Cell-cell communication via LuxR solos in *Photorhabdus* species

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PUBLICATIONS AND MANUSCRIPTS ORIGINATING FROM THIS THESIS

CHAPTER 2

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CHAPTER 3

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CHAPTER 5

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CONTRIBUTIONS TO PUBLICATIONS AND MANUSCRIPTS PRESENTED IN THIS THESIS

CHAPTER 2

A.O.B. performed PPY isolation, structure elucidation and quantification and identified *ppyS*. S.B. performed qRT-PCR, analyzed and quantified PPY bioactivity and specificity for PluR, performed cell clumping assays, generated amino acid replacements in PluR and measured the influence on pyrone sensing. Y.K. constructed PPY-producing *E. coli* strains. D.K. modeled PluR structure and performed PPY docking and *in silico* mutagenesis. I.H. performed two-dimensional PAGE, northern blot analyses and *Photorhabdus* pathogenicity assays. C.M. characterized chemical properties of the PluR signal and performed first *E. coli* cell clumping assays. K.S. performed preliminary fractionations of *P. luminescens* supernatants via HPLC and analyzed them for PluR-signal presence. H.B.B. and R.H. coordinated and designed the experiments, analyzed the data and wrote the manuscript.

CHAPTER 3

S.B. constructed *P. asymbiotica* mutants and reporter strains, analyzed and quantified dialkylresorcinol bioactivity and specificity for PauR, performed cell clumping assays, generated amino acid replacements in PauR, measured the influence on DAR sensing, and performed insect pathogenicity assays. D.K. modelled PauR structure, performed docking experiments, *in silico* mutagenesis, and HPLC/MS and phylogenetic analysis of *Photorhabdus* strains. H.B.B. and R.H. coordinated and designed the experiments, analyzed the data and wrote the manuscript.

CHAPTER 4

S.B. performed bioinformatics analyses and quorum quenching assay. S.B. and R.H. coordinated the experiments and analysed the data. S.B., D.K., H.B.B and R.H. and wrote the manuscript.

CHAPTER 5

S.B. generated amino acid substitutions in PluR and PauR and measured their functionality and ability to sense different signalling molecules. S.B. performed western blot analysis. S.B. and R.H. coordinated and designed the experiments, analysed the data and wrote the manuscript.

I hereby confirm the above statements:

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NOMENCLATURE

Amino acid substitutions in proteins are termed as follows: The amino acid introduced by (site-directed) mutagenesis is terminally added in one-letter code (Example: PluR-Y66A).

Deletions of genes are marked by "Δ".

Supplementary figures are described for example with Fig. S1.

ABBREVIATIONS

AHL	acyl-homoserine lactone
DBD	DNA-binding domain
DNA	deoxyribonucleic acid
n-His tag	affinity tag composed of n histidine residues
HSL	homoserine lactone
IM	inner membrane
LB	lysogeny broth
mCherry	red fluorescent protein mCherry
MFS	major facilitator superfamily of transporters
M63	Minimal Medium 63
OM	outer membrane
PAGE	polyacrylamide gel electrophoresis
PAS	protein domain present in Per, Arnt, Sim proteins
SBD	signal-binding domain
SD	standard deviation
TM	transmembrane domain
QS	quorum sensing
QQ	quorum quenching

SUMMARY

Bacteria constantly need to monitor their environment and adapt the bacterial group-coordinated behaviour to changing habitats like nutrition alterations or host variations. Commonly cell-cell communication via acyl homoserine lactones (AHLs) is used to synchronise the behaviour of a bacterial population dependent on cell size. This process is referred to as quorum sensing (QS) and predominantly occurs in Gram-negative bacteria. The typical QS system consists of a LuxI-synthase that synthesises AHLs, and a LuxR-type receptor, which then responds to these AHLs. Upon AHL-binding, the LuxR-type receptor regulates the expression of different target genes and thus influences several processes, like biofilm formation, virulence, antibiotic production or cell-cell interaction. Interestingly, many proteobacteria possess additional LuxR homologs, but lack a cognate LuxI-type synthase. Those LuxR-type receptors are referred to as LuxR orphans or LuxR solos and can expand the regulatory QS network. *Photorhabdus* species are insect pathogenic bacteria, living in symbiosis with entomopathogenic nematodes. They all possess an exceptionally high number of LuxR solos, but lack LuxI homologs and therefore do not produce AHLs. The function of these LuxR solos, their role in cell-cell communication and the identification of their cognate signalling molecules in *Photorhabdus* species is the main focus of this work.

In this thesis a novel signalling molecule used for QS could be identified for the first time in *P. luminescens*. This novel QS molecule is an α-pyrone named photopyrone (PPY) and produced endogenously by the photopyrone synthase (PpyS). The PPYs are specifically recognized by the LuxR solo regulator PluR, which then activates expression of the *pcf* (*Photorhabdus* clumping factor) operon leading to cell clumping of *P. luminescens* cells. Moreover, the PpyS/PluR quorum sensing system and its induced cell clumping contribute to the overall toxicity of *P. luminescens*.

Furthermore, a second novel signalling molecule sensed by a LuxR solo of *Photorhabdus* species could be identified besides PPYs. The insect and human pathogenic bacteria *P. asymbiotica* lacks a PpyS homolog as well as a LuxI homolog, but harbours a *pcf* operon and a homologue to PluR, which is named PauR. The signalling molecule sensed by the LuxR-type receptor PauR could be identified, which is neither an AHL nor a PPY. PauR recognises a 2,5-dialkylresorcinol (DAR) produced by the DarABC pathway. Upon binding of the cognate signalling molecule,

PauR activates expression of the *pcf* operon. This also leads to cell clumping in *P. asymbiotica*. Furthermore, the DarABC/PauR QS system also contributes to the overall pathogenicity of *P. asymbiotica* against *Galleria mellonella* insect larvae.

A bioinformatics approach revealed a high number of LuxR solos present in *P. temperata* and *P. asymbiotica* like in *P. luminescens*. Thereby, several conserved motives of amino acids could be identified, which are potentially important for signal-binding and -specificity. Variations in these amino acid motifs are assumed to reflect the overall variety of signals that can be sensed by LuxR solos.

Furthermore, the specificity of the two LuxR solos PluR and PauR towards their cognate signalling molecules, PPYs and DARs, respectively, was analysed. Thereby, it could be shown that the previously identified conserved amino acid motives in the signal-binding domain (SBD), the TYDQCS-motif of PluR and the TYDQYI-motif of PauR, are essential but not sufficient for ligand-binding.

Similar as for AHLs, it was unclear how the signalling molecules PPYs and DARs can cross the bacterial cell membrane. In the last part of this thesis the import mechanism for the *Photorhabdus*-specific signalling compounds PPYs and DARs were identified. Initial evidence could be provided that the membrane-integrated transporter FadL is mainly involved in the import of these hydrophobic compounds, and that they are not transported via simple diffusion across the cell membrane, which is assumed for AHLs.

In conclusion, the data that is compiled presents two LuxR solos of *Photorhabdus* species adapted to sense and respond to novel non-AHL signalling molecules used for QS. Therefore, this thesis reveals that cell-cell communication via LuxR-type receptors goes far beyond AHL-signalling in nature.

ZUSAMMENFASSUNG

Bakterien beobachten ständig ihre Umwelt, um ihr Gruppenverhalten der sich stetig verändernden Umgebung, wie Nahrungszusammensetzung oder wechselnde Wirte, anzupassen. Normalerweise werden Acyl-homoserin-Laktone (AHL) für die Zell-Zell-Kommunikation genutzt, wodurch das Verhalten einer bakteriellen Population synchronisiert werden kann. Dieses System wird als „Quorum Sensing" (QS) bezeichnet und tritt vorzugsweise in Gram-negativen Bakterien auf. Ein typisches QS System besteht aus einer LuxI-Synthase, welche konstant AHL produziert, und einem LuxR-ähnlichem Rezeptor, der das AHL wahrnimmt. Durch Binden des AHLs reguliert der LuxR-ähnliche Regulator die Expression verschiedener Zielgene und beeinflusst dadurch diverse Prozesse wie Biofilmbildung, Virulenz, Antibiotikaproduktion oder Zell-Zell-Interaktionen. Interessanterweise, besitzen viele Proteobakterien zusätzliche LuxR-Homologe, die jedoch keine zugehörige LuxI-ähnliche Synthase aufweisen. Daher werden diese LuxR-ähnlichen Rezeptoren als LuxR-Waise oder LuxR-Solo bezeichnet und können das regulatorische QS Netzwerk der Zelle erweitern. *Photorhabdus* Spezies sind insektenpathogene Bakterien, die auch in Symbiose mit Entomopathogenen Nematoden leben. Sie besitzen eine außergewöhnlich hohe Nummer an LuxR-Solos, jedoch keine LuxI-Homologe und können daher keine AHLs produzieren. Die Funktionsweise dieser LuxR-Solos, ihre Rolle in Zell-Zell-Kommunikation und die Identifizierung der zugehörigen Signalmoleküle in *Photorhabdus* Spezies war Gegenstand dieser Arbeit.

In dieser Arbeit wurde zum ersten Mal ein neuartiges Signalmolekül identifiziert, welches für QS in *P. luminescens* genutzt wird. Dieses neue QS-Signalmolekül ist ein α-Pyron namens Photopyron (PPY), welches endogen von der Photopyron-Synthase (PpyS) produziert. Die PPY werden spezifisch von dem LuxR-Solo Regulator PluR erkannt, welcher daraufhin die Expression des *pcf* (*Photorhabdus* clumping factor) Operons aktiviert, so dass einem Verklumpen der *P luminescens* Zellen induziert wird. Darüber hinaus trägt das PpyS/PluR QS System und dessen induziertes Zellklumpen zur Pathogenität von *P. luminescens* bei.

Außerdem wurde in dieser Arbeit ein zweites neues Signalmolekül neben PPY identifiziert, welches ebenfalls von einem LuxR-Solo erkannt wird. Das insekten- und

humanpathogene Bakterium *P. asymbiotica* besitzt weder ein PpyS-Homolog noch eine LuxI-ähnliche Synthase. Jedoch besitzt *P. asymbiotica* ein homologes *pcf* Operon und ein PluR-Homolog, welches PauR benannt wurde. Das von PauR wahrgenommene Signalmolekül konnte identifiziert werden, welches weder ein AHL noch ein PPY ist. PauR nimmt ein 2,5-Dialkylresorcinol (DAR) wahr, das von dem DarABC System synthetisiert wird. Nach dem Binden des Signalmoleküls aktiviert PauR die Expression des *pcf* Operons. Dies führt ebenfalls zu einem Verklumpen der Zellen. Außerdem trägt das DarABC/PauR QS System zur Pathogenität von *P. asymbiotica* gegenüber *Galleria mellonella* Insektenlarven bei.

Ein bioinformatischer Ansatz zeigte eine hohe Anzahl an LuxR-solos in *P. temperata* und *P. asymbiotica* auf, wie vormals beschrieben für *P. luminescens*. Außerdem konnten verschiedene konservierte Aminosäure-Motive bestimmt werden, welche möglicherweise für Signal-Bindung und -Spezifität verantwortlich sind. Variationen in diesen Motiven spiegeln möglicherweise die Vielfalt der diversen wahrgenommenen Signalmoleküle wider.

Des Weiteren wurde die Spezifität der zwei LuxR-Solos PluR und PauR gegenüber ihren jeweiligen Signalmolekülen, dem Photopyron beziehungsweise dem Dialkylresorcinol, untersucht. Dadurch konnte gezeigt werden, dass die vorher identifizierten konservierten Aminosäuremotive der Signal-Binde Domäne (SBD), das TYDQCS-Motiv von PluR und das TYDQYI-Motiv von PauR, essentiell aber nicht ausreichend für die Bindung des Liganden sind.

Wie die Signalmoleküle PPYs und DARs die bakterielle Zellmembran überwinden, ist ebenso unklar wie für AHLs. Daher wurde in dem letzten Teil dieser Arbeit der Importmechanismus der *Photorhabdus*-spezifischen Signalmoleküle PPY und DAR identifiziert. Initiale Ergebnisse zeigten, dass der membranintegrierte Transporter FadL vornehmlich für den Import dieser hydrophoben Signalmoleküle verantwortlich ist. Deshalb ist anzunehmen, dass diese Verbindungen, ebenso wie AHLs, nicht einfach durch Diffusion die Zellmembran passieren können.

Abschließend lässt sich sagen, dass diese Arbeit zwei LuxR-Solos von *Photorhabdus* präsentiert, welche jeweils zwei neu identifizierte Signalmoleküle wahrnehmen. Diese Signalmoleküle sind keine AHLs und werden für QS genutzt. Außerdem zeigt sich, dass Zell-Zell-Kommunikation mittels LuxR-ähnlichen Rezeptoren über die Grenzen der AHL-abhängigen Signalweiterleitung hinaus reicht.

1. INTRODUCTION

1.1 *Photorhabdus* species

Photorhabdus species are Gram-negative, bioluminescent, entomopathogenic bacteria belonging to the family Enterobacteriaceae. The first isolates of *Photorhabdus* species were reported in 1977 by Khan and Brooks (1977) and Poinar et al. (1977). *Photorhabdus* were characterised due to their bioluminescence and their symbiotic association with entomopathogenic *Heterorhabditis* nematodes (EPN) of the family Heterorhabditidae. *Photorhabdus* species only interact with nematodes of *Heterorhabditis* species, which suggests a co-phylogeny between these two symbiotic partners (Maneesakorn et al., 2011). Furthermore, *Photorhabdus* species are able to infect a wide range of insect hosts, like *Galleria mellonella* or *Manduca sexta* (Akhurst and Boemare, 1988). Moreover, *Heterorhabditis*-nematodes are widely distributed over the earth and are adapted to nearly every ecological niche, including aqueous habitats, soils and animal tissues. Therefore, all known animal groups including themselves can be colonised. Association of *Heterorhabditis*-nematodes with *Photorhabdus* bacteria has certainly played a part in this evolutionary distribution (Andrassy, 1976). Accordingly, *Photorhabdus* species are also widely distributed. Up until today, three different species are known: *Photorhabdus luminescens* (Fischer-Le Saux et al., 1999), *Photorhabdus temperata* (Tailliez et al., 2010) and *Photorhabdus asymbiotica* (Wilkonson et al., 2009). In addition to the symbiotic lifestyle with nematodes, the three species are highly pathogenic towards insects. Furthermore, *P. asymbiotica* additionally infects men and is associated with severe soft-tissue in humans (Gerrard et al., 2003). Therefore, *P. asymbiotica* is considered as an emerging threat (Gerrard et al., 2004).

1.1.1 Life cycle of *Photorhabdus* species

Photorhabdus species have a complex life cycle, being on the one hand as symbiont in the gut of *Heterorhabditis* species nematodes and on the other hand also pathogenic in the insect or human body (Fig. 1). *Photorhabdus* species are commonly

found in association with the infective juvenile (IJ) stage of nematodes, however they are also able to live independently in the soil (Joyce et al., 2011). After colonising the gut of the IJ stage of nematodes, the bacteria are directly injected into the insect's hemolymph. At this point the bacteria have to sense their current host to switch their behaviour to a pathogenic lifestyle. Then the bacteria reproduce rapidly inside the insect and produce a broad range of different toxins, like Toxin complexes (Tcc's) or Makes caterpillar floppy (Mcf) toxins. Within 48 hours the insect is killed due to a septicaemia. Furthermore, several potential virulence factors are synthesised, including lipases, proteases and lipopolysaccharides. Moreover, in order to prevent invasion of the dead larvae with other bacteria several antibiotics are synthesised. The chemical nature of two of the produced antibiotics has already been solved. One is a stilbene antibiotic (3,5-dihydroxy-4-isopropylstilbene), which has an antimicrobial activity especially against Gram-positive bacteria. The other is a β-lactam carbapenem antibiotic known to have a broad spectrum against diverse bacteria. Additionally, the enzyme luciferase is produced, which causes dying insect larvae to glow. Via bacterial exoenzymes, the insect larvae are converted into a rich nutrient source, both for the bacteria and the nematodes. At this point, the bacteria are switching to the symbiotic life phase and are supporting the growth of the nematodes, probably by providing essential nutrients that are required for efficient nematode proliferation (Han and Ehlers, 2000). When the insect cadaver is depleted, the nematodes and bacteria re-associate and emerge from the insect's cadaver in search for a new insect host. A single IJ infecting one insect larvae results in the production of >100,000 IJs within 2 to 3 weeks, showing that the *Photorhabdus-Heterorhabditis* interaction is highly efficient (Forst et al., 1997; Clarke, 2008; Waterfield et al., 2009). Furthermore, *Photorhabdus* is required but not essential for nematode growth. Several metabolic interactions are suggested to be important for the symbiosis with nematodes, like the crystalline inclusion proteins CipA and CipB and iso-branched fatty acids (BCFAs). Additionally, BCFAs produced by *Photorhabdus* in a significant amount by the *bkdABC* operon, may represent an adaptation to symbiosis as these are required by its nematode partner. The production of BCFAs is not widespread among *Enterobacteriaceae* (Joyce et al, 2008). Moreover, *Photorhabdus* synthesises a broad range of secondary metabolites necessary for the symbiosis with the nematodes and insect larvae (Kontnik et al., 2010).

As *P. asymbiotica* is additionally pathogenic to men, it can cause local infection of skin and soft tissue, with the formation of subcutaneous nodules (Costa et al., 2010). The bacteria are also transmitted via *Heterorhabditis* species into the host. Invasion is often associated with minor skin traumata, however whether the nematodes are able to penetrate intact human skin or skin wounds are needed as entry is not clear (Gerrard et al., 2006).

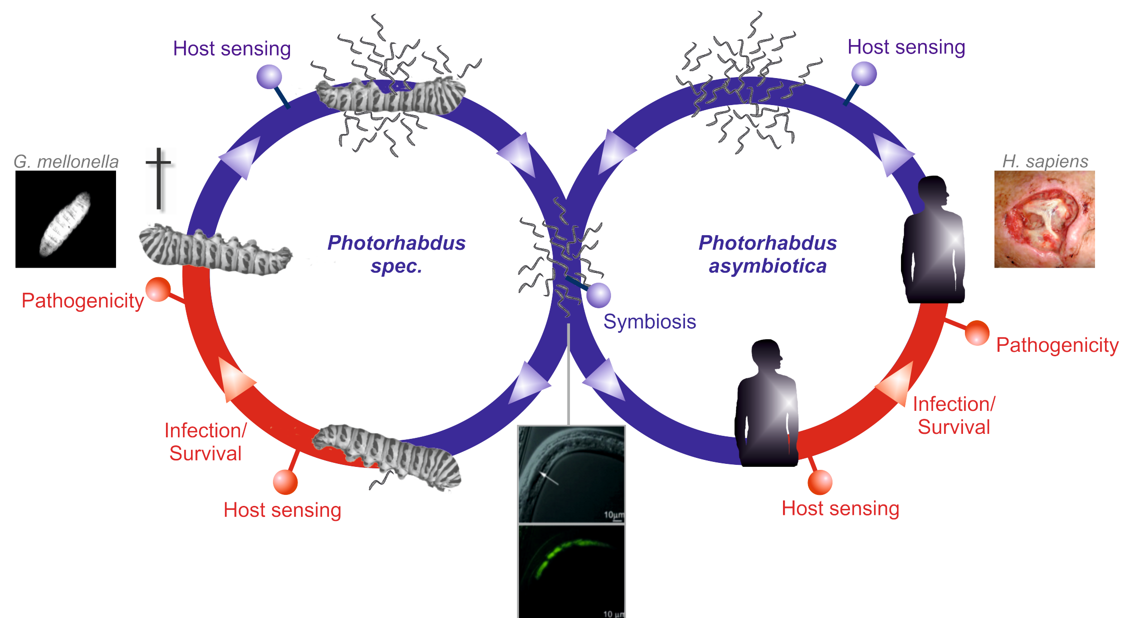

Figure 1: Life cycle of *Photorhabdus* species. During the symbiotic life style (purple), *Photorhabdus* species are colonizing the gut of the infective juvenile (IJ) stage of the nematodes of *Heterorhabditis* species. Then the bacteria are directly injected into the insect's hemolymph (left side) or also into the human host (right side), which is additionally the case for *P. asymbiotica*. Then the pathogenic life style (red) starts, therefore the bacteria rapidly proliferate and produce toxins, antibiotics, lipases, exoenzymes and bioluminescence. Within 48 hours the insect larvae is converted into a rich food source and the bacteria and the nematodes are re-associating to search for a new host (Waterfield et al., 2009; Gerrard et al., 2006). Picture of GFP-labeled *P. luminescens* cells in the intestines of *H. bacteriophora* (Ciche and Ensign, 2003). Picture of the hand infected with *P. asymbiotica* (Gerrard et al., 2006).

Besides infecting insects or men, *Photorhabdus* species constantly have to monitor their surrounding environment during the complex life cycle. Furthermore,

Photorhabdus continually have to communicate with each other to perfectly adapt to the respective conditions and lifestyles so that the whole bacterial population is simultaneously either symbiotic or pathogenic. Due to the multifaceted lifestyle, *Photorhabdus* species are a good model system for the study of bacteria-host interactions and its regulation.

1.1.2 Phenotypic heterogeneity of *Photorhabdus* species

Apart from the symbiotic and pathogenic life style of *Photorhabdus* species, two phenotypic variants occur, which are designated as phase I (primary) and phase II (secondary) cells. Both cell variants are genetically homogeneous but differ in many morphologically and physiologically traits (Forst et al., 1997; Waterfield et al., 2009). Several primary-specific phenotypes are lacking or are reduced in secondary cells, like the production of the crystal proteins CipA and CipB (Bintrim and Ensign, 1998). Furthermore, both cell variants are equally pathogenic against insects, but only the primary cell variant is able to be symbiotic with nematodes (Joyce et al., 2011). The reason for phenotypic heterogeneity of *Photorhabdus* cell populations and its regulation is not yet understood, but it is assumed that secondary cells are better adapted for a life in the soil, in absence of the nematodes. Therefore, phenotypic switching might be an adaptation for survival of the bacterial population that remains in the insect cadaver after the new generation of IJs has dispersed (Smigielski et al., 1994). Furthermore, a tight regulation of the switching process from primary to secondary cells is needed, as it has yet only been found to be unidirectional. In 2003, the LysR-type transcriptional regulator HexA was identified to be necessary for the maintenance of secondary-specific phenotypes. As secondary-specific phenotypes are achieved via repression of primary-specific genes by HexA, however the regulation mechanisms of HexA are not yet fully understood. Furthermore, primary-specific features or metabolites are suggested to support symbiosis and to be required for nematode growth and development, as only primary cells are symbiotic with *Heterorhabditis*-nematodes (Joyce and Clarke, 2003).

1.1.3 Genome architecture of *Photorhabdus* species

Each *Photorhabdus* species shares common and unique genes, however *P. luminescens* and *P. temperata* are closer related to each other than to *P. asymbiotica* (Tailliez et al., 2010).

The genome of *P. luminescens*, as an example of insect pathogenicity, was compared against the genome of *P. asymbiotica*, which is an example of an insect and human pathogenic bacterium. The comparison of both genomes revealed that the reduced size of *P. asymbiotica* genome is due to a smaller diversity of insecticidal genes, like those encoding the Tc's and Mcf toxins. Each strain carries approximately one megabase of DNA that is unique to each species. However, *P. asymbiotica* harbours several pathogenicity islands including a novel type-three secretion system (T3SS), potentially important for virulence against men. The lack of insecticidal genes in *P. asymbiotica* does not correlate with a reduced pathogenicity towards insects. Therefore, *P. asymbiotica* is possibly better equipped for virulence towards men via the acquisition of a plasmid, named pPAU1, and specific virulence factors (Wilkonson et al., 2009).

Yersinia enterocolitica is primarily pathogenic against human. However, *Y. enterocolitica* is also virulent against insects but less virulent than *P. luminescens*. A comparison of their genomes revealed that both share different factors important within insect hosts, like common degradative metabolic pathways to access nutrients in the insect gut or hemolymph. Furthermore, both harbour a yet uncharacterised two-component system, unique for the species *Photorhabdus* and *Yersinia* and possibly playing a major part in the interaction of pathogen and insect. Moreover, *P. luminescens* and *Y. enterocolitica* share different toxins, like Tc's, however each strain harbours unique virulence factors. *P. luminescens* contains in total a higher number of genes encoding insecticidal toxins and virulence factors reflecting its higher virulence against insects (Heermann and Fuchs, 2008). As *P. temperata* is closely related to *P. luminescens*, the knowledge stated above may presumably be adapted. In general, pathogenicity is an important feature of *Photorhabdus* species, therefore all three species, *P. luminescens*, *P. temperata* and *P. asymbiotica*, contain several pathogenicity islands distributed throughout their genomes. These pathogenicity islands encode different groups of toxins including Tc's, *Photorhabdus*

insect-related (Pir) proteins, Mcf toxins and the *Photorhabdus* virulence cassettes (PVC) proteins.

Interestingly, *P. luminescens* harbours an exceptional high number of LuxR-type receptors potentially involved in cell-cell communication or host sensing (Heermann and Fuchs, 2008).

1.2 Bacterial communication

Bacteria are part of a complex environment in nature and additionally live in close association with other organism. Therefore, they need to monitor and communicate with their surrounding mixed community to adapt their behaviour. Thus, bacteria are able to coordinate the bacterial group behaviour in response to environmental changes and requirements using complex intercellular communication networks. Regulation of bacterial behaviour in a population density-dependent manner via the use of small signalling molecules is called quorum sensing (QS) (Nealson and Hastings, 1979). Quorum sensing pathways are widespread and several processes are regulated via QS systems in bacteria, like bioluminescence, antibiotic production, virulence, biofilm formation, motility or sporulation, usually benefitting the QS-producing population (Waters and Bassler, 2005). The major signalling molecules used for QS can be classified into two different groups among Gram-negative and Gram-positive bacteria. Typically, Gram-negative bacteria use N-acyl homoserine lactones (AHLs), derived from fatty acids, as signalling molecules for quorum sensing regulated processes, whereas Gram-positive bacteria mainly use peptide-derived signalling molecules. However, recently also a Gram-positive bacterium belonging to the *Exiguobacterium* genera isolated from marine water was found to synthesize AHLs, though (Biswa and Doble, 2013). Furthermore, the autoinducer-2 (AI-2) synthesised by LuxS, present in Gram-negative as well as Gram-positive bacteria, was proposed as a universal QS signalling molecule in bacteria (Winzer et al., 2002).

As *P. luminescens* harbours several LuxR solos potentially involved in cell-cell communication, we want to focus here on communication systems via LuxR-type regulators.

1.2.1 LuxR-type regulators used for quorum sensing

The first quorum sensing (QS) system was studied in the marine bacterium *Vibrio fischeri*, in which bioluminescence is regulated dependent on cell density via the signalling molecule 3-oxo-hexanoyl-L-homoserine lactone (3OC6 HSL). Therefore, the first classical QS system was described consisting of the transcriptional LuxR receptor and the AHL-synthase LuxI (Nealson and Hastings, 1979).

Therefore, the molecular basic of QS systems consists of two proteins, the AHL-synthase LuxI, constantly synthesising the signalling molecule at a low basal level, and the LuxR-type receptor, recognising its cognate signalling molecule (Fuqua et al., 1996). Upon binding of the signalling molecule the LuxR-type transcriptional regulator undergoes conformational changes and is then able to bind target promoter regions, which control the expression of different target genes (Fig. 2). Therefore, the whole bacterial population responds in a group-coordinated behaviour (Waters and Bassler, 2005).

LuxI synthases are able to synthesise distinct AHLs, hence the lengths of the acyl moieties of the AHLs can vary between four and 18 C atoms. The third C atom in the acyl chain can be either a carbonyl group, a hydroxyl group or a methylene moiety (Whitehead et al., 2001). These structural differences among the AHLs play a crucial role in the signalling specificity of QS LuxR-type receptors in different bacterial species (Kim et al., 2013). Moreover, if transcription of *luxI* is positively regulated via the LuxR-type regulator in complex with its cognate signalling molecule, further amplifying AHL synthesis, the signalling molecule is termed autoinducer (Fuqua et al., 1994).

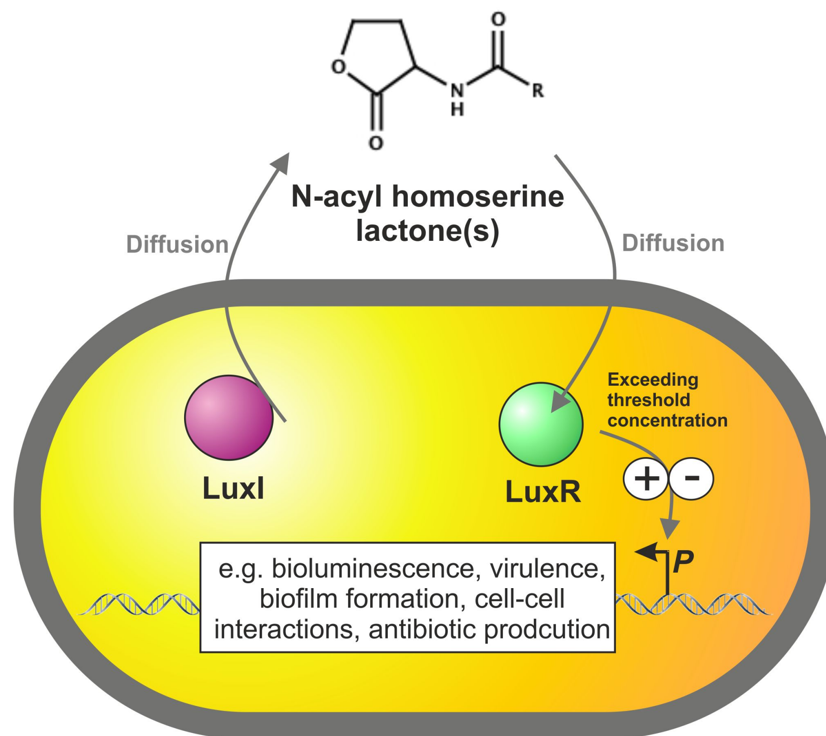

Figure 2: Model of the LuxI/LuxR quorum sensing system in Gram-negative bacteria. The AHL-synthase LuxI, constantly synthesise N-acyl homoserine lactones (AHLs), at a low basal level, which accumulates outside of the cell probably via diffusion. After exceeding a certain threshold concentration, the LuxR-type receptor recognises the AHL signalling molecule and regulates the expression of different target genes/operons influencing the behaviour of the whole population dependent on cell size.

LuxR-type receptors are composed of two functional domains, a N-terminal signal-binding domain (SBD) and a C-terminal DNA-binding domain (DBD). The DBD contains a helix-turn-helix motif, the HTH LUXR motif, which is typical for LuxR-type proteins. The N-terminal SBD is important for signal-binding, binding-specificity and shaping the ligand pocket. Additionally, LuxR-type proteins share a low protein sequence identity (18%-25%), however nine highly conserved amino acid are described. In the DBD are three conserved amino acids (E178, L182 and G188, with respect to TraR) present, important for DNA-binding. The SBD harbours six conserved amino acids (W57, Y61, D70, P71, W85 and G113, with respect to TraR) important for AHL-binding (Fuqua et al., 1996; Patankar and González, 2009).

Additionally, QS regulatory systems might be complicated as several distinct QS circuits can be present in one bacterium. For example, *Pseudomonas aeruginosa* harbours three QS systems, the two systems LasI/LasR and RhlI/RhlR respond to AHLs and the third system is dependent on the *Pseudomonas* quinolone signal (PQS) sensed by PqsR and primary synthesised by *pqsABCD* (Pearson et al., 2000; Gallagher et al., 2002). Deletion of genes encoding its QS systems reduced *P. aeruginosa* virulence in mice. Moreover, these QS regulatory network regulates in a hierarchical relationship the expression of different virulence determinants (Lee and Zhang, 2014; Martínez, 2014). Furthermore, QS circuits can also be encoded on plasmids, like the TraI/TraR QS system from *Agrobacterium tumefaciens* carried on the tumour-inducing (Ti) plasmid, which is important for successful colonization of the plant host. Upon recognising N-3-oxooctanoyl-L-homoserine lactone (3OC8 HSL), TraR controls the conjugation of the Ti plasmid important to promote virulence and opine metabolism (Fuqua and Winans, 1994).

1.2.2 Influencing quorum sensing systems

Many animal or plant pathogens use QS systems to regulate their virulence or biofilm formation. Therefore, a possibility to inhibit the infection or colonization process of pathogens gained via the disruption of QS circuits is a promising target for antimicrobial drugs (Waters and Bassler, 2005; Marx, 2014). The mechanism that interferes with QS-regulated processes of neighbouring bacteria is termed quorum quenching (QQ). Mainly three targets for QQ strategies are addressed: inhibition of the LuxI synthase, inhibition of the LuxR-type receptor or degradation of the signalling molecule (Galloway et al., 2011).

To modulate the activity of the LuxI synthase, competitive inhibitors of LuxI can be used, like analogues of the AHL precursor S-adenosylmethionine (SAM) (Parsek et al., 1999). The central metabolite SAM is also used as a precursor in AI-2 pathways (Xavier and Bassler, 2003).

Furthermore, several antagonists of LuxR-type regulators are known, which are either naturally or synthetically produced (Defoirdt et al., 2007; Galloway et al., 2011). The first example of AHL-mimicking compounds inhibiting LuxR-type regulators were halogenated furanones produced by the marine algae *Delisea pulchra*

(Gram et al., 1996). Halogenated furanones are structurally similar to AHLs and have strong antimicrobial properties and an inhibiting activity against LuxR homologs (Givskov et al., 1996). Moreover, the QS-regulated phenotypes in *A. tumefaciens* are tightly regulated but are also interfered by low molecular weight compounds produced by plants. These compounds are supposed to act as antagonists or agonists to the bacterial AHL QS systems (Teplitski et al., 2000; Subramoni et al., 2014).

Many bacteria produce enzymes to degrade the signalling molecules in order to interrupt AHL-mediated processes. For this purpose AHL-lactonases and/or AHL-acylases are synthesised, which hydrolyze the lactone ring or cleave the amide bond of AHLs, respectively. These enzymes are found in both Gram-negative AHL producers and non-AHL producers as well as in Gram-positive bacteria (Williams, 2007). For example, the virulence of the human pathogen *P. aeruginosa* and of the plant pathogens *P. solanacearum* and diverse *Erwinia* species is influenced via QQ enzymes (Dong et al., 2000).

In summary, studying of QQ mechanism could be important to identify novel antimicrobial drugs. These might function via interfering with the QS systems either via blocking the QS receptor or degradation of QS signals, and thereby attenuate the virulence of pathogenic bacteria.

1.2.3 Role of LuxR solos in cell-cell communication

Complete QS circuits are present only in 26% of the 265 proteobacterial genomes analysed in 2008. However, additional LuxR-type proteins can be present beside a complete QS system (Case et al., 2008). These LuxR-type proteins show the modular structure of QS LuxR family members, but do not possess a cognate LuxI synthase and therefore these are referred to as LuxR orphans (Patankar and González, 2009) or LuxR solos (Subramoni and Venturi, 2009). In fact, 66% of the known proteobacterial genomes posses more LuxR than LuxI homologs and further 45 of the 265 proteobacterial genomes do not harbour a *luxI*-like AHL synthase encoding gene, but posses *luxR* homologs. So far, these LuxR-type proteins have only been identified in proteobacteria and their role is often associated with regulation of virulence, for example in *P. aeruginosa* or *Agrobacterium tumefaciens* (Case et al., 2008).

LuxR solos are present in AHL-producing bacteria and in non-AHL-producing bacteria. In AHL-producing bacteria, LuxR solos can either sense endogenous or exogenous AHLs and therefore extending the QS regulon to additional target genes. Furthermore, they could respond with different specificities towards AHLs or eventually recognize ligands produced by the surrounding bacterial community. Well-characterised LuxR solos in AHL-producing bacteria are BisR of *Rhizobium leguminosarum* bv. *viciae*, ExpR of *S. meliloti* and QscR of *P. aeruginosa* (Subramoni and Venturi, 2009). Having a closer look at the LuxR solo QscR, it remains to be present beside the QS circuits in the human pathogen *P. aeruginosa* (Oinuma and Greenberg, 2011). These QS systems are accurately regulated in a hierarchical cascade, however QscR modulates the activity of the Las and Rhl regulons. QscR responds to 3OC12 HSL, sharing it with LasR (Lee et al., 2006), and deletion of *qscR* results in hypervirulent strains (Chugani et al., 2001). Therefore QscR is a QS inhibitor via acting as a Las- and Rhl-antagonist (Fuqua, 2006).

In non-AHL-producing bacteria, LuxR solos are able to bind exogenous signalling molecules, either AHLs or even other signals produced by eukaryotes, like hormones. These LuxR solos enable the bacteria to listen to their bacterial neighbours and benefit from this information to adapt their behaviour according to their environment. Moreover, recognizing signals produced by eukaryotes may inform the bacteria about the current host or habitat to coordinate bacterial group behaviour, like virulence. However, they are not necessarily involved in QS processes and might only collect information about the surrounding area (Subramoni and Venturi, 2009). The LuxR solo SdiA is one example of LuxR homologs present in non-AHL producing bacteria, like *Escherichia coli* or *Salmonella enterica*. SdiA homologs are known to detect exogenously produced AHLs enabling these organisms to sense and respond to mixed microbial communities (Michael et al., 2001). At first, SdiA (suppressor of cell division inhibitor) of *E. coli* was described as a transcriptional activator of the *ftsQAZ* operon, which encodes essential cell division proteins (Wang et al., 1991). Lately, it could be shown that SdiA is involved in regulating the expression of several genes responsible for diverse functions such as metabolism, motility, virulence, survival and defence mechanisms in the presence of AHLs (Kim et al., 2013).

In summary, LuxR solos function in diverse roles in bacterial interspecies and interkingdom communication and are expanding the regulatory networks. Therefore,

the several LuxR solos of *P. luminescens* might also be involved in communication processes.

1.2.4 LuxR solos of plant-associated bacteria

In plant-associated bacteria (PAB), AHL-regulated QS systems are used to regulate a wide range of phenotypes including virulence, rhizosphere competence, conjugation, secretion of hydrolytic enzymes, and production of antimicrobial secondary metabolites. Interestingly, in several PABs LuxR homologs are present and recently a subfamily of LuxR solos has been described, whose regulators bind to plant low molecular weight molecules rather than to AHLs. These LuxR solos are present in beneficial and pathogenic PABs, including members of xanthomonads, rhizobia, agrobacteria and pseudomonads, and are important for plant-bacteria interactions. Furthermore, this new subfamily of LuxR solos is highly related to classical QS LuxR family members, however they form phylogenetic distinct cluster. These LuxR solos contain substitutions of one or two conserved amino acids in the AHL-binding domain, possibly allowing these proteins to bind to other signalling molecules than AHLs (González and Patel, 2013). Therefore, LuxR solos of PABs are suggested to play an important role in interkingdom signalling circuits. Accordingly, LuxR solos from *Photorhabdus* species might also respond to non-AHL signalling compounds, eventually synthesised by the eukaryotic hosts.

Different *P. putida* strains appear to be unique in varying the presence and conservation of their AHL-producing QS system. The majority of the soil-borne or plant-associated *P. putida* strains does not produce AHLs and only one third harbours a complete QS system (Elasri et al., 2001; d'Angelo-Picard et al., 2005). This is one exception of the strict classification into AHL- or non-AHL-producing bacterial strains. Interestingly, one LuxR solo, PpoR, is highly conserved in all tested *P. putida* strains. PpoR binds AHLs, either exogenously or endogenously produced, and regulates several loci, but it is not involved in rhizosphere colonization. Furthermore, expression of *ppoR* is growth-phase dependent and influenced, if present, by the corresponding QS system. However, the presence and sequence similarity of PpoR and its orthologous suggests a significant role for *P. putida*, potentially in sensing and

responding to bacterial endogenous and/or exogenous signalling molecules (Subramoni and Patel, 2009).

1.2.5 Communication via LuxR solos in *Photorhabdus* species

Bioinformatics analyses revealed the presence of an exceptionally high number of LuxR-type transcriptional regulators in *P. luminescens*, which are over-represented among the transcriptional regulators (Heermann and Fuchs, 2008). As *P. luminescens* contains no *luxI* homologous genes, these 39 LuxR-type transcriptional regulators are referred to as LuxR solos. They all share the typical domain modularity of QS LuxR-type regulators, with a N-terminal signal-binding domain (SBD), important for signal-binding and -specificity, and a C-terminal DNA-binding domain (DBD) harbouring the conserved "HTH LUXR" motif. Furthermore, they all comprise the conserved length of about 250 amino acids (Fuqua and Winans, 1994; Hanzelka and Greenberg, 1995). The 39 LuxR solos of *P. luminescens* were grouped into five different types due to the diversity of the SBD. The majority of the LuxR solos of *P. luminescens* harbour N-terminal a PAS4-domain, however the signals recognised by these receptors are yet not known. Only two of the LuxR solos of *P. luminescens*, Plu0320 and Plu4562, are predicted to possess an N-terminal AHL-binding domain of AHL-sensors. Therefore, these two LuxR solos potentially sense exogenously produced AHLs (Heermann and Fuchs, 2008). However, the LuxR solo Plu4562 shows modifications within the SBD, possibly allowing the perception of a different signalling molecule than AHLs.

The diverse LuxR solos of *P. luminescens* represent a huge cell-cell communication capacity via the recognition of diverse signalling molecules, either exogenous AHLs, endogenous and/or exogenous non-AHLs or eukaryotic signals, deriving from the nematode or insect host. Therefore, the LuxR solos are proposed to play an important role in intra-, inter- or interkingdom-signalling. Of the three *Photorhabdus* species only the genome of *P. luminescens* has been analysed for the presence of LuxR-type regulators, however it is likely that *P. temperata* and *P. asymbiotica* contain several LuxR-type receptors as well. However, up to date no signalling molecules are known to be used for the intercellular communication of *Photorhabdus* species.

1.3 Scope of this work

The main objective of this thesis was to elucidate the capacity of LuxR solo regulators of *Photorhabdus* species in cell-cell communication and the elucidation of their signalling molecules. In order to address these questions, the function of the LuxR solos PluR of *P. luminescens* and PauR of *P. asymbiotica* were characterised and their cognate signalling molecules were identified. *P. luminescens* and *P. asymbiotica* are both insect pathogens, whereas *P. asymbiotica* is additionally human pathogenic, therefore understanding of their communication opens new possibilities to identify potential drug targets.

Primarily, this thesis concentrates on the function of the LuxR solo PluR of *P. luminescens*. The target of PluR, the adjacent *pcf* (*Photorhabdus* clumping factor) operon, was previously identified via a proteomic analysis, however the signalling compound was not known. In order to identify the signalling molecule recognised by PluR, HPLC-fractionated *P. luminescens* supernatant was analysed using a fluorescence-based reporter approach. Furthermore, the role of PluR in cell-cell communication, quorum sensing or interkingdom signalling should be elucidated. Possibly, PluR also might be involved in the pathogenicity or the symbiotic competence of *P. luminescens*, which can be analysed with the insect larvae *Galleria mellonella* assay.

Interestingly, *P. asymbiotica* contains a similar LuxR solo to PluR, named PauR, and a homologous *pcf* operon, but does not harbour a PpyS. Therefore, in the second part of this thesis, the function of the LuxR solo PauR should be identified and its possibly role as an activator of the *pcf* operon. PauR either might respond to non-AHL signalling molecules or to exogenous AHLs. Furthermore, the potential role of PauR in communication systems should be discovered and its importance for the overall pathogenicity or the symbiotic capability of *P. asymbiotica*.

As formerly *P. luminescens* was identified to harbour a high number of LuxR solos, the next part of this thesis concentrates on all three *Photorhabdus* species. Therefore, the genomes of the other two *Photorhabdus* species, *P. temperata* and *P. asymbiotica*, should be analysed for the presence and diversity of LuxR solos and compared to *P. luminescens*. Furthermore, a specific focus on the SBD of potential LuxR solos was made, to identify important amino acids or motifs for binding distinct signalling molecules.

Additionally, the possibility of specific transport mechanism of the signalling molecules besides diffusion recognised by PluR and PauR should be investigated.

1.4 References of introduction

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2. PYRONES AS BACTERIAL SIGNALING MOLECULES

Reproduced from Nature Chemical Biology, (2013) Brachmann, A. O., Brameyer, S., Kresovic, D., Hitkova, I., Kopp, Y., Manske, C., Schubert, K., Bode, H. B., and Heermann, R. Pyrones as bacterial signaling molecules. 9: 573–578. doi:10.1038/nchembio.1295.

Abstract

Bacteria communicate via small diffusible molecules and thereby mediate group-coordinated behavior, a process referred to as quorum sensing. The prototypical quorum sensing system found in Gram-negative bacteria consists of a LuxI-type autoinducer synthase that produces N-acyl homoserine lactones (AHLs) as signals and a LuxR-type receptor that detects the AHLs to control expression of specific genes. However, many proteobacteria have proteins with homology to LuxR receptors yet lack any cognate LuxI-like AHL synthase. Here we show that in the insect pathogen *Photorhabdus luminescens* the orphan LuxR-type receptor PluR detects endogenously produced α-pyrones that serve as signaling molecules at low nanomolar concentrations. Additionally, the ketosynthase PpyS was identified as pyrone synthase. Reconstitution of the entire system containing PluR, the PluR-target operon we termed *pcf* and PpyS in *Escherichia coli* demonstrated that the cell-cell communication circuit is portable. Our research thus deorphanizes a signaling system and suggests that additional modes of bacterial communication may await discovery.

Full-text article:

http://www.nature.com/nchembio/journal/v9/n9/full/nchembio.1295.html

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http://www.nature.com/nchembio/journal/v9/n9/extref/nchembio.1295-S1.pdf

3. DIALKYLRESORCINOLS AS BACTERIAL SIGNALING MOLECULES

Reproduced from Proceedings of the National Academy of Sciences (2014) Brameyer, S., Kresovic, D., Bode, H. B., and Heermann, R. Dialkylresorcinols as bacterial signaling molecules. XXX. doi:10.1073/pnas.1417685112

Abstract

It is well recognized that bacteria communicate via small diffusible molecules, a process termed quorum sensing. The best understood quorum sensing systems are those that use acylated homoserine lactones (AHLs) for communication. The prototype of those systems consists of a LuxI-like AHL synthase and a cognate LuxR receptor that detects the signal. However, many proteobacteria possess LuxR receptors, yet lack any LuxI-type synthase, and thus these receptors are referred to as LuxR orphans or solos. In addition to the well-known AHLs, little is known about the signaling molecules that are sensed by LuxR solos. Here, we describe a novel cell-cell communication system in the insect and human pathogen *Photorhabdus asymbiotica*. We identified the LuxR homolog PauR to sense dialkylresorcinols (DARs) and cyclohexanediones (CHDs) instead of AHLs as signals. The DarABC synthesis pathway produces the molecules, and the entire system emerged as important for virulence. Moreover, we have analyzed more than 90 different *Photorhabdus* strains by HPLC/MS and showed that these DARs and CHDs are specific to the human pathogen *P. asymbiotica*. On the basis of genomic evidence, 116 other bacterial species are putative DAR producers, among them many human pathogens. Therefore, we discuss the possibility of DARs as novel and widespread bacterial signaling molecules and show that bacterial cell-cell communication goes far beyond AHL signaling in nature.

Full-text article:

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4. LUXR SOLOS IN *PHOTORHABDUS* SPECIES

Reproduced from Frontiers in Cellular and Infection Microbiology, (2014) Brameyer S, Kresovic D, Bode HB, Heermann R LuxR solos in *Photorhabdus* species. 4: 1–23. doi:10.3389/fcimb.2014.00166.

Abstract

Bacteria communicate via small diffusible molecules to mediate group-coordinated behavior, a process designated as quorum sensing. The basic molecular quorum sensing system of Gram-negative bacteria consists of a LuxI-type autoinducer synthase producing acyl-homoserine lactones (AHLs) as signaling molecules, and a LuxR-type receptor detecting the AHLs to control expression of specific genes. However, many proteobacteria possess one or more unpaired LuxR-type receptors that lack a cognate LuxI-like synthase, referred to as LuxR solos. The enteric and insect pathogenic bacteria of the genus *Photorhabdus* harbor an extraordinarily high number of LuxR solos, more than any other known bacteria, and all lack a LuxI-like synthase. Here, we focus on the presence and the different types of LuxR solos in the three known *Photorhabdus* species using bioinformatics analyses. Generally, the N-terminal signal-binding domain (SBD) of LuxR-type receptors sensing AHLs have a motif of six conserved amino acids that is important for binding and specificity of the signaling molecule. However, this motif is altered in the majority of the *Photorhabdus*-specific LuxR solos, suggesting the use of other signaling molecules than AHLs. Furthermore, all *Photorhabdus* species contain at least one LuxR solo with an intact AHL-binding motif, which might allow the ability to sense AHLs of other bacteria. Moreover, all three species have high AHL-degrading activity caused by the presence of different AHL-lactonases and AHL-acylases, revealing a high quorum quenching activity against other bacteria. However, the majority of the other LuxR solos in *Photorhabdus* have a N-terminal so-called PAS4-domain instead of an AHL-binding domain, containing different amino acid motifs than the AHL-sensors, which potentially allows the recognition of a highly variable range of signaling molecules that can be sensed apart from AHLs. These PAS4-LuxR solos are proposed to be involved in host sensing, and therefore in inter-kingdom signaling. Overall, *Photorhabdus* species are perfect model organisms to study bacterial communication via LuxR solos and their role for a symbiotic and pathogenic life style.

Full-text article:

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5 # 5. SPECIFICITY OF SIGNAL-BINDING VIA
6 # NON-AHL LUXR-TYPE RECEPTORS
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Specificity of signal-binding via non-AHL LuxR-type receptors

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Abstract:	Quorum sensing is a typical communication system among Gram-negative bacteria used to control group-coordinated behavior via small diffusible molecules dependent on cell number. The key components of a quorum sensing system are a LuxI-type synthase, producing acyl-homoserine lactones (AHLs) as signaling molecules, and a LuxR-type receptor that detects AHLs to control expression of specific target genes. Six conserved amino acids are present in the signal-binding domain of AHL-sensing LuxR-type proteins, which are important for ligand-binding and -specificity as well as shaping the ligand-binding pocket. However, many proteobacteria possess LuxR-type regulators without a cognate LuxI synthase, referred to as LuxR solos. The two LuxR solos, PluR and PauR from Photorhabdus luminescens and Photorhabdus asymbiotica, respectively, are not sensing AHLs. Instead PluR and PauR sense α-pyrones and dialkylresorcinols, respectively, and are part of cell-cell communication systems contributing to the overall virulence of these Photorhabdus species. However, PluR and PauR both harbor substitutions in the conserved amino acid motif compared to AHL sensors, which appeared to be important for binding the corresponding signaling molecules. Here we analyze the role of the conserved amino acids in the signal-binding domain of these two non-AHL LuxR-type receptors for their role in signal perception. Our studies reveal that the conserved amino acid motif alone is essential but not solely responsible for ligand-binding.
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Abstract

Quorum sensing is a typical communication system among Gram-negative bacteria used to control group-coordinated behavior via small diffusible molecules dependent on cell number. The key components of a quorum sensing system are a LuxI-type synthase, producing acyl-homoserine lactones (AHLs) as signaling molecules, and a LuxR-type receptor that detects AHLs to control expression of specific target genes. Six conserved amino acids are present in the signal-binding domain of AHL-sensing LuxR-type proteins, which are important for ligand-binding and -specificity as well as shaping the ligand-binding pocket. However, many proteobacteria possess LuxR-type regulators without a cognate LuxI synthase, referred to as LuxR solos. The two LuxR solos, PluR and PauR from *Photorhabdus luminescens* and *Photorhabdus asymbiotica,* respectively, are not sensing AHLs. Instead PluR and PauR sense α-pyrones and dialkylresorcinols, respectively, and are part of cell-cell communication systems contributing to the overall virulence of these *Photorhabdus* species. However, PluR and PauR both harbor substitutions in the conserved amino acid motif compared to AHL sensors, which appeared to be important for binding the corresponding signaling molecules. Here we analyze the role of the conserved amino acids in the signal-binding domain of these two non-AHL LuxR-type receptors for their role in signal perception. Our studies reveal that the conserved amino acid motif alone is essential but not solely responsible for ligand-binding.

Introduction

Cell-cell communication via small diffusible molecules to mediate bacterial group-coordinated behavior dependent on the cell size is referred to as quorum sensing (QS). The basic molecular QS system of Gram-negative bacteria consists of a LuxI-like autoinducer synthase and a LuxR-type receptor that detects the signaling molecule to control expression of specific target genes [1]. Typically, Gram-negative bacteria use acyl-homoserine lactones (AHLs) for communication, which are constantly synthesized by LuxI at a basal level, and sensed by the cognate LuxR-like receptor when exceeding a threshold concentration. However, several LuxR-type proteins show the modular domain structure of QS LuxR family members, but do not possess a cognate LuxI synthase. These LuxR proteins are referred to as LuxR orphans [2] or solos [3]. Strikingly, the three enteric and insect pathogenic *Photorhabdus* species, *P. luminescens*, *P. temperata* and *P. asymbiotica*, harbor an exceptional high number of LuxR solos [4], however no *luxI* homologous genes are present. Therefore, all *Photorhabdus* species found so far do not producing AHLs [5].

Recently, we identified the two homologous LuxR-type proteins PluR and PauR of *P. luminescens* and *P. asymbiotica*, respectively, detecting each an endogenous signaling molecule used for cell-cell communication. Formerly, both PluR and PauR were classified as LuxR solos, however, since the cognate synthase systems were identified, these receptors are designated as LuxR-type receptors in the following. The LuxR-type receptor PluR of *P. luminescens* senses α-pyrones, named photopyrones (PPYs), as signaling molecules at nanomolar concentrations. Moreover, PPYs are produced by the photopyrone synthase PpyS, which is a ketosynthase-like enzyme. *P. temperata* possess a PluR-homolog and a PpyS-homolog revealing a similar cell-cell communication via pyrones [6]. Contrarily, *P. asymbiotica* comprises neither a LuxI nor a PpyS homolog, thus PauR detects dialkylresorcinols (DARs) and cyclohexanediones (CHDs) as signaling molecules instead of AHLs or PPYs [5]. These signaling compounds are used as well for cell-cell communication and are synthesized by the DarABC operon. Moreover, CHDs are intermediates of the DAR pathway [7]. Upon signal recognition, both LuxR-type receptors activate expression of the cognate *pcfABCDEF* operon leading to cell clumping and contributing to the overall virulence of *Photorhabdus* species

1 [5, 6]. *Photorhabdus* species are highly pathogenic toward insects, whereas
2 *P. asymbiotica* is additionally able to colonize and to infect men.
3 Certainly, both the LuxR-type receptors PluR and PauR both share the typical
4 domain modularity of QS LuxR proteins, with a N-terminal signal-binding domain
5 (SBD) and a C-terminal helix-turn-helix DNA-binding domain (DBD) [8] (Fig. 1A).
6 Upon binding the cognate signaling molecule to the SBD of a LuxR-type regulator, a
7 conformational change is induced, commonly followed by the recognition of target
8 promoter regions by the DBD and transcriptional activation [9]. Furthermore, AHL-
9 binding to AHL-LuxR family proteins is necessary for stability, correct folding [10]
10 or dimerization [11]. LuxR-type proteins share a low protein sequence identity (18 %-
11 25 %), however, nine highly conserved amino acids are identical in at least 95% of
12 LuxR-type proteins. The SBD harbors six conserved amino acids (W57, Y61, D70,
13 P71, W85 and G113, with respect to TraR, Fig. 1C), reflecting a conserved motif for
14 AHL-sensors, which is important for signal-specificity and shaping of the ligand-
15 binding pocket [2]. Three conserved amino acids are located in the C-terminal DBD
16 important for DNA-binding [2] (Fig. 1C). The highest conservation of primary
17 structure of several QS LuxR family members is located in the DBD, as its function
18 and mechanism is similar in all LuxR receptors. Whereas the SBD is quite diverse,
19 potentially evolved to an adaptation to specific signaling molecules [12]. The non-
20 AHL sensors PluR and PauR both share a high protein sequence identity among each
21 other compared to typical AHL-LuxR QS family members like TraR from
22 *Agrobacterium tumefaciens* (Fig. 1B). Furthermore, PluR and PauR both harbor four
23 substitutions at the similar positions in the conserved WYDPWG-motif of AHL-
24 sensors, displaying a TYDQCS-motif and a TYDQYI-motif, respectively [5].
25 However, single substitution of the conserved positions Y66 and D75 with alanine in
26 PluR as well as PauR prevented activation by the cognate signaling molecules [5, 6].
27 In this study we focused on the function of the amino acids of the TYDQCS-motif
28 of PluR and the TYDQYI-motif of PauR in the SBD for binding and/or specificity of
29 the cognate signaling molecule and the functionality of the ligand-binding pocket.
30 Specific amino acids were replaced either against A or the respective conserved
31 amino acid at the similar position of QS LuxR family proteins. Furthermore, the
32 conserved motifs of the non-AHL LuxR solos PluR and PauR were restored to

1 possibly sense unrelated signaling molecules to locate the adequate amino acids for
2 AHL- or PPY-sensing.
3

Materials and Methods

Bacteria and growth conditions

The bacterial strains used in this study were *E. coli* DH10a [13] and *E. coli* LMG194 [14]. The plasmids used in this study are listed in S1 Table and oligonucleotides in S2 Table. *E. coli* strains were grown aerobically at 37°C in LB medium [10% (w/v) peptone, 5% (w/v) yeast extract, 10% (w/v) NaCl] or in M63 minimal medium [15] with appropriate antibiotics. Carbenicillin and ampicillin were used at 100 µg/ml and gentamicin was used at 20 µg/ml final concentration. Synthetic C8-HSL (N-3-oxooctanoyl-L-homoserinelactone) was purchased from Sigma-Aldrich and dissolved in methanol. Photopyrone D (PPYD) were isolated and purified from *P. luminescens* TT01 supernatant and dissolved in isopropanol [6]. 2,5-dialkylresorcinol (DAR), 2,5-dialkylcyclohexane-1,3-diones (CHDA and CHDB) and isopropylstilbene (IPS) were isolated and purified from *P. asymbiotica* PB68.1 supernatant and dissolved in isopropanol [5].

Generation of plasmids

To monitor the effect of amino acid substitutions in PluR and PauR site-directed mutagenesis was performed to generate *pluR* and *pauR* derivatives. This was achieved with two-step PCR using the appropriate primer pairs and *P. luminescens* or *P. asymbiotica* genomic DNA, respectively, as template (e.g. PluR_T62W_fwd and PluR_T62W_rev for pBAD24-His-*pluR*-T62W). The overlap PCR was performed using the primers Plu4562-6HisNcoIs and 4562_SalI_rev for *pluR* derivatives and the primers PAU4062-His-NheI_fwd and 4062_SalI_rev for *pauR* derivatives, and the respective DNA-fragment was cloned into plasmid pBAD24 [14] using restriction sites NcoI and SalI or NheI and SalI for *pluR* or *pauR*, respectively. Correct insertion was verified by sequence analyzes using primer pBAD24_Seq_fwd.

1 **Reporter plasmid assays**

2 To test the specificity of PluR or PauR towards different signaling molecules,
3 *E. coli* LMG194 was transformed with the plasmids pBAD24-His-*pluR* and the
4 reporter plasmid pBBR1-$pcfA_{P.l.}$-*luxCDABE* or pBAD24-His-*pauR* and the reporter
5 plasmid pBBR1-$pcfA_{P.a.}$-*luxCDABE*, respectively. As controls, *E. coli* LMG194 was
6 transformed with the plasmids pBAD24-His-*pluR* or pBAD24-His-*pauR* and pBBR1-
7 MCS5-TT-RBS-*lux* (no promoter) or pBAD24 (empty plasmid) and pBBR1-P$pcfA_{P.l.}$-
8 *lux* or pBBR1-P$pcfA_{P.a.}$-*lux*, respectively. Overnight cultures were grown in M63
9 minimal medium, adjusted to $OD_{620\ nm}$ = 0.05 and then aerobically cultivated in 96-
10 well plates at 37 °C. At $OD_{620\ nm}$ = 0.1, different signaling molecules were separately
11 added, and the $OD_{620\ nm}$ and the luminescence were monitored every hour in a Sunrise
12 plate reader (Tecan, Crailsheim) and a Centro luminometer (Berthold Technologies,
13 Bad Wildbad), respectively. The signaling molecules photopyrone D (PPYD), 2,5-
14 dialkylresorcinol (DAR), 2,5-dialkylcyclohexane-1,3-diones (CHDA and CHDB),
15 isopropylstilbene (IPS) were added in a final concentration of 3.5 nM. The AHL N-3-
16 oxooctanoyl-L-homoserinelactone (C8-HSL) was added in a final concentration of
17 100 nM. Nomenclature of PPYD is used according to [6] and nomenclature of IPS,
18 CHDA, CHDB and DAR is used according to [5]. As control the same amount of
19 isopropanol or methanol was added. For all strains the relative light unit (RLU) was
20 calculated and subtracted from the respective control strain where only isopropanol or
21 methanol was added. Moreover, highest induction was determined at time point 2 h
22 after addition of substances.

23 Amino acid substitutions in PluR or PauR might affect the spatial structure of the
24 proteins and influence their functionality to bind the cognate *pcfA* promoter. To
25 quantify the structural influence of amino acid replacements in the signal-binding
26 domain (SBD) of PluR or PauR, the ability of PluR wild type or PauR wild type and
27 different derivatives to activate *pcfA* promoter activity was determined. Therefore, the
28 similar method was used as described above, however 0.1 % (w/v) arabinose was
29 added and also derivatives of PluR or PauR were used. For better comparison, the
30 values of PluR wild type or PauR wild type was set as 100% in the figures 3 and 4.

31 To quantify the influence of amino acid replacements in the SBD of PluR or PauR
32 on sensing of signaling molecules, the similar plasmid combinations as described
33 above were used, however the distinct signaling molecules PPYD, CHDA, CHDB and

DAR were added. The same final concentration of substances was used as described above. For better comparison, the values of PluR wild type or PauR wild type was set to 100% in the figures 3 and 4.

Generation of αPluR and αPauR antibodies

To generate specific antibodies against full-length PluR, *E. coli* BL21 was transformed with the plasmid pBAD24-His-*pluR*, cultivated at 30 °C and expression was induced at $OD_{600\ nm} = 0.5$ with 0.1% (w/v) arabinose. Whole cells were subjected to SDS-PAGE [16], and the amount of 6His-PluR was detected by staining with coomassie solution [40% (v/v) ethanol; 10% (v/v) acetic acid; 0.2% (w/v) coomassie brilliant blue R250] and destained with destaining solution [40% (v/v) ethanol; 10% (v/v) acetate] [17]. The according band with a size of 27.03 kDa was cut and used as an antigen to produce polyclonal antisera and antibodies in two rabbits (Biogenes, Berlin). Furthermore, total IgG of αPluR antibody was purified using Protein-A column (Biogenes, Berlin). Highest specificity of αPluR antibody was given in 3 % BSA and a dilution of 1:10.000.

To generate specific antibodies against PauR, polyclonal antibodies were generated in two rabbits against a peptide of PauR (amino acids 62-75: CTMGNYDKNDNHDSD) (Biogenes, Berlin). Total IgG of αPauR antibody was purified using Protein-A column (Biogenes, Berlin). Highest specificity of αPauR antibody was given in 5 % milk powder and a dilution of 1:10.000.

Western Blot analysis

For control of protein production of PluR, PauR and the respectively derivatives, *E. coli* strains harboring pBAD-His-*pluR*, pBAD-His-*pauR* or derivatives were cultivated at 37°C in LB. Cells were harvested 2 h after addition of 0.1% (w/v) arabinose and then adjusted to $OD_{600\ nm} = 1$. After SDS-PAGE [16], the proteins were blotted onto a nitrocellulose membrane (Whatman, Germany) in a Mini Trans-Blot cell (Bio-Rad, USA) chamber using a constant power of 100 mA over night. Then, the membranes were incubated with the αPluR antibody (Biogenes, Berlin) or the αPauR

1 antibody (Biogenes, Berlin), as the primary antibody. The α-rabbit alkaline
2 phosphatase-conjugated antibody (Rockland Immunochemicals, Hamburg) was used
3 as the secondary antibody according to the manufacturer's recommendations.
4 Localization of the secondary antibody was visualized using colorimetric detection of
5 alkaline phosphatase activity with 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and
6 nitro blue tetrazolium chloride (NBT).
7

Results

PluR and PauR specifically sense their cognate signaling molecule

The SBD of QS LuxR family members mediates specificity towards the respective signaling molecules, on the one hand by shaping the ligand pocket and on the other hand via amino acids that are essential for signal-binding. Primarily, we tested the specificity of PluR and PauR to sense different endogenous or exogenous signaling compounds, like their native signaling molecules or AHLs. For that reason, PluR and PauR were each tested on their ability to sense photopyrone D (PPYD) from *P. luminescens*, the *P. asymbiotica*-specific signaling molecules dialkylresorcinol (DAR) and its precursors, dialkylcyclohexane-1,3-diones (CHDA and CHDB) and isopropylstilbene (IPS), and the AHL N-3-oxooctanoyl-L-homoserinelactone (C8-HSL). For this purpose the PluR- and PauR-specific reporter plasmid systems, pBAD24-His-*pluR* and pBBR1-*pcfA*$_{P.l.}$-*lux* or pBAD24-His-*pauR* and pBBR1-*pcfA*$_{P.a.}$-*lux*, respectively, were applied.

To prove suitability of the reporter system, we proved that no luminescence induction occurred either in the presence of one regulator, PluR or PauR, with a promoter-less reporter plasmid or on the other hand in the absence of PluR or PauR with a reporter plasmid containing the *pcfA* promoter of *P. luminescens* or of *P. asymbiotica*, respectively (Fig. 2). Then, PluR as well as PauR were tested for induction of reporter gene activity in the presence of different signaling molecules. PluR senses specifically PPYD with a concentration of 3.5 nM and not unrelated signaling molecules like C8-HSL or the signaling compounds DAR, CHDA, CHDB and IPS from *P. asymbiotica* (Fig. 2A). Similar to PluR, also PauR showed specificity for it´s native signaling molecules. PauR neither recognizes PPYD nor C8-HSL, but most specifically senses DAR with a concentration as low as of 3.5 nM. Furthermore, PauR senses the DAR precursors CHDA, CHDB and IPS with decreasing sensitivity, tested each with a concentration of 3.5 nM (Fig. 2B). Summing up, both LuxR-type regulators PluR and PauR specifically sense the signaling molecule produced by its own species and not to exogenous signaling molecules. None of the two LuxR-type

receptors senses C8-AHL, even when added in higher concentrations than their native signaling molecules. Although chemically different, the tested signaling molecules appear structurally somewhat similar in their size, so that their would potentially all fit into the SBD ligand-binding pocket of both PluR and PauR (Fig. 2C).

Specificity of conserved amino acids towards signaling molecule sensing

The SBD of QS LuxR family members harbors six conserved amino acids constituting a conserved WYDPWG-motif for AHL-sensors, which is essential for signal-specificity and shaping of the ligand-binding pocket. PluR and PauR both share only two of the conserved amino acids within AHL-sensors, Y66 and D75, and display a TYDQCS-motif and a TYDQYI-motif at the similar amino acid positions, respectively. Although, the sequence identity between PluR and PauR represents 83% (Fig. 1), both sense different signaling molecules. To identify amino acids within each conserved motif essential for signal binding for PluR and PauR, the amino acids within the TYDQCS- and TYDQYI-motif were individually replaced either with alanine or the respective amino acids conserved in AHL-sensors or in PluR. As above, the ability of PluR or PauR and their derivatives were tested to bind and activate the corresponding *pcfA* promoter in presence of the native signaling molecule. To exclude structural influences on the receptor by the amino acid replacements, PluR and PauR wild type and their derivatives were first tested on their general functionality to bind and activate the corresponding *pcfA* promoter. This was tested by activation of *pcfA* promoter activity due to simple overproduction independent of the cognate signaling molecule (Fig. 3, lower axis). The amino acid substitutions D75E and S115G in PluR dramatically decreased its ability to activate expression of *pcfA* promoter region to 50% compared to PluR wild type (Fig. 3, left lower quadrant). This decreased ability to bind and activate the corresponding *pcfA* promoter, which is possibly caused by conformational defects within one monomer or defects in dimerization. However, all other PluR derivatives that have been tested showed no general functional defects. Furthermore, all tested amino acid replacements in PluR did not influence protein production since protein amounts comparable to the wild type could be detected after

1 overproduction (S1 Fig.). Then, the ability of all PluR derivatives was tested for
2 PPYD-sensing. It emerged that the substitutions Y66A, D75A, D75N, Q76A and
3 Q76P in PluR most dramatically impaired signaling molecule sensing (Fig. 3, right
4 lower quadrant). The PluR derivative C90W showed a 50% reduced reporter gene
5 activity compared to the wild type. Only the replacement T62W did not affect PPYD-
6 sensing of PluR (Fig. 3, upper right quadrant).
7 Likewise, PauR and its derivatives carrying amino acid substitutions in the
8 TYDQYI-motif were analyzed for their functionality and ability of DAR-sensing.
9 Additionally, the amino acids S38 and Y40 were also analyzed for their impact on
10 DAR-sensing as these have been predicted to be as well involved in DAR-sensing [5].
11 The amino acid replacements Y40A, Y40F, D75E, D75N and Q76A in PauR
12 dramatically influenced the general functionality of PauR. Thus, over-production of
13 these proteins dramatically reduced *pcfA*$_{P.a.}$ promoter activity compared to wild type
14 (Fig. 4, lower left quadrant). The most drastic effect appeared when position D75 was
15 replaced with E and when position Y40 was substituted against A (7-25% compared
16 to PauR wild type) (Fig. 4, lower left quadrant). Therefore, these amino acids might
17 be important for shaping the SBD and substitutions of these amino acids affect either
18 the monomer structure or dimerization prior to *pcfA* promoter binding. Protein over-
19 production of PauR wild type and all derivatives were comparable revealing that the
20 amino acid substitutions did not effect protein production (S1 Fig.). All tested amino
21 acids replacements within the TYDQYI-motif as well as S38A decreased the ability
22 of PauR to sense DAR (Fig. 4, lower right quadrant). Replacement of T62A in PauR
23 completely prevented DAR-sensing (5 %) without affecting its overall functionality.
24 Furthermore, drastic effects were gained on DAR-sensing with the replacement of
25 S38A, Y66A, D75A and D75N in the SBD of PauR (33-65 %) and a decreased effect
26 on DAR-sensing were gained with the replacement of Y90C and I113S in PauR (53-
27 64%) (Fig. 4, lower right quadrant).
28 In both LuxR-type receptors the size of amino acid at position 75 seems to be
29 crucial. When D75 is substituted with a bigger amino acid (D75E) the general
30 functionality of both proteins PluR and PauR is affected (Figs. 3 and 4). Whereas
31 charge reversion at position D75N impaired signal-binding and not the general
32 functionality of both PluR and PauR. Furthermore, amino acid Y66 in both proteins is
33 essential for recognizing of the corresponding signaling molecule. Interestingly, also

position S38 of PauR is crucial for DAR-sensing (Fig. 4). However, this amino acid is not comprised in the conserved TYDQYI-motif but predicted to be involved in DAR-sensing in a DAR-docking model of the SBD of PauR [5]. Overall, all amino acids within the conserved amino acid motif in PluR as well as PauR are important for signal-sensing or functionality, supporting the idea that all these amino acids are located in the respective signal-binding pocket.

In another approach, we changed the TYDQYI-motif of PluR to the WYDPWG-motif of AHL-sensors to possibly achieve AHL-sensing instead of PPYs. For that reason, we generated quadruple substitutions within the SBD of PluR, resulting in the derivative PluR-T62W/Q76P/C90W/S115G, which was stepwise performed via introducing the next amino acid exchange in PluR-T62W. With successive introduction of more amino acid substitutions within the SBD of PluR, the general functionality of these derivatives is stepwise decreased. However, these derivatives showed comparable protein amounts like wild type (S1 Fig.). The quadruple derivative PluR-T62W/Q76P/C90W/S115G strongly impaired the ability to activate the *pcfA*$_{\mathrm{P.l.}}$ promoter tested by simple over-production of the protein (Fig. 5A). Likewise, PPYD-sensing is stepwise impaired with progressive amino acid substitutions in PluR, but AHL-sensing could not be gained (Fig. 5B). Furthermore, the reporter gene activities after addition of C8-HSL were lower than the background values of unspecific signaling molecules (compare with Fig. 2A). PluR shows the highest protein sequence identity to QscR of *Pseudomonas aeruginosa* of LuxR-type regulators with known crystal structures. The LuxR solo QscR responds to multiple AHLs, like C8-HSL [18], which was therefore used in our study. In conclusion, more amino acids must make up the specificity for AHL-sensing besides the conserved WYDPWG-motif.

Since PluR is very homologous to PauR with a protein sequence identity of 83 % (Fig. 1), we tried to convert PauR to a PPY-sensor. For that reason, the conserved TYDQYI-motif of PauR was converted to the TYDQCS-motif of PluR to potentially gain PPYD-sensing, though also the general functionality of PauR-Y90C/I113S was affected and reduced to approximately 50 % compared to PauR wild type (Fig. 5C). However, PauR-Y90C/I113S still had the ability to sense DAR, although this was dramatically reduced about 70 % compared to PauR wild type (Fig. 5D). Protein amounts of PauR-Y90C/I113S were comparable to PauR wild type (S1 Fig.).

Thus, these six conserved amino acids in the SBD, displaying either the WYDPWG-motif of AHL-sensors, the TYDQCS-motif of PluR or the TYDQYI-motif of PauR, are all essential for shaping the specific ligand pocket and ligand-binding, however, these are not sufficient for signal-sensing and -specificity. The solely insertion of the specific amino acid motif for AHL-, PPY- or DAR-sensors into a LuxR-type receptor is therefore not sufficient to convert the signal specificity of the sensor. This reveals that other amino acids in the SBD must also be essential for forming the ligand-binding pocket and for signal perception, although these are not highly conserved.

Discussion

The two LuxR-type regulators PluR and PauR are both part of a quorum sensing system in *P. luminescens* and *P. asymbiotica* depending on non-AHL compounds as signals. Both receptors sense different endogenous signaling molecules, PPYs or DARs, respectively, but both activate the expression of the corresponding *pcfABCDEF* operon. Expression of the *pcfABCDEF* operon in turn leads to cell clumping and contributes to the virulence of *Photorhabdus* species [5, 6]. In this study we focused on signal specificity of the two non-AHL sensing LuxR-type receptors PluR and PauR.

PluR and PauR harbor two of the conserved amino acids of the conserved WYDPWG-motif of AHL-sensors, comprising a TYDQCS-motif and a TYDQYI-motif, respectively. However, our studies reveal that these motifs are as important for signal-specificity and conformation as for AHL-sensing of QS LuxR family proteins. Substitution of the conserved amino acid D75 of each PluR and PauR highly decreased the recognition of the cognate signaling molecule. Since D75 of PluR and PauR is deduced to form a hydrogen bond to the hydroxy group attached to the pyrone and the DAR-hydroxy group, respectively [5, 6]. Likewise, in the AHL-sensor TraR this position (D70) is known to be important for binding the amide group of the N-3-oxooctanoyl-L-homoserine lactone [19]. Certainly in PluR and PauR the size and charge of the amino acid at position D75 mediates correct signaling molecule binding as substitution against glutamic acid impaired conformation and substitution against asparagine either affect binding of PPYD or DAR. Docking experiments with PauR and DAR as ligand revealed an arene-arene interaction between T62 and Y66 and the DAR aromatic ring [5]. This was also confirmed by single replacements of both amino acids with alanine, which decreased the ability to sense the ligand (Fig. 4). However, in PluR only Y66 was deduced to form an arene-arene interaction with the pyrone ring [6]. Accordingly, substitution of Y66A in PluR showed a dramatically reduced ability to sense PPYD, whereas substitution of T62 against A showed no effect (Fig. 3). The appropriate position to Y66 of PluR and PauR is Y61 in AHL-sensors and this amino acid is known to be involved in binding of the acyl chain of the signaling molecule via hydrophobic interactions, e.g. in TraR [19] or LuxR [11]. In general, the six conserved amino acids in the SBD are essential for shaping the

ligand pocket and ligand-binding, either in QS LuxR family members binding AHLs or non-AHLs like PluR and PauR. Also the subfamily of LuxR solos of plant-associated bacteria have conserved substitutions in the WYDPWG-motif of AHL-sensors, which are W57M and Y61W (with respect to TraR), however the specific signaling molecules are yet unknown. These substitutions are assumed to allow the binding of plant signal molecules rather than AHLs [2, 20]. Furthermore, several amino acids beside the WYDPWG-motif are known to be involved either in ligand-binding, dimerization or DNA-binding in SdiA from *E. coli* [21], TraR from *A. tumefaciens* [22] and LuxR from *V. fischeri* [23, 24]. This is also true for PauR, in which the position S38 outside of the conserved motif is important for DAR-sensing.

In conclusion, the conserved TYDQCS- and TYDQYI-motifs of PluR and PauR, respectively, are essential but not only sufficient for ligand-binding. Hence, other amino acids of the SBD must also contribute to the signal sensing specificity, although these are not highly conserved. Similarly, QS LuxR family members sensing AHLs contain several important amino acids in the SBD that are important for AHL-binding beside the conserved WYDPWG-motif. Therefore, each QS LuxR-type protein potentially evolved special amino acids to bind its specific signaling molecule to regulate diverse cellular processes. However, PluR and PauR regulate the expression of the cognate *pcfABCDEF* operon leading to cell clumping. Therefore, the question remains why both organisms, *P. luminescens* and *P. asymbiotica*, use different molecules for this QS-regulated process, which results in a similar phenotype. Possibly, both adapted to their different host, and therefore PPY-signaling might be more appropriate for infection of invertebrate hosts and DAR-signaling might be a better choice for infecting vertebrates. This idea is underline by the fact that genomic analysis revealed that many human pathogens are putative DAR producers and that these pathogens might also constitute a DAR-dependent QS system, possibly besides an intact AHL QS system [5].

In summary, our studies reveal that specific amino acid motifs in the SBD of LuxR-type receptors are important for signal-sensing, but not alone sufficient for signal-specificity. The replacement of diverse amino acids within the SBD allow LuxR-type receptors to sense diverse families of signaling molecules beside AHLs. The specific amino acid motifs for AHL as well as for PPY and DAR sensors are incomplete to date. Future work has to be performed to identify the complete amino

acid motifs in the SBD of LuxR-type receptors, which can then be used for signal prediction of yet un-investigated LuxR solos.

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1 # Supplementary information
2
3
4 ## Tables
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7 **S1 Table: Plasmids used in this study.**

Plasmid	Characteristics	Reference
pBAD24	Expression vector, arabinose inducible promoter, Amp^R	[14]
pBAD24-His-*pluR*	*pluR* (*plu4562*) in pBAD24 with N-terminal His-tag	[6]
pBAD24-His-*pluR*-T62W	Substitution of T62W in *pluR* (*plu4562)* in pBAD24	This study
pBAD24-His-*pluR*-Y66A	Substitution of Y66A in *pluR* (*plu4562)* in pBAD24	[6]
pBAD24-His-*pluR*-D75A	Substitution of D75A in *pluR* (*plu4562)* in pBAD24	[6]
pBAD24-His-*pluR*-D75N	Substitution of D75N in *pluR* (*plu4562)* in pBAD24	This study
pBAD24-His-*pluR*-D75E	Substitution of D75E in *pluR* (*plu4562)* in pBAD24	This study
pBAD24-His-*pluR*-Q76P	Substitution of Q76P in *pluR* (*plu4562)* in pBAD24	This study
pBAD24-His-*pluR*-C90W	Substitution of C90W in *pluR* (*plu4562)* in pBAD24	This study
pBAD24-His-*pluR*-S115G	Substitution of S115G in *pluR* (*plu4562)* in pBAD24	This study
pBAD24-His-*pauR*	*pauR* (*pau_04062*) in pBAD24 with N-terminal His-tag	[5]
pBAD24-His-*pauR*-S38A	Substitution of S38A in *pauR* (*pau_04062)* in pBAD24	This study
pBAD24-His-*pauR*-Y40A	Substitution of Y40A in *pauR* (*pau_04062)* in pBAD24	This study
pBAD24-His-*pauR*-Y40F	Substitution of Y40F in *pauR* (*pau_04062)* in pBAD24	This study
pBAD24-His-*pauR*-T62A	Substitution of T62A in *pauR* (*pau_04062)* in pBAD24	[5]
pBAD24-His-*pauR*-Y66A	Substitution of Y66A in *pauR* (*pau_04062)* in pBAD24	[5]
pBAD24-His-*pauR*-D75A	Substitution of D75A in *pauR* (*pau_04062)* in pBAD24	[5]
pBAD24-His-*pauR*-D75E	Substitution of D75E in *pauR* (*pau_04062)* in pBAD24	This study
pBAD24-His-*pauR*-D75N	Substitution of D75N in *pauR* (*pau_04062)* in pBAD24	This study
pBAD24-His-*pauR*-Q76A	Substitution of Q76A in *pauR* (*pau_04062)* in pBAD24	This study

pBAD24-His-*pauR*-Y90A	Substitution of Y90A in *pauR* (*pau_04062)* in pBAD24	This study
pBAD24-His-*pauR*-1113A	Substitution of I113A in *pauR* (*pau_04062)* in pBAD24	This study
pBBR1-MCS5-TT-RBS-*lux*	*luxCDABE* and terminators lambda T0 rrnB1 T1 cloned into pBBR1-MCS5 for plasmid-based transcriptional fusions; Gm^R	[25]
pBBR1-P*pcfA*$_{P.l.}$-*lux*	*pcfA* (*plu4568*) promoter in pBBR1-MCS5-TT-RBS-*lux*	[6]
pBBR1-P*pcfA*$_{P.a.}$-*lux*	*pcfA* (*pau_04068*) promoter in pBBR1-MCS5-TT-RBS-*lux*	[5]

S2 Table. Oligonucleotides used in this study. Underlined nucleotides indicate the position of the site-directed mutagenesis.

Oligo	Sequence
Plu4562-6HisNcoIs	5'-GAGGAACCATGGCGCACCACCATCATCACCAT AGGAAAATCTTATGAACACATC-3'
4562_SalI_rev	5'-TAGCCGTCGACTTATATGATTAGATTATATGC-3'
PluR_T62W_fwd	5'-TAAATATAGAATGGGCAAGTAAT-3'
PluR_T62W_rev	5'-ATTACTTGCCCATTCTATATTTA-3'
PluR_Y66A_fwd	5'-GCAAGTAATGCTAATCAAG-3'
PluR_Y66A_rev	5'-CTTGATTAGCATTACTTGC-3'
PluR_D75A_fwd	5'-CATGACAGCGCACAATTAATG-3'
PluR_D75A_rev	5'-CATTAATTGTGCGCTGTCATG-3'
PluR_D75E_fwd	5'-CATGACAGCGAGCAATTAATG-3'
PluR_D75E_rev	5'-CATTAATTGCTCGCTGTCATG-3'
PluR_D75N_fwd	5'-CATGACAGCAACCAATTAATG-3'
PluR_D75N_rev	5'-CATTAATTGGTTGCTGTCATG-3'
PluR_Q76P_fwd	5'-GACAGCGACCCATTAATGAATG-3'
PluR_Q76P_rev	5'-CATTCATTAATGGGTCGCTGTC-3'
PluR_C90W_fwd	5'-CCGTCAGTTTGGAATGATAAA-3'
PluR_C90W_rev	5'-TTTATCATTCCAAACTGACGG-3'
PluR_S115A_fwd	5'-GTCAAAAATGCTCTTTCAA-3'
PluR_S115A_rev	5'-TTGAAAGAGCATTTTTGAC-3'
PluR_S115G_fwd	5'-GTCAAAAATGGTCTTTCAA-3'
PluR_S115G_rev	5'-TTGAAAGACCATTTTTGAC-3'
PAU4062-His-NheI_fwd	5'GAGGAAGCTAGCCGCACCACCATCATCACCATCCCG GGATCTTATGAATACTTTATT-3'
4062_SalI_rev	5´-TAGCCGTCGACTTATATGATTAGATTATATGC-3´
PauR_S38A_fwd	5´-GAATTTTACGCAATTTATCAGG-3'
PauR_S38A_rev	5´-CCTGATAAATTGCGTAAAATTC-3'
PauR_Y40A_fwd	5´-TTACTCAATTGCTCAGGAAG-3'
PauR_Y40A_rev	5´-CTTCCTGAGCAATTGAGTAA-3'
PauR_Y40F_fwd	5´-TTACTCAATTTTCCAGGAAG-3'
PauR_Y40F_rev	5´-CTTCCTGGAAAATTGAGTAA-3'
PauR_D75E_fwd	5´-CATGACAGTGAACAACTAATG-3'
PauR_D75E_rev	5´-CATTAGTTGTTCACTGTCATG-3'
PauR_D75N_fwd	5´-CATGACAGTAATCAACTAATG-3'

PauR_D75N_rev	5´-CATTAGTTGATTACTGTCATG-3’
PauR_Q76A_fwd	5´-CATGACAGTGATGCACTAATG-3’
PauR_Q76A_rev	5´-CATTAGTGCATCACTGTCATG-3’
PauR_Y90A_fwd	5´-CTTCAATTGCTGATGAAAAAAC-3’
PauR_Y90A_rev	5´-GTTTTTTCATCAGCAATTGAAG-3’
PauR_I113A_fwd	5´-GATTAAAAACGCTATTTCC-3’
PauR_I113A_rev	5´-GGAAATAGCGTTTTTAATC-3’

S3 Table: Influence of amino acid substitutions within the SBD of PluR on general functionality and PPYD-sensing. PluR wild type and PluR derivatives were tested for their ability to activate $pcfA_{P.l.}$ promoter activity controlling the *luxCDABE* operon in the presence of 0.1% (w/v) arabinose or 3.5 nM PPYD. Reporter gene activity was quantified 2 h after addition of 0.1% (w/v) arabinose (functionality [%]) or 3.5 nM PPYD (PPYD-sensing [%]) and compared to PluR wild type, which values were set to 100%. RLU, relative light units. Std, standard deviation of three biological experiments.

	Functionality [%]	std	Sensing of 3.5nM PPYD [%]	std
PluR wt	100.0	± 7.4	100.0	± 7,4
PluR-T62W	113.0	± 6.8	95.4	± 6,8
PluR-Y66A	87.7	± 5.7	35.4	± 5.7
PluR-D75A	100.4	± 11.8	25.5	± 1.8
PluR-D75E	48.4	± 3.2	30.4	± 3.2
PluR-D75N	96.4	± 8.3	35.5	± 8.3
PluR-Q76A	97.2	± 9.1	2.2	± 0.4
PluR-Q76P	109.1	± 5.9	50.4	± 5.9
PluR-C90W	120.7	± 9.3	15.4	± 3.3
PluR-S115A	102.7	± 10.1	25.3	±10.1
PluR-S115G	47.3	± 7.6	12.4	± 4.6

1 **S4 Table: Influence of amino acid substitutions within the SBD of PauR on**
2 **general functionality and DAR-sensing.** PauR wild type and PauR
3 derivatives were tested for their ability to activate *pcfA*$_{P.a.}$ promoter activity
4 controlling the *luxCDABE* operon in the presence of 0.1% (w/v) arabinose or
5 3.5 nM DAR. Reporter gene activity was quantified 2 h after addition of 0.1%
6 (w/v) arabinose (functionality [%]) or 3.5 nM DAR (DAR-sensing [%]) and
7 compared to PauR wild type, which values were set to 100%. RLU, relative
8 light units.
9

	Functionality [%]	std	Sensing of 3.5nM DAR [%]	std
PauR wt	100.0	± 8.3	100.0	± 7.7
PauR-S38A	92.2	± 8.9	25.5	± 2.6
PauR-Y40A	7.8	± 1.6	2.1	± 0.5
PauR-Y40F	49.8	± 5.4	57.4	± 11.1
PauR-T62A	98.6	± 10.5	5.4	± 0.3
PauR-Y66A	98.9	± 10.8	25.4	± 2.8
PauR-D75A	92.6	± 8.1	33.4	± 4.9
PauR-D75E	25.7	± 2.0	2.2	± 0.5
PauR-D75N	75.2	± 4.4	10.5	± 1.9
PauR-Q76A	49.3	± 6.1	10.4	± 1.0
PauR-Y90C	91.6	± 8.1	64.2	± 3.7
PauR-I113S	89.5	± 5.1	53.4	± 7.6

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Figures and Legends

Figure 1: Protein sequence comparison of QS LuxR family members and the non-AHL sensors PluR and PauR. (**A**) Modular domain structure of LuxR-type regulators, with a N-terminal signal-binding domain (SBD) and a C-terminal DNA-binding domain (DBD) [8], containing the helix-turn-helix "HTH LUXR" motif (SMART00421) [26]. (**B**) Comparison of the protein sequence identity of PluR from *P. luminescens*, PauR from *P. asymbiotica* and TraR from *A. tumefaciens*. The identity was compared either of the full-length protein sequence, only the signal-binding domain (SBD) and only the DNA-binding domain (DBD). To calculate identity of the protein sequences the LALIGN software from SIB (Swiss Institute of Bioinformatics) was used [27]. (**C**) Sequence alignment of the protein sequences of PauR from *P. asymbiotica* (*P.a.*), PluR from *P. luminescens* (*P.l.*), QscR from *Pseudomonas aeruginosa* (*P.a.*), SdiA from *Escherichia coli* (*E.c.*), TraR from *Agrobacterium tumefaciens* (*A.t.*) and LuxR from *Vibrio fischeri* (*V.f.*). The SBD is depicted with a blue bar and the DBD with a green bar. Within the SBD the six conserved amino acids, displaying the WYDPWG-motif of AHL-sensors, are marked with red asterisks and the three conserved amino acids in the DBD are marked with blue asterisks. Amino acids with a consensus of 60-100% are shown, positions with a lower coverage are marked with a dot. The RasMol colouring of the amino acids and the alignment was generated with CLC Mainworkbench 7 (CLC Bio Qiagen, Hilden, Germany).

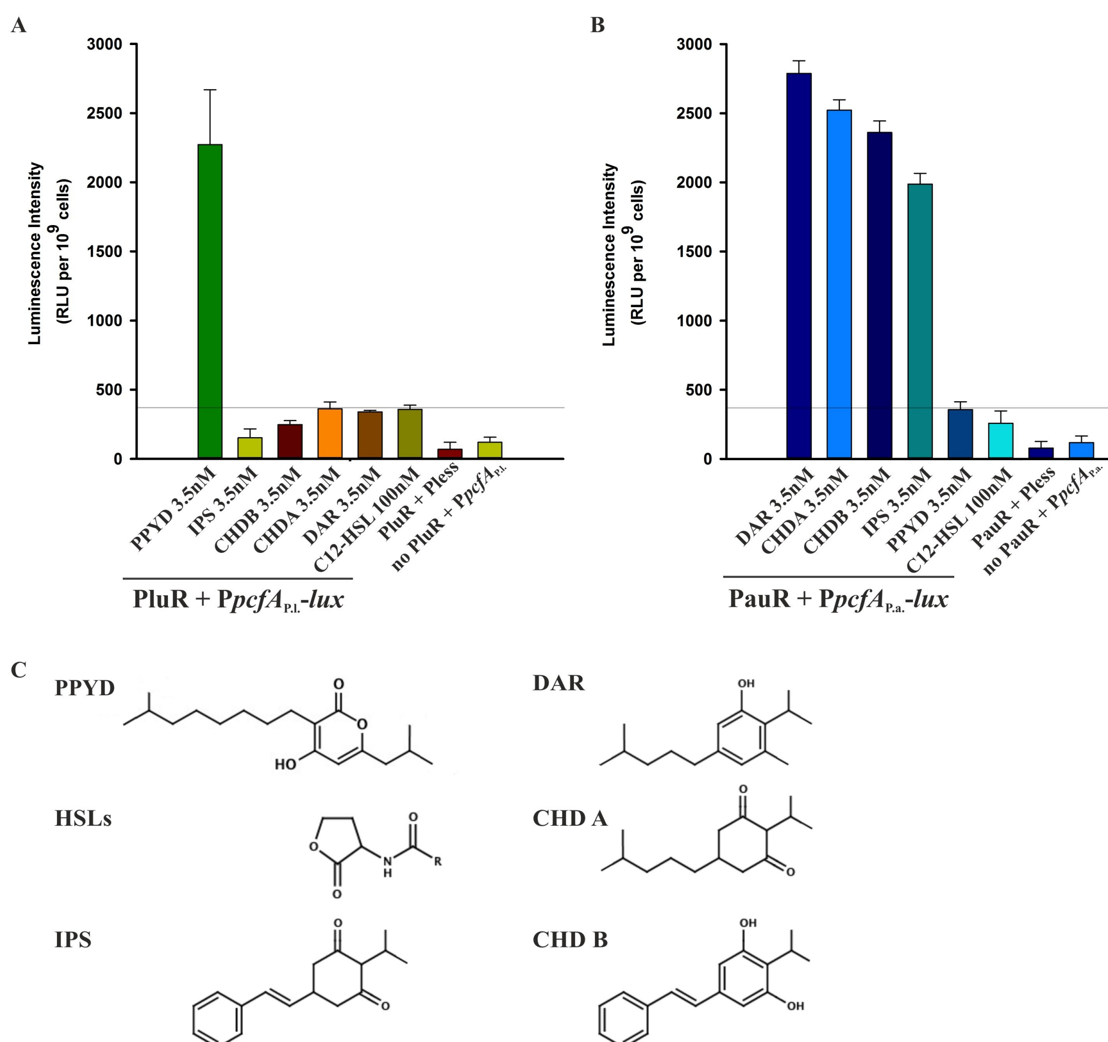

Figure 2: Specificity of PluR and PauR towards different signaling molecules. PluR (**A**) senses its cognate signaling molecule PPYD, whereas unrelated signaling molecules, like C8-HSL or DAR, CHDA, CHDB and IPS, are not sensed. To test the specificity of PluR the reporter system pBAD24-His-*pluR* and pBBR1-*pcfA*$_{P.l.}$-*lux* was used. Similarly, PauR (**B**) specifically senses its native signaling molecules with the highest specificity towards DAR compared to the DAR-precursors, CHDA, CHDB and IPS. The PauR-specific reporter plasmid system composed of pBAD24-His-*pauR* and pBBR1-*pcfA*$_{P.a.}$-*lux* was used. Cells harboring the promoter-less reporter plasmid in combination with each PluR and PauR did not exhibit significant *pcfA* promoter activity. Furthermore, cells harboring the empty pBAD24 plasmid, and therefore no *pluR* or *pauR*, with the respective reporter plasmid as well did not exhibit significant *pcfA* promoter activity. RLUs are shown for 2 h after addition of the depicted signaling molecule. Reference line was set to 370 RLUs to underline the background of the system. RLU, relative light units. (**C**) Comparison of the structures of the signaling molecules used in this study.

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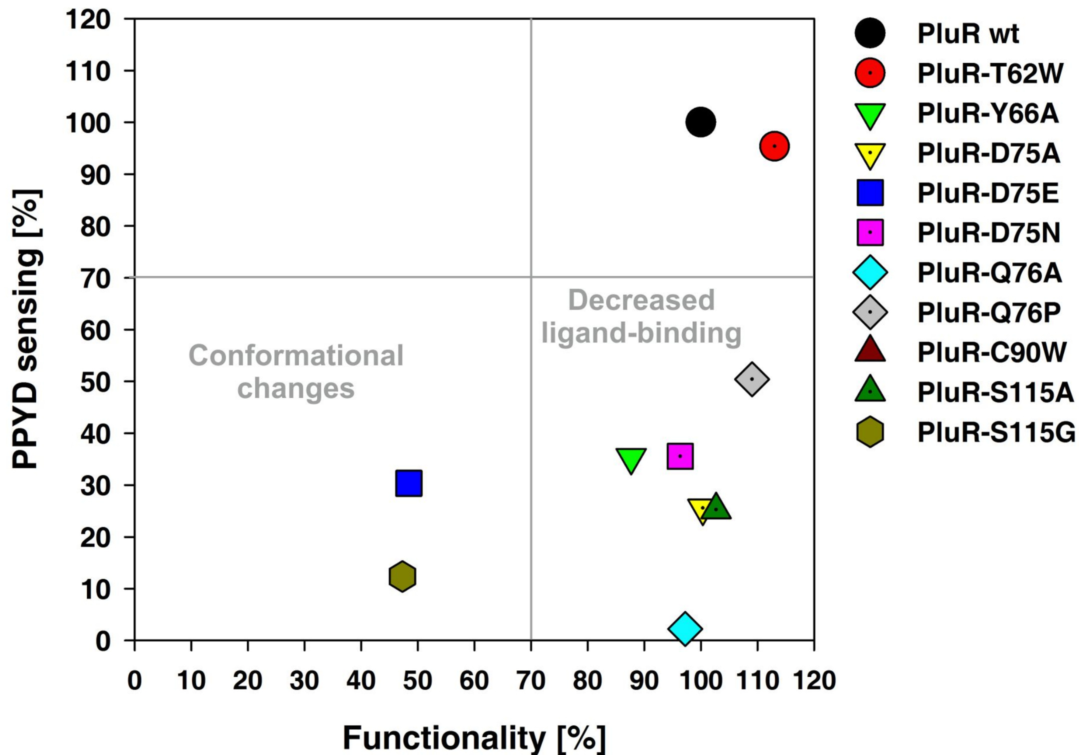

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4 **Figure 3: Amino acid replacements within the SBD of PluR caused either**
5 **functionality or impaired PPYD-sensing.** The PluR derivatives D75E and S115G
6 dramatically decreased functionality and hence decreased its ability to bind and
7 activate *pcfA*P.l. promoter (lower left quadrant). Replacements within the TYDQCS-
8 motif of PluR decreased the ability of PluR to sense PPYD. The most drastic
9 influence on PPYD-sensing is detectable with the replacement of Y66A, D75A,
10 D75N, Q76A, Q76P and S115A in PluR (lower right quadrant). Only the
11 replacement T62W showed no effect and same induction levels as PluR wild type
12 (upper right quadrant). The activity of the *pcfA*P.l. promoter was measured via
13 luminescence as read-out and the depicted values were taken 2 h after addition of
14 0.1% (w/v) arabinose (lower axis) or 3.5 nM PPYD (left axis) and compared to PluR
15 wild type, which values were set to 100%. To evaluate the different PluR derivatives,
16 a cut-off of 70% was set for each value. RLU (relative light units) values for all PluR
17 derivatives and PluR wild type are depicted in S3 Table.
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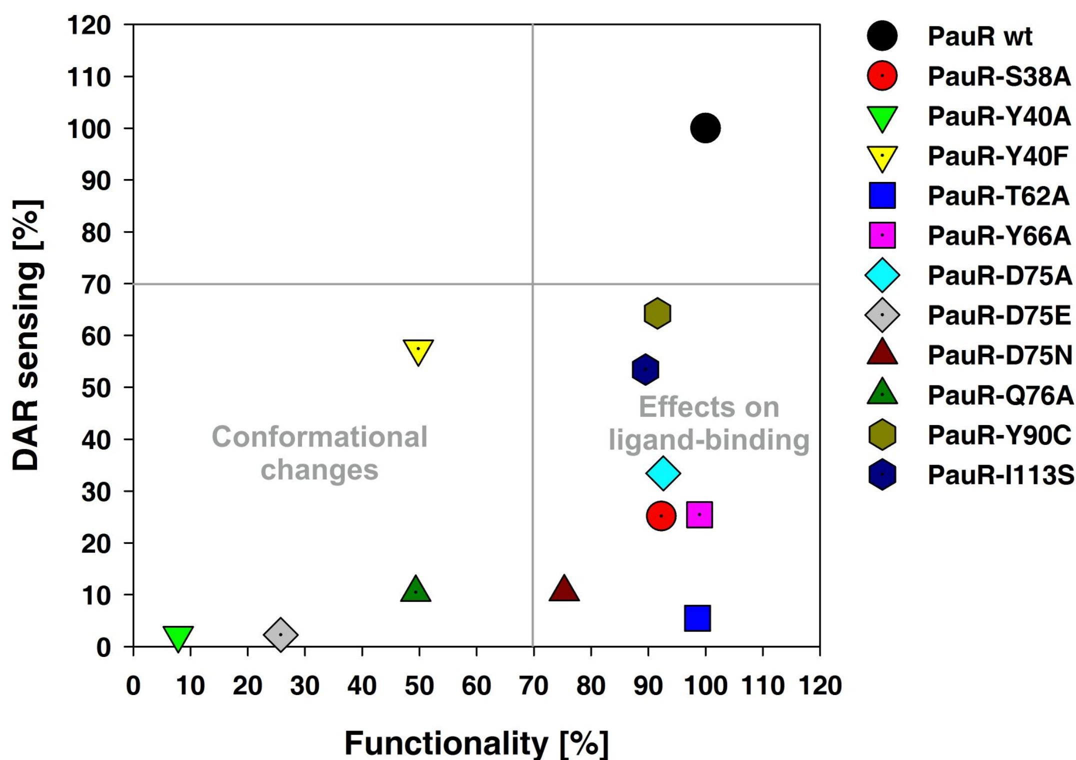

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3 **Figure 4: The TYDQYI-motif in the SBD of PauR is essential for the overall**
4 **functionality of the receptor and DAR-sensing.** The most drastic effects on DAR-
5 sensing were gained with the replacement of S38A, T62A, Y66A, D75A and D75N
6 in the SBD of PauR and a decreased effect on DAR-sensing were gained with the
7 replacement of Y90C and I113S in PauR (lower right quadrant). The PauR
8 derivatives Y40A, Y40F, D75E and Q76A dramatically influenced the structure of
9 PauR and decrease its ability to bind and activate $pcfA_{\mathrm{P.a.}}$ promoter (lower left
10 quadrant). The activity of $pcfA_{\mathrm{P.a.}}$ promoter was measured via luminescence as read-
11 out and pictured values were taken 2 h after addition of 0.1% arabinose (lower axis)
12 or 3.5 nM DAR (left axis) and compared to PauR wild type, which values were set to
13 100% (upper right quadrant). To evaluate the different derivatives, a cut-off of 70%
14 was set for each value. RLU (relative light units) values for all PauR derivatives and
15 PauR wild type are depicted in S4 Table.

16

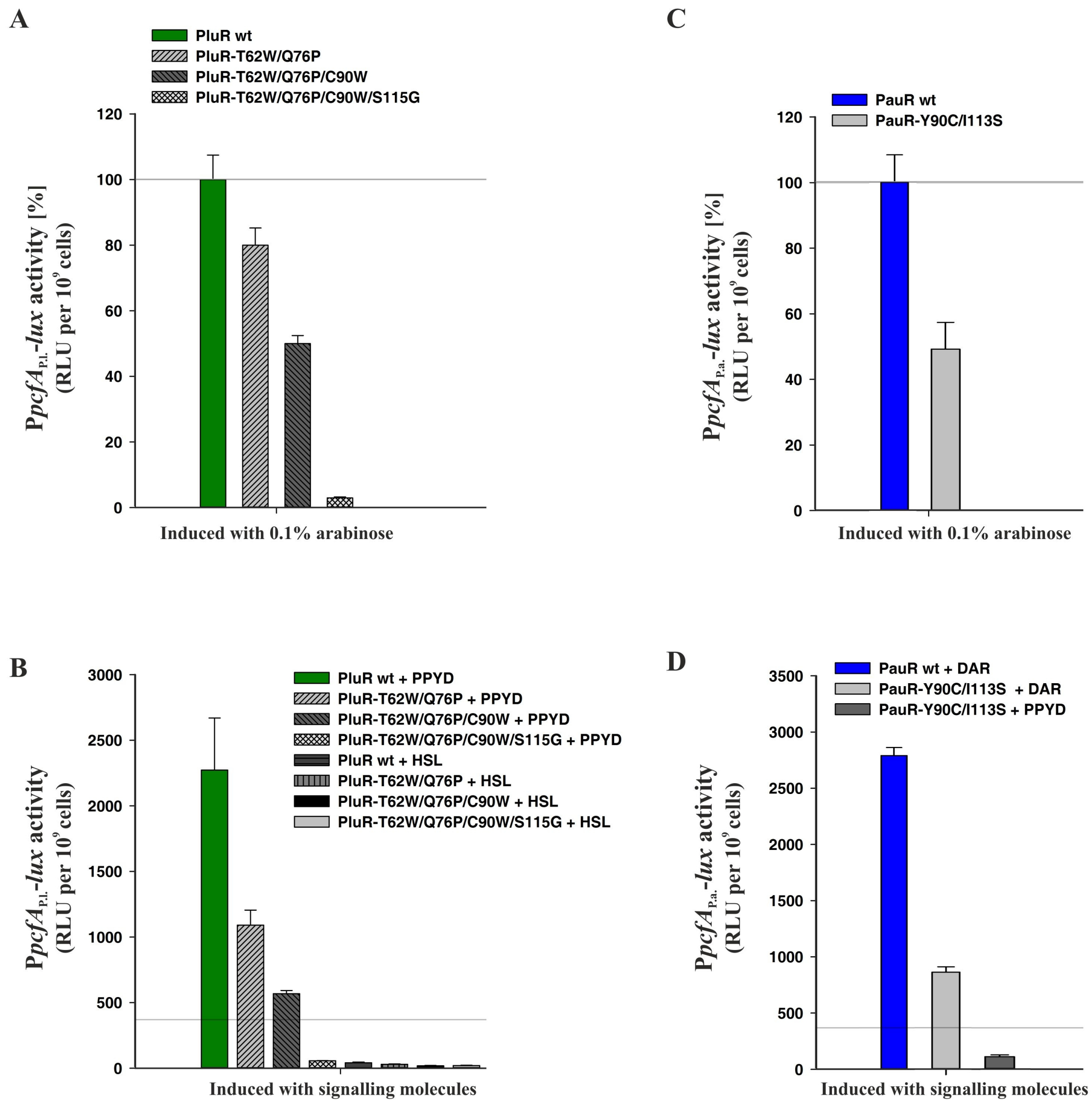

Figure 5: The conserved motifs in the SBD of PluR and PauR are essential but not sufficient for ligand-binding specificity. (**A**) Stepwise replacement of the non-conserved amino acids in PluR to the conserved WYDPWG-motif of AHL-sensors effects the conformation and decreases its ability to activate *pcfA*P.l. promoter compared to PluR wild type (wt). The quadruple replacement of PluR-T62W/Q76P/C90W/S115G effects most dramatically the conformation compared to PluR wild type. (**B**) Stepwise replacement of the non-conserved amino acids of PluR decreases its ability to sense its native signaling molecule PPYD, however C8-HSL-sensing could not be gained. **(C)** Replacement of Y90C and I113S in PauR decreased the ability of PauR-Y90C/I113S to activate *pcfA*P.a. promoter activity approximately to 50% compared to PauR wild type. (**D**) DAR-sensing was decreased approximately about 70% in the PauR-Y90C/I113S derivative compared to PauR wild type (wt), however, PPYD-sensing could not gained. (**A**) and (**C**): RLUs are shown 2 h after induction and value of PluR or PauR wild type was set to 100% and compared to the respective derivatives. (**B**) and (**D**): The RLU values are depicted 2 h after the addition of the distinct signaling molecules, either 3.5 nM PPYD, 3.5 nM DAR or 100 nM C8-HSL. Reference line was set to 370 RLUs to compare better with Fig. 2.

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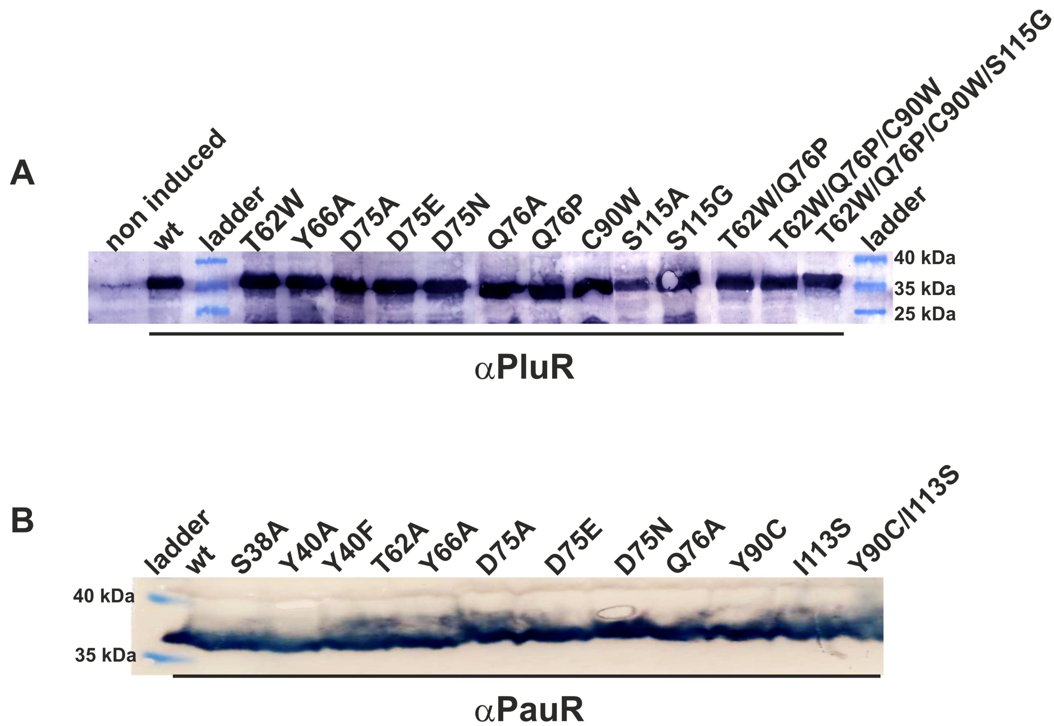
3 # Supporting information



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5 **S1 Fig.: Protein production of PluR and PauR and their respective derivatives.**
6 For analysis of protein production of PluR and its respective derivatives (**A**) and of
7 PauR and its respective derivatives (**B**), *E. coli* strains harboring pBAD-His-*pluR*,
8 pBAD-His-*pauR* or variants were cultivated at 37°C in LB medium. Cells were
9 harvested 2 h after addition of 0.1 % (w/v) arabinose, as a control no arabinose was
10 added. The figure shows the immunoblots of 12.5 % SDS gels. Antibodies directed
11 against the respective protein were used to detect PluR or PauR. PluR has an estimated
12 size of 27.03 kDa and PauR has an estimated size of 27.14 kDA. The PageRuler
13 prestained protein ladder (Thermo Fischer, Schwerte) was used to estimate protein
14 sizes.

6. IDENTIFICATION OF THE IMPORT SYSTEM FOR THE SIGNALLING MOLECULES PPYs AND DARs

6.1 Introduction

External signalling molecules, like AHLs, used for quorum sensing (QS) regulated processes have to cross the cell envelope, because these are sensed internally by cytosolic LuxR-type receptors. Commonly, short-chain AHLs are supposed to be mediated via diffusion. However, the outer membrane is an efficient barrier for diffusion of hydrophobic, long-chain AHLs and therefore the mechanism of internalization of long-chained molecules is yet unknown. The hydrophobicity of AHLs is affected by its nature of the C3 substituent (H, O or OH) and its length and number of insaturations of the acyl chain (Kaplan and Greenberg, 2014).

The FadL family members are known to transport hydrophobic compounds across the bacterial outer membrane, as shown for the outer membrane (OM) integrated transporter FadL of *Escherichia coli* transporting long-chain fatty acids (LCFAs). Furthermore, as FadL homologs are widespread in Gram-negative bacteria this suggests a general function in other organisms. LCFAs enter the inner membrane (IM) subsequently and “flip-flop” to the inner leaflet that is thought to occur spontaneously. In the next step, the LCFAs are removed from the IM and activated via the IM-associated fatty acyl-CoA synthetase FadD (van den Berg, 2005). Furthermore, the long-chain acyl-CoA responsive transcription factor FadR activates expression of *fadL* and *fadD* as well as fatty acid degradative genes at high intracellular long-chain acyl-CoA concentrations. This allows the cell a quick adaptation of its metabolism in response to extracellular LCFA levels (Dirusso and Black, 2004).

Moreover, systems for the transport of long-chain AHLs has only been identified in *Pseudomonas aeruginosa* and in *Burkholderia pseudomallei* up until now, which is an active efflux system (Boyer and Wisniewski-Dye, 2009).

Similar to AHLs, the signalling molecules PPYs and CHDs/DARs used for cell-cell communication by *P. luminescens* and *P. asymbiotica*, respectively, are

accumulating outside of the cell. Thus, recognition by the LuxR-type receptors PluR and PauR, respectively, occurs intracellular (Brachmann et al., 2013; Brameyer et al., 2014). Moreover, PPYs and CHDs/DARs share the hydrophobic properties of AHLs, but show structural differences to AHLs. Therefore, the question arises how PPYs and CHDs/DARs are passing the cell membrane in *Photorhabdus* species. These compounds might be transported across the cell membrane either via diffusion, passive mechanisms like porines or channels or active transport mechanism, like known transporters. Furthermore, the LuxR solo regulators PluR and PauR might influence the expression of the transport system used for their cognate signalling molecule. In order to gain initially insights into those potential regulatory mechanisms, the transcriptome of the Δ*pluR* mutant was analysed and compared against the transcriptome of *P. luminescens* wild type. Thereby, a global overview was achieved of differentially expressed genes due to the deletion of *pluR*.

6.2 Material and methods

6.2.1 RNA extraction and RNA-Sequencing

For RNA-sequencing (RNA-Seq), triplicates of total RNA was isolated via a chloroform-phenol extraction at OD_{600} = 12 (50 h) from *P. luminescens* TT01 wild type and TT01Δ*pluR*. Residual genomic DNA was removed by DNase digestion (DNase I, RNase-free, Fermentas). The quality and integrity of the total RNA was verified via the Agilent bioanalyzer and only samples with an RNA Integrity Number (RIN) > 7 were used. Furthermore, a ratio of 2:1 for 23SrRNA to 16SrRNA was achieved, which is regular for Gram-negative bacteria. Ribosomal RNA (rRNA) was removed from total RNA with the Ribo-Zero rRNA Removal Kits for Gram-Negative Bacteria (Epicentre, Madison, USA). Again, the quality and integrity of the remaining RNA was verified via the Agilent bioanalyzer and a RIN > 7 was sufficient. The mRNA-seq library for sequencing was prepared using the ScriptSeq v2 RNA-seq kit (Epicentre, Madison, USA) and AMPure purification (Agencourt, Krefeld) kit was used to purify synthezised cDNA and the library in the end. The quantity of the library was determined with the Qubit Fluorometer (Life Technologies). Sequencing results were obtained using 10 mM of each cDNA library and the Illumina Genome Analyzer platform and the 100 bp reads with the Illumina primers recommended in the ScriptSeq kit. Sequenced reads were processed with the CLC Genomics Workbench (Hilden, Germany). First, sequenced reads were trimmed and mapped against the reference genome of *P. luminescens* subsp. *laumondii* TT01 (NCBI reference sequence NC_005126.1) (Fischer-Le Saux et al., 1999). In order to identify differentially expressed genes, the RPKM (Reads per kilobase transcript per million reads) values were first calculated for each gene per sample (Mortazavi et al., 2008). As RPKM values are log-normally distributed, it is convenient to express them as $\log_2$(RPKM) (Chaudhuri et al., 2011). Therefore, differentially expressed genes in Δ*pluR* were identified via determination of the $\log_2$-fold change in Δ*pluR* compared to TT01 wild type (wt):

$$\log_2\left(\frac{\mathbf{RPKM}_{\Delta pluR}}{\mathbf{RPKM}_{\mathbf{wt}}}\right) = fold\ \ change$$

Furthermore, genes with a log_2-fold change > -2 and > 2 of TT01/Δ*pluR* were judged as a significant change in gene expression. Putative proteins encoded by the differentially expressed genes were analysed using the browser software (http://genolist.pasteur.fr/PhotoList/) (Lechat et al., 2008).

In order to verify results of the RNA-Seq approach selected genes were analysed via qRT-PCR. Therefore, the same triplicates of total RNA samples as used for RNA-Seq were used for reverse transcription, which was performed via the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Schwerte) using oligo (dt)18 primer, and cDNA was used for subsequent qRT-PCR reactions. qRT-PCR was conducted on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, München) using the Maxim SYBR Green/ROX qPCR Master Mix (Bio-Rad, München) and analyzed with the iQ5 Optical System Software (Bio-Rad). The gene *recA* was used as a reference gene and sequences of the primers are listed in Table 1. All of the reactions were performed on three biological replicates. The transcript amount was calculated using the ΔΔCt method (Livak and Schmittgen, 2001).

Table 1: Sequences of primers used for qRT-PCR.

Oligo	Sequence
0169_qPCR_sense	5´-GTAAAATAGTTGAACAATGGCC-3´
0169_qPCR_anti	5´-GCCAATTTATTTTCAGTCTCACC-3´
0258_qPCR_sense	5´-GCTGGTATCGAATATAAAGAGTC-3´
0258_qPCR_anti	5´-GCACGTAAAGATAGTGCCACAATG-3´
plu0925_qPCR_sense	5´-CGTCATTACGTTTTTATCATTAG-3´
plu0925_qPCR_anti	5´-GCTTTATTACAGCGGCCTCC-3´
plu1563_qPCR_sense	5´-GTTCAAGCTTTATTATCTGATG-3´
plu1563_qPCR_anti	5´-CGCTATTATGCTGACGCAGAAT-3´
plu3720_qPCR_sense	5´-CATACATATGTGTTTCAAGCG-3´
plu3720_qPCR_anti	5´-GGGTGGTATGATTATGAACTTC-3´
plu3721_qPCR_sense	5´-CCAGTGAAGTGACGCGTTGCAT-3´
plu3721_qPCR_anti	5´-CGATATTATAGGGCTATCTATG-3´
plu4301_qPCR_sense	5´-CTGGAAAAACTAGGTTCCGGCT-3´
plu4301_qPCR_anti	5´-CCAGGTTTGAGCTCGTTCTT-3´
FadL_qPCR_sense	5´-GCTGATAACGCAGCGGTAGGAAG-3´
FadL_qPCR_anti	5´-CTGGGGATTTACCGGTAATATC-3´
FadD_qPCR_sense	5´-CCTTCCGTAAGTTGGAAGAGC-3´
FadD_qPCR_anti	5´-GCAACGGGATATTGCAGCAGG-3´
RecA_qPCR_sense	5´-GCGAAGACCGTTCAATGGACG-3´
RecA_qPCR_anti	5´-CGAAGATTCCGGGCCATAGATTTC-3´

6.2.2 Reporter-based assay to quantify influence of Δ*fadD* and Δ*fadL* on the import of the signalling molecules sensed by PluR and PauR

The ability of PluR or PauR to activate its cognate *pcfA* promoter in the presence of different signalling molecules was measured via *luxCDABE* expression.

In order to analyse the influence of FadD and FadL on the import of the signalling molecule PPYD sensed by PluR, the strains *E. coli* wild type, Δ*fadD* and Δ*fadL* harbouring a *PpcfA*$_{P.l.}$-*luxCDABE* (*pcfA* promoter of *P. luminescens*) fusion and producing PluR were cultivated in LB and induced with 3.5 nM PPYD or with 0.1% (w/v) arabinose (ara) as positive control.

In order to analyse the influence of FadD and FadL on the import of the signalling molecules CHDs/DARs, sensed by PauR, *E. coli* wild type, Δ*fadD* and Δ*fadL* cells harbouring a *PpcfA*$_{P.a.}$-*luxCDABE* (*pcfA* promoter of *P. asymbiotica*) fusion and producing PauR were cultivated in LB and induced with 3.5 nM of IPS, CHDA, CHDB, DAR or with 0.1% (w/v) arabinose (ara) as positive control. Luminescence value of *E. coli* wild type induced with different ligands was set to 100 %. In order to calculate RLU (relative light unit) the values of luminescence were divided by the optical density (OD). Error bars represent standard deviation of at least three independently performed experiments. Nomenclature of IPS, CHDA, CHDB and DAR is according to Brameyer et al. (2014) and nomenclature of PPYD is according to Brachmann et al. (2013). Reference line was set to 100 %.

E. coli BW25113Δ*fadD* and BW25113Δ*fadL* strains were received from the Keio collection (Baba et al., 2006).

6.3 Results

6.3.1 Deletion of *pluR* results in up-regulation of *fadD* and *fadL* in *P. luminescens*

In order to analyse the regulatory role of the LuxR-type regulator PluR, the transcriptome of *P. luminescens* wild type and Δ*pluR* strain at the stationary phase was analysed using a RNA-sequencing (RNA-Seq) approach. The total RNA was isolated in the stationary phase at OD_{600} = 12 (50 h) and used for RNA-Seq to identify differentially expressed genes due to PluR, as it was previously shown via qRT-PCR that *pcfA* was up-regulated at this time point in wild type compared to D*pluR* (Brachmann et al., 2013).

In total, 57 genes are up-regulated and 18 genes are down-regulated in Δ*pluR* compared to wild type (Table 2). Several genes encoding for enzymes belonging to different metabolic processes and putative transport systems, like the universal stress protein B (UspB), glutamate/aspartate transport ATP-binding protein GltL, glutamate/aspartate transport system permease GltI and lipase 1 precursor (Lip-1), are differentially expressed in Δ*pluR* compared to wild type. However, several genes encoding proteins with unknown function are also expressed differentially in Δ*pluR* compared to wild type. Moreover, the expression of three genes, *plu0925*, *plu3720* and *plu3721*, coding for LuxR solos are down-regulated in Δ*pluR* compared to wild type. Often whole operons are differentially regulated upon deletion of *pluR* like *plu0277-plu0282*, *plu3025-plu3037* and *plu4301-plu4305*, however the functions of these gens are hypothetical.

Additionally, *fadD* and *fadL*, coding for the long-chain-fatty-CoA-ligase FadD and the long-chain-fatty-acid transport precursor FadL, respectively, are both up-regulated in Δ*pluR*, suggesting a regulation of the expression of these two genes via PluR, either direct or indirect.

To verify the accuracy of the RNA-Seq approach, the expression of selected genes (*plu0169*, *plu0258*, *plu0925*, *plu1563*, *plu3720*, *plu3721*, *plu4301*, *fadD* and *fadL*) were analysed, at the same time point as used for RNA-Seq, using qRT-PCR. The candidate genes, *plu0169*, *plu0258, plu1563* and *plu4301*, to be analysed with qRT-

PCR were chosen based on their maximal up-regulation or maximal down-regulation of expression upon deletion of *pluR*. The genes *plu3720*, *plu3721* and *plu0925* were chosen to verify with qRT-PCR as they are coding for LuxR solos. Furthermore, the expression of *fadD* and *fadL* were analysed, since both genes might influencing the import of PPYs and CHDs/DARs. In summary, the results of differentially expression of the selected genes gained with the RNA-Seq method could be confirmed for all selected genes with qRT-PCR (Fig. 5).

Table 2: Differentially expressed gene in *P. luminescens* Δ*pluR* compared to wild type in late exponential phase. The total RNA was isolated at OD_{600} = 12 (50 h) and used for RNA-sequencing (RNA-Seq), as *pcfA* was differentially expressed at this time point in wild type compared to Δ*pluR* shown via qRT-PCR (Brachmann et al., 2013). The RPKM of each transcript was calculated using the CLC Genomics Software (Hilden, Germany) and compared between *P. luminescens* TT01 wild type and TT01Δ*pluR* and presented as a fold change (log_2) of the genes or operons differentially expressed in Δ*pluR*. In the left panel, the genes or operons differentially expressed in Δ*pluR* are listed, along with putative functions for the gene products (middle panel). At the right panel, the fold change (log_2) of the genes or operons differentially expressed in Δ*pluR* are shown. The fold change (log_2) of operons differentially expressed in Δ*pluR* are shown as a mean of the values of all genes of the operon. Bold numbers emphasizes negative fold change values.

Gene/operon	Putative function of gene product(s)	Δ*pluR*: fold change
Metabolism / Transport		
rbsK (*plu0059*)	Ribokinase	2.2
uspB (*plu0120*)	Universal stress protein B	2.0
gor (*plu0375*)	Glutathione oxidoreductase (GR) (GRase)	2.4
rof (*plu0690*)	Rof protein	2.7
gltL (*plu1304*)	Glutamate/aspartate transport ATP-binding protein GltL	2.1
gltI (*plu1307*)	Glutamate/aspartate transport system permease protein GltI	2.2
artI (*plu1587*)	Arginine-binding periplasmic protein 1 precursor	2.5
fabA (*plu1772*)	D-3-hydroxydecanoyl-(acyl carrier-protein) dehydratase	1.9
fadD (*plu2134*)	Long-chain-fatty-acid-CoA ligase (long-chain acyl-CoA synthetase)	0.9
lpp (*plu2615*)	Major outer membrane lipoprotein precursor (murein-lipoprotein)	2.9
fabH (*plu2835*)	3-oxoacyl-[acyl carrier-protein] synthase III (beta-ketoacyl-ACP synthase III)	1.9
rcsB (*plu3048*)	Capsular synthesis regulator component B	1.9
fadL (*plu3202*)	Long-chain fatty acid transport protein precursor (outer membrane FadL protein)	2.5
lip-1 (*plu3510*)	Lipase 1 precursor (triacylglycerol lipase)	2.1

sctQ (*plu3770*)	Type III secretion component protein SctQ	**-1.8**
cyoE (*plu3879*)	Protoheme IX farnesyltransferase (heme O synthase)	2.3
hemB (*plu4407*)	Delta-aminolevulinic acid dehydratase (porphobilinogen synthase)	2.1
argI (*plu4490*)	Ornithine carbamoyltransferase chain I (OTCase-1)	2.2
LuxR-type regulator		
plu0925	Similar to transcriptional regulator LuxR family	**-3.1**
plu3720	Similar to transcriptional regulator LuxR family	**-0.7**
plu3721	Similar to transcriptional regulator LuxR family	**-1.8**
Unknown function		
plu0169	Unknown function	3.2
plu0257-plu0258	Similar to unknown protein of *Photorhabdus luminescens*	~ 3.2
plu0265-plu0268	Similar to outer membrane usher protein precursor	~ **-2.3**
plu0277-plu0282	Similar to hypothetical secreted protein of *Photorhabdus luminescens*	~ 2.9
plu0860	Probable hypoxanthine phosphoribosyltransferase	2.0
plu1563	Similar to putative oxidoreductase YiaY of *Escherichia coli*	**-4.1**
plu1818	Similar to unknown protein YceP of *Escherichia coli*	2.1
plu1990-plu1991	Some similarities with unknown protein YhfS of *Escherichia coli*	~ 2.9
plu2058	Highly similar to unknown protein of *Photorhabdus luminescens*	2.0
plu2142	Some similarities with hypothetical phage protein	2.0
plu2169	Similar to unknown protein	2.2
plu2213	Unknown	**-2.1**
plu2769	Weakly similar to 3-phosphoshikimate 1-carboxyvinyltransferase	**-2.3**
plu2838	Highly similar to probable membrane protein YceD of *Escherichia coli*	2.2
plu2865	Similar to unknown protein YejG of *Escherichia coli*	2.6
plu3008-plu3009	Some similarities with phage tail fiber assembly protein	~ 2.3
plu3020	Hypothetical protein	2.3
plu3023	Similar to unknown protein YfdK of *Escherichia coli*	2.9
plu3025 -plu3037	Similar to unknown bacteriophage protein	~ 2.2
plu3863	Similar to unknown protein YbaW of *Escherichia coli*	2.1
plu3915	Similar to to unknown protein and to putative transposase	2.4
plu4034	Highly similar to ABC transporter, ATP-binding protein YrbF of *Escherichia coli*	2.1
plu4301-plu4305	Similar to putative membrane protein	~ **-2.2**
plu4369-plu4370	Similar to bacteriophage tail fiber assembly protein	~ **-4.2**
plu4558	Similar to unknown protein YtfJ precursor of *Escherichia coli*	2.2
plu4601	Some similarities with VgrG and VgrE proteins	2.2

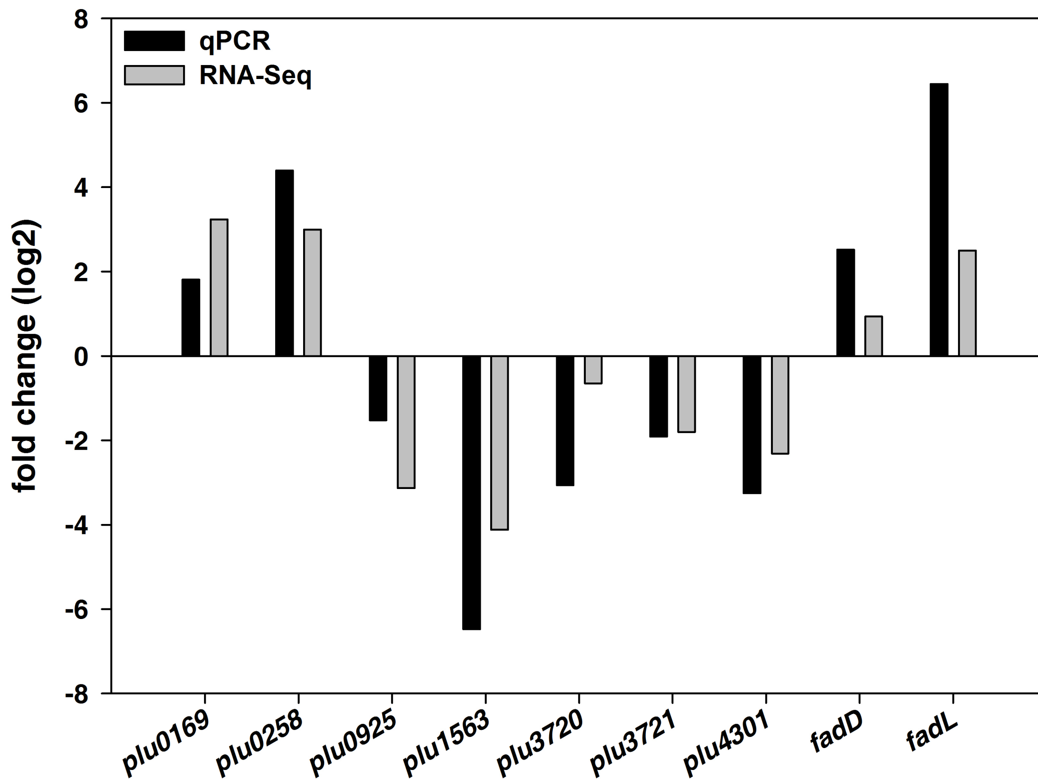

Figure 5: Comparison of results from qRT-PCR (qPCR) and RNA-Sequencing (RNA-Seq) of differentially expressed genes in TT01Δ*pluR*. The expression of the selected genes is calculated as a log_2-fold change. The genes *plu0169*, *plu0258*, *plu0925*, *plu1563*, *plu3720*, *plu3721*, *plu4301*, *fadD* and *fadL* were chosen to compare the results of RNA-Seq (grey) with the qPCR method (black).

6.3.2 FadL influence internal sensing of PPYD and DAR via the LuxR-type receptors PluR and PauR

In order to gain insights on the effect of FadL and FadD on the internalisation of PPYs and CHDs/DARs sensed by PluR and PauR, respectively, the system was analysed heterologously in *E. coli* strains lacking either FadL or FadD. Therefore, the PPYD-PluR reporter system (Brachmann et al., 2013) and CHDs/DARs-PauR reporter system (Brameyer et al., 2014) were used in the three different backgrounds. The reporter plasmids were transformed into *E. coli* wild type, Δ*fadD* and Δ*fadL* and upon addition of each signalling molecule; the influence of FadD and FadL on the import could be monitored, as the LuxR-type receptors PluR and PauR and their

cognate *pcfA* promoter fusion are in the cytoplasm. Thereby, it could be shown that deletion of *fadD* decreased dramatically the internalization of PPYD sensed by PluR, whereas deletion of *fadL* had a minor effect on PPYD import (Fig. 6). Furthermore, deletion of *fadD* and *fadL* each decreased dramatically the internalization of the signalling molecules sensed by PauR, which are IPS, CHDA, CHDB and DAR (Fig. 6). Hence, we initially were able to show that FadD and FadL might also be involved in the transport of hydrophobic compounds like the signalling molecules PPYD and CHDs/DARs in *Photorhabdus* species.

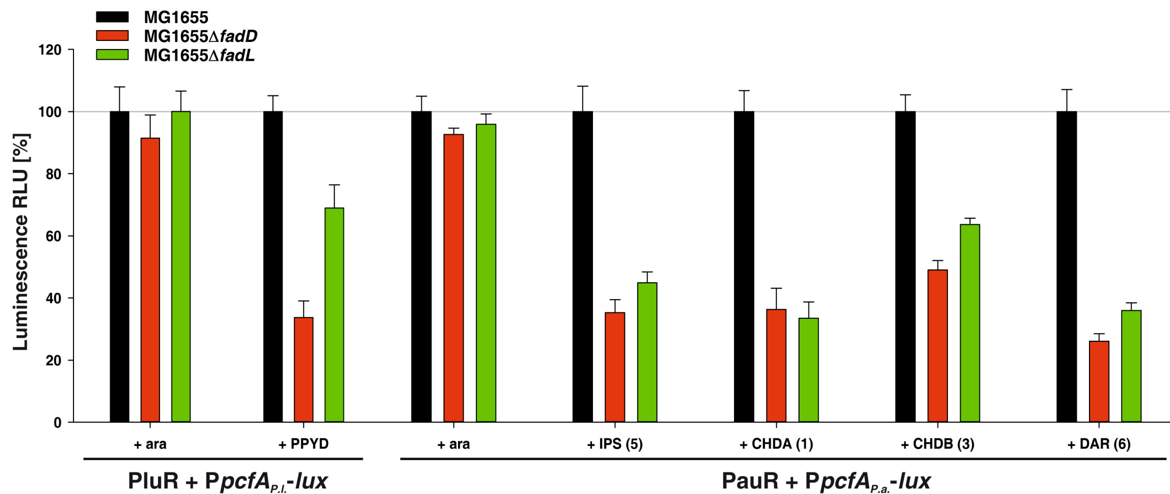

Figure 6: Influence of the lack of FadD and FadL on import of signalling molecules sensed by PluR and PauR. The ability of PluR or PauR to activate its cognate *pcfA* promoter in the presence of different signalling molecules was measured via *luxCDABE* expression and therefore luminescence as readout. To analyse the influence of FadD and FadL on the import of the signalling molecules, sensed by PluR or PauR, *E. coli* wild type, Δ*fadD* and Δ*fadL* cells harbouring the PPYD-PluR plasmid reporter system (Brachmann et al., 2013) or CHDs/DARs-PauR plasmid reporter system (Brameyer et al., 2014), respectively, were used. To analyse the sensing of PPYD via PluR in the absence of *fadD* and *fadL*, cells harbouring a *PpcfA*$_{P.l.}$*-luxCDABE* (*pcfA* promoter of *P. luminescens*) fusion and producing PluR were cultivated in LB and induced with 3.5 nM PPYD or with 0.1% arabinose (ara) as positive control. And to analyse the influence of FadD and FadL on the import of the signalling molecules CHDs/DARs, sensed by PauR, *E. coli* wild type, Δ*fadD* and Δ*fadL* cells harbouring a *PpcfA*$_{P.a.}$*-luxCDABE* (*pcfA* promoter of *P. asymbiotica*) fusion and producing PauR were cultivated in LB and induced with 3.5 nM of IPS, CHDA, CHDB, DAR or with 0.1% arabinose (ara) as positive control. Luminescence value of MG1655 induced with different ligands was set to 100 %. Error bars represent standard deviation of at least three

independently performed experiments. RLU, relative light unit. Reference line was set to 100 %.

Moreover, the FadL homologs of *P. luminescens* and *P. asymbiotica* show a high protein sequence identity to FadL of *E. coli* (63 % and 61 %, respectively), however not to the FadL homolog of the rhizobial bacterium *Sinorhizobium meliloti* (21 % and 22 %, respectively) (Fig. 5). Potentially, FadL homologs of *Photorhabdus* species possess a similar transport mechanism as used for transporting LCFAs via FadL in *E. coli*.

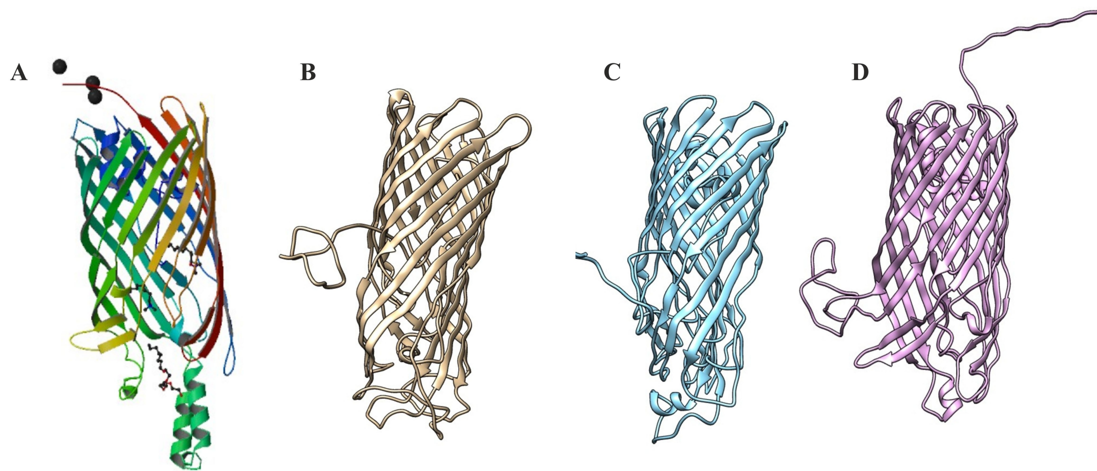

Figure 5: The FadL homologs of *E. coli*, *S. meliloti*, *P. luminescens* and *P. asymbiotica* share the characteristic β-barrel structure of FadL family members. Modelled structures of FadL of *E. coli* (**A**), of *S. meliloti* (strain SM11) (**B**), of *P. luminescens* (**C**) and of *P. asymbiotica* (**D**) are predicted to comprise the common β-barrel structure of the FadL family members. The structures of the FadL homologs of *S. meliloti*, of *P. luminescens* and of *P. asymbiotica* were modelled based on crystal structure of FadL of *E. coli* (van den Berg et al., 2004) using the HHpred tool (Homology detection & structure prediction by HMM-HMM comparison (Söding et al., 2005)) and visualised using UCSF Chimera (Pettersen et al., 2004). The PBD identifier 1T1L was used for the crystal structure of FadL of *E. coli* (van den Berg et al., 2004).

6.4 Discussion

Up until now, the mechanism of importing or exporting the hydrophobic AHLs used in QS systems is not elucidated. This is also the case for the *Photorhabdus*-specific signalling molecules PPYs and CHDs/DARs.

The RNA-Sequencing (RNA-Seq) approach revealed in total 75 genes that are differentially expressed at late exponential phase in Δ*pluR* compared to *P. luminescens* TT01 wild type. 57 genes are up-regulated and 18 genes are down-regulated (Table 2). These are mostly genes with unknown functions, some of the fatty acid metabolism and three LuxR solo regulators Plu0925, Plu3720 and Plu3721, which are down-regulated in Δ*pluR*. Furthermore, the expression of *fadD* and of *fadL* is up-regulated in Δ*pluR*. The changed expression of *fadD* and *fadL* might be mediated either directly or indirectly via the LuxR-type regulator PluR. FadL family members are known to transport hydrophobic compounds across the bacterial outer membrane. And *fadD* encodes the IM-associated fatty acyl-CoA synthetase FadD important for the activation of imported LCFAs (van den Berg, 2005). Furthermore, the rhizobial FadL homolog of *S. meliloti* was identified to facilitate the perception of long-chain AHLs and was therefore suggested to form channels and act as long-chain AHL transporter (Krol and Becker, 2014). As PluR is involved in the regulation of expression of *fadD* and of *fadL*, these might play a role in importing the *Photorhabdus*-specific signalling molecules PPYs and CHDs/DARs. Indeed, deletion of *fadD* and of *fadL* in *E. coli* decreased dramatically the internalization of the tested signalling molecules, PPYD, IPS, CHDA, CHDB and DAR. This demonstrated a potential role of both FadD and FadL in importing the signalling molecules PPYD and CHDs/DARs in *Photorhabdus* species.

The FadL homologs of *P. luminescens* and *P. asymbiotica* were predicted to share the characteristic β-barrel structure of FadL channels (Fig. 5). This β-barrel structure is conserved among FadL homologs and comprises a long, hydrophobic tunnel through the polar lipopolysaccharide layer. Then the hydrophobic substrates can diffuse into the periplasm. Thereby, hydrophobic substrates can passage via diffusion along the hydrophobic passageway from the extracellular environment into the lipid bilayer, which was demonstrated for the FadL proteins of *E. coli* and *P. aeruginosa*. Using FadL as pores allows hydrophobic compounds to move through the hydrophilic

LPS layer of the OM without moving through the aqueous phase, which would be energetically unfavourable for substrates with low aqueous solubility coefficients (Hearn et al., 2009).

Moreover, FadL homologs of *P. luminescens* and *P. asymbiotica* show a high protein sequence identity to FadL of *E. coli*, however not to the FadL homolog of *S. meliloti* (Fig. 5). Thus, FadL of *S. meliloti* contains a conserved extracellular loop 5 (L5) that is important for specificity for long-chain AHLs. However, the L5 loop is not conserved in the FadL homologs of *P. luminescens* and *P. asymbiotica*, but in FadL of *A. tumefaciens* (Krol and Becker, 2014). Potentially, FadL homologs of *Photorhabdus* species possess a different mechanism for specificity.

In conclusion, FadL homologs of *P. luminescens* and *P. asymbiotica* might be involved in diffusion-based internalization of the *Photorhabdus*-specific signalling molecules PPYs and CHDs/DARs across the OM. However, this mechanism needs to be further investigated.

6.5 References of chapter 6

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7. CONCLUDING DISCUSSION

For a long time, *Photorhabdus* species were thought to only to eavesdrop on the LuxR-based communication of other bacteria. Furthermore, the ability to synthesise signalling molecules used for the own cell-cell communication was not described in *Photorhabdus*. However, the identification of an exceptionally high number of LuxR-type regulators in *P. luminescens* suggested a huge capacity for cell-cell communication via these regulators (Heermann and Fuchs, 2008). During its life cycle, *Photorhabdus* is exposed to different environments and changing hosts. Therefore, the bacteria need to adapt their bacterial group-coordinated behaviour. The function of these LuxR-type regulators of the three *Photorhabdus* species and their role in cell-cell communication was the main objective of this study. For this purpose, a combination of different fluorescence- and luminescence-based reporter systems, transcriptomics and bioinformatics approaches were chosen. In the course of this work, two novel non-AHL signalling molecules of LuxR solos could be identified. Furthermore, these are the first endogenous QS signals sensed by LuxR solos that have ever been described in *Photorhabdus* species. These signaling molecules are used either by the LuxR solo PluR of *P. luminescens* or by the LuxR solo PauR of *P. asymbiotica* for cell-cell communication. Both systems are contributing to the overall toxicity of each *Photorhabdus* species. Moreover, specific amino acid motifs were identified in the signal-binding domain of the two LuxR solos PluR and PauR, which are essential but not sufficient for ligand-binding and -specificity. Moreover, this work gives first insight in how these signals are potentially transported into the cell.

7.1 Quorum sensing via the LuxR-type receptor PluR

Little was known about cell-cell communication systems in *Photorhabdus* species, however the bacterial behaviour of the individuals needs to be adapted to react to the changing environments or hosts. Furthermore, *Photorhabdus* species does not encode any AHL-based quorum sensing (QS) systems, as no homologous LuxI-type or LuxM-type AHL-synthase could be identified (Joyce et al., 2011). Therefore, no AHLs can be produced by *Photorhabdus* species. However, *Photorhabdus* species

harbour several LuxR solos potentially used for communications systems (Chapter 4, Fig. 1).

Initially, the LuxR-type receptor PluR was thought to respond to exogenous AHLs, as an AHL-domain was predicted in the signal-binding domain (SBD), which is typical for AHL-sensing LuxR-type receptors. However, this work clearly showed that PluR does not sense AHLs. Moreover, the chemical nature of the PluR-specific signal could be identified, which are α-pyrones named photopyrones (PPYs), which are produced by the photopyrone synthase (PpyS) (Fig. 6A). Production of PPYs increases with cell number, which is typical for classical QS systems, (Chapter 2, Fig. S16). The PpyS/PluR communication system can be influenced on distinct layers, for example at the transcriptional or translational level of the QS circuit components. Furthermore, the stability or turnover of these proteins might be influenced.

The transcriptional expression of all three components of the system, *pluR*, *pcfA* and *ppyS*, were analysed via qRT-PCR and the promoter activity was analysed using mCherry-promoter fusions (Chapter 2, Fig. 1). However, transcriptional analysis of *ppyS* showed no positive regulation via PluR (Chapter 2, Fig. S15). Commonly, a positive feedback loop on the expression of the synthase gene via the LuxR-type regulator exists in typical QS systems. One example is the QS system of *Vibrio fischeri*, comprised of the LuxR regulator and the LuxI-synthase. Upon AHL-binding, LuxR positively regulates transcription of *luxI*, thereby creating a positive feedback loop that amplifies AHL synthesis (Fuqua et al., 1994). In this case, the signalling molecule can be entitled as autoinducer. A lack of this positive feedback regulation does not exclude the system to be termed QS system, though. The term QS is defined as controlling or synchronizing of gene expression within individual cells in response to population density (Nealson and Hastings, 1970; Nealson and Hastings, 1979; Hagen et al., 2010). Furthermore, a basic QS system consists of two regulatory proteins, the AHL-synthase LuxI, synthesising the signalling molecule, and the LuxR-type receptor, recognizing the signalling molecules (Engebrecht et al., 1983). These attributes also apply for the PpyS/PluR system, which therefore can be termed a QS system based on PPYs.

Thus, *P. luminescens* harbours a LuxS-synthase homolog and has been shown to produce the autoinducer-2 (AI-2) molecule *in vitro* (Joyce et al., 2011). However, this compound is suggested to act as a universal QS signalling molecule in bacteria

(Winzer et al., 2002). Furthermore, the role of AI-2 as a signal is not clear in *P. luminescens*. Some bacteria like *E. coli* have specific transport systems, encoded by the *lsr* region, that facilitate the uptake of the AI-2 molecule into the cell where it can be metabolised (Taga and Bassler, 2003; Joyce et al., 2011).

The ketosynthase-like PpyS can synthesize eight different PPYs dependent on the precursors of the fatty acid metabolism, which are 5-methyl-3-oxohexanoyl thioester and different thioesters of straight-chain and iso-branched chain fatty acids (Chapter 2 Fig. 2, Fig. S13). This reaction is catalysed via a claisen condensation and a lactonization. One specific LuxI synthase can also synthesize several different AHLs. Certainly the precursors of AHL-production are different, which are S-adenosylmethionine (SAM) and the appropriate charged acyl carrier protein (Williams, 2007). Moreover, PluR recognises most specifically PPYD, but is also able to sense in a decreased manner the other PPYs produced by the PpyS. The LuxR solo QscR of *Pseudomonas aeruginosa* also responds to multiple signalling molecules, which can be C8-, C10-, 3OC10-, C12-, 3OC12- and C14-HSLs (Chugani and Greenberg, 2014).

P. luminescens produces PPYs in LB medium as well as in the insect larvae, suggesting that QS via the PpyS/PluR system occurs during the infection process of the larvae (Chapter 2, Fig. S16). Pyrones are a class of heterocyclic compounds with two isomers found in nature, which are denoted as 2-pyrone (a-pyrone) and 4-pyrone (γ-pyrone) (Streitwieser and Heathcock, 1985). *P. luminescens* is not the only organism able of synthesising α-pyrones, but they are also well-known secondary metabolites in species like pseudomonads (Chu et al., 2002; Kong et al., 2005), fungi (Elbandy et al., 2009) and Streptomycetes (Chemler et al., 2012). In Streptomycetes pyrones are involved in spore germination (Aoki et al., 2011). Thus, these organisms might use pyrones also as signalling molecules beside their known function. Furthermore, *Photorhabdus temperata* ssp. *thracensis* contains a PpyS homolog (71% identity and 84% similarity) and a PluR homolog. These observations suggest a similar PpyS/PluR QS system used in *P. temperata* to control cell clumping (Chapter 2).

Deletion of *pluR* in *P. luminescens* showed no decreased pathogenicity compared to TT01 wild type (Chapter 2, Fig. S17). In contrast expression of the *pcf* operon in normally harmless *E. coli* cells resulted in a pathogenic strain against

Galleria mellonella larvae (Chapter 2, Fig. 4). The *pcf*-dependent cell clumping and therefore the PpyS/PluR QS system contribute to the overall toxicity of *P. luminescens* towards insect larvae.

7.2 Quorum sensing via the LuxR-type receptor PauR

A PluR-homologous LuxR-type regulator was identified to be present in the genome of *P. asymbiotica*, which was named PauR. Furthermore, this work showed that PauR activates the expression of the *pcfABCDEF* operon upon binding to its cognate signalling molecule present in the supernatant of *P. asymbiotica*. However, *P. asymbiotica* produces neither PPYs nor AHLs, since no pyronesynthase or LuxI synthase homologs are present. Thus, the signalling molecule sensed by PauR could be identified in this work, which is an endogenous non-AHL signalling molecule, which belongs to the class of dialkylresorcinols (DARs). Upon binding of the signalling molecule DAR, cell clumping is induced due to the activation of *pcf* operon's expression via PauR (Chapter 3) (Fig. 6B). This cell-cell communication system is analogous to the PPY-based QS system mechanism of *P. luminescens*.

DARs and its precursors cyclohexanediones (CHDs) are synthesised by the *darABC* operon. Plenty of bacteria possess DarABC homologs, several of them being pathogenic to animals, plants or humans, like various *Neisseria* strains (Fuchs et al., 2013). Therefore, the presence of the *darABC* operon and its products is often linked with virulence. Furthermore, dialkylresorcinols produced by *Pseudomonas* sp. can have antimicrobial properties (Pohanka et al., 2006). Among the 116 bacterial strains containing a *darB* homolog, not all strains harbouring *luxR* homologs, suggesting another role of DARs besides acting as a QS signal as shown for *P. asymbiotica* (Chapter 3). Recently, the first LuxI without a cognate LuxR-type regulator was identified in the marine sponge symbiont *Ruegeria* species influencing swimming motility (Zan et al., 2014). Furthermore, the presence of *darABC* homologous genes does not ensure the production of DARs and/or CHDs, as shown for the GameXPeptide production. Although the GxpS enzyme, responsible for the production of GameXPeptides, is widespread amongst *Photorhabdus* and *Xenorhabdus* strains, however only *Photorhabdus* species produce GameXPeptides. However, the synthesis occurs only in the presence of *G. mellonella* larvae (Nollmann

et al., 2014). Moreover, *P. asymbiotica* produces a specific subset of DAR, CHDA, CHDB and IPS (Chapter 3, Fig. 1), depending on the precursors derived from two fatty acids (Fuchs et al., 2013). Among these compounds, PauR most specifically recognises DAR at nanomolar concentrations. This is a common range as shown also for LuxR homologs, which sense their corresponding AHLs at concentrations of about 25 nM (Liu et al., 2011). Furthermore, PauR also senses the remaining compounds, but with decreasing affinities of the following order: CHDB > CHDA > IPS. Induction with CHDB, CHDA or IPS also leads to cell clumping, but also in a decreased manner (Chapter 3, Fig. S5). The entire QS circuit, consisting of *pauR*, *darABC* and the *pcfABCDEF* operon, can be heterologously expressed in *E. coli* leading to cell clumping, thereby confirming nature of this cell-cell communication system. Furthermore, the *pcf* operon is sufficient to generate cell clumping either in *Photorhabdus* species or in *E. coli* (Chapter 3, Fig. S7).

Analysing the promoter activities of the components of the DAR-dependent QS system showed an auto-regulation of PauR on its own transcription (Chapter 3, Fig. S6). This positive auto-regulation is typical for QS systems, but it could not be detected in the PPY-based QS system of *P. luminescens*. Furthermore, the DAR synthesis operon *darABC* is expressed only at a low level and is, however, not influenced by PauR. A similar phenomenon was found for the expression of the photopyrone synthase *ppyS* in *P. luminescens*. Therefore, both LuxR-type regulators of *Photorhabdus* species, either PauR or PluR, are not influencing the expression of their cognate signalling molecule synthesis system, in contrast to the most AHL QS systems.

Deletion of *pauR* in *P. asymbiotica* resulted in a drastic decrease of pathogenicity against *G. mellonella* larvae. This finding was in contrast to the deletion of *pluR* in *P. luminescens* on the other hand that showed no significant effect on pathogenicity in *G. mellonella*. Therefore, it might be possible that DAR-dependent regulation of virulence in general might be more important for colonization of humans. *G. mellonella* larvae are discussed as a cheap and convenient model host for human pathogenic bacteria. They are used as host systems in order to analyse bacterial and fungal pathogens (Cook and McArthur, 2013). The innate immune systems of *G. mellonella* larvae share a high degree of structural and functional homology to the vertebrates. Furthermore, both cellular and humoral defences are present in

G. mellonella comparable to the ones of humans (Hoffmann, 1995; Lionakis, 2011). It is conceivable that *P. asymbiotica* might have been adapted to the pathogenicity towards human via the use of DAR as QS signal, instead of PPYs or AHLs.

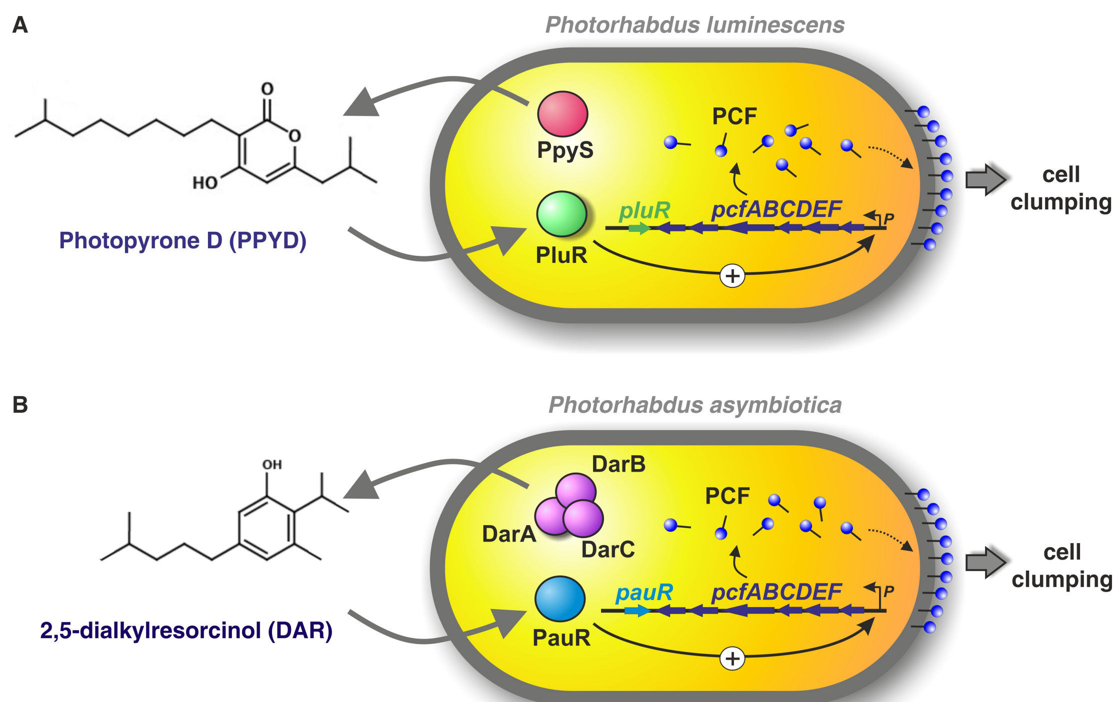

Figure 6: PPYD- or DAR-dependent cell-cell communication in *P. luminescens* or *P. asymbiotica*, respectively. In *P. luminescens* (**A**) the LuxR solo PluR senses photopyrone D (PPYD) as signalling molecules and then activates the expression of the *pcfABCDEF* operon, which leads to cell clumping. PPYD is endogenously produced by the photopyrone synthase (PpyS). Similarly, the LuxR solo PauR activates the *pcfA* promoter and thereby induces expression of the *pcfABCDEF* operon, which leads to a cell clumping in *P. asymbiotica* (**B**). However, PauR senses 2,5-dialkylresorcinol (DAR) synthesized via the DarABC operon.

7.3 Definition of a LuxR solo protein

The first quorum sensing (QS) system, the LuxR/LuxI pair, was described in *Vibrio fischeri* regulating the expression of bioluminescence dependent on cell size (Nealson and Hastings, 1979). Based on this first description, the typical QS system in Gram-negative bacteria was defined to consist of a LuxR-type receptor and a LuxI-type synthase producing the signalling molecule AHL. Furthermore, the LuxR-type

receptor contains a N-terminal AHL-binding domain, important for AHL-sensing, as AHLs were the first known signalling molecules, termed autoinducers (Fuqua et al., 1994). Later on, LuxR-type receptors were identified, which do not possess a cognate LuxI-synthase, but share the domain modularity of LuxR-type proteins and homology to LuxR receptors. These receptors were referred to as LuxR solos or LuxR orphans (Subramoni and Venturi, 2009; Fuqua, 2006) . Up to date, LuxR-type proteins not associated with a LuxI synthase are called LuxR solos, however many of them might also comprise a QS pair with a yet unknown synthase or synthesis system. This is the case for PluR from *P. luminescens* and PauR from *P. asymbiotica* have formerly also been classified as a LuxR solos, however, cognate synthase systems have been identified and it is therefore questionable whether these LuxR-type receptors can actually still be designated as LuxR solos. They can therefore better been reclassified them as LuxR-type receptors being part of a QS system, even though they are not associated with a LuxI synthase. Furthermore, QS systems are usually but not necessarily genetically linked, like the TraI/TraR pair of *Agrobacterium tumefaciens* and the RhiI/RhiR proteins of *Rhizobium leguminosarum* are not adjacently positioned, despite the fact that these function as cognate pairs (Subramoni and Venturi, 2009; Gray and Garey, 2001). This is also the case for the LuxR-type receptors PluR and PauR, which are not genetically linked to their synthases, but these are both adjacent located to their target operon.

7.4 Structure and activity of the LuxR-type receptors PluR and PauR

Several parameters can influence the condition of LuxR-type receptors like PluR and PauR. The activity, stability and structure of PluR and PauR might be altered via the presence or absence of the cognate signalling molecule. Thereby the expression of the *pcf*-dependent cell clumping is modified.

7.4.1 Modulation of the activity of PluR and PauR

The activity of LuxR-type receptors can be influenced via the presence and amount of its cognate signalling molecules. LuxR-type receptors usually act as

activators upon binding of the corresponding signalling molecule. Thereby, the RNA polymerase is recruited to the promoter and the transcription of the target genes is induced (Nasser and Reverchon, 2006). Likewise, PluR and PauR both activate expression of their corresponding *pcf* operon upon binding of the corresponding signalling molecule (Chapter 2, Fig. 2; Chapter 3, Fig. 1). Moreover, some LuxR-type proteins act in the opposite way as transcriptional repressors, whose DNA-binding activity is reduced by the interaction with AHL (Horng et al., 2002). One exception is EsaR that binds DNA in the absence of AHL, acting either as an activator or a repressor of transcription, and is deactivated when bound to AHL (Shong et al., 2013; Schu et al., 2014).

So far, binding-affinities of the LuxR homologs PluR or PauR to its specific signalling molecules could not yet been determined. Due to the overall hydrophobicity of PluR and PauR, gaining a high yield of active and correct folded protein was challenging (data not shown), as also known for other LuxR homologs (Oinuma and Greenberg, 2011). PluR and PauR are both recognising their cognate signalling molecules in low concentration of 3.5 nM, which was heterologously determined in *E. coli* and *P. luminescens* or *P. asymbiotica*, respectively. For the LuxR homologs TraR, LasR and QscR a half maximal effective concentration (EC_{50}) of ~25 nM of the corresponding AHL were determined. The EC_{50} values were determined in three different strain background revealing the same results (Liu et al., 2011). The modelled structures of PluR and PauR both display the typical three-dimensional fold of LuxR-type proteins (Fig. 8), which suggests a binding stoichiometry of signalling-molecule-to-monomer of one-to-one, which is true for several LuxR homologs (Welch et al., 2000; Schuster et al., 2004; Oinuma and Greenberg, 2011). PluR most specifically senses PPYD of the eight different photopyrones (Chapter 2, Fig. S10). Furthermore, PluR might comprise diverse binding-affinities towards the several PPYs. The same might be true for PauR, which most specifically recognises DAR and might also have different binding-affinities towards the four tested CHDs and DARs (Chapter 3, Fig. S5). The *P. aeruginosa*-specific LuxR homolog QscR exhibits different affinities to several AHLs. Moreover, also the opposite case exists, as QscR and LasR from *P. aeruginosa* show different binding-affinities to the same AHL (Oinuma and Greenberg, 2011). The *Photorhabdus*-specific signalling molecules PPYs and CHDs/DARs appear to have

similar binding affinities to their cognate receptor, however, the structural differences of these compounds allow a specific recognition by the distinct LuxR homologs PluR and PauR (Chapter 5, Fig. 4).

Binding of the corresponding signalling molecule to the LuxR-type regulator often promotes a conformational change that allows the protein to dimerise. Thus, the homodimer can then bind to its promoter target regions and induce transcriptional regulatory processes, whereby the signal-free monomer are subsequently proteolysed (Whitehead et al., 2001; Oinuma and Greenberg, 2011). Likewise, it is therefore conceivable that dimerization of the LuxR homologs PluR and PauR might also be stimulated in the presence of their native signalling molecules (Fig. 7).

7.4.2 Protein stability of PluR and PauR

Generally, LuxR-type proteins can be classified into three groups based on the interactions with AHL (Liu et al., 2011). In Class I, the LuxR-type protein binds irreversibly its cognate AHL and requires it for proper folding during protein synthesis. Examples of Class I receptors are LasR of *P. aeruginosa* and TraR of *A. tumefaciens* (Schuster et al., 2004). Class II activator proteins require AHL to be produced in a soluble and active form, but signal-binding is reversible, which is for example the case for QscR of *P. aeruginosa* (Liu et al., 2010). Class III proteins bind reversible to its corresponding AHL. The signalling molecule is not needed for folding, which was demonstrated for RhlR and EsaR (Medina et al., 2003; Minogue et al., 2005). PluR and PauR both need their cognate signalling molecule to interact with its DNA target regions. Furthermore, heterologous overproduction of *pluR* in *E. coli* yielded in a higher amount and soluble form, when the *ppyS* and the *bkd* operon were produced simultaneously (data not shown). The LuxR solo QscR of *P. aeruginosa* also requires AHLs to an achieve significant levels of soluble and active proteins (Oinuma and Greenberg, 2011). Thus, it can be concluded that PluR and PauR both belong either to Class I or Class II as both need their cognate signalling compound for an soluble, active protein. However, it is not fully understood whether the signalling molecules are bound reversibly or irreversibly. Moreover, PluR and PauR are both able to recognise their corresponding *pcfA* promoter regions in a signal-independent manner when heterologously overproduced in *E. coli* (Chapter 5, Fig. 3). The same

effect was shown for QscR of *P. aeruginosa*, which binds to the target site in a signal-independently active form at sufficiently high concentrations, which can be achieved by overproduction (Oinuma and Greenberg, 2011; Park et al., 2013). Furthermore, absence of the cognate signalling molecules might induce degradation of PluR and PauR as it is also the case for QscR (Chugani and Greenberg, 2014).

Recently, a post-transcriptional regulation of the QS regulator ExpR of *S. meliloti* via the RNA binding protein Hfq was described. Thereby, Hfq controls production and perception of the QS signals mediated at high cell densities via a direct interaction with *expR* (Gao et al., 2014). This adds another example of possible layers on controlling QS-dependent gene expression, which might also affect PluR or PauR.

7.4.3 Ligand-binding pocket of PluR and PauR

The structure of each PluR and PauR were modelled based on the crystal structure of QscR, revealing that both share the common three-dimensional fold of LuxR-type receptors (Chapter 2, Fig. S11; Chapter 3, Fig. S8). The LuxR solo QscR shows the highest identity to both PluR (32 %) and PauR (30 %) of the available crystal structures of LuxR homologs. Furthermore, docking experiments of the cognate signalling molecule with the ligand-binding pockets of PluR and PauR identified amino acid residues important for ligand-binding (Chapter 2, Fig. 3; Chapter 3, Fig. 3). In general, the SBD of LuxR-type receptors harbour six conserved amino acids (W57, Y61, D70, P71, W85 and G113, with respect to TraR), comprising a conserved WYDPWG-motif for AHL-sensors. This motif is important for ligand-binding and shaping the ligand-pocket (Patankar and González, 2009). PluR and PauR both share only two of these amino acid (Y66, D75). As the remaining four amino acids are substituted, PluR harbours a TYDQCS-motif and PauR a TYDQYI-motif in the SBD (Chapter 4, Fig. 2). Thus, both motives are contributing to correct ligand-sensing and the shape of the ligand-pocket of PluR and PauR (Chapter 5, Fig. 5, Fig. 6). However, these motives are essential but not sufficient for ligand-binding. Thus it can be concluded that also non-conserved amino acids of the SBD contribute the specificity of these regulators to their cognate signalling molecules, although these are not highly conserved (Chapter 5, Fig. 7). Recently, a novel subfamily of LuxR solos present in plant-associated bacteria has been identified, which contains substitutions within the

WYDPWG-motif that are W57M and Y61W (with respect to TraR). Although the specific signalling molecules are yet unknown, these substitutions might allow the binding of plant signal molecules rather than AHLs (Patel et al., 2013). Likewise, the several LuxR solos of *Photorhabdus* species show alterations in the conserved WYDPWG-motif, potentially allowing the LuxR homologs to evolve and adapt to distinct compounds besides AHLs (Chapter 4, Fig. 2).

7.5 The *Photorhabdus* clumping factor PCF

The *pcfABCDEF* operon of *P. luminescens* and *P. asymbiotica* both contribute to the overall pathogenicity of the bacteria. Both *pcf* operons display a high identity among each other (Chapter 3, Fig. 1). The genes of the *pcfABCDEF* operon are predicted to encode proteins of the amino acid metabolism and one putative efflux transporter (PcfE). These enzymes might synthesise an unknown factor, named *Photorhabdus* clumping factor (PCF), which is transported to the surface of the cells via PcfE, and then letting the cells clump together (Fig. 7). *Pcf*-induced cell clumping is also functional in *E. coli* (Chapter 2, Fig. 4; Chapter 3, Fig. S7), suggesting the presence of common precursors for the synthesis of PCF in these organisms. Also the mechanism of presenting the PCF on the surface of the cells should be similar in *E. coli* and *Photorhabdus* species. Although, the nature of the PCF is still unknown, it is conceivable that it either might be a toxin presented on the surface of the cells or that clumping cells occlude the inner gut system of the larvae. Furthermore it is possible that the cells clumps are simply better protected from the immune system of the larvae than single cells. *E. coli* cells expressing the *pcf* operon are stainable with congo red (Janosz, 2012), which might indicate an amyloid-like structure and is often associated with pathogens (Sipe, 1994; Cangelosi et al., 1999). Furthermore, a congo red staining phenotype suggests alterations in cell wall components, which might indicate changes in virulence or drug susceptibility (Parrish et al., 2004).

The expression of both *pcf* operons of *P. luminescens* and *P asymbiotica* is activated via the homologous LuxR-type receptors PluR or PauR, respectively, which sense different signalling molecules. Therefore, adaption of *P. luminescens* and *P. asymbiotica* to their different host, insects or men, respectively, possibly has occurred on the level of signal-input diversity, PPY or DAR, whereas the phenotype

cell clumping has remained similar as the output. However, PauR also might regulate the expression of different target genes than PluR and thereby generates pathogenicity towards men.

Furthermore, the LysR-type regulator HexA inhibits expression of the *pcf* operon in secondary cells, which prevents cell clumping in *P. luminescens* secondary cells (Personal communication with Angela Glaeser, data not shown) (Fig. 7). In general, HexA is responsible for secondary-specific phenotypes via repression of the expression of primary-specific genes (Joyce and Clarke, 2003).

In conclusion, beside PluR and PauR also several other factors might influence the expression of the *pcf* operon in *P. luminescens* and *P. asymbiotica* and thereby cell clumping (Fig. 7). Therefore, several direct and indirect mechanism influence the expression of the *pcf* operon, like the stability of the LuxR-type regulator PluR or PauR, which is influenced by the presence or absence of the cognate signalling molecule in *P. luminescens* or *P. asymbiotica*, respectively. Furthermore, PluR seems to control the expression of the putative importer *fadL* and thereby controlling the import of PPYs. Moreover, HexA potentially negatively controls the expression of the *pcf* operon. Additionally, the putative efflux transporter PcfE seems to be important for the secretion of PCF (Fig. 7).

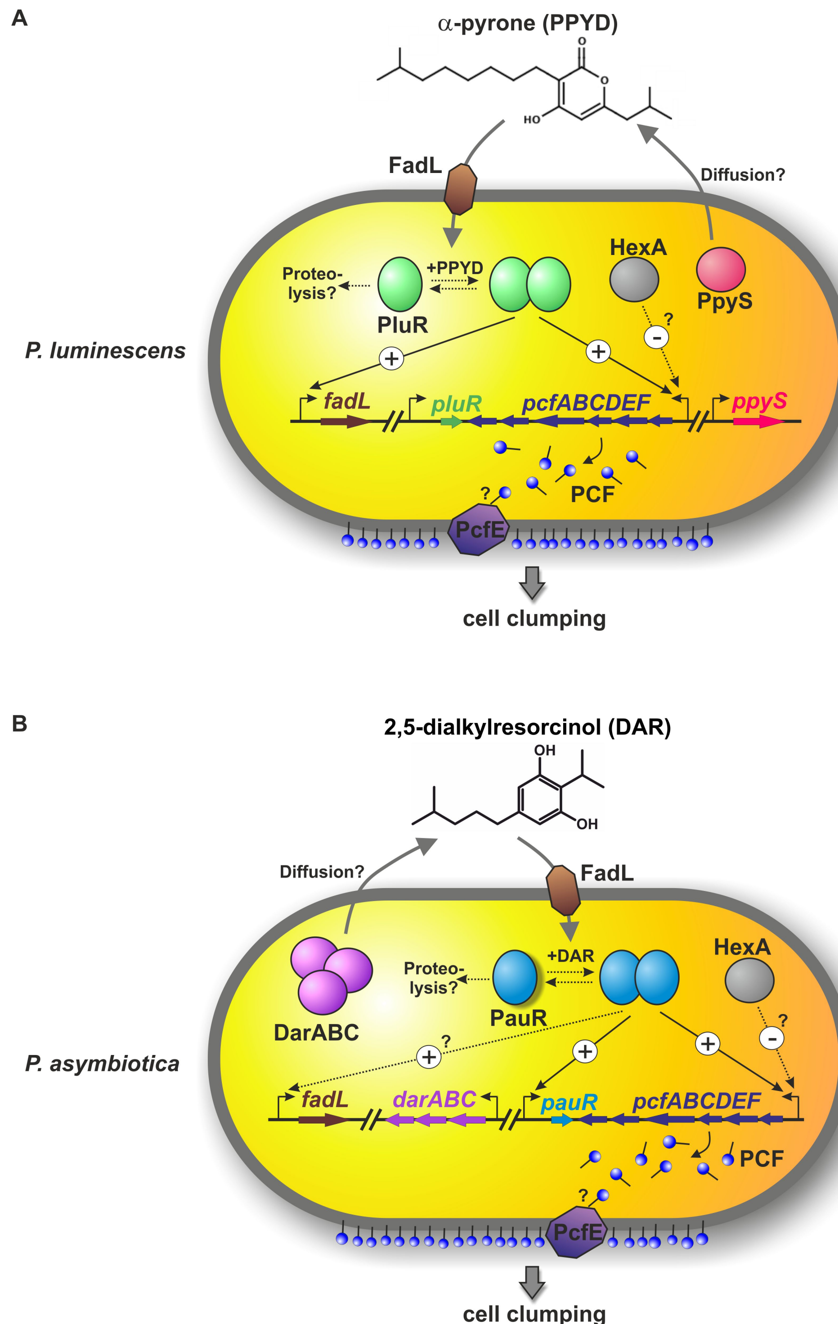

Figure 7: Putative extended model of regulation mechanisms of the *pcf*-derived cell clumping in *P. luminescens* and *P. asymbiotica.* **(A)** Upon binding of the signalling molecule PPYD, the LuxR-type regulator PluR can dimerize and thereby can activate the expression of *fadL* and the *pcf* operon. PPYD is synthesised by the photopyrone synthase PpyS and is possible imported via FadL. In the absence of the signalling molecule, the PluR monomer can be degraded. Expression of the *pcf* operon leads to the production of the PCF (*Photorhabdus* clumping factor), which is potentially exported via the putative transporter PcfE. Furthermore, the LysR-type regulator HexA, responsible for the repression of phase I-specific phenotypes, potentially negatively controls expression of the *pcf* operon. However, cell

clumping contributes to pathogenicity of *Photorhabdus* species. (**B**) Similarly, the LuxR-type regulator PauR recognises its signalling molecule a 2,5-dialkylresorcinol (DAR) and the actvates expression of the *pcf* operon. Contrarily, PauR auto-regulates its own expression. Black arrows indicate regulation, either positively (+) or negatively (-). Grey arrows indicate transport. Black arrows or with a dashed line a question mark indicate putative regulation mechanisms. The genes, *fadL*, *pluR*, *pcfABCDEF*, *ppyS*, *darABC* and *pauR* are indicated in their orientation on the genome. The promoter of each gene/operon is indicated with a black arrow (↱).

7.6 Regulatory network of LuxR solos?

All three *Photorhabdus* species harbour a high number of LuxR solos, which are grouped according to their N-terminal domain into three types (Chapter 4, Fig. 1). The variety of LuxR solos present in *Photorhabdus* species may reflect their diversity of signals, which can be recognised.

In LuxR-type regulators responding to AHLs, the conserved amino acid WYDPWG-motif is important for specificity towards their cognate signalling molecule. This conserved WYDPWG-motif is only intact in the SdiA homologs Plu0320 of *P. luminescens*, Pte2206 of *P. temperata*, Pau0252 and Pau0255 of *P. asymbiotica* (Chapter 4, Fig. 2). Therefore, these LuxR solos might sense exogenous AHLs to eavesdrop on the surrounding Gram-negative bacterial community. Potentially, these are involved in QQ mechanism via regulation of the expression of AHL-latonases and/or AHL-acylases to inhibit the AHL-based communication of the neighbouring bacteria (Chapter 4, Table 1). Quite a few organisms show the ability to degrade AHLs in order to disturb the communication of neighbouring bacteria, whereas QS systems have been reported from a wide range of organisms (Kalia et al., 2011). Furthermore, production of AHL-latonases and/or AHL-acylases does not disturb the own QS-based communication of *Photorhabdus* species as only non-AHL-based signalling molecules are used.

The majority of the LuxR solos of *Photorhabdus* species contain a PAS4-domain with at least two substitutions within the WYDPWG-motif. These are possibly used to sense eukaryotic signals either from the nematode or the insect host. Thereby, the current host can be identified and symbiotic or pathogenic behaviour may be induced. As all three *Photorhabdus* species are able to infect different insects, orthologous LuxR solos with a PAS4-domain might sense similar eukaryotic signals when each

species resides in the same insect host. Moreover, PAS4-domain-containing LuxR solos of *P. asymbiotica* tend to the substitution D70N in the conserved amino acid motif in the SBD (Chapter 4, Fig. 2). These might sense and respond to eukaryotic signals predominantly stemming a human host. However, LuxR solos with a PAS4-domain do not necessarily contribute to a cell-size dependent cell-cell communication, but contribute to the recognition of the habitat (Chapter 4, Fig. 5).

Several organisms evolved hierarchical regulation among their different QS circuits. One example is *P. aeruginosa*, which shows a sophisticated hierarchy regulation of its QS network. This network consists of the two classical QS systems LasI/LasR and RhlI/RhlR and the LuxR solo QscR, which negatively regulates both the *las* and *rhl* QS system by binding their cognate AHLs (Ledgham et al., 2003). The first indication of a regulatory network among LuxR solos might be the positive regulation of three LuxR solos via PluR. The expression of the LuxR solos encoding genes *plu0925* and *plu3720-plu3721* is down-regulated in Δ*pluR* in the late stationary phase (Chapter 6, Table 2). Moreover, no binding motif could be identified in the promoter of these three LuxR solos encoding genes or the remaining genes that are differentially expressed in the Δ*pluR* mutant. However, the regulation via PluR might be an indirect or direct effect. For several LuxR-type regulators a specific binding site was identified. LuxR of *V. fischeri* is known to bind to the "*lux"* box, a 20 bp inverted repeat present in the *luxI* promoter, and thereby activates transcription of the *lux* operon (Egland and Greenberg, 1999; Trott and Stevens, 2001). Furthermore, each unit of a QscR dimer binds to one half of its palindromic 18-bp binding site present in the promoter region of *traI* in *P. aeruginosa* (Lintz et al., 2011).

So far, no regulation circuit among the several LuxR homologs from *Photorhabdus* species is known. Thus, as the high number of homologous LuxR solos possibly responding to similar signals might activate similar biological pathways, hierarchical regulatory processes might be assumed.

7.7 FadL mediates import of hydrophobic signalling molecules

The cell envelope is an efficient barrier for diffusion of hydrophobic substrates. Therefore, strategies are needed for the import and export of long-chain AHLs, which are synthesised by several α-proteobacteria (Kaplan and Greenberg, 1985; (Boyer and Wisniewski-Dye, 2009). Accordingly, the *Photorhabdus*-specific hydrophobic signalling compounds PPYs and CHDs/DARs might not simply be internalised via diffusion, potentially a specific transport mechanism might be required. Interestingly, the transcriptome analysis of the Δ*pluR* mutant revealed a regulation of the expression of the *fadL* homolog via PluR, either direct or indirect. The FadL family members encode proteins responsible for the transport of hydrophobic compounds across the bacterial outer membrane (van den Berg, 2005). Therefore, the influence of FadL on the internalization of the *Photorhabdus*-specific signalling molecules was heterologously tested in *E. coli*. Thereby, it could be shown that a lack of FadL decreased the amount of signalling molecules sensed by the LuxR-type receptors PluR and PauR (Chapter 6, Fig. 6). Moreover, the FadL homologs of *Photorhabdus* species display the typical β-barrel structure potentially forming a long, hydrophobic tunnel through the polar lipopolysaccharide layer. Hence, FadL of *Photorhabdus* species mediates transport of PPYs and CHDs/DARs across the outer membrane into the periplasm. Furthermore, also deletion of *fadD* in *E. coli* decreased the amount of signalling molecules sensed by PluR and PauR. However, the signalling molecules PPYs and CHDs/DARs do not need to be activated like long-chain fatty acids (LCFAs). In *E. coli* FadD is important in order to remove long-chain fatty acids (LCFAs) from the inner membrane (IM) and to activate these to long-chain acyl-CoAs (van den Berg, 2005). Perhaps, in *Photorhabdus* species FadD is required to remove the signalling molecules PPYs and CHDs/DARs from the IM without any further processing. Furthermore, recently the FadL homolog of *S. meliloti* was described to perceive long-chain AHLs and to form channels for transporting these hydrophobic compounds (Krol and Becker, 2014). A similar function might also be performed by the FadL homologs of *Photorhabdus* species. However, potentially other possibilities might influence the internalisation of the hydrophobic *Photorhabdus*-specific signalling molecules PPYs and DARs, as the deletion of *fadL*

or *fadD* in *E. coli* not completely prevent activation of the reporter plasmid (Chapter 6, Fig. 4). Furthermore, the question of the export mechanism of these signalling molecules remains open.

In *E. coli* the product of FadL-mediated transport, the long chain acyl-CoAs, activates the DNA-binding activity of FadR. In turn, FadR regulates the expression of *fadL* and *fadD* (Dirusso and Black, 2004). In *P. luminescens* PluR interacts with PPYs, the product of FadL-mediated import, and then also regulates expression of *fadD* and *fadL*. Suggesting a different regulation layer of PluR to control the amount of signalling molecules, which occurs not at the level of the transcription of *luxI*, but on the transcription of the importer system.

7.8 Outlook

Of the remaining questions regarding the function of the several LuxR solos in *Photorhabdus* species, some of the most pressing are the following: What are the binding affinities of PluR and PauR towards their cognate signalling molecules? Is a specific export mechanism needed for PPYs and CHDs/DARs? Do PluR and PauR bind to a specific promoter motif? What is the role of HexA in the *pcf* operon-mediated cell clumping? Which signals are sensed by the multiple LuxR solos containing an PAS4-domain? Which set of genes do these LuxR solos regulate? Why are these LuxR solos redundant?

Binding affinities of PluR and PauR to their native signalling molecule can be determined using a Surface Plasmon Resonance Spectroscopy assay. For this approach, a high yield and a high purity of PluR and PauR are needed, however, due to their overall hydrophobicity overproduction and purification of LuxR homologs is challenging (Oinuma and Greenberg, 2011). Therefore, also a fluorescence-based thermal stability assay could be used, which determines an alteration of the conformation of the LuxR homologs PluR and PauR in the presence or absence of their corresponding signalling molecules (Boivin et al., 2013). Furthermore, additional amino acids in the SBD of PluR and PauR should be analysed, to extend the knowledge about critical amino acids important for sensing of the respective signalling molecule and to define specific binding motifs for the different signalling molecules to their cognate LuxR-type receptor.

In order to gain insights into a possible promoter motif essential for PluR or PauR DNA-binding, successive truncation of the *pcfA* promoter regions of *P. luminescens* and *P. asymbiotica* could be investigated using the luminescence-based reporter plasmids. Moreover, nucleotide substitutions or deletions could be performed in the promoter region to identify a specific promoter motif important for recognition via PluR or PauR. Moreover, an electrophoretic mobility shift assay (EMSA) could be used to analyse binding of PluR or PauR to different *pcfA* promoter fragments. Furthermore, the question remains whether HexA and PluR or PauR show a competitive or cooperative behaviour in binding to the *pcfA* promoter regions. Therefore, the reporter plasmids with the *pcfA* promoter fusion could be analysed in the presence of different ratios of HexA and PluR or PauR.

In order to determine which compounds are recognised by the PAS4-domain-containing LuxR solos, liquid chromatography and mass spectrometric analysis of homogenised insect or nematode fluid could be used to identify the signals. Moreover, fluorescence-based or luminescence-based reporter systems should be developed for each LuxR solo to analyse HPLC-derived fractions or pure compounds. In order to identify target promoter regions a transcriptome analysis of the respective *luxR* solo mutant compared to wild type could be utilised. Redundancy of these LuxR solos might be investigated by successive deletion of the large gene clusters and testing their potential additive effect on pathogenicity or symbiosis.

7.9 References of discussion

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