. The wildtype *P. fluorescens* A506 exhibited a weak protease activity (Figure 15). The deletion of genes encoding AprA (the protease), AprD (ABC transporter), AprE (membrane fusion protein), AprF (outer membrane protein) as well as deletion of all the genes encoding the T1SS components (AprAIDEF) lead to slightly less protease activity in the environment of the colonies on skim milk agar. In contrast to this, the deletion of *aprI* resulted in an increased protease activity. This indicated that the protease AprA was somehow involved in extracellular protein degradation and AprI was inhibiting this protease activity.

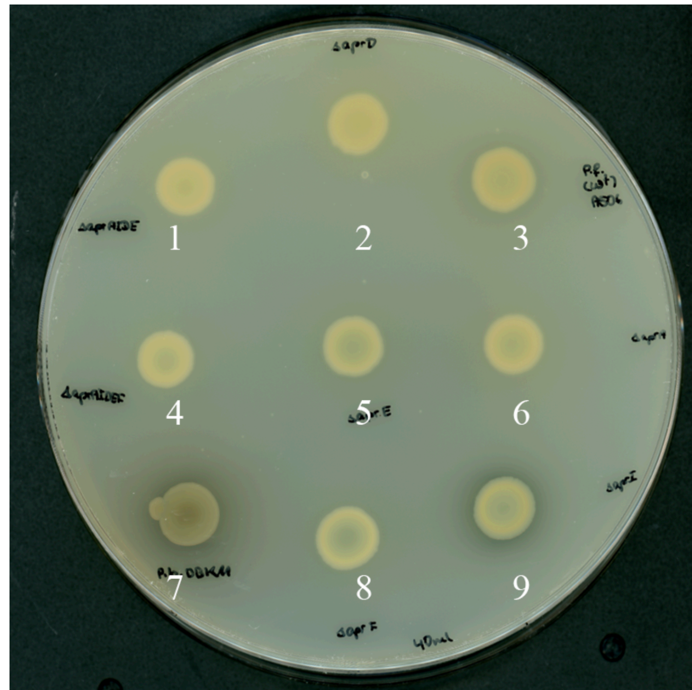

Figure 15: Growth of different *Pseudomonas* strains on skim milk agar for detection of extracellular protease activity. 1: *P. fluorescens* A506 Δ*aprAIDE*. 2: *P. fluorescens* A506 Δ*aprD.* 3: *P. fluorescens* A506 (wildtype). 4: *P. fluorescens* A506 Δ*aprAIDEF*. 5: *P. fluorescens* A506 Δ*aprE*. 6: *P. fluorescens* A506 Δ*aprA*. 7: *P. brassicacearum* DBK11 (wildtype). 8: *P. fluorescens* A506 Δ*aprF*. 9: *P. fluorescens* A506 Δ*aprI*. Wildtype strain *P. fluorescens* A506 showed weak protease activity in the environment of *P. fluorescens* A506 colonies and deletion of *aprI* increased this observed protease activity. The other PGPR strain *P. brassicacearum* DBK11 showed an even stronger protease activity in the environment of the colonies and was only used for comparison.

3.4 Heterologously expressed *aprA* elongates exponential growth phase of *Escherichia coli*

As a first approach to get further information on localization of the T1SS components, the *aprAIDEF* genes were heterogeneously expressed in Gram-negative *E. coli*. Expression of the genes in *E. coli* had the advantage that different vectors were available for different purposes. The pBW-based vector system with a rhamnose-inducible and glucose-repressible promoter system allowed tightly controlled expression of *aprA* and *aprI*. The pABS-based vector with a constitutive promoter optimized for membrane proteins was used.
By comparing the growth behavior of the strains (Figure 16), the recombinant strains could be classified into two groups.

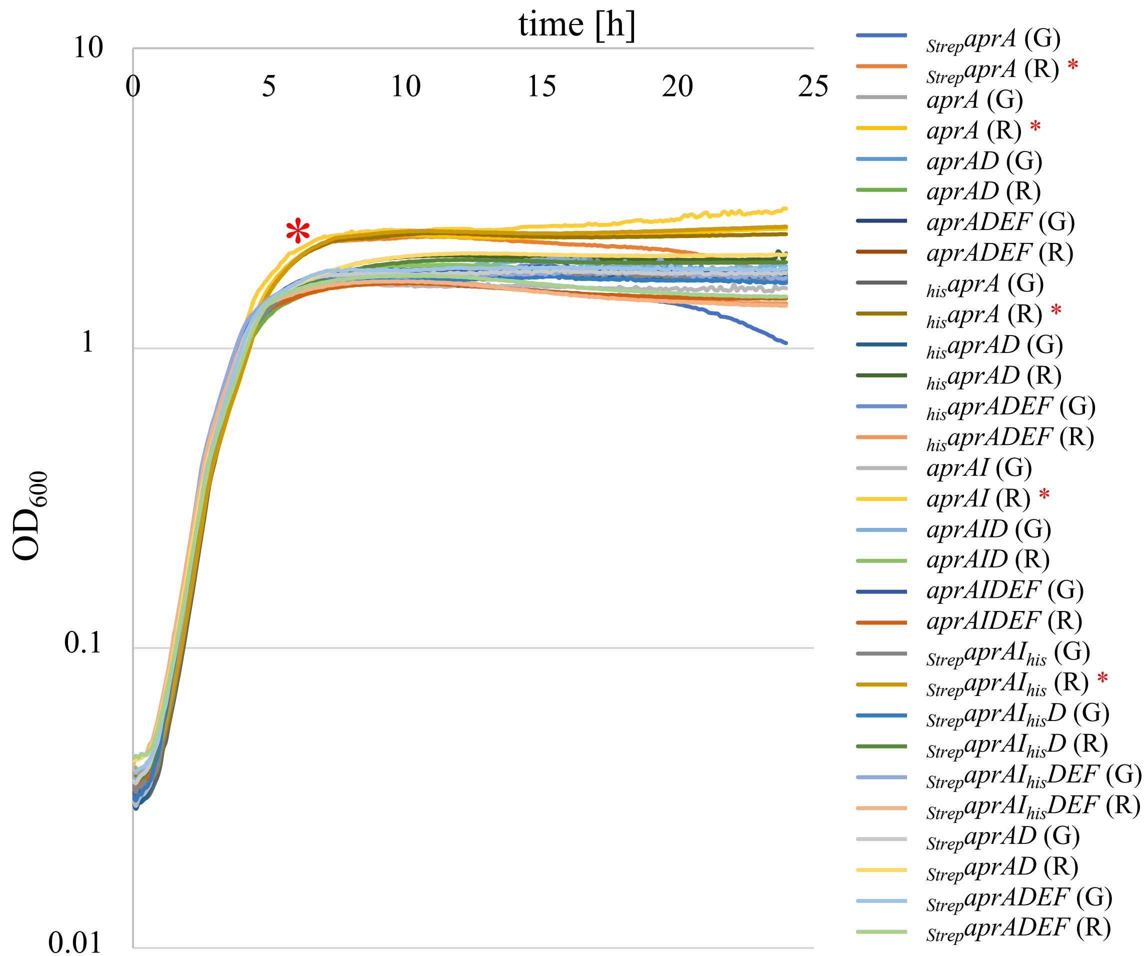

Figure 16: Growth curves of *E. coli* with components of the T1SS from *P. fluorescens* A506 encoded on plasmids. Components of the translocations machinery (*aprDEF*) were encoded on a pABS-based plasmid while *aprA* and *aprI* (with and without C-terminal or N-terminal fused tags) were encoded on a pBW-based plasmid and under the control of a L-rhamnose-inducible promoter. During cultivation there was either 1 % (w/v) glucose (G) or 0.1 % L-rhamnose (R) in the growth medium for repression respectively induction of the L-rhamnose-inducible promoter. Cultures showing an elongated exponential growth phase are indicated by *.

Five cultures achieved a higher optical density during cultivation (Figure 16). This group derived from cultures where rhamnose was contained in the medium for induction of the gene expression of $_{Strep}$*aprA*, *aprA*, $_{his}$*aprA*, *aprAI* or $_{Strep}$*aprAI*$_{his}$. The same strains grown with glucose (for gene repression) instead of rhamnose were not reaching such an elongated growth phase (Figure 17). The strains showing this elongated growth phase did not contain a pABS-derivate with genes encoding the ABC transporter (*aprD*) or the whole translocation machinery (*aprDEF*). When the genes of the translocation machinery components were additionally expressed in the *E. coli* strains, the growth phase was not elongated.

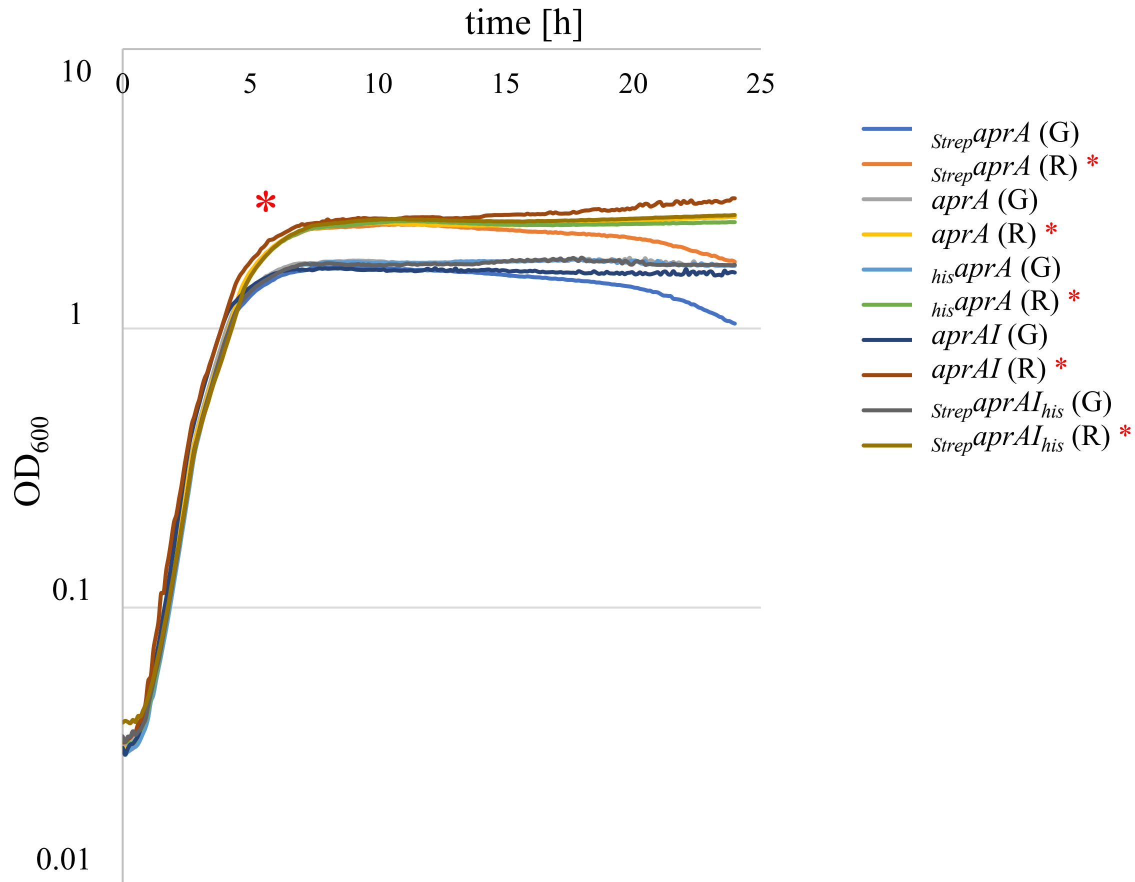

Figure 17: Growth curves of *E. coli* with components of the T1SS from *P. fluorescens* A506 encoded on plasmids. The T1SS components *aprA* and *aprI* (with and without C-terminal or N-terminal fused tags) were encoded on a pBW-based plasmid and under the control of a L-rhamnose-inducible promoter. During cultivation there was either 1 % (w/v) glucose (G) or 0.1 % L-rhamnose (R) in the growth medium for repression respectively induction of the L-rhamnose-inducible promoter. Cultures showing an elongated exponential growth phase are indicated by *.

To sum up the observations, the exponential growth phase was only elongated when *aprA* was expressed. Tags for later detection and additional expression of *aprI* had no influence on cell growth. When the ABC transporter *aprD* or the whole translocation machinery *aprDEF* was expressed, this effect was not observed.

3.5 Heterologously produced AprI is transported into the periplasm of *Escherichia coli*

Because it was still unclear whether the heterologous gene expression of the T1SS leads to a functional translocation system in *E. coli*, further experiments were done. The genes encoding the whole translocation machinery were constitutively expressed (pABS-*aprDEF*) and the expression of *aprA* and *aprI* was under control of the rhamnose-inducible system on pBW-vectors, thus was induced by adding rhamnose during cell cultivation. For later detection AprA and AprI were fused with a Strep-Tag® II sequence or a hexa-histidine tag. The cytoplasmic fraction, the membrane fraction, the periplasmic fraction and the cell debris were used for antibody detection.

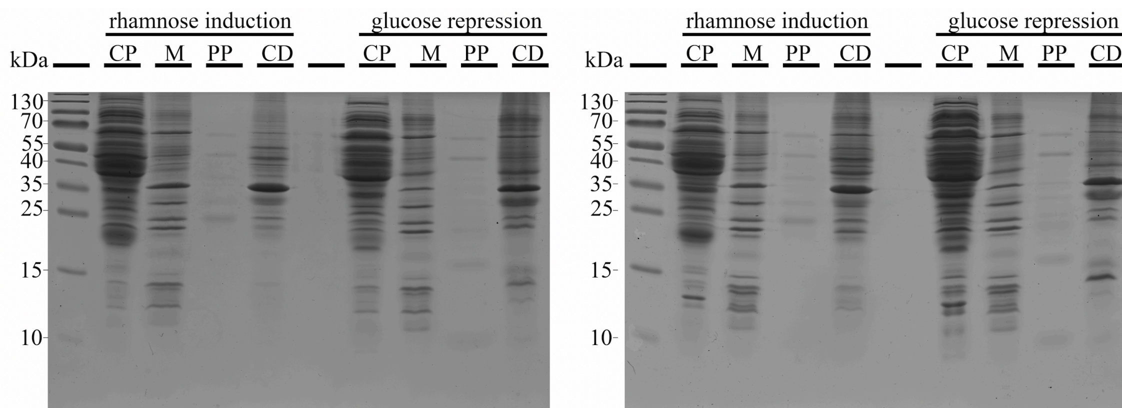

Figure 18: Coomassie stained acrylamide gels with samples derived from heterogenous expression of the T1SS in *E. coli*. A 15 % SDS-PAGE acrylamide gel was used for separation of proteins in the different cell fractions. Samples deduced from cultivation of DH5α pBW-$_{Strep}$*aprAI*$_{his}$ pBW-*aprDEF* (left) or DH5α pBW-$_{his}$*aprAI*$_{Strep}$ pBW-*aprDEF* (right) with either 1 % (w/v) glucose or 0.1 % (w/v) L-rhamnose (for repression or induction of $_{Strep}$*aprAI*$_{his}$ respectively $_{his}$*aprAI*$_{Strep}$ gene expression) added during cultivation. 3 µl of the prestained protein ladder and 10 µl of each cell sample were loaded onto the gel. CP: cytoplasmic fraction. M: membrane fraction. PP: periplasmic fraction. CD: Cell debris sample.

The fractionation of *E. coli* cells into cytoplasmic fraction, membrane fraction and periplasmic fraction worked well (Figure 18). No major contaminations from one fraction into another were observed.

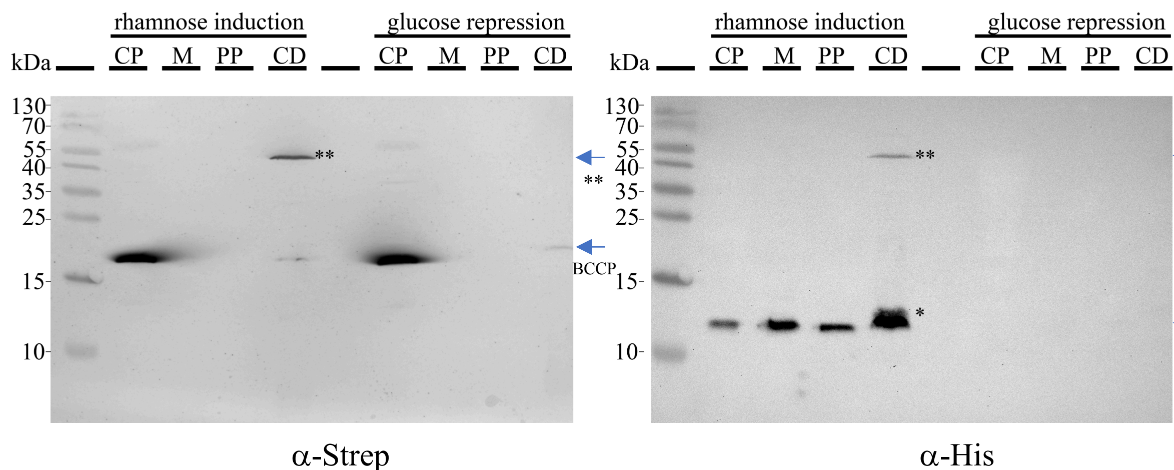

Figure 19: Semi-dry Western blots of samples derived from heterogenous expression of the T1SS in *E. coli.* A 15 % SDS-PAGE acrylamide gel was used for separation of proteins in the different cell fractions. Samples deduced from cultivation of DH5α pBW-$_{Strep}$*aprAI*$_{his}$ pBW-*aprDEF* with either 1 % (w/v) glucose or 0.1 % (w/v) L-rhamnose (for repression or induction of $_{Strep}$*aprAI*$_{his}$ gene expression) added during cultivation. 3 µl of the prestained protein ladder and 10 µl of each cell sample were loaded onto the gel. CP: cytoplasmic fraction. M: membrane fraction. PP: periplasmic fraction. CD: Cell debris sample. BCCP: cytoplasmic biotin carboxyl carrier protein. *: AprI-His. **: Strep-AprA/AprA. The left Western blot was developed with the recombinant Strep-Tactin-AP-conjugate. The right Western blot was developed with anti-His-tag (mouse) and goat-anti-mouse-HRP-conjugate.

DH5α carriing the plasmids pBW-$_{Strep}$*aprAI*$_{his}$ and pABS-*aprDEF* were cultivated and fractionated. As the gene expression of $_{Strep}$*aprAI*$_{his}$ was under control of the rhamnose-inducible promoter, samples deduced from cultivation with added inducer and cultivation with added glucose (for promoter repression) were compared (Figure 19). AprI has a size of 13.3 kDa and was detected via a C-terminal fused hexa-histidine tag that made the protein only slightly larger. The anti-His antibody detected only proteins within the samples that deduced from cultivation with added inducer, indicating no unspecific protein detection. The small protein AprI$_{His}$ was found in all cell fractions tested. The cytoplasm signal was the weakest signal compared with the others. The signals in the membrane fraction and periplasmic fraction were stronger. A small shift of the signal of AprI$_{His}$ in the periplasmic sample might indicate processed AprI$_{His}$.

The T1SS substrate AprA has a size of 49.3 kDa and was N-terminally fused with a Strep-Tag® II sequence for detection. In cytoplasmic samples deduced from cultivations with and without induction of the rhamnose-inducible promoter system, the small protein BCCP protein was detected. Only small amounts of BCCP were detected in the cell debris samples indicating a good cell disruption. Thus, the strong signal in the cell debris samples detected with anti-His

antibodies were not caused by incomplete cell disruption or contamination with the cytoplasmic fraction. More likely it was caused by aggregated proteins.

The only protein detected that was dependent on rhamnose-induction, was shown by a weak signal in the cell debris sample. The detected sample had a size of approximately 50 kDa and thus was expected to be Strep-AprA. A protein with the same size in the same sample was also found by the anti-His antibody. A possible reason for this was the histidine-rich sequence of AprA, thus the used antibody might have already detected the native AprA.

To conclude, AprI was detected in small amounts in the cytoplasm and in higher amounts in the membrane fraction and in the periplasm. This indicated a translocation of AprI into the periplasm. As the Coomassie stained gel showed, the proteins that could be found in the membrane fraction were also found in small amounts in the cytoplasmic fraction (Figure 18). This might have indicated a not complete removal of the cytoplasm from the membrane fraction. AprA was only found in the cell debris sample, thus the production of soluble AprA clould not be shown.

The same experiment was repeated with changed tags for detection. DH5α pBW-$_{his}$*aprAI*$_{Strep}$ pABS-*aprDEF* was cultivated and the cells were fractionated. As the gene expression of $_{his}$*aprAI*$_{Strep}$ was under control of the rhamnose-inducible promoter, samples deduced from cultivation with added inducer and cultivation with added glucose (for promoter repression) were compared.

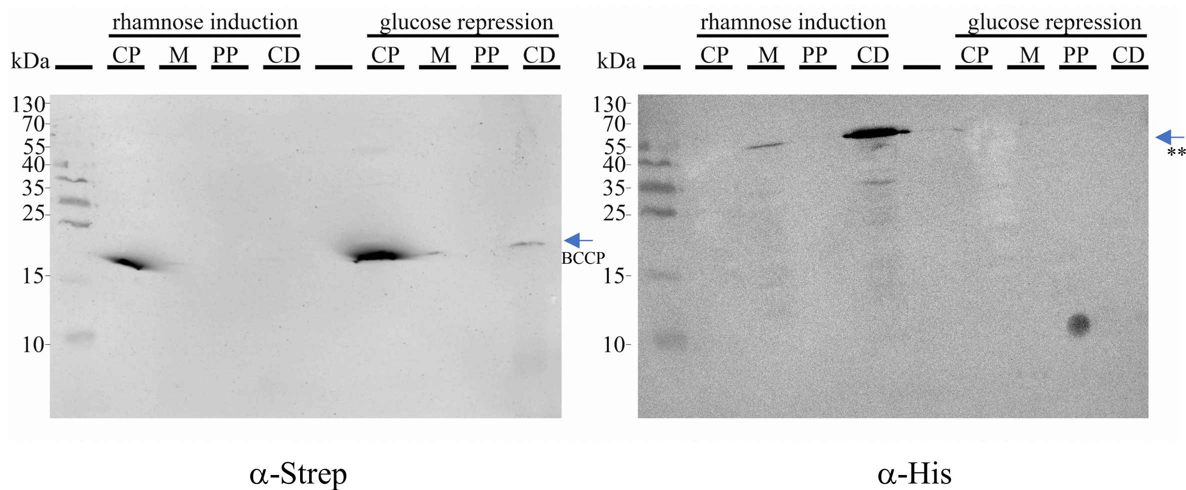

Figure 20: Semi-dry Western blots of samples derived from heterogenous expression of the T1SS in *E. coli*. A 15 % SDS-PAGE acrylamide gel was used for separation of proteins in the different cell fractions. Samples deduced from cultivation of DH5α pBW-$_{his}$*aprAI*$_{Strep}$ pBW-*aprDEF* with either 1 % (w/v) glucose or 0.1 % L-rhamnose (for repression or induction of $_{his}$*aprAI*$_{Strep}$ gene expression) added during cultivation. 3 µl of the prestained protein ladder and 10 µl of each cell sample were loaded onto the gel. CP: cytoplasmic fraction. M: membrane fraction. PP: periplasmic fraction. CD: Cell debris sample. BCCP: cytoplasmic biotin carboxyl carrier protein. **: His-AprA. The left Western blot was developed with the recombinant Strep-Tactin-AP-conjugate. The right Western blot is developed with anti-His-tag (mouse) and goat-anti-mouse-HRP-conjugate.

The anti-Strep antibodies only detected large amounts of periplasmic BCCP in the cytoplasmic fractions and a small BCCP amount in the cell debris samples that indicated an efficient cell disruption. AprI$_{Strep}$ was not detected in any sample (Figure 20). The anti-His antibodies detected an approximately 50 kDa sized protein in samples that deduced from cultivation with an induced rhamnose promoter, indicating that this might be $_{His}$AprA. A small amount of $_{His}$AprA was detected in the membrane fraction and a larger amount was detected in the cell debris sample. Soluble $_{His}$AprA was not detected (Figure 20). One possible reason for not detected AprI$_{Strep}$ via the Strep-Tag® was that the detection via alkaline phosphatase was much less sensitive than the horse reddish peroxidase detection.

The heterologous gene expression of this T1SS deriving from *P. fluorescens* in *E. coli* was only a first approach to get more information about this translocation system. Whereas AprI was translocated in *E. coli*, AprA was not soluble at all. This indicated, that a further investigation of this T1SS system in *E. coli* was not useful. To get more information about the T1SS of *P. fluorescens*, investigations should be done in *Pseudomonas* itself or a closely related organism.

3.6 Development of a tunable gene expression system for physiological analysis of *Pseudomonas fluorescens*

During this study it became necessary to tightly control protein production in the strain *P. fluorescens* A506. A regulatory system for gene expression was needed that allowed a low expression and a high gene expression if necessary. For that purpose, the genome of *P. fluorescens* strain A506 was screened for promoters that were likely to be regulated by an AraC-family regulator. To facilitate the use of the promoter for later experiments, the inducer should not be expensive. The search was focused on AraC-family regulators that might regulate the expression proteins that are degrading cheap and small molecules. Another requirement for the possible promoter system is the sugar-independency. Sugars can serve as inducers but they may interfere with the cell metabolism and non-metabolized sugars need specific uptake mechanisms.

By screening the *Pseudomonas* genome database pseudomonas.com for AraC-family regulators in *P. fluorescens* strain A506, 45 hits are found (Table 19).

Table 19: List of AraC family regulators found in strain *P. fluorescens* A506 (Hoffmann *et al.*, 2020).

Locus Tag	Orf length (nt)	protein size (residues)	annotation
PflA506_0206	1005	334	AraC family transcriptional regulator
PflA506_0557	789	262	AraC family transcriptional regulator
PflA506_0688	810	269	AraC family transcriptional regulator
PflA506_0827	996	331	AraC family transcriptional regulator
PflA506_1989	969	322	AraC family transcriptional regulator
PflA506_2163	969	322	AraC family transcriptional regulator
PflA506_2234	1176	391	AraC family transcriptional regulator
PflA506_2273	939	312	AraC family transcriptional regulator
PflA506_2310	771	256	AraC family transcriptional regulator
PflA506_2318	897	298	AraC family transcriptional regulator
PflA506_2342	1038	345	AraC family transcriptional regulator
PflA506_2443	894	297	AraC family transcriptional regulator
PflA506_2585	1017	338	AraC family transcriptional regulator
PflA506_2600	729	242	transcriptional regulator, AraC family

PflA506_2609	810	269	AraC family transcriptional regulator
PflA506_2816	810	269	AraC family transcriptional regulator
PflA506_2822	1011	336	AraC family transcriptional regulator
PflA506_2841	906	301	AraC family transcriptional regulator
PflA506_2889	924	307	AraC family transcriptional regulator
PflA506_2892	822	273	AraC family transcriptional regulator
PflA506_2972	840	279	AraC family transcriptional regulator
PflA506_3058	846	281	AraC family transcriptional regulator
PflA506_3074	399	132	AraC family transcriptional regulator
PflA506_3077	951	316	AraC family transcriptional regulator
PflA506_3080	972	323	AraC family transcriptional regulator
PflA506_3108	948	315	AraC family transcriptional regulator
PflA506_3113	777	258	AraC family transcriptional regulator
PflA506_3120	996	331	AraC family transcriptional regulator
PflA506_3125	789	262	AraC family transcriptional regulator
PflA506_3292	1035	344	AraC family transcriptional regulator
PflA506_3408	942	313	AraC family transcriptional regulator
PflA506_3408	1086	361	DJ-1/PfpI family protein / transcriptional regulator, AraC family
PflA506_3578	996	331	AraC family transcriptional regulator
PflA506_3768	750	249	AraC family transcriptional regulator
PflA506_3984	894	297	AraC family transcriptional regulator
PflA506_4025	999	332	AraC family transcriptional regulator
PflA506_4167	1059	352	AraC family transcriptional regulator
PflA506_4211	741	246	AraC family transcriptional regulator
PflA506_4269	930	309	AraC family transcriptional regulator
PflA506_4438	876	291	AraC family transcriptional regulator
PflA506_4486	**993**	**330**	**AraC family transcriptional regulator**
PflA506_4789	972	323	AraC family transcriptional regulator
PflA506_4968	945	314	AraC family transcriptional regulator
PflA506_5132	966	321	AraC family transcriptional regulator
PflA506_5358	993	330	AraC family transcriptional regulator

The result of the screening of the genome of *P. fluorescens* A506 for putative AraC family transcriptional regulators is shown in table 19. Because the gene with the locus tag PflA506_4486 is predicted to be involved in the regulation of degradation of anthranilate, this genomic region may serve as a promoter region with a cheap not-sugar-based inducer (anthranilate).

Figure 21: Localization of the gene encoding PflA506_4486 in *P. fluorescen*s A506 (pseudomonas.com).

The gene with the locus tag PflA506_4486 is predicted to encode a cytoplasmic protein and is located upstream of *antABC* that are predicted to be involved in anthranilate degradation. Oglesby *et al.* (2008) have suggested that AntR is an activator for the *antABC* operon in *P. aeruginosa* PAO1 (Oglesby *et al.*, 2008). In 2011 Choi *et al.* showed that AntR is induced by anthranilate in *P. aeruginosa* PAO1 (Choi *et al.*, 2011). In *P. aeruginosa* PAO1 *antR* is described divergently from *antA* in the genome next to the *antABC* operon (Figure 22).

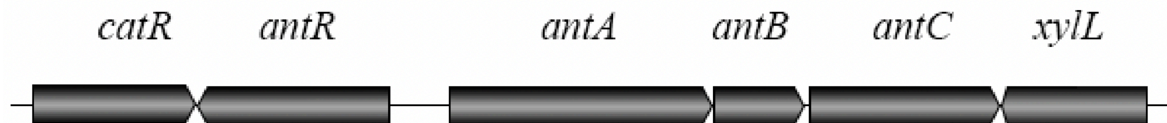

Figure 22: Localization of *antR* in *P. aeruginosa* PAO1 (pseudomonas.com).

Because the location of *antR* in *P. aeruginosa* PAO1 looked similar to the location of the gene with the locus tag PflA506_4486 in *P. fluorescens* A506 (Figure 21 and Figure 22), the primary structures of the two corresponding proteins were aligned. The encoded protein of the gene with the locus tag PflA506_4486 showed 61 % identity to AntR of *P. aeruginosa* PAO1 (Figure 23).

```
PflA506_4486      -----MSSQTRDIHIQRFDLEGARSWMSGICGPHRLATATPERLRFHHSANVFKSRATTL 55
PAO1_AntR         MMRTHPVADRGDLHADHLDLAAARSWMSKVCGPHRLEAASPGLVQFQHHGNVLKSMCTTL 60
                         ::  *:* :::** .****** :****** :*:*  ::*:* .**:** .***

PflA506_4486      GVIEYGTDVTIDIEDAEHFRSYSLSLPLVGEQELSKNGERLSSNRDQGVIISPNEHQVLA 115
PAO1_AntR         GYIGYGTDVTITVEDAAAFNAYSLSLPLSGEQELCRGGLRLLSDVRRGVIIAPNERQELS 120
                  * * ******* :***  *.:******* *****.:.* ** *:  :****:***:* *:

PflA506_4486      ISGDCRKLQVVITRAAMSESLEGLLQRPIDAPLRFESVMDAVDGAPASWWRMARYFIAEL 175
PAO1_AntR         IAGDCRKLQVVIGRTAMRKVLEEMLQRPIDTPLRFDPEMDALDGASASWWRTVRHISEE- 179
                  *:********** *:** : ** :******:****:  ***:*** ***** .*::  *

PflA506_4486      ECSSELYEQAAFTRDLESSLIKGLILAQPNNYSEELREVLGVKLPHYLIRARQFIHDNAR 235
PAO1_AntR         MARSELYAQAFFSSDLERALIKGLILAQPNNYSEALQQGLGGRPPHYLLRAREFLQANAR 239
                   . **** ** *: *** :*************** *:: ** : ****:***:*:: ***

PflA506_4486      EVLHLEDLEAAAGVSRFKLFDAFRKYFALSPMAYLKKHRLGAVRQEILEQGSMRTISEIA 295
PAO1_AntR         ETLSLEDVERAAGVSRFKLFEGFRRYFGVSPMSYLKHYRLAAVREEILASGGARSISTIA 299
                  *.* ***:* **********:.**:**.:***:***::**.***:***  .*. *:** **

PflA506_4486      LGWGFTHLGRFSAEYRKLFDESPSQTLQRKRLRIT 330
PAO1_AntR         LGWGFSHLGRFSVDYRKRFEETPSMTQRRAARRS- 333
                  *****:******.:*** *:*:** * :*   *
```

Figure 23: Alignment of the amino acid sequence of PflA506_4486 from *P. fluorescens* A506 with the amino acid sequence of AntR from *P. aeruginosa* PAO1 (Hoffmann *et al.*, 2020).

Because of the similarity between the gene product of the gene with the locus tag PflA506_4486 of *P. fluorescens* A506 and AntR of *P. aeruginosa* PAO1, the gene of *P. fluorescens* A506 with the locus tag PflA506_4486 was named *antR* in this study.

The idea was, that the region upstream of *P. fluorescens* A506 might serve as an anthranilate-inducible promoter region with *antR* as an AraC family transcription regulator in *P. fluorescens* A506. The AntR regulated P_{antA} promoter was then investigated for recombinant gene expression in *Pseudomonas* and later on it was compared with another inducible system that was already used for recombinant gene expression in some pseudomonads.

3.6.1 Activity of the P_{antA} promoter can be monitored by fluorescence microscopy

To get first information about the inducibility of the P_{antA} promoter of *P. fluorescens* A506, the P_{antA} promoter was cloned into pUCP20, a shuttle vector that replicated in both organisms, *E. coli* (as the cloning organism) and *P. fluorescens* (West *et al.*, 1994). For the observation of P_{antA} promoter activity, a *gfp* gene encoding a green fluorescent protein was put under the control of P_{antA}. GFP served as a reporter in the following experiments. An empty vector that only carried the promoter region served as negative control for the experiments. To be more

flexible in later experiments, the same plasmids carrying another resistance cassette and with the *gfp* replaced by a multiple cloning site, were produced.

The plasmids that were created for the investigation of the anthranilate-inducible promoter system are listed in table 20, two vector maps as an example for the vector construction are shown in figure 24.

Table 20: Plasmids that were created for investigation of the anthranilate-inducible promoter (Hoffmann *et al*., 2020).

Vector	characteristics	reference
pUCP20-ANT1	pUCP20-based *Escherichia-Pseudomonas* shuttle vector for anthranilate-regulated gene expression, amp^R, *lacZ alpha*, ColE1 ori (*E. coli*), *antR*, *antA* promoter	This study
pUCP20-ANT1-*gfp*	pUCP20-ANT1 with *gfp* as reporter gene for the anthranilate-regulated *antA* promoter	This study
pUCP20-ANT1-MCS	pUCP20-ANT1 with multiple cloning site of pUCP20	This study
pUCP20-ANT2	As pUCP20-ANT1, but amp^R exchanged by kan^R	This study
pUCP20-ANT2-*gfp*	pUCP20-ANT2 with *gfp* as reporter gene for the anthranilate-regulated *antA* promoter	This study
pUCP20-ANT2-MCS	pUCP20-ANT2 with MCS from pUCP20	This study

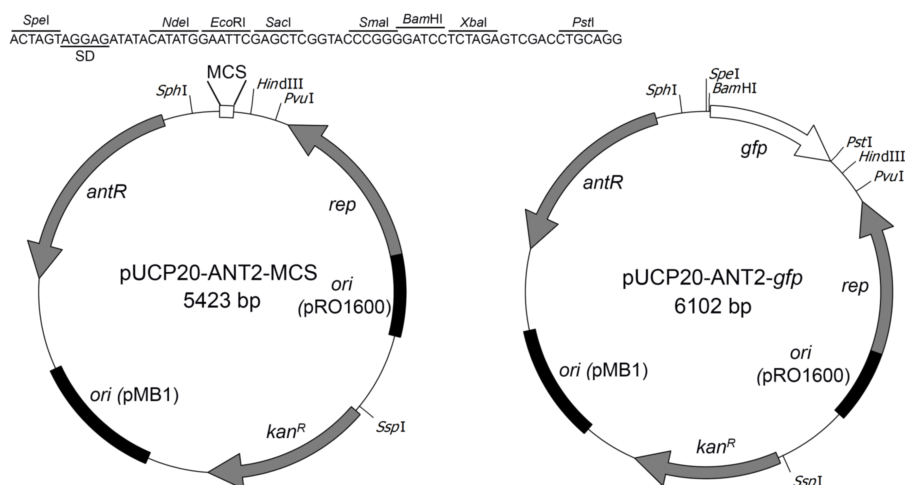

Figure 24: Vector maps of two pUCP20 plasmids with the anthranilate-inducible promoter region with either the multiple cloning site (left) or *gfp* as a reporter gene (right). SD: Shine Dalgarno site (underlined) (Hoffmann et al., 2020).

To get information about inducibility of P_{antA} respectively the promoter activity under two different growth conditions, two standard growth media were chosen: LB as a complex medium and M9 as a minimal medium. Different concentrations of anthranilate as an inducer were tested: 0 mM, 0.001 mM, 0.01 mM, 0.1 mM, 1 mM and 10 mM anthranilate. The GFP-fluorescence was analyzed by epifluorescence microscopy at four time points: 0 h, 1h, 2 h and 3 h after induction.

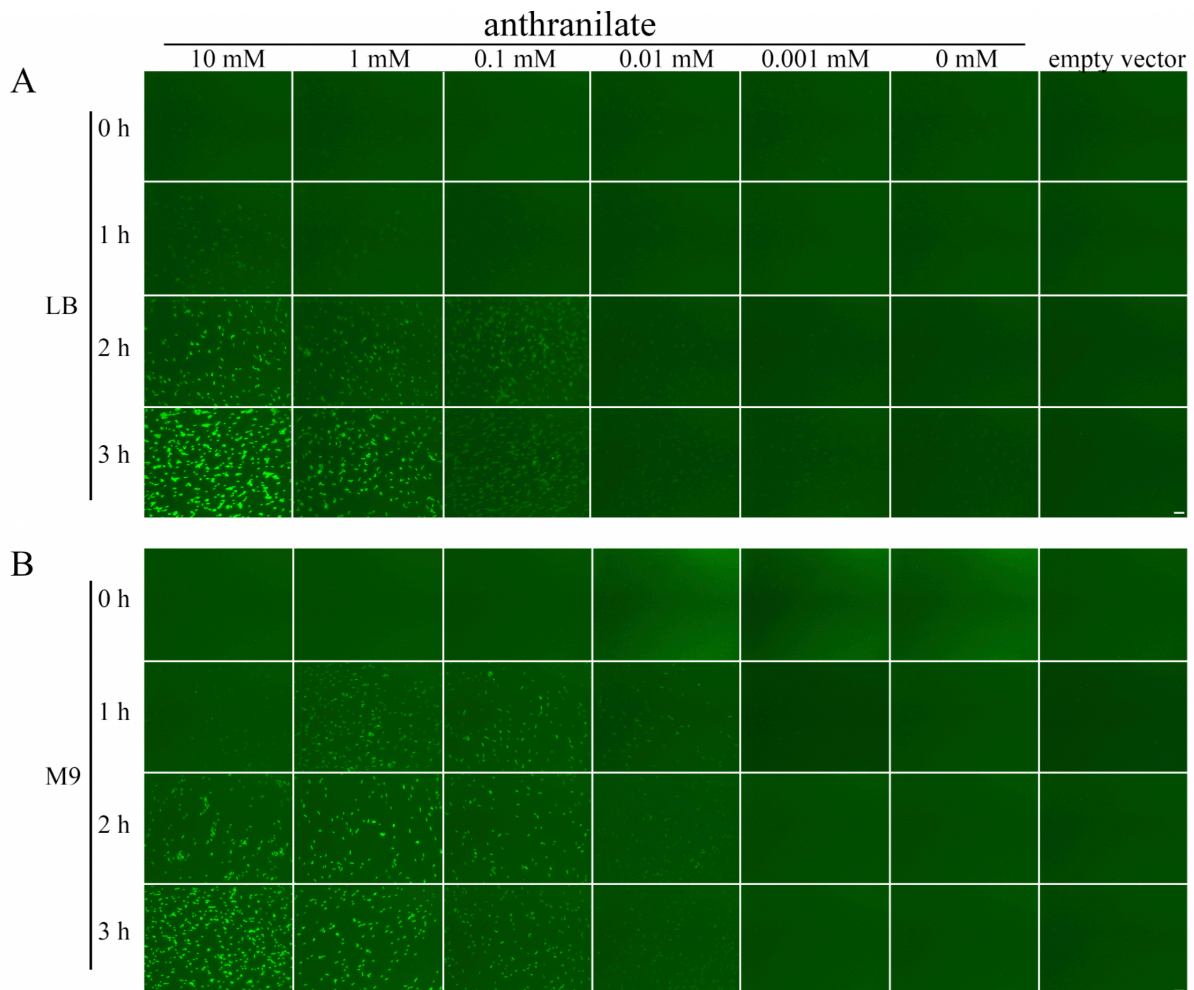

Figure 25: Epifluorescence microscopy of the anthranilate-regulated expression system with GFP as a reporter protein. GFP-fluorescence was analyzed in *P. fluorescens* A506 pUCP20-ANT2-*gfp*. Different concentrations of anthranilate (10 mM, 1 mM, 0.1 mM, 0.01 mM, 0.001 mM and 0 mM) were used for promoter induction as indicated at the top. Samples were analyzed at four time points (0 h, 1 h, 2 h and 3 h). The empty vector control derived from cultures carrying the empty plasmid pUCP20-ANT2. A, Cell cultures were grown in LB medium. B, Cell cultures were grown in M9 medium (Hoffmann *et al.*, 2020, modified).

The GFP-fluorescence intensity in bacteria cultivated in both media tested (Figure 25 A and B) was similar and GFP-fluorescence intensity, respectively the correlated promoter activity was not depending on the growth medium.

The intensity of the GFP-fluorescence depended on the time points and the inducer concentration that was added during cultivation at time point 0 h. The cell samples deriving from cultures that contained 10 mM inducer showed an increasing fluorescence level over time and the highest fluorescence level could therefore be observed in the samples deriving from the 3 h induced cell cultures. The induction with 1 mM and 0.1 mM inducer lead to similar fluorescence levels at 2 h and 3 h time points, both showing intermediate fluorescence, indicating an intermediate expression level that was constant for hours. Induction with 0.01 mM anthranilate lead only to very weak GFP-fluorescence observation.

To conclude, anthranilate could serve as an inducer for P_{antA}. The gene expression level was dependent on inducer concentration and at high inducer concentrations also on induction time.

3.6.2 P_{antA} promoter activity is tunable by inducer concentration

Flow cytometry was used to better compare the promoter activity within the population of the cell cultures. This technique allowed a much more sensitive detection of fluorescence than with the microscope. A possible variance or spreading of fluorescence intensity within a population was investigated. The same growth conditions as in 3.6.1 were used for flow cytometry and samples were analyzed at three time points: 1 h, 2 h and 3 h after induction.

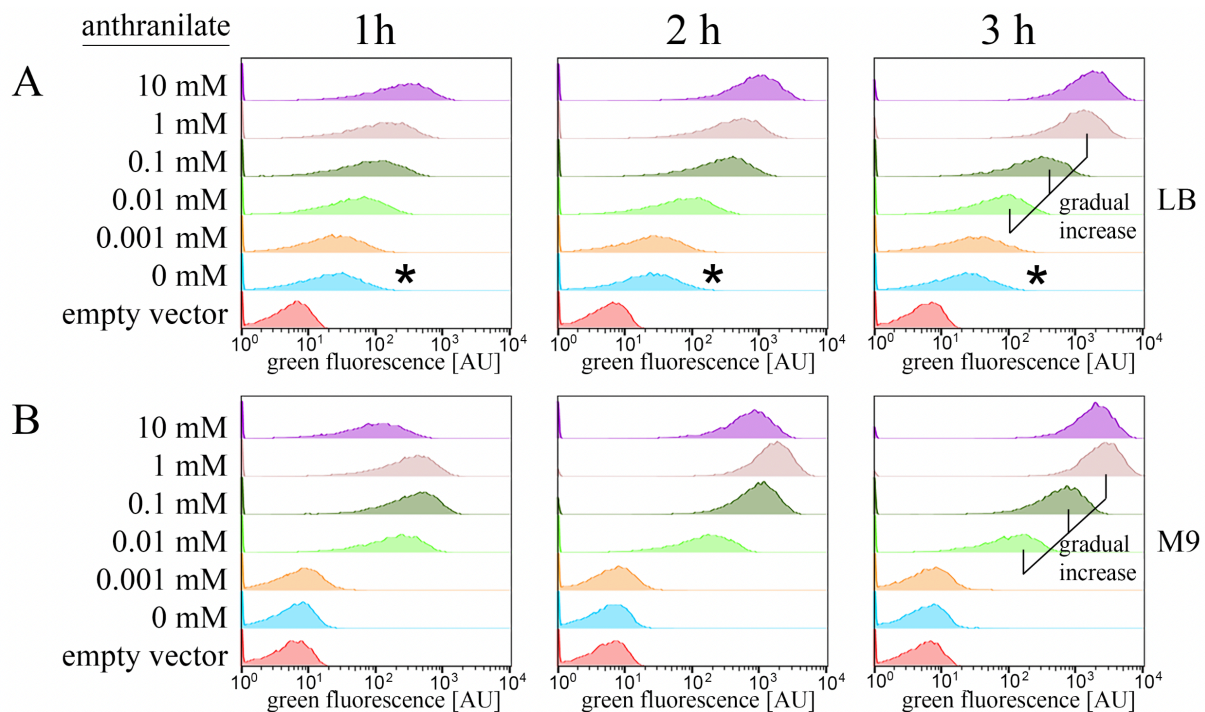

Figure 26: Flow cytometry analyses of the anthranilate-regulated expression system with GFP as a reporter protein. GFP-fluorescence was analyzed in *P. fluorescens* A506 pUCP20-ANT2-*gfp*. Different concentrations of anthranilate (10 mM, 1 mM, 0.1 mM, 0.01 mM, 0.001 mM and 0 mM) were used for promoter induction as indicated on the left side. Samples were analyzed at three time points (1 h, 2 h and 3 h). The empty vector control derived from cultures carrying the empty plasmid pUCP20-ANT2. Lines indicate the tuned expression levels in the 3 h diagram. * indicates autoinduction. A, Cell cultures were grown in LB medium. B, Cell cultures were grown in M9 medium (Hoffmann *et al*., 2020, modified).

The results of the flow cytometry indicated a dependency of the promoter activity on the inducer concentration (Figure 26). After 3 h of induction with 0.01 mM, 0.1 mM and 1 mM anthranilate, a gradual increase in fluorescence signal intensity in the respective samples was observed for both growth conditions (Figure 26 A, B). At the 3 h time point, 1 mM and 10 mM induction lead to similar gene expression levels. The gene expression observed was similar in the samples deriving from complex medium and minimal medium cultivation for an inducer concentration of 0.01 mM up to 1 mM. 10 mM induction in M9 medium is less effective than the 1 mM induction at 1 h and 2 h time points.

The samples deriving from complex medium and minimal medium cultivation with 0 mM up to 0.1 mM inducer concentration lead to constant fluorescence levels over the three time points indicating a stable P_{antA} activity for hours (Figure 27 D, E, F, J, K, L). The induction with 1 mM and 10 mM anthranilate lead to an increasing fluorescence signal over time, reaching similar and high fluorescence levels at the 3 h time point (Figure 27 A, B, C, G, H, I). Same fluorescence levels in the respective media were measured in the 0.001 mM and 0 mM

(negative control) inducer samples. This indicated the useful range for P_{antA} promoter induction started above 0.001 mM inducer. A difference in the activity of the promoter depending on the growth media could be observed by comparing the fluorescence levels of the not-induced sample (0 mM inducer) and the empty vector control (Figure 26 A, B). In the samples deriving from the minimal medium M9 the same intensity of fluorescence could be detected whereas the intensity of the fluorescence of the samples deriving from the 0 mM inducer sample in LB showed a slightly stronger fluorescence signal than the sample from the empty vector control. This indicated a slight promoter activity and therefore a slight leakiness of the P_{antA} promoter.

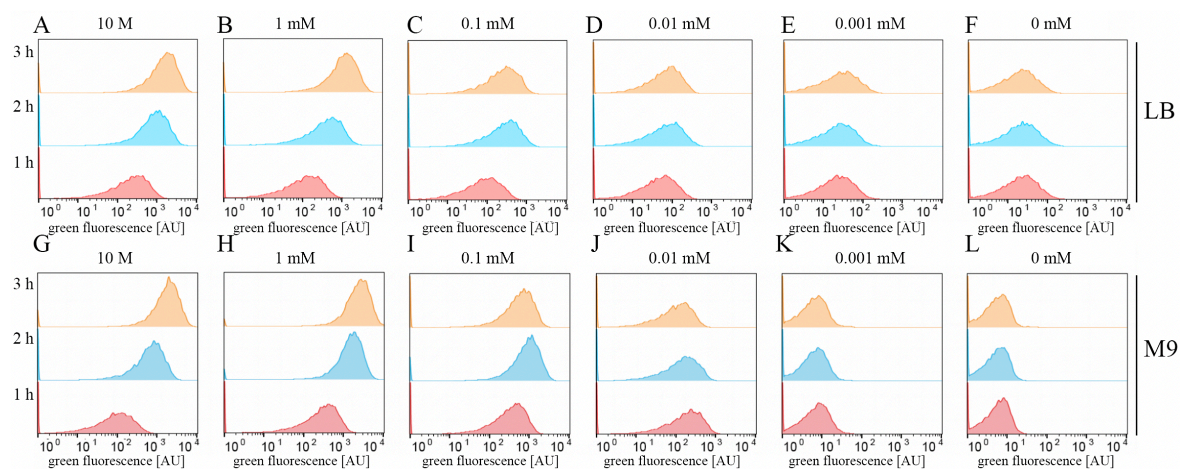

Figure 27: Flow cytometry analyses of the anthranilate-regulated expression system with GFP as a reporter protein. GFP-fluorescence was analyzed in *P. fluorescens* A506 pUCP20-ANT2-*gfp*. Samples were analyzed at three time points (1 h, 2 h and 3 h). The upper data set derived from cultivation in LB medium, the lower data set derived from cultivation in M9 medium. A, induction with 10 mM inducer. B, induction with 1 mM inducer. C, induction with 0.1 mM inducer. D, induction with 0.01 mM inducer. E, induction with 0.001 mM inducer. F, no induction during cultivation. G, induction with 10 mM inducer. H, induction with 1 mM inducer. I, induction with 0.1 mM inducer. J, induction with 0.01 mM inducer. K, induction with 0.001 mM inducer. L, no induction during cultivation.

To conclude, a gradual increase of the fluorescence signal with increasing inducer concentrations from 0.01 mM up to 1 mM was possible, and by choosing M9 minimal medium for cultivation the P_{antA} promoter was not showing a detectable leakiness (with GFP as a reporter).

3.6.3 P_{antA} promoter leakiness is dependent on growth conditions

Because previous data indicated a slight leakiness of the P_{antA} promoter when cultivated in LB complex medium, the GFP-fluorescence intensity was measured during cell cultivation in both

media. No external inducer was added to the cultures. The fluorescence level of the cultures in M9 minimal medium was the same and stable over the whole cultivation period (Figure 28 B). No leakiness of the P_{antA} promoter was observed in the samples deriving from M9 minimal medium cultures.

When the cells were cultivated on LB complex medium, the result differed. During exponential growth phase, almost no promoter activity was observed. The culture with the reporter gene behaved like the negative control with the empty vector (Figure 28 A). In stationary phase, there was a strong autonomous induction of the P_{antA} promoter shown by the strongly increasing GFP-fluorescence level. These data fitted to the previous results shown in 3.6.2 and indicated a leakiness of the P_{antA} promoter cultivated in LB complex medium.

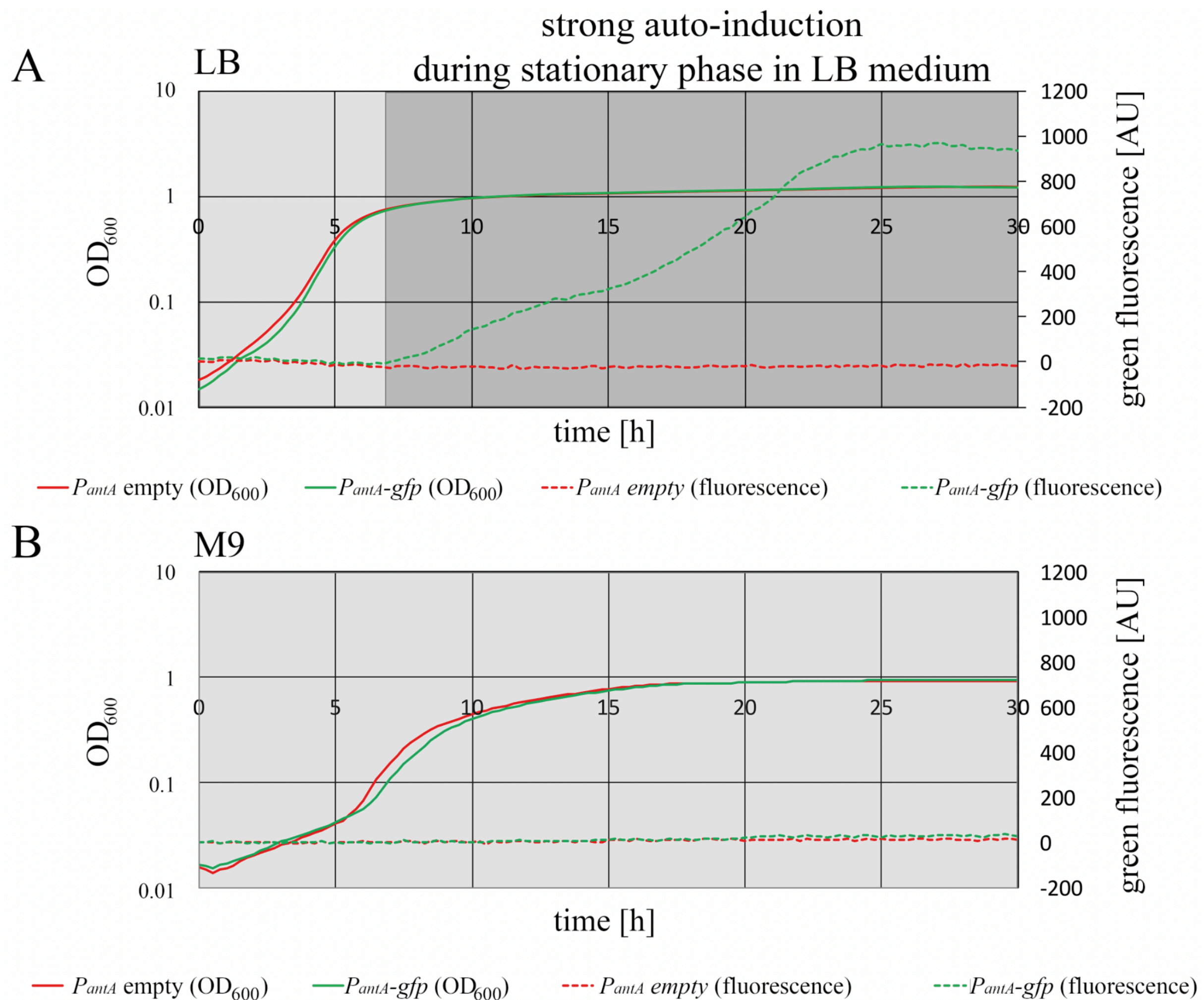

Figure 28: Analysis of P_{antA} autoinduction during growth of *P. fluorescens* A506 pUCP20-ANT2-*gfp*. The GFP-fluorescence served as a reporter for promoter activity. Cultivation of *P. fluorescens* A506 pUCP20-ANT2 served as a reference (negative control). A, analysis of possible autoinduction in cultures grown in LB medium without externally added inducer. B, analysis of possible autoinduction in cultures grown in M9 medium without externally added inducer. OD_{600} and green fluorescence (excitation at 488 nm, detection at 509 nm) were plotted (Hoffmann *et al*., 2020, modified).

To sum up the observations, the leakiness of the P_{antA} promoter system depended on cultivation medium.

3.6.4 The anthranilate-inducible system allows better finetuning of expression levels than the rhamnose-inducible system

To compare the anthranilate-inducible promoter system with another inducible promoter system used in pseudomonads, epifluorescence microscopy and cell cytometry experiments were repeated with pUCP20 vectors carrying the reporter gene *gfp* under the control of another promoter. Meisner and Goldberg (2016) compared some induced systems in *P. aeruginosa* and concluded the RhaSR/P_{rhaB} works well in *P. aeruginosa* (Meisner and Goldberg, 2016). This promoter system is originally from *E. coli* and requires the two regulatory proteins RhaS and RhaR (Table 21). The regulatory proteins are encoded on the genome of *E. coli* upstream of the promoter in opposite direction (Egan and Schleif, 1993).

Table 21: Properties of the anthranilate- and rhamnose-inducible promoter systems that were compared in this study.

property	P_{antA}	P_{rhaB}
Originally from this organism	*P. fluorescens* A506	*E. coli*
Regulatory proteins	AntR	RhaS, RhaR
Inducer	anthranilate	rhamnose

The vectors were created as described above for P_{antA} (3.6.1). An empty vector containing the promoter and no reporter gene *gfp* served as negative control.

The experimental setup was the same as for the experiments with the anthranilate-inducible system in 3.6.1. Inducer concentrations from 0 mM, 0.001 mM, 0.01 mM, 0.1 mM, 1 mM to 10 mM were tested and samples taken at the same four points (0 h, 1 h, 2 h, 3h after induction) were used.

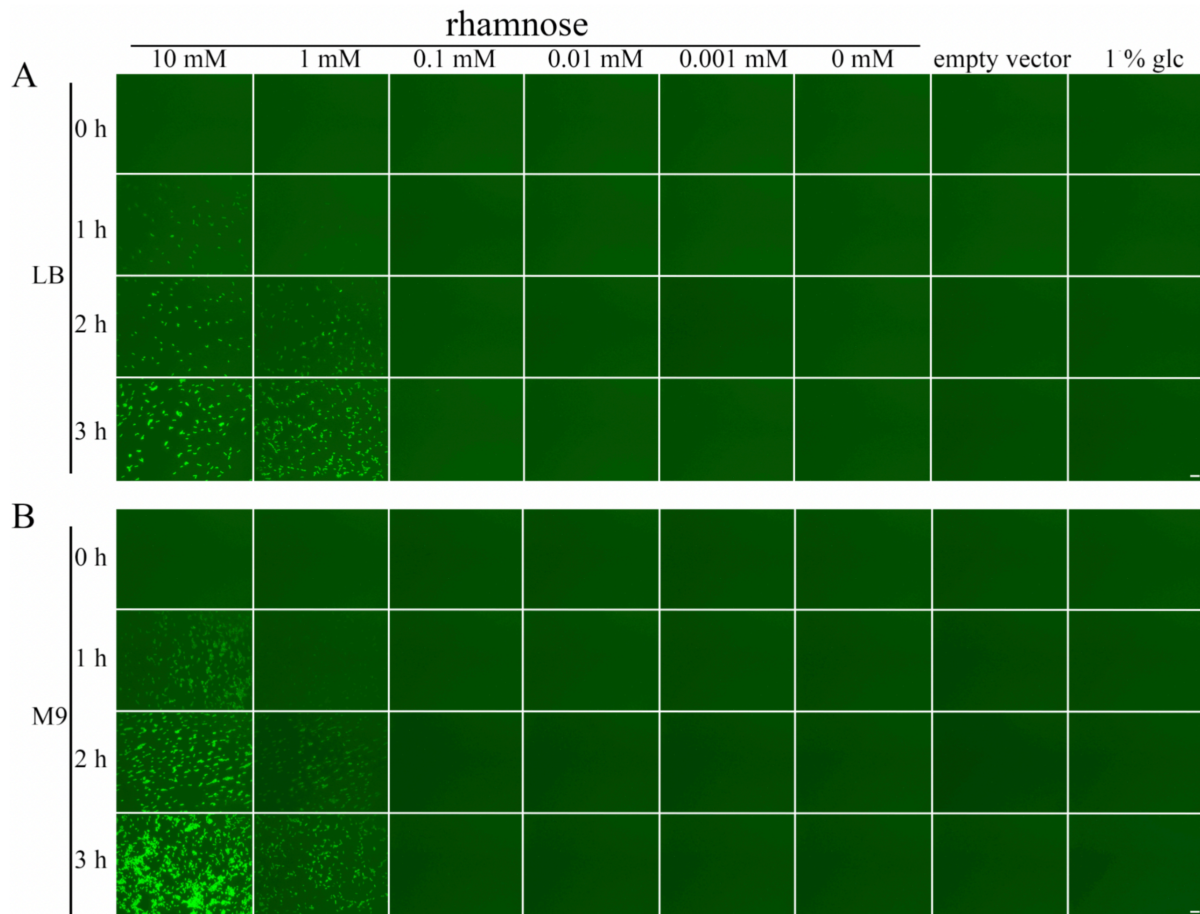

Figure 29: Epifluorescence microscopy of the rhamnose-regulated expression system with GFP as a reporter protein. GFP-fluorescence was analyzed in *P. fluorescens* A506 pUCP20-RHA2-*gfp*. Different concentrations of rhamnose (10 mM, 1 mM, 0.1 mM, 0.01 mM, 0.001 mM and 0 mM) were used for promoter induction as indicated at the top. Samples were analyzed at four time points (0 h, 1 h, 2 h and 3 h). The empty vector control derived from cultures carrying the empty plasmid pUCP20-ANT2. 1 % glc: The rhamnose-inducible promoter was repressed by 1 % (w/v) glucose. A, Cell cultures were grown in LB medium. B, Cell cultures were grown in M9 medium (Hoffmann *et al*., 2020, modified).

The highest inducer concentration of 10 mM lead to a strong gene expression respectively to a strong GFP-fluorescence signal (Figure 29). A weaker GFP-fluorescence intensity was observed in the 1 mM samples and induction with inducer concentration lower than 1 mM lead to the observation of no fluorescent bacteria and thus no promoter activity was observed. The GFP-fluorescence intensity in both the 10 mM rhamnose and the 1 mM rhamnose samples was increasing over time resulting in the highest fluorescence level after 3 h of induction.

To compare these results with the results derived from the P_{antA} promoter constructs (3.6.1), two differences could be observed: (1) the GFP-fluorescence level increased over time and (2) only the two highest inducer concentration lead to a well visible GFP-fluorescence observed with the epifluorescence microscope.

By measuring the GFP-fluorescence intensity of the single cells via flow cytometry it became clear that promoter activity could not be adjusted to a specific level as previously seen for the anthranilate-inducible system (Figure 30).

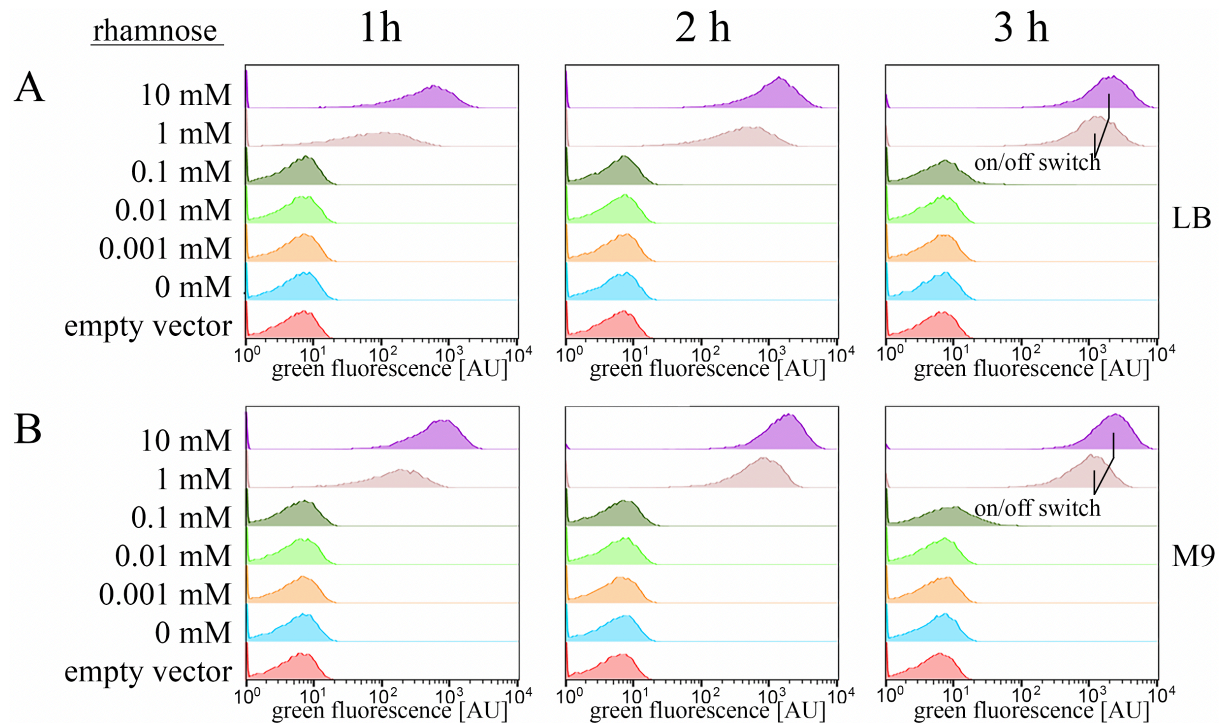

Figure 30: Flow cytometry analyses of the rhamnose-regulated expression system with GFP as a reporter protein. GFP-fluorescence was analyzed in *P. fluorescens* A506 pUCP20-RHA2-*gfp*. Different concentrations of rhamnose (10 mM, 1 mM, 0.1 mM, 0.01 mM, 0.001 mM and 0 mM) were used for promoter induction as indicated on the left side. Samples were analyzed at three time points (1 h, 2 h and 3 h). The empty vector control derived from cultures carrying the empty plasmid pUCP20-RHA2. Lines indicate the on/off switch in the 3 h diagram. A, Cell cultures were grown in LB medium. B, Cell cultures were grown in M9 medium (Hoffmann *et al.*, 2020, modified).

The cultivation medium had no influence on the P_{rhaB} promoter activity (Figure 30 A and B). Only 1 mM and 10 mM inducer lead to a detectable *gfp* gene expression, the other samples with lower rhamnose concentrations (0 mM, 0.001 mM, 0.01 mM ans 0.1 mM rhamnose) showed the same GFP-fluorescence level as the empty vector control samples. The activity of the P_{rhaB} promoter was almost the same when 1 mM or 10 mM rhamnose was used for induction. The only difference of the samples from cultures with 1 mM and 10 mM induction was, that 10 mM inducer lead slightly faster to the very strong fluorescence signal. This means, there was no difference in fluorescence signal intensity at the 2 h and the 3 h time point when 10 mM inducer was used, whereas fluorescence intensity was slightly increasing over time in the samples deriving from the 1 mM induced (Figure 31 A, B, D, E).

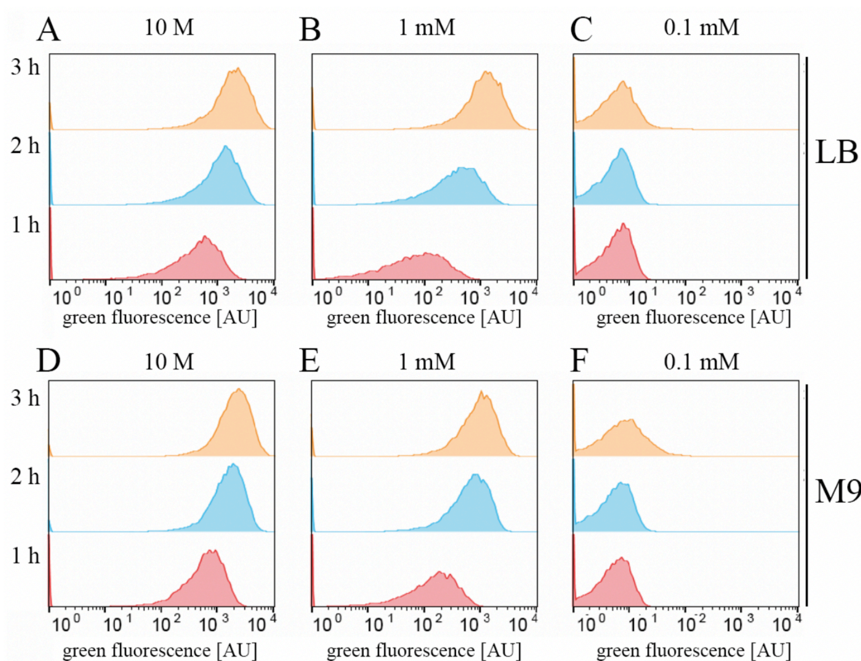

Figure 31: Flow cytometry analyses of the rhamnose-regulated expression system with GFP as a reporter protein. GFP-fluorescence was analyzed in *P. fluorescens* A506 pUCP20-RHA2-*gfp*. Samples were analyzed at three time points (1 h, 2 h and 3 h). The upper data set derived from cultivation in LB medium, the lower data set derived from cultivation in M9 medium. A, induction with 10 mM inducer. B, induction with 1 mM inducer. C, induction with 0.1 mM inducer. D, induction with 10 mM inducer. E, induction with 1 mM inducer. F, induction with 0.1 mM inducer.

After 3 h of induction with 1 mM rhamnose, the GFP-fluorescence intensity was almost the same as with 10 mM inducer.

To sum up, no leakiness could be observed for the rhamnose-inducible system in both media tested but only higher concentrations of inducer switched the gene expression fully on. No fine tuning was observed, the gene expression was increasing over time until the maximum was reached. The gene expression was either switched on or off, no intermediate level that stayed stable over time could be reached

3.6.5 The anthranilate-inducible system from *Pseudomonas fluorescens* A506 can be used in other pseudomonads

Previously performed experiments made clear that gene expression could be tightly controlled in *P. fluorescens* A506 with the anthranilate-inducible system. To examine whether this promoter system also allows a tightly controlled gene expression in other related pseudomonads two often used reference strains were chosen for analysis: *P. aeruginosa* PAO1 and *P. putida* type strain DSM291^T. As in the experiments with *P. fluorescens* A506 the same vectors were

used, one encoding GFP under the control of the P_{antA} promoter, the other vector lacking the reporter gene. As with *P. fluorescens* A506 the gene expression was tested in two different media, one complex medium and one minimal medium. The same inducer concentrations as with *P. fluorescens* A506 were tested: 0 mM, 0.001 mM, 0.01 mM, 0.1 mM, 1 mM and 10 mM anthranilate. As before the fluorescence signal was measured at three time points: 1 h, 2 h and 3 h after induction. Flow cytometry was chosen for analysis of GFP-fluorescence intensity and promoter activity.

3.6.5.1 Use of the system in *Pseudomonas aeruginosa*

For *P. aeruginosa* PAO1 LB medium was chosen as complex medium and M9 medium as minimal medium. The gene expression of *gfp* was strongly influenced by the cultivation medium (Figure 32). While no *gfp* gene expression observed in the not-induced sample grown on minimal medium, a stronger GFP-fluorescence signal was detected in the not-induced sample grown on LB indicating autoinduction of the P_{antA} promoter. The fluorescence intensity was increasing over time although no inducer was added. At time point 3 h, all the samples derived from LB medium cultures showed almost the same intense GFP-fluorescence, except the one derived from the empty vector control. This indicated a strong autoinduction of the anthranilate promoter in cultures grown on LB medium.
When the bacteria were grown on M9 minimal medium, no leakiness of the P_{antA} promoter was observed. The samples of the empty vector control showed the same fluorescence signal as the not-induced cell sample did. To detect fluorescence caused by GFP and thus to detect P_{antA} promoter activity, at least 0.01 mM inducer had to be used.

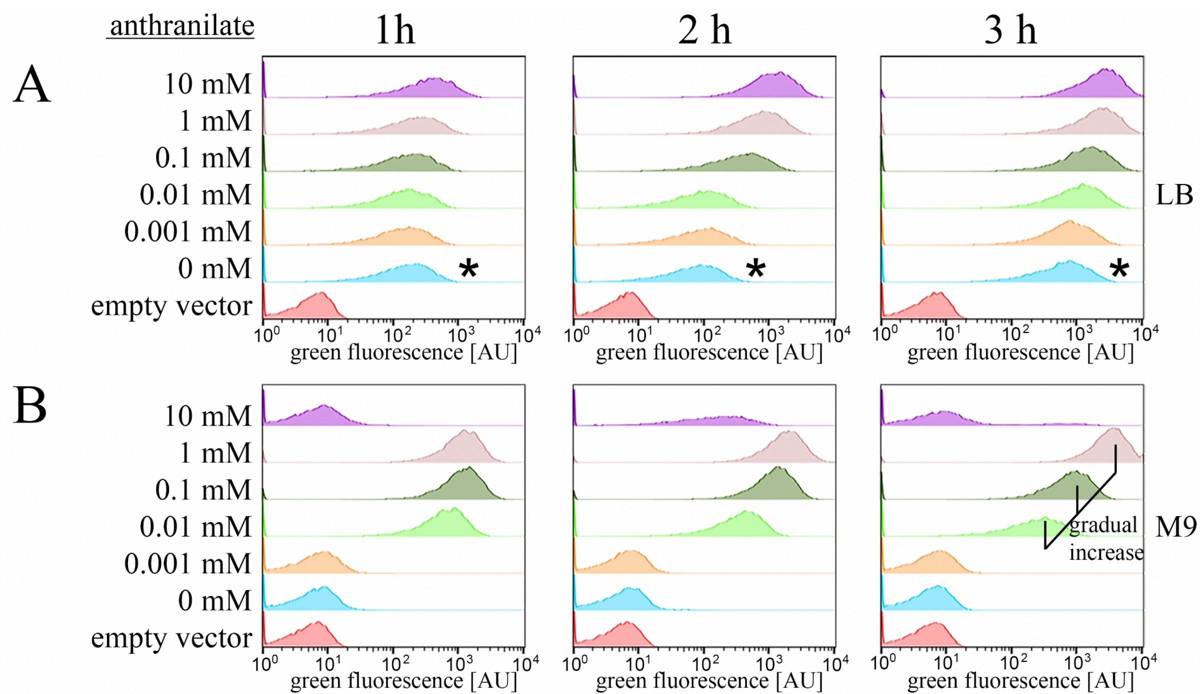

Figure 32: Flow cytometry analyses of the anthranilate-regulated expression system with GFP as a reporter protein. GFP-fluorescence was analyzed in *P. aeruginosa* PAO1 pUCP20-ANT1-*gfp*. Different concentrations of anthranilate (10 mM, 1 mM, 0.1 mM, 0.01 mM, 0.001 mM and 0 mM) were used for promoter induction as indicated on the left side. Samples were analyzed at three time points (1 h, 2 h and 3 h). The empty vector control derived from cultures carrying the empty plasmid pUCP20-ANT1. Lines indicate the tuned expression levels in the 3 h diagram. * indicates autoinduction. A, Cell cultures were grown in LB medium. B, Cell cultures were grown in M9 medium (Hoffmann *et al*., 2020, modified).

The fluorescence intensity not only depended on the inducer concentration but also varied over induction time in M9 minimal medium (Figure 32). The strongest fluorescence signal indicating the strongest P_{antA} promoter activity in this experiment was observed at the 3 h time point with 1 mM inducer. After one hour of induction, a strong fluorescence signal was detected. The fluorescence signal was slightly increasing over time (Figure 33 B). The 10-fold higher inducer concentration (10 mM) was not leading to a strong fluorescence signal. It obviously showed a toxic effect of high inducer concentration on the cells and only slight fluorescence was detected after 2 h of induction. This fluorescence was disappearing again after three hours of induction (Figure 33 A). A reason for this effect might be that at high concentrations the anthranilate might negatively uncouple the energetization of the cell.

Weaker inducer concentrations lead to a strong fluorescence signal at the 1 h time point (Figure 33 C and D). Afterwards, the fluorescence signal was slowly decreasing. This lead to the suggestion of an influence of turn-over of the inducer anthranilate on induction in M9 minimal medium.

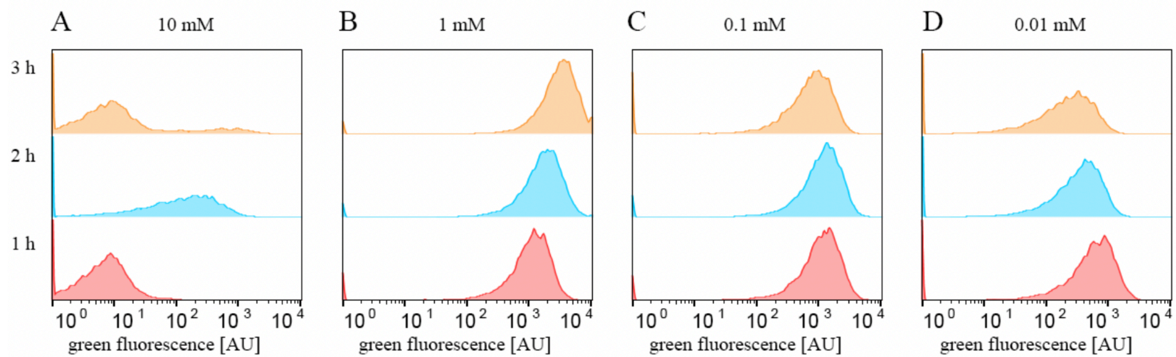

Figure 33: Flow cytometry analyses of the anthranilate-regulated expression system with GFP as a reporter protein. GFP-fluorescence was analyzed in *P. aeruginosa* PAO1 pUCP20-ANT1-*gfp*. Cell cultures were grown in M9 medium and samples were analyzed at three time points (1 h, 2 h and 3 h). Different concentrations of anthranilate were used for promoter induction as indicated on top: A, 10 mM anthranilate, B, 1 mM anthranilate, C, 0.1 mM anthranilate, and D, 0.01 mM anthranilate.

To conclude, the results indicate that the P_{antA} promoter system from *P. fluorescens* A506 can also be used in *P. aeruginosa*. For adjusting the promoter activity to a specific level, the M9 minimal medium can be recommended. The expression level can be adjusted via the inducer concentration that should be between 0.01 mM and 1 mM.

When only a strong promotor activity and no specific induction time point is wanted, the LB complex medium may also be used.

3.6.5.2 Use of the system in *Pseudomonas putida*

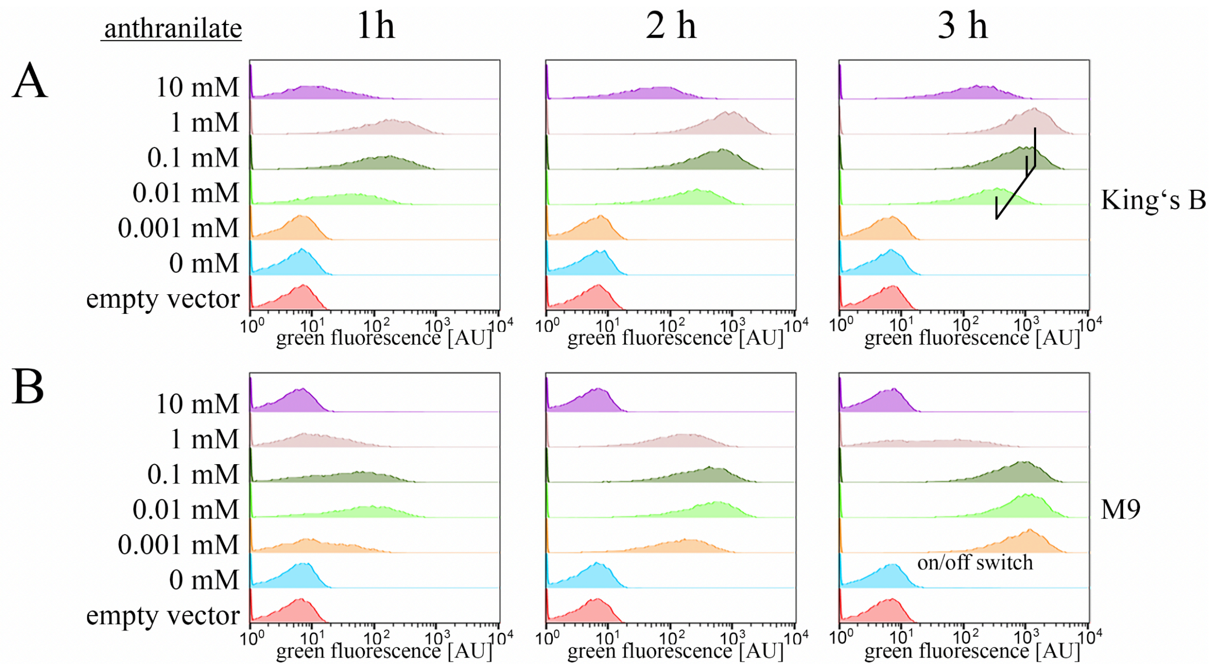

Figure 34: Flow cytometry analyses of the anthranilate-regulated expression system with GFP as a reporter protein. GFP-fluorescence was analyzed in *P. putida* DSM291 pUCP20-ANT2-*gfp*. Different concentrations of anthranilate (10 mM, 1 mM, 0.1 mM, 0.01 mM, 0.001 mM and 0 mM) were used for promoter induction as indicated on the left side. Samples were analyzed at three time points (1 h, 2 h and 3 h). The empty vector control derived from cultures carrying the empty plasmid pUCP20-ANT2. Lines indicate the tuned expression levels in the 3 h diagram. A, Cell cultures were grown in King's B medium. B, Cell cultures were grown in M9 medium (Hoffmann *et al*., 2020, modified).

Pseudomonas putida was grown in King's B medium as complex medium, and in M9 medium as minimal medium. As in previous experiments, the promoter activity depended on the growth medium. In contrast to previous experiments, no leakiness of the promoter was detected in both media. The samples derived from the empty vector control showed the same signal as the sample deriving from the not-induced cultures (Figure 34).

The high inducer concentration of 10 mM anthranilate (both media) and 1 mM (M9 minimal medium) were not capable to induce the promoter strongly (Figure 34). No fluorescence was detected in the 10 mM inducer samples at any time point in M9 medium.

In complex medium, inducer concentrations from at least 0.01 mM lead to detected gene expression. The intensity of P_{antA} promoter induction depended on inducer concentration and induction time. When the inducer concentration was even or above 0.01 mM GFP-fluorescence was detected, indicating promoter induction (Figure 34). 1 mM inducer leads to the strongest fluorescence signal detected (Figure 34). Induction with 10 mM anthranilate was much less

effective than induction with 0.01 mM to 1 mM inducer, indicating toxic effects of the high anthranilate concentration. The P_{antA} promoter induction was slightly increasing over time, resulting in the maximum fluorescence signal at 3 h time point (Figure 35 A, B, C, D).

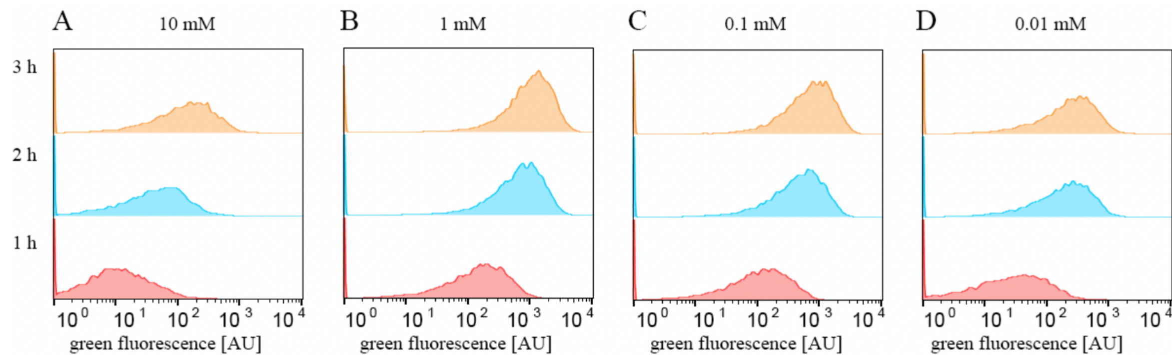

Figure 35: Flow cytometry analyses of the anthranilate-regulated expression system with GFP as a reporter protein. GFP-fluorescence was analyzed in *P. putida* DSM291 pUCP20-ANT2-*gfp*. Cell cultures were grown in King's B medium and samples were analyzed at three time points (1 h, 2 h and 3 h). Different concentrations of anthranilate were used for promoter induction as indicated on top: A, 10 mM anthranilate, B, 1 mM anthranilate, C, 0.1 mM anthranilate, and D, 0.01 mM anthranilate.

The expression of *gfp* under the control of the P_{antA} promoter in *P. putida* grown in M9 minimal medium was induced by 0.001 mM, 0.01 mM, 0.1 mM and 1 mM anthranilate concentration. Induction with 10 mM anthranilate did not induce gene expression and induction with 1 mM anthranilate a weak induction at 2 h time point and an even weaker induction at 3 h time point (Figure 36 A). This indicated toxic effects of anthranilate in M9 minimal medium for concentrations of 10 mM and 1 mM. No difference in gene expression was observed in samples derived from cell cultures induced with 0.001 mM, 0.01 mM or 0.1 mM inducer. In all three cases, the fluorescence signal detected was increasing over time, indicating a high expression level at 3 h time point (Figure 36 A to D).

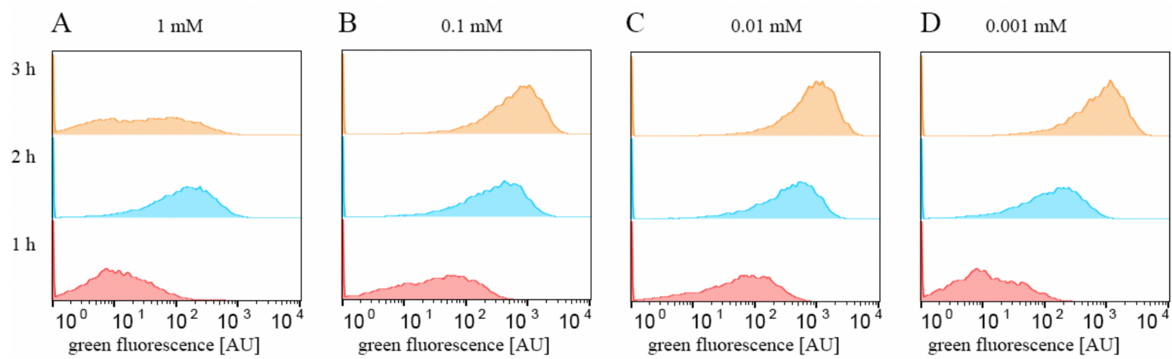

Figure 36: Flow cytometry analyses of the anthranilate-regulated expression system with GFP as a reporter protein. GFP-fluorescence was analyzed in *P. putida* DSM291 pUCP20-ANT2-*gfp*. Cell cultures were grown in M9 medium and samples were analyzed at three time points (1 h, 2 h and 3 h). Different concentrations of anthranilate were used for promoter induction as indicated on top: A, 1 mM anthranilate, B, 0.1 mM anthranilate, C, 0.01 mM anthranilate, and D, 0.001 mM anthranilate.

To sum up, the P_{antA} promoter activity is dependent upon growth medium. In complex medium (King's B) the P_{antA} promoter activity can be adjusted via an inducer concentration between 0.01 mM and 1 mM. In M9 minimal medium only an on/off switch can be achieved by inducer concentrations between 0.001 mM and 0.1 mM.

4 Discussion

This study was split into two topics. First, the influence of deleted genes encoding translocation system components was investigated. The investigation of a specific T1SS found in *P. fluorescens* was limited. Until then, no tightly controlled and fine-tunable expression system for *P. fluorescens* was available that could also be used for physiological analyses. Thus, in a second part of this study, a new tightly controlled gene expression system for *P. fluorescens* was established and compared with another published expression system.

4.1 The T1SS encoded by *aprAIDEF*

It could be shown that deletion of *aprE* encoding a T1SS component negatively influenced biofilm formation of *P. fluorescens* A506. Later on, it became clear that this effect was not caused by the T1SS substrate AprA. Mistargeted protease into the cytoplasm caused by deleting a component of the translocation machinery was suggested as a reason.

Usually T1SS are encoded on one operon and are specific to one substrate. T1SS translocate their substrates in a one-step mechanism, thus the substrate enters the translocation machinery in the cytoplasm and leaves it directly in the extracellular space. It is not expected to be located in the periplasm of Gram-negative bacteria at any time. The operon of the investigated Apr T1SS of *P. fluorescens* A506 encodes five proteins: the substrate AprA, the translocation machinery AprDEF and a small protein AprI. AprI was shown to inhibit protease activity on skim milk agar (chapter 3.3). In 2014, Pel *et al.* already compared the operons from different bacteria encoding this T1SS (Pel *et al.*, 2014).

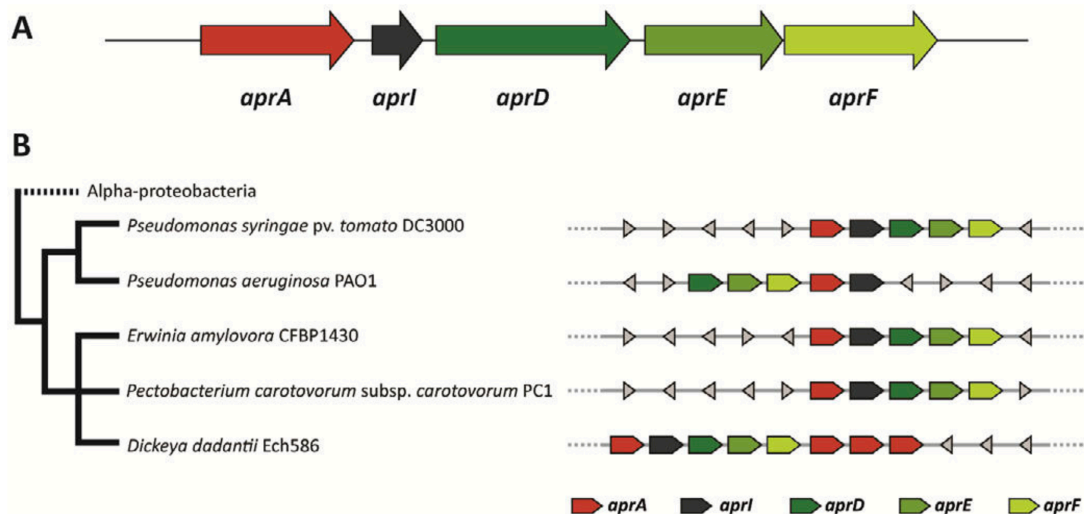

Figure 37: Genomic organisation of the genes encoding the T1SS of the substrate AprA varies in different proteobacteria. A, genomic organisation of aprAIDEF in *Pseudomonas syringae* pv. *tomato* DC3000 (the same as in *P. fluorescens* A506). B, genomic organisation of the *apr* genes in the genomes of *P. syringae* pv. *tomato* DC3000 (NP_793115), *P. aeruginosa* PAO1 (NP_249940), *Erwinia amylovora* CFBP1430 (YP_003532934), *Pectobacterium carotovorum* subsp. *carotovorum* PC1 (YP_003017132), and *Dickeya dadantii* Ech586 (YP_003333619). Genes encoding homologous Apr components appear in the same color (red: *aprA*, black: *aprI*, dark green: *aprD*, medium green: *aprE*, light green: *aprF*) and other genes are shown in grey (Pel *et al.*, 2014).

The genes encoding the T1SS components are found in different organisms, although the order of the genes and the number of genes encoding substrates vary (Figure 37). Guzzo *et al.* (1991) and Létoffé *et al.* (1990) showed that the secretion of ArpA depends on the gene products of *aprD*, *aprE* and *aprF* which encode a T1SS (Guzzo *et al.*, 1991, Létoffé *et al.*, 1990).

In *P. aeruginosa* the T1SS is already well investigated. The alkaline protease AprA is the substrate of this T1SS and belongs to the serralysin family of the zinc metalloproteases (Miyoshi and Shinoda, 2000). Duong *et al.* (1996) showed that the C-terminus of AprA has a secretion signal that is required for recognition of the ABC transporter AprD (Duong *et al.*, 1996). The secreted AprA from *P. aeruginosa* is shown to be involved in degradation of flagellin monomers (Bardoel *et al.*, 2011). Generally, AprA secretion is suggested to be connected with virulence, and AprA is suggested to play an important role as a virulence factor in *P. syringae* pv. *tomato* DC3000 (Pel *et al.*, 2014, Liehl *et al.*, 2006, Parmely *et al.*, 1990, Tommassen *et al.*, 1992). For the plant pathogen bacteria *P. syringae* pv. *tomato* DC3000 AprA secretion is advantageous for infecting plants. The plant's own defense system can be activated via flagellin receptors. AprA degrades the bacteria's own flagellin monomers leading to a reduced host defense system of the plant (Pel *et al.*, 2014). The AprA of *P. aeruginosa* is also

degrading monomeric flagellin to avoid the recognition by such flagellin receptors of the host (Bardoel *et al.*, 2011). Other targets of *P. aeruginosa* AprA in hosts are the central complement protein C3 and cytokines that are important for activation of the host immune response (Hong and Ghebrehiwet, 1992, Parmely *et al.*, 1990). In the case of *P. aeruginosa*, AprA is described as a virulence factor that helps the pathogen to evade the host's immune system (Bardoel *et al.*, 2011).

Pel *et al.* (2014) identified 134 AprA homologues in 102 different and mainly pathogenic bacterial strains, including strains belonging to α-proteobacteria, β-proteobacteria, γ-proteobacteria, cyanobacteria and chlorobi (Pel *et al.*, 2014).
In *P. aeruginosa* it was shown that another protein of unknown function is also secreted by this T1SS (Duong *et al.*, 2001), the corresponding gene *aprX* is located upstream of *aprD* in *P. aeruginosa* PAO1.

The operon encoding the T1SS components encodes the translocation machinery AprDEF, the substrate AprA and another small protein AprI. In *P. aeruginosa* this protein is already further described as a protease inhibitor for AprA. Bardoel *et al.* (2011) investigated the *P. aeruginosa* AprA-AprI inhibitory mechanism. They already wondered about the function of the inhibitor in *P. aeruginosa* as they said that "AprI is predicted to be a periplasmic protein" (Bardoel *et al.*, 2011) and AprA as the T1SS substrate is directly translocated from the cytoplasm to the extracellular space. They presumed that "AprI is part of a safety mechanism that provides protection of self-proteins for degradation by AprA prior to secretion" (Bardoel *et al.*, 2011).

Feltzer *et al.* (2000) showed that AprA and AprI form a strong enzyme-inhibitor complex and that the N-terminus of Apr I is crucial for interaction. By deleting the five N-terminal amino acids of AprI, the enzyme-inhibitor complex is not formed anymore (Feltzer *et al.*, 2000).

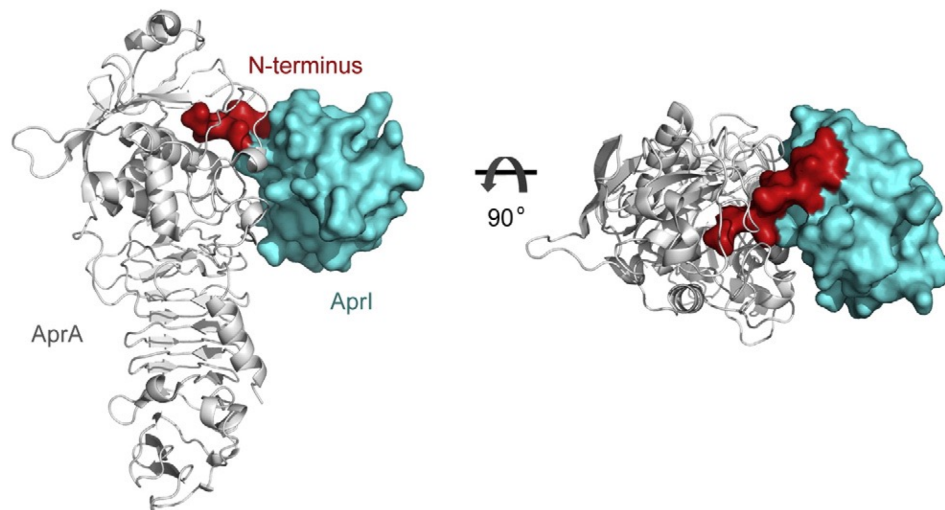

Figure 38: Visualization of the AprA-AprI interaction. The N-terminal region of AprI (N-terminus is colored red, the rest of the protein is colored light blue) is interacting in the active site of AprA (colored in grey) (Bardoel *et al.*, 2012).

The N-terminus of AprI (Figure 38, red) is located inside the active site cleft of AprA (Figure 38, grey). An exchange of the fourth amino acid leads to an even stronger AprA-AprI complex (Feltzer *et al.*, 2003) but the N-terminal region of AprI alone is not sufficient for an interaction of AprI with AprA (Bardoel *et al.*, 2011). The amino acid sequence of AprA and AprI from different species are similar but not identical. Bardoel *et al.* (2011) showed that the inhibitor AprI of one organism is not inhibiting the AprA protease of another organism. They assumed a direct correlation between species specificity of AprA and AprI and the ability to form enzyme–inhibitor complexes (Bardoel *et al.*, 2011).

To sum up, it was already shown that this T1SS is found in many species. The corresponding operon encodes a substrate, a translocation machinery and an inhibitor. The order of genes varies and, in some species, more substrates for the T1SS are found, all of them encoded in the neighborhood of the translocation machinery. The target of the inhibitor AprI is the T1SS substrate AprA, and a strong enzyme-inhibitor complex can be formed. AprA is often described as a virulence factor. As a T1SS substrate, AprA is translocated from the cytoplasm to the extracellular space by a one-step mechanism, it is not expected to be located in the periplasm at any time. AprI is suggested to be located in the periplasm. Mainly, this information was deduced from experiments with pathogenic *Pseudomonas* species (Bardoel *et al.*, 2011).
In this study the T1SS of non-pathogenic *P. fluorescens* A506 was investigated. In a first approach the localization of AprI was investigated by heterogenous expression in *E. coli*. AprI

could be detected in the periplasmic fraction. This indicated that there must be a secretion system that translocated AprI across the cytoplasmic membrane into the periplasm of *E. coli*.

Figure 39: N-terminal Sec-signal peptide prediction of AprI with SignalP 5.0 (Emanuelsson *et al.*, 2007).

By scanning the amino acid sequence of AprI with the bioinformatic tool SignalP 5.0 (Emanuelsson *et al.*, 2007), an N-terminal Sec-signal peptide was found (Figure 39). Thus, AprI of *P. fluorescens* A506 is predicted to be translocated via the Sec-pathway.

As a side aspect, it could be observed that heterologous expression of *aprA* in *E. coli* lead to an elongated exponential growth phase, when the ABC transporter AprD was not produced. When no translocation system for AprA was available, AprA was expected to remain in the cytoplasm of *E. coli*. A possible explanation for this growth phenotype is, that the unspecific protease AprA degrades peptides that regulate or inhibit cell growth during late-exponential growth phase, respectively proteins that regulate the transition from exponential to stationary growth phase.

4.2 The anthranilate-inducible promoter system

The promoter system originally found in *P. fluorescens* A506 is working fine in the strain itself and in the two other tested *Pseudomonas* strains *P. aeruginosa* PAO1 and *P. putida* type strain DSM291^{T}. Although the gene expression could be induced in all three strains both in complex and minimal medium, data showed a strong influence on induction behavior and sensitivity to anthranilate concentration by the chosen medium (Table 22).

Table 22: Summary of the experimental observations of P_{antA} promoter induction.

	P. fluorescens		***P. aeruginosa***		***P. putida***	
	LB	**M9**	**LB**	**M9**	**KB**	**M9**
observed induction autoinduction	yes slightly	yes no	yes very strong	yes no	yes no	yes no
useful range of inducer concentrations [mM]	0.01 – 10	0.01 – 10	none	0.01–1	0.01–1	0.001 – 0.1
promoter activity is adjustable	yes	yes	no	yes	yes	no
promoter activity is only switched on/off	no	no	yes (by auto-induction)	no	no	yes
induction level is uniform in the whole cell culture	yes	yes	yes	yes	yes	yes
toxic effect of high inducer concentration	no	slightly	no	yes	yes	yes
recommended medium	**M9**		**M9**		**KB**	

4.2.1 Influence of cultivation media on P_{antA} properties and anthranilate metabolization in *Pseudomonas*

During cultivation of *P. putida*, a stronger sensitivity to anthranilate concentration compared with the two other *Pseudomonas* strains was observed. The results in 3.6.5.2 showed that gene expression can be well controlled in *P. putida* when cultivated in King's B medium and no autoinduction could be observed. The activity of P_{antA} could be adjusted by the inducer concentration with an inducer concentration between 0.01 mM and 1 mM. Thus, the use of the promoter system in King's B medium can be recommended.

For *P. fluorescens* and *P. aeruginosa* LB medium was chosen as complex medium because it is an often-used standard medium for cultivation of these strains. In both cases, the P_{antA} promoter was induced during cultivation although no inducer was added, clearly indicating an autoinduction of the promoter. A possible reason for this might be L-tryptophan containing proteins in LB medium. These serve as an energy source for the bacteria and since LB medium

contains a lot of tryptophan, some bacteria are able to use tryptophan to catabolize it to other metabolites such as anthranilate.

In 1975, Bouknight and Sadoff already suggested that L-tryptophan is catabolized in *Bacillus megaterium* leading to intermediates such as kynorenine, catechol and anthranilic acid (Bouknight and Sadoff, 1975). Later in 1980, Salcher and Lingens showed L-tryptophan degradation via an anthranilic acid intermediate in *Pseudomonas aureofaciens* (Salcher and Lingens, 1980). In 2003 Kurnasov *et al.* predicted a three-step mechanism (Figure 40) for L-tryptophan catabolism and verified enzymatic activity of kynurenine formamidase involved in this degradation process. Intermediate products from this degradation pathway are formylkynurenine and kynurenine (Kurnasov *et al.*, 2003).

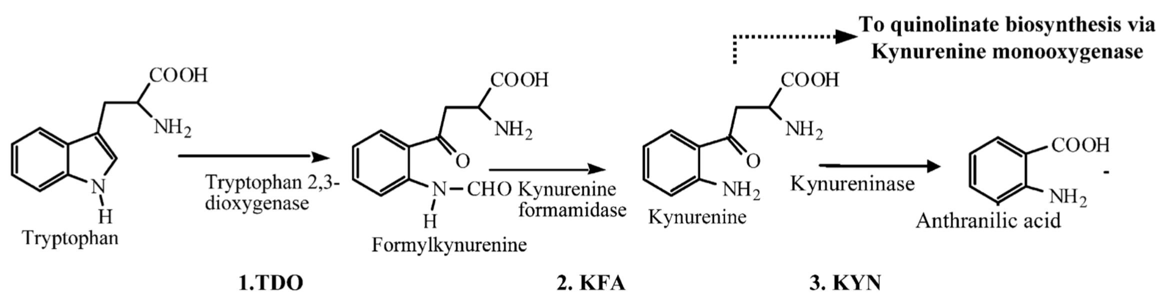

Figure 40: Degradation pathway of tryptophan to anthranilic acid (Kurnasov *et al.*, 2003).

Since LB complex medium offers many tryptophan containing proteins, the medium clearly offers a lot of tryptophan that can be metabolized into anthranilate, the inducer of the P_{antA} promoter. This results in an autoinduction of the promoter although no inducer was added.

Due to the autoinduction of the P_{antA} promoter in LB, this medium is not recommendable for a tightly controlled gene in *P. fluorescens* and *P. aeruginosa*. In contrast to this, the defined minimal medium M9 does not offer peptides to the bacteria, explaining no observed autoinduction in this medium. Although *P. fluorescens* and *P. aeruginosa* seem to be more sensitive in M9 minimal medium to the toxic effects of high anthranilate concentrations, this medium allows a fine tunable gene expression with the P_{antA} promoter and therefore it can be recommended.

As these results showed, P_{antA} promoter system worked fine in all three tested *Pseudomonas*. Growth conditions and inducer concentrations were established, allowing a tightly controlled gene expression in the respective strain. The promoter system could also work in other *Pseudomonas* strains, that had not been tested so far. It has to be kept in mind, that the cultivation medium had a strong influence on inducibility of the P_{antA} promoter. Gene

expression in different *Pseudomonas* behaved differently in different media in relation to anthranilate sensitivity and autoinduction of the promoter. If another strain is tested for the usability of this promoter system, different cultivation media should be tested and compared, because there is not a general medium for cultivation of all *Pseudomonas* recommendable.
Even if no promoter leakage could be detected in the experiments, it does not mean that there was no promoter leakage at all. Faint leakage below detection level cannot be excluded. Sometimes a very low gene expression can even be desired for e.g. enzymatic experiments.

In *P. aeruginosa* anthranilate plays an even more central role in the metabolism because it is involved in formation of the *Pseudomonas* quinolone signal (PQS) (Pesci *et al.*, 1999). Farrow *et al.* (2015) described the production of PQS as "a key component of the *P. aeruginosa* cell-to-cell signaling network, impacts multiple physiological functions, and is required for virulence" (Farrow *et al.*, 2015). PQS is involved in the regulation of virulence genes and under specific iron limiting conditions that are typical for hosts, PQS is activated (Farrow and Pesci, 2007). Besides PQS, two main cell-to-cell signaling systems are known, the *las* and the *rhl* systems that are homoserine lactone-based systems which can also be found in other Gram-negative bacteria (Déziel *et al.*, 2004, Wagner *et al.*, 2003, Schuster *et al.*, 2003). The *las* system, the *rhl* system and the PQS are linked with each other. The *las* system can positively regulate PQS production, whereas the *rhl* system can negatively impact it. The *rhl* system is positively regulated by PQS and *las* system (Pesci *et al.*, 1999, Latifi *et al.*, 1996, Pesci *et al.*, 1997, McKnight *et al.*, 2000, Diggle *et al.*, 2003, McGrath *et al.*, 2004, Jensen *et al.*, 2006, Hazan *et al.*, 2010).

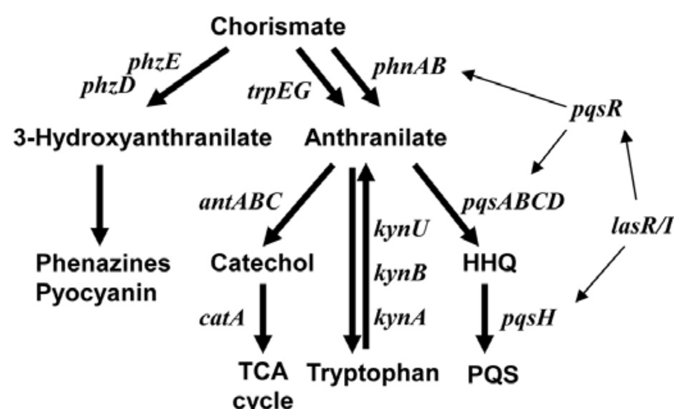

Figure 41: Metabolic pathways inside bacteria with anthranilate as an intermediate product (Choi *et al.*, 2011).

The *Pseudomonas* quinolone signal (PQS) of *P. aeruginosa* may also influence anthranilate concentration during cultivation. Anthranilate is a central intermediate in the metabolism of *P. aeruginosa* (Figure 41). Choi *et al.* (2011) said that “anthranilate is an important intermediate at the metabolic branch point during the PQS biosynthesis” (Choi *et al.*, 2011). It can be metabolized by degradation of tryptophan and used as a building block for other products such as catechol, tryptophan or PQS (Calfee *et al.*, 2001, Essar *et al.*, 1990, Oglesby *et al.*, 2008, Choi *et al.*, 2011).

For the metabolism of anthranilate to PQS, *pqsABCDE* and *pqsH* are encoding enzymes in *P. aeruginosa*. So far, the production of PQS and the *pqs* genes were only found in *P. aeruginosa*.

Studies have shown that iron has a strong influence on physiology of *P. aeruginosa*. Oglesby *et al.* (2008) showed that the small PrrF RNAs PrrF1 and PrrF2 repress genes encoding anthranilate degradation under iron limiting conditions, thus the anthranilate availability for PQS is increased. The small RNAs PrrF inhibit the translation of *antR* mRNA and thus the expression of *antABC* is inhibited. In 2008, Oglesby *et al.* assumed an interaction between PrrF and the *antR* mRNA as responsible for the repression of *antA* by PrrF (Oglesby *et al.*, 2008). They showed that there is a complementary region between the PssF RNAs and translation initiation site of *antR*. In 2018, Djapgne *et al.* could demonstrate an interaction of the PrrF RNAs with the 5’ UTR of *antR* RNA resulting in an overlapped translational start site of this mRNA (Djapgne *et al.*, 2018). The annealing of the PrrF RNAs with the 5’ UTR of *antR* RNA is mediated by Hfq (a regulatory factor) and the binding potential of Hfq plays a major role for catabolite repression control (Crc) of *antR* (Sonnleitner *et al.*, 2017, Djapgne *et al.*, 2018).

By this negative regulation of *antR*, AntR is not produced and gene expression of *antABC* for catechol synthesis from anthranilate is inhibited (Figure 42).

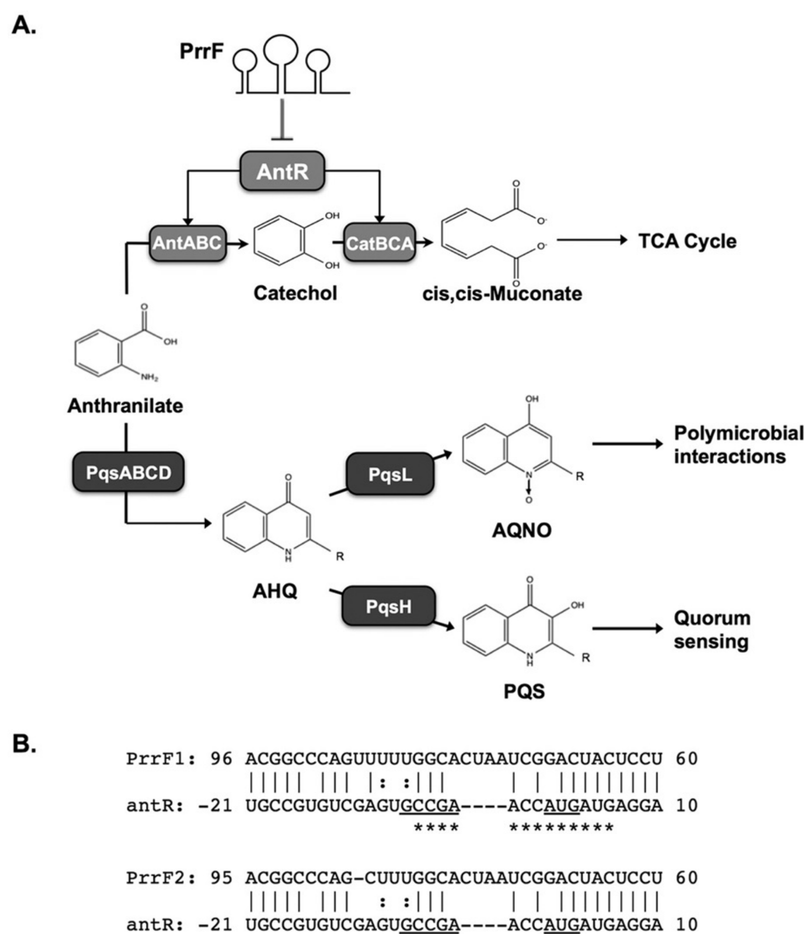

Figure 42: Regulation of anthranilate metabolism by the small RNAs PrrF. A, Regulation by PrrF leads to increased alkylquinolone production in *P. aeruginosa*. The expression of *antR* is repressed by PrrF and thus, the expression of *antABC* and *catBCA* is not induced. This leads to a repression of anthranilate degradation via catechol and an improvement of using anthranilate to build quinolone metabolites. B, Complementary regions of small RNA PrrF1 respectively PrrF2 and *antR* leader sequence. The predicted start codon of the mRNA of *antR* is underlined and the numbers indicate the orientation of the RNA in relation to the transcriptional (for PrrF1 and PrrF2) respectively the translational start site (*antR* leader sequence). Vertical lines indicate base pairing. Asterisks indicate the region of *antR* that was protected by the small RNA PrrF1 in Rnase degradation assays (Djapgne *et al.*, 2018).

In this study, the anthranilate promoter sequence from *P. fluorescens* was used. Synthesis of PQS and the *pqs* genes were only found in *P. aeruginosa* and not in *P. fluorescens* and the 5' UTR of *antR* RNA from *P. fluorescens* does not contain the PrrF1 and PrrF2 binding site (Figure 43). Thus, the tested expression system is not expected to be affected by the PrrF small RNA regulatory system.

5’UTR of *antR* (PAO1): **UGCCG**U**GUC**GAGUG**CCG**AACCAUGAUGAGGACCCAU
5’UTR of *antR* (A506): AACCGCCGUGGGUGCACCCUGAUGAGUAGCCAGACA

Figure 43: The 5’ UTR of *antR* RNA from *P. aeruginosa* PAO1 and *P. fluorescens* A506. Letters in bold indicate the bases involved in base pairing of 5’UTR of *antR* (*P. aeruginosa* PAO1) with PrrF1 and PrrF2. Underlined region indicates start codon.

4.2.2 Anthranilate and its transport into the cell

To induce a promoter system the first requirement for an inducer is to get into the cell and pass the membrane. In this study it was shown that anthranilate is works fine even at low concentrations as an inducer for the P_{antA} promoter system in all *Pseudomonas* strains tested. This indicates that anthranilate was somehow able to get across the membrane to enter the cell. No specific uptake mechanism is known for anthranilate. Rioux *et al.* (1986) showed that anthranilate is secreted by rhizobia and is involved in iron uptake under specific iron limiting conditions (Rioux *et al.*, 1986). This means that there may be a mechanism for anthranilate secretion and uptake in this organism. Anthranilate may also be transported via an amino acid transporter or a transporter for small acids with low specificity. Pajor and Randolph (2007) found that anthranilate derivatives (such as N-(p-amylcinnamoyl) anthranilic acid or 2-(p-amylcinnamoyl) amino-4-chloro benzoic acid) inhibit Na+/dicarboxylate cotransporters (Pajor and Randolph, 2007). It may be possible that the non-modified anthranilate also interacts with these transporters and may even be transported by them.

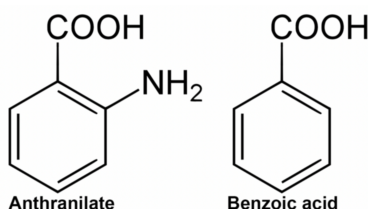

Figure 44: Chemical structure of anthranilate and benzoic acid.

Anthranilate is a small molecule. Its structure can be described as a benzoic acid molecule with an amino group next to the carboxyl group (Figure 44). For benzoic acid Salmond *et al.* (1984) showed that it lowers the intracellular pH of *E. coli* and thus negatively influences cell growth (Salmond *et al.*, 1984). In this study a high anthranilate concentration lead in some cases to a weaker cell growth or toxic effects, this might be caused by acidification of the cytoplasm as it was observed for benzoic acid.

In *P. fluorescens* no rhamnose import mechanism is known. Rhamnose may have a low affinity to other transporters which enable the rhamnose uptake. This might explain the high concentration of rhamnose that is necessary for a promoter induction. To compare this with anthranilate as an inducer, anthranilate is advantageous over rhamnose because it seems to be taken up also at lower concentrations.

The flow cytometry experiments gave information about the induction behavior of the cell culture. All data from the flow cytometry experiments showed a single Gaussian distribution of fluorescence intensity. This indicates a cell culture with homogeneous gene expression level. Thus, both promoter systems led to a homogeneously induced cell culture. In cases of a promoter system that is only capable of switching the gene expression on or off, two populations would be visualized in flow cytometry. An example for such an all-or-nothing expression system is the arabinose system in *E. coli*. Here, the transporter for the inducer is under the control of the inducer itself (Siegele and Hu, 1997, Khlebnikov *et al.*, 2000). Because of no known rhamnose importer in *P. fluorescens*, it was expected that the uptake of rhamnose is not activated or influenced by rhamnose itself.

4.2.3 Comparison of the two tested promoter systems

In this study two inducible promoter systems were compared in *P. fluorescens*. The anthranilate-inducible promoter system was compared with the rhamnose-inducible RhaSR/P_{rhaB} system deriving from *E. coli*. With the anthranilate-inducible promoter system, the gene expression was adjustable by inducer concentration and lead to almost constant lower or higher gene expression levels over time. And an important observation was that the whole culture behaved similarly, so that all cells were homogeneously induced.
The rhamnose-inducible promoter system only lead to strong gene expression that increased over time until it reached its maximum. Also, the rhamnose-inducible promoter was less sensitive to inducer concentration. It could only be switched on with higher inducer concentrations. This led to the conclusion that the anthranilate-inducible system is well suitable for liquid culture experiments with a desired constant gene expression level over time such as physiological assays. The rhamnose-inducible system is not useful for experiments with constant lower gene expression levels. When only a strong gene expression is desired, both promoter systems tested are suitable and in some cases a sugar-free inducer may be

advantageous for cultivation. At least, this conclusion applies for experiments with *P. fluorescens* A506.

The anthranilate-inducible promoter behaved in *P. putida* and *P. aeruginosa* similarly as in *P. fluorescens*. In this study the rhamnose-inducible system was not tested in the other strains. Previous studies have reported the functionality of the heterologous *E. coli* rhamnose system in other *Pseudomonas* strains. In *P. putida*, Jeske and Altenbuchner (2010) showed that this rhamnose-inducible system leads to high gene expression level when the promoter is induced with high inducer concentration (~12 mM) (Jeske and Altenbuchner, 2010) and in 2016, Meisner and Goldberg tested the rhamnose-inducible system in *P. aeruginosa* (Meisner and Goldberg, 2016). But it has to be noted that no information was given about whether the cell culture showed homogeneous gene expression as in the case of *P. fluorescens*.

To conclude, both expressions systems are useful for gene expression in *Pseudomonas* but the anthranilate-inducible system from *P. fluorescens* is advantageous over the rhamnose-inducible system from *E. coli* for gene expression in *Pseudomonas*. The investigated P_{antA} promoter system allows a fine-tunable gene expression that enables the adjustment of the gene expression level via the inducer concentration. In an applicable cultivation medium, no promoter leakage is detectable and the gene expression is stable over time. Anthranilate as an inducer is cheap, non-toxic and stable.

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List of publications

1. Hoffmann L, Sugue M-F, and Brüser T (2020). A tunable anthranilate-inducible gene expression system for *Pseudomonas* species. App. *Microbiol. Biotechnol*. 105:247–258.