RNA-SEQ BASED IDENTIFICATION AND BIOCHEMICAL CHARACTERIZATION OF THE *PSEUDOMONAS AERUGINOSA* LASR AND QUORUM SENSING ANTI-ACTIVATOR PA2226

DISSERTATION

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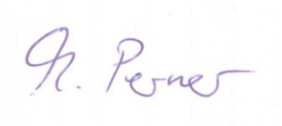
English language declaration

As a native English speaker, I hereby declare that I have checked the thesis "RNA-seq based identification and biochemical characterization of the *Pseudomonas aeruginosa* LasR and Quorum Sensing anti-activator PA2226" by Christian Utpatel for grammatically correct English and the scientific accuracy of the language.

Sincerely,



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Abstract

Many pathogenic bacteria regulate their virulence factor expression by Quorum Sensing (QS), the cell density dependent release and perception of small diffusible molecules, which are called autoinducers (AI). In the opportunistic pathogenic microorganism *Pseudomonas aeruginosa* PAO1, in which nearly 10 % of the annotated genes are involved in regulation, Quorum Sensing regulated gene expression contributes to the formation and maintenance of biofilms and their tolerance to conventional antimicrobials. This makes it a promising target for infection control. Earlier it was shown that the enzymatic degradation of AI molecules is an efficient approach to control virulence of *Pseudomonas*. Acylases, lactonases and oxidoreductases are now known to cleave or modify the AI and thereby prevent activation of QS and in succession the controlled phenotypes like elastase, pyocyanin, hydrogen cyanide and biofilm formation. Within this framework, we have shown that the expression of a metagenome-derived short-chain dehydrogenase/reductase (SDR, BpiB09) resulted in significantly reduced pyocyanin production, decreased motility, poor biofilm formation and decreased paralysis of nematodes. In this study and with the aim to unravel the impact on the complicated regulatory system upon the expression of *bpiB09*, RNA sequencing (RNA-seq) was employed to get detailed insight into the genetic alterations caused by BpiB09 and their impact on the bacterial metabolism. By analyzing the global transcriptome of PAO1 pBBR::*bpiB09* via RNA-seq it was observed that the upregulation of the hypothetical protein *PA2226* was not only associated with AI modification, but also with a 19 kb deletion in the bacterial chromosome. Within this thesis I could show, that the upregulation of *PA2226* was most likely responsible for several strong phenotypes connected to QS, including pyocyanin, elastase and 3-oxo-C12-HSL formation. For further verification of these phenotypes, several mutant strains with deletions in genes essential for QS were constructed. These strains included newly constructed *lasI*, *rhlI* and a double deletion mutant thereof as well as a mutant with a deleted *PA2226*. These mutations were verified by genetic complementation and PCR. Surprisingly, none of these mutants showed an altered biofilm phenotype compared to the parent strain. However, ΔlasI, ΔrhlI and ΔlasI/rhlI showed strong phenotypes for motility, elastase, pyocyanin and 3-oxo-C12-HSL production. With a coordinated approach of RNA-seq of a PA2226 overproducer, QS reporter gene assays in *E. coli* and further phenotypical testing, PA2226 was established as responsible agent and QS core mechanisms were suspected as its target. The obtained RNA-seq data were in good concordance with the recently defined QS core regulon of *P. aeruginosa* PAO1. Among the differentially expressed genes the elastase *lasB*, the QS regulator *rsaL*, the AI synthase *rhlI*, the genes for hydrogen cyanide formation *hcnABC* and *ambBCDE*, which are involved in IQS formation, were observed. Furthermore, it became

evident that the overexpression of PA2226 had a major impact on genes belonging to the functional classes of secreted factors and protein secretion/export. Additional overexpression of PA2226 in *E. coli* showed no activation of the included QS reporter, exposing QS active promotors and LasR as essential parts of the mode of action. After excluding the potential interference of recombinant PA2226 with QS active promotors via electrophoretic mobility shift assays (EMSA), LasR emerged to be the most likely target. It was possible to demonstrate that PA2226's regulatory function is closely linked to this QS response regulator by coprecipitation of recombinant PA2226 and LasR from crude cell extracts, indicating that both proteins strongly interact in *P. aeruginosa*. This correlates with the finding that QS phenotypes were partially rescued, when LasR and PA2226 were co-expressed, but likewise opens the possibility for at least one other interaction partner. Taking advantage of next generation sequencing to analyze a PAO1 strain with strong Quorum Quenching phenotypes enabled the identification and characterization of a novel major QS regulator, extending the knowledge of the complex QS network of *P. aeruginosa*.

Zusammenfassung

Viele pathogene Bakterien regulieren ihre Virulenz durch Quorum Sensing (QS), der zelldichteabhängigen Ausschüttung und Detektion kleiner diffusionsfähiger Moleküle, genannt Autoinducer (AI). In dem opportunistisch pathogenen Mikroorganismus *Pseudomonas aeruginosa,* in welchem nahezu 10 % aller annotierter Gene in Regulationsprozessen involviert sind, trägt die Quorum Sensing regulierte Genexpression auch zur Bildung und Erhaltung von Biofilmen und der Toleranz entgegen Antibiotika bei. Dies macht es zu einem vielversprechenden Ziel zur Infektionskontrolle. Es wurde bisher gezeigt, dass der enzymatische Abbau der Autoinducermoleküle ein effizienter Ansatz zur Kontrolle der Virulenz in *Pseudomonas* ist. Acylasen, Laktonasen und Oxidoreduktasen degradieren oder modifizieren Autoinducermoleküle und verhindern damit die Aktivierung von QS und davon kontrollierten Phänotypen wie Elastase-, Pyocyanin-, Hydrogencyanid- und Biofilmbildung. In diesem Zusammenhang haben wir gezeigt, dass die Expression einer metagenomisch gewonnenen Dehydrogenase/Reduktase (BpiB09) zu signifikant reduzierter Produktion von Pyocyanin, geringer Biofilmbildung und reduzierter Paralyse von Nematoden führte. In dieser Studie wurde RNA-Sequenzierung (RNA-Seq) verwendet, um den Einfluss der Expression von *bpiB09* auf das komplexe regulatorische Netzwerk zu enträtseln, sowie detaillierte Einsicht in durch BpiB09 hervorgerufene genetische Modifikationen und ihren Einfluss auf den bakteriellen Metabolismus zu bekommen. Bei der Analyse des globalen Transkriptoms von PAO1 pBBR::*bpiB09* mittels RNA-Seq wurde beobachtet, dass die Hochregulierung des hypothetischen Proteins *PA2226* nicht nur in Verbindung mit der Modifikation des Autoinducers steht, sondern ebenso mit einer 19 kb Deletion im bakteriellen Chromosom. In dieser Studie konnte ich zeigen, dass die Hochregulierung von *PA2226* für einige starke QS Phänotypen verantwortlich ist, darunter Pyocyanin-, Elastase- und 3-oxo-C12-HSl Bildung. Zur Verifizierung dieser Phänotypen wurden eine Reihe von mutierten Stämmen generiert, welche Deletionsmutationen für essentielle QS Gene beinhalten. Diese generierten Stämme beinhalten Einzeldeletionsmutanten für *lasI* und *rhlI*, eine Doppelmutante aus diesen, sowie eine Mutante mit deletiertem *PA2226*. Die Mutationen wurden mittels Komplementation und PCR überprüft. Überraschenderweise zeigte keine dieser Mutanten einen veränderten Biofilm Phänotyp im Vergleich zum Wildtyp. Andererseits zeigten die Mutanten ΔlasI, ΔrhlI und ΔlasI/rhlI starke Phänotypen für Motilität, sowie Elastase, Pyocyanin und 3-oxo-C12-HSL Produktion. In einem abgestimmten Ansatz aus RNA-Seq von einem PA2226 Überproduzierer, QS Reporter Gen Assays in *E. coli* und weiteren phänotypischen Tests, konnte PA2226 als verantwortliche Ursache identifiziert und zentrale QS Mechanismen als potenzielles Ziel ausgemacht werden. Die erhaltenen RNA-Seq Daten waren in großer Übereinstimmung mit dem kürzlich beschriebenen zentralem QS Regulon von

P. aeruginosa PAO1. Unter den differenziell exprimierten Genen konnten die Elastase *lasB*, der QS Regulator *rsaL*, die AI Synthase *rhlI*, die Gene für Hydrogencyanidbildung *hcnABC* und *ambBCDE*, welche in der Synthese von IQS involviert sind, beobachtet werden. Darüber hinaus wurde deutlich, dass die Überexpression von PA2226 großen Einfluss auf die Genexpression von Genen hat, welche zu den funktionellen Klassen der „Sekretierten Faktoren“ und der „Protein Sekretion/Export“ gehören. Weitere Überexpression von PA2226 in *E. coli* zeigte, dass der hier beinhaltende QS Reporter nicht aktiviert wird, was QS aktive Promotoren und LasR als essentielle Bestandteile des Mechanismus identifiziert. Nachdem eine Interaktion von rekombinantem PA2226 mit QS aktiven Promotoren mittels Electrophoretic Mobility Shift Assay (EMSA) ausgeschlossen werden konnte, erschien LasR als wahrscheinlichstes Ziel. Im Nachfolgenden konnte durch Kopräzipitation von rekombinantem PA2226 und LasR aus Zellrohextrakten gezeigt werden, dass die regulatorische Funktion von PA2226 eng mit diesem QS Regulator verbunden ist. Dies korreliert mit der Beobachtung, dass QS Phänotypen partiell wiederhergestellt werden konnten wenn LasR und PA2226 gleichzeitig exprimiert wurden, öffnet aber die Möglichkeit mindestens eines weiteren Interaktionspartners. Indem sich Next Generation Sequencing bei der Analyse eines PAO1 Stammes mit starken Quorum Quenching Phänotypen zu Nutze gemacht wurde, konnte ein neuer QS Regulator identifiziert und charakterisiert werden, womit das Wissen um das komplexe QS Netzwerk von *P. aeruginosa* erweitert wurde.

1 Introduction

1.1 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative bacterium and belongs to the class of γ-proteobacteria. It is a ubiquitous opportunistic pathogen which grows in soil, coastal marine habitats, on plants and animal tissues. It has a genome size of 6.3 Mbp and its genome analysis revealed that remarkable 10 % of all annotated genes are involved in regulation (Stover et al. 2000). The average GC-content with 67 % is slightly elevated, compared to other γ-proteobacteria. Aerobically growing *P. aeruginosa* is capable of utilizing a wide range of organic compounds like sugars, fatty acids, amino acids or aromatic compounds (Lessie and Phibbs 1984). *P. aeruginosa* can also be grown anaerobically if an alternative electron acceptor is available, which are usually nitrogen compounds.

P. aeruginosa can be found in opportunistic infections and is responsible for approximately 10-20 % of all nosocomial infections (Ikeno et al. 2007, Van Delden and Iglewski 1998), but is also found in chronical infections like cystic fibrosis (CF) where it significantly increases morbidity and mortality (Murray et al. 2007, Emerson et al. 2002). However, treatment can be complex. *P. aeruginosa* has a high intrinsic resistance to several antibiotics, because of its low membrane permeability and active export of antibiotics (Hancock 1998). Moreover biofilm formation often leads to complications in CF and other infections because of the elevated antibiotic resistance of bacteria embedded in these (Wagner and Iglewski 2008, Costerton et al. 1995, Costerton et al. 1999). Therefore, as alternative to antibiotic-mediated killing or growth inhibition the attenuation of bacteria with respect to pathogenicity has been suggested (Bjarnsholt and Givskov 2007). In *P. aeruginosa* the expression of several virulence factors, motility, biofilm formation and up to 11 % of the genome are controlled by Quorum Sensing (Schuster et al. 2003, Juhas et al. 2005).

1.2 Quorum Sensing

Quorum Sensing (QS) is a form of bacterial intercellular communication to sense the population density and alter gene expression accordingly (Fuqua et al. 1994, Coggan and Wolfgang 2012). QS is based on the production and perception of small diffusible molecules called autoinducers (AI) (Kaplan and Greenberg 1985, Atkinson and Williams 2009). Autoinducers are formed constitutively by an AI synthase and are released from

cells by diffusion or active transport. Its concentration in the surrounding resembles therefore a certain cell density (Whitehead et al. 2001). When a distinct threshold concentration is reached, a receptor protein perceives autoinducers that diffused back into the cells and a biological response is triggered. QS allows the bacterial population to synchronize the activation of processes that are advantageous when executed in large numbers, which is for example important for the infection of a host (Smith and Iglewski 2003, Van Delden and Iglewski 1998, Figure 1). Apart from this first defined concept, it is now known, that QS is not only dependent on the cell density and rather is the result of the combination of different factors such as diffusion rate and spatial distribution. In consequence, alternative terms like “diffusion sensing” or “efficiency sensing” have been proposed (Hense et al. 2007, Redfield 2002). Supporting this expanded concepts, it has been shown for *P. syringae* that even the small number of 10 cells is sufficient to accumulate autoinducers up to the QS activating threshold concentration (Dulla and Lindow 2008).

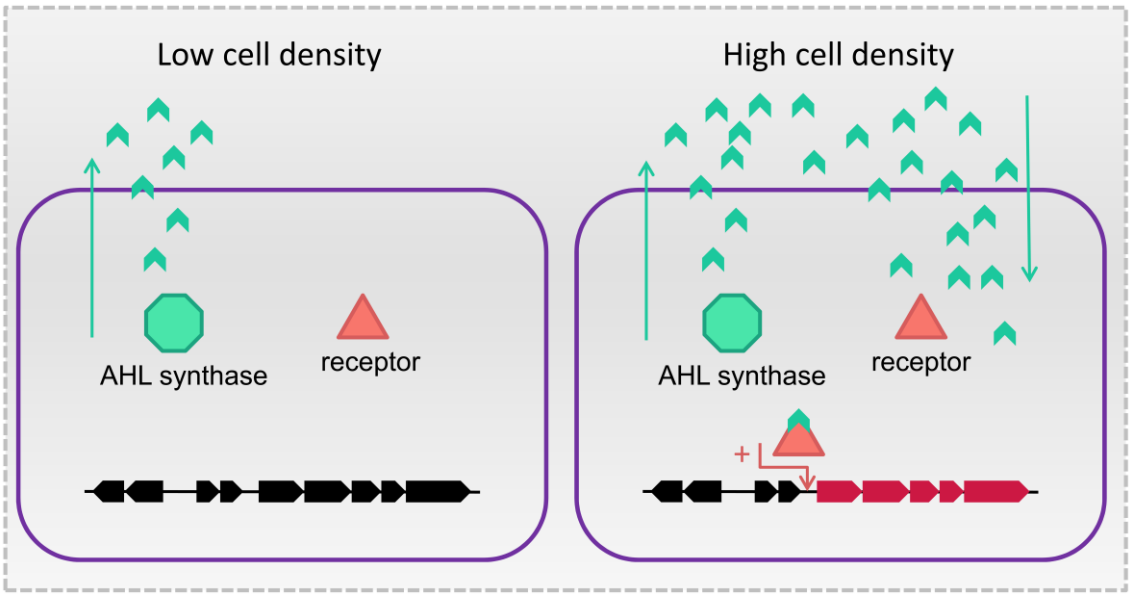

Figure 1: AHL based Quorum Sensing model for Gram-negative bacteria. AHLs produced by the signal synthase leave the cell by diffusion or active transport. With rising population density and reaching a threshold concentration, AHLs are bound by the receptor and the active complex regulates transcription through designated QS promoters (modified from Czajkowski and Jafra 2009).

In general two systems can be differentiated. Gram-negative bacteria mainly use *N*-acyl homoserine lactones (AHLs) whereas Gram-positive bacteria use modified linear or cyclic peptides (Miller and Bassler 2001). The so-called autoinducer 2 system (AI-2) is shared by both classes and is considered as an universal “language” (Winans 2002). The signal molecules are derivatives of 4,5-dihydroxy-2,3-pentanedione and AI-2

systems were shown in over 50 species (Schauder et al. 2001, Winzer et al. 2002, Xavier and Bassler 2003, Vendeville et al. 2005). The probably least characterized signal molecule and system is AI-3 which has been described for *E. coli* and *Salmonella typhimurium*, though the structure and gene responsible for AI-3 production remain unknown (Walters and Sperandio 2006, Tay and Yew 2013). For Gram-negative bacteria also the DSF (***d***iffusible ***s***ignal ***f***actor) signaling pathway is described, which signal is a cis-unsaturated fatty acid (Wang et al. 2004). First described for *Xanthomonas campestris* it also was found in other *Xanthomonas* species, *Stenotrophomonas maltophilia*, and structural similar compounds in *Burkholderia* species (Barber et al. 1997, Fouhy et al. 2007, Deng et al. 2010).

QS was first described in the marine bacterium *Vibrio fischeri*, where bioluminescence depends on the population density (Nealson et al. 1970, Eberhard 1972). The signal molecule was later identified as acyl homoserine lactone (Eberhard et al. 1981) the synthase as LuxI and the receptor as LuxR. LuxRI homologue systems were described for over 70 different Gram-negative species, among others *Agrobacterium tumefaciens* (TraRI), *Chromobacterium violaceum* (CviRI) und *Pseudomonas aeruginosa* (LasRI and RhlRI) (Williams et al. 2007, Boyer and Wisniewski-Dye 2009). AHL molecules are in general specific to individual bacterial species and they differ in their acyl chain length, saturation and substitutions.

1.3 QS in *Pseudomonas aeruginosa*

The two main QS systems, the Las and Rhl systems, have been well studied in *P. aeruginosa*. The Las system includes the LasI and LasR, and the Rhl system comprises RhlI and RhlR. They were named after the first targets they were discovered to control, the elastase *lasB* and the rhamnolipids synthase genes *rhlABC*. The synthase LasI catalyzes the formation of the N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) signal molecule, which binds to the transcriptional response regulator LasR (Pearson et al. 1994). RhlI catalyzes the formation of N-butanoyl-L-homoserine lactone (C4-HSL), which binds to RhlR (Pearson et al. 1997). Both molecules contain a homoserine lactone ring and an acyl side chain of variable length. The length of the acyl chain is an important factor to the receptor specificity. The acyl chain length of the autoinducers synthesized by LasI and RhlI is determined by the synthases acyl-chain binding pocket, which restricts the acyl chain length of the substrate (Gould et al. 2004). Because of its short acyl chain length the C4-HSL is able to diffuse free through the membrane, but the 3-oxo-C12-HSL is, beside a very slow diffusion, actively transported by the multidrug efflux pump MexAB-OprM (Pearson et al. 1999). This also seems to play a role in controlling

the access to non-cognate AHLs with different acyl chain lengths in *Pseudomonas*, as a *mexAB oprM* deletion mutant could respond to 3-oxo-C10-HSL and 3-oxo-C9-HSL. It was suggested that the AHLs entry into the cell is likely to be passive and AHLs were extruded by MexAB-OprM, leading to a non-natural response in the MexAB-OprM mutant on accumulation of these AHLs (Minagawa et al. 2012).

Analysis of amino acid sequences of LasR and RhlR suggests that both of them, like LuxR, contain an autoinducer binding site in the N-terminal part of the protein and a DNA binding domain in the C-terminal region (Fuqua et al. 1994, Latifi et al. 1995). Both, LasR and RhlR bind to their cognate autoinducer molecules with high specificity and in succession multimerize to their active forms (Kiratisin et al. 2002, Lamb et al. 2003). The LasR monomer binds to 3-oxo-C12-HSL and becomes a dimer, which binds to its target promoters (Schuster et al. 2004). RhlR dimerizes and binds to DNA, both in the presence and absence of C4-HSL (Ventre et al. 2003), which is only needed for activation (Medina et al. 2003). The target DNA is for example the so-called las box, an conserved palindromic DNA sequence which has been found upstream of some genes known to be directly regulated by the Las or the Rhl system, e.g. the AHL synthases (Schuster and Greenberg 2006, Whiteley et al. 1999) In consequence both systems regulate their own synthases through a positive feedback loop, which results in enhanced signal production (Seed et al. 1995). Moreover, the systems are organized in a hierarchical structure in which the Las system controls the Rhl system (de Kievit et al. 2002, Pesci et al. 1997, Latifi et al. 1996), although Rhl activation independent from LasR has been reported for growth in phosphate limited medium (Dekimpe and Deziel 2009). A negative feedback reaction involves RsaL and in its absence uncontrolled production of 3-oxo-C12-HSL is the consequence (Rampioni et al. 2006). The gene *rsaL* is located in reverse orientation in the intergenic region between *lasI* and *lasR*, shares the bi-directional promoter with *lasI* and acts as direct repressor to *lasI* by binding to the *lasI* promoter at a distinct binding site from LasR and thereby inhibits transcription even when LasR is bound (de Kievit et al. 1999).

A third QS molecule is the 2-heptyl-3-hydroxy-4-quinolone, named *Pseudomonas* quinolone signal (PQS), which can be regulated by the Las system (Pesci et al. 1999), regulates the Rhl system (McKnight et al. 2000) and is itself regulated by the Rhl system (Brouwer et al. 2014). LasR positively regulates *pqsA*, *pqsH* and *pqsR* whereas RhlR also activates transcription but regulates *pqsA* by using an alternative transcriptional start site and induces expression of a longer transcript that forms a secondary structure in the 5' untranslated leader region. In consequence access of the ribosome to the Shine-Dalgarno sequence is restricted and translation efficiency is reduced (Brouwer et al. 2014). Under certain conditions PQS signaling can overcome the dependency on LasR and can

activate the expression of downstream QS factors, which seems to be mainly dependent on RhlR (Diggle et al. 2003, Dekimpe and Deziel 2009).

It is generally accepted that 4-hydroxy-2-heptylquinoline (HHQ), the precursor of PQS, is synthesized from anthranilate by *pqsABCD* (Gallagher et al. 2002). Furthermore, it was reported recently that also *pqsE* has a role in HHQ synthesis (Drees and Fetzner 2015). Anthranilate itself is produced by either the PhnAB or TrpEG synthases (Essar et al. 1990). PqsH (Gallagher et al. 2002 53) afterwards converts the HHQ to PQS. The cognate transcriptional regulating protein for HHQ and PQS is the LysR-type regulator PqsR (Wade et al. 2005), which autoregulates *pqsABCDE* (McGrath et al. 2004). Beside the recently discovered role in HHQ synthesis for *pqsE*, it was found before that it is necessary for pyocyanin and hydrogen cyanide production (Gallagher et al. 2002). PqsE induces the production of these virulence factors by enhancing RhlR activity in a HHQ and PQS signal independent manner (Farrow et al. 2008).

PQS exhibits iron-chelating activities. Consequently, when PQS is added to the growth medium PQS depletes iron from the medium so that the bacteria experience iron deprivation and react with an increased expression of the iron acquisition systems, PQS production and *rhlR* expression (Bredenbruch et al. 2006).

Recently, a fourth inter-cellular communication signal has been discovered to be capable of integrating environmental stress with the Quorum Sensing network (Lee et al. 2013). IQS belongs to a new class of QS molecules and its structure was identified as 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde. The genes that are involved in IQS synthesis belong to the non-ribosomal peptide synthase gene cluster *ambBCDE*. When IQS was disrupted, this resulted in a decreased production of PQS and C4-HSL signals, as well as in virulence factor concentration of pyocyanin, rhamnolipids and elastase. Under conditions of phosphate depletion stress, IQS is able to partially take over the functions of the central Las system (Lee et al. 2013, Lee and Zhang 2015).

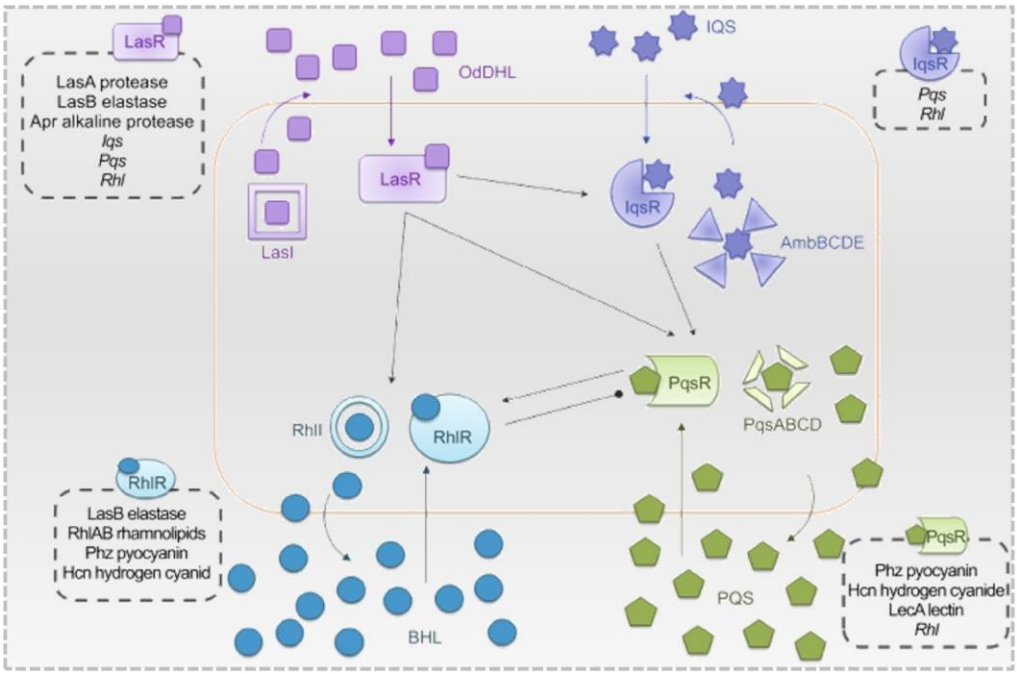

Figure 2: Schematic representation of the four QS signaling networks in *P. aeruginosa* and their respective regulons. Arrows indicate a stimulatory effect. Perpendicular lines indicate an inhibitory effect (modified from Lee and Zhang 2015).

P. aeruginosa is capable to detect signal molecules of other mircobacteria in its surrounding. Producing itself no AI-2, it can detect AI-2 and gene expression is altered upon its perception (Duan et al. 2003). In addition, DSF from *S. maltophilia* is able to influce the biofilm structure of *P. aeruginosa* in dual species biofilms. DSF is perceived by the sensor kinase PA1396 which input domain has a related amino acid sequence to the sensor domain of RpfC, the DSF sensor kinase of *S. maltophilia* (Ryan et al. 2008).

P. aeruginosa possesses two more LuxR homologues, which have no own signal synthase connected to them. QscR is able to interact and to form heterodimers with the complex compromised out of LasR or RhlR and their cognate AHL, preventing their binding to their target DNA and dampening the QS response (Ledgham et al. 2003), apart from binding the free 3-oxo-C12-HSL molecule and controlling its own regulon (Chugani et al. 2001, Fuqua 2006, Lee et al. 2006). VqsR (***v***irulence ***q***uorum ***s***ensing ***r***egulator) is a positive regulator of QS and is itself positively regulated by the Las-system (Li et al. 2007). VqsR mutants show reduced production of quorum sensing signals and virulence factors (Juhas et al. 2004) and as a first direct target QscR could be identified. VqsR negatively regulates expression of *qscR* by binding independently from 3-oxo-C12-HSL to its promotor (Liang et al. 2012).

The advantages of this regulation cascade are not fully resolved, but it is clear that genes on different levels can be regulated through this hierarchical system depending on

growth phase, cell density or environmental conditions (Venturi 2006). In total, over 600 genes seem to be controlled by QS in *P. aeruginosa* (Schuster et al. 2003, Hentzer et al. 2003, Wagner et al. 2003). A significant proportion of the regulated genes encode for secreted products, which is in agreement with findings from proteomics of extracellular protein regulated by QS (Nouwens et al. 2003).

1.3.1 Other QS related regulators

QS is a global regulatory system that controls many different cell functions, which are not only determined by *P. aeruginosa's* LuxR-like regulators. Other regulators involved in QS control and global regulation are Vfr, GacSA, RpoS, VqsM, MvaT among others (Venturi 2006).

Direct transcriptional regulators of QS include VqsM, AlgR and PmpR. VqsM (***v***irulence ***q***uorum ***s***ensing ***m***odulator) is reported to act activating upstream of VqsR, whereas AlgR only negatively regulates the promotors of *rhlI* and *rhlA*, as well as PmpR only negatively regulates *pqsR* (Dong et al. 2005, Morici et al. 2007, Liang et al. 2008).

Vfr (***v***irulence ***f***actor ***r***egulator) is a member of the cAMP receptor protein family and a homologue of *E. coli*'s CRP, which is involved in catabolite repression (Gosset et al. 2004). In *Pseudomonas* Vfr is not involved in catabolite regulation and seems to be located at the top of the QS cascade and activates transcription of *lasR*, which is indepent of cAMP and controls several virulence factors, like exotoxin A and the type III secretion system (Suh et al. 2002, Albus et al. 1997, Fuchs et al. 2010). In the regulation of Vfr itself RpoN seems to be involved (Heurlier et al. 2003). Moreover it was shown, that Vfr directly regulates *rhlR* transcription through its binding to several Vfr binding sites present in the *rhlR* promotor (Croda-Garcia et al. 2011).

The GacSA two-component system positively controls QS in *P. aeruginosa* and upregulates the production of C4-HSL, hydrogen cyanide (HCN), pyocyanin and elastase via the small RNAs RsmYZ (Pessi et al. 2001, Reimmann et al. 1997, Kay et al. 2006). GacA directly only controls the two genes *rsmY* and *rsmZ* and this finally results in only the modulation of RsmA activity, which is a translational regulator and prevents or facilitates translational initiation (Brencic and Lory 2009, Pessi et al. 2001, Heurlier et al. 2004). The small RNA RsmZ, but not RsmY, is co-regulated by MvaT and is repressed upon the biding of MvaT to its adjacent DNA region. This two-histone-like nucleoid structuring protein (H-NS-like) binds to regions of relatively high AT % and is often counteracted by the activities of specific transcriptional regulators (Brencic et al. 2009, Castang et al. 2008). For MvaT repression of pyocyanin, 3-oxo-C12-HSL and C4-HSL has been reported, but although it seems to repress QS some phenotypes like elastase, lasA

protease and swarming are induced (Diggle et al. 2002). It is also suggested, that MvaT plays an important role in the growth phase dependent regulation and is involved in the timing of QS activation. MvaT directly regulates *ptxS* which interferes with the enhancing effect of *ptxR* on *toxA,* encoding exotoxin A (Westfall et al. 2004, Colmer and Hamood 1998).

1.3.2 Quorum Sensing controls virulence in *P. aeruginosa*

P. aeruginosa expresses a broad spectrum of virulence factors that contribute to its pathogenicity and various infection models were used in a range of studies to demonstrate the importance of the QS systems during pathogenesis. It was shown for PQS that it is present in the lungs of *P. aeruginosa* infected CF patients (Collier et al. 2002) and its importance for virulence was demonstrated using *Caenorhabiditis elegans*, *Arabidopsis* and mice models (Cao et al. 2001, Gallagher and Manoil 2001). Accordingly, for the inhibition of AHL-dependent QS a reduction in virulence could be proven (Rumbaugh et al. 2000).

Multiple factors mediate virulence including secreted enzymes and toxins as well as the more complex type III and type VI secretion systems. QS also plays a possible role in biofilm formation. It was observed that when QS was disrupted in *P. aeruginosa* the formed biofilms were flat, undifferentiated and susceptible to SDS treatment, unlike the 3-dimensional, mushroom-like structured biofilm of the wild type (Davies et al. 1998). Several QS controlled factors are linked to biofilm formation. However, the role of QS in the activation of biofilm formation is still not fully resolved and seems to be also highly dependent on nutritional and hydrodynamic conditions. It was given evidence that QS is for example not involved in the formation of biofilms when bacteria are grown with glucose and glutamate as sole carbon source, or when grown under turbulent flow where QS molecules fail to accumulate (Purevdorj et al. 2002, Shrout et al. 2006).

For tissue invasion, several virulence fators are produced and secreted from the cells. Among these secreted factors are elastases, alkaline proteases, rhamnolipids, exotoxin A, exoenzym S, hemolysin and pyocyanin (Van Delden and Iglewski 1998).

Exotoxin A one of the most virulent proteins secreted by *P. aeruginosa* is an ADP-ribosyl transferase enzyme that catalyses the transfer of the ADP-ribosyl moiety of NAD^+ onto elongation factor 2 in eukaryotic cells leading to a cessation of protein synthesis and eventual cell death (Iglewski and Kabat 1975, Westfall et al. 2004).

Elastase is capable of degrading or inactivating important biologic tissues and immune system components, including immunoglobulin, collagen, fibrin, and elastin, causing tissue damage and invasion as well as immunomodulation (Rust et al. 1996, Cathcart et

al. 2011). Together with alkaline proteases the inactivation of interferon gamma is reported (Horvat et al. 1989).

Rhamnolipids are glycolipids and required for a form of surface motility called swarming, where it functions as a wetting agent and reduces surface tension (Caiazza et al. 2005, Deziel et al. 2003). It is also suggested that in the early stages of infection of human airway epithelium, the incorporation of rhamnolipids within the host cell membrane leads to tight-junction alterations (Zulianello et al. 2006).

Pyocyanin (1-hydroxy-N-methylphenazine) is a redox-active, blue-green phenazine produced by *P. aeruginosa*. Another yellow-green fluorescent pigment is the siderophore pyoverdin. These two pigments are responsible for the green color of *P aeruginosa* cultures. Because of its reductive properties, pyocyanin causes oxidative stress in human cells, especially in human lung epithelial cells and leads to the production and accumulation of reactive oxygen species, such as superoxide and hydrogen peroxide, by inhibiting catalase activity (O'Malley et al. 2003). It has also antibacterial activity by interacting with the electron transport chain, depleting oxygen supply and negatively influencing the active transport mechanisms (Jayaseelan et al. 2014), but little is known on how *Pseudomonas* itself withstands pyocyanin (Hassett et al. 1992). In addition, pyocyanin is a terminal signaling factor in the QS network, activated in stationary phase and modulating the expression of genes involed in transport, redox control and iron acquisition (Dietrich et al. 2006).

1.4 Inhibition of QS as therapeuthic target

Based on the facts that QS is of great importance for the virulence of *P. aeruginosa* and after it was proven that the deletion of QS regulators leads to significant attenuation of this virulence, QS was soon proposed to be an unique and ideal target for novel antimicrobial drugs and the inhibition of *Pseudomonas* infections. Taking into account that multidrug resistant *Pseudomonas* strains are becoming more prevalent and current treatments are not able to eradicate *Pseudomonas* from CF infections, therapeutics which target alternative mechanisms and attenuate virulence could assist the host immune response in clearing the infection (Smith and Iglewski 2003, Hoiby 2011). The most promising target would be the development of AHL analogues that act as antagonists for 3-oxo-C12-HSL and C4-HSL, but also increased efforts on the research on enzymes degrading or modifying the QS signals were made. On the other hand it was already shown, that for one of the best characterized antagonist compound, brominated furanone C-30, rapidly bacterial resistance developed (Maeda et al. 2011). In a broad range of studies lactonases, acylases and oxidoreductases were identified, which activities were shown

to cleave the autoinducers and in consequence reduced the virulence of *Pseudomonas*. This enormous number of nearly 50 Quorum Quenching enzymes has been extensively reviewed in Chen et al. 2013 and Fetzner 2015. Specifically BpiB09 has to be mentioned. BpiB09 is one of the rare Quorum Quenching enzymes from the group of oxidoreductases, for which up date only four members have been reported (Fetzner 2015). Its expression in *P. aeruginosa* resulted in significantly reduced pyocyanin production, decreased motility, poor biofilm formation and absent paralysis of *Caenorhabditis elegans* (Bijtenhoorn et al. 2011). On the other hand, while measuring the reducing activity of BpiB09 on the autoinducer molecule 3-oxo-C12-HSL by HPLC only limited turnover was observed. We suggested that AHLs are probably not the native substrate of this enzyme and proposed an alternative activity on the synthesis pathway of homoserine lactones, the intermediates of the fatty acid cycle.

1.5 RNA-seq

Massive parallel cDNA sequencing or RNA-seq is a method for transcriptome profiling which uses deep-sequencing technologies. The transcriptome is the complete set of transcripts present in a cell for specific growth stage or physiological condition. Understanding the transcriptome is essential for interpreting the functional elements of the genome, the molecular constituents of cells and understanding diseases. RNA-seq also provides a far more precise measurement of levels of transcripts than other methods and overcomes the limited dynamic range of detection of the microarray technologies as well as the difficulties with cross experiment comparability (Wang et al. 2009). RNA is converted to a library of cDNA fragments and sequenced by high throughput sequencing methods like 454, Illumina or SOLID systems to obtain up to millions of short sequencing reads. Reads are mapped to a reference genome or transcriptome or can be assembled *de novo.* RNA-seq has low background signal and no upper limit for quantification and has in consequence a large dynamic range of expression levels it can detect. For *Saccharomyces cerevisiae* a range of more than 9000-fold was estimated (Nagalakshmi et al. 2008). Moreover, high accuracy as well as high reproducibility have been reported (Mortazavi et al. 2008, Cloonan et al. 2008). With RNA-seq several novel approaches to characterize the genome and transcriptome are now possible and applications like transcriptional start site mapping, strand specific RNA-seq, discovery of splicing patterns or small RNA profiling are reviewed in Ozsolak and Milos 2011.

1.6 Intention of this work

The aim of this work was to elucidate the underlying mechanism of the strong Quorum Quenching activity of BpiB09, despite only observing limited turnover of 3-oxo-C12-HSL. For this purpose the rather new technology of RNA-seq was expected to be established and employed to study the global transcriptome of the BpiB09 overproducing *P. aeruginosa* PAO1. The impact of BpiB09 on the regulatory network is intended to be characterized on the single gene level and promising key genes and mechanisms are supposed to be further studied and analyzed. If necessary, proceeding RNA-seq experiments are planned to be employed on key genes to deepen the knowledge on the regulatory network of *P. aeruginosa*.

2 Material & Methods

2.1 Bacterial strains, vectors, constructs and oligonucleotides

Bacterial strains, vectors, constructs and oligonucleotides used in this work are specified in Table 1, Table 2, Table 3, Table 4 and Table 5. Unless otherwise indicated in the respective sections, constructs were verified by restriction analysis and Taq polymerase was used for PCRs.

Table 1: Bacterial strains, vectors and constructs used in this study

Strain/Plasmid	Description/Genotype	Source/Reference
Strains		
E. coli		
DH5α	F-, Φ80*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *recA1*, *endA1*, *hsdR17*(rk$^-$, mk$^+$), *phoA*, *supE44*, *thi-1*, *gyrA96*, *relA1*, λ-	Invitrogen (Karlsruhe, Germany)
MT102	Sm^R, F-, *thi*, *araD139*, *ara-leuΔ7679*, Δ(*lacIOPZY*), *galU*, *galK*, r$^-$, m$^+$	T. Hansen, Novo Nordisk A/S (Bagsvaerd, Denmark)
ST18	*E. coli* S17 λ*pir*, Δ*hemA*	Thoma and Schobert 2009
Rosetta-gami 2(DE3)	Tc^R, Sm^R, Cm^R, expression strain	Novagen®, Merck (Darmstadt, Germany)
BL21 (DE3)	Expression strain	Studier and Moffatt 1986
P. aeruginosa		
PAO1	Parent strain	Holloway 1955
PBBR	PAO1 containing pBBR1MCS-5	This study
PBBRc	PAO1 containing pBBR::*celA*	Schipper et al. 2009
ΔlasI	*lasI* deletion mutant of PAO1	This study
ΔrhlI	*rhlI* deletion mutant of PAO1	This study
ΔlasI/ΔrhlI	*lasI* deletion mutant of ΔrhlI	This study
ΔPA2226	*PA2226* deletion mutant of PAO1	This study
ΔlasI-C	ΔlasI containing pBBR::*lasI*	This study
ΔrhlI-C	ΔrhlI containing pBBR::*rhlI*	This study
PA2226	PAO1 containing pBBR1MCS-5::*PA2226*	This study
VqsM	PAO1 containing pBBR1MCS-5::*vqsM*	This study

Strain/Plasmid	Description/Genotype	Source/Reference
A. tumefaciens		
NTL4 *(pCF218) (pCF372)*	Sp^R, Tc^R, AHL reporter strain, p*traI::lacZ*, carrying pCF218 and pCF372	Fuqua and Winans 1994, Fuqua and Winans 1996, Luo et al. 2001
Plasmids		
pBluescript II SK (+)	*E. coli* cloning vector, Ap^R	Agilent Technologies (Santa Clara, CA, USA)
pDrive	*E. coli* TA-cloning vector, Ap^R	Quiagen (Hilden, Germany)
pMAL-c2X	Ap^R, Maltose-binding protein fusion vector	New England Biolabs (Ipswich, MA, USA)
pET-28a	Km^R, expression vector	Novagen®, Merck (Darmstadt, Germany)
pBBR1MCS-5	Gm^R, broad-host-range (bhr)	Kovach et al. 1995
pSRKGm	Gm^R, pBBR1MCS-5 derived bhr expression vector, *lacI*	Khan et al. 2008
pEX18Ap	Ap^R, *oriT*$^+$, *sacB*$^+$, gene replacement vector with MCS from pUC18	Hoang et al. 1998
pPS858	Ap^R, Gm^R, source of Gm^R-GFP FRT cassette	Hoang et al. 1998
pFLP2	Ap^R, bhr, Flp recombinase	Hoang et al. 1998
pUCP20T	Ap^R; mobilizable bhr cloning vector	West et al. 1994
pHERD20T	pUCP20T with *lacZp* replaced by *araC-badp*	Qiu et al. 2008
pMAL-c2X::*PA2226*	pMAL-c2X containing in frame *PA2226*	This study
pET28a::*lasR*	Km^R, *lasR* gene in pET-28a	Liu et al. 2009
pHERD20T::*lasR*	pHERD20T containing *lasR*	This study
pKR-C12	Gm^R, pBBR1MCS-5 carrying *lasBp-gfp*(ASV), *lacZp-lasR*	Riedel et al. 2001
pSK::*PA2226*	pBluescript II SK (+) containing PCR fragment from the primers PA2226_xhoI_fw / PA2226_hindIII_rv in EcoRV site	This study
pKR-C12Δ*lasR*	Gm^R, pKR-C12 with deletion of *lasR* by BamHI	This study
pUCP20T::*PA2226*	pUCP20T containing XbaI/HindIII fragment from pSK::*PA2226*	This study
pBBR::*PA2226*	pBBR1MCS-5 containing *PA2226*	This study
pBBR::*vqsM*	pBBR1MCS-5 containing *vqsM*	This study
pBBR::*PA2222*	pBBR1MCS-5 containing *PA2222*	This study
pBBR::*PA2223*	pBBR1MCS-5 containing *PA2223*	This study
pBBR::*PA2224*	pBBR1MCS-5 containing *PA2224*	This study

Strain/Plasmid	Description/Genotype	Source/Reference
pBBR::*PA2225*	pBBR1MCS-5 containing *PA2225*	This study
pBBR::*PA2228*	pBBR1MCS-5 containing *PA2228*	This study
pSRKGm::*PA2226*	pSRKGm containing XbaI/HindIII fragment from pSK::*PA2226*	This study
pBBR::*malE-PA2226*	pBBR1MCS-5 containing *malE-PA2226* fusion from pMAL-c2X::*PA2226*	This study
pBBR::*lasR*	pBBR1MCS-5 containing *lasR*	This study
pBBR::*lasI*	pBBR1MCS-5 containing *lasI*	This study
pBBR::*rhlI*	pBBR1MCS-5 containing *rhlI*	This study
pBBR::*celA*	pBBR1MCS-5 containing 2 kb cellulase gene, experimental control	Schipper et al. 2009
pBBR::*bpiB09*	pBBR1MCS-5 containing *bpiB09*	Bijtenhoorn et al. 2011
pSK::H1_*lasI*	pBluescript II SK (+) with *lasI* 5' homologous fragment in KpnI/HindIII sites	This study
pSK::Δ*lasI*	pSK::H1_*lasI* with *lasI* 3' homologous fragment in PstI/BamHI sites	This study
pSK::Δ*lasI*_gm	pSK::Δ*las* with Gm^R/GFP-cassette from pSP858 in EcoRV site	This study
pSK::H1_*rhlI*	pBluescript II SK (+) with *rhlI* 5' homologous fragment in KpnI/HindIII sites	This study
pSK::Δ*rhlI*	pSK::H1_*rhlI* with *rhlI* 3' homologous fragment in PstI/BamHI sites	This study
pSK::Δ*rhlI*_gm	pSK::Δ*rhlI* with Gm^R/GFP-cassette from pSP858 in EcoRV site	This study
pEX18Ap::Δ*lasI*_gm,	pEX18Ap containing KpnI/BamHI fragment from pSK::Δ*lasI*_gm	This study
pEX18Ap::Δ*rhlI*_gm	pEX18Ap containing KpnI/BamHI fragment from pSK::Δ*rhlI*_gm	This study
pEX18Ap::Δ*PA2226*_gm	pEX18Ap containing *PA2226* 5' homologous fragment in XmaI/BamHI site, 3' homologous fragment in BamHI/XbaI site and BamHI fragment from pSP858	This study

Table 2: Primers used for cloning and construct validation

Name	Sequence 5' – 3'[a]	Annealing temperature [°C]	Product length [bp]
M13-20	GTAAAACGACGGCCAGT	55	-[b]
M13 rev	CAGGAAACAGCTATGACC		
PA2222_xhoI_fw	CTCGAGCGATGGCCGAGATCAATA	59	665
PA2222_hindIII_rv	AAGCTTACACTAGATCGCTGCAAA		
PA2223_xhoI_fw	CTCGAGAAGCAGAGGAGACTAAGG	55	1058
PA2223_hindIII_rv	AAGCTTTCGCCACCTCTAGAGTC		
PA2225_xhoI_fw	CTCGAGGGAAGAGCGTTGAGGA	55	446
PA2225_hindIII_rv	AAGCTTAACGCTGCTCACCTGA		
PA2226_xhoI_fw	CTCGAGTGGGCAAGGAGCATCT	55	573
PA2226_hindIII_rv	AAGCTTCGCTCTTCCTCCTTGAAA		
PA2224_xhoI_fw	CTCGAGTCATGCAGGGCATAAAC	57	778
PA2224_hindIII_rv	AAGCTTTGCTTAGTGAAGCCAATT		
PA2227_xhoI_fw	CTCGAGAGGGGTGAGGTGAATAT	57	1035
PA2227_hindIII_rv	AAGCTTTTGCCCAATCTCAAACT		
PA2228_xhoI_fw	CTCGAGAGAGATCGACAAGAATGA	57	1239
PA2228_hindIII_rv	AAGCTTTCACCTCACCCCTACT		
PA2226_pmal_ecoRI_fw	GGGGAATTCATGAATTTCAA-TAATCGTA	55	512
PA2226_pmal_hindIII_rv	CCCAA-GCTTTATCTAAAAATGACTCTTG		
lasR_xbaI_fw	TCTAGAAAGAAGAACGTAGCGC-TATG	67	759
lasR_hindIII_rv	AAGCTTAGAGGCAAGATCAGA-GAGTA		
PA2226mbp_xhoI_fv	CTCGAGTTCACCAACAAGGACCATA	65	1712
PA2226mbp_hindIII_rv	AAGCTTTATCTAAAAATGACTCTTG		
lasI_hindIII_fw	AAGCTTCGGGTTCACCGAAATC	57	752
lasI_bamHI_rv	GGATCCTCCCTCTCCGACAGAGAAC		
rhlI_hindIII_fw	AAGCTTGTGCGCGAAACGG	57	768
rhlI_bamHI_rv	GGATCCCGTTCATCCTCCTTTAGTC		
PAO1_19kbdel_fw	CACCTCACCCCTACTGTTC	53	861[c]
PAO1_19kbdel_rv	AGCACGTCACAGGTTAGC		
PslL_fw	AGCGGGAGTTGCAAAGTG	53	1316[d]

[a] Restriction site is underlined.

[b] Depending on fragment size.

[c] With 19 kb deletion, no product in the wild type.

[d] Wild type, no product with 19 kb deletion.

Table 3: Primers used for deletion mutant generation

Name	Sequence 5' – 3'[a]	Annealing temperature [°C]	Product length [bp]
5' homologue fragment of *lasI*			
A_kpnI_lasI_fw	GGTACCTGCTGAAGCCAATGTG	59	512
B_hindIII_lasI_rv	AAGCTTCGCCGACCAATTTGTACG		
3' homologue fragment of *lasI*			
C_pstI_lasI_fw	CTGCAGGACTGGCGGTTTCATGAC	59	389
D_bamHI_lasI_rv	GGATCCTGATCGGCAACCTTACCC		
5' homologue fragment of *rhlI*			
A_kpnI_rhlI_fw	GGTACCCGCTTCTTCCGCTACAGAG	59	571
B_hindIII_rhlI_rv	AAGCTTGGTCGTCAGCCGTTTC		
3' homologue fragment of *rhlI*			
C_pstI_rhlI_fw	CTGCAGTCAGTCGGAGGACATACC	59	470
D_bamHI_rhlI_rv	GGATCCGCCGAACAATTTGCTCAG		
5' homologue fragment of *PA2226*			
A_xmaI_PA2226_fw	CCCGGGAGACGTCATTGAGCATGAG	57	562
B_bamHI_PA2226_rv	GGATCCTTGGCTTAGACGTCAAGAG		
3' homologue fragment of *PA2226*			
C_bamHI_PA2226_fw	GGATCCATGCTCCTTGCCCAATCTC	59	622
D_xbaI_PA2226_rv	TCTAGAGCCTCTGCAACCTACAAG		
Gm^r/GFP-cassette			
pPS858_FRT_fw	GAGCTCGAATTGGGGATC	50	ca. 1,800
pPS858_FRT_rv	GAGCTCGAATTAGCTTCA		

[a] Restriction site is underlined.

Table 4: Primers used for deletion mutant validation

Name	Sequence 5' – 3'	Annealing temperature [°C]	Product length [bp]
Outside the homologous recombination fragments of *lasI*			
A_1_lasI_fw	CGAGCTGGCGATCGGTAATTTG	59	*2613*[a]
D_1_lasI_rv	TGGTCAGCCATCCGCAATTC		**2201**[b]
Outside the homologous recombination fragments of *rhlI*			
A_1_rhlI_fw	GCGCCAACGAAGAAATAACCG	59	*2141*
D_1_rhlI_rv	CCACGACCAGTTCGACAATG		**1618**
Outside the homologous recombination fragments of *PA2226*			
A_1_PA2226_fw	GTGTCCAGTGCTTCGTATC	59	*2669*
D_1_PA2226_rv	CGTCCTGGACGTTACATAG		4130[c]
Within *lasI*			
X_lasI_fw	AAACTGCTGGGCGAGATG	54	*527*
Y_lasI_rv	TAAAGCGCGATCTGGGTC		ND[d]
Within *rhlI*			
X_rhlI_fw	GTCTCGCCCTTGACCTTCTG	59	*433*
Y_rhlI_rv	ACCGGCATCAGGTCTTCATC		ND
Within *PA2226*			
X_PA2226_fw	GACAAGTCGCTCGATTACTC	57	*371*
Y_PA2226_rv	TGATTGCCGAGACTCAGTTC		ND
5' – 3' ends of homologous sites *lasI*			
A_kpnI_lasI_fw	GGTACCTGCTGAAGCCAATGTG	59	2833
D_bamHI_lasI_rv	GGATCCTGATCGGCAACCTTACCC		*1455*
			1043
5' – 3' ends of homologous sites *rhlI*			
A_kpnI_rhlI_fw	GGTACCCGCTTCTTCCGCTACAGAG	59	2973
D_bamHI_rhlI_rv	GGATCCGCCGAACAATTTGCTCAG		*1706*
			1183
5' – 3' ends of homologous sites *PA2226*			
A_xmaI_PA2226_fw	CCCGGGAGACGTCATTGAGCATGAG	54	3114
D_xbaI_PA2226_rv	TCTAGAGCCTCTGCAACCTACAAG		*1647*

[a] Italic: product length of the wild type.

[b] Bold: product length with deleted gene.

[c] Underlined: product length with integrated Gm^R/GFP-cassette and deleted gene.

[d] Not detectable (ND) in mutants.

Table 5: Oligonucleotides used for EMSA DNA probe assembly

Name	Sequence 5' – 3'[a]	5' Modification
plasb_op1_cy5	AATCAAGGCTACCTGCCAGTTCTGGCAGGTTT-GGCCGCGG	Cy5
plasb_op1_cpl	CCGCGGCCAAACCTGCCAGAACTGGCAGGTAGCCTT-GATT	

2.2 Culture conditions

If not otherwise indicated, media were autoclaved and supplemented afterwards with antibiotics and/or other reagents (Table 6) at appropriate temperatures, if necessary.

Table 6: Antibiotics and supplements

Reagent	Solvent[a]	Final conc.[b] [µg/ml]		
		E. coli	*P. aeruginosa*	*A. tumefaciens*
Ampicillin	EtOH (70 %)	100		
Carbeniccelin	EtOH (70 %)	100	250	
Chloramphenicol	EtOH	32		
Spectinomycin	H_2O			50
Tetracycline	EtOH			4.5
Gentamycin	H_2O	10	50	
5-Aminolevulinic acid	H_2O	50		
IPTG	H_2O	100		
X-Gal	DMF	40		

[a] Stocks with H_2O were filtered sterile (0.22 µm membrane filter); all stocks were stored at -20 °C.

[b] Stock concentration ×1000.

2.2.1 Cultivating *E. coli* and *P. aeruginosa*

Cultures were grown in lysogeny broth (LB) medium (Bertani 1951) overnight at 37 °C and 200 rpm. Plasmid containing strains were grown with appropriate antibiotics (Table 1 and Table 6).

2.2.2 Cultivating *A. tumefaciens* NTL4 (pCF218) (pCF372)

Precultures were grown overnight in LB medium from glycerol stocks at 28 °C. The next day, AT minimal glucose medium (Tempe et al. 1977) containing 0.5 % (wt/vol) glucose,

0.079 M KH_2PO_4, 0.015 M $(NH_4)_2SO_4$, 0.6 mM $MgSO_4 \cdot 7H_2O$, 0.06 mM $CaCl_2 \cdot 2H_2O$, 0.027 mM $FeSO_4 \cdot 7H_2O$, and 0.0071 mM $MnSO_4 \cdot H_2O$ in H_2O, pH 7, was inoculated with 1-5 % of the preculture and grown overnight at 28 °C and 150 rpm with addition of 50 µg/ml spectinomycin and 4.5 µg/ml tetracycline.

2.3 Transcriptome of PAO1 pBBR::*bpiB09*

In order to analyze the transcriptome at a defined growth phase the growth of PAO1 pBBR::*bpiB09* and PAO1 pBBR1MCS-5 (PBBR; PAO1 transformed with pBBR1MCS-5 as described in 2.4.5) was measured over time. Overnight cultures were used to inoculate 100 ml LB with a starting optical density (OD_{600}) of 0.001. Cultures were grown at 37 °C and 200 rpm. Aliquots of 1 ml were removed every hour for 15 h from the cultures and the OD_{600} was measured. Experiments were run in duplicates three times. After determination of exponential-, transient- and stationary growth phase, cells were grown 5 h for transcriptome analysis. Cultures were immediately chilled on ice, centrifuged for 5 min at 10,000 g and stored at −80 °C. RNA extraction, rRNA depletion, cDNA synthesis, 454-shotgun library construction and 454 sequencing was performed by the Göttingen Genomics Laboratory as described in detail in Bijtenhoorn et al. 2011 for the 5 h sample. Total reads of all samples were mapped on the reference genome sequence of PAO1 extended by the *bpiB09* sequence (ORF3 of GenBank entry EF530730.1) and were evaluated with respect to the hits per gene, proportionately to the gene length. Values from the two parallels were averaged and normalized by total read counts. A magnitude of change ≥10fold was considered significant.

2.3.1 Genomic sequence analysis of PAO1 pBBR::*bpiB09*

Taking advantage of the 454 sequencing, these acquired long reads were used to confirm the genomic integrity of PAO1 pBBR::*bpiB09* and to investigate diverging phenotypic testing results from PAO1 pBBR::*bpiB09* retransformants (data not shown) to the original strain. Reads from parallel experiments were combined and the GS Reference Mapper 2.7 (Roche, Basel, Switzerland) with default settings was used for remapping onto the genome of PAO1 (GenBank accession no.: AE004091) and following analysis. A major 19 kb chromosomal deletion of PAO1 pBBR::*bpiB09* was further analyzed and verified by colony PCR using Taq polymerase and the primer pairs PAO1_19kbdel_fw / PAO1_19kbdel_rv, targeting outside the deletion, and PslL_fw / PAO1_19kbdel_rv, with PslL_fw targeting inside the deletion (Table 2), with following conditions: 95 °C for 30 s, 53 °C for 30 s and 72 °C for 1:30 min (30 cycles). PAO1 was used in a control experiment.

2.4 Deletion mutant generation in PAO1

The main workflow for deletion mutant generation is described in Hoang et al. 1998 and is specified subsequently in detail for the different deletion mutants. A schematic overview is shown in Figure 4.

2.4.1 Generation of the suicide constructs for ΔlasI and ΔrhlI

For validation of Quorum Quenching phenotypes, autoinducer synthase single and double deletion mutants of PAO1 were generated. Suicide construct workflow is demonstrated schematically in Figure 3 (I, III). 5' and 3' homologous regions of *lasI* and *rhlI* were PCR-amplified by Pfu polymerase from PAO1 genomic DNA. Genomic DNA from PAO1 was isolated by using the peqGOLD Bacterial DNA Kit (VWR, Erlangen, Germany) according to the manufacturer's protocol. For *lasI* the primer pairs A_kpnI_lasI_fw / B_hindIII_lasI_rv for the 5' and C_pstI_lasI_fw / D_bamHI_lasI_rv for the 3' region were used. For *rhlI* the primer pairs A_kpnI_rhlI_fw / B_hindIII_rhlI_rv for the 5' and C_pstI_rhlI_fw / D_bamHI_rhlI_rv for the 3' region were used (Table 3). The PCR conditions were: 95 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min (30 cycles). Purified DNA fragments were cloned in pBluescript II SK (+) (pSK) and were transformed in competent *E. coli* DH5α by heat shock. Positive clones containing plasmids with inserts were selected by blue-white screening. 5' and 3' homologous fragment pairs were subsequently subcloned into the same pBluescript II SK (+) (for details see Table 1; pSK::H1_*lasI*, pSK::Δ*lasI*; pSK::H1_*rhlI*, pSK::Δ*rhlI*) by using the restriction sites indicated in the primer names. Insertion of the second fragment was checked via restriction analysis. For selection purposes a Gm^R/GFP (***g***reen ***f***luorescent ***p***rotein)-cassette, flanked by FRT-sites was inserted between the 5' and 3' homologous fragments. The Gm^R/GFP-cassette was PCR-amplified with Pfu polymerase by using the primer pair pPS858_FRT_fw / pPS858_FRT_rv (Table 3) and using pPS858 as template with the following conditions: 95 °C for 30 s, 50 °C for 30 s and 72 °C for 4 min (30 cycles). The purified DNA fragment was cloned in the EcoRV-site between the 5' and 3' homologous fragments in pBluescript II SK (+) (pSK::Δ*lasI*_gm, pSK::Δ*rhlI*_gm) and was selected on LB medium with 10 µg/ml gentamycin (Gm). The KpnI/BamHI-fragment containing the 5' and 3' homologous sites as well as the Gm^R/GFP-cassette was subcloned in pEX18Ap, transformed in competent *E. coli* DH5α by heat shock and selected on LB medium with 10 µg/ml Gm and by green fluorescence, yielding pEX18Ap::ΔlasI_gm and pEX18Ap::ΔrhlI_gm (Table 1).

2.4.2 Generation of the suicide construct for ∆2226

For further exploration of *PA2226* (see 2.6 following) a gene deletion mutant in PAO1 was established. Suicide construct workflow is demonstrated schematically in Figure 3 (II, III). 5' homologous region of *PA2226* was PCR-amplified from PAO1 genomic DNA by using the primer pairs A_xmaI_PA2226_fw / B_bamHI_PA2226_rv (Table 3), the conditions were: 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s (30 cycles). For the 3' region Pfu polymerase and the primer pair C_bamHI_PA2226_fw / D_xbaI_PA2226_rv with following parameters were used: 95 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min (30 cycles). The 5' fragment was cloned in pDrive, the 3' fragment in pBluescript II SK (+) and were transformed in *E. coli* DH5α by heat shock and selected by blue-white screening. Constructs were sequenced utilizing primers M13-20 and M13_rev. The 5' and 3' fragments were excised from plasmids by the restriction sites indicated in the amplification primers. The Gm^r/GFP-cassette was excised from pPS858 by BamHI. The three fragments were cloned in a single step into pEX18Ap restricted with XbaI and XmaI. After transformation in *E. coli* DH5α by heat shock and selection on LB medium with 10 µg/ml Gm the construct pEX18Ap::∆PA2226_gm was verified by BamHI restriction analysis.

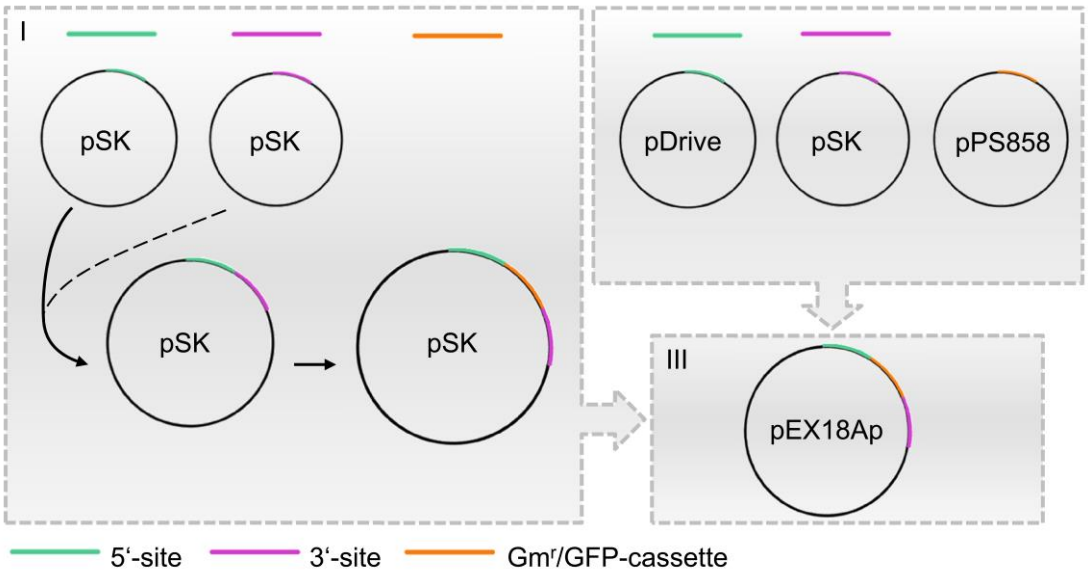

Figure 3: Suicide vector construction. PCR products (colored lines) were cloned in pBluescript II SK (+) (pSK) or pDrive and the suicide constructs for ∆*las*I and ∆*rhlI* were built in succeeding steps (I, III, 2.4.1) or were constructed in a single step (II, III, 2.4.2), as for ∆*PA2226*. Vector or backbone names are depicted within the schematic plasmid representations. Illustrations shown do not correspond in every case to the original size.

2.4.3 Homologous recombination and mutant selection

Electrocompetent *E. coli* ST18 (ST18), auxotrophic for 5-aminolevulinic acid (ALA), cells were transformed by electroporation with the suicide constructs pEX18Ap::ΔlasI_gm, pEX18Ap::ΔrhlI_gm and pEX18Ap::ΔPA2226_gm, respectively. Transformants were selected on LB medium containing 50 µg/ml ALA, 100 µg/ml carbenicillin (Carb) and 10 µg/ml Gm. The strains ST18 pEX18Ap::ΔlasI_gm, ST18 pEX18Ap::ΔrhlI_gm and ST18 pEX18Ap::ΔPA2226_gm were used in a biparental mating with PAO1. 100 µl of a PAO1 overnight culture were mixed with 1 ml overnight culture of the respective ST18 strain and centrifuged 1 min at 13,000 g. The supernatant was discarded and the pellet resuspended in 100 µl fresh LB medium and 1 µl ALA (50 mg/ml). 50 µl drops were placed on LB medium plates and were incubated 6 h at 37 °C after drying for 1 h. Cells were resuspended from the plates in 1 ml LB medium and merodiploids were selected on LB medium containing 250 µg/ml Carb and 50 µg/ml Gm, but no ALA for counterselection of ST18 cells. Double-crossover events were selected on LB medium plates containing 80 µg/ml Gm and 5 % (w/v) sucrose and clones were checked subsequently for Carb sensitivity. Mutants were additionally checked by colony PCR using the primer pairs A_kpnI_lasI_fw / D_bamHI_lasI_rv for Δ*lasI* (Figure 4, II) and A_kpnI_rhlI_fw / D_bamHI_rhlI_rv for Δ*rhlI*, yielding product sizes of 2833 bp and 2973 bp. The conditions were: 95 °C for 30 s, 59 °C for 30 s and 72 °C for 3 min (30 cycles). For Δ*PA2226* and a product size of 3114 bp verification primers A_xmaI_PA2226_fw / D_XbaI_PA2226_rv and following conditions were used: 95 °C for 30 s, 54 °C for 30 s and 72 °C for 3 min (30 cycles) (Table 4).

2.4.3.1 Gm^R/GFP-cassette excision from deletion mutants

In order to excise the Gm^R/GFP-cassette from deletion mutants ST18 was transformed by electroporation with pFLP2. Biparental mating of ST18 pFLP2 and the respective mutant was applied as described in 2.4.3 and clones were selected by 250 µg/ml Carb. For pFLP2 counterselection cells were grown on LB medium plates with 5 % (w/v) sucrose. Carb and Gm sensitive, but $sucrose^R$ cells yielded ΔlasI, ΔrhlI and Δ2226.

2.4.3.2 Generation of ΔlasI/rhlI

Generation of ΔlasI/rhlI was carried out as described in 2.4.3 and 2.4.3.1, but using ST18 pEX18Ap::ΔlasI_gm and ΔrhlI in the biparental mating.

2.4.4 Deletion mutant verification

For reliable verification of the deletion mutations of *lasI*, *rhlI* and *PA2226* several PCRs were performed (Figure 4, Table 4).

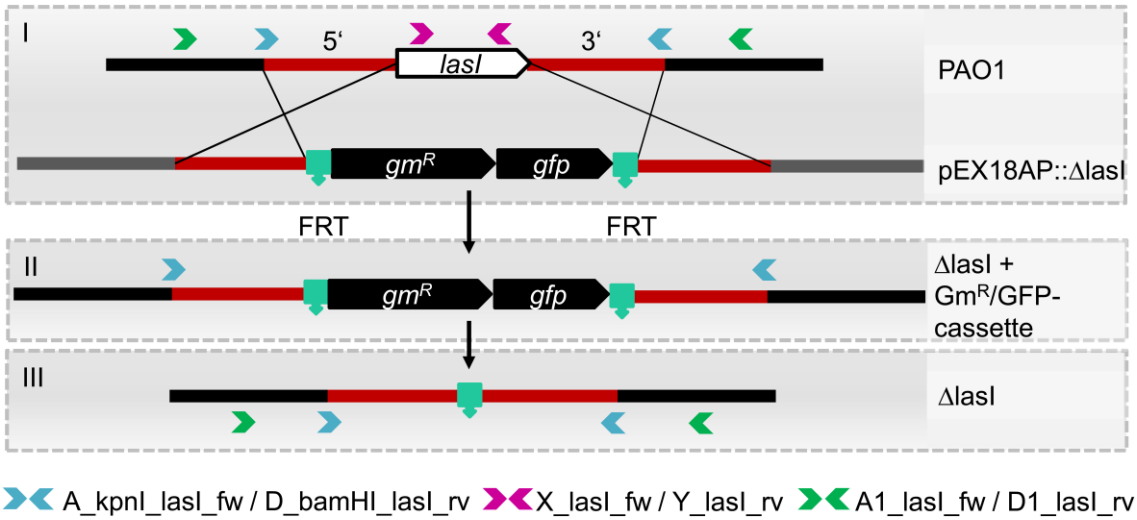

Figure 4: Mutant generation and validation illustrated by the example of ΔlasI. Mutations were introduced via homologous recombination (I) and selection for double-crossover events (II). Gm^r/GFP-cassette was excised by a flipase targeting the FRT-sites (***f***lipase ***r***ecognition ***t***arget) (III). Primer pairs for validation PCRs are indicated by colored arrows. Illustrations shown do not correspond in every case to the original size.

Genomic DNA was isolated from ΔlasI, ΔrhlI, ΔlasI/rhlI and Δ2226gm (Δ2226 still carrying the Gm^r/GFP-cassette) (2.4.1). PAO1 genomic DNA (2.4.1) was used in control experiments. Expected PCR product lengths for the different primer pair and strain combinations are listed in Table 4. At first, primer pairs A_kpnI_lasI_fw / D_bamHI_lasI_rv for ΔlasI and ΔlasI/rhlI, as the double mutant was generated from a verified ΔrhlI strain, A_kpnI_rhlI_fw / D_bamHI_rhlI_rv for ΔrhlI and A_xmaI_PA2226_fw / D_XbaI_PA2226_rv for Δ2226 from the homologues recombination cassette construction were used. PCR conditions were: 95 °C for 30 s, 59 °C for 30 s and 72 °C for 3 min (30 cycles) for ΔlasI, ΔlasI/rhlI and ΔrhlI. For Δ2226 the conditions were: 95 °C for 30 s, 54 °C for 30 s and 72 °C for 3 min (30 cycles). Deletion mutants with excised Gm^R/GFP-cassette carry additional 65 bp to what would be expected. This fragment, encoding for a FRT-site (***f***lipase ***r***ecognition ***t***arget), is left over after Gm^R/GFP-cassette excision (Hoang et al. 1998) and is included in the product lengths in Table 4. The second set of PCRs targeted the respective gene itself, checking for total genomic loss of the gene, leading to an amplification product in the wild type and to no product in the deletion mutants. The primer pairs X_lasI_fw / Y_lasI_rv for ΔlasI and ΔlasI/rhlI, X_rhlI_fw / Y_rhlI_rv for ΔrhlI and X_PA2226_fw / Y_PA2226_rv for Δ2226 were used with following conditions: 95 °C for 30 s, 54 °C (ΔlasI and ΔlasI/rhlI), 59 °C (ΔrhlI) or 57 °C (Δ2226) for 30 s and 72 °C for 30 s (30 cycles). For the last verification step primer pairs targeting sites outside the homologous recombination cassette, checking for recombination at the accurate genomic location, were used.

A1_lasI_fw / D1_lasI_rv for ΔlasI and ΔlasI/rhlI, A1_rhlI_fw / D1_rhlI_rv for ΔrhlI and A1_PA2226_fw / D1_PA2226_rv for ΔPA2226 were used with following conditions: 95 °C for 30 s, 59 °C for 30 s and 72 °C for 3 min (ΔlasI and ΔlasI/rhlI), 2 min (ΔrhlI) or 4 min (Δ2226) (30 cycles).

2.4.5 Complementation of deletion mutants

The ΔlasI and ΔrhlI deletion mutants were complemented with wild type gene copies to show the recovery of wild type phenotypes in the mutants. DNA fragments containing *lasI* and *rhlI* were PCR-amplified with Pfu polymerase from PAO1 genomic DNA. The fragment containing *lasI* was amplified using the primer pair lasI_hindIII_fw / lasI_bamHI_rv, the fragment containing *rhlI* by using the primer pair rhlI_hindIII_fw / rhlI_BamHI_rv with following conditions: 95 °C for 30 s, 57 °C for 30 s and 72 °C for 90 s. DNA fragments were cloned in pBBR1MCS-5 (Table 1) by using the restriction sites indicated in the primer names and were transformed in the respective chemically competent deletion mutant as follows: 50 ml LB medium were inoculated with 500 µl *P. aeruginosa* overnight culture and grown to OD_{600} 0.5 and cooled 10 min on ice. After centrifugation for 10 min at 4000 g the cell pellet was washed in 25 ml 10 mM NaCl and centrifuged again. The pellet was resuspended in 25 ml $CaCl_2$ and placed on ice for 20 min. After repeated centrifugation the pellet was resuspended in 500 µl $CaCl_2$.100 µl competent cells were mixed with 5 µl plasmid (≥100 ng/µl) and placed on ice for at least 20 min. Cells were heat shocked for 2 min at 42 °C and cooled 10 min on ice. After addition of 900 µl LB medium and subsequent incubation for 60 min at 37 °C the cell suspension was centrifuged 60 s at 5000 g, the supernatant except for 100 µl was discarded, cells were resuspended in these 100 µl and plated on selective LB medium. Phenotypical tests for pyocyanin formation, swarming and 3-oxo-C12-HSL production as controls for successful complementation are described in 2.5.2.3, 2.5.2.2 and 2.5.2.6.

2.5 Identification of Quorum Sensing active genes

Only eight genes were found to be upregulated in the transcriptome of PAO1 pBBR::*bpiB09*. In addition, seven of these genes were succeeding each other and were adjacent to a 19 kb chromosomal deletion. For further analyses according to this gene regions function, the seven genes were cloned separately.

2.5.1 Cloning of *PA2222* to *PA2228*

Single genes from the PAO1 genomic region *PA2222* to *PA2228* encoding for hypothetical proteins and the ***v***irulence ***q***uorum ***s***ensing ***m***odulator *vqsM* (*PA2227*) were PCR-

amplified from genomic DNA using Pfu DNA polymerase. Genomic DNA from PAO1 was isolated by using the peqGOLD Bacterial DNA Kit (VWR, Erlangen, Germany) according to the manufacturer's protocol. The DNA fragment encoding for *PA2222* was PCR-amplified using the primer pair PA2222_xhoI_fw / PA2222_hindIII_rv with following conditions: 95 °C for 30 s, 59 °C for 30 s and 72 °C for 2 min (30 cycles). Fragments encoding *PA2223*, *PA2225* and *PA2226* were amplified using the primer pairs PA2223_xhoI_fw / PA2223_hindIII_rv, PA2225_xhoI_fw / PA2225_hindIII_rv and PA2226_xhoI_fw / PA2226_hindIII_rv with the following conditions: 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min (30 cycles). For the fragments encoding for *PA2224* and *PA2227* primer pairs PA2224_xhoI_fw / PA2224_hindIII_rv and PA2227_xhoI_fw / PA2227_hindIII_rv with following conditions were used: 95 °C for 30 s, 57 °C for 30 s and 72 °C for 2 min (30 cycles). The fragment encoding *PA2228* was amplified using primers PA2228_xhoI_fw / PA2228_hindIII_rv and following conditions: 95 °C for 30 s, 57 °C for 30 s and 72 °C for 2:30 min (30 cycles) (Table 2). PCR fragments containing the respective genes were cloned blunt-end in pBluescript SK (+) and subcloned in pBBR1MCS-5 (Table 1) by using the restriction sites indicated in the primer names and were transformed in chemically competent PAO1 (see 2.4.5).

2.5.2 Phenotypic testing of *P. aeruginosa*

Several phenotypic tests were performed with the established mutants, complemented mutants and *P. aeruginosa* strains over-expressing separately the genes PA2222 to PA2228 to assess the Quorum Quenching potential.

2.5.2.1 Biofilm formation of *P. aeruginosa* in flow cells

P. aeruginosa strains were grown overnight, allowed to sediment for 10 min at 5000 g and resuspended in 5 ml modified AP medium (mAP) (modified from Ohman and Chakrabarty 1981) containing 10 mM sodium gluconate, 10 mM KNO_3, 1.25 mM $NaH_2PO_4 \cdot H_2O$, 2.8 mM K_2HPO_4 and 1 mM $MgSO_4 \cdot 7H_2O$ in H_2O. Cells were diluted in fresh mAP medium to a cell density of 10^7 cells/ml. Biofilms were cultivated on glass slides in 2-channel flow cells as has been described before and is represented in Figure 5 (Bijtenhoorn et al. 2011). Visualization and image acquisition were performed according to Bijtenhoorn et al. 2011, with the exception of using as objective a Zeiss LD LCI Plan-Apochromat 25x/0.8 (Carl Zeiss AG, Oberkochen, Germany).

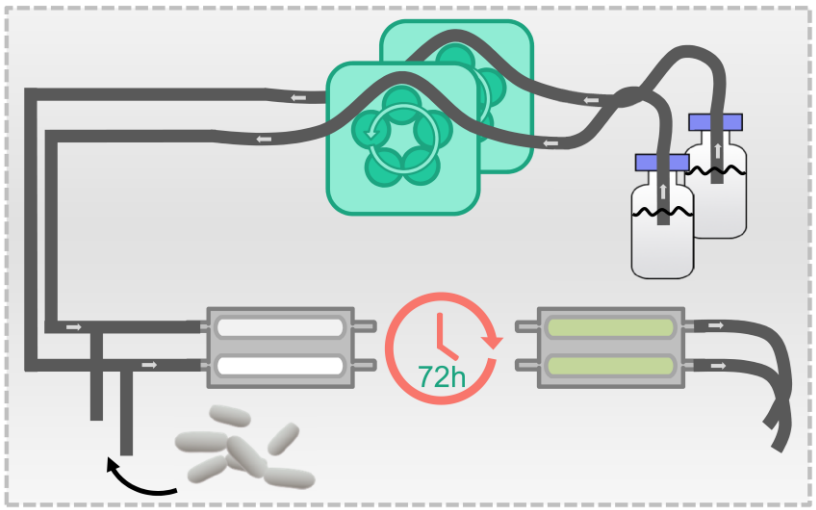

Figure 5: Setup of biofilm growth experiments in flow cells. Cells were cultivated under laminar flow. The medium was transported unidirectional through flow cells by using a peristaltic pump (green box). Flow cells were inoculated with bacteria through a T-junction right before the chambers. Biofilm growth was analyzed after 72 h.

2.5.2.2 Swarming activity

Swarming behavior of *P. aeruginosa* strains was studied as described before (Bijtenhoorn et al. 2011). Plates were incubated for 20 h at 30 °C.

2.5.2.3 Pyocyanin formation

Pyocyanin content in supernatants of *P. aeruginosa* strains was measured according to Gallagher et al. 2002.

2.5.2.4 Elastase activity

Elastase production was quantified using the elastin Congo red (ECR) elastolysis assay described in Pearson et al. 1997 with modifications. 1 ml of culture supernatants filtrates from cultures grown for 16 h at 37 °C from an initial inoculum at OD_{600} of 0.02 was added to 1 ml of ECR buffer (100 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.2) containing 20 mg of ECR. Tubes were incubated for 3 h or 16 h at 37 °C with shaking at 250 rpm. After incubation, 0.2 ml of 0.12 M Na_2EDTA was added to stop the reaction. Insoluble ECR was separated by centrifugation at 3,500 g for 10 min and the absorbance of the supernatant at 495 nm was measured and normalized by the OD_{600} from the initial 16 h cultures. Absorbance of samples incubated in the absence of culture filtrate was considered background activity and was subtracted from all samples.

2.5.2.5 Protease activity

Protease activity was determined by halo size on casein agar (2.5 g Hammerstein casein, 3 g meat extract, 5 g peptone from meat, 5 g NaCl, 0.15 g $Ca(OH)_2$, 0.05 g $CaCl_2 \cdot 2H_2O$, 12 g agar in 1000 ml H_2O, pH 7, autoclaved at 112 °C for 30 min). 1 µl overnight culture was dropped on the agar and plates were incubated at 30 °C for 16 h.

2.5.2.6 3-oxo-C12-HSL formation

3-oxo-C12-HSL content in supernatants of 20 h *P. aeruginosa* cultures was measured as described in Bijtenhoorn et al. 2011 with following additions: 1 ml of the *A. tumefaciens* cultures with added supernatants was mixed with 20 µl toluol. After phase separation 800 µl of the aqueous phase were mixed with fresh ONPG-solution (4 mg/ml in Z-Buffer, 1.61 g $Na_2HPO_4 \cdot 7H_2O$, 0.55 g $Na_2HPO_4 \cdot H_2O$, 0.075 g KCl, 0.025 g $MgSO_4 \cdot 7H_2O$, 0.27 ml mercaptoethanol, ad 100 ml H_2O) and incubated for 20 min at RT. 400 µl 1 M Na_2CO_3 solution was added to quench the reaction. After centrifugation for 2 min at 13,000 g the absorbance at 420 nm was measured. *A. tumefaciens* cultures with no added supernatant were used as controls and values were subtracted from measurements after calculating the OD_{420}/OD_{600} ratio.

2.6 Molecular mechanism of action of PA2226

As seen from the phenotypic tests, *PA2226* alone seems to be responsible for a strong Quorum Quenching effect. Therefore, *PA2226* was further analyzed to elucidate the molecular mechanism of action.

2.6.1 Expression from an inducible promoter

To test if the Quorum Quenching effect is dependent on gene expression levels pSRKGm::*PA2226* was constructed. An XbaI/HindIII fragment from a pSK::*PA2226* with an insert orientation at which *PA2226* is encompassed by these restriction sites was cloned into the respective sites of pSRKGm. Pyocyanin formation was tested as described (2.5.2.3) with cultures induced by 10, 20 and 50 µM IPTG. Swarming activity was tested according to 2.5.2.2 with cultures induced by 5, 10, 20, 50, 100, 200, 500 and 1000 µM IPTG.

2.6.2 AHL complementation of *P. aeruginosa* strains

To investigate, if the Quorum Quenching of *PA2226* could be overcome with excess AHL addition, strains were complemented with 3-oxo-C12-HSL and C4-HSL. Overnight cultures were diluted 10^{-3} and 10 µM 3-oxo-C12-HSL and/or 100 µM C4-HSL were added.

Pyocyanin formation and elastase activity was assessed qualitatively after overnight incubation at 37 °C and 120 rpm as already described (2.5.2.3, 2.5.2.4).

2.6.3 AHL reporter assay

Because of the strong global effect of Quorum Quenching, the possibility of an interaction with LasR was tested.

2.6.3.1 Reporter assay using pKR-C12Δ*lasR*

Reporter assays from known LasR inhibitors show an early activation of Quorum Sensing in the respective deletion mutant strains. Therefor, cultures of PAO1, PAO1 and ∆2226 (2.4) harboring pKR-C12Δ*lasR* (Table 1) were kept in early log phase (OD_{600}<0.2) for at least 3 h by repeated dilution before used for inoculation (Siehnel et al. 2010). To monitor promoter activity throughout growth, cultures were inoculated with an initial OD_{600} of 0.005. 200 µl aliquots of cultures were grown in opaque-walled micro titer plates at 37 °C in a Synergy™ HT microplate reader (BioTek, Winooski, VT, USA). The plate was shaken 5 min at medium speed prior to each measurement every 30 min using $\lambda_{ex}/\lambda_{em}$ of 485/528 nm and normalization to OD_{600}.

2.6.3.2 Reporter assay using pKR-C12

To test the effect of *PA2226* uncoupled from the *P. aeruginosa* background and possible interferences, *E. coli* MT102 and the pKR-C12 reporter plasmid (Table 1) were used in an AHL reporter test. The plasmid pUCP20t::*PA2226* was created by ligating an XbaI/HindIII fragment from a pSK::*PA2226* with an insert orientation at which *PA2226* is encompassed by these restriction sites into the respective sites of pUCP20T. Chemically competent MT102 pKR-C12 cells were either transformed with pUCP20t::*PA2226* or pUCP20T as control. Overnight cultures were diluted 1:5 and 200 µl with either 1 µM, 10 µM or no added AHL for each strain were incubated in opaque-walled micro titer plates at 30 °C in a Synergy™ HT microplate reader (BioTek, Winooski, VT, USA). The plate was shaken 5 min at medium speed prior to each measurement every 15 min using $\lambda_{ex}/\lambda_{em}$ of 485/528 nm and normalization to OD_{600}.

2.6.4 Protein overexpression and purification

PA2226 was overexpressed as maltose-binding-protein (MBP) fusion. A DNA fragment containing *PA2226* was PCR-amplified from pBBR::*PA2226* by using the primer pair PA2226_pmal_ecoRI_fw / PA2226_pmal_hindIII_rv (Table 2) and following conditions: 95 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min (30 cycles). The PCR fragment was cloned in pMAL-c2X by in the primer names indicated restriction sites. Competent

E. coli Rosetta-gami 2(DE3) cells were transformed with the construct pMAL-c2X::*PA2226* (Table 1). Overexpression cultures were grown according to the pMAL™ Protein Fusion and Purification System's manual (NEB, Ipswich, MA, USA) with the exception of an elonganted overnight cultivation at 37 °C. Cells were harvested and resuspended as described in the manufacturer's manual. Protein was released from cells using a French Pressure Cell Press (American Instrument Company) and was further purified as described in the manual. If necessary, protein was concentrated in 30 kDa Vivaspin-6-Concentrators (Sartorius Stedim Biotech GmbH, Göttingen, Germany). Successful overexpression and purification was analyzed by 12 % SDS-PAGE and gel staining with Coomassie Brilliant Blue. Due to the large size of the MBP it was tested if the MBP-PA2226 fusion is still folding to an active conformation. A DNA fragment was PCR-amplified from pMAL-c2X::*PA2226* by using the primer pair PA2226mbp_xhoI_fv / PA2226mbp_hindIII_rv (Table 2), Phusion polymerase and following conditions: 95 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s (30 cycles). The fragment containing *malE-PA2226* was cloned in pBBR1MCS-5 by using the restriction sites indicated in the primer names and competent PAO1 cells were transformed with pBBR::*malE-PA2226* (see 2.4.5). The activity of the fusion protein was confirmed by qualitatively assessing the pyocyanin formation, by observing the presence of the blue pigment. LasR was overexpressed from pET28a::*lasR* in *E. coli* BL21 (DE3) as His-tag fusion according to Liu et al. 2009 (Table 1). After cells had been harvested and resuspended in 1× LEW buffer (Macherey-Nagel, Düren, Germany), His-tagged fusion protein was released from cells by French Pressure Cell Press and purified by using Protino® Ni-TED 2000 columns (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. If necessary, protein was concentrated in 10 kDa Vivaspin-6-Concentrators (Sartorius, Göttingen, Germany). All protein concentrations were determined by the method of Bradford (Bradford 1976).

2.6.5 Electrophoretic Mobility Shift Assay (EMSA)

Oligonucleotides containing the operator OP1 site of the *lasB* promoter (Table 5), one of these labeled with Cy5, were annealed to a 40 bp double stranded DNA probe. 100 pmol/µl of each oligonucleotide and 1× annealing buffer (AB; 10× AB: 100 mM Tris-HCl, 1 M NaCl, 10 mM EDTA, pH 7.5) were heated 5 min at 95 °C and cooled to RT for 1 h. For shift assays 0.25 pmol of the DNA probe was incubated for 30 min at RT with 0-50 pmol of LasR, MBP-PA2226 or MBP in buffer containing 50 mM Tris, 100 mM NaCl, 0.2 mg/ml BSA, 10 µM 3-oxo-C12-HSL and 2 % glycerol. The samples were analyzed by neutral DNA PAGE on 8 % 1× TBE gels and the Cy5 labeled DNA probes were visualized afterwards with a VersaDoc 4000 MP (Bio-Rad, Hercules, CA, USA).

2.6.6 Protein-protein interaction

To test for the possibility of a protein-protein interaction co-precipitation experiments were performed. The basic concept is described in Wu et al. 2006. 100 ml PA2226 and LasR overexpression cultures were grown as described in 2.6.4. LasR expression cultures were supplemented with 2 µM 3-oxo-C12-HSL. As a control *E. coli* Rosetta-gami 2(DE3) pMAL-c2X was equally grown like PA2226 overexpressing cultures to yield MBP. Cells were harvested and pellets were resuspended in 2 ml binding buffer (BB; 20 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5) per 0.5 g wet mass. To keep LasR soluble additional 0.5 µM 3-oxo-C12-HSL were added to the BB for LasR release from cells. All overexpression cell suspensions were sonicated three times for 2 min (cycle 0.5, 50 % amplitude) and centrifuged 30 min at 13,000 g and 4 °C. For both PA2226 and MBP 50 µl amylose resin was washed with BB and 1.5 ml of the particular supernatants from the sonicated suspensions were added and afterwards incubated for 1 h at 4 °C with gentle shaking. The resin was washed with 1 ml BB 3 to 4 times or until the OD_{280} was less than 0.05. 1 ml of LasR crude extract supernatant was added to the washed and protein bound resin and additionally to 50 µl washed amylose resin and all samples were incubated 1 h at 4 °C with gentle shaking. Subsequently the resin was washed 5 times with 1 ml BB. Protein was eluted from the resin by addition of 75 µl BB + 10 mM maltose and incubation for 10 min at 4 °C and gentle shaking. After a short centrifugation 10 µl of the supernatants were mixed with 10 µl SDS sample buffer and were boiled for 5 min. 10 µl of each sample were separated by 12 % SDS-PAGE. As additional controls the crude extract supernatants were separated as well. Gels could be used in immunoblots or were Coomassie Brilliant Blue stained.

2.6.6.1 Protein immunoblot

Proteins were transferred onto nitrocellulose membranes by a semidry transfer method and were washed 10 min in TBS (100 mM Tris, 0.9 % (w/v) NaCl, pH 7.5) and afterwards blocked overnight in TBST (TBS + 0.1 % (v/v) Tween-20) + 5 % (w/v) milk powder at RT. Membranes were incubated with an anti-His antibody (polyclonal, made in rabbit) at 1:5,000 dilution for 1 h at 4 °C with gentle shaking, followed by 3 times washing in TBST for 10 min. Membranes were incubated with alkaline phosphatase labeled anti-rabbit IgG (made in goat) at 1:10,000 dilution in TBST + 5 % (w/v) milk powder for 1 h at RT and were washed afterwards extensively. After equilibration for 3 min in detection buffer (DB; 100 mM Tris-HCl, 100 mM NaCl, pH 9.5) signals were developed in staining solution (66 µl NBT-solution, 75 mg/ml NBT in 70 % DMF; 22 µl BCIP-solution, 50 mg/ml BCIP in DMF; ad 10 ml DB) at RT in the dark. After extensive washing of the membranes a second immunoblot could be applied by using anti-MBP (made in mouse) and alkaline

phosphatase labeled anti-mouse IgG (made in rabbit) antibodies applying the same procedure as for the anti-His antibody.

2.6.6.2 Complementing strain PA2226 by LasR

To evaluate if excess LasR could restore wild type phenotypes, *lasR* was PCR-amplified from genomic PAO1 DNA by using Phusion polymerase and the primer pair lasR_xbaI_fw / lasR_hindIII_rv and following conditions: 95 °C for 30 s, 67 °C for 30 s and 72 °C for 30 s (35 cycles) (Table 2). The fragment containing *lasR* was cloned in pHERD20T by the restriction sites indicated in the primer names and was transformed in strain PA2226. Elastase activity and pyocyanin formation were evaluated qualitatively as already described (2.5.2.3, 2.5.2.4). For induction of pHERD20T::*lasR* 0.1 % arabinose was added to cultures.

2.7 Transcriptome of strain PA2226

In order to reveal the influence of PA2226 on transcription levels in *P. aeruginosa*, the transcriptome of strain PA2226 and PBBR was analyzed. Overnight cultures were used to inoculate 10 ml LB with a starting optical density (OD_{600}) of 0.02. Cultures were grown at 37 °C and 125 rpm to an OD_{600} of 2 in duplicate. Two 2 ml aliquots were harvested by 1 min centrifugation at 13,000 g. The supernatant was discarded and the pellet resuspended in 1 ml RNA*later* (QIAGEN, Hilden, Germany) and stored at 4 °C until RNA isolation. For each of the four samples RNA isolation was performed twice. RNA was isolated using the UltraClean Microbial RNA Isolation Kit (MO BIO, Carlsbad, CA, USA) according to the manufacturer's instructions with following modifications: Cells in MR1 solution and added DTT were heated for 20 min at 65 °C and vortexed afterwards for 20 min. Residual DNA was removed by the RTS DNase Kit (MO BIO, Carlsbad, CA, USA) following the manufacturer's protocol. RNA Clean & Concentrator™-5 (ZYMO RESEARCH, Irvine, CA, USA) was used for purification and concentration of RNA samples. RNA was eluted with 8 µl RNase free H_2O twice and RNA isolations from technical replicates were pooled, yielding 32 µl total RNA per sample. Concentration and purity was evaluated by using the Take3 plate in a Synergy™ HT microplate reader (BioTek, Winooski, VT, USA). Concentrations ranged from 70-160 ng/µl. The RNA integrity was controlled on a denaturing formaldehyde gel by checking for sharp 23S and 16S rRNA bands with a 2:1 ratio. The rRNA was depleted by using the Ribo-Zero rRNA Removal Kit (Gram-Negative Bacteria) (epicentre®, Madison, WI, USA) according to the manufacturer's instructions. RNA Clean & Concentrator™-5 was used afterwards for sample concentration. The mRNA was finally eluted twice with 6 µl RNase free H_2O, yielding a concentration of ca. 42 ng/µl per sample. Successful rRNA depletion was controlled on a

denaturing formaldehyde gel. The cDNA library preparation using the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA) and 50 bp single read sequencing on an Illumina HiSeq2000 (Illumina, San Diego, CA, USA) were performed by GATC Biotech (Konstanz, Germany). Quality of reads was assessed with FastQC and reads were mapped with Bowtie 2 (Langmead and Salzberg 2012) to the PAO1 transcriptome (GenBank accession no.: AE004091). SAM files were converted to BAM format and sorted by SAMtools 0.1.17 (Li et al. 2009). Cuffdiff from the Cufflinks 2.1.1 package was used to analyze differential expression. A more detailed description of the “tuxedo suite” tools is given in Trapnell et al. 2012. The tRNAs, rRNAs and pseudogenes were masked by a mask GTF file. Genes with a ≥2-fold change and a, by False Discovery Rate (FDR controlled by Benjamini-Hochberg procedure), corrected p-value ≤0.05 (q-value) were considered differential expressed. For graphical display of expression levels reads were mapped to the genome sequence and SAM files were converted to BAM format and sorted as described before. Graphs were calculated using SAMtools and a perl script from http://www.ecseq.com/support.html (Appendix Script A 1). Resulting wig files were converted to bigWig (wigToBigWig from http://genome.ucsc.edu/) and were normalized and converted to wig by normalize_bigwig.py from RSeQC (Wang et al. 2012). In the Integrated genome Browser (IGB, Nicol et al. 2009) tracks from the respective parallels were averaged and the final graphs of PA2226 and PBBR transcript levels were overlaid. Transcription units were predicted from read data by Rockhopper 2 (McClure et al. 2013). Global transcriptome maps were visualized with circos 0.64 (Krzywinski et al. 2009).

3 Results

3.1 Transcriptome of PAO1 pBBR::*bpiB09*

In a previous study it was observed that PAO1 carying an AI reducing oxidoreductase was attenuated in its virulence. The protein had been designated BpiB09 and had been identified as rather pormiscous enzyme acting on a wide range of substrates (Bijtenhoorn 2012). To unravel the impact of *bpiB09* and to better understand the metabolic changes within PAO1 in response to this proteins expression the global transcriptome was analyzed. In order to identify an appropriate point in time of growing *P. aeruginosa* cultures for sampling, growth curves for PAO1 pBBR::*bpiB09* and strain PBBR were recorded (2.3). Figure 6 shows that the strains were growing equally until the 5 h time point and PAO1 pBBR::*bpiB09* kept growing decelerated from here on until reaching the stationary phase after approximately 12 h, whereas PBBR cell density remained static between the 6 h and 7 h time points and continued afterwards growing until reaching the stationary phase after 13-15 h.

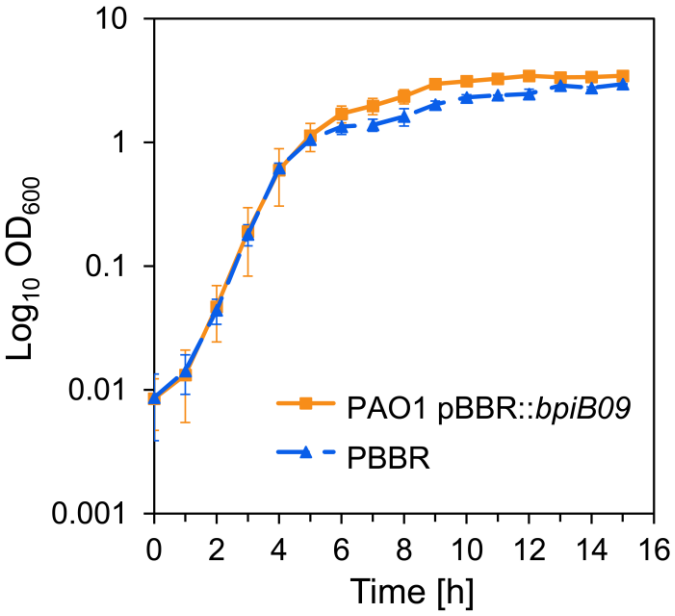

Figure 6: Growth curves of *P. aeruginosa* pBBR::*bpiB09* and PBBR. Strains showed equal growth until the 5 h time point at the ending of the exponential growth phase. In contrast to *P. aeruginosa* pBBR::*bpiB09* the growth of strain PBBR remained static between the 6 h and 7 h time point and continued growing afterwards. Both strains kept growing decelerated until reaching the stationary phase between the 12 h and 15 h points in time.

The 5 h time point was chosen as sampling point for the transcriptomic analysis to assess the transcriptome at the same growth phase and comparable cell densities. In

addition, this offered the possibility to record the change in transcription, which possibly led to diverging growth.

In total 567,003 reads were achieved for the four cDNA libraries. In detail this were 103,894, 236,530, 115,765 and 110,814 reads for the B09_1, B09_2, PBBR_1 and PBBR_2 samples, respectively.

The cDNA levels of cells expressing *bpiB09* from its native promoter on pBBR::*bpiB09* were compared to the control carrying an empty vector (PBBR). Evaluation of the acquired data showed that *bpiB09* (gene coverage 46) and the expression of ribosomal genes like *rplR*, *rpsL* and *rplM* (gene coverage 46.6, 47.9 and 48.1) was comparable, making *bpiB09* one of the 70 most abundant mRNAs. Overall the level of gene expression of housekeeping genes (for example *recA, rpoD, gyrA, gyrB* and *dnaA,* data not shown) was similar and differed not more than 2.5-fold in the reference samples and the PAO1 carrying the *bpiB09* gene.

The global expression analysis revealed a significant fraction of differentially regulated genes. In total 823 genes were down-regulated in PAO1 pBBR::*bpiB09* compared to PBBR, whereas only 8 genes were up-regulated (see Table S2 and Table S3 from Bijtenhoorn et al. 2011). A focus on putative Quorum Sensing linked genes, according to Schuster et al. 2003, Hentzer et al. 2003 and Wagner et al. 2003, revealed 38 at least 10-fold down-regulated genes (Table 7). Among these genes *lasI, rhlI, ambCD* and *pqsABCD* could be identified, thus including autoinducer synthesis genes for both AHLs, PQS, as well as for the recently discovered fourth Quorum Sensing molecule IQS. In line with earlier experimental data of PAO1 pBBR::*bpiB09* phenotypes, genes like *hcnA* (involved in hydrogen cyanide synthesis), *lasB* (elastase) and *phzM* (involved in pyocyanin synthesis) were found.

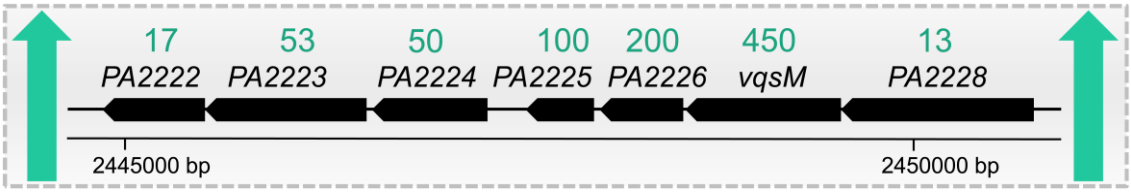

Figure 7: Cluster of up-regulated genes in PAO1 pBBR::*bpiB09*. Genomic region of PAO1 compromising the seven up-regulated consecutive genes. The *vqsM* gene is described as QS modulator. Green numbers indicate the fold-change in gene expression (rounded to two significant figures) of PAO1 pBBR::*bpiB09* compared to a control strain with empty vector.

Remarkable was the low number of up-regulated in contrast to down-regulated genes, as well as that seven of these genes were consecutive (Figure 7). One of these genes

was *vqsM*, described as activating Quorum Sensing modulator, whereas the other six genes were annotated as hypothetical genes. The up-regulation of *vqsM* conflicted with the overall detected down-regulation of QS-dependent genes and the current knowledge about *vqsM* (Dong et al. 2005, Liang et al. 2014).

Table 7: QS-controlled and ≥10-fold repressed genes/ORFs in PAO1 expressing *bpiB09*[a]

PA Orf no.[b]	Gene name	Description	Change in PAO1 pBBR::*bpiB09* compared to PBBR (fold)[c]
PA0007		hypothetical protein	-12
PA0122		hypothetical protein	-12
PA0179		two-component response regulator	ND[d]
PA0572		hypothetical protein	-12
PA0996	*pqsA*	coenzyme A ligase	-55
PA0997	*pqsB*	hypothetical protein PqsB	-130
PA0998	*pqsC*	hypothetical protein PqsC	-46
PA0999	*pqsD*	3-oxoacyl-(acyl carrier protein) synthase III	-94
PA1003	*mvfR*	transcriptional regulator	-12
PA1130	*rhlC*	rhamnosyltransferase 2	-12
PA1248	*aprF*	Alkaline protease secretion outer membrane protein AprF precursor	-37
PA1250	*aprI*	alkaline proteinase inhibitor	-13
PA1432	*lasI*	autoinducer synthesis protein	-150
PA1784		hypothetical protein	-30
PA2069		carbamoyl transferase	-49
PA2193	*hcnA*	hydrogen cyanide synthase HcnA	-24
PA2300	*chiC*	chitinase	-15
PA2303	*ambD*	hypothetical protein	-79
PA2304	*ambC*	hypothetical protein	-30
PA2591	*vqsR*[e]	transcriptional regulator	-16
PA2592		periplasmic spermidine/putrescine-binding protein	-20
PA3328		FAD-dependent monooxygenase	ND
PA3329		hypothetical protein	-24
PA3331		cytochrome P450	-22
PA3332		hypothetical protein	-29
PA3333	*fabH2*	3-oxoacyl-(acyl carrier protein) synthase III	-35
PA3334		acyl carrier protein	-34
PA3335		hypothetical protein	ND
PA3476	*rhlI*	autoinducer synthesis protein	-16
PA3478	*rhlB*	rhamnosyltransferase chain B	-15
PA3479	*rhlA*	rhamnosyltransferase chain A	-25
PA3724	*lasB*	elastase	-840
PA3906		hypothetical protein	-50
PA3907		hypothetical protein	-15
PA3908		hypothetical protein	-20
PA4209	*phzM*	phenazine-specific methyltransferase	-15
PA4211	*phzB1*	phenazine biosynthesis protein	-16
PA5059		transcriptional regulator	-13

[a] Genes listed as being QS-regulated refer to the studies of Schuster et al. 2003, Wagner et al. 2003 and Hentzer et al. 2003. Listed are genes common to all three studies, which were repressed in this transcriptomic analysis with a magnitude of change of ≥10fold. Additionally *lasI* and *rhlI* are listed, which could not be measured in the three mentioned trancriptomic analyses,

because of the use of a *lasI-rhlI*-mutant strain.

[b] Gene Number, Name and description are from the Pseudomonas genome project (Winsor et al. 2011).

[c] Changes in gene expression (rounded to two significant figures) of *P. aeruginosa* PAO1 expressing *bpiB09* compared to a control-strain with empty vector. The displayed values are the averages out of two independent transcriptomic analyses, sample point 5 h.

[d] ND, no transcripts detectable.

[e] Name refers to the study by Juhas et al. 2004.

3.1.1 Genomic sequence analysis of PAO1 pBBR::*bpiB09*

In the first report on BpiB09 it was speculated that the strong QQ phenotypes observed were possibly not only dependent on BpiB09 and its reducing activity on 3O-C12-HSL and that some additional not yet identified processes were involved.

Taking adavatage of the 454 sequencing of the transcriptome the reads achieved for the two samples of PAO1 pBBR::*bpiB09* were pooled and used in the genomic sequence analysis. In total 271,875 reads could be uniquely aligned to the *P. aeruginosa* PAO1 reference sequence (GenBank accession no.: AE004091) with a total sequence coverage breadth of 99.16 %, confirming the physical map of the PAO1 genome. Moreover, in the strain PAO1 pBBR::*bpiB09* two major structural rearrangements in respect to the reference sequence could be detected in the alignment (Table 8), one shorter 96 bp and one major 18,917 bp deletion.

Table 8: Structural rearrangement in the PAO1 pBBR::*bpiB09* genome

Type	Position 1 [bp]	Position 2 [bp]	Supporting reads	Non-supporting reads	Length [bp]
Deletion	48,557	48,654	7	0	96
Deletion	2,449,905	2,468,823	5	0	18,917

The 96 bp deletion was located in the middle of the 10,608 bp spanning ORF *PA0041*, annotated as probable hemagglutinin gene. This surprisingly large gene harbored domains for hemagglutination activity and regions of homology to exoproteins involved in heme utilization or adhesion. One of these regions was affected by the deletion. *In silico* GC-frame-plot analysis in the artemis genome browser (Carver et al. 2012) supported the annotation as one large ORF.

More striking was a major 18,917 bp deletion, which started in ORF *PA2228,* encompassed the majority of the PSL exopolysaccharide synthesis cluster involved in biofilm

formation in PAO1 (Figure 8, I) and was located right in front of the significant upregulated gene cluster (Figure 7). Additional to *PA2228* and *pslA-pslL* the two ORFs *PA2229* and *PA22230*, annotated as hypothetical genes were included in the deletion. This significant structural rearrangement could be validated by PCR (Figure 8, II). Detailed single nucleotide polymorphism (SNP) analysis was omitted, because of the too low average read depth of 15.1 and therewith lacking reliability. Hence, it cannot be excluded that further SNP based differences which impact the global expression are present.

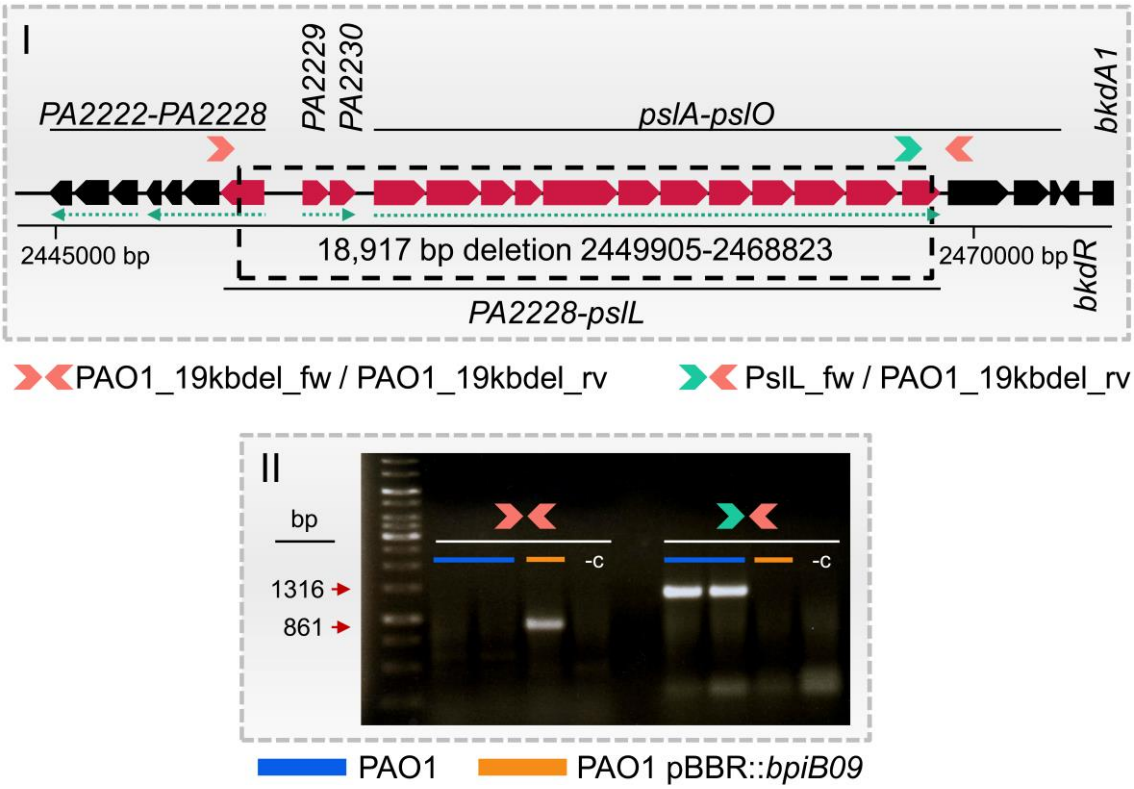

Figure 8: The 18,917 bp deletion of strain PAO1 pBBR::*bpiB09*. (I) The *psl*-operon codes for the major exopolysaccharide of PAO1 used in biofilm formation. *PA2228-PA2230* are annotated as hypothetical genes. The dotted green arrows show operon structures, predicted with Rockhopper from RNA-seq data. The colored arrows indicate primer pairs used for deletion validation (I, II). (II) The deletion was validated via PCR with primer pairs binding to the PAO1 genome as shown in (I), no DNA was used in the negative control (-c). The DNA ladder used was the GeneRuler 1 kb DNA Ladder (Thermo Scientific).

The identification of this influential deletion made previous results look suspicious and fresh PAO1 cultures were transformed with pBBR::*bpiB09*. In fresh transformed PAO1 pBBR::*bpiB09* no inhibition of pyocyanin formation could be observed anymore (data not shown). It is noteworthy that pBBR::*bpiB09* was transformed several times in PAO1 strains with different origin. This indicated that indeed the major deletion is responsible for specific observed phenotypes.

3.2 Identification of Quorum Sensing active genes

The exclusive transcriptional up-regulation of the gene cluster *PA2222-PA2228* in combination with a large 19 kb deletion right in its front led to further analyses to understand the impact of this gene cluster. Qualitatively pyocyanin formation analyses of PAO1 clones overproducing one of the operon's genes at a time (data not shown) revealed two candidates with strong from the control differing phenotypes. PAO1 expressing *PA2226* (strain PA2226) showed no visible pyocyanin formation, whereas PAO1 expressing *vqsM* (strain VqsM) showed increased production. To further evaluate the importance of the observed large deletion with respect to QS-dependent processes in PAO1, gene deletions of the QS genes were constructed and analyzed in the background of the parent strain and the mutated strain. For this purpose AI synthase single and double deletion mutants as well as a *PA2226* deletion mutant were created.

3.2.1 Deletion mutant generation in PAO1

Deletion mutants were established by homologous recombination. The created PAO1 deletion mutants ΔlasI, ΔrhlI, ΔrhlI/lasI and ΔPA2226 could be verified by PCRs for successful deletion of the wild type gene as well as for correct location of the mutation, as shown in Figure 4, and are summarized in Figure 9. The resulting band sizes correlated in every case to the theoretical sizes (Table 4). As expected PCR products for the deletion mutants were shorter than in the wild type or in the case of ΔPA2226 longer than in the wild type because of the integrated Gm^R/GFP-cassette. The cassette was excised afterwards (data not shown). PCRs with primer targets within the genes sequences showed no products for the mutants and distinct bands for the correlating genes in the wild type.

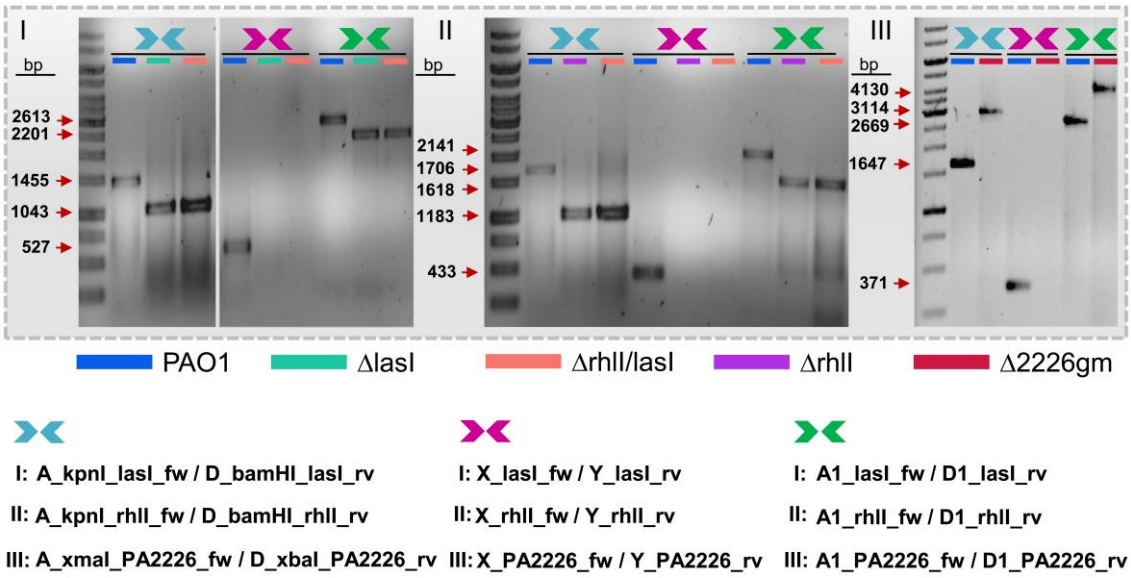

Figure 9: Deletion mutant verification. The mutants ΔlasI (I), ΔrhlI (II), ΔrhlI/lasI (I,II) and ΔPA2226 (III) were verified by multiple PCRs for the correct location and success of the deletion of the wild type genes. The strain ΔPA2226 still harbored the Gm^R/GFP-cassette (ΔPA2226gm), which was excised afterwards (data not shown). Numbers show the expected band sizes for the respective primer pair and strain combination. Primer names starting with A/D targeted the 5' and 3' start and end of the homologous recombination cassette, X/Y targeted inside the wild type gene and A1/D1 outside of the recombination cassette (compare Figure 4). The DNA ladder used was the GeneRuler 1 kb DNA Ladder (Thermo Scientific).

3.2.2 Phenotypic testing of Quorum Sensing active genes

To assess the full range of Quorum Sensing phenotypes affected by *PA2226* in *Pseudomonas* several phenotypic tests with the overproducing strain PA2226, the established mutants ΔlasI, ΔrhlI, ΔrhlI/lasI and ΔPA2226 were performed. The *vqsM* overproducer VqsM was included to clarify the influence of *vqsM* on the regulation circuits. Phenotypes tested were pyocyanin formation, elastase activity, 3-oxo-C12-HSL concentration (Figure 10), protease and swarming activity (Figure 11) as well as biofilm formation (Figure 14, Figure 13).

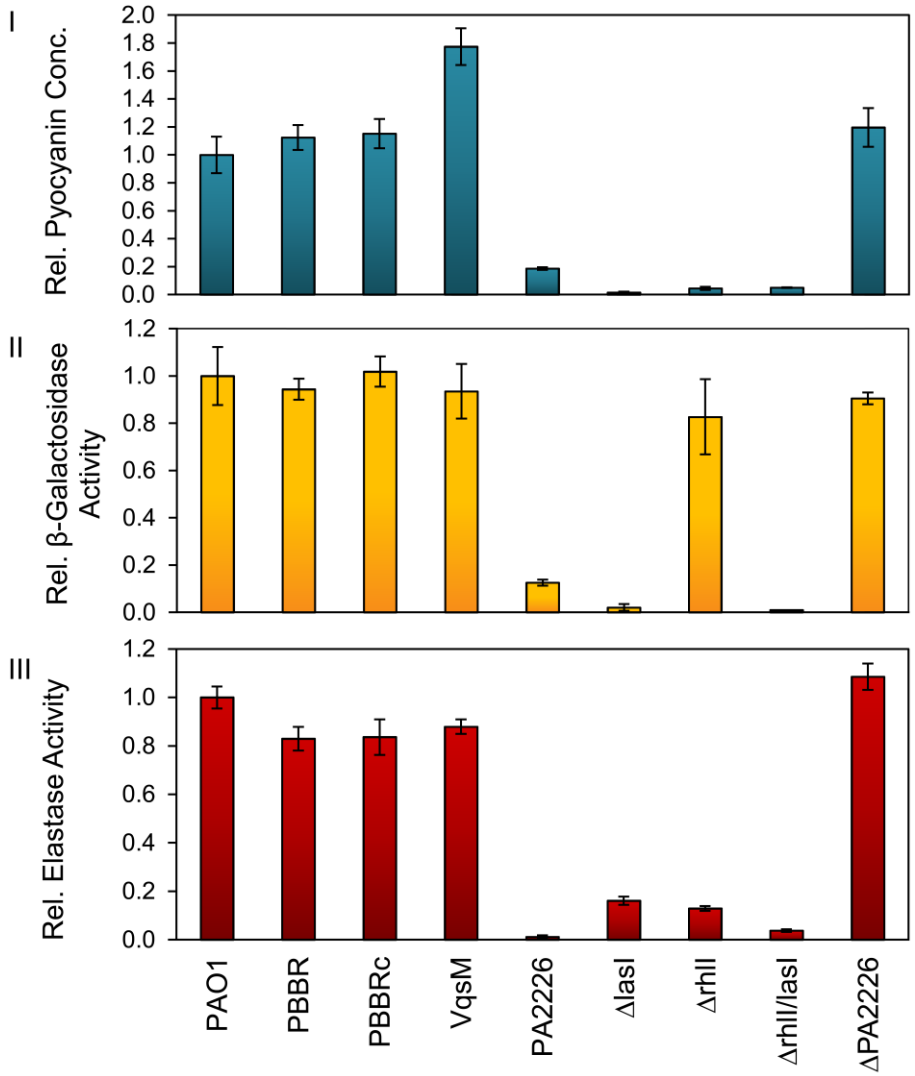

Figure 10: Pyocyanin formation, 3-oxo-C12-HSL concentration and elastase activity. (I) Pyocyanin was chloroform extracted from culture supernatants, acidified and the degree of coloring was measured. (II) 3-oxo-C12-HSL concentrations in supernatants were determined by using an AHL-reporter strain with a *traR-lacZ* fusion and measuring β-galactosidase activity. (III) Elastase activity was measured by the release of Congo red from elastin Congo red. Results are representatives from at least two independent experiments performed in triplicate. Measurements of wild type *P. aeruginosa* PAO1 (PAO1) were set to 1. For negative control PAO1 strains harboring empty pBBR1MCS-5 (PBBR) or pBBR1MCS-5::*celA* (PBBRc), containing a cellulase gene for extra protein load, were used. Positive controls were autoinducer synthase single and double deletion mutants (ΔlasI, ΔrhlI, ΔrhlI/lasI). Test strains were expressing *PA2226* (PA2226), *vqsM* (VqsM) or contained a *PA2226* deletion (ΔPA2226).

As already noticed in the qualitative analysis of pyocyanin formation of the strains overproducing the single genes from the prominent regulated gene cluster, VqsM showed a 77 ± 13 % higher pyocyanin concentration in the supernatant than the PAO1 wild type, whereas PA2226 showed only 19 ± 1 %. No significant change was noticeable in the

Δ*PA2226* mutant, but as expected nearly no pyocyanin could be detected in the autoinducer synthase deletion mutants (Figure 10, I). No significant change in 3-oxo-C12-HSL concentration was detectable for VqsM and ΔPA2226, but PA2226 only had 13 ± 1 % of the wild type level. The mutants ΔlasI and ΔrhlI/lasI showed almost no 3-oxo-C12, whereas ΔrhlI showed no significant change (Figure 10, II). The lowest elastase activity possessed PA2226 (1 ± 1 %), comparable to the double mutant ΔrhlI/lasI (4 ± 1 %). The strains ΔlasI and ΔrhlI showed slight activity, 16 ± 2 % and 13 ± 1 %, respectively. For VqsM and ΔPA2226 no significant change was visible.

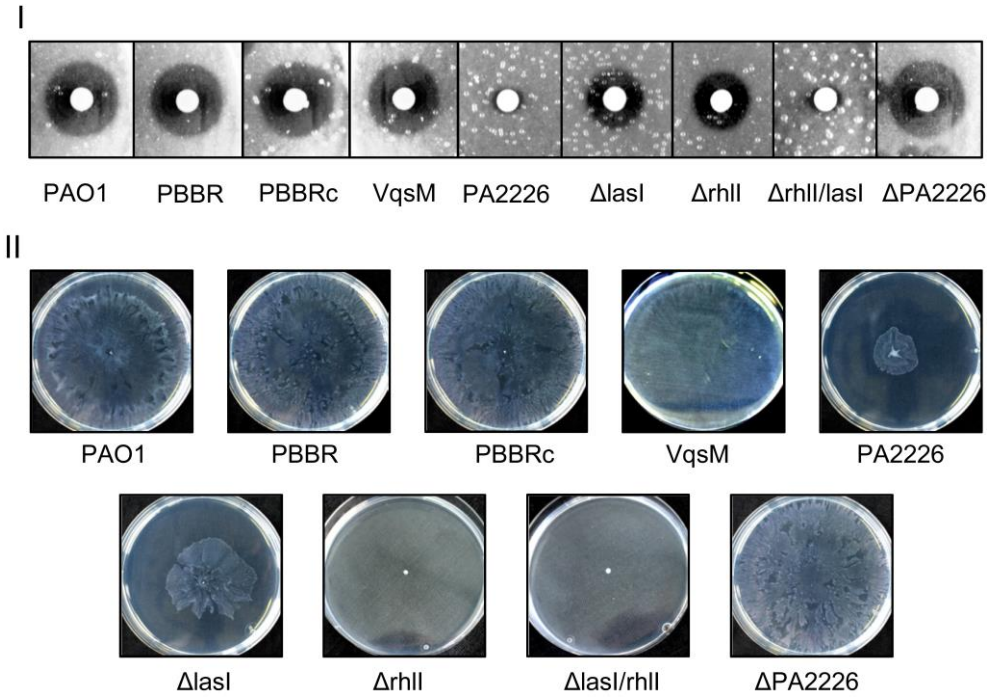

Figure 11: Protease and swarming activity. (I) Protease activity was assessed by patching bacteria on casein plates and enzyme activity was indicated after incubation by clear halos surrounding the bacteria. (II) Swarming activity was evaluated by the degree of spreading over the plates. Results are representatives from at least two independent experiments performed in triplicate. Wild type *P. aeruginosa* PAO1 (PAO1), PAO1 strains harboring empty pBBR1MCS-5 (PBBR) or pBBR1MCS-5::*celA* (PBBRc), containing a cellulase gene for extra protein load, were used for negative control. Positive controls were autoinducer synthase single and double deletion mutants (ΔlasI, ΔrhlI, ΔrhlI/lasI). Test strains were expressing *PA2226* (PA2226), *vqsM* (VqsM) or contained a *PA2226* deletion (ΔPA2226)

Evaluation of the protease activity showed similar results (Figure 11, I). Protease activity was not detectable for PA2226 and ΔrhlI/lasI, but ΔlasI and ΔrhlI showed reduced activity, whereas the halo size did not change for VqsM or ΔPA2226. Concerning the swarming activity PA2226 resembles the reduced phenotype of the *lasI* mutant, whereas ΔrhlI and

ΔrhlI/lasI showed no swarming. Again, no difference to the controls was detectable for VqsM and ΔPA2226 (Figure 11, II).

Credibility of the used autoinducer synthase deletion mutants and their respective phenotypes was validated by complementation with the wild type genes in *trans* (Figure 12). It could be shown that complementation rescued the wild type phenotypes for pyocyanin formation (Figure 12, I), autoinducer 3-oxo-C12-HSL production (Figure 12, II) and swarming (Figure 12, III). Because of the uncontrolled transcription an overshoot of pyocyanin concentration and an only partly restored phenotype for swarming activity could be observed.

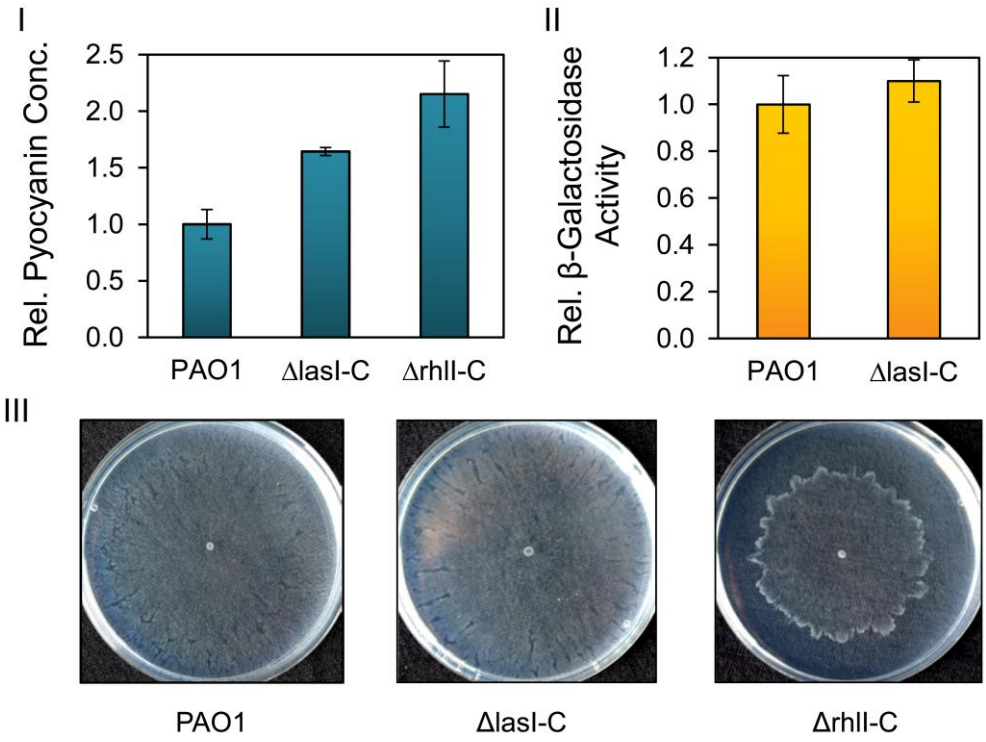

Figure 12: Complementation of ΔlasI and ΔrhlI. Complementation of ΔlasI and ΔrhlI by wild type genes in *trans* were measured for pyocyanin (I) and 3-oxo-C12-HSL (II) concentration, as well as for swarming activity (III). Wild type *P. aeruginosa* PAO1 (PAO1) was used as positive control and measurements were set to 1. Complemented mutants ΔlasI (ΔlasI-C) and ΔrhlI (ΔrhlI-C) harbored pBBR::*lasI* or pBBR::*rhlI*, respectively. Results are representatives from at least two independent experiments performed in triplicate.

As biofilm formation is in part influenced by Quorum Sensing, the impact of PA2226 overproduction and its deletion on biofilm growth was measured (Figure 13). In control experiments employing the newly constructed AI synthase deletion mutants the degree of biofilm reduction would be expected to be significant (Figure 14).

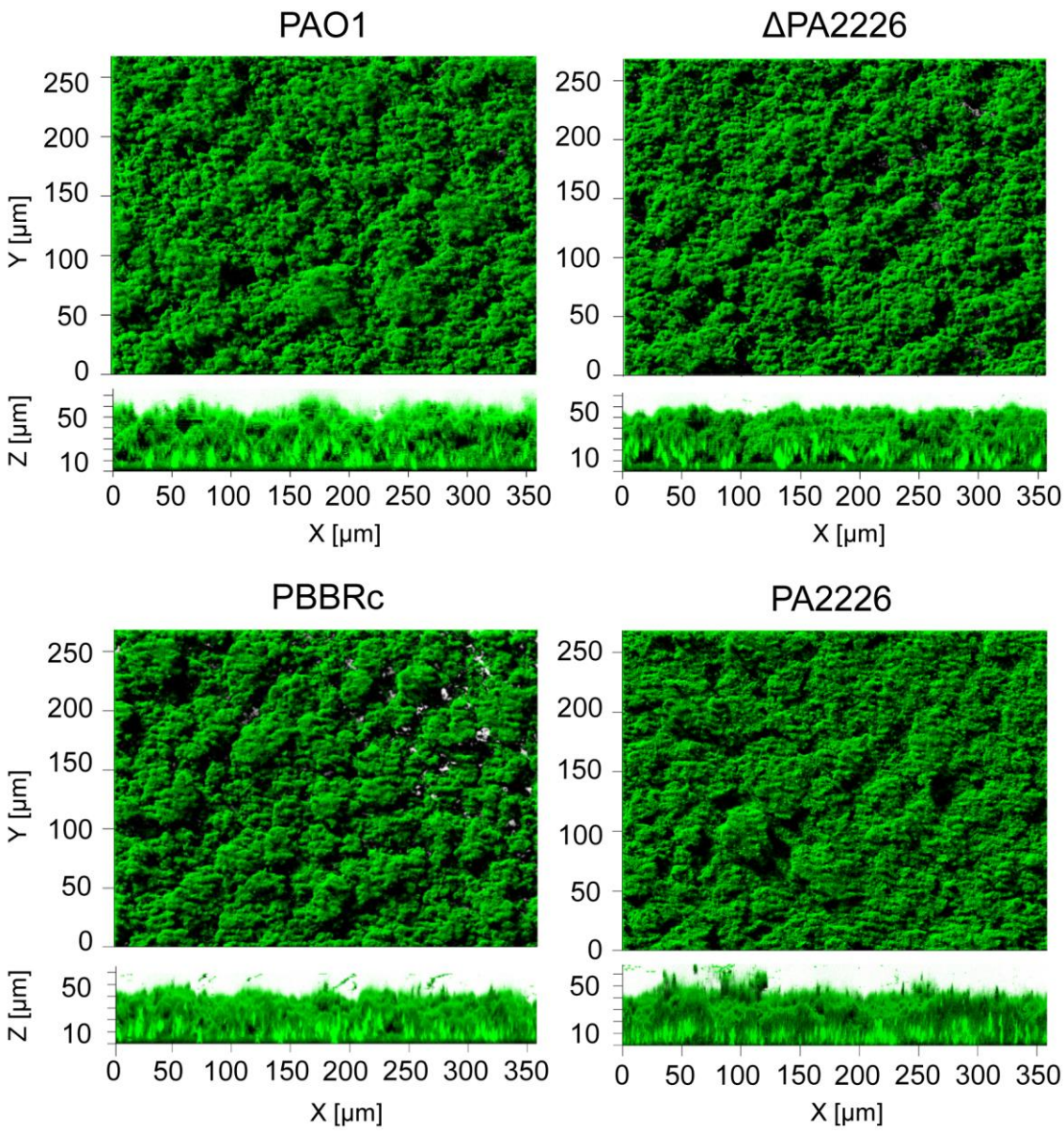

Figure 13: Biofilm formation of PA2226 and ΔPA2226. Biofilms were grown in flow cells for 72 h and were stained with SYTO 9 for fluorescence image acquisition. The PA2226 deletion mutant (ΔPA2226) was compared to the wild type (PAO1) and the PA2226 overproducer (PA2226) to PAO1 carrying pBBR1MCS-5::*celA* (PBBRc), containing a cellulase gene for extra protein load. Results are representatives from at least three independent experiments performed in duplicate.

Despite the strong effects on the other Quorum Sensing controlled phenotypes no difference in the spatial distribution of the formed biofilms of PA2226 and ΔPA2226 could be detected. Comparable to the controls the overall thickness reached ca. 40-50 µm. It is noteworthy that also for ΔlasI, ΔrhlI and even ΔrhlI/lasI no differences in the topology of the biofilm compared to the wild type could be determined. The overall biofilm thickness was ca. 50 µm.

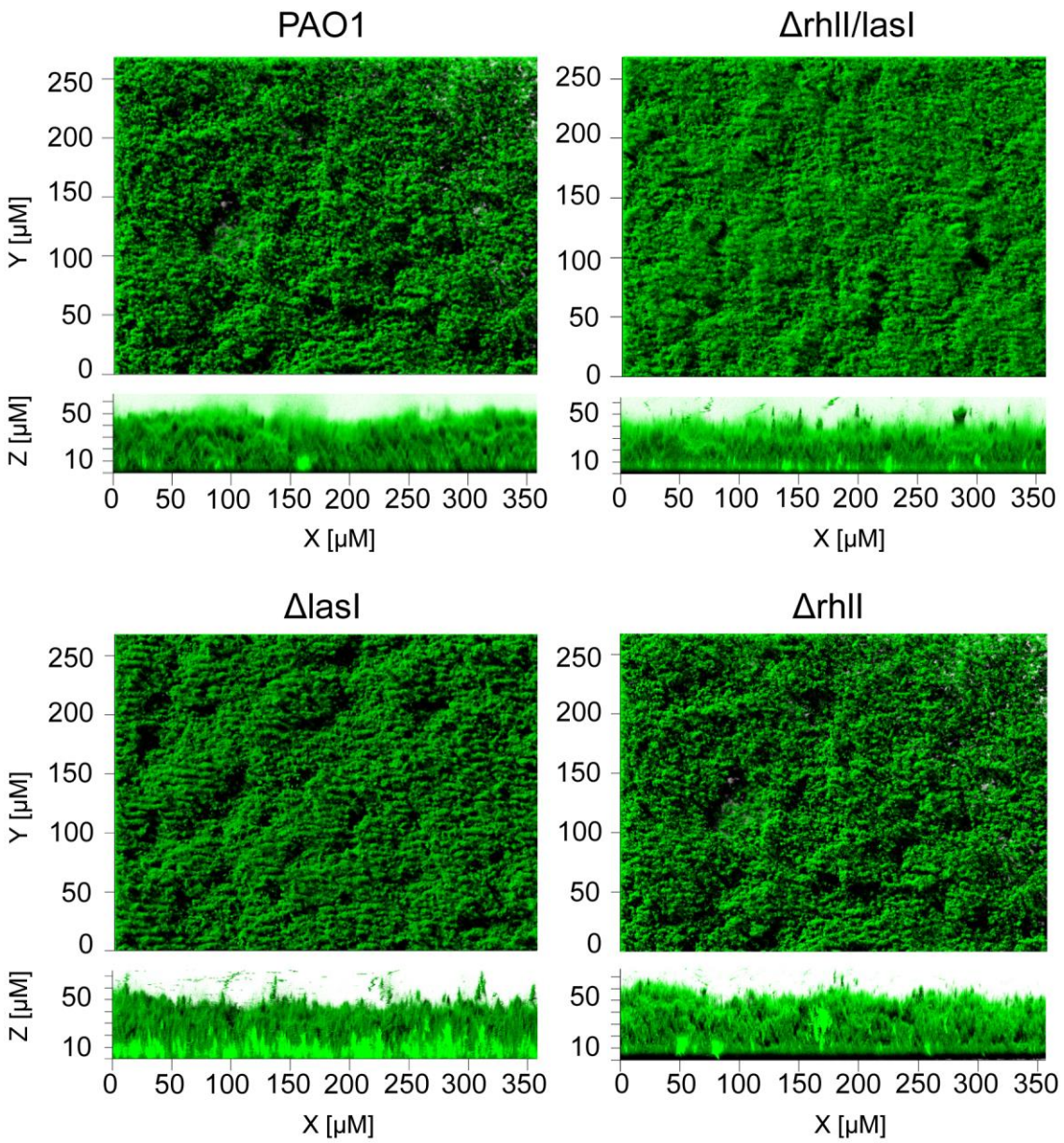

Figure 14: Biofilm formation of autoinducer synthase deletion mutants. Biofilms were grown in flow cells for 72 h and were stained with SYTO 9 for fluorescence image acquisition. The autoinducer synthase single and double deletion mutants (ΔlasI, ΔrhlI, ΔrhlI/lasI)) were compared to the wild type (PAO1). Results are representatives from at least three independent experiments performed in duplicate.

3.3 Molecular mechanism of action of PA2226

The strong impact on Quorum Sensing regulated phenotypes in PAO1 pBBR::*bpiB09* was further analyzed by elucidating the molecular mechanism of action of PA2226. Thereby, the above described experiements provided strong evidence that part of the observed phenotypes in PAO1 pBBR::*bpiB09* might indeed be caused by the observed

large deletion and not primarily attributed to the *bpiB09* gene. To further verify this PA2226 was expressed from an inducible promoter.

3.3.1 *PA2226* expression from an inducible promoter

By testing the expression of *PA2226* from an inducible promoter on the vector pSRKGm in PAO1 it could be shown for pyocyanin formation (Figure 15, I) and swarming activity (Figure 15, II) that the Quorum Quenching effect is based on the expression strength.

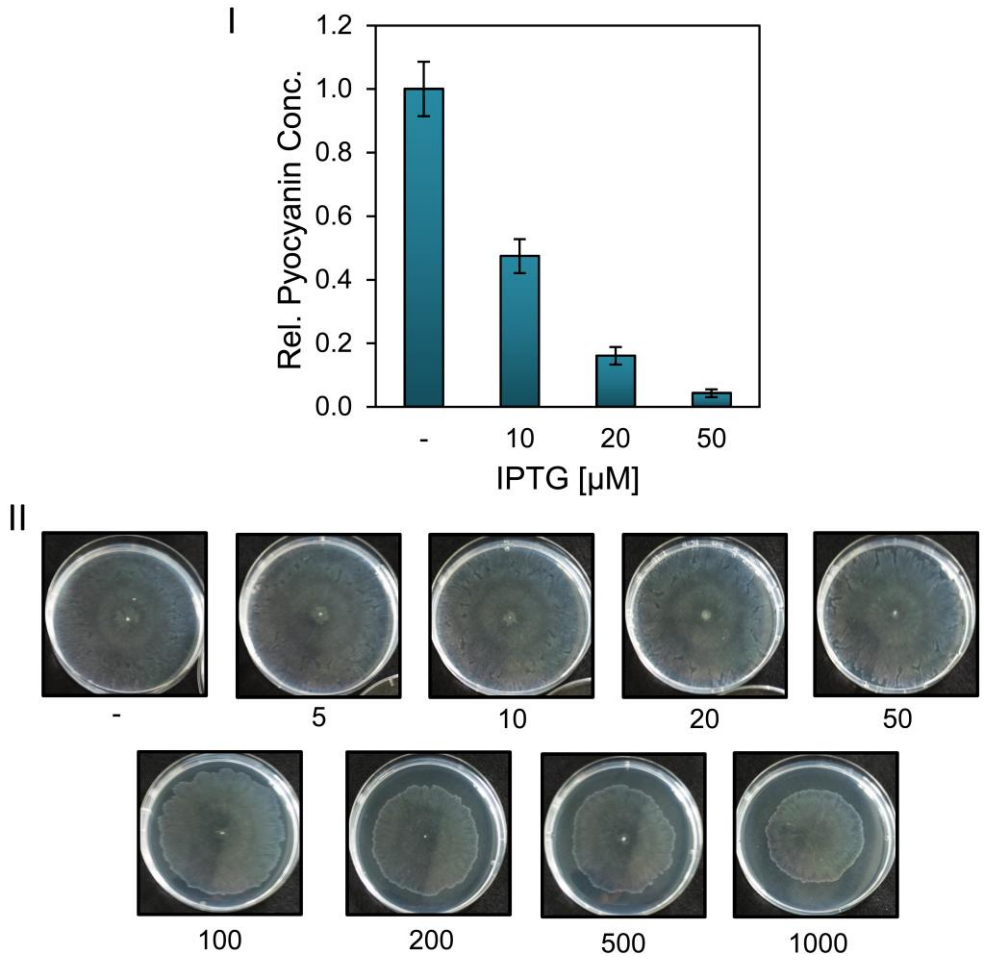

Figure 15: Pyocyanin formation and swarming activity influenced by *PA2226* expressed from an inducible promoter. (I) PAO1 harboring pSRKGm::*PA2226* was induced with rising concentrations of IPTG (numbers under the pictures) and pyocyanin was chloroform extracted from culture supernatants, acidified and quantified. (II) Swarming activity was evaluated by the degree of spreading over the plates. Results are representatives from at least two independent experiments performed in triplicate.

For pyocyanin formation an induction with 10 µM IPTG was sufficient to reduce the concentration to 47 ± 4 % of the uninduced culture, which was reduced further to 4 ± 1 % by induction with 50 µM IPTG.

The swarming activity was not as strong affected as the pyocyanin formation which could be anticipated from the previous experiments, but showed with rising IPTG concentration distinct reduction in swarming. With induction by 50 µM IPTG first disturbance of swarming was visible and increased with rising induction concentrations. The tested maximum induction was 1000 µM IPTG and showed an impairment in swarming comparable to a Δ*lasI* mutant (Figure 11, II).

3.3.2 AHL complementation

Given that the overproduction of PA2226 led to an attenuation of 3-oxo-C12-HSL and sufficient autoinducer is essential for Quorum Sensing controlled factors in *P. aeruginosa* the effect of external complementation with AHLs was tested. Strains were complemented by exogenous addition of 10 µM 3-oxo-C12-HSL, 100 µM C4-HSL or a combination of both and pyocyanin concentration and elastase activity were assayed (Figure 16). Autoinducer synthase mutants were used as positive controls and could be likewise verified for complementation not only by the wild type genes but also for their AHL products. Within first experiments it could be shown that a concentration of 10 µM C4-HSL was not sufficient to complement the *rhlI* deletion mutant whereupon the test concentration was raised to 100 µM.

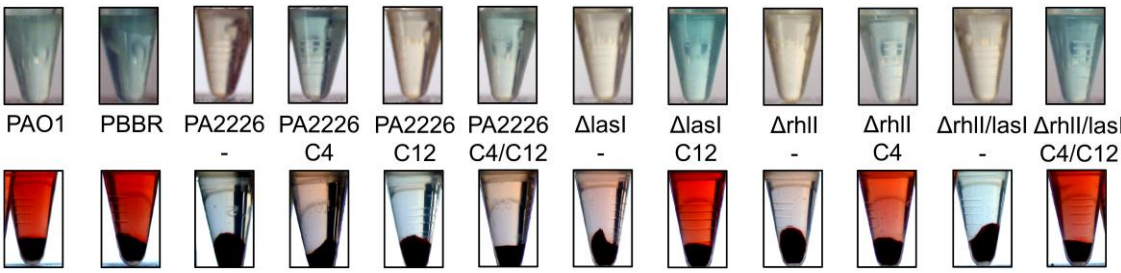

Figure 16: Complementation of PA2226 by exogenous addition of AHLs. Cultures were complemented by exogenous addition of 10 µM 3-oxo-C12-HSL (C12) and/or 100 µM C4-HSL (C4) and were tested for pyocyanin formation (upper row) and elastase activity (lower row). Elastase activity was observed by the release of Congo red from elastin Congo red for 3 h. Pyocyanin was chloroform extracted from culture supernatants. Results are representatives from at least two independent experiments.

Wild type phenotypes could be restored for pyocyanin and elastase by the corresponding AHLs in the autoinducer synthase mutants. For strain PA2226 a complementation could only be shown for the addition of C4-HSL. In the case of pyocyanin nearly wild type levels could be observed, whereas for elastase an activity level equal to the autoinducer synthase single mutants was monitored. This effect was not enhanced by the addition of

both AHL molecules. Complementation with saturating levels of 3-oxo-C12-HSL had no effect at all, which indicated a major impact of PA2226 on the Las-system other than signal degradation.

3.3.3 AHL reporter assay

Strain PA2226 did not respond to exogenous 3-oxo-C12-HSL and did not show autoinduction even though the 3-oxo-C12-HSL synthase was not impaired (Figure 27) and autoinducer was produced at a basal level (Figure 10). As the LasR-3-oxo-C12-HSL complex controls the regulation of the autoinducer synthases and other Quorum Sensing controlled genes, the role of the LasR protein was further investigated. The AHL reporter plasmid pKR-C12, comprised of *lasR* controlled by *lacZp* and GFP controlled by the QS active *lasBp*, was used to study the basic regulatory mechanism and components of the Las-system. The sensor plasmid was studied in *E. coli* uncoupled from the *P. aeruginosa* genetic background to further investigate if PA2226 blocks Quorum Sensing independently from other regulatory elements of the QS system.

As presented in Figure 17 heterologous expression of PA2226 blocked QS depended GFP expression as it did for the tested QS phenotypes of *P. aeruginosa.* GFP expression is shown 6 h after induction with saturating levels of 3-oxo-C12-HSL, for complete curves see Appendix Figure A 1.

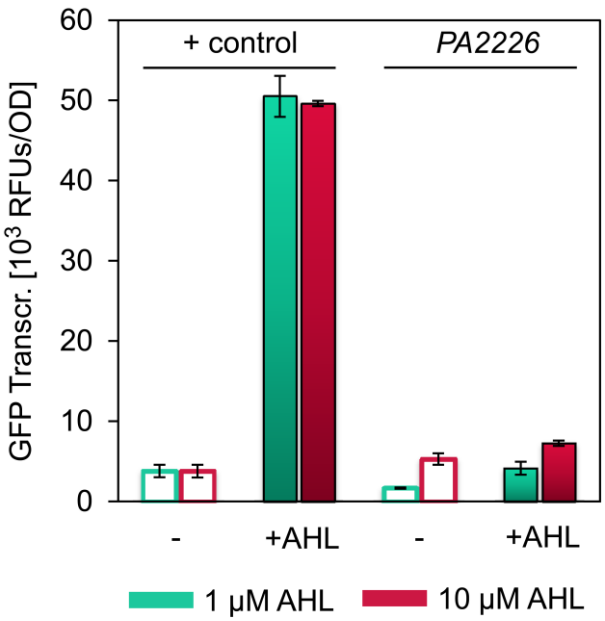

Figure 17: Heterologously expressed PA2226 blocks QS. *E. coli* expressing *lasR*, a *lasB* reporter and harboring either control or *PA2226* expressing vectors were incubated with inducing concentrations of 1 µM (filled green bars) or 10 µM (filled red bars) exogenous 3-oxo-

C12-HSL and fluorescence was measured after 6 h. Not induced cultures were used as controls (unfilled bars). Results are representatives from at least two independent experiments in triplicate.

As shown PA2226 seems to be involved in signal perception rather than signal degradation leaving two remaining options. PA2226 could bind to the QS active promoter and inhibit transcription or interact directly with LasR to inhibit QS activation. Earlier activation of QS has been reported for deletion mutants of LasR binding proteins, therefore, an QS reporter was measured in the background of ΔPA2226 and the wild type. Activation of GFP from *lasBp* occurred to the same point in time as for the wild type but reached finally higher expression in ΔPA2226 throughout growth.

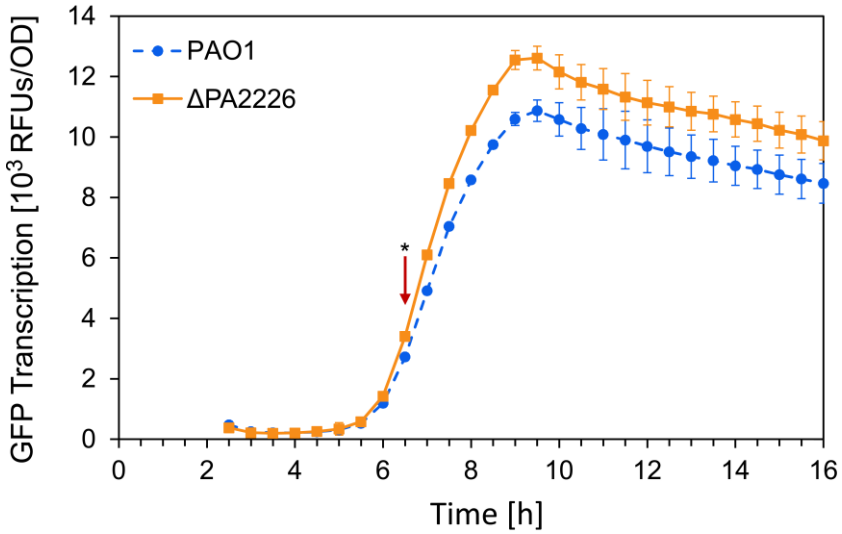

Figure 18: QS activation in ΔPA2226. Reporter activity was measured in *P. aeruginosa* PAO1 and ΔPA2226 expressing a transcriptional *lasB* reporter. Results are representatives from at least two independent experiments with seven measurements for each data point. Difference in expression was significant (p≤0.001) after 6.5 h of growth (*).

3.3.4 DNA binding ability of PA2226

PA2226 was used in DNA binding studies to test its ability to block QS by binding to QS depending promoters and alter their transcription. Therefore soluble PA2226 was purified as MBP fusion protein. The MBP-LacZα was equally purified to exclude an interfering DNA binding activity of the MBP (Figure 19, I). Protein separated on a SDS-gel after MPB affinity purification yielded distinct bands of ca. 61 kDa for MBP-PA2226 and 50 kDa for MBP-LacZα, matching the theoretical protein sizes. LasR was purified as described by Liu et al. 2009 as positive control (data not shown). The physiological activity of the MBP-PA2226 fusion was reinsured by its expression in PAO1 and qualitatively

assessing the pyocyanin concentration (Figure 19, II). The fusion inhibited the pyocyanin formation to the same extend as the unmodified PA2226.

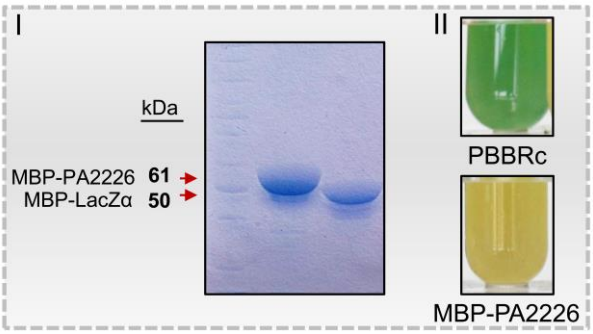

Figure 19: Purification of recombinant MBP-PA2226 and verification of its activity. (I) SDS-PAGE of recombinant protein after amylose affinity purification. Soluble PA2226 could be expressed and purified as MBP fusion protein with a theoretical size of 61 kDa. As control MBP-LacZα with a size of 50 kDa was purified. The protein ladder used was the PageRuler™ Unstained Protein Ladder 26614 (Thermo Scientific). (II) Pyocyanin formation after overnight incubation in PAO1 expressing the MBP-2226 fusion protein from pBBR1MCS-5 (MBP-2226) or harboring pBBR1MCS-5::*celA* (PBBRc), containing a cellulase gene for extra protein load as control.

The promoter OP1 site of *lasBp* was shifted completely by 25 and 50 pmol of purified LasR. In contrast, 42 pmol of PA2226 showed likewise as the negative control of 48 pmol MBP-LacZα no interaction with the DNA and hence no shifted band nor a reduction in band intensity (Figure 20).

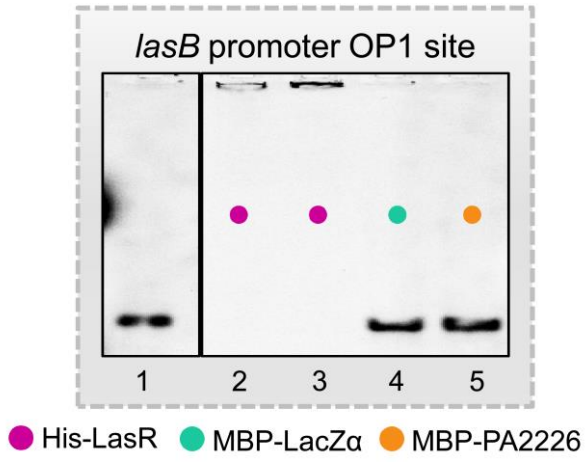

Figure 20: DNA binding affinity of PA2226. 0.25 pmol of a Cy5 labelled DNA probe containing the OP1 site of *lasBp* (1) was incubated with 25 pmol (2), 50 pmol (3) His-LasR, 48 pmol MBP-LacZα (4) or 42 pmol MBP-PA2226 (5). Formed protein-DNA complexes were separated on 8 % 1× TBE gels and the Cy5 label was visualized.

3.3.5 Protein-protein interaction of LasR and PA2226

After excluding a possible DNA binding ability of PA2226 to QS active promoters and thereby inhibiting QS activation, the direct interaction of PA2226 and LasR was tested. Crude extracts of heterologously expressed MBP-PA2226 and His-LasR protein were used in co-precipitation experiments. Co-precipitates eluted from MBP binding amylose resin were separated by SDS-PAGE (Figure 21, I) and immunoblotted with anti-His (Figure 21, II) and subsequently with anti-MBP antibodies (Figure 21, III).

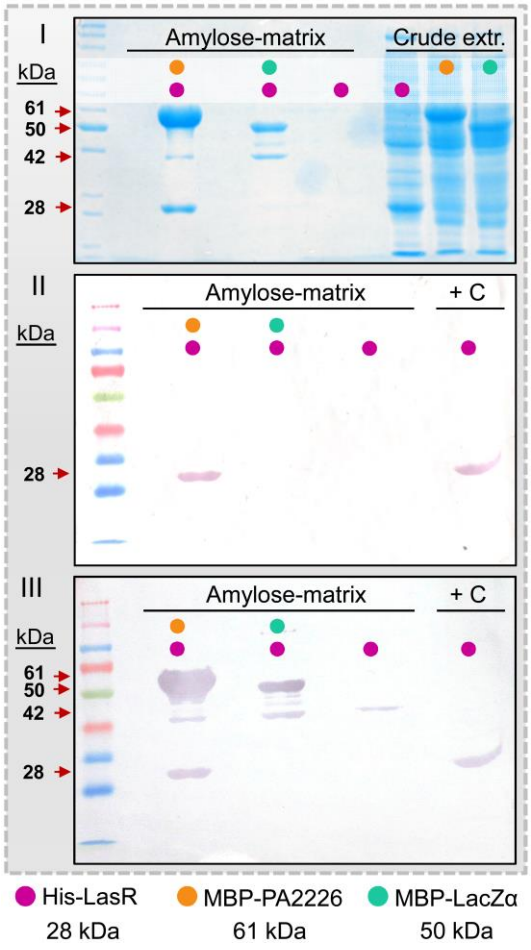

Figure 21: Analysis of PA2226 and LasR co-precipitation. Crude extracts containing His-LasR (magenta circle), MBP-PA2226 (orange circle) and MBP-LacZα (teal circle) were used in co-precipitation experiments with anti-MBP affinity chromatography. (I) SDS-PAGE of crude extracts and after affinity purification. (II) Immunoblot with anti-His antibody. His-LasR crude extract was used as positive control (+ C). (II) Anti-His treated immunoblot with anti-MBP antibody. PageRuler™ Unstained Protein Ladder 26614 (Thermo Scientific) was used for Coomassie stained SDS-gels and Spectra™ Multicolor Broad Range Protein Ladder 26634 (Thermo Scientific) for immunoblots.

Coomassie stained SDS-gel co-precipitation of MBP-PA2226 and His-LasR extracts resulted in two strong protein bands with a size of approximately 61 and 28 kDa. Additionally, a weaker band with the size of ca. 42 kDa was apparent (Figure 21). A band with the same size additional to a 50 kDa band was also visible in the control co-precipitation of MBP-LacZα and His-LasR. Another weak band appeared between these. As a specificity control for anti-MBP affinity chromatography no band was visible after incubation of His-LasR with the anti-MBP affinity matrix (Figure 21, I). In the immunoblot with anti-His antibody this specificity could be confirmed. No signal was detectable for the incubation with His-LasR and for MPB-LacZα and His-LasR although His-LasR was present in the crude extract, which could be seen from the positive signal in the control.

By this, a 28 kDa band from an assumed co-precipitation of MBP-PA2226 and His-LasR could be confirmed as His-LasR (Figure 21, II). In the subsequent immunoblot with anti-MBP antibody it could be shown for the remaining bands from the SDS-gel to contain MBP. Even in the affinity chromatography with only His-LasR containing crude extract a faint band at ca. 42 kDa was visible (Figure 21, III). Altoghether these data indicate that LasR specifically interacts with PA2226.

LasR as target for PA2226 was further tested by complementing strain PA2226 with a LasR encoding vector controlled by an inducible promoter (Figure 22). It is noteworthy, that although elastase activity could be restored after LasR induction (Figure 22, II), pyocyanin formation remained hampered (Figure 22, I).

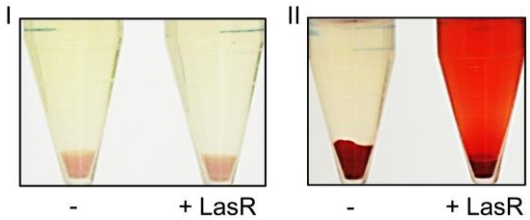

Figure 22: Recovery of QS by LasR complementation. Strain PA2226 was complemented by LasR expression from the vector pHERD20T::*lasR* after induction with 0.01 % arabinose. Pyocyanin formation (I) and elastase activity (II) were evaluated after overnight growth.

3.3.6 Transcriptome of strain PA2226

Parallel to the molecular characterization of PA2226 the global gene expression profile was studied in the background of a PA2226 overproducer. At this it was expected to see strong downregulation in expression for the genes involved in the tested QS phenotypes and indications for the reason of these regulations to assist in the molecular characterization, beside the elucidation of the impacted regulon.

For the four sequenced libraries, the biological duplicates from PBBR (PB_1, PB_2) and PA2226 (26_1, 26_2) cultures from exponential phase, read numbers between 22 and 51 million were sequenced and 60 to 66 % were successfully mapped to the *P. aeruginosa* PAO1 transcriptome. Overall rtRNA contamination was with 0.5 to 4.3 % very low in the aligned reads (Table 9). The average Phred quality scores over the read length were between 32 and 38.

Table 9: Sequencing and mapping statistics

Sample	Tot. Reads	Tot. Mapped	Unique Mapped	% Tot. Mapped	% mRNA	% rtRNA
PB_1	38,234,119	25,405,728	23,964,348	66.45	65.97	0.48
PB_2	24,695,273	14,847,183	13,378,222	60.12	58.26	1.86
26_1	51,020,046	32,679,851	30,648,855	64.05	63.63	0.42
26_2	22,635,549	14,1394,20	12,617,881	62.47	58.18	4.29

First, FPKM (***f***ragments ***p***er ***k***ilobase of exon per ***m***illion reads) values from the Cuffdiff output were compared for the two test conditions.

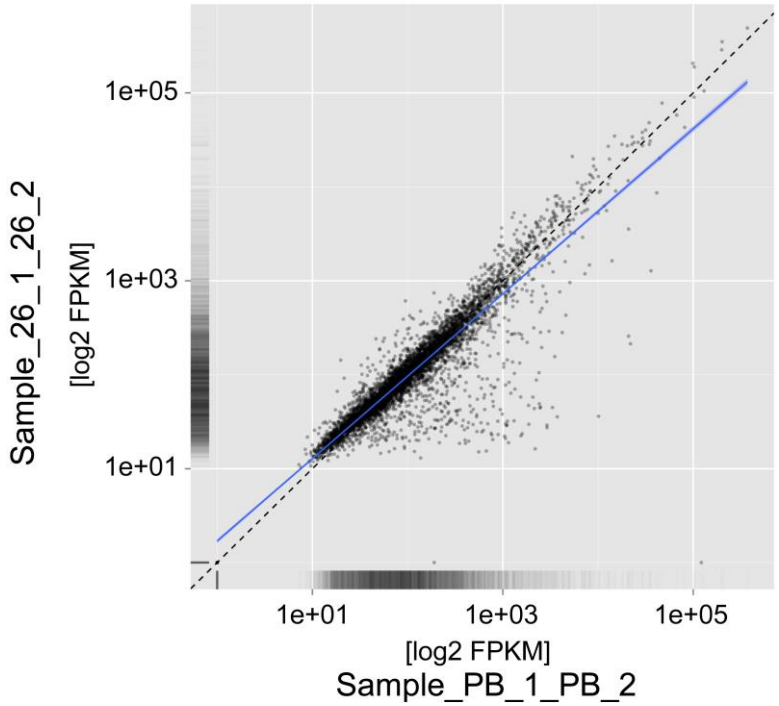

Figure 23: Pairwise sample comparison. Scatterplot of pairwise condition comparison of the mean FPKM values.

The majority of the genes scattered near 1 (dashed line), but the pattern showed already an imbalance towards the PB_1 and PB_2 samples (Figure 23) indicating reduced transcription for the most differentially regulated genes. The genes for rtRNA and pseudogenes were excluded from further analyses and apart from these three more genes could not be further evaluated in the analysis for significant differentially expressed genes because of too high data values in one of the samples. Cuffdiff skips loci with more than 10^6 aligned reads. Manual inspection of *PA2226*, *ssrA*, coding for the tmRNA, and *crcZ,* coding for a RNA involved in carbon catabolite repression control, in IGB revealed only for *PA2226* a visually significant difference in read coverage (Figure 24). As Cuffdiff overestimates FPKM values for very short transcripts the outcome for the most abundant gene transcripts in the samples has to be treated carefully. Among the 50 highest expressed genes in both strains were many small RNAs like *RsmY* and *RsmZ*, outer membrane proteins like *oprFGHI* and ribosomal proteins like *rmpHIJ*. Some highly expressed genes involved in QS like *lasB*, *lecB* and *rsaL* were only present in the control PBBR.

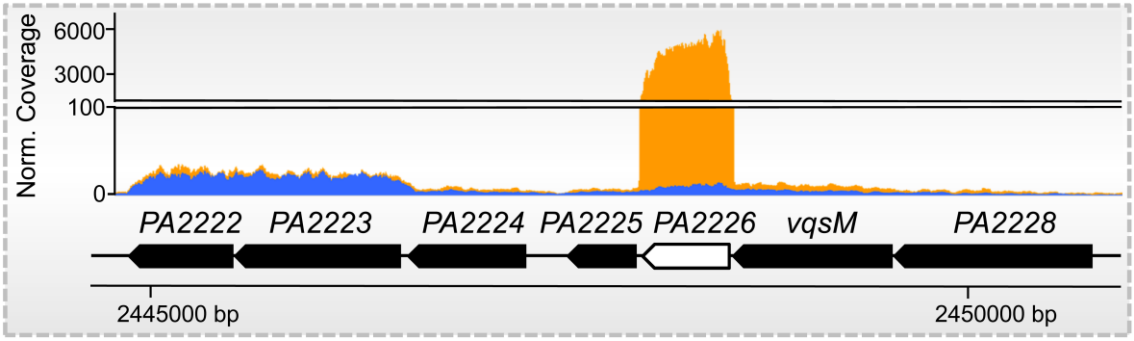

Figure 24: Transcription profile for the *PA2226* gene region. Normalized read coverage for the gene region surrounding *PA2226.* Transcription activity for strain PA2226 is displayed in orange, in blue for the control PBBR.

Differential gene expression analysis revealed 208 significantly regulated genes, 3.66 % of the PAO1 genome, scattered over the whole replicon. As could be presumed from Figure 23 the majority, with 190 genes (91.35 %), was down- and only 18 genes (8.65 %) were up-regulated (Appendix Table A 1, Table A 2). Hypothetical genes were distributed with the same rate to up- and down-regulated groups with 5 (27.78 %) and 60 genes (31.58 %), respectively. In Figure 25 a map of the PAO1 genome including the gene wise change in expression between conditions is shown. With more than 256-fold down-regulation the gene for the elastase LasB was the strongest affected gene. In contrast the highest up-regulated gene, the probable transcriptional regulator *PA1864*, was only 8-fold increased. Most genes only showed a fold change from 2 to −2 and thereby no

biological significant change in expression (Figure 25, white area). Some genes with a greater fold change and not marked as significant could be included by lowering the q-value to 0.1, but for others the biological variability between replicates is too high to call significance.

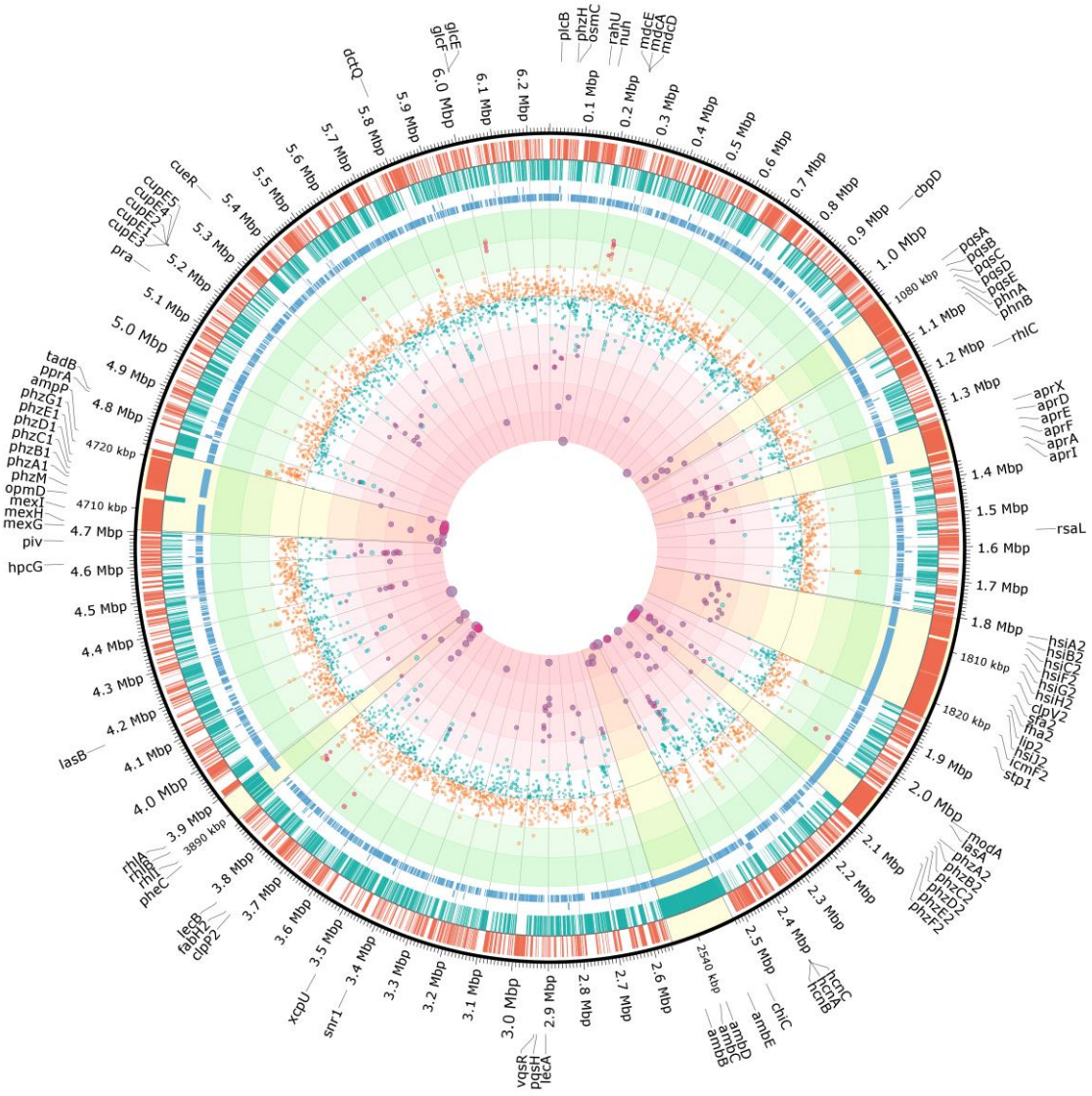

Figure 25: Map of the PA2226 transcriptome. Description from outermost to innermost circle: Labelings for significant regulated genes, if gene name available. Genomic coordinates in mega base pairs (Mbp), ORFs on the leading strand (dark orange), ORFs on the lagging strand (seagreen), operon structures (blue), log2 3, 2, 1 indicated in shades of green to white and log2 −1, −2, −3, −4, −5 in shades from white to light pink. Log2 fold change values of differences in gene expression of PA2226 compared to PBBR are plotted as orange dots, if up-regulated, as cyan dots, if down-regulated and are overlaid with magenta, if regulation is significant. Log2 cutoff for down-regulated genes is at −5. Dot sizes correlate with strength in expression change.

A classification to functional groups according to the *Pseudomonas aeruginosa* PAO1 Genome Project Function Class (Figure 26) taken from the Pseudomonas Genome Database (Winsor et al. 2011) exposed that a striking 33 % of genes belonging to the group of secreted factors is impaired in its transcription, followed by the group of genes involved in protein secretion and export with 15 %. Up-regulated genes were almost exclusively found in the two groups of carbon compound catabolism and central intermediary metabolism. It is noteworthy, that in gene groups essential for cell metabolism like cell division, DNA replication and repair, as well as in transcription not a single gene was affected. Likewise it may appear remarkable that not a single ncRNA seemed to be altered in its expression, but this is put into the perspective by the fact that for small RNAs a different sequencing protocol is needed for trustworthy evaluation.

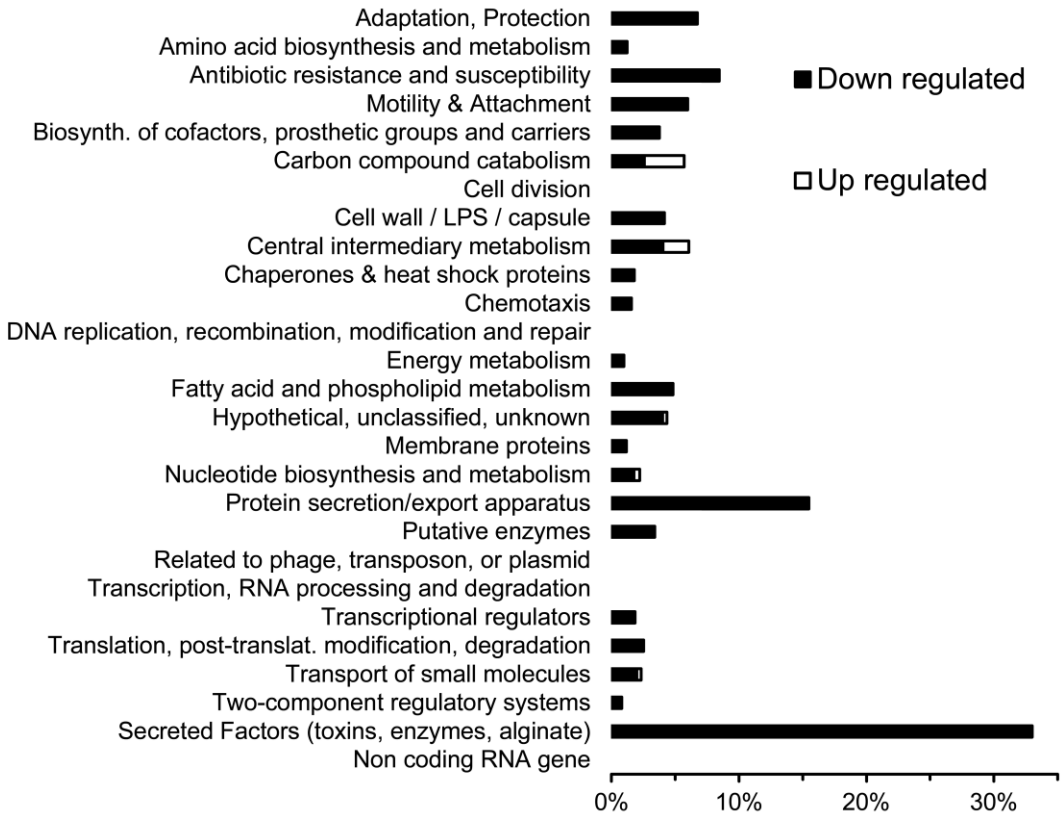

Figure 26: Functional classes of PAO1 affected by PA2226. Differentially expressed genes were assigned to functional classes and the fraction of affected genes was evaluated in respect to the total number of genes belonging to a class. Functional classes were taken from Pseudomonas Genome Database (Winsor et al. 2011). Genes can be assigned to more than one class.

If the distribution to classes and its evaluation was limited to only the total number of differential expressed genes ca. 50 % belonged to hypothetical genes and putative

enzymes. But even then, second and third most abundant classes were secreted factors (15 %) and protein secretion (11 %) (Appendix Figure A 2).

The affected enzymes from the secreted factors class were mainly proteases, like the alkaline protease, protease IV and elastases LasAB. Furthermore, the synthesis genes for pyocyanin and rhamnolipids belong to this class and were down-regulated as well as the *ambBCDE* cluster, which encodes for two nonribosomal peptide synthetases. The fraction of regulated genes in the class of protein secretion were mostly the genes for the second type VI secretion system of PAO1, probable secretion proteins and *xcpU* which is part of the Xcp-related type II secretion system (Nunn and Lory 1993, Filloux et al. 1998).

Strain PA2226 showed these strong inhibitions in QS related phenotypes, but the *lasRI* genes were not part of the differentially regulated genes, neither were it *rhlR* nor *pqsR*. A closer look at the transcription coverage profiles revealed indeed same transcription rates for *lasRI* but elevated levels of *rsaL*, the *lasI* negative regulator, in the control (Figure 27, I).

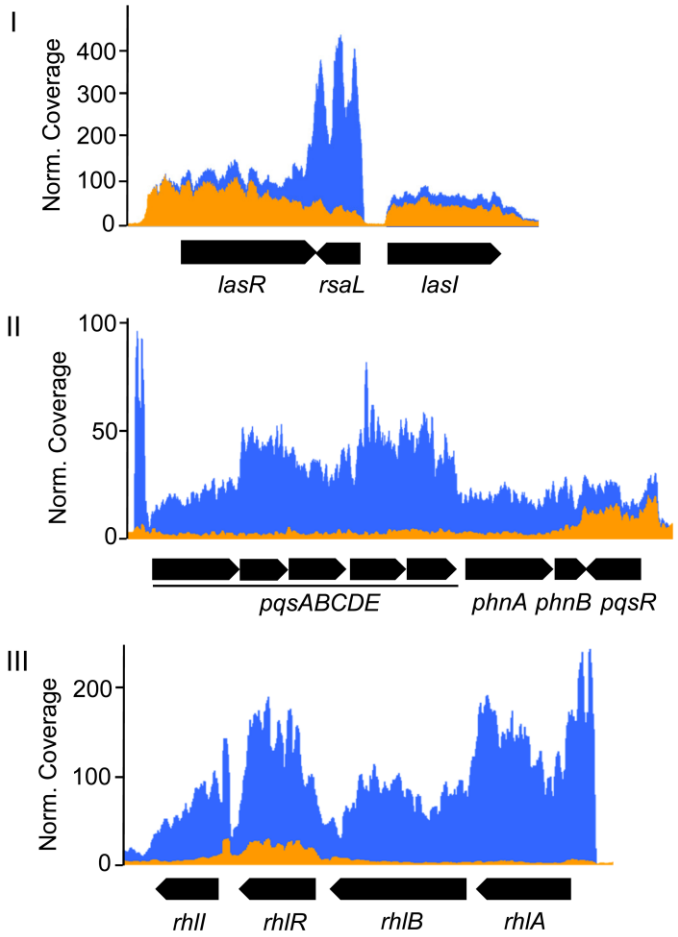

Figure 27: Transcription profiles of the autoinducer synthase clusters. Normalized read coverage for the gene regions of *lasRI* (I), *pqsABCDE* (II) and *rhlRI* (III). Transcription activity for strain PA2226 is displayed in orange, in blue for the control PBBR.

The synthesis genes for the PQS signal were not activated in strain PA2226, as well as the synthase genes *phnAB* for the PQS precursor anthranilate although the transcriptional regulator *pqsR* seemed to be equally transcribed in both strains (Figure 27, II). For the *rhl* operon a difference in the transcription of *rhlR* is visible (Figure 27, III) and *rhlR* could be added to the list of differentially expressed genes by lowering the q-value to 0.1.

Recently, the QS regulon of PAO1 and the core QS regulon were examined by assessing the diversity in strain dependent QS gene expression (Chugani et al. 2012) by RNA-seq. A comparison of these datasets with the regulon of strain PA2226 revealed a great overlap when compared to the regulon of PAO1 (Figure 28, I, left panel) and a match of nearly 100 % with the found core QS-regulon (Figure 28, I, right panel). As the up-regulated genes were not reported a comparison was not possible. Comparisons presented in Figure 28 II and III are with data sets from a *vqsM* and an *mvaT* deletion mutant in which *PA2226* appeared up-regulated. The overlap between down-regulated and up-regulated

datasets from the *mvaT* mutant was virtually non-existent (Figure 28, II), just as the overlap with the up-regulated set of genes in the *vqsM* mutant, whereas here 88 regulated genes were shared in the down-regulated set (Figure 28, III).

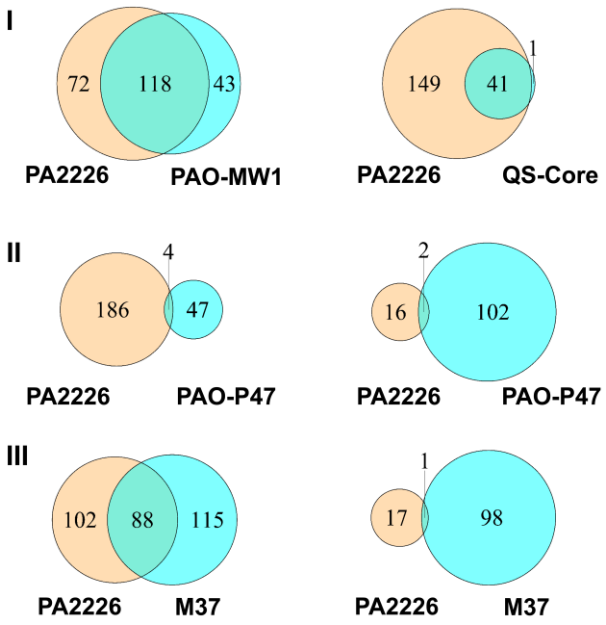

Figure 28: Comparisons of transcription regulons. The set of down-regulated genes from PA2226 was compared to the regulon of a *lasI/rhlI* deletion mutant (PAO-MW1, I, left panel) and to a core QS regulon (I, right panel). Comparisons with transcriptomic studies of *mvaT* (PAO-P47) and *vqsM* deletion mutants (M37), in which *PA2226* was found to be up-regulated, are presented for the down-regulated (II,III, left panel) and up-regulated gene sets (II, III, right panel).

4 Discussion

Pseudomonas aeruginosa PAO1 was first isolated in 1954 and has become the reference strain in *Pseudomonas* genetics and functional analyses (Holloway 1955, Holloway et al. 1979). In the year 2000 it was the first *Pseudomonas* which had its full genome sequenced (Stover et al. 2000). This analysis revealed that nearly 10 % of all annotated genes are involved in regulation. Nearly 60 years later PAO1 is one of the best studied bacteria of our time, but it is hardly surprising that we are still far away from a deep understanding of the complex regulatory network of this bacterium and that year after year new regulating factors and new functions and interconnections are found. Up to date there are still 2025 hypothetical genes with no distinct function assigned leaving room for further discoveries.

The QS system in *Pseudomonas* contributes to the virulence of this opportunistic pathogen which makes it a promising target for infection control. Extensive research has expanded this system more and more and has moved far from the start of its elucidation making it one of the most complex QS systems studied.

It has been shown that the enzymatic degradation of autoinducer molecules is an efficient approach (Schipper et al. 2009, Bijtenhoorn et al. 2011, Sio et al. 2006, Czajkowski and Jafra 2009) to control virulence of *Pseudomonas*. Acylases, lactonases and oxidoreductases have been shown to cleave or modify the autoinducer molecule and thereby preventing activation of QS and the controlled phenotypes like elastase, pyocyanin, hydrogen cyanide and biofilm formation. For the metagenome derived oxidoreductase BpiB09 nearly complete deactivation of these controlled phenotypes was shown when expressed in PAO1. On the other hand, while measuring the direct activity of BpiB09 on reducing the autoinducer molecule 3-oxo-C12-HSL by HPLC only limited turnover was observed.

In this study the global transcriptome of the BpiB09 overproducing PAO1 was analyzed, to better understand the influence of BpiB09 on the regulatory network of *Pseudomonas* apart from degrading the autoinducer. The subsequent analysis of the genomic sequence identified a major deletion in the strain carrying the *bpiB09*, possibly responsible for many of the observed effects rather than BpiB09 itself. By combining results from the transcriptomic and the genomic analysis a gene cluster for further analysis was selected and finally the hypothetical protein PA2226 as responsible effector identified. The molecular mechanism of action was elucidated for this novel QS regulator and its distinct regulon was analyzed.

4.1 Transcriptome and genomic analyses of PAO1 pBBR::*BpiB09*

To better understand the strong QQ influence of BpiB09 on PAO1, which was not satisfactorily explained by the low turnover rate of the autoinducer 3-oxo-C12-HSL, the transcriptome was analyzed by NGS from exponentially growing planktonic cultures. Cells were harvested after 5 h growth in rich medium since from here on growth of the cultures started to diverge and the control PBBR remained static for an hour and reached afterwards never the same high density as PAO1 pBBR::*bpiB09.* At the sampling time point the OD of the cultures was approximately the same, allowing direct comparisons of gene expression between the strains. Despite a slight difference in growth being visible, it was not significant. That the QS deficient PAO1 pBBR::*bpiB09* was not hampered in its growth is in line with findings for other QS deficient PAO1 strains. It has been shown for *lasR, rhlR* and *pqsR* deletion mutants that growth in not QS requiring media is overall the same and only in QS requiring media the growth of the *lasR* and *rhlR* mutants was impaired mainly because of the significantly lesser production of proteases Wilder et al. 2011.For each cDNA library at least 100,000 reads were obtained by 454 sequencing and mapped to the PAO1 genome. 454 sequencing has been used in other transcriptome studies to identify novel transcripts in *Sinorhizobium meliloti* and to assess the primary transcriptome of *Helicobacter pylori* by sequencing between 19,000 and 500,000 reads (Mao et al. 2008, Sharma et al. 2010), thus the achieved read numbers were assumed as adequate. In total, 831 genes were found to be influenced in PAO1 pBBR::*bpiB09* which are 14.6 % of all genes and is slightly higher as the reported 11 % for the transcriptome of a *lasI/rhlI* double mutant (Wagner et al. 2003). Other studies reported between 2.9 and 6 % differentially expressed genes (Schuster et al. 2003, Hentzer et al. 2003) for this double mutant and although all three studies used microarrays this shows the variability dependent on the used experimental setup. By comparing the shared subset of 85 QS activated genes from these studies with the 831 found genes, 38 genes connected to QS were identified (Table 7). Among these genes *lasI, rhlI, ambCD* and *pqsABCD* could be identified, thus including autoinducer synthesis genes for both AHLs, for the Pseudomonas quinolone signal, as well as for the recently discovered fourth Quorum Sensing molecule IQS, suggesting none or only weak transcription of these genes in *P. aeruginosa* cells carrying copies of *bpiB09* in comparison to the control strain. Hence, QS controlled genes for a virulent phenotype were suppressed, like *hcnA* (involved in hydrogen cyanide synthesis)*, lasB* (elastase) and *phzM* (involved in pyocyanin synthesis), which is concordant with experimental data of the PAO1 pBBR::b*piB09* phenotypes (Bijtenhoorn et al. 2011). This once again confirms the broad impact BpiB09 seemed to have on the entire core QS elements. It is noteworthy

that the only genes involved in biofilm formation affected were genes for motility like the gene for the major flagellin protein *fliC*, some other flagellar genes and the rhamnolipid synthases *rhlAB*. The swarming motility as well as the biofilm formation is impaired in PAO1 pBBR::*bpiB09*, whereas other work has demonstrated an inverse regulation of these phenotypes (Kuchma et al. 2007, Shrout et al. 2006, Kirisits et al. 2005).

Surprisingly, only 8 up-regulated genes were found and 7 of these were even consecutive (Table S3 Bijtenhoorn et al. 2011, Figure 7). 6 of these were annotated as hypothetical genes and a BLAST search did not provide any further information. The remaining gene was *vqsM* (*PA2227*), which up-regulation conflicted with the overall detected down-regulation of QS-dependent genes and the studies about *vqsM* describing it as activating Quorum Sensing modulator (Dong et al. 2005, Liang et al. 2014). Deletion of *vqsM* lead to reduced AHL production and virulence, which was restored after complementation. Moreover, the gene expression analysis showed among other up-regulated genes the same cluster from *PA2222-PA2226* with an up-regulation between 20 to 120-fold, which was not further evaluated (Dong et al. 2005).

The genomic PAO1 pBBR::*bpiB09* sequence was analyzed by using the 454 reads from the transcriptome analysis. 271,875 reads were aligned to the reference genome of PAO1 resulting in nearly complete sequence coverage of 99.16 % with an average read depth of 15.1. Two structural rearrangements in respect to the reference sequence were detected with 100 % confidence in PAO1 pBBR::*bpiB09* which were not found in the control strain with the empty vector (Table 8). The short 96 bp deletion was located in the middle of *PA0041* which is annotated as probable hemagglutinin. The exoprotein PA0041 is genetically and functionally linked to its transporter PA0040 and increased levels were observed in culture supernatants of QS mutants (Jacob-Dubuisson et al. 2001, Nouwens et al. 2003), however no regulating activity for itself is known. The second deletion encompassed remarkable 19 kb, nearly the complete *psl*-operon from *pslA* to *pslL* and the three hypothetical genes *PA2228-PA2230* and could be additionally verified by PCR (Figure 8). The *psl* genes were not suspicious in the transcriptome in the first place, which can be explained by the fact that planktonic cultures were sampled for which genes involved in biofilm formation are not active since planktonic and sessile cells have distinct gene expression patterns (Dotsch et al. 2012). PSL is beside of PEL one of the major structural components of the non-mucoid *P. aeruginosa* biofilm matrix (Ryder et al. 2007) and different from PEL the extracellular polysaccharide (EPS) PSL is essential for the biofilm maturation of PAO1, even if both synthesis clusters are present (Colvin et al. 2011, Colvin et al. 2011). For the *pslBCD* deletion mutant it was shown that the ability to form mature biofilms was lost. The same genes are affected in PAO1 pBBR::*bpiB09* by the 19 kb deletion and it can be concluded that despite other

QS influences this is the main reason for the poor biofilm formation of this strain, thereby explaining the inverse regulation of motility and biofilm formation. In addition, the deleted region lies right beside *PA2222-PA2227* and affects approximately half of *PA2228*, which are the same genes up-regulated in PAO1 pBBR::*bpiB09* and in a *vqsM* deletion mutant with strong impairment in QS regulated genes. Further, PAO1 strains retransformed with pBBR::*bpiB09* showed no impairment in pyocyanin formation. Therefore it was speculated that one of the genes from *PA2222* to *PA2228* is responsible for the complete QS shut down rather than BpiB09.

4.2 Identification of a QS active protein and its molecular mechanism of action

The genes from *PA2222* to *PA2228* were cloned separately and were overexpressed in PAO1 to resemble their regulation in PAO1 pBBR::*bpiB09.* Even if the impact of the deletion of *vqsM* is already described (Dong et al. 2005) *vqsM* was nevertheless included to measure the specific consequences of its overexpression in a non-mutated background. As a first indicator for interference with QS the pyocyanin formation was assessed qualitatively after overnight growth and PA2226 alone was found to inhibit and VqsM to raise the pyocyanin formation. Elevated levels of pyocyanin where expected for *vqsM* expression, because of its nature of being a QS activator. The other hypothetical proteins showed no difference to the control. In consequence the subsequent work was focused on *PA2226*. *VqsM* was included for control purposes in some experiments.

Prior to in depth analysis of *PA2226* and in terms of evaluating its impact on QS *lasI* and *rhlI* signal single and double deletion mutants were created via homologous recombination as positive controls. Likewise a *PA2226* deletion mutant was created to determine not only the effects of overproduction but also of absence of *PA2226.* Mutations could be confirmed via PCR (Figure 9) and for the signal deletion mutants it was additionally demonstrated that the relevant phenotypes were impaired and accordingly restored by wild type gene expression in *trans* as well as by exogenous added autoinducer (Figure 12, Figure 16).

Phenotypes connected to QS and virulence were measured for the PA2226 overproducer, the *PA2226* deletion mutant and the VqsM overproducer (Figure 10, Figure 11).

4.2.1 Overexpression of VqsM

Apart from a strong ca. 80 % elevated pyocyanin formation, which was already observed in the qualitative testing, VqsM had no measurable enhancement effect on protease

activity, swarming or concentration of 3-oxo-C12-HSL in the supernatant beyond wild type levels. It has been shown that a *vqsM* transposon mutant was deficient for several Quorum Sensing phenotypes and that a complementation was possible, but no increased pyocyanin formation was reported (Dong et al. 2005). Elsewise than the reported direct binding to the *lasI* promoter (Liang et al. 2014) and thereby proposed regulation of QS, in this study the only increased phenotype was an RhlRI controlled one. The Las-system activates the Rhl-system thus activation of pyocyanin formation is possible in this way. But this is rather unlikely because no other QS controlled phenotype is enhanced, hence it seems more convincing that VqsM controls pyocyanin in another way. The interaction of VqsM with the *lasI* promoter was shown *in vitro* but no consequences of this were shown *in vivo*, thus the direct influence of VqsM on QS remains unclear. Otherwise it was shown that VqsM binds to its own promoter region and that in the *vqsM* deletion mutant transcription of *PA2228* is up-regulated and when complemented with *vqsM* down-regulated to wild type level. An operon structure is computationally predicted for *PA2226*, *VqsM* and *PA2228*, which is here supported by the operon prediction from the RNA-seq read data from the PA2226 transcriptome analysis, which additionally designates *PA2225* belonging to this operon. In this framework transcription of the operon is activated by a so far unknown factor and negatively regulated by VqsM, which explains elevated expression of *PA2225-PA2228* in a *vqsM* deletion mutant. Because of the assumably deleted operator region in front of *PA2228* high expression of *vqsM* had no influence on the operon's transcription in PAO1 pBBR::*bpiB09* but the positive regulation of pyocyanin formation is nevertheless inhibited, indicating another strong deactivating factor in the regulation cascade, which is described in the following.

4.2.2 Overexpression of PA2226

Overexpression of PA2226 resulted in an impairment of all tested phenotypes to levels of ΔlasI or even ΔrhlI/lasI. In detail elastase and other protease activities were completely abolished, pyocyanin and 3-oxo-C12-HSL formation were reduced to basal levels and swarming was narrowed, reasoning that PA2226 alone is responsible for an almost complete shut-down of QS in *P. aeruginosa*. It is generally accepted that biofilm formation is guided by QS (de Kievit 2009, Fazli et al. 2014) and it was at first shown for a *lasI* mutant to be unable to form fully matured biofilms (Davies et al. 1998). However, despite the strong impact of PA2226 on other QS controlled phenotypes the biofilm formation remained intact, but likewise signal deletion mutants showed the same growth as the wild type, questioning the importance of QS in biofilm formation (Figure 13, Figure 14). The detection of reduced biofilm formation is possible under the here used conditions as can be seen from the results of the tested PAO1 pBBR::*bpiB09* (Bijtenhoorn et al. 2011),

which, as discovered in this work, is deficient in PSL production. On the other hand biofilm formation is strongly influenced by the used carbon source (Shrout et al. 2006). Differences in the biofilm structure between QS mutants and the wild type were observed with succinate as sole carbon source, whereas biofilm formation did not differ with glucose and glutamate. Also indistinguishable biofilm formation between the wild type and QS mutants has been shown before for growth under different flow conditions and only slightly different experimental changes to Davies et al. (Purevdorj et al. 2002, Heydorn et al. 2002). The biofilm formation seems to be very dependent on the distinct experimental parameters and environmental conditions and assumptions on QS and biofilm behavior should be made carefully. Under the here used conditions QS appears not relevant for biofilm formation.

When *PA2226* was expressed under the control of an inducible promoter the impact on QS is proportional to the inducing IPTG concentrations. Therefore, the degree of QS inhibition depends directly on the concentration of mRNA or formed protein, is tunable and no on-off switch (Figure 15).

4.2.3 Molecular mechanism of action of PA2226

PA2226 orthologs are found exclusively among other *Pseudomonas aeruginosa* strains (Appendix Table A 3) and likewise blastn and blastx searches only returned hypothetical protein hits from *P. aeruginosa* strains. The lowest blastn identity was 78 % with 94 % query coverage and an E-value of 2e-72. PA2226 and its orthologs are highly conserved among *P. aeruginosa* but up to date have not been characterized.

Nearly no 3-oxo-C12-HSL was measured in cultures expressing PA2226 and given that sufficient autoinducer is essential for Quorum Sensing activation and the LasRI system is the highest ranked QS system in *P. aeruginosa* the possibility was raised that PA2226 interferes with signal synthesis or degrades the autoinducers. Although neither domains nor motifs were found in the protein or DNA sequences, which encouraged this. As signal synthases themselves are QS controlled (de Kievit et al. 2002, Seed et al. 1995) the low signal level could also be the consequence of action at other central points of the regulatory network. Cultures of PA2226 were complemented with inducing concentrations of C4-HSL and/or 3-oxo-C12-HSL and recovery of QS phenotypes was possible only for C4-HSL (Figure 16). Activity of autoinducers was confirmed by complementing the respective signal single and double mutants. Pyocyanin formation was complemented by C4-HSL in the same way as in ΔrhlI and additional 3-oxo-C12-HSL had no increasing effect. Otherwise elastase activity was complemented only to the level of a non-induced signal single mutant. This indicates that for the inhibition of the Las-system degraded

autoinducer or impaired production of these is not the reason, which can also be seen from the transcriptome analysis of PA2226, where *lasI* is transcribed at a constitutive level and the basal concentration of 3-oxo-C12-HSL, which was measured in the supernatant. Different from this the Rhl-system is deactivated, because not sufficient C4-HSL is present. The C4-HSL production is controlled by the Las-system which could not be complemented and remained inactive, therefore QS inhibition is likely due to 3-oxo-C12-HSL perception.

The cognate 3-oxo-C12-HSL response regulator is LasR and its role in QS inhibition in PA2226 was assessed via an AHL reporter assay. In addition the pKR-C12 AHL-reporter was used in *E. coli* so the only present parts of the *P. aeruginosa* QS system were constitutively expressed LasR and the promoter *lasBp* with a fused GFP, so PA2226 could be studied detached from the PAO1 genetic background. The *lasBp* is well studied (Rust et al. 1996) and 3-oxo-C12-HSL is most efficient in stimulating its activity (Passador et al. 1996). Parallel expression of PA2226 inhibited GFP expression from *lasBp* even at high levels of 3-oxo-C12-HSL (Figure 17). 10 µM were about three times the concentration which has been measured for stationary phase cultures supernatants (Chugani et al. 2012). This shows that the inhibition of QS in strain PA2226 is only dependent on PA2226, LasR and promoters regulated by QS. In the transcriptomic analysis it could be seen that the *lasR* transcription in strain PA2226 was the same as in the control, which could exclude the hypothesis that PA2226 inhibits *lasR* expression. On the other hand, *lasI* transcription was also the same in both strains, but in the control the negative regulator *rsaL* was already highly up-regulated and *lasI* thereby negatively regulated to basal expression. The *lasR* expression is dependent on and controlled by *vfr* (Albus et al. 1997, Fuchs et al. 2010) for which same expression levels in both strains could be measured. Moreover, in the QS-reporter pKR-C12 *lasR* is transcribed under control of *lacZp* demonstrating that PA2226 inhibits LasR post transcriptionally and inhibited *lasR* transcription is not the reason for QS impairment. As the signal concentration has already been ruled out via the complementation assays, two main possibilities remained: PA2226 binds to QS active promoters and inhibits transcription of genes activated by the LasR-3-oxo-C12-HSL complex or interacts with LasR and thereby prevents QS activation.

First, the DNA binding ability of PA2226 was tested and the main OP1 binding site of *lasBp*, the same promoter as in the QS-reporter, was therefor used. The protein PA2226 was expressed as MBP fusion protein and to reassure that MBP had no negative effect on the activity of PA2226 the fusion protein was also expressed in PAO1 (Figure 19). Pyocyanin formation was inhibited in the same way as in strain PA2226, concluding that the fusion of MBP had no negative influence on the molecular mechanism of PA2226. But even with no added competitor DNA to the shift assay PA2226 showed no binding

activity, entirely different than the used positive control LasR, which bound the promoter and shifted the DNA probe (Figure 20). This result was expected as PA2226 does not contain any distinct DNA-binding domain.

Finally, a protein-protein interaction of PA2226 and LasR could be shown by co-precipitation (Figure 21). His-LasR was exclusively eluted from an anti-MBP matrix when MBP-PA2226 was present, which was confirmed by immunoblotting. Additional MBP containing bands to the main MBP and MBP-22226 protein bands were detected, but as stated by the manufacturer such degraded fusion proteins are common. In that case several bands could be observed between the size of the fusion protein and 42 kDa, the size of MBP. The single detected MBP containing band with only LasR crude extract used can be explained by the fact that the *E. coli* host exhibits its own *malE* gene, whose protein was detected.

Anti-activators which regulate QS by interacting with LuxR-type regulators have been observed before. For *A. tumefaciens* TrlR, TraM and its homologue TraM2 have been described to interfere with QS by protein-protein interaction with TraR (Chai et al. 2001, Hwang et al. 1999, Wang et al. 2006). For *P. aeruginosa* there are so far two proteins known to interact with LasR and to control QS. It was shown that QteE influences the stability of LasR and that QslA controls the 3-oxo-C12-HSL concentration needed for activation by binding to LasR and thereby both raise and define the QS threshold (Siehnel et al. 2010, Seet and Zhang 2011). QslA interacts with the ligand binding domain and it was further shown that QslA disrupts LasR dimerization and thereby preventing LasR from binding to its target DNA Fan et al. 2013). For their deletion mutants loss of the QS threshold and premature activation of QS was demonstrated. PA2226 binds equal to QslA to LasR, but for ΔPA2226 loss of the QS threshold could not be confirmed (Figure 18). Activation of GFP from *lasBp* occurred to the same point in time as for the wild type but reached finally higher expression in ΔPA2226 throughout growth, suggesting a different role of PA2226 from the other two known regulators in PAO1. Judging from these results PA2226 could be potentially a deactivator or tuner of QS to prevent excessive production of QS controlled factors, otherwise the deletion of *PA2226* had no measurable effect for the tested phenotypes. PA2226 shows no sequence homology (Table A 4) to other QS anti-activators but it remains possible that PA2226 functions similar to one of the so far known proteins, because anti-activators may share little sequence similarity (Lazdunski et al. 2004).

To reassure that the observed protein-protein interaction between LasR and PA2226 is the reason for the complete shutdown of QS in strain PA2226, it was complemented by LasR in *trans* (Figure 22). Indeed, induction of *lasR* transcription could restore elastase activity to wild type levels, showing that the block of QS by PA2226 can be overcome by

excess LasR. On the other hand the pyocyanin concentration could not be restored. This suggests that LasR is not the only target in PAO1 and that PA2226 has at least one more interaction partner which is LasR independently regulated. For QteE apart from its ability to lower LasR concentrations an independent inhibition of RhlR was observed. This is not surprising because LasR and RhlR are both LuxR-type regulators and share high similarity in their C-terminal HTH-domain. For PA2226 this second interaction is also possible but would not explain the lack of pyocyanin activation, because with activation of the Las system the Rhl system should be activated accordingly. And this can be seen from the completely restored elastase activity. Activation of *lasB* by only one system would have led to only half the activity level as shown in Figure 16. The pyocyanin formation is beside from the Rhl system controlled by the PQS system and is highly dependent on PqsE (Farrow et al. 2008). In the AHL complementation tests C4-HSL at a concentration of 10 µM was not successful in restoring the wild type phenotypes. The successful concentration of 100 µM seems very high and is more than 3 times the concentration reported in supernatants of *Pseudomonas* cultures (Chugani et al. 2012), but resembles the concentration which was at least needed to complement a *pqsE* deletion mutant (Farrow et al. 2008). The conclusion was that a *pqsE* mutant is less sensitive to C4-HSL but that it can respond to C4-HSL, suggesting that RhlR is active in a *pqsE* mutant. This is also congruent with the inability to complement the VqsM mutant, in which *PA2226* is upregulated, with only 50 µM C4-HSL (Dong et al. 2005). The importance of *pqsE* to the QS circuit has already been described earlier. The PQS controlled regulon is not only dependent on PqsR and produced HAQs/PQS, but highly relies on *pqsE.* It was demonstrated that the loss of PqsE function, and not only PQS production, was responsible for the phenotypes of *lasR* or *pqsR* mutants previously solely attributed to PQS production deficiency (Deziel et al. 2005).

This implies that PA2226 has, other than only on LasR, presumably an impact on PqsE or the entire PQS operon either through direct interaction with PqsE or PqsR or other unknown intermediary factors.

During completion of this study a first report on *PA2226* was published (Kohler et al. 2014). Independently from this study the upregulation of *PA2226* in consequence of an adjactend deletion was found to be responsible for strong QQ phenotypes, while studing “QS-cheaters”, bacteria which do not respond to QS but use public goods produced by QS-cooperators. It was also found that the deletion of *vqsM* was not affecting QS regulation, by this confirming also the results from this study, that upregulation of *vqsM* does not result in broad QS activation. The authors were not able to rescue any of the QS phenotypes by expressing LasR in single copy from its own promotor. This shows that the distinct stoichiometry is a crucial factor for the inhibitory activity of PA2226 and

strengthens the finding of direct protein-protein interaction of this study. In consequence the authors were not able to provide any evidence of how *PA2226* affects the QS system. Apart from this the concerted action of PA2226 and PA2225 on inhibition of the type III protein secretion system (TTSS) was demonstrated.

4.3 Transcriptome of strain PA2226

Simultaneously to the elucidation of the molecular mechanism of PA2226 its distinct regulon was specified by the use of RNA-seq. Cultures grown to an OD of 2.0 were used to make results comparable to a study in which the Quorum Sensing regulon was determined with NGS as well (Chugani et al. 2012). For each sample at least 12 million uniquely mapped reads were achieved, up to 30 million for a single sample. For *E. coli* sized genomes 5-10 million reads were suggested to detect all but a few of the lowest expressed genes (Haas et al. 2012). If calling differential expressed genes from biological replicates numerous genes can be detected with high statistical significance even when the number of fragments per sample is reduced to 2-3 million. Hence, the here achieved read numbers are more than sufficient to identify differential expressed genes with high confidence. All samples were isolated in one run and sequenced on the same lane of the same flow cell therefor results did not have to be corrected for batch effects. In total 208 regulated genes were identified which corresponds to 3.66 % of the PAO1 genome. 190 of these were down-regulated which corresponds well to the 161 down-regulated genes of a *lasI/rhlI* double mutant (Chugani et al. 2012). In addition 118 genes are shared between the two sets. The overlap between the described core QS regulon is even greater with 41 out of 42 in the PA2226 transcriptome (Figure 28). Among these genes are the elastase *lasB*, the QS regulator *rsaL*, *rhlI*, the genes for hydrogen cyanide formation *hcnABC* and the *ambBCDE* genes. Up-regulated genes could not be compared as they were not reported in the other study. Apart from the core QS regulon many other genes described before as being QS related and controlled were found to be down-regulated like the pyocyanin synthesis genes, the lectins *lecA* and *lecB, vqsR* and the resistance-nodulation-cell division (RND) efflux pump MexGHI-OpmD, which is controlled through QS and as terminal signaling factor by pyocyanin (Dietrich et al. 2006). Likewise, PA2274, a putative flavin-dependant monooxygenase, also controlled by pyocyanin could be retrieved (Deziel et al. 2005).

When divided into functional categories it became evident that PA2226 overexpression has a major impact on genes belonging to the secreted factors group and congruent with that on the group of genes involved in protein secretion export (Figure 26). As many of the *Pseudomonas* virulence factors are secreted and controlled by QS, together with the

great regulon overlap with autoinducer mutants the whole transcriptome analysis of PA2226 showed a distinct impact on QS. Apart from the factors revealed by phenotypical testing, many other key factors of the QS regulon could be identified.

Interestingly four key genes could not be identified, *lasRI*, *rhlR* and *pqsR.* But as presented in Figure 27 *rhlR* is not included only because of stringent filter criteria but can be identified as strongly regulated. For *lasI* it can be seen that its negative regulator *rsaL* is strongly induced in the control indicating a progressed state in QS, where *lasI* transcription is already deactivated. The detected transcription levels resemble therefor the equal basal transcription activity in both strains. This shows that the general transcription of *lasI* is not impaired and autoinduction may be possible. That *rsaL* is not elevated in strain PA2226 shows that here this progressed state never was reached and induction never took place. The *lasR* transcription level is the same in both strains. This corresponds to equal expression levels of *vfr*, which in return regulates *lasR* expression (Albus et al. 1997, Fuchs et al. 2010). Hence, insufficient LasR was excluded as reason for QS deficiency, but LasR was chosen as target for further analyses to reveal the molecular mechanism of PA2226. Based on the progressed state of QS in the control strain, it can be also assumed for *pqsR* that it is regulated back to its basal transcription level.

The gene *PA2226* already has been reported in other studies analyzing the complex PAO1 regulation. *PA2226* was highly up-regulated in two studies who examined a QS-deficient *vqsM* and an *mvaT* deletion mutant, respectively (Dong et al. 2005, Vallet et al. 2004). Global transcription profiles were measured by microarray in these studies so comparisons have to be treated carefully. Indeed, the overlap between down-regulated and up-regulated datasets from the *mvaT* mutant was virtually non-existent (Figure 28, II), just as the overlap with the up-regulated set of genes in the *vqsM* mutant, whereas here 88 regulated genes were shared in the down-regulated set (Figure 28, III). As both transcription factors have their own regulon and especially MvaT is known as global regulator on expression of virulence genes and influences QS positively and negatively, shared regulations are hard to distinguish (Diggle et al. 2002). But it is obvious that the observed effects on QS for both regulators seem not solely based on PA2226 overexpression and its interplay with other global regulators needs further experiments to elucidate how these factors are interweaved.

5 Conclusion & Outlook

Within this study it was shown that the upregulation of the hypothetical protein *PA2226* in PAO1 pBBR::*bpiB09* is linked to a deletion of 19 kb in the bacterial chromosome. It was demonstrated that the exclusive upregulation of *PA2226* is responsible for several strong phenotypes connected to QS, including pyocyanin, elastase and 3-oxo-C12-HSL formation. Furthermore, it was given evidence that PA2226's regulatory function is closely linked to the QS response regulator LasR and a protein-protein interaction could be demonstrated. In addition, the complete PA2226 regulon was assessed using next generation sequencing's RNA-seq.

It remains unclear under which physiological conditions this regulator is of importance, as its deletion had no impact under laboratory conditions and further experiments will have to show this. Moreover, it became evident that LasR is presumably not the only interaction partner of PA2226. Expression of maltose tagged PA2226 in *P. aeruginosa* itself and subsequent affinity purification would help to identify other proteins directly interacting with PA2226. Although it is obvious that QS is inhibited upon the binding to LasR the immediate mechanism needs further elaboration. It is unclear if PA2226 prevents dimerization of LasR or binds to the dimer and prevents its DNA binding ability. It is also possible, that PA2226 interacts in a way which prevents autoinducer binding to LasR and thereby inhibits the formation of an active complex. Protein-protein interaction experiments with truncated LasR could help to identify the needed epitopes. Furthermore, co-crystallaziation and subsequent resolution of the structur could directly identify the binding pattern. If other interaction partners are found, it will be interesting to see through which epitopes PA2226 interacts with its other tragets and where they are located in the global QS circuit of *P. aeruginosa*.

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7 Appendix

Script A 1: Perl script for genome transcription coverage in wig format

```
samtools mpileup -BQ0 sorted.bam | perl -pe '($c, $start, undef,
$depth) = split;if ($c ne $lastC || $start != $lastStart+1) {print
"fixedStep chrom=$c start=$start step=1 span=1\n";}$_ =
$depth."\n";($lastC, $lastStart) = ($c, $start);' | gzip -c >
run.wig.gz
```

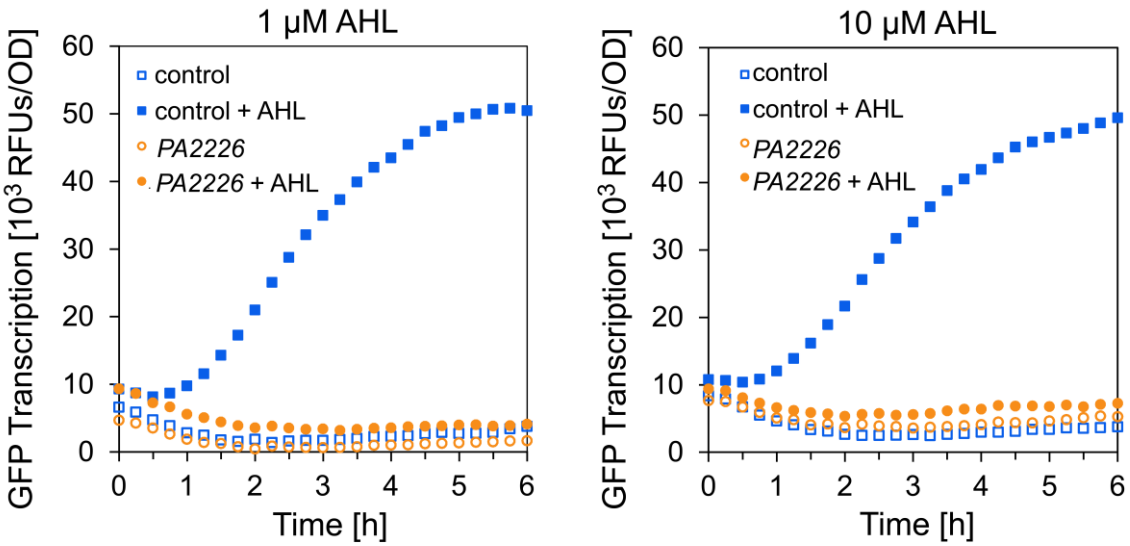

Figure A 1: Heterologously expressed PA2226 blocks QS. *E. coli* expressing *lasR*, a *lasB* reporter and harboring either control (■) or *PA2226* expressing vectors (●) were incubated with inducing concentrations of 1 µM or 10 µM exogenous 3-oxo-C12-HSL and fluorescence was measured every 15 min. Not induced cultures were used as controls (□ and ○). Results are representatives from at least two independent experiments in triplicate.

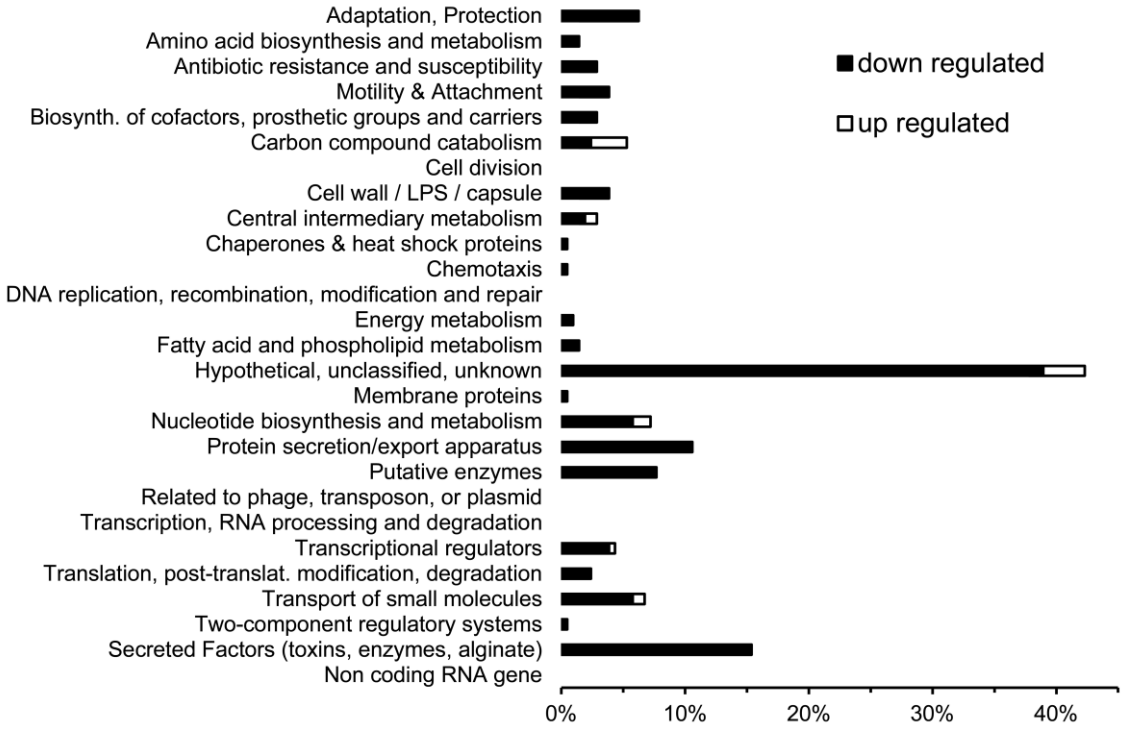

Figure A 2: Functional classes of PAO1 affected by PA2226. Differentially expressed genes were assigned to functional classes and the fraction from the total of differentially expressed genes was evaluated for each class. Functional classes were taken from Pseudomonas Genome Database (Winsor et al. 2011). Genes can be assigned to more than one class.

Table A 1: Up-regulated genes in strain PA2226

PA Orf no.[a]	Gene name	Description	Change in PA2226 compared to PBBR (log2 fold)[b]
PA0201	-	hypothetical protein	1.6
PA0208	*mdcA*	malonate decarboxylase alpha subunit	2.1
PA0212	*mdcE*	malonate decarboxylase gamma subunit	2
PA0213	-	hypothetical protein	2
PA0214	-	probable acyl transferase	1.7
PA1863	*modA*	molybdate-binding periplasmic protein precursor ModA	2.4
PA1864	-	probable transcriptional regulator	2.9
PA3283	-	conserved hypothetical protein	2.1
PA3284	-	hypothetical protein	2.7
PA3431	-	conserved hypothetical protein	1.9
PA3432	-	hypothetical protein	1.9
PA3445	-	conserved hypothetical protein	1.6
PA3446	-	conserved hypothetical protein	1.8
PA4918	-	hypothetical protein	2
PA5168	*dctQ*	DctQ	1.6
PA5352	-	conserved hypothetical protein	1.8
PA5353	*glcF*	glycolate oxidase subunit GlcF	1.9
PA5354	*glcE*	glycolate oxidase subunit GlcE	2.1

[a] Gene Number, Name and description are from the Pseudomonas genome project (Winsor et al. 2011).

[b] Changes in gene expression (rounded to two significant figures) of *P. aeruginosa* PAO1 expressing *PA2226* compared to a control-strain with empty vector.

Table A 2: Down-regulated genes in strain PA2226

PA Orf no.[a]	Gene name	Description	Change in PA2226 compared to PBBR (log2 fold)[b]
PA0026	*plcB*	phospholipase C, PlcB	-1.9
PA0027	-	hypothetical protein	-2.5
PA0028	-	hypothetical protein	-2.5
PA0051	*phzH*	potential phenazine-modifying enzyme	-2.1
PA0052	-	hypothetical protein	-3.8
PA0059	*osmC*	osmotically inducible protein OsmC	-2.2
PA0122	*rahU*	rahU	-6.3
PA0123	-	probable transcriptional regulator	-3.5
PA0143	*nuh*	purine nucleosidase Nuh	-1.7

PA0144	-	hypothetical protein	-2
PA0572	-	hypothetical protein	-3.5
PA0852	*cbpD*	chitin-binding protein CbpD precursor	-5.7
PA0996	*pqsA*	probable coenzyme A ligase	-3.6
PA0997	*pqsB*	PqsB	-4.7
PA0998	*pqsC*	PqsC	-4
PA0999	*pqsD*	3-oxoacyl-[acyl-carrier-protein] synthase III	-4.3
PA1000	*pqsE*	Quinolone signal response protein	-3.9
PA1001	*phnA*	anthranilate synthase component I	-3.2
PA1002	*phnB*	anthranilate synthase component II	-1.8
PA1130	*rhlC*	rhamnosyltransferase 2	-3.2
PA1131	-	probable major facilitator superfamily (MFS) transporter	-2.8
PA1213	-	hypothetical protein	-2.6
PA1214	-	hypothetical protein	-3.5
PA1215	-	hypothetical protein	-3.6
PA1216	-	hypothetical protein	-3.8
PA1217	-	probable 2-isopropylmalate synthase	-3.2
PA1218	-	hypothetical protein	-2.6
PA1219	-	hypothetical protein	-2.5
PA1220	-	hypothetical protein	-3
PA1221	-	hypothetical protein	-2.1
PA1245	*aprX*	AprX	-3.1
PA1246	*aprD*	alkaline protease secretion protein AprD	-2.8
PA1247	*aprE*	alkaline protease secretion protein AprE	-3
PA1248	*aprF*	Alkaline protease secretion outer membrane protein AprF precursor	-3.1
PA1249	*aprA*	alkaline metalloproteinase precursor	-3.7
PA1250	*aprI*	alkaline proteinase inhibitor AprI	-3.3
PA1251	-	probable chemotaxis transducer	-2.5
PA1323	-	hypothetical protein	-2.3
PA1324	-	hypothetical protein	-2.3
PA1431	*rsaL*	regulatory protein RsaL	-3.1
PA1656	*hsiA2*	HsiA2	-3.2
PA1657	*hsiB2*	HsiB2	-3.5
PA1658	*hsiC2*	HsiC2	-2.8
PA1659	*hsiF2*	HsiF2	-2.8
PA1660	*hsiG2*	HsiG2	-2.7
PA1661	*hsiH2*	HsiH2	-2.3
PA1662	*clpV2*	clpV2	-2.9
PA1663	*sfa2*	Sfa2 regulator	-2.7

PA1665	*fha2*	Fha2	-3.1
PA1666	*lip2*	Lip2	-3
PA1667	*hsiJ2*	HsiJ2	-2.5
PA1668	*dotU2*	DotU2	-2.4
PA1669	*icmF2*	IcmF2	-2.4
PA1670	*stp1*	Stp1	-2.6
PA1784	-	hypothetical protein	-3.4
PA1869	-	probable acyl carrier protein	-4.5
PA1871	*lasA*	LasA protease precursor	-6.7
PA1872	-	hypothetical protein	-2.8
PA1874	-	hypothetical protein	-4.5
PA1875	-	probable outer membrane protein precursor	-4.3
PA1876	-	probable ATP-binding/permease fusion ABC transporter	-3.6
PA1877	-	probable secretion protein	-3.9
PA1878	-	hypothetical protein	-2.5
PA1894	-	hypothetical protein	-2.1
PA1899	*phzA2*	probable phenazine biosynthesis protein	-4
PA1900	*phzB2*	probable phenazine biosynthesis protein	-6.2
PA1901	*phzC2*	phenazine biosynthesis protein PhzC	-5.8
PA1902	*phzD2*	phenazine biosynthesis protein PhzD	-6.7
PA1903	*phzE2*	phenazine biosynthesis protein PhzE	-6.2
PA1904	*phzF2*	probable phenazine biosynthesis protein	-5.7
PA1905	*phzG2*	probable pyridoxamine 5'-phosphate oxidase	-5.2
PA1906	-	hypothetical protein	-3.4
PA1907	-	hypothetical protein	-2.2
PA1914	-	conserved hypothetical protein	-4.1
PA2030	-	hypothetical protein	-3.2
PA2031	-	hypothetical protein	-3.7
PA2066	-	hypothetical protein	-3.3
PA2067	-	probable hydrolase	-3.9
PA2068	-	probable major facilitator superfamily (MFS) transporter	-4.4
PA2069	-	probable carbamoyl transferase	-5.5
PA2159	-	conserved hypothetical protein	-2.2
PA2162	-	probable glycosyl hydrolase	-1.9
PA2163	-	hypothetical protein	-2.2
PA2164	-	probable glycosyl hydrolase	-1.6
PA2165	-	probable glycogen synthase	-1.8
PA2166	-	hypothetical protein	-2.6
PA2171	-	hypothetical protein	-2.2

PA2172	-	hypothetical protein	-2.4
PA2173	-	hypothetical protein	-2.3
PA2193	*hcnA*	hydrogen cyanide synthase HcnA	-5.3
PA2194	*hcnB*	hydrogen cyanide synthase HcnB	-5.3
PA2195	*hcnC*	hydrogen cyanide synthase HcnC	-5.2
PA2274	-	hypothetical protein	-3.9
PA2299	-	probable transcriptional regulator	-1.8
PA2300	*chiC*	chitinase	-7.2
PA2301	-	hypothetical protein	-2.8
PA2302	*ambE*	AmbE	-5.1
PA2303	*ambD*	AmbD	-4.7
PA2304	*ambC*	AmbC	-4.5
PA2305	*ambB*	AmbB	-4.5
PA2328	-	hypothetical protein	-2.1
PA2331	-	hypothetical protein	-2.2
PA2414	-	L-sorbosone dehydrogenase	-2.9
PA2423	-	hypothetical protein	-1.9
PA2433	-	hypothetical protein	-3.1
PA2448	-	hypothetical protein	-2.3
PA2563	-	probable sulfate transporter	-2.2
PA2564	-	hypothetical protein	-2.9
PA2565	-	hypothetical protein	-3.3
PA2566	-	conserved hypothetical protein	-3.5
PA2570	*lecA*	LecA	-4.8
PA2587	*pqsH*	probable FAD-dependent monooxygenase	-3.1
PA2588	-	probable transcriptional regulator	-3.2
PA2591	*vqsR*	VqsR regulator	-2.5
PA2592	-	probable periplasmic spermidine/putrescine-binding protein	-2.6
PA2593	*qteE*	quorum threshold expression element, QteE	-2
PA2747	-	hypothetical protein	-2.4
PA2939	-	probable aminopeptidase	-4.3
PA3032	*snr1*	cytochrome c Snr1	-2.4
PA3100	*xcpU*	General secretion pathway outer membrane protein H precursor	-1.6
PA3325	-	conserved hypothetical protein	-1.8
PA3326	*clpP2*	ClpP2	-3.9
PA3327	-	probable non-ribosomal peptide synthetase	-4.3
PA3328	-	probable FAD-dependent monooxygenase	-5.3
PA3329	-	hypothetical protein	-4.6
PA3330	-	probable short chain dehydrogenase	-6

PA3331	-	cytochrome P450	-5.3
PA3332	-	conserved hypothetical protein	-5.4
PA3333	*fabH2*	3-oxoacyl-[acyl-carrier-protein] synthase III	-5.3
PA3334	-	probable acyl carrier protein	-4.7
PA3335	-	hypothetical protein	-3.6
PA3361	*lecB*	fucose-binding lectin PA-IIL	-6.7
PA3475	*pheC*	cyclohexadienyl dehydratase precursor	-1.9
PA3476	*rhlI*	autoinducer synthesis protein RhlI	-3.8
PA3478	*rhlB*	rhamnosyltransferase chain B	-4.7
PA3479	*rhlA*	rhamnosyltransferase chain A	-6
PA3520	-	hypothetical protein	-3.4
PA3535	-	probable serine protease	-1.9
PA3677	-	probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein precursor	-2
PA3724	*lasB*	elastase LasB	-8.2
PA3734	-	hypothetical protein	-2.7
PA3891	-	probable ATP-binding component of ABC transporter	-1.4
PA3904	-	hypothetical protein	-3.6
PA3905	-	hypothetical protein	-2.9
PA3906	-	hypothetical protein	-3.1
PA3907	-	hypothetical protein	-2.7
PA3908	-	hypothetical protein	-3
PA4078	-	probable nonribosomal peptide synthetase	-3.2
PA4127	*hpcG*	2-oxo-hept-3-ene-1,7-dioate hydratase	-2
PA4128	-	conserved hypothetical protein	-3.3
PA4129	-	hypothetical protein	-3
PA4130	-	probable sulfite or nitrite reductase	-3.5
PA4131	-	probable iron-sulfur protein	-3
PA4132	-	conserved hypothetical protein	-3.1
PA4134	-	hypothetical protein	-3.4
PA4141	-	hypothetical protein	-4.8
PA4142	-	probable secretion protein	-2.2
PA4175	*piv*	protease IV	-2.2
PA4205	*mexG*	hypothetical protein	-5.2
PA4206	*mexH*	probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein precursor	-4.8
PA4207	*mexI*	probable Resistance-Nodulation-Cell Division (RND) efflux transporter	-4.6
PA4208	*opmD*	probable outer membrane protein precursor	-5
PA4209	*phzM*	probable phenazine-specific methyltransferase	-3.9
PA4210	*phzA1*	probable phenazine biosynthesis protein	-3.2

PA4211	*phzB1*	probable phenazine biosynthesis protein	-5.6
PA4212	*phzC1*	phenazine biosynthesis protein PhzC	-5.8
PA4213	*phzD1*	phenazine biosynthesis protein PhzD	-6.8
PA4214	*phzE1*	phenazine biosynthesis protein PhzE	-6.1
PA4215	*phzF1*	probable phenazine biosynthesis protein	-5.6
PA4216	*phzG1*	probable pyridoxamine 5'-phosphate oxidase	-5.3
PA4217	*phzS*	flavin-containing monooxygenase	-4.5
PA4218	*ampP*	AmpP	-2.7
PA4293	*pprA*	two-component sensor PprA	-2.5
PA4294	-	hypothetical protein	-3
PA4301	*tadB*	TadB	-1.8
PA4590	*pra*	protein activator	-2
PA4648	*cupE1*	Pilin subunit CupE1	-2.7
PA4649	*cupE2*	Pilin subunit CupE2	-2.3
PA4650	*cupE3*	Pilin subunit CupE3	-1.9
PA4651	*cupE4*	Pilin assembly chaperone CupE4	-2.9
PA4652	*cupE5*	Fimbrial usher protein CupE5	-1.9
PA4677	-	hypothetical protein	-2.7
PA4738	-	conserved hypothetical protein	-2.6
PA4778	*cueR*	CueR	-2.3
PA4877	-	hypothetical protein	-1.8
PA4917	-	hypothetical protein	-1.6
PA5059	-	probable transcriptional regulator	-1.6
PA5220	-	hypothetical protein	-4.1
PA5481	-	hypothetical protein	-2.4
PA5482	-	hypothetical protein	-2.5

[a] Gene Number, Name and description are from the Pseudomonas genome project (Winsor et al. 2011).

[b] Changes in gene expression (rounded to two significant figures) of *P. aeruginosa* PAO1 expressing *PA2226* compared to a control-strain with empty vector.

Table A 3: PA2226 orthologs[a] from pseudomonas.com[b]

P. aeruginosa **Strain**	**Locus Tag**	**Description**
2192	PA2G_05512	hypothetical protein
B136-33	G655_23355	hypothetical protein
C3719	PACG_04962	hypothetical protein
DK2	PADK2_23670	hypothetical protein
PA7	PSPA7_4439	hypothetical protein
PACS2	PaerPA_01000893	hypothetical protein
RP73	M062_05370	hypothetical protein
UCBPP-PA14	PA14_58930	hypothetical protein

[a] Orthologs were identified by reciprocal-best-BLAST (RBBH) analysis using an E-value cutoff of 1E-004.

[b] Winsor et al. 2011

Table A 4: QS anti-activators - Percent Identity Matrix[a]

Protein	PA2226	QslA	QteE	TraM
PA2226	-	20.51	20.97	20
QslA	20.51	-	16.3	15.69
QteE	20.97	16.3	-	23.61
TraM	20	15.69	23.61	-

[a] created by Clustal2.1[Clustal Omega]

Declaration on oath

I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids.

Christian Utpatel Hamburg, 5. March 2016

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