Adaptation and regulation of the alternative lifestyle of insect pathogenic *Photorhabdus luminescens* in the soil and its potential as biocontrol agent

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Nazzareno Dominelli

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1. Berichterstatter: Univ.-Prof. Dr. Ralf Heermann

Tag der mündlichen Prüfung:

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Nomenclature

Both phenotypic cell variants of *Photorhabdus luminescens* subsp. *laumondii* DJC are called primary and secondary cells and are termed DJC 1° or 1° cells and DJC 2° or 2° cells.

Gene deletions in DJC 1° and 2° are marked with the symbol "Δ". Gene integration in DJC 1° and 2° are marked with "::" followed by the respective vector used.

Affinity tags are stated previous promoter, gene, or protein names. For genes and proteins: first the tag name is stated, followed by the corresponding terminus letter and the gene/protein name (e.g.: 6xHis-N-SdiA) and for promoters the tag name is stated in square brackets prior the promoters name (e.g.: [Btn]-P_{sdiA}).

Abbreviations

AA	amino acid
AHL	acyl homoserine lactone
ALA	5-amino levulinic acid
AQ	anthraquinone
bp	base pairs
Btn	biotin
Carb	carbenicillin
Cbp	chitin binding protein
Chi	chitinase
DBD	DNA binding domain
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	1,4-Dithiothreitol
gDNA	genomic DNA
Gent	gentamycin
6xHis	affinity tag composed of 6 histidine
HTH	helix turn helix
IJs	infective juveniles
IKS	interkingdom signaling
IPS	isopropyl stilbene
Kan	kanamycin
LB	lysogeny broth
mRNA	messenger RNA
MST	microscale thermophoresis
nanoDSF	nano differential scanning fluorimetry
Ni-NTA	nickel nitrilotriacetic acid
PAGE	polyacrylamide gel electrophoresis
Pcf	*Photorhabdus* clumping factor
PCR	polymerase chain reaction
PGBs	plant associated bacteria
PGPRs	plant growth promoting rhizobacteria
PpyS	photopyrone synthase

QQ	quorum quenching
QS	quorum sensing
RNA	ribonucleic acid
Rif	rifampicin
SBD	signal binding domain
SRSM	switching responsible signal molecule
Suc	sucrose
SPR	surface plasmon resonance
YMG	yeast malt glucose
X-Gal	5-Brom-4-chlor-3-indoxyl-β-D-galactopyranoside

Publications and manuscripts presented in this thesis

Chapter 2:

Eckstein S, **Dominelli N**[1], Brachmann A, and Heermann R (2019). Phenotypic heterogeneity of the insect pathogen *Photorhabdus luminescens*: Insights into the fate of secondary cells. *Appl Environ Microbiol* 85. doi:10.1128/AEM.01910-19.

Chapter 3:

Regaiolo R*, **Dominelli N***, Andresen K, Heermann R (2020). The biocontrol agent and insect pathogen *Photorhabdus luminescens* interacts with plant roots. *Appl Environ Microbiol* 86(17):e00891-20: doi: 10.1128/AEM.00891-20.

* Authors contributed equally. First author order was determined by which author had more seniority.

Chapter 4:

Dominelli N, Platz F, Heermann R (2022). The insect pathogen *Photorhabdus luminescens* protects plants from phytopathogenic *Fusarium graminearum* via chitin degradation. Accepted. *Appl Environ Microbiol.*

Chapter 5:

Dominelli N*, Regaiolo A*, Heermann R. SdiA mediated interkingdom communication of *Photorhabdus luminescens* with plants and its role in biofilm and motility

Chapter 6:

Dominelli N, Jäger HJ, Langer A, Brachmann A, Heermann R[1] (2022). High throughput sequencing analysis reveals genomic similarity in phenotypic heterogeneous *Photorhabdus luminescens* cell population. Accepted. *Ann Microbiol*

Chapter 7:

Dominelli N and Heermann R (2020). “Small Talk” – Die stille Kommunikation der Bakterien. *Biol Unserer Zeit* **50:6,** 414-423. https://doi.org/10.1002/biuz.202010720

Chapter 8:

Dominelli N and Heermann R (2021). Freund oder Feind? — Die zwei Gesichter von *Photorhabdus luminescens*. *Biospektrum* **27,** 690–692. https://doi.org/10.1007/s12268-021-1662-9

Contributions to publications and manuscripts presented in this thesis

Chapter 2:

S.E. and R.H. designed the experiments. A.B. conducted the RNA-sequencing. N.D. performed the chemotactic movement assays and the growth experiments with temperature shifts. S.E. prepared the RNA for RNA-sequencing, analyzed the data and performed qRT-PCR and the swimming motility assays. S.E. and R.H. analyzed the data and wrote the manuscript.

Chapter 3:

A.R. and N.D designed the experiments. R.H. coordinated the project. A.R. and N.D. carried out the RNA-sequencing, K.A. processed the raw sequencing data and A.R. and N.D. interpreted these data. A.R. and N.D. carried out all the other experiments and analyzed the data. A.R., N.D. and R.H. wrote the manuscript.

Chapter 4:

N.D. designed the experiments. R.H. coordinated the project. N.D. carried out the bioinformatic analysis, carried out the *in planta* tests and checked for Chi2A activity against fungi. N.D. and F.P. performed protein purification and nanoDSF analysis and checked for chitin degradation, created deletion and complementation mutants, and performed the antifungal growth assays with the deletion mutants. N.D. carried out the antifungal growth assays with the complementation mutants. F.P. performed the antifungal growth assays with *E. coli* cells. N.D. and F.P. performed hyphae colonization assays. N.D. analyzed the data. N.D. and R.H. wrote the manuscript.

Chapter 5:

N.D., A.R. and R.H. designed the experiments. R.H. coordinated the project. N.D. and A.R. performed the experiments and wrote the manuscript

Chapter 6:

A.L. and R.H. designed the cultivation experiments; N.D. and R.H. designed the molecular experiments. R.H. coordinated the project. A.L. cultivated *P. luminescens* 1°

picked the switched cells and isolated the genomic DNA for HTS sequencing. A.B. carried out the sequencing and H.Y.J. performed bioinformatic analysis and processed the data. N.D. analyzed the processed data, isolated DNA from bacteria and performed PCR-analyses to confirm the HTS-results. N.D. and R.H. wrote the manuscript.

Chapter 7:

N.D. and R.H. wrote the review.

Chapter 8:

N.D. and R.H wrote the review.

Summary

Soil living and plant beneficial bacteria rose in importance as biocontrol agents in sustainable agriculture as many pests and diseases harshly reduce crop yields. *Photorhabdus luminescens* is a Gram-negative bacterium living in symbiosis with entomopathogenic nematodes (EPNs) and is highly pathogenic towards a wide range of insect larvae. The EPNs-*Photorhabdus* complex is already employed in agriculture as biocontrol agent. *P. luminescens* exists in two phenotypically different forms, the primary (1°) and the secondary (2°) cell variants, however only the 1° cells live in mutualistic symbiosis with EPNs. Once the nematodes invade insects and release *P. luminescens* into the hemocoel, the bacteria effectively kill the larvae. During the infective lifecycle up to 50% of 1° cells switch to the 2° phenotype. Since 2° cells cannot reassociate with EPNs they are left in soil when the insect cadaver is depleted. Both cell variants are believed to share identical genomes, but they differ in many phenotypic traits, which is referred to as phenotypic heterogeneity. However, the fate of 2° cells in the soil and therefore the biological reason for phenotypic heterogeneity is unclear. Moreover, the genetical identity of both cell variants has not been confirmed yet. For that purpose, this work focuses on the biological role of 2° cells in the rhizosphere.

First, to understand the regulation processes that are involved in phenotypic switching and to obtain a first idea for the fate of 2° cells a comparative transcriptome analysis of *P. luminescens* DJC 1° cells and 2° cells was performed. First of all, it could be proved that the different 1° and 2° specific phenotypes are regulated at transcriptional level. In fact, the respective genes coding for 1°-specific features like e.g., pigmentation, bioluminescence, clumping factors were downregulated in 2° cells. Furthermore, differently expressed genes (DEGs) coding for different LuxR solos were identified, indicating that a yet unknown circuit of cell-cell communication could exist in 2° cells. For example, the major regulator of quorum sensing (QS) in 1° cells, *pluR*, was downregulated in 2° cells, whereas genes encoding PAS4-LuxR-solos PluDJC_10415-PluDJC_10460 and two LuxR-solos with an undefined signal binding domain PluDJC_09555 and PluDJC_21150 were upregulated in 2° cells. This also points out a putative regulatory role of QS in *P. luminescens* phenotypic heterogeneity. Furthermore, DEGs involved in stress-response such as starvation related genes were upregulated, while genes involved in metabolism were differently modulated in 2° cells

indicating an adaptation to the nutrient-limited availability in the soil. Moreover, increased swimming and twitching motility as well as chemotaxis, which are essential for rhizosphere colonization, were observed for 2° cells. This supports the hypothesis of an alternative lifecycle of *P. luminescens* in the rhizosphere. Remarkably, 2° cells chemotactically responded to plant root exudates (PRE), showed increased biofilm formation, and specifically interacted with plant roots. Additionally, plant growth promoting ability of this cell variant could be determined. To further understand the adaptability of 2° cells to plant roots, a comparative transcriptome analysis was performed comparing 2° cells supplemented with and without PRE. Here, DEGs involved in e.g., biofilm formation, motility, or chitin degradation were identified to be upregulated in the presence of PRE. Two of the most upregulated genes were those encoding a putative chitin binding protein and a putative chitinase (Chi2A) suggesting the chitin degrading and therefore fungicidal activity of 2° cells in the soil. Indeed, 2° cells specifically inhibited growth of phytopathogenic *Fusarium graminearum* after physical contact. This ability was impaired in *P. luminescens* 2° Δ*chi2A* and Δ*cbp* deletion mutants. Furthermore, *in planta* assays using tomato plants infected with *F. graminearum* proved that 2° cells could protect the plant from infection and therefore promoted plant growth, which was not the case using *P. luminescens* 1° wildtype and the 2° Δ*chi2A* and Δ*cbp* deletion mutants. Moreover, effective chitin degradation was verified using purified Chi2A enzyme. This indicates a role of 2° cells in protecting plants from phytopathogens upon root colonization.

Moreover, a SdiA-like LuxR solo was identified as an essential player in interkingdom signaling (IKS) communication between plants and the bacteria. SdiA could play a role in the first steps of root colonization as a decreased motility and increased biofilm formation of *P. luminescens* 2° Δ*sidA* was observed compared to wildtype 2° cells. A plant-derived signaling molecule is assumed to be sensed by SdiA, which could lead to expression of genes important for the 2° cells-plant interaction. Furthermore, putative binding of long and short chain *N*-acyl homoserine lactones (AHLs) to SdiA were suggested, as different folding conformations occurred upon binding. Using surface plasmon resonance spectroscopy a direct and high affine interaction of purified SdiA to its own promoter as well as to the promoter of the gene adjacent to *sdiA*, *aidA*, could be demonstrated. This indicated a bidirectional transcriptional regulation of the intergenic *aidA*-*sdiA* promoter region. Furthermore, a putative role of AidA in microbe-host interaction and an accurate self-regulatory mechanism of SdiA could be assigned.

Lastly, to verify phenotypic heterogeneity in *P. luminescens* subs. *laumondii* DJC strain high-throughput sequencing data of the respective genomes were analyzed. With that the genetic similarity of both cell variants should be confirmed to exclude genotypic heterogeneity. Indeed, it could be confirmed that *P. luminescens* DJC 1° and 2° are genetical identical, and that large genome rearrangements are not involved in the switch from the 1° to the 2° phenotype.

In conclusion, the presented thesis gives direct evidence for an alternative lifestyle of *P. luminescens* 2° in the rhizosphere for the first time. The bacteria show a specific adaptation to plant roots protecting them from phytopathogenic fungi. Besides the biotechnological use of *P. luminescens* 1° cells as bioinsecticides, 2° cells could be used as plant growth promoting organism and as biopesticide for plant protection in the future.

Zusammenfassung

Pflanzennützliche Bodenbakterien gewinnen als Biofungizide und Bioinsektizide in der nachhaltigen Landwirtschaft zunehmend an Bedeutung, da viele Schädlinge und Krankheiten die Ernteerträge stark beeinträchtigen. *Photorhabdus luminescens* ist ein Gram-negatives Bakterium, das in Symbiose mit entomopathogenen Nematoden (EPN) lebt und für eine Vielzahl von Insektenlarven hoch pathogen ist. Der EPN-*Photorhabdus*-Komplex wird bereits in der Landwirtschaft als Bioinsektizid eingesetzt. *P. luminescens* existiert in zwei phänotypisch unterschiedlichen Formen, der primären (1°) und der sekundären (2°) Zellvariante, wobei nur die 1°-Zellen in Symbiose mit den EPN leben. Sobald die Nematoden in die Insekten eindringen und *P. luminescens* in das Hemocoel freisetzen, werden die Larven durch die Bakterien schnell und effizient abgetötet. Im Lebenszyklus wechseln während der Infektion bis zu 50 % der 1°-Zellen zum 2°-Phänotyp. Da 2°-Zellen nicht mit den EPN reassoziieren können, verbleiben sie im Boden, wenn die Nährstoffe im Insektenkadaver aufgebraucht sind. Es wird angenommen, dass beide phänotypisch unterschiedlichen Zellvarianten genetisch identisch sind, was als phänotypische Heterogenität bezeichnet wird. Über den Verbleib der 2°-Zellen im Boden und damit der biologische Hintergrund für die phänotypische Heterogenität ist nichts genaues bekannt. Außerdem ist die genetische Identität der beiden Zellvarianten noch nicht bestätigt worden. Aus diesem Grund fokussiert sich diese Arbeit auf die biologische Rolle der 2°-Zellen in der Rhizosphäre.

Um die Regulationsprozesse zu verstehen, die an dem phänotypischen Phasenwechsel beteiligt sind, und um Hinweise über das Schicksal der 2°-Zellen zu erhalten, wurde zunächst eine vergleichende Transkriptomanalyse von *P. luminescens* DJC 1°-Zellen und 2°-Zellen durchgeführt. Zunächst konnte nachgewiesen werden, dass die unterschiedlichen 1°- und 2°-spezifischen Phänotypen tatsächlich auf transkriptioneller Ebene reguliert werden. Die Expression der entsprechenden Gene, die für 1°-spezifische Merkmale wie z. B. Pigmentierung, Biolumineszenz und Verklumpungsfaktoren kodieren, war in 2°-Zellen herunterreguliert. Darüber hinaus wurden unterschiedlich exprimierte Gene (DEGs) identifiziert, die für LuxR-Solos kodieren. Dies deutet darauf hin, dass in 2°-Zellen eine noch unbekannte Art der Zell-Zell-Kommunikation existieren könnte. So wurde beispielsweise die Expression des wichtigsten Regulator-Gens des Quorum Sensing (QS) in 1°-Zellen, *pluR*, in 2°-Zellen

herunterreguliert. Hingegen war die Expression der Gene, die für die PAS4-LuxR-Solos PluDJC_10415-PluDJC_10460 und zwei LuxR-Solos mit einer nicht definierten Signalbindungsdomäne PluDJC_09555 und PluDJC_21150 kodieren, in 2°-Zellen hochreguliert. Dies weist ebenfalls auf eine mutmaßliche regulatorische Rolle von QS bei der phänotypischen Heterogenität von *P. luminescens* hin.

Darüber hinaus wurden DEGs, die bei der Stressantwort beteiligt sind, identifiziert. Darunter waren Gene, die mit Nährstoffmangel und dem Primärstoffwechsel in Verbindung stehen, in der Expression hoch- bzw. herunterreguliert, was auf eine Anpassung der Bakterien an die begrenzte Nährstoffverfügbarkeit im Boden hindeutet. Darüber hinaus wurden bei 2°-Zellen eine erhöhte Schwimm- und Zuckungsmotilität sowie Chemotaxis beobachtet, die für die Besiedlung der Rhizosphäre von essenzieller Bedeutung sind. Dies unterstützt die Hypothese eines bisher nicht bekannten Lebenszyklus von *P. luminescens* in der Rhizosphäre. Weiterhin reagierten die 2°-Zellen chemotaktisch auf Pflanzenwurzelexsudate (PRE), zeigten eine verstärkte Biofilmbildung und interagierten spezifisch mit Pflanzenwurzeln. Darüber hinaus konnte die Fähigkeit dieser Zellvariante zur Förderung des Pflanzenwachstums beobachtet werden. Um die Anpassungsfähigkeit von 2°-Zellen an Pflanzenwurzeln besser zu verstehen, wurde eine vergleichende Transkriptomanalyse durchgeführt, bei der 2°-Zellen mit und ohne PRE verglichen wurden. Dabei wurde festgestellt, dass die Expression von Genen, die z. B. an der Biofilmbildung, der Motilität oder dem Abbau von Chitin beteiligt sind, in Gegenwart von PRE hochreguliert waren. Zwei der am stärksten in der Expression induzierten Gene waren diejenigen, die für ein potenzielles Chitin-bindendes Protein (CBP) und eine potenzielle Chitinase (Chi2A) kodieren, was auf eine chitinolytische und damit fungizide Aktivität der 2°-Zellen im Boden schließen lässt. Tatsächlich hemmten 2°-Zellen nach physischem Kontakt spezifisch das Wachstum von phytopathogenen *Fusarium graminearum*. *In planta* Tests mit *F. graminearum* infizierten Tomatenpflanzen zeigten außerdem, dass 2°-Zellen die Pflanze vor einer Infektion mit dem Pilz schützen konnten und somit das Pflanzenwachstum förderten. Diese Fähigkeit war bei *P. luminescens* 2° Δ*chi2A*- und Δ*cbp*-Deletionsmutanten beeinträchtigt. Eine chitinolytische Aktivität von Chi2A wurde mit gereinigtem Enzym bestätigt. Insgesamt deuteten diese Experimente auf eine wichtige Rolle der 2°-Zellen beim Schutz der Pflanzen vor Phytopathogenen bei der Wurzelbesiedlung hin.

In einem weiteren Teil der Arbeit wurde das *Interkingdom*-Signaling (IKS) zwischen *P. luminescens* und der Pflanze untersucht. Dazu wurde ein SdiA-ähnlicher LuxR-Solo als wesentlicher Rezeptor im IKS zwischen Pflanzen und den Bakterien identifiziert. SdiA könnte eine Rolle bei den ersten Schritten der Wurzelbesiedlung spielen, da eine verringerte Motilität und eine erhöhte Biofilmbildung von *P. luminescens* 2° Δ*sidA* im Vergleich zu Wildtyp 2°-Zellen beobachtet wurde. Es wird angenommen, dass ein von der Pflanze stammendes Signalmolekül von SdiA wahrgenommen wird, was zur Expression von Genen führen könnte, die für die Interaktion zwischen 2°-Zellen und Pflanze wichtig sind. Darüber hinaus wurde eine mögliche Bindung von lang- und kurzkettigen *N*-Acylhomoserinlaktonen (AHLs) an SdiA vermutet, da bei der Bindung dieser Moleküle unterschiedliche Thermostabilitäten des Proteins beobachtet wurden. Mittels Oberflächenplasmonenresonanzspektroskopie konnte eine direkte und hochaffine Wechselwirkung von gereinigtem SdiA mit seinem eigenen Promotor sowie mit dem Promotor des Nachbargens von *sdiA*, *aidA*, nachgewiesen werden. Dies deutete auf eine bidirektionale Transkriptionsregulation der intergenen *aidA*-*sdiA*-Promotorregion hin. Darüber hinaus konnte eine mutmaßliche Beteiligung von AidA bei der Interaktion zwischen Bakterium und dem Pflanzenwirt sowie ein genauer Selbstregulierungsmechanismus von SdiA nachgewiesen werden.

Zuletzt wurden zur Überprüfung der phänotypischen Heterogenität in *P. luminescens* subsp. *laumondii* DJC-Stamm Hochdurchsatz-Sequenzierungsdaten der jeweiligen Genome analysiert. Damit sollte die genetische Ähnlichkeit beider Zellvarianten bestätigen werden, um genotypische Heterogenität auszuschließen. In der Tat konnte bewiesen werden, dass *P. luminescens* DJC 1° und 2° genetisch identisch sind und dass große Genom-Umlagerungen nicht am phänotypischen Phasenwechsel vom 1°- zum 2°-Phänotyp beteiligt sind.

Zusammenfassend zeigt die vorliegende Arbeit zum ersten Mal einen direkten Beweis für eine neue Lebensweise von *P. luminescens* 2° in der Rhizosphäre. Die Bakterien besiedeln spezifisch Pflanzenwurzeln im Boden und schützen ihren neuen Wirt vor einer Infektion mit phytopathogenen Pilzen. Neben der biotechnologischen Nutzung von *P. luminescens* 1°-Zellen als Bioinsektizid könnten 2°-Zellen daher in Zukunft als effiziente pflanzenwachstumsfördernde Mikroorganismen sowie als Biopestizide im Pflanzenschutz eingesetzt werden.

1. Introduction

1.1 The genus *Photorhabdus*

"The black death": designation of a pandemic where humans experienced death via septicemia or plaque caused by Gram-negative *Yersinia pestis*. In contrast to *Yersinia*, close related insect pathogenic bacteria are responsible for a so called "bright death". In 1977 Khan and Brooks reported for the first time the appearance of a Gram-negative chromogenic bioluminescent bacterium associated with entomopathogenic nematodes (EPNs). Initially these bacteria were assigned to the genus of *Xenorhabdus* and were therefore named *Xenorhabdus luminescens* (Poinar et al., 1977). However, based on phenotypic and genotypic differences, in 1993 the creation of a new genus *Photorhabdus* was suggested and therefore the bacteria were later renamed as *Photorhabdus luminescens* (Boemare et al., 1993; Fischer-Le Saux et al., 1999). Bacteria from the genus *Photorhabdus* live in a close mutualistic symbiosis with *Heterorhabditidiae* EPNs and are highly pathogenic towards insects (Akhurst, 1980; Akhurst and Boemare, 1988). *P. luminescens* (Fischer-Le Saux et al., 1999), *P. temperata* (Tailliez et al., 2010), and *P. asymbiotica* (Wilkinson et al., 2009) were described as representative strains of *Photorhabdus spec*. Later, *P. asymbiotica* also occurred to be pathogenic against humans causing soft tissue skin infections (Gerrard et al., 2004, 2006). Moreover, in the last years new species and subspecies of the taxonomy of *Photorhabdus* were revised upon whole-genome sequencing (Machado et al., 2018). *P. luminescens* subs. *laumondii* TT01 is a well characterized and commonly used strain in research, but in the last decades a spontaneously mutated rifampicin resistant TT01 strain emerged and was designated as TT01^{Rif} (Bennett and Clarke, 2005). Both TT01 strains differ in their phenotypic traits, and their genomes display dissimilarities, for which reason, *P. luminescens* subs. *laumondii* TT01^{Rif} was renamed to *P. luminescens* subs. *laumondii* DJC to clearly distinguish both subspecies (Bager et al., 2016; Engel et al., 2017; Langer et al., 2017; Zamora-Lagos et al., 2018), and at the same time *P. luminescens* subs. *laumondii* was suggested to be renamed as *P. laumondii* (Machado et al., 2018).

1.1.1 The lifecycle of *P. luminescens*

P. luminescens [*P. laumondii*] undergoes a dualistic life cycle. The bacteria enter a mutualistic symbiosis with *Heterorahabditidae* EPNs colonizing the upper gut of infective juveniles (IJs) (Forst et al., 1997). In the soil the nematodes actively search for an insect prey invading susceptible larvae of, e.g. the great wax moth *Galleria mellonella*. Once inside the larvae, the nematodes regurgitate *P. luminescens* into the haemocoel of the prey, where *P. luminescens* enters the pathogenic lifestyle. The bacteria start producing a wide range of toxins, e.g. insecticidal toxin complexes (Tc) and the "makes caterpillar floppy" (Mcf) toxin as major pathogenicity factors, leading to oral toxicity, apoptosis of epithelial cells and septicemia, respectively. The larvae are thereby effectively killed within 48 h (Waterfield et al., 2001; Daborn et al., 2002; Watson et al., 2005). Successively, *P. luminescens* cells protect themselves and the insect cadaver from other microorganisms via the production of antimicrobial substances, such as the β-lactam antibiotic carbapenem, or 3,5-dihydroxy-4-isopropylstilbene (IPS), both with high biocidal activity against several microbes (Derzelle et al., 2002). Additionally, luciferase activity of *P. luminescens* leads to bioluminescence causing glowing of the insect cadaver, the "bright death" (Forst et al., 1997; Daborn et al., 2001). Furthermore, the bacteria produce and secret several exoenzymes to degrade the insect cadavers' tissue providing nutrients for both themselves and the nematodes. At this point of the lifecycle, the bacteria change to symbiotic behavior, providing essential nutrients and secondary metabolites that support nematode development (Han and Ehlers, 2001). Once the nutrients of the insect carcass are depleted, nonfeeding infective juveniles (IJs) of nematodes emerge and reassociate with *P. luminescens,* thus leaving the carcass and searching for a new prey (ffrench-Constant et al., 2003) **(Fig. 1-1)**.

Also strains of *P. temperata* and *P. asymbiotica* undergo a similar lifecycle. However, as *P. asymbiotica* emerged to be additionally pathogenic towards humans, here infection occurs via invading human skin, where nematodes release *P. asymbiotica,* which then causes local infection often associated with minor skin traumata (Gerrard et al., 2004, 2006).

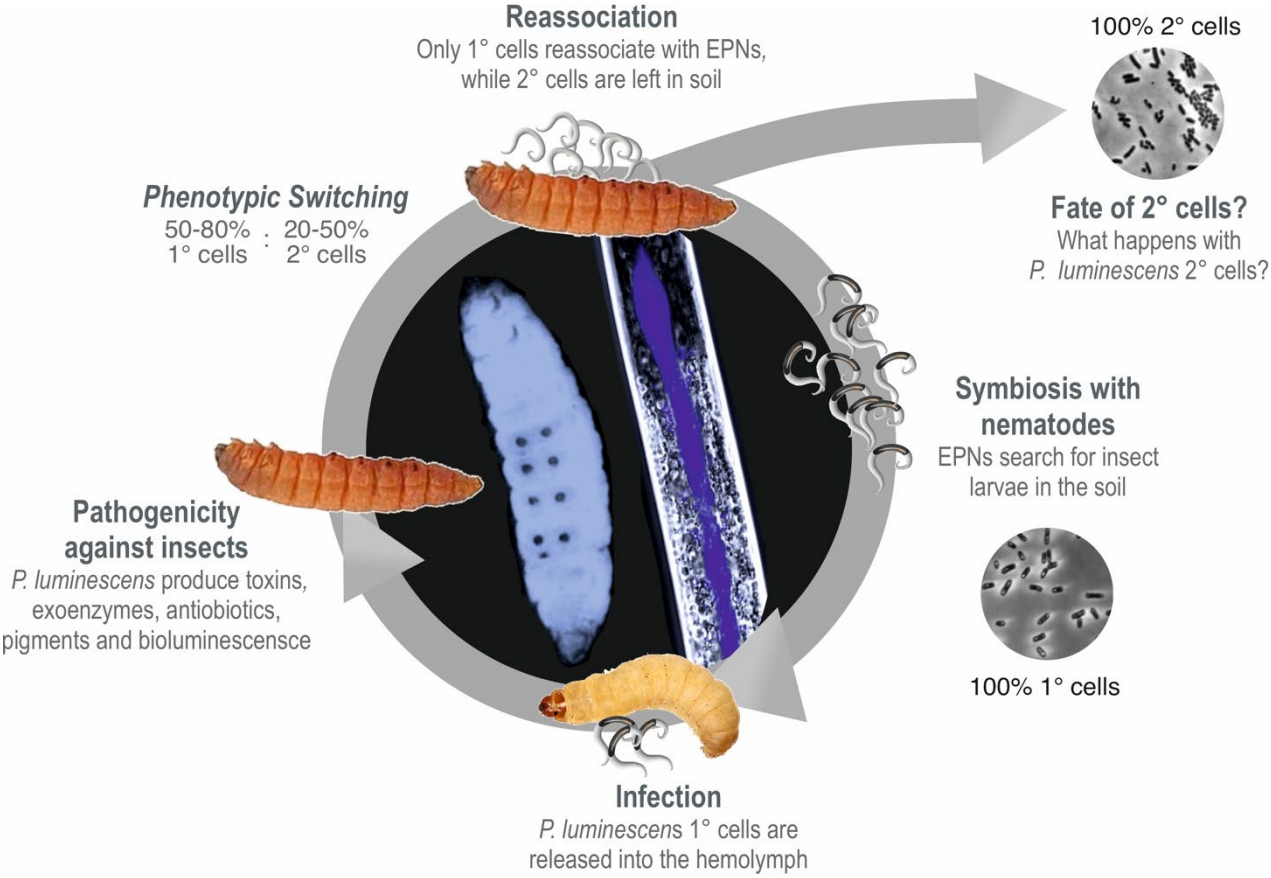

Figure 1-1: Lifecycle of *Photorhabdus luminescens*. i) *P. luminescens* 1° cells colonize the upper gut of entomopathogenic nematodes (EPNs). ii) EPNs-*P. luminescens* 1° search for and invade insect prey in the soil. iii) *P. luminescens* cells are regurgitated into the insects' hemolymph, proliferating, and producing toxins thus overcoming the larva immune system defenses and killing it. The bacteria further produce bioluminescence, pigments, and antibiotics. iv) *P. luminescens produce* exoenzymes thus bioconverting the cadaver into nutrients used by themselves and the ENPs. v) At this point of the infective lifecycle, 20-50% of *P. luminescens* switch into the 2° cell variant. Once the nutrients of the cadaver are depleted only 1° ells reassociate with EPNs and emerge from the insect cadaver, whereas 2° cells are left in soil with an unknown fate.

1.2 Bacterial phenotypic heterogeneity

Bacteria need to cope with constantly occurring environmental stresses and to better adapt to them they evolved different strategies like DNA methylation or genome rearrangements (Smits et al., 2006). Moreover, non-genetic strategies like the appearance of different phenotypic cells within a genetic homogenous cell population is another tactic bacteria evolved, which is designated as phenotypic heterogeneity (Avery, 2006; Davidson and Surette, 2008; Grote et al., 2015). Under evolutionary pressure single cells display different phenotypic traits resulting in phase variations with a major beneficial fitness; hence, this phase variation mostly correlates with

altering gene expression (Elowitz et al., 2002; van der Woude, 2011; Davis and Isberg, 2016). This phenomenon of heterogeneity is found in different behaviors within bacterial populations, such as biofilm formation, DNA uptake, motility, bacterial competence, sporulation, and antibiotic resistance in Gram-positive and -negative bacteria. In some cases, bacterial phenotypic heterogeneity was observed to be under control of quorum sensing (QS) (Grote et al., 2015). QS-based bioluminescence of *Vibrio harveyi* was the first heterogenous QS-response observed (Anetzberger et al., 2009; Pérez and Hagen, 2010). QS describes bacterial communication at high cell density, however, different studies reveled for different bacteria like *Pseudomonas, Vibrio* and *Xanthomonas,* QS-reversible non-genetic phenotypic heterogeneity responses whereupon two distinct sub-population evolved: i) the QS-responsive and ii) QS-non-responsive cells (Anetzberger et al., 2009; Pradhan and Chatterjee, 2014; Cárcamo-Oyarce et al., 2015; Bauer et al., 2017). However, little is known about the molecular dynamics between QS and phenotypic heterogeneity, especially during host-pathogen interaction. Recent studies suggested a transition from heterogeneity to homogeneity upon QS-response when *Xanthomonas campestris* population was involved in virulence towards the plant host (Samal and Chatterjee, 2019). One strategy following phenotypic heterogeneity is the so-called bet-hedging, where single individuals display an increased fitness, resulting in an adaptation to environmental changings. Therefore, bet-hedging is hypothesized to be advantageous in harsh and unpredictable environments (Cohen, 1966; Veening et al., 2008; Olofsson et al., 2009) and it is also found in bacteria. A well-studied example is the occurrence of persister cells (dormant variants of vital cells), where a very small portion of induvial cells in a population can persist against antibiotic activity (Helaine and Kugelberg, 2014). Another example is the sporulation of *Bacillus subtilis*, where some of the bacterial cells start to undergo sporulation upon nutrient limitation in order to survive starvation (Veening et al., 2008). Interestingly, also for *P. luminescens* bet-hedging was described to be important during the lifecycle: an antimicrobial peptide-resistant subpopulation of *P. luminescens* was described to be responsible for virulence (Mouammine et al., 2017). Generally, phenotypic heterogeneity occurs in *P. luminescens* and is important for the lifecycle of the bacteria.

1.3 Phenotypic heterogeneity in *P. luminescens*

During the dualistic life cycle of *P. luminescens* two phenotypic cell variants occur: the pigmented primary (1°) cells and the non-pigmented secondary (2°) cells. Both are suggested to be genetically similar, however, they differ in many phenotypic traits. Differences can be found in cell morphology, since 1° cells are long rod-shaped, and 2° cells are smaller rod-shaped (Wang et al 2006). Further prominent differences in phenotypic traits are: i) 1° cells exhibit strong bioluminescence and red pigmentation, ii) they produce many different secondary metabolites and antibiotics, and iii) they produce crystalline inclusion proteins (CipA and CipB) and the *Photorhabdus* cell clumping factor (PCF); all these traits are absent from 2° cells (Akhurst, 1980; Akhurst and Boemare, 1988; Richardson et al., 1988; You et al., 2005; Langer et al., 2017). Interestingly, both cell variants are equally pathogenic towards insects, however, at the beginning of the life cycle only 1° cells exist. During the infective part of the life cycle some 1° cells start to switch into the 2° cell variant, and once all the nutrients are depleted up to 50% of 1° cells switch to the 2° phenotype. As only 1° cells support nematode growth and are able to reassociate with their symbiosis partner, 2 ° cells are left in soil after the infective cycle (Han and Ehlers, 2001) **(Fig. 1-1)**.

So far, phenotypic switching of *P. luminescens* seems to occur only from 1° to 2° cells, as a switch back from 2° to 1° was never observed. The phenotypic switch from 1° to 2° does not only occur in infected insects but also after prolonged cultivation suggesting nutrient limitation or global stress as major signal for the switching process (Joyce et al., 2006). However, the reasons why only a portion of the 1° cell population switches to the 2° cell form and therefore how phenotypic switching is regulated at single cell level is not fully understood.

There are still several open questions regarding the phenotypic heterogeneous lifestyle of *P. luminescens.* Until now, there is no evidence whether both cell variants are truly genetically identical. Furthermore, light on the sociobiological aspects of the co-existence of both cell variants has still to be shed, to understand when and why 1° cells switch to the 2° cell variant and to characterize the fate of 2° cells once they emerge during the lifecycle and left in soil. Therefore, these questions are addressed in the present thesis.

1.3.1 Regulation of phenotypic switching in *P. luminescens*

Different phenotypic traits in a genetically homogenous cell population must be tightly controlled, especially when a population probably “irreversibly” switches. This population switch can occur after the infective life cycle as well as after prolonged cultivation in the laboratory, therefore this process must be strictly regulated to prevent a break-down of the insect pathogenic life cycle of *P. luminescens*. Several regulators have already been identified that play a major role in controlling the switching process. These are the master regulator HexA, the two-component system AstS/AstR and the XRE-like transcriptional regulators XreR1 and XreR2 that are mainly involved in controlling expression of different *P. luminescens* phenotypic traits (Joyce and Clarke, 2003; Joyce et al., 2006; Langer et al., 2017; Eckstein et al., 2021).

HexA - a LysR-type transcriptional regulator (LTTR) consisting of an N-terminal helix-turn-helix (HTH) DNA-binding domain and a LysR substrate-binding domain (Maddocks and Oyston, 2008) - is a versatile regulator controlling phenotypic heterogeneity in *P. luminescens* (Joyce and Clarke, 2003; Langer et al., 2017). 1°-specific traits are downregulated by HexA in *P. luminescens*. Overexpression of *hexA* in 1° cells led to the 2°-specific phenotype, while deletion of *hexA* in 2° cells led to the 1°-specific phenotype (Joyce and Clarke, 2003; Langer et al., 2017). 2° cells showed increased amounts of HexA and is therefore believed to act as repressor of 1°-specific genes. HexA has been demonstrated to directly interact with the promoter region of the *pcfABCDEF* operon and thereby repressing *P. luminescens* cell clumping factor (PCF) production, usually highly upregulated in 1° cells upon high cell density (Langer et al., 2017; Eckstein and Heermann, 2019). Although HexA binding to further promotor regions of genes related to other 1°-specific traits has not been shown yet, the *hexA* deletion mutant displayed impaired traits such as bioluminescence suggesting that the respective *luxCDABE* operon is repressed at post-transcriptional level (Langer et al., 2017). Further studies revealed a posttranscriptional regulation of HexA by a Hfq dependent regulatory small RNA (sRNA), ArcZ, that was discovered to directly basepair to the HexA-mRNA, thus repressing metabolite production and probably 1° cell specific traits (Neubacher et al., 2020). In this perspective, HexA directly and indirectly acts as master regulator of 1° cell specific phenotype. However, neither a putative substrate signal that binds to HexA nor the complete molecular regulatory mechanism of HexA are known to date.

The two-component system AstS/AstR was described as timer of phenotypic switching in *P. luminescens* and was found to be homologous to a two-component system found in *E. coli*. Indeed, *P. luminescens* mutant cells lacking the respective genes, switched several days before the wildtype strain, revealing that AstS/AstR is involved in stress response regulation, motility, and antibiotic production and controls timing of the switching process (Derzelle et al., 2004). However, the exact signal that is sensed by the histidine kinase AstS is unknown.

Furthermore, the phenotypic switching was shown to be controlled by xenobiotic response elements (XRE) (Eckstein et al., 2021). XRE-type regulators are one of the most frequently occurring regulators in bacteria and suspected to be activated by environmental signals (Bai et al., 1993; Fisher and Wray, 2002; Barragán et al., 2005). *P. luminescens* harbors in total 27 putative XRE-like regulators. For two of these, XreR1 and XreR2 a regulatory role in the control of phenotypic switching of *P. luminescens* was demonstrated (Eckstein et al., 2021). Both XreR1 and XreR2 harbor a highly conserved HTH domain similar to the λ phage Cro/C1 repressor within the N-terminal region (Hsiang et al., 1977; Sauer et al., 1982; Barragán et al., 2005), responsible for DNA binding (Eckstein et al., 2021; Aggarwal et al.). The C-terminal region instead harbors a regulatory domain that is variable (Kulinska et al., 2008). The transcriptional analysis comparing the transcriptomes of 1° and 2° cells spotlighted *xreR1* as upregulated in 1° cells and *xreR2* in 2° cells. Deletion of *xreR1* in 1° cells and *xreR2* in 2° cells as well as insertion of extra copies of *xreR2* in 1° cells and *xreR1* in 2° cells led to the opposite phenotype in the respective cell form, thus playing an important regulatory role in phenotypic switching of *P. luminescens*. Furthermore, XreR1 represses the expression of *xreR2*, while XreR2 seems to indirectly induce its own gene expression by binding to XreR1 (Eckstein et al., 2021) (**Fig. 1-2**). The exact regulatory mechanism of these regulators and how they are involved in phenotypic switching is still under study.

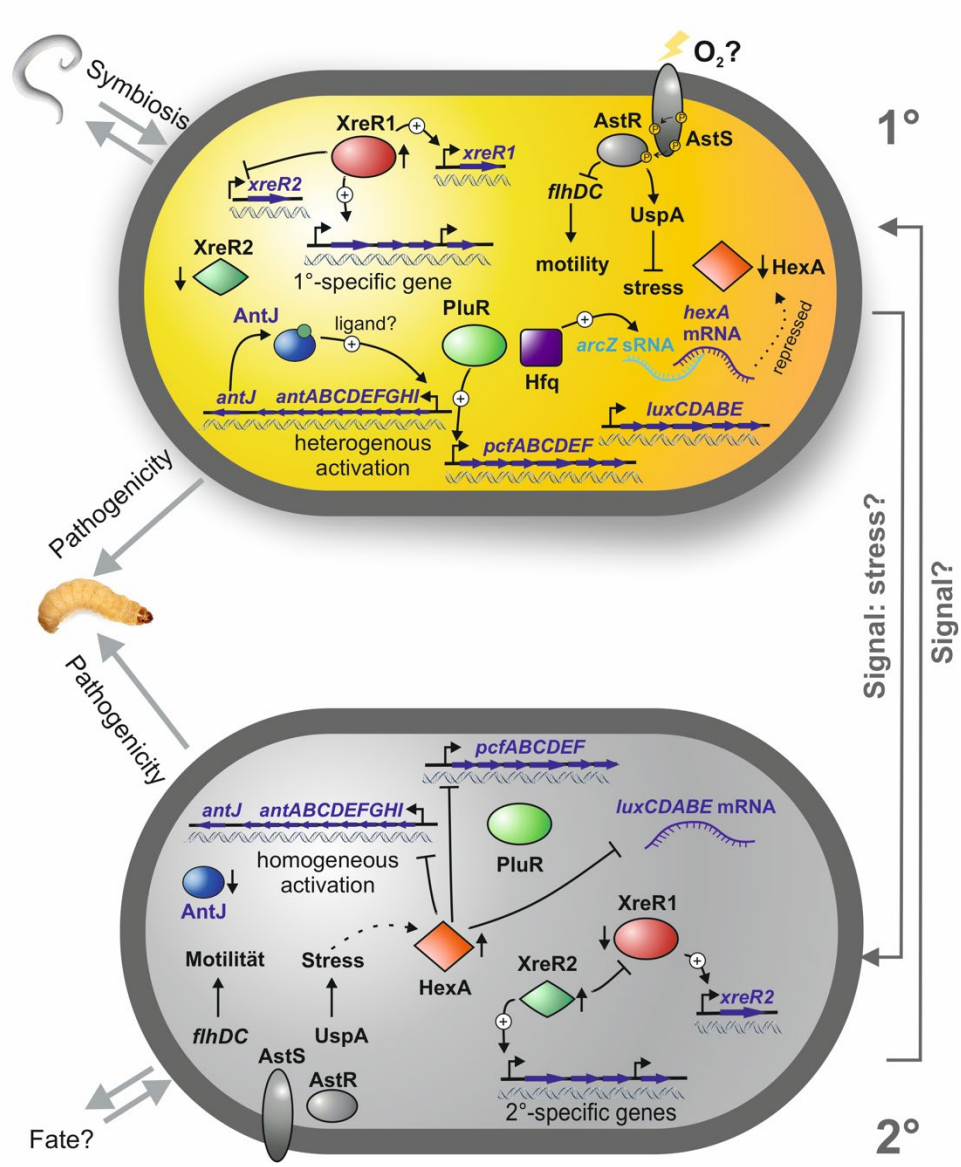

Figure 1-2: Regulation of phenotypic heterogeneity in *P. luminescens*. The regulatory mechanism of phenotypic switching from 1° to 2° is a complex interaction between different regulators. HexA (orange) is a versatile master regulator playing an essential role in this process, as it is highly present in 2° cells (grey cell) and inactivates 1°-specific genes coding for bioluminescence (*luxCDABE)*, pigmentation (*antABCDEFGHI*), cell clumping (*pcfABCDEF*) and nematode interaction. In 1° cells instead, *hexA* is posttranscriptionally regulated by an Hfq dependent small RNA, ArcZ, that directly basepairs to mRNA encoding HexA. Moreover, XRE-regulators play an essential role in the switch from 1° to 2°. In 1° cells, XreR1 (red) is highly produced and activates 1°-specific genes, which in turn is repressed in 2° cells by XreR2 (green). Subsequently, in 2° cells, 2°-specific genes are activated. Lastly, AstS/AstR sensor kinase/response regulator system is involved in timing of the switching process. This figure was modified after (Eckstein and Heermann, 2019).

1.3.2 Different phenotypic traits of *P. luminescens* 1° and 2° cells

Phenotypic heterogeneity implies different phenotypic appearances and behaviors in a genetically homogenous cell population. For *P. luminescens*, among the most predominant and visible differences are the production of secondary metabolites including pigmentation and the bioluminescence resulting from luciferase reaction encoded by *luxCDABE* in 1° cells (**Fig. 1-3**). In general, 1° cells produce more secondary metabolites than the 2° cells (Clarke, 2016), like anthraquinones (AQ), which are responsible for the red pigmentation of 1° cells (Richardson et al., 1988). AQ biosynthesis is driven by a type II polyketide synthase acting together with several further enzymes encoded by *antABCDEFGHI* operon (Brachmann et al., 2007). *P. luminescens* is the only yet known Gram-negative bacterium producing AQ, since AQ production has only been described in fungi, plants and streptomyces before. The *antABCDEFGHI* operon is positively regulated by AntJ, a ligand-dependent activator harboring an HTH-domain. However, the signal molecule that binds and modulates AntJ is yet unidentified but is putatively only present in 1° cells (Heinrich et al., 2016). Additionally, among many different antibiotics, *P. luminescens* 1° cells produce polyketide stilbenes (usually only found in plants), which are synthesized by phenylalanine ammonia lyase StlA (Derzelle et al., 2002; Williams et al., 2005; Joyce et al., 2008). Stilbenes play an important role in many stages of the *P. luminescens* 1° cells' life cycle: it acts against fungi and Gram-positive bacteria, it supports the bacteria to overcome the immune system of insects and lastly it is important for nematode development, a trait only found in 1° cells (Eleftherianos et al., 2007; Joyce et al., 2008). Furthermore, crystal inclusion bodies CipA and CipB, only found in 1° cells, were reported to be involved in nematodes' development, as 1° cells lacking the single respective genes were not able to support nematodes growth (Bintrim and Ensign, 1998; You et al., 2005).

Another 1° cell specific trait lacking in 2° cells is the *Photorhabdus* clumping factor (PCF), which mediates cell clumping, a virulence factor contributing to *P. luminescens* higher pathogenicity (Brachmann et al., 2013).

For *P. luminescens* 1° cells also the production of exoenzymes was described to be enhanced compared to 2° cells (Joyce and Clarke, 2003). So far, phenotypic traits were only described for 1° cells, but not for 2° cells. For better understanding the full lifecycle of *P. luminescens* and the role of 2° cells in the soil, it is of great importance to determine 2°-cell specific traits.

Phenotype	1° cells	2° cells
Bioluminescence	+++	+
Cell clumping (PCF)	+	-
Protease production	+++	?
Pigmentation	+++	-
Crystal proteins	+	-
Pathogenicity	+++	+++
Symbiosis	+++	-

Figure 1-3: Phenotypic differences between *P. luminescens* 1° and 2°. Both cell variants display different phenotypes. Among all depicted 1°-specific phenotypes, 2° cells lack almost all of them with some exceptions. 2° cells are only slightly bioluminescent, and they are equally pathogenic towards insect larvae. Protease ability of 2° cells was not determined, yet. The table was modified after (ffrench-Constant et al., 2003; Langer et al., 2017).

1.3.3 The role of *P. luminescens* 2° cells

The mutualistic symbiosis with EPNs and the pathogenic part of *P. luminescens* towards insects is well investigated. However, this part of the life cycle only fits the 1° cell variant. After nutrient depletion of insect cadaver, a portion of 1° cells switch to the 2° cell form which lack symbiosis with EPNs and do not re-enter the life cycle (Han and Ehlers, 2001) (**Fig. 1-1**). Therefore, it was assumed, that 2° cells adapt to a free lifestyle in soil (Smigielski et al., 1994). 2° cells are better suited to survive altering nutrient availability, as they adapt faster to nutrient addition after a period of starvation compared to 1° cells (Smigielski et al., 1994), a trait that is essential to live in a soil environment with nutrient limitation. Indeed, a proteome analysis revealed enzymes involved in metabolisms, such as respiratory enzymes, and the transmembrane proton motive force to be upregulated in 2° cells further proposing their better adaptability to a soil lifestyle (Smigielski et al., 1994; Turlin et al., 2006). Nevertheless, the mechanisms of 2° cells soil adaptation and interaction with other soil-living organisms

are still unclear. The role of 2° cells in the soil is therefore investigated in the present thesis.

1.4 Quorum sensing and interkingdom signaling via LuxR-type receptors

Bacteria can colonize a plethora of environments, like soil, water, plants, animals, and humans. They must perceive different environments and hosts and quickly adapt their behavior to changing environmental conditions or when changing hosts. Furthermore, bacteria do not act as 'loner' but they are mostly organized as communities: they communicate with each other using small diffusible signaling molecules, a process that is designated as quorum sensing (QS) (Nealson' and Hastings, 1979). These signaling molecules are produced in very low amounts and secreted into the environment. Concomitant with increasing cell count also the concentration of the signaling molecules increase until reaching a certain concentration. Once the minimal threshold (called quorum) for signal detection is reached, the signaling molecule(s) bind(s) to their respective cognate receptor, which activates or represses the expression of different genes (Ng and Bassler, 2009). QS based communication is widespread among bacteria and many biological processes such as virulence, biofilm formation, motility, metabolite production, bioluminescence, and sporulation are strongly regulated by this process (Waters and Bassler, 2005). QS differs between Gram-positive and Gram-negative bacteria. Gram-positive bacteria use peptide-derived signaling molecules, that are usually actively secreted out of the cell via export systems and sensed by typical bacterial two-component systems. In contrast, Gram-negative bacteria typically use fatty acid derived *N*-acyl homoserine lactones (AHLs) for communication, and the AHLs are sensed by a receptor of the so-called LuxR-family (Waters and Bassler, 2005). However, recent studies revealed members of the Gram-positive bacteria to produce acylated signaling molecules as well (Biswa and Doble, 2013; Rajput and Kumar, 2017).

1.4.1 The canonical QS communication of Gram-negative bacteria

Nelson and Hastings described for the first-time communication in bacteria through studying the bioluminescence mechanisms of marine bacterium *Vibrio fischeri* and pointing out that it was mediated by the LuxI/LuxR QS-based system. Detailly, a small

diffusible signaling molecule C_6-AHL is produced by the AHL-synthase LuxI and sensed by the receptor LuxR. After reaching the threshold concentration, C_6-AHL binds to LuxR, which then regulates the expression of the luciferase biosynthesis genes resulting in bioluminescence (Nealson' and Hastings, 1979). LuxI synthases constantly produce low levels of hydrophobic AHLs, that can easily pass bacterial membrane into the environment (Fuqua et al., 1996, 2001; Waters and Bassler, 2005). The length of the acyl moieties of the AHLs synthesized by LuxI varies between 4 and 18 carbon residues. Additionally, acyl chain of AHLs can carry a carbonyl, hydroxyl, or methylene group, thus increasing the LuxR-type receptors recognition (Whitehead et al., 2001; Kim et al., 2014). These receptors consist of two domains: i) The N-terminal signal binding domain (SBD) perceiving and binding the AHLs-like signal molecule with high specificity and ii) the C-terminal DNA-binding domain (DBD) with a helix-turn-helix motif (HTH LUXR) (**Fig. 1-5**) acting as transcriptional regulator thus modulating gene expression (Choi and Greenberg, 1991; Hanzelka and Greenberg, 1995). LuxR receptors undergo conformational changes upon AHL binding that lead to binding on its target promoters thus controlling the respective gene expression (Waters and Bassler, 2005). Furthermore, transcription of *luxI* is positively regulated by cognate LuxR upon AHLs binding, enhancing AHL production thus designating these molecules as autoinducer (Fuqua et al., 1994) (**Fig.1-4A**).

This LuxI/LuxR cell-cell communication of *V. fischeri* represents a prototype that is widespread among Gram-negative bacteria (Nasser and Reverchon, 2007). Even though all known LuxR-type receptors only 25% homology, nine amino acids were described to be highly conserved in either SBD or DBD of at least 95% of LuxR-type proteins (Fuqua et al., 1996; Whitehead et al., 2001; Zhang et al., 2002; Patankar and González, 2009). Bacteria can be "multilingual" by harboring several LuxI/LuxR QS systems. For nosocomial *Pseudomonas aeruginosa* two AHL-QS systems are well described, e.g., virulence traits are controlled by the LasI/LasR (via 3-oxo-C_{12}-AHL) and RhlI/RhlR (via C_4-AHL) QS systems (Pearson et al., 2000; Miller and Bassler, 2001). Additionally, *P. aeruginosa* harbors a third QS system depended on a quinolone signal (PQS) which is synthesized by PqsABCD and sensed by LysR-receptor PqsR (Gallagher et al., 2002). There are several LuxI/LuxR homologs found in proteobacteria involved in virulence regulation, like CviI/CviR of *Chromobacterium violaceum* (Swem et al., 2009; Chen et al., 2011) or YenI/YenR of *Yersinia enterocolica* (Ng et al., 2018) and so forth. Interestingly, QS systems can also be found encoded on plasmids, i.e.,

the *Agrobacterium tumefaciens* TraI/TraR QS system, that regulates plant host infection, which is encoded on the tumor-inducing (Ti) plasmid (Fuqua and Winans, 1994). However, many proteobacteria harbor LuxR type receptors, lacking a cognate LuxI synthase, thus such LuxR homologs are designated as LuxR orphans or solos (Patankar and González, 2009; Subramoni and Venturi, 2009) (**Fig. 1-4B**). *P. aeruginosa* for example harbors also a third LuxR-type receptor, QscR, which lacks a cognate LuxI-type synthase, but senses the 3-oxo-C_{12}-AHL (produced by LasI) regulating further virulence genes (Lee et al., 2006; Subramoni and Venturi, 2009). *P. luminescens* counts so far 40 LuxR-type receptors lacking a cognate LuxI synthase (Brameyer et al., 2014) and they could be involved in different processes during the lifecycle of *P. luminescens* helping the bacteria to cope in different host environments.

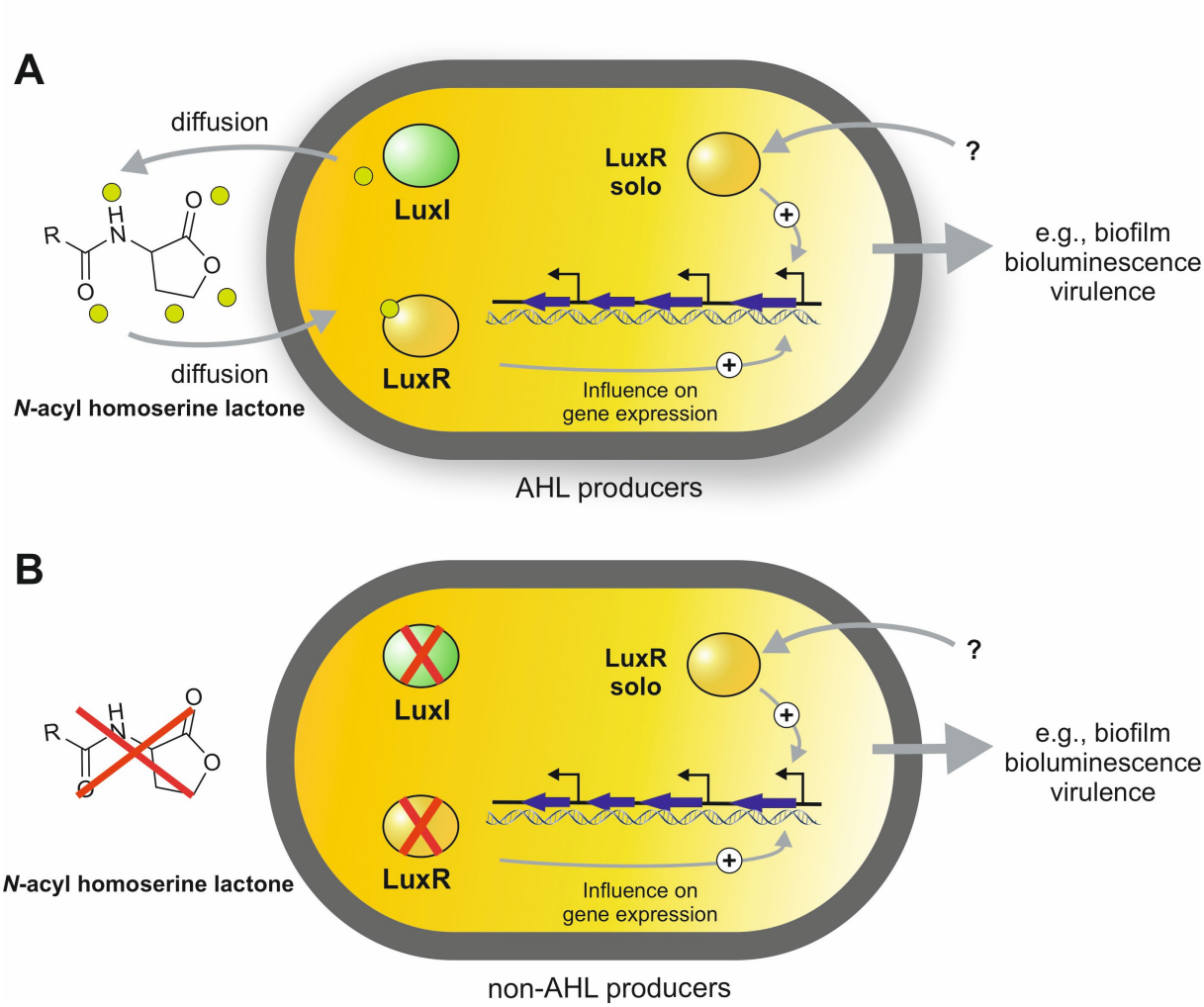

Figure 1-4: The canonical LuxI/LuxR quorum sensing (QS) system in Gram-negative bacteria. (A) *N*-acyl homoserine lactones (AHLs) are small diffusible molecules that are constantly produced at low basal level by AHL-synthase LuxI. After exceeding a certain concentration threshold, cognate LuxR-type receptors recognize the AHL and subsequently acts as transcriptional regulator modulating the expression of different target genes influencing the behavior of the bacterial population dependent on cell count. However, LuxR-type receptors can occur without a cognate LuxI and are designated as LuxR

solos. They can be found in either AHL producing and (B) non-AHL producing bacteria sensing AHLs from the environment (modified after: Brameyer and Heermann 2015).

1.4.2 LuxR solos in cell-cell communication and interkingdom signaling

Studying QS-based communication of bacteria led to identification of many LuxI/LuxR homologous in Gram-negative bacteria. Usually, the respective genes are found in proximity on the genomes. However, the presence of additional LuxR-type receptors lacking a cognate LuxI synthase became more apparent as more and more genomes were analyzed (Patankar and González, 2009; Subramoni and Venturi, 2009). LuxR solos can be found in AHL producing and non-producing bacteria. In AHL producing bacteria these additional receptors extend the regulatory network targeting further genes as they sense either exogenous or endogenous AHLs as it was described for QscR of *P. aeruginosa* (Lee et al., 2006). Interestingly, the QS systems of *P. aeruginosa* are strongly regulated in a hierarchical cascade, where i.e., QscR also influences LasR, a LuxR strongly inducing virulence. QscR also responds to 3-oxo-C_{12} AHL produced by LasI thereby infouencing LasR activity (Lee et al., 2006). Since deletion of *qscR* leads to hypervirulence it was suggested that QscR acts as QS antagonist of LasR and RhlR (Chugani et al., 2001; Fuqua, 2006). LuxR solos found in non-AHL producing bacteria were suggested to either sense exogenous AHLs, hormone-like signals produced by eukaryotes or both. Subsequently, bacteria sense and communicate with neighboring bacteria or recognize their eukaryotic hosts or habitat and consequently adapt their behavior (Subramoni and Venturi, 2009). A common LuxR solo present in non-AHL producing bacteria is the transcriptional regulator SdiA found in enteric bacteria like *Escherichia* and *Salmonella*. For SdiA of *E. coli* transcriptional activation of *ftsQAZ,* an operon coding for cell division proteins, was described and was therefore designated as ‘suppressor of cell division inhibitor’ (Wang et al., 1991). Furthermore, all SdiA homologues harbor an AHL signal binding domain and therefore detect exogenic AHLs produced by neighboring bacteria enabling interbacterial communication (Michael et al., 2001). Upon AHL binding, SdiA regulates expression of several genes involved in metabolism, motility, virulence, and different survival mechanism (Kim et al., 2014). Docking analysis revealed higher binding affinity of long chain C_{12}-AHLs to SdiA compared to AHLs with smaller side chains (Almeida et al., 2016), and similar LuxRs were found in plant associated bacteria like *Kosakonia,* designated as LoxR, which binds AHLs (Mosquito et al.,

2020). QS based communication is not only restricted to bacterial cell-to-cell communication, but it also enables communication between bacteria and their eukaryotic hosts by sensing respective signal molecules mechanism called interkingdom signaling (IKS) communication. The bacteria usually sense hormone-like signals, whereupon they change their behavior to promote host colonization (Hughes and Sperandio, 2008). Moreira et al. described one of the first IKS in enterohemorrhagic *E. coli* (EHEC). After human host infection EHEC sense hormones epinephrine and norepinephrine, as well as an autoinducer molecule produced in the human gut via the QseC/QseB two-component system. Subsequently, expression of genes encoding another two-component-system (QseE/QseF) are induced, which then activate expression of virulence genes (Moreira et al., 2016). Furthermore, recent studies indicate some LuxR solos in plant associated bacteria to be involved in IKS.
In summary, bacterial communication via LuxR solos is not only restricted in interspecies communication but also includes interkingdom communication in a complex regulatory network.

1.4.3 LuxR solos in plant associated bacteria

A common phenomenon of interkingdom interaction occurs in the rhizosphere, where microorganisms interact with the plant hosts. In some plant associated bacteria, LuxR homologs belonging to a different subgroup of LuxR solos, which harbor an AHL signal binding domain (SBD) were noticed (Patel et al., 2013; Venturi and Fuqua, 2013) (Venturi and Fuqua, 2013, González and Patel 2013). Thereby, two conserved amino acids are substituted in the SBD, which is in agreement with the evidence that these proteins bind low-molecular weight molecules different from AHLs (Ferluga and Venturi, 2009). Therefore, LuxR solos of plant associated bacteria might play an important role in IKS. For *Xanthomonas* a role of the LuxR solo OryR in IKS with rice plants and the regulation of genes coding for virulence or motility was reported (Ferluga et al., 2007; González et al., 2013). Moreover, LuxR solos involved in IKS with plants are found in further plant associated bacteria such as XccR, XagR in Xanthomonades, NesR in Rhizobia or PipR and PsrR in Pseudomonades (Ferluga et al., 2007; Zhang et al., 2007; Ferluga and Venturi, 2009; Patankar and González, 2009; Subramoni and Venturi, 2009; Coutinho et al., 2018). Recently, for endophytic *Kosakonia* spec. two LuxR solos were described not only to play a role in interspecies, but also interkingdom signaling with plants (Mosquito et al., 2020).

1.4.4 LuxR solos in *P. luminescens*

Several bioinformatic studies revealed in total 40 LuxR-type receptors in *P. luminescens*, all lacking a cognate LuxI synthase and therefore referred to as LuxR solos (Heermann and Fuchs, 2008; Brameyer et al., 2014). Generally, *Photorhabdus* spec. harbor three different types of LuxR solos all sharing the typical C-terminal helix-turn-helix motif “HTH” acting as DNA-binding domain (DBD) and a N-terminal signal binding domain (SBD). However, differences in the SBD of *Photorhabdus* LuxR have been found, for which reason they were grouped in three subgroups: LuxR solos with PAS4-domain, AHL-domain, and with a yet undefined SBD domain (Brameyer et al., 2014) (**Fig. 1-5A**). The LuxR solos of *P. luminescens* were already suggested to enable the bacteria to sense different types of signals, like exogenous AHLs, exogenous and endogenous non-AHLs, or eukaryotic signals (Subramoni and Venturi, 2009). Generally, the high number of divers LuxR solos in *P. luminescens* gives hints for an extremely high capacity of communication in intra- and interbacterial signaling as well as IKS, especially recognizing exogenous AHLs and/or non-AHLs deriving from e.g., nematodes or insect hosts. Indeed, first indications for IKS between *P. luminescens* and insect hosts were given by PAS4-LuxR solos PikR1/PikR2 (Plu2018/Plu2019; now: PluDJC_10520/PluDJC_10530) which sense stearic and palmitic acid two fatty acids identified in *G. mellonella* insect homogenate (Brehm, 2021) (**Fig. 1-5B**). It was suggested that *P. luminescens* can specifically identify the insect host by detecting host specific signals sensed by the different PAS4-LuxR solos and thereby adapt to the specific insect species (Heermann and Fuchs, 2008; Brehm, 2021).

P. luminescens harbors two LuxR solos with an AHL-domain, PluR (Plu4562, or PluDJC_22590 in *P. luminescens* DJC strain) and SdiA-like (Plu0320; here: PluDJC_01675). Modification in SBD domain of PluR suggested perception of molecules different than AHLs and indeed corresponding to the ability of PluR to sense endogenously produced photopyrones (α-pyrones, PPYD) which are synthesized by pyrone synthase PpyS and controlling cell clumping in *P. luminescens* (Brachmann et al., 2013) (**Fig. 1-5B**). But for the second AHL-LuxR solo SdiA-like of *P. luminescens* no signal molecule has yet been identified, although the SBD contains the conserved amino acid WYDPWG-motif important for AHL binding highlighting that the *P. luminescens* SdiA-like LuxR solo could sense exogenous AHLs (Brameyer et al., 2014).

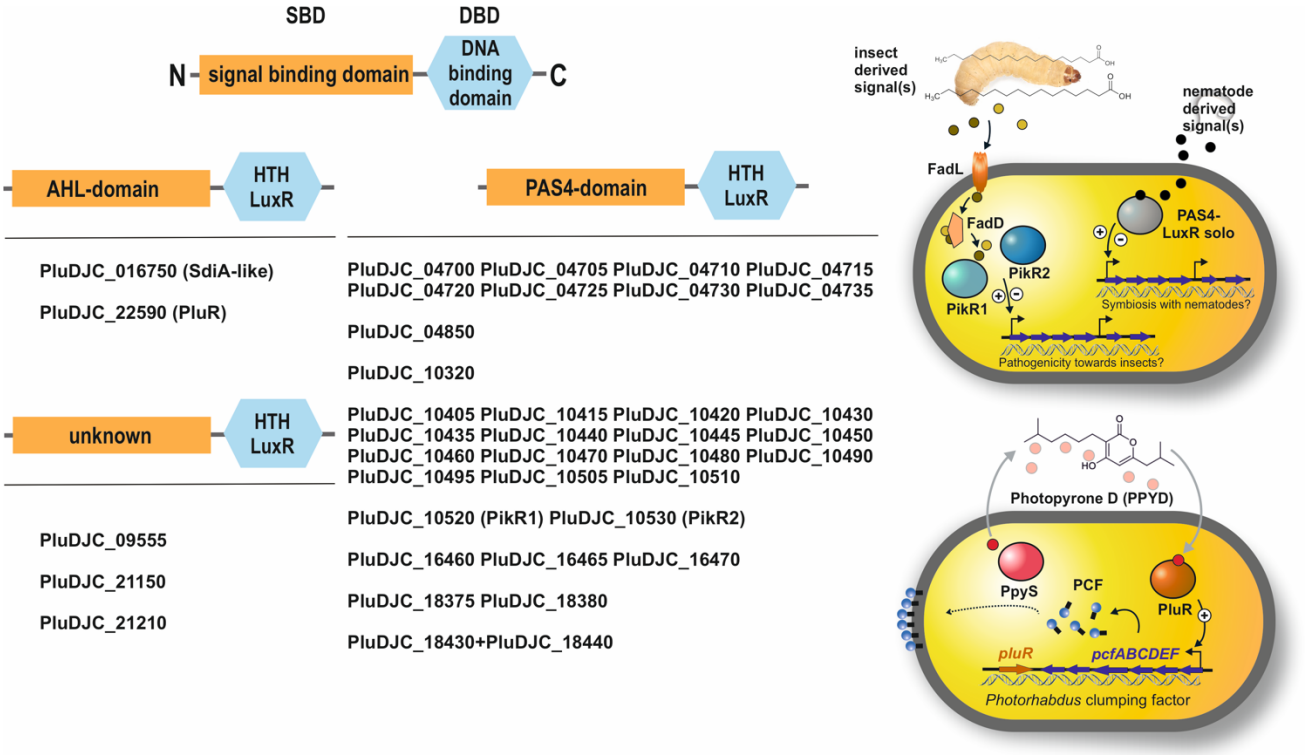

Figure 1-5: LuxR solos in *P. luminescens*. (A) LuxR-type receptors consist of two modular domains, the N-terminal signal binding domain (SBD; orange) and the C-terminal HTH LUXR (SMART00421) DNA-binding domain (DBD; blue). The LuxR solos of *P. luminescens* are classified in three subclasses, according to their SBD: AHL-domain (PFAM03472: Autobind_bind), the PAS4-domain (PFAM08448: PAS_4) and an unknown domain. The list indicates LuxR solos homologous in *P. luminescens* subs. *laumondii* DJC and was created considering (Brameyer et al., 2014). (B) Putative PikR1/PikR2 mediated interkingdom signaling (IKS) in *P. luminescens*. The bacteria putatively sense insect derived fatty acids, that are transported via FadD/FadL to LuxR solos PikR1 and PikR2, which then actives genes putatively involved in insect pathogenicity and nematode symbiosis (Brehm, 2021). (C) PPY-dependent cell-cell communication in *P. luminescence*. Although PluR is described as LuxR solo lacking a cognate LuxI synthase, it senses the endogenously produced PPYs (signal molecule photopyrone D, PPYD, with highest affinity) produced by photopyrone synthase PpyS, which activates the expression of *pcfABCDEF* operon leading to cell clumping (modified after Brachmann et al., 2013).

1.5 Scope of the dissertation

Understanding the occurrence of phenotypic heterogeneity and the fate of *P. luminescens* 2° cells is still a striving work. For that purpose, the main objective of this thesis was to elucidate the alternative lifestyle that *P. luminescens* 2° undergoes after phenotypic switching and to elucidate a putative IKS communication mechanism of *Photorhabdus* in the rhizosphere. Therefore, this work focuses on to address the queries regarding the fate of 2° cells in the rhizosphere and understanding their communication with the environment.

Primarily, this thesis focuses on the phenotypic heterogeneity of *P. luminescens* subs. *laumondii* 1° and 2° cells, which so far were only suggested to be genetically identical, however, no evidence was provided yet. Therefore, the first steps were performing genomic (HTS-Seq) and transcriptomic (RNA-Seq) analyses to elucidate the genetical identity of both cell variants and to understand the origin of the different phenotypes. The resulting data of the RNA-Seq should provide information about genes mediating 1°-specific phenotypes in 1° cells and should give hints in genes involved in phenotypic switching and highlight genes involved in 2°-specific traits. Since 2° cells are left in soil after an insect infection cycle, they encounter different stress conditions like nutrient availability, temperature shifts, oxidative stress, whereupon it would be very essential to determine genes involved in adaptation of 2° to the new environment.

Especially, due to the altered nutrient availability 2° cells would have to adapt to different sources, which are very likely not in proximity in the soil. Most nutrients in the soil derive from plants which are dominantly present in the rhizosphere, and *P. luminescens* 2° cells might get in contact with. Therefore, the second part of the thesis concentrates on whether 2° cells react to plants and their root exudates (PRE). Phenotypic tests like plant root colonization, motility, or biofilm formation should provide first indications about the *Photorhabdus* 2°-plant interaction. Whether an effect can be observed, it would be of great importance to investigate which genes are involved in this interaction. Therefore, knowledge about differentially expressed genes (DEGs) upon PRE in 2° cells is required, which should be achieved via comparative RNA-Seq analysis considering PRE exposed cells. To finally elucidate the role of 2° cells in the rhizosphere, DEGs found in RNAseq analysis, should be selected and their putative role in e.g., plant colonization, growths promotion, or protection should be investigated.

Bacterial communication with eukaryotes like plants is often referred to as IKS communication and is driven by LuxR regulators, which are highly represented in *P. luminescens.* Therefore, in the last part of the thesis, the role of AHL-LuxR solo SdiA, which is homologous to LuxRs found in plant associated bacteria, in IKS with plants was investigated. For that purpose, the effect of the respective gene on plant colonization specific phenotypes should be analyzed. Furthermore, PRE should be screened for potential signaling molecule that binds purified SdiA, as well as its DNA binding capacity upon signal binding should be examined using surface plasmon resonance spectroscopy.

Although always assumed, the genetical identity of *P. luminescens* 1° and 2° cells has never been proved. For that purpose, the genomes of single 2° clones switched from the 1° variant should be compared using bioinformatics analyses to prove that phenotypic switching from 1° to 2° cells is due to bacterial phenotypic heterogeneity.

1.6 References of introduction

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2. Phenotypic heterogeneity of insect pathogenic *Photorhabdus luminescens* - insights into the fate of secondary cells in the soil

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ENVIRONMENTAL MICROBIOLOGY

Phenotypic Heterogeneity of the Insect Pathogen *Photorhabdus luminescens*: Insights into the Fate of Secondary Cells

Simone Eckstein,[a,b] Nazzareno Dominelli,[a] Andreas Brachmann,[c] Ralf Heermann[a]

[a]Institut für Molekulare Physiologie, Mikrobiologie und Weinforschung, Johannes-Gutenberg-Universität Mainz, Mainz, Germany
[b]Biozentrum, Bereich Mikrobiologie, Ludwig-Maximilians-Universität München, Martinsried, Germany
[c]Biozentrum, Bereich Genetik, Ludwig-Maximilians-Universität München, Martinsried, Germany

ABSTRACT *Photorhabdus luminescens* is a Gram-negative bacterium that lives in symbiosis with soil nematodes and is simultaneously highly pathogenic toward insects. The bacteria exist in two phenotypically different forms, designated primary (1°) and secondary (2°) cells. Yet unknown environmental stimuli as well as global stress conditions induce phenotypic switching of up to 50% of 1° cells to 2° cells. An important difference between the two phenotypic forms is that 2° cells are unable to live in symbiosis with nematodes and are therefore believed to remain in the soil after a successful infection cycle. In this work, we performed a transcriptomic analysis to highlight and better understand the role of 2° cells and their putative ability to adapt to living in soil. We could confirm that the major phenotypic differences between the two cell forms are mediated at the transcriptional level as the corresponding genes were downregulated in 2° cells. Furthermore, 2° cells seem to be adapted to another environment as we found several differentially expressed genes involved in the cells' metabolism, motility, and chemotaxis as well as stress resistance, which are either up- or downregulated in 2° cells. As 2° cells, in contrast to 1° cells, chemotactically responded to different attractants, including plant root exudates, there is evidence for the rhizosphere being an alternative environment for the 2° cells. Since *P. luminescens* is biotechnologically used as a bio-insecticide, investigation of a putative interaction of 2° cells with plants is also of great interest for agriculture.

IMPORTANCE The biological function and the fate of *P. luminescens* 2° cells were unclear. Here, we performed comparative transcriptomics of *P. luminescens* 1° and 2° cultures and found several genes, not only those coding for known phenotypic differences of the two cell forms, that are up- or downregulated in 2° cells compared to levels in 1° cells. Our results suggest that when 1° cells convert to 2° cells, they drastically change their way of life. Thus, 2° cells could easily adapt to an alternative environment such as the rhizosphere and live freely, independent of a host, putatively utilizing plant-derived compounds as nutrient sources. Since 2° cells are not able to reassociate with the nematodes, an alternative lifestyle in the rhizosphere would be conceivable.

KEYWORDS bacterium-host interaction, cell-cell communication, entomopathogenic bacteria, PpyS/PluR

Photorhabdus luminescens is a Gram-negative, entomopathogenic bacterium belonging to the family *Enterobacteriaceae* (1, 2). The bacteria undergo a dualistic life cycle including mutualistic symbiosis with *Heterorhabditidae* nematodes and a pathogenic relationship in which they infect and kill insects (1). *P. luminescens* was first isolated from the gut of *Heterorhabditis bacteriophora* nematodes, found in temperate climates. The bacteria exist in two phenotypically different forms, which are designated primary

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Address correspondence to Ralf Heermann, heermann@uni-mainz.de.

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TABLE 1 Genes corresponding to 1° cell-specific features downregulated in 2° cells[a]

Phenotype and gene	1° cells	2° cells	FC by growth phase (2° wt/1° wt)[b]	
			Exp	Stat
Bioluminescence	+++	+		
luxC			NS	−11.56
luxD			NS	−10.85
Pigmentation	+	−		
antA			−19.71	−25.95
antB			−19.52	−57.15
antC			−36.69	−15.25
antD			−35.16	−13.11
antE			−20.61	NS
antF			−30.46	−26.52
antG			−20.86	NS
antH			−12.11	−10.52
antI			−12.73	−30.02
Crystal proteins	+	−		
cipA			−5.01	−27.91
cipB			NS	−16.21
PluDJC_07765			−4.20	−47.76
Antibiotic production	+	−		
PluDJC_04580			−11.29	NS
PluDJC_045805			−5.06	NS
PluDJC_04590			−4.90	NS
PluDJC_15990			NS	−5.47
PluDJC_16670			−5.58	NS
stlA			−4.95	NS
Cell clumping	+	−		
pcfA			NS	−64.84
pcfB			NS	−87.19
pcfC			NS	−110.61
pcfD			NS	−100.05
pcfE			NS	−10.98
pcfF			NS	−10.52
Protease production	++	+		
prtA			−8.47	NS
Lipase production	+	−		
pdl			NS	−6.33

[a]Genes were differentially transcribed between 1° and 2° cells in exponentially growing or stationary phase cultures. The presence (+) or absence (−) of the phenotype as it is described in the literature is indicated.
[b]Fold change (FC) was calculated as the level of expression in wild-type 2° cells/expression in wild-type 1° cells. An FC value of less than −3 or greater than 3 was considered significant ($P \leq 0.05$). NS, not significant. Exp, exponential growth phase; Stat, stationary growth phase; wt, wild type.

(1°) and secondary (2°) cells. After prolonged cultivation, a large portion of single 1° cells undergo phenotypic switching and convert into 2° cells, which differ from 1° cells in various phenotypic traits (3) (Table 1). Most predominant is that 2° cells are less bioluminescent than 1° cells, do not produce red pigments, and are unable to live in symbiosis with the nematode partner (4–7). So far, phenotypic switching of *P. luminescens* cells has been observed only unidirectionally from the 1° to the 2° cell form (1, 3; our unpublished observations). Previously, phenotypic switching of *Photorhabdus* has been referred to as phase variation (8). However, this phenomenon differs from classical bacterial phase variation as both variants are genetically identical (1; our own unpublished observations) and has therefore been termed phenotypic heterogeneity (9). The exact regulatory mechanism behind phenotypic switching and the biological role of *P. luminescens* 2° cells still remain elusive. As 2° cells are known not to be capable of reassociating with nematodes and support their growth and development (6), it has

been assumed that they might be better adapted to a life in soil (10, 11). However, 2° cells have thus far not been isolated from soil. The fact that they are found only after prolonged cultivation of 1° cells led to the assumption that the switch occurs as a response to environmental or metabolic stress (12). It was also observed that, after a period of starvation, 2° cells adapted faster to the addition of nutrients and grew faster than 1° cells. Furthermore, proteome analysis demonstrated that 2° cells experience an upregulation of several metabolic enzymes (11). According to this observation, major respiratory enzymes and also the transmembrane proton motive force were found to be upregulated in 2° cells, supporting the assumption that this cell variant might be more adapted for a life in soil (11, 13).

The purpose of the present study was to shed light on the general function of *P. luminescens* 2° cells and their fate when they are left behind in the soil after an infection cycle. For that reason, we compared the transcriptomes of *P. luminescens* DJC 1° and 2° cells. Based on the description of the transcriptomic variation observed, we performed various follow-up investigations and bring evidence for an alternative life cycle of 2° cells in soil.

RESULTS AND DISCUSSION

Phenotypic heterogeneity of *P. luminescens* DJC 1° and 2° cells. As a first step, we analyzed the phenotypic differences between *P. luminescens* strain DJC 1° and 2° cells with respect to symbiosis, insect pathogenicity, anthraquinone (pigment) production, and antibiotic, lipase, and protease activities. As also observed for other *Photorhabdus* strains (6, 14, 15), 2° cells were no longer able to support nematode development (Fig. 1A), whereas insect pathogenicity was comparable to that of 1° cells (Fig. 1B). Furthermore, pigment (anthraquinone) as well as light production was absent from 2° cells (Fig. 1C and D). Antibiotic production and proteolytic activity were strongly decreased while lipase activity, cell clumping, and crystal inclusion proteins were not detectable in 2° cells (Fig. 1E, G, and H). In contrast to the rod-shaped 1° cells that form mucoid colonies, 2° cells are smaller coccoid rods forming nonmucoid colonies (Fig. 1F). The different phenotypes of *P. luminescens* DJC 1° and 2° cells show that they are comparable to the phenotypic heterogeneity that has been described previously for other *Photorhabdus* species, such as *Photorhabdus temperata* (15).

Comparative transcriptome analysis of *P. luminescens* 1° and 2° cells. To gain more insights into the differences between *P. luminescens* 1° and 2° cells, we performed transcriptome sequencing (RNA-Seq) analysis. Thereby, 638 differentially expressed genes (DEGs) were found in 1° and 2° cells, including 373 genes present during exponential growth phase, 178 in early stationary phase, and 87 in both growth phases (see Table S1 in the supplemental material). Ignoring the genes whose function is unclear, the remaining DEGs were divided into 18 subgroups corresponding to their specific functions (Fig. 2A). The subgroup referred to as "others" contains genes that were predicted to be truncated or even pseudogenes, together with genes not yet classified.

First, we looked for genes that correlate with the distinct phenotypic differences of 1° and 2° cells described above. We found genes responsible for all phenotypic traits mentioned above, such as bioluminescence (*luxCD*), pigmentation (*antABCDEFGHI*), crystal inclusion proteins (e.g., *cipA*), cell clumping (*pcfABCDEF*), antibiotic production (e.g., *PluDJC_04580*), proteases (*prtA*), and lipases (*pdl*), to be downregulated in 2° cells (Table 1).

2° cells of *P. luminescens* DJC are unable to reassociate with the nematodes and are therefore left behind in the soil. Thus, phenotypic switching has to be tightly regulated as a switching frequency of 100% would lead to a breakdown of the bacterium's life cycle. However, the exact mechanism is still unclear. Our transcriptome analysis revealed 35 DEGs encoding transcriptional regulators, of which two-thirds are of unknown function (Table S1). Consequently, one or more of these regulatory genes could be involved in the regulation of phenotypic heterogeneity in *P. luminescens* DJC cell populations.

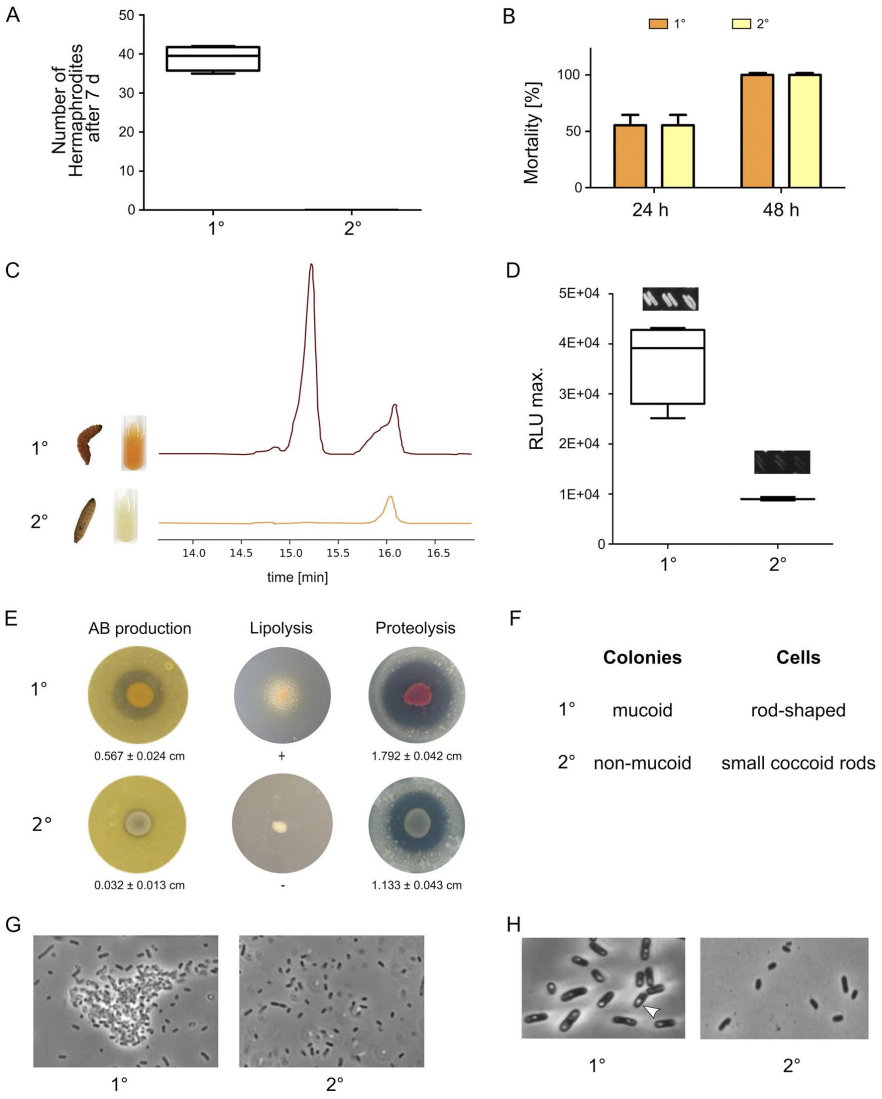

FIG 1 Phenotypic comparison of *P. luminescens* DJC 1° and 2° cells. (A) Nematode bioassay. Fifty axenic *Heterorhabditis bacteriophora* IJs were spotted on 1° or 2° cells grown on lipid agar plates. After 7 days the number of developed hermaphrodites was counted. (B) Pathogenicity assay. Approximately 2,000 of the 1° or 2° cells were injected into 10 *G. mellonella* larvae each. Mortality was monitored over 48 h. (C) Pigmentation of both phenotypic cell forms was visually monitored over 5 days, and anthraquinone production was quantified from culture supernatant extracts via HPLC. (D) Bioluminescence of 1° and 2° cells was monitored over 24 h using a luminescence plate reader. Additionally, single colonies were streaked, and light production was visually analyzed by taking pictures with 5 min of exposure time. (E) To test for antibiotic production both 1° and 2° cells were spotted onto *B. subtilis* germ-agar plates. Furthermore, lipolytic or proteolytic activity was tested by spotting both phenotypic cell forms onto Tween agar or skim milk agar plates, respectively. (F) The colony morphology of both cell forms was analyzed by streaking single colonies with a toothpick. The shape of the cells as well as formation of cell clumps (G) and crystal inclusion proteins (H) was investigated via phase-contrast microscopy. Error bars represent standard deviations of three independently performed experiments.

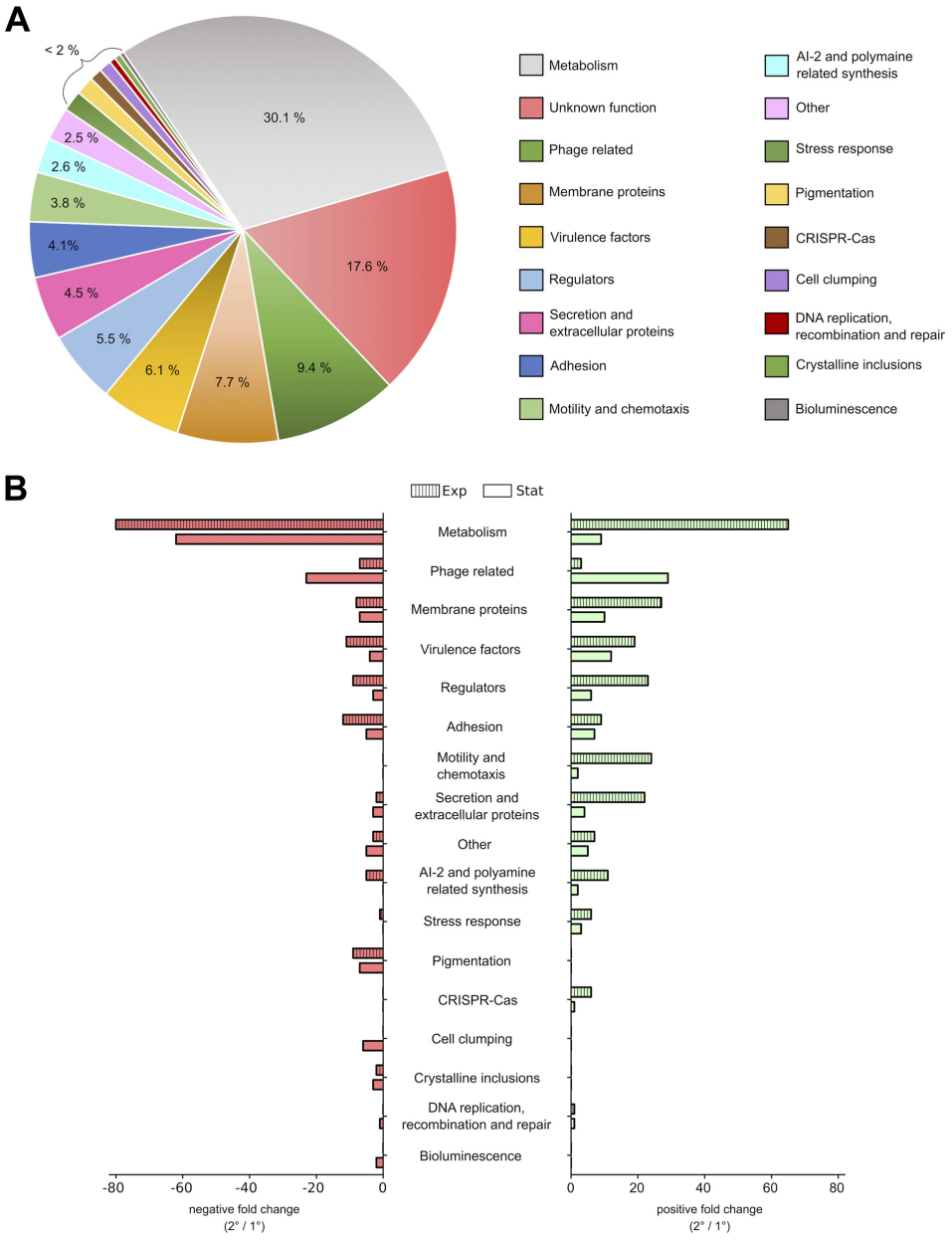

FIG 2 Overview of genes differentially expressed in 2° cells compared to levels in 1° cells. (A) Division of the 638 DEGs according to their functionality into 19 subgroups. (B) Negative (red) and positive (green) FCs of all DEGs obtained at the exponential (Exp; striped bars) as well as stationary (Stat; plain bars) growth phase.

2° cells of *Photorhabdus* sp. are commonly described as cell variants that lack several phenotypes. However, our transcriptome analysis revealed that several of the DEGs were upregulated in 2° cells, including genes involved in the cells' metabolism, stress response, motility, and chemotaxis (Fig. 2B). This indicates that 2° cells are adapted to living in an environment other than that of the symbiotic host. Due to the incapability of 2° cells to reassociate with the nematodes, it seems likely that they are adapted to a free life in soil or the rhizosphere.

As the fate of 2° cells is a crucial missing piece to understanding phenotypic heterogeneity of *P. luminescens*, we therefore focused on genes that could support 2° cells to deal with alternative environmental conditions such as those of the soil and the rhizosphere.

Changes in signaling and cell-cell communication. Among the genes with affected expression in 2° cells were various genes encoding regulators involved in signaling and cell-cell communication. Two of these are *pluR and ppyS*, which code for the LuxR solo (16) and the photopyrone synthase, respectively, were also downregulated in 2° cells. PpyS/PluR is the quorum sensing system used by *P. luminescens* to control expression of the *pcfABCDEF* operon and, therefore, cell clumping via PluR (17). This explains the diminished *pcfABCDEF* transcription and therefore the absence of cell clumps in 2° cells, since PluR positively regulates expression of the *pcf* operon. However, downregulation of *pluR* would also affect the cells' ability to communicate with each other. Since *P. luminescens* harbors 40 LuxR solo receptors, which are supposed to be involved in cell-to-cell communication as well as interkingdom signaling (18, 19), it is likely that 2° cells use an alternative to the PpyS/PluR communication system.

Transcriptome analysis revealed upregulation of 12 LuxR solo genes in 2° cells: the 8 genes of the *PluDJC_10415-PluDJC_10460* operon, which are part of the largest PAS4-LuxR solo cluster of *P. luminescens*; two single PAS4-LuxR solos (*PluDJC_04850* and *PluDJC_18380*); and the only two LuxR solos with a yet undefined signal binding domain (SBD) (*PluDJC_09555* and *PluDJC_21150*). The LuxR solos of *P. luminescens* can be divided into four subgroups corresponding to their SBDs. The largest group comprises 34 LuxR solos harboring a PAS4 signal binding domain (19). PAS4 domains *of P. luminescens* are homologous to the PAS3 domain of the fruit fly *Drosophila melanogaster*, in which it has been shown that this domain acts as a juvenile hormone (JH) receptor (20). Therefore, it is suggested that PAS4 domains of *P. luminescens* play an important role in interkingdom signaling and also bind hormone-like molecules (21). Moreover, it has also been shown that LuxR solos of plant-associated bacteria can respond to plant signaling molecules (22, 23), which might also be true for one or more LuxR solos upregulated in 2° cells. However, no specific signal sensed by the PAS4-LuxR solos of *P. luminescens* has been identified yet.

In summary, the DEGs encoding LuxR receptors strongly suggest that 2° cells utilize other cell-cell communication systems for intra- as well as interkingdom signaling than 1° cells and thereby are able to adapt to an alternative lifestyle. Future work will investigate to which signals the LuxR solos respond and if they support the adaptation of 2° cells to a life in the soil and the rhizosphere.

Differences in LPS composition. We observed an alteration in expression of six *wbl* genes, which were either up- or downregulated, that play a role in the O-antigen biosynthesis of lipopolysaccharide (LPS) in the cells (24) (Table S1). For host-associated microbes, changes in LPS composition have previously been associated with differences in host niche (25, 26). Therefore, we hypothesize that the change in LPS composition in 2° cells strongly indicates a specificity for environmental conditions other than those to which 1° cells are adapted. Whether the differences in LPS composition could support the idea that the 2° cells live free in soil that is in contact with plants remains to be tested.

Metabolic changes. Our transcriptome analysis of 1° and 2° cells revealed a large set of DEGs involved in the cells' metabolism, which already gives hints of an adaption of 2° cells to alternative nutrients. Among these DEGs were, e.g., genes playing a role

in cobalamin biosynthesis or fumarate degradation (Table S1). The complete set of genes involved in hydroxyphenylacetate (HPA) metabolism were expressed at higher levels in 2° cells. 4-HPA is a common fermentation product of aromatic amino acids. Several bacteria, such as *Escherichia coli*, are able to degrade 4-HPA over several converting steps to finally metabolize it to pyruvate and succinate. Furthermore, it is also often found in soil as a result of plant material degradation by animals (27). Therefore, an enhanced capability to degrade 4-HPA could help 2° cells to grow in soil as it can be used as a carbon source.

In contrast, 2° cells seem to have less affinity for phenylpropanoid compounds than 1° cells as we found the respective cluster (*hcaCFE*, *hcaB*, and *hcaD*) (28) to be downregulated. However, as phenylpropanoids most commonly originate from proteins (28), which are the main nutrient source inside the larvae, reorientation of 2° cells after leaving the cadaver would be obligatory.

Furthermore, the genes *astABDE* and *PluDJC_15875*, encoding enzymes for arginine degradation (29), are upregulated in 2° cells. In *E. coli* the arginine succinyltransferase (AST) pathway is induced when nitrogen is limited and aspartate and arginine are present (30). Again, this could be a mechanism allowing 2° cells to overcome starvation in soil as in the rhizosphere large amounts of amino acids, which are secreted, e.g., from plant roots, are present (31).

As the bacterium-nematode complex, which comprises only 1° cells, emerges from the cadaver when all nutrients of the larvae are depleted, 2° cells might be exposed to starvation. An increase in motility and a higher sensitivity to nutrients and, therefore, enhanced chemotaxis would be an essential strategy for the bacteria to overcome nutrient limitation.

Increased motility and chemotaxis of 2° cells. The general function of *P. luminescens* 2° cells is still unclear, but it is assumed that they might be better adapted to a life in soil (10, 11). Since the nutrients present in the rhizosphere differ from the those present in the bioconverted insect cadaver and may not always be easily available, an increase in motility and a higher sensitivity to alternative nutrients could therefore be of great advantage for the whole cell population.

As flagellum formation and directed or nondirected motility are highly complex, including many different operons, we evaluated this group of data considering fold change (FC) values above 1.5 or below −1.5 to include all DEGs involved in these processes. Indeed, we found several DEGs involved in motility and chemotaxis that were upregulated in 2° cells.

(i) Motility. The transcriptome analysis demonstrated increased expression of 22 genes involved in flagellum formation with an FC of >3 and an additional 13 genes with fold changes ranging from 2.0 to 2.98. We found *flhD* and *flhC*, the two parts of the transcriptional activator complex FlhDC (32), to be upregulated in 2° cells (Table 2). Furthermore, we found that several structural genes involved in flagellar hook-basal body complex assembly, which are designated class 2 flagellar genes, (32) were upregulated. In detail, expression levels of either parts of or the complete operons *flgBCDEFGHIJ*, *flhBAE*, *fliFGHIJK*, and *fliLMNOPQR* as well as the gene encoding FliE were higher in 2° cells. Furthermore, two class 3a structural gene clusters, *fliDST* and *flgKL*, as well as *fliC* (class 3b), which encodes flagellin, exhibited increased expression in exponentially growing 2° cells (Table 2) (32).

As a representative for motility genes, *fliC*, the major driving force for flagellum formation, was chosen for RNA-Seq data validation via reverse transcription-quantitative PCR (RT-qPCR). Thereby, we could confirm upregulation of *fliC* in 2° cells during the exponential growth phase (Fig. 3).

Previously, for *Xenorhabdus nematophila* and *Photorhabdus temperata* strains, motility was described to be a specific feature of 1° cells (33). However, we found upregulation of motility-related genes in *P. luminescens* 2° cells and therefore analyzed whether motility is truly increased in 2° cells. For that purpose, we performed swimming assays by spotting the respective cell forms onto soft-agar swimming plates and

TABLE 2 Motility- and chemotaxis-related genes transcribed at higher levels in 2° cells than in 1° cells in exponential or stationary growth phase[a]

Category and locus tag	Operon	Gene	Protein(s)	FC by growth phase (2° wt/1° wt)[b]	
				Exp	Stat
Flagellum formation					
Class 1					
PluDJC_09685	*flhDC*	*flhD*	Flagellar transcriptional activator	3.15	NS
PluDJC_09685		*flhC*	Flagellum biosynthesis transcription activator	2.09	
Class 2					
PluDJC_09860	*flhBA*	*flhB*	Flagellar biosynthesis protein	3.41	NS
PluDJC_09865		*flhA*	Flagellar biosynthesis protein	2.78	
PluDJC_09935	*flgAMN*	*flgN*	Flagellar synthesis protein	2.97	
PluDJC_09940		*flgM*	Negative regulator of flagellin synthesis	2.00	
PluDJC_09945		*flgA*	Flagellar basal body P-ring formation protein precursor	2.17	
PluDJC_09950	*flgBCDEFGHIJ*	*flgB*	Flagellar basal body rod protein	6.74	NS
PluDJC_09955		*flgC*	Flagellar basal body rod protein	6.63	NS
PluDJC_09960		*flgD*	Basal body rod modification protein	5.76	NS
PluDJC_09965		*flgE*	Flagellar hook protein	5.35	NS
PluDJC_09970		*flgF*	Flagellar basal body rod protein	4.87	NS
PluDJC_09975		*flgG*	Flagellar basal body rod protein	4.95	NS
PluDJC_09980		*flgH*	Flagellar L-ring protein precursor	3.26	NS
PluDJC_09985		*flgI*	Flagellar P-ring protein precursor	3.28	NS
PluDJC_09990		*flgJ*	Peptidoglycan hydrolase	2.66	
PluDJC_10070	*fliLMNOPQR*	*fliO*	Flagellar protein	2.39	
PluDJC_10075		*fliN*	Flagellar motor switch protein	2.56	
PluDJC_10080		*fliM*	Flagellar motor switch protein	2.98	
PluDJC_10085		*fliL*	Flagellar protein	3.71	NS
PluDJC_10090	*fliFGHIJK*	*fliK*	Flagellar hook-length control protein	2.97	
PluDJC_10095		*fliJ*	Flagellar protein	3.06	NS
PluDJC_10100		*fliI*	Flagellum-specific ATP synthase	3.31	NS
PluDJC_10105		*fliH*	Flagellar assembly protein	2.83	
PluDJC_10110		*fliG*	Flagellar motor switch protein	3.04	NS
PluDJC_10115		*fliF*	Flagellar basal body M-ring protein	4.00	NS
PluDJC_10120	*fliE*	*fliE*	Flagellar hook-basal body 11-kDa protein	4.75	NS
Class 3a					
PluDJC_09935	*flgMN*	*flgN*	Flagellar synthesis protein	2.97	
PluDJC_09940		*flgM*	Negative regulator of flagellin synthesis	2.00	
PluDJC_09995	*flgKL*	*flgK*	Flagellar hook-associated protein 1 (HAP1)	8.57	4.53
PluDJC_10000		*flgL*	Flagellar hook-associated protein 3 (HAP3)	8.92	NS
PluDJC_10140	*fliDST*	*fliT*	Flagellar protein FliT	5.08	NS
PluDJC_10145		*fliS*	Flagellar protein FliS	8.56	NS
PluDJC_10150		*fliD*	Flagellar hook-associated protein 2 (HAP2)	15.77	4.21
Class 3b					
PluDJC_09695	*mocha*	*motA*	Chemotaxis protein, motor rotation	2.70	
PluDJC_09700		*motB*	Chemotaxis protein, motor rotation	2.68	
PluDJC_09705		*cheA*	Chemotaxis protein	1.83	
PluDJC_09710		*cheW*	Purine-binding chemotaxis protein	2.19	
PluDJC_10155	***fliC***	***fliC***	**Flagellin**	**25.47 (32.42)**	**NS (2.48)**
Chemotaxis					
PluDJC_09715		***cheD***	**Methyl-accepting chemotaxis protein I (MCP-I), highly similar to serine chemoreceptor *tsr***	**5.93 (4.79)**	**NS (1.40)**
PluDJC_09720			MCP-I, highly similar to *tar* (maltose/aspartate chemoreceptor)	4.01	NS

[a]A set of flagellum formation genes and chemoreceptor homologues were differentially expressed between 1° and 2° cells in exponentially growing or stationary phase cultures. Gray-shaded rows indicate genes that belong to the respective structural operon whose transcriptional changes did not fit into our initial filter criteria of fold change values greater than 3 or less than −3 ($P < 0.05$). The genes chosen for qRT-PCR validation are in boldface.

[b]Fold change (FC) was calculated as the level of expression in wild-type 2° cells/expression in wild-type 1° cells. Values in parentheses indicate the fold change after qRT-PCR validation. Exp, exponential growth phase; Stat, stationary growth phase; wt, wild type; NS, not significant.

measuring the zone of colonization at two different time points. Previously, growth rates of 1° and 2° cells were confirmed to be similar in the medium that was used for the swimming assays (data not shown). In fact, 2° cells exhibited a significantly increased swimming motility compared to that of 1° cells after 18 h of incubation. However, after 36 h the difference between the two cell forms decreased to a nonsignificant level (Fig. 4). This is in accordance with the transcriptome data, which

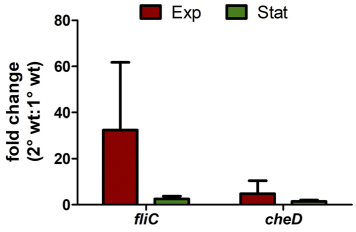

FIG 3 RT-qPCR data on *fliC* and *cheD* displaying higher transcription in 2° than in 1° cells. RT-qPCR revealed a higher level of transcription of *fliC* and *cheD* in 2° cells than in 1° cells either in the exponential growth phase (red) or in the stationary phase (green); the fold change is significantly higher in the exponential growth phase for both genes. The data are presented as the fold change ratio of 2° cells to 1° cells with *recA* used as the housekeeping gene. Values are means of three independent biological replicates and were calculated using the Pfaffl method. wt, wild type.

showed that changes in expression of almost all motility-related genes occurred only in the exponential growth phase and were not significant during the stationary growth phase (Table 2).

As the transcriptome analysis was performed under noninducing conditions, increased motility seems to be a specific feature of 2° cells of the *P. luminescens* DJC strain. In *E. coli* the master activator of flagella formation, *flhDC*, is directly repressed by *lrhA* (34). *P. luminescens* harbors a homologue of this LysR-type transcriptional regulator, HexA, which was identified to act as a master repressor of 1°-cell-specific genes and is highly upregulated in 2° cells of *P. temperata* (15). However, in *X. nematophila*, which is closely related to *P. luminescens*, *lrhA* positively regulates motility (35). Thus, the *flhDC* operon might also be activated by *hexA* in *P. luminescens* 2° cells. High levels of *flhDC*, in turn, could cause the increased swarming of 2° cells as positive regulation of swarming motility via FlhDC was observed for *X. nematophila* (36). We also found *hexA* upregulated in *P. luminescens* DJC 2° cells. However, due to the strong cutoff criteria we used, it is not listed.

(ii) Chemotaxis. As motility and chemotaxis go hand in hand, we next analyzed if increased motility in 2° cells subsequently leads to an enhanced chemotactic behavior of the cells. We found upregulation of the complete *mocha* operon described for *E. coli* (37) with fold changes in 2° cells of between 1.83 and 2.7 (Table 2). This operon (class 3b flagellar genes) comprises four genes, *motA*, *motB*, *cheA*, and *cheW*, and is an important part of the chemotaxis systems as it drives motor rotation and attractant sensing (38, 39).

In *E. coli* the last part of the chemotaxis system is the *meche* or *tar* operon, which consists of four sensory (*cheRBYZ*) and two receptor (*tar* and *tap*) genes (40, 41). Transcriptome analysis of *P. luminescens* DJC 1° and 2° cells revealed one homologue of *tar*, *PluDJC_09720*, as upregulated in 2° cells. Despite that, *PluDJC_09715*, which is highly similar to *tsr* of *E. coli*, was also expressed at a higher level in 2° cells (Table 2). Tsr, a type

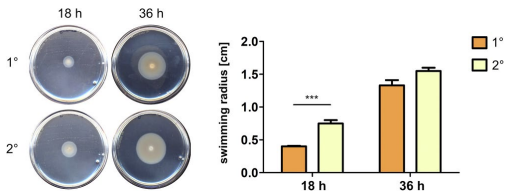

FIG 4 Enhanced swimming motility of 2° cells in comparison to that of 1° cells. Upon spotting 5×10^6 1° or 2° cells onto semisolid swimming agar plates, 2° cells showed significantly increased swimming activity compared to that of 1° cells after 18 h of incubation. Error bars represent standard deviations of three independently performed experiments. ***, $P < 0.001$.

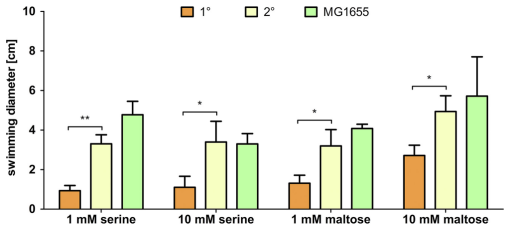

FIG 5 Swimming diameters after addition of different putative attractants. Attractant-dependent motility of *P. luminescens* DJC 1° and 2° cells and *E. coli* MG1655 cells, as indicated, was determined. Error bars represent standard deviations of at least three independently performed experiments. *, $P < 0.05$; **, $P < 0.01$.

l methyl-accepting chemotaxis protein (MCP-I), is a primary chemoreceptor for the transduction of the attractant serine, while Tar, a type II MCP, is a chemoreceptor for the transduction of aspartate and maltose in *E. coli* (42). Gene expression of *PluDJC_09715* was exemplarily verified via RT-qPCR (Fig. 3).

In order to investigate the difference in the chemotaxis-driven motilities of *P. luminescens* 1° and 2° cells, swarming assays were performed. For that purpose, a single bacterial colony was spotted onto the center of a semisolid agar plate containing 1 mM or 10 mM serine or maltose, respectively. *E. coli* MG1655 wild type served as a positive control for chemotactic swarming, while the nonmotile *P. luminescens* 2° Δ*fliC* strain was used as a negative control.

P. luminescens 1° cells showed only a low response to both concentrations of serine as well as 1 mM maltose. However, there was increased movement on the soft-agar plates containing 10 mM maltose. In contrast, 2° cells showed a significantly stronger response to both serine and maltose. Here, a higher sensibility to serine was observed as the swarming diameter on serine plates was significantly bigger than the diameters on plates supplemented with maltose. *E. coli* MG1655 cells were slightly more motile than *P. luminescens* 2° cells with 1 mM serine as well as with both concentrations of maltose (Fig. 5 and Fig. S1).

By increasing the serine concentration, a negative effect could be perceived for *E. coli*. Here, supplementing the plates with 10 mM instead of 1 mM serine led to a 30.9% shrinkage of the swimming diameter. This effect could be observed only for *E. coli* and has been reported before as a result of saturation of the serine-sensing transducer Tsr in *E. coli* (43). However, the swimming diameter of 2° cells did not increase by raising the serine concentration from 1 mM to 10 mM but was similar to the value obtained with the lower serine concentration (Fig. 5 and Fig. S1). Therefore, the Tsr homolog of *P. luminescens* PluDJC_09715 might be able to cope with a higher concentration of serine. The 2° cells of the Δ*fliC* strain, which does not produce any flagellin, served as a negative control and were nonmotile upon addition of any putative attractant (data not shown).

The putative role of plants in the life cycle of 2° cells. The main producers of nutrients in the soil are plants, as the majority of compounds in the rhizosphere, such as amino acids or sugars as organic acids peptides, proteins, or lipids, derive from root exudates (44–46). Therefore, we investigated whether *P. luminescens* cells also respond to plant root exudates. For that purpose, we used soft-agar swimming plates supplemented with root exudates of the pea plant *Pisum sativum* extracted in methanol (MeOH-Ex) and spotted *P. luminescens* 1° and 2° cells on the plates. The plant-pathogenic strain *Pseudomonas fluorescens* WS1750 served as a positive control. Effects of methanol on swimming activity were excluded by solely adding the solvent (data not shown). Analysis of the swimming diameters after 24 h or 48 h revealed a significantly higher response of 2° cells to MeOH-Ex than that of 1° cells (Fig. 6A and B). The compositions of compounds contained in the root exudates are unknown. Comparing

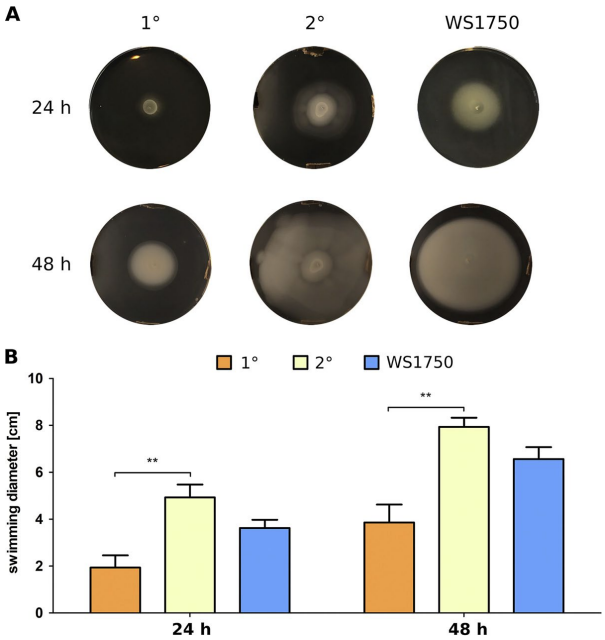

FIG 6 Effects of plant root exudates on swimming motility of *P. luminescens* 1° and 2° cells. On plates containing MeOH-Ex, 2° cells showed a significantly stronger response in terms of increased swimming activity than 1° cells. The recorded swimming diameters were even bigger than those observed with the positive-control *P. fluorescens* WS1750. (A) Pictures of soft-agar swimming plates supplemented with MeOH-Ex after 24 h and 48 h. (B) Graphical depiction of swimming diameters of 1° and 2° cells as well as the WS1750 strain. Error bars represent standard deviations of three independently performed experiments. **, $P < 0.01$.

the swimming activities in the presence of MeOH-Ex to those in the presence of serine and maltose showed them to be comparable or even higher for 2° cells. However, we already applied serine and maltose in excess, as this amino acid and sugar are usually excreted from plants in micromolar or nanomolar amounts (47, 48). Thus, a stronger response of 2° than 1° cells toward other compounds derived from the plant seems likely. Here, further evaluation of the exudate ingredients to resolve the structure and thus the specific signal to which 2° cells respond is needed.

The sensing of plant root exudates by 2° cells might be attributable to PluDJC_09715 and PluDJC_09720, as they are MCPs not only for serine and maltose but also for the amino acids alanine/glycine and aspartic acid/glycine, respectively. Furthermore, *fruAB* was upregulated in 2° cells, which indicates a higher affinity for taking up and utilizing fructose. In addition to galactose, arabinose, raffinose, rhamnose, xylose, and sucrose, fructose and also maltose are the dominant sugars found in root exudates (49). Therefore, a higher-level response of 2° cells than of 1° cells to maltose underlines the suggestion of an increased affinity of 2° cells toward compounds primarily derived from plants.

However, in addition to sugars, vitamins, and amino acids, plants also secrete a wide variety of organic acids that are known to attract bacteria and serve as a nutrient source (50). Thus, additional, as-yet-unknown MCPs involved in the response of 2° cells to plant root exudates might be present in *P. luminescens*.

Increased temperature tolerance of 2° cells. Our findings that *P. luminescens* 2° cells are better adapted to different nutrients than 1° cells support the theory of

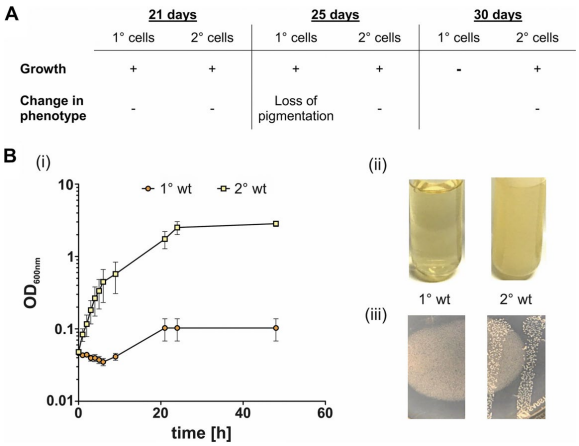

FIG 7 Growth and phenotype of 1° and 2° cells at high and low temperatures. (A) 1° cells do not recover growth after being incubated for 30 days at 4°C and already show loss of pigmentation after 25 days at 4°C. In contrast, 2° cells restart growth after 30 days of exposure to cold and are not affected at all in their fitness or phenotype. (B) 2° cells were capable of growing at 37°C when cultivated in liquid culture while growth of 1° cells was highly decreased under this condition (i and ii). Upon streaking both cell forms onto agar plates and incubating them at 37°C, only 2° cells were able to form colonies (iii). All experiments were independently performed three times. Error bars represent standard deviation.

free-living 2° cells in soil. Additionally, although cultures were grown in rich medium, our transcriptome analysis revealed that several genes involved in the stress response were upregulated in 2° cells (Fig. 1B). Among them, the majority of genes we found are usually induced upon starvation (e.g., *dppABCDF*, *phoH*, *cstA*, or *cspD*).

However, it has already been described that 2° cells recover faster from periods of starvation than 1° cells (11), although outside the host, 2° cells would also be more exposed to changing temperatures. Therefore, we attempted to examine whether 2° cells show a higher tolerance to low and high temperatures. As we performed the RNA-Seq analysis under noninducing conditions, no relevant genes were found. For that purpose, we cultivated both cell forms at low temperatures. Here, neither 1° nor 2° cells showed growth when cultivated at 4°C (data not shown). However, we observed an advantage for 2° cells upon storing LB plates with colonies of each cell form at 4°C for 30 days. Every 4 to 5 days, a single colony was inoculated into LB medium and cultivated at 30°C to determine whether the cells were able to recover and to restart growth. While 2° cells grew perfectly well at all tested time points (Fig. 7A), 1° cells were not able to grow after 30 days. Furthermore, although the 1° cells grew after 25 days of incubation at 4°C, we observed a loss of pigmentation (Fig. 7A), which indicates decreased fitness of the cells, as they remained 1° cells with respect to all other phenotypes (data not shown). Even though we did not find upregulation of any genes encoding heat shock proteins, we also tested the capability of both cell forms to deal with higher temperatures. We found that 2° cells grew significantly better in terms of reaching higher cell densities than 1° cells when they were cultivated at 37°C (Fig. 7B, panels i and ii). Furthermore, only 2° and not 1° cells formed colonies when plated onto LB plates and incubated at 37°C (Fig. 7B, panel iii). Growth at different temperatures is much more important for a free life in soil than for a life inside a host. Night and day as well as the different seasons have a great impact on soil temperature. Therefore, the larger temperature tolerance of 2° cells further supports the idea that they are better adapted for a life in soil than 1° cells.

Conclusion. We could confirm that the most prominent phenotypic traits of *P. luminescens* DJC 1° and 2° cells are mediated at the transcriptional level. Furthermore,

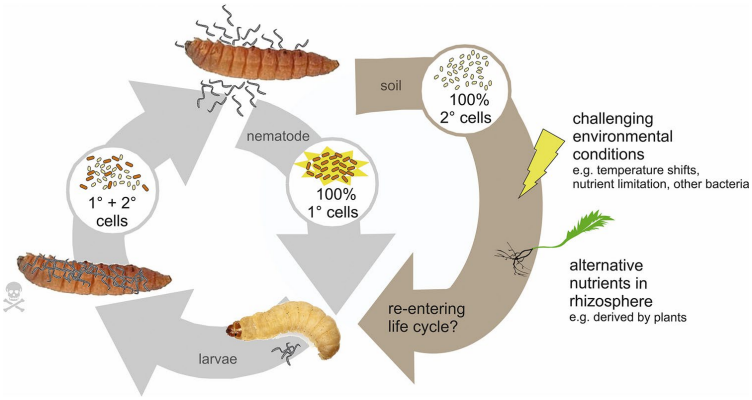

FIG 8 Model of extended life cycle of 2° cells in soil. As only 1° cells are able to reassociate with the nematodes and emerge from the cadaver, 2° cells are left behind in the soil. Based on our transcriptome data, it seems likely that 2° cells are better adapted to free living in soil and thereby are able to survive changing and challenging environmental conditions but also develop strategies to utilize alternative nutrients which are present in soil and which are most likely derived from plants. Eventually, they may find a yet unknown way to reenter the life cycle of *P. luminescens*.

our transcriptome data support the idea that 2° cells are better adapted to an alternative environment outside insect hosts. We found evidence that 2° cells change their metabolism in order to be better adapted to alternative nutrients. Furthermore, 2° cells highly express genes that deal with stress situations, and we could show that they are less sensitive to high or low temperatures than 1° cells. These data thereby strongly support the theory of free-living 2° cells in soil where they withstand challenging environmental conditions and feed from nutrients present in the soil (Fig. 8). Furthermore, we found evidence that 2° cells might somehow be associated with plants or feed on plant-derived nutrients in the rhizosphere.

If and how 2° cells can reenter the pathogenic life cycle or can convert to the 1° phenotype again still remain elusive. However, since the bacteria are already used as a bio-insecticide in agriculture, further investigation of a putative interaction of *Photorhabdus sp.* 2° cells with plant roots is of great importance for biotechnology and agriculture.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strains MG1655 and DH5α λ*pir* were used in this study. They were routinely grown at 37°C in LB medium [1% (wt/vol) NaCl, 1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract]. If necessary, 50 μg/ml antibiotic was added into the medium. *P. luminescens* DJC (2) 1° and 2° cells were obtained from the lab of David Clarke (University College Cork, Ireland) and were cultivated aerobically in either LB medium or CASO medium (0.5% [wt/vol] NaCl, 0.5% [wt/vol] peptone from soy, 1.5% [wt/vol] tryptone) at 30°C. If necessary, the growth medium was supplemented with 50 μg/ml rifampin (Sigma-Aldrich). For preparation of agar plates, 1.5% (wt/vol) agar was added to the respective medium.

Bioluminescence bioassays. Luminescence measurements were performed by cultivation of *P. luminescens* DJC 1° and 2° cells in black 96-well plates with transparent bottoms (Corning, Bodenheim, Germany) and recording of optical density (OD) as well as luminescence using an Infinite-500 reader (Tecan, Salzburg, Austria). Additionally, single colonies of the respective *P. luminescens* variants were streaked onto LB plates and incubated at 30°C for 48 h. Subsequently, bioluminescence was monitored using a chemiluminescence imager (Peqlab, Erlangen, Germany) with a 5-min exposure time.

Pathogenicity bioassays. Fifth-instar larvae of *Galleria mellonella* (reared in our lab) were incubated on ice for 10 min to reduce movement and surface sterilized in a 70% (vol/vol) ethanol bath, followed by a bath of sterile water. Larvae were infected via subcutaneous injection of approximately 2,000 *P. luminescens* DJC 1° or 2° cells using a sterilized microsyringe (1702 RN, 25 μl; Hamilton). The infected larvae were then incubated at 30°C, and the mortality rate was determined by counting dead and live animals after 24 h and 48 h.

Protease bioassays. *P. luminescens* DJC 1° and 2° cells were grown overnight in LB medium at 30°C. Then, an aliquot of 50 μl (OD at 600 nm [OD_{600}] of 1.0) was dropped onto the middle of a skim-milk agar plate (1% [wt/vol] skim milk, 0.3% [wt/vol] yeast extract, 1.2% [wt/vol)] agar), and the plates were incubated for 2 days at 30°C.

Lipase activity bioassays. *P. luminescens* DJC 1° and 2° cells were grown overnight in LB medium at 30°C. Then, an aliquot of 50 μl (OD_{600} of 1.0) was dropped onto the middle of a Tween 20 agar plate (1% Tween 20 [vol/vol)], 1% [wt/vol] tryptone, 0.5% [wt/vol] $NaCl_2$, 0.1% [wt/vol] $CaCl_2{\cdot}2\ H_2O$, 2% [wt/vol] agar], and the plates were incubated for 2 days at 30°C. The precipitation of the calcium salt was visually monitored.

Antibiotic bioassays. For testing antibiotic activity, we used soft-agar plates supplemented with *Bacillus subtilis* as a test strain. For that purpose, an overnight culture of *B. subtilis* at an OD_{600} of 2 to 3 in a 1:100 dilution was added to liquid hand-warm LB agar medium (0.8% [wt/vol] agar). After the plates were polymerized, an aliquot of 30 μl (OD_{600} of 1.0) of the respective *P. luminescens* DJC 1° or 2° cells was dropped onto the middle of the agar plate and incubated for 48 h at 30°C.

Symbiosis bioassays. An aliquot of 50 μl of an overnight culture of *P. luminescens* DJC 1° and 2° cells, diluted to an OD_{600} of 1.0, was spread in a Z pattern onto the surface of a lipid agar plate (1% [vol/vol] corn syrup, 0.5% [wt/vol] yeast extract, 5% [vol/vol] cod liver oil, 2% [wt/vol] $MgCl_2{\cdot}6\ H_2O$, 2.5% [wt/vol] Difco nutrient agar [Becton, Dickinson, Heidelberg, Germany]) using an inoculating loop. The plates were incubated at 30°C for 3 days before addition of 50 surface-sterilized axenic *Heterorhabditis bacteriophora* infective juvenile (IJ) nematodes to the bacterial biomass. Nematodes were surface sterilized by washing in a solution (0.4% [wt/vol]) of hyamine (Sigma-Aldrich, Deisenhofen, Germany). The plates were kept at room temperature. Nematode recovery was assessed 7 to 8 days after addition of IJ nematodes by counting the number of hermaphrodites on the lipid agar plate.

Pigmentation. The development of red pigments was visually noted after 3 days of growth of *P. luminescens* DJC 1° and 2° cells on LB plates at 30°C or 3 days after injection of the bacteria into *G. mellonella* larvae. Additionally, pigmentation was quantified by determining the anthraquinone (AQ) production via high-performance liquid chromatography (HPLC). To this end, 100 ml of LB medium was inoculated to an OD_{600} of 0.1 using overnight cultures of *P. luminescens* DJC 1° and 2° cells. After 72 h of growth at 30°C, 15 ml of each culture was centrifuged for 5 min at 5,000 rpm (at room temperature [RT]). Then, 10 ml of the resulting supernatant was transferred into a new reaction tube and mixed with 10 ml of ethyl acetate plus 0.1% (vol/vol) formic acid (FA) and shaken for 1 h at RT. Subsequently, the reaction tube was kept standing for 1 h and briefly centrifuged in order to separate the organic (upper phase) from the hydrophilic phase. The latter was removed with a vacuum evaporator (Heidolph) at 240×10^5 Pa at 42°C. The extracts were resuspended in 750 μl of methanol and analyzed by HPLC-UV (Thermo Scientific) using a C_{18} Hypersil Gold column (particle size, 5 μm; 250 by 4.6 mm), with detection achieved by measuring UV absorbance at 430 nm. Acetonitrile (ACN) plus 0.1% (vol/vol) FA was used as the mobile phase. With that, a gradient from 5% (vol/vol) to 95% (vol/vol) ACN–0.1% (vol/vol) FA in a period of 25 min was followed by an isocratic step (95% [vol/vol] ACN plus 0.1% FA) with a flow rate of 0.5 ml/min. The column temperature was set at 30°C. The resulting peak areas were normalized against the optical density of the culture measured at the harvesting step.

RNA preparation. Total RNA from three independent cultures of DJC 1° or DJC 2° cells in the exponential growth phase (6-h culture, 3×10^9 CFU/ml) and early stationary phase (18-h culture, 10×10^9 CFU/ml) grown at 30°C was extracted. The pellets of harvested cells were resuspended in 500 μl of ice-cold AE buffer (20 mM NaAc, pH 5.2; 1 mM EDTA, pH 8.0), and then 500 μl of Roti-Aqua-P/C/I (where P/C/I is phenol, chloroform, and isoamyl alcohol) (Roth) and 25 μl of 10% SDS were added. After vortexing, the mixture was incubated for 30 min at 60°C with shaking. Subsequently, the samples were placed into a refrigerator for one night. On the next day, the samples were centrifuged at 16,100 relative centrifugal force units (rcf) for 20 min at 0°C. Afterwards, the supernatant was transferred into 5PRIME Phase Lock gel tubes (Quantabio), supplemented with 500 μl of P/C/I and 50 μl of 3 M NaAc, pH 5.2, and after mixing the tubes were centrifuged at 16,100 rcf for 10 min at 0°C. Then the supernatants were mixed with 1 ml of 96% ethanol (EtOH) and held at −80°C for overnight precipitation. On day 3 samples were again centrifuged at 16,100 rcf for 30 min at 0°C, but this time the supernatant was discarded. To wash the pellet, 1 ml of 80% EtOH was added and subsequently removed by centrifugation at 16,100 rcf for 10 min at 0°C. This washing step was repeated two times. Then the pellet was air dried for 60 min with an open lid and resolved in 100 μl of diethyl pyrocarbonate (DEPC)-treated water. Five micrograms of RNA was then treated with DNase I (ThermoFisher) to remove genomic DNA. Integrity and quantity of total RNA samples were tested with an Agilent 2100 Bioanalyzer system. To eliminate rRNA, a Ribo-Zero rRNA removal kit for Gram-negative bacteria was used according to the protocol provided by the manufacturer (Illumina). Afterwards, an additional quality check with the Agilent 2100 Bioanalyzer system was performed.

Transcriptome analysis. To sequence RNA samples, cDNA libraries were generated using an NEBNext Ultra II RNA Library Prep kit for Illumina (New England Biolabs [NEB]), according to the manufacturer's instructions, starting from 50 ng of rRNA-depleted RNA. The libraries were quality controlled by analysis on an Agilent 2000 Bioanalyzer with an Agilent High Sensitivity DNA kit (Agilent Technologies) for fragment sizes of around 200 to 500 bp. Libraries were pooled, and sequencing on a MiSeq sequencer (2- by 75-bp paired-end sequencing; version 3 chemistry [Illumina]) was performed at the Genomics Service Unit (Ludwig-Maximilians-Universität [LMU] Biocenter, Martinsried, Germany). CLC Genomics Workbench (version 11.0.0; Qiagen) was used to analyze the data. Raw reads were trimmed for quality and adapter sequences, mapped to the reference genome (*P. luminescens* DJC; GenBank accession number NZ_CP024900.1), and analyzed using an RNA-Seq analysis tool. We selected differen-

tially expressed genes having a *P* value of ≤0.05, and the filter for the fold change was set to values of less than −3 or greater than 3. To exclude single outliers, the limit for the maximum group mean was set to ≥20. The functions of the genes of interest were extracted from the UniProt (https://www.uniprot.org) and NCBI (https://www.ncbi.nlm.nih.gov) databases.

RT-qPCR. To validate the whole-transcriptome data, reverse transcription-quantitative PCR (RT-qPCR) was carried out on three independent total RNA preparations, in each case in triplicate. cDNAs were synthesized during the run using a Luna Universal One-Step RT-qPCR kit (NEB), and the reactions were performed according to the protocol provided by the manufacturer. Reactions and melting curves were monitored in a LightCycler (Bio-Rad). Differences in gene expression levels were calculated using the Pfaffl method (51) with *recA* serving as a housekeeping gene. All data are presented as a ratio of three independent biological replicates. Values are means ± the standard deviations.

Generation of knockout mutants. The *fliC* gene was deleted in *P. luminescens* 2° cells as described previously (52). In brief, 500 bp upstream and downstream of genomic *fliC* (*PluDJC_10155*) were amplified by PCR using the primer pair BamHI_fliC FA fwd (ACGGGATCCGGCAACGAATGCATCATG) and FliC FA ovl FB rev (CCCTAGCTGAGCGATTAACGTGCCATAGTTAGAGTTCC) and the pair FliC FB ovl FA fwd (GGAACT CTAACTATGGCACGTTAATCGCTCAGCTAGGG) and fliC FB_EagI rev (ACTCGGCCGCAATCACGGCTCCTTA AC), introducing BamHI and EagI restriction sites (underlined) into the 5′ end of the upstream fragment and the 3′ end of the downstream fragment, respectively. Overlap extension PCR was used to fuse the two PCR products, which were then cloned into pNPTs138-R6KT using the BamHI and EagI restriction sites, resulting in pNPTS-FAB Δ*fliC*. Correctness of the plasmid was confirmed by PCR using primers Check pNPTS-FA FB FWD (TGCTTCCGGCTCGTATG) and Check pNPTS-FA FB REV (GTAAAACGACGGCCAGTCC). This plasmid was then conjugated from *E. coli* S17-1 λ*pir* into 2° cells, and exconjugants were selected as Rif^r Km^r colonies. The pNPTs138-R6KT plasmid contains the *sacB* gene, and after growth in LB broth (with no selection), putative mutants were identified by screening for Rif^r Suc^r Km^s colonies. The deletion of *fliC* was confirmed by PCR using the primer pair BamHI_fliC FA fwd/fliC FB_EagI rev, followed by DNA sequencing.

Swimming assays. Swimming assays were performed using soft-agar plates containing 0.3% (wt/vol) agar, 1% tryptone (wt/vol), and 0.5% NaCl (wt/vol). Overnight cultures of 1° and 2° cells were set to an OD_{600} of 1, and 5 μl was spotted into the center of a soft-agar swimming plate. Without any further movement, the plates were incubated at RT. After 18 and 36 h the diameters of the colonies representing swimming were documented and evaluated using the ImageJ tool for measuring distances. The data were obtained from three independently performed biological and technical replicates.

Chemotaxis movement assays. Soft-agar swarming assays were performed using agar plates containing 0.3% (wt/vol) agar, 1% tryptone (wt/vol), 1% NaCl (wt/vol), and the putative attractant. After autoclaving, the soft agar was kept at 60°C. Right before use, 20 ml of soft agar was supplemented with either 1 mM or 10 mM L-serine or maltose. As the concentration of the plant root exudate was unknown, 600 μl of exudate dissolved in methanol (MeOH-Ex) was added to 20 ml of 0.3% soft agar. After the plates were polymerized, 10 μl of *P. luminescens* DJC 1° and 2° wild-type (WT), DJC 2° Δ*fliC*, and *E. coli* MG1655 cells at an OD_{600} of 0.1 were spotted into the center of the soft-agar plates. Swarming plates were incubated for 24 h and at 30°C without motion. The swimming diameters, representing chemotaxis-dependent movement, were documented and analyzed via the ImageJ tool for measuring distances. The data were obtained from three independently performed biological and technical replicates.

Extraction of plant root exudates. To extract plant root exudates, 75 *Pisum sativum* plants were put in flasks containing 250 ml of methanol. After 16 h of shaking at RT, the liquid was collected, filter sterilized, and stored at 4°C until further use.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01910-19.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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S.E. and A.B. performed and evaluated RNA-Seq analysis. S.E. conducted the phenotypic comparison of the two cell forms and performed the swimming assays as well as RT-qPCR analysis. N.D. generated the *P. luminescens* 2° Δ*fliC* mutant and performed the growth and chemotaxis assays. S.E. generated the figures. S.E. and R.H. designed the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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3. The biocontrol agent and insect pathogen *Photorhabdus luminescens* interacts with plant roots

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* Authors contributed equally. First author order was determined by which author had more seniority.

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The Biocontrol Agent and Insect Pathogen *Photorhabdus luminescens* Interacts with Plant Roots

Alice Regaiolo,[a] Nazzareno Dominelli,[a] Karsten Andresen,[a] Ralf Heermann[a]

[a]Johannes-Gutenberg-University Mainz, Institute of Molecular Physiology (imP), Microbiology and Wine Research, Mainz, Germany

Alice Regaiolo and Nazzareno Dominelli contributed equally to the manuscript. First author order was determined by which author had more seniority.

ABSTRACT The number of sustainable agriculture techniques to improve pest management and environmental safety is rising, as biological control agents are used to enhance disease resistance and abiotic stress tolerance in crops. Here, we investigated the capacity of the *Photorhabdus luminescens* secondary variant to react to plant root exudates and their behavior toward microorganisms in the rhizosphere. *P. luminescens* is known to live in symbiosis with entomopathogenic nematodes (EPNs) and to be highly pathogenic toward insects. The *P. luminescens*-EPN relationship has been widely studied, and this combination has been used as a biological control agent; however, not much attention has been paid to the putative lifestyle of *P. luminescens* in the rhizosphere. We performed transcriptome analysis to show how *P. luminescens* responds to plant root exudates. The analysis highlighted genes involved in chitin degradation, biofilm regulation, formation of flagella, and type VI secretion system. Furthermore, we provide evidence that *P. luminescens* can inhibit growth of phytopathogenic fungi. Finally, we demonstrated a specific interaction of *P. luminescens* with plant roots. Understanding the role and the function of this bacterium in the rhizosphere might accelerate the progress in biocontrol manipulation and elucidate the peculiar mechanisms adopted by plant growth-promoting rhizobacteria in plant root interactions.

IMPORTANCE Insect-pathogenic *Photorhabdus luminescens* bacteria are widely used in biocontrol strategies against pests. Very little is known about the life of these bacteria in the rhizosphere. Here, we show that *P. luminescens* can specifically react to and interact with plant roots. Understanding the adaptation of *P. luminescens* in the rhizosphere is highly important for the biotechnological application of entomopathogenic bacteria and could improve future sustainable pest management in agriculture.

KEYWORDS entomopathogenic bacteria, bacteria-plant interaction, entomopathogenic nematodes, phenotypic heterogeneity

Pests and diseases considerably reduce crop yields. Without prevention programs using chemical pesticides, 70% of agricultural production would be lost (1). The use of agrochemicals ensures adequate crop yields that allow us to feed an increasingly growing population (2). While the use of pesticides has profited agricultural production and management, promiscuous use has led to environmental damage and toxicity toward nontarget organisms (i.e., bees and other wildlife) and human beings (3). Indeed, agricultural workers and people exposed to agrochemicals through occupational use (eating food, drinking liquids containing agrochemical residues, or inhalation or contact with pesticide-contaminated air) are at increasing risks of leukemia and myeloma (4). Furthermore, pesticides in the soil can interact with the rhizosphere microbiome, negatively impacting its composition, metabolism, and growth (5).

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Address correspondence to Ralf Heermann, heermann@uni-mainz.de.

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During the last decade, new sustainable agriculture techniques, e.g., use of beneficial microorganisms (plant growth-promoting rhizobacteria [PGPR]) and entomopathogenic nematodes (EPNs), have arisen to improve pest management, low energy consumption, and environmental and human safety (6). Beneficial microorganisms can protect plants from pests, enhancing disease resistance (i.e., induced systemic resistance [ISR]) and abiotic stress tolerance. In fact, plants can recognize the presence and activities of PGPR in the roots and respond with hormonal and metabolic changes to a wide range of pathogens, without impairing their fitness (7). EPNs from Steinernematidae and Heterorhabditidae became effective and popular biological control agents during the last 3 decades. They have direct effects on plant pathogens, plant parasitic nematodes, and pest insect populations, and they can indirectly improve the soil quality (8). A unique characteristic of EPNs is their symbiotic relationship with bacteria of the *Xenorhabdus* and *Photorhabdus* genera.

Photorhabdus luminescens is a Gram-negative entomopathogenic enterobacterium living in mutualistic symbiosis with EPNs. *P. luminescens* is characterized by a complex dualistic life cycle, i.e., (i) it is able to symbiotically interact with nematodes of the Heterorhabditidae family, and (ii) it is highly pathogenic toward a wide range of insect species since it produces a wide range of high-molecular-weight toxins and secondary metabolites that effectively kill insect larvae within 48 h (9, 10).

P. luminescens exists in two phenotypically different cell forms: the symbiosis phenotypic variant (primary [1°] cells) and the symbiosis "deficient" phenotypic variant (secondary [2°] cells) (11). The 1° and 2° cells are genetically identical (12) (R. Heermann, unpublished data) and equally pathogenic toward insect larvae. However, they differ in diverse phenotypic traits and in the success of their relationship with nematodes since 2° cells can neither support their development nor reassociate with them (13, 14). Furthermore, 1° cells display different distinct phenotypic characteristics as follows: (i) toxins, extracellular enzymes, and pigment production; (ii) secondary metabolites like antibiotics; (iii) bioluminescence; (iv) cell clumping factor; and (v) crystalline inclusion proteins (the majority of which are missing or have a reduced level in 2° cells) (12, 15). Since 2° cells are unable to live in symbiosis with EPNs, we have suggested earlier that they could adapt to a free life in soil and hence better respond to different environmental stress conditions, nutrient poverty, and plant-derived molecules (16, 17). Indeed, it has been reported that 2° cells had a more active cellular metabolism and accumulation of stock proteins to be responsive to new environments (18), such as those represented by the rhizosphere and plant roots.

The rhizosphere is characterized by plant root exudates that can act as a signal(s), influencing specific bacterial gene expression patterns and, thus, impacting the microbial ability to colonize roots and to survive in the rhizosphere (19). Despite the application of *P. luminescens* EPNs as a biopesticide, very little is known about the role of *P. luminescens* 2° cells in the rhizosphere.

For that reason, here we investigate the capacity of the *P. luminescens* strain DJC 2° variant (*P. luminescens* 2°) to interact with plant roots, their chemotactic response to plant-derived compounds, and their effect toward phytopathogenic microorganisms (e.g., pathogenic fungi). First, we examined the response of *P. luminescens* 2° cells to plant root exudates using RNA-seq transcriptome sequencing analysis, allowing the identification of putative genes involved in 2° *P. luminescens*-plant root interaction, adaptation, and colonization. Understanding the role and the function of this bacterium in the rhizosphere will contribute to the understanding of phenotypic heterogeneity in *P. luminescens* cell populations and will have profound implications on bioagriculture and pest management using EPNs.

RESULTS AND DISCUSSION

Transcriptome profile of *P. luminescens* 2° cells in response to plant root exudates. In order to identify genes in *P. luminescens* 2° cells that are important for the interaction of the bacteria with plant roots, we performed a comparative transcriptome analysis of *P. luminescens* 2° cells in the presence and absence of pea root exudates with

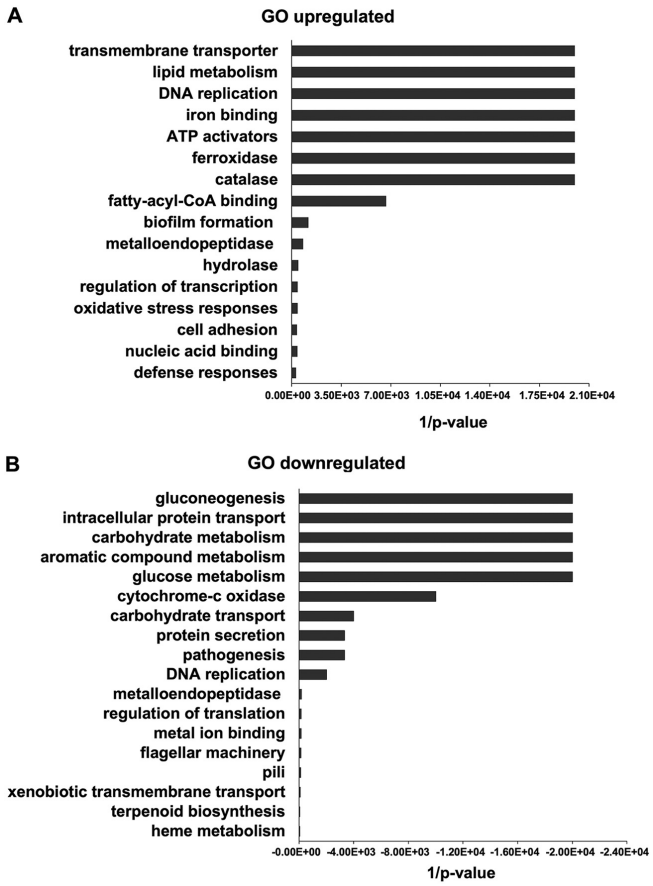

FIG 1 Overview of DEG functional analysis of *P. luminescens* 2° cells in response to pea root exudates. Most significant gene ontology (GO) categories of DEGs upregulated (A) and downregulated (B) in *P. luminescens* 2° cells in the presence of pea root exudates.

cells collected at the logarithmic (6 h) as well as at the late stationary growth phase (24 h). Since we intended to gather all bacterial genes affected by the root exudates, we pooled the differentially expressed genes (DEGs) of both time points. Analysis of the transcriptome profile showed that the expression of 741 genes (see Fig. S1 and Table S1 in the supplemental material), representing ~6% of the transcriptome, were significantly altered ($-1 \leq \log_2$ fold change ≥ 1; $P \leq 0.05$) in response to the root exudates; specifically, 233 DEGs were upregulated and 508 showed downregulation. The DEGs were analyzed to identify their function and the respective gene ontology (GO) terms. The GO terms highlighted as the most important functional classes of the DEGs upregulated in response to the root exudates are putative transmembrane transporters, lipid metabolic enzymes, transcriptional regulators, iron-binding proteins, ATP activators, ferroxidase, and catalase (Fig. 1a), whereas many of the significantly downregulated genes showed GO functional classes involved in gluconeogenesis, carbohydrate metabolism, protein and carbohydrate transport, and aromatic compound metabolism (Fig. 1b).

The putative functions of the identified DEGs indicate a profound switch in the lifestyle of the bacteria, especially in metabolism. Particularly, the downregulation of gluconeogenesis and changes in carbohydrate metabolism support the idea of a switch in sugar metabolism when the cells are faced with the plant roots after an insect infection cycle where preferentially other carbohydrates are used. This is in accordance with the different sugars that we identified in the pea root exudates (see Table S2 in the supplemental material). In the presence of the exudates, we found that the gene *PluDJC_05975*, which is homologous to *csrA*, is upregulated. CsrA is a glycolysis activator and a gluconeogenesis repressor in *Escherichia coli*, and the corresponding gene was also found to be upregulated in the presence of spinach root exudates (20). Therefore, it is likely that PluDJC_05975 has a similar function to regulate sugar metabolism in *P. luminescens* 2° cells.

Moreover, the transcriptome analysis presented here spotlights a drastic transcriptional reprogramming of *P. luminescens* 2° cells, probably due to root signaling molecules contained in the medium. Indeed, the modulation of a large set of genes encoding transcriptional regulators, which represents ~5.5% of all DEGs, was influenced by root exudates as, e.g., observed for XRE- and LuxR-like transcriptional regulator proteins (of which the majority showed positive regulation) (Fig. 2a; see also Table S1). The relationship between plant-derived molecules and LuxR- and XRE-like regulators has been demonstrated for other bacteria before. A plant compound from leaf macerate, an ethanolamine-derived small molecule, activates the LuxR-like receptor PipR and, therefore, its regulated genes in *Pseudomonas* GM79 (21, 22). Additionally, the LuxR-like protein OryR of *Xanthomonas oryzae* possesses an acyl homoserine lactone (AHL)-binding domain that specifically responds to a plant-derived molecule (23). XRE regulators can be associated with carbon metabolism (24), and thus, these regulators might also be involved in the regulation of carbohydrate metabolism and transport, processes found negatively modulated in the transcriptome analysis presented here. In addition, downregulation of protein transport and changes in carbohydrate metabolism as well as differential expression of several regulatory genes were also observed for *Bacillus mycoides* in response to potato root exudates (25).

Genes involved in flagellar motility and chemotaxis, i.e., *flgG*, *flgE*, and *fliC*, were downregulated (Fig. 2a), showing that root exudate attractants and their concentration could play a role in motility and chemotaxis for a successful colonization of the rhizosphere by *P. luminescens* 2° cells. In a transcriptional profiling of *Pseudomonas aeruginosa* PAO1, genes encoding FlgE and FliC were found to be downregulated in response to sugar beet root exudates (26). Moreover, for *Pseudomonas putida* KT2440, an enhanced chemotaxis at a certain distance to the roots could be demonstrated. Indeed, low root exudate concentration increased chemoreceptor transcription levels, thus positively modulating the motility and chemotaxis related genes. This process was reversed at root proximity, where the concentration of root exudates is higher (27). This observation could reflect the capacity of *P. luminescens* 2° cells to detect concentration differences of root exudates in the rhizosphere. Another interesting gene found positively modulated by the root exudates was *fliZ*. FliZ contains a DNA-binding domain that could play a direct role in type II flagellar gene transcriptional regulation by direct binding to the *flhD* promoter as reported for *Xenorhabdus nematophila* (28). Additionally, FliZ of *Xenorhabdus* could also be involved in the regulation of motility and mutualism. Moreover, FliZ together with RpoS promotes the adhesion of *Xenorhabdus* in the intestinal region of the soil-dwelling nematodes (29). Therefore, FliZ could also trigger in cooperation with the RpoS-encoding gene *PluDJC_03680*, which was upregulated in response to the root exudates, the adhesion of *P. luminescens* 2° cells onto plant roots.

Bacterial type VI secretion systems (T6SSs) play a key role in interbacterial competition. They are molecular weapons projected to deliver toxic effectors into prey cells, thus providing advantages for T6SS active strains in polymicrobial environments (30). For *P. putida*, it has been reported that the T6SS is important for the fight against competitors like *Xanthomonas campestris*, thereby reducing leaf necrosis of the plant

A

	log_2 fold-change	Gene	Function
Type IV Secretion	2.65	*PluDJC_04230*	Type VI secretion system tip protein VgrG
	2.10	*PluDJC_01825*	Hcp family type VI secretion system effector
	-2.47	*PluDJC_00025*	Hcp family type VI secretion system effector
	-2.90	*PluDJC_22815*	Type VI secretion system baseplate subunit TssF
	-2.93	*PluDJC_16640*	Hcp family type VI secretion system effector
	-3.17	*PluDJC_01815*	Type VI secretion system contractile sheath large subunit
Flagella	1.85	*PluDJC_10165*	Flagellar regulatory protein FliZ
	-2.41	*PluDJC_09970*	Flagellar basal body rod protein FlgF
	-2.18	*PluDJC_09690*	Flagellar transcriptional regulator FlhC
	-2.02	*PluDJC_09975*	Flagellar basal-body rod protein FlgG
	-3.34	*PluDJC_09965*	Flagellar hook protein FlgE
	-3.95	*PluDJC_10155*	Flagellin FliC
Transcription factors	5.19	*PluDJC_03960*	XRE family transcriptional regulator, MrfJ Protein, repressor
	2.88	*PluDJC_19160*	Transcriptional regulator BolA
	2.65	*PluDJC_06425*	Ferric iron uptake transcriptional regulator
	2.53	*PluDJC_19360*	Phosphate regulon transcriptional regulatory protein PhoB
	2.34	*PluDJC_13835*	HTH-domain-containing protein, AraC transcriptor regulator
	2.19	*PluDJC_20235*	DNA-binding transcriptional regulator Fis
	1.82	*PluDJC_14965*	LysR family transcriptional regulator
	1.79	*PluDJC_22695*	HTH-type transcriptional repressor, nsrR
	1.68	*PluDJC_20450*	LysR family transcriptional regulator
	1.66	*PluDJC_10030*	XRE family transcriptional regulator
	1.40	*PluDJC_04730*	HTH transcriptional regulator
	1.40	*PluDJC_13425*	Fatty acid metabolism transcriptional regulator FadR
	1.37	*PluDJC_10415*	LuxR family transcriptional regulator
	1.36	*PluDJC_19735*	XRE family transcriptional regulator
	1.19	*PluDJC_22740*	LysR family transcriptional regulator
	1.14	*PluDJC_05665*	LysR family transcriptional regulator
	1.00	*PluDJC_10415*	LuxR family transcriptional regulator
	-1.08	*PluDJC_21240*	Transcriptional regulator
	-1.13	*PluDJC_19300*	Transcriptional regulator NrdR
	-2.18	*PluDJC_09690*	Flagellar transcriptional regulator FlhC
	-2.38	*PluDJC_09505*	MurR/RpiR family transcriptional regulator
	-3.31	*PluDJC_04850*	LuxR family transcriptional regulator
Others	6.88	*PluDJC_11885*	Chitinase
	5.36	*PluDJC_12460*	Chitin-binding protein
	3.10	*PluDJC_14950*	Iron ABC transporter permease
	2.25	*PluDJC_09560*	Biofilm formation regulator BssS
	1.93	*PluDJC_14955*	Iron ABC transporter substrate-binding protein
	-6.76	*PluDJC_20195*	Bcr/CflA family efflux MFS transporter

B

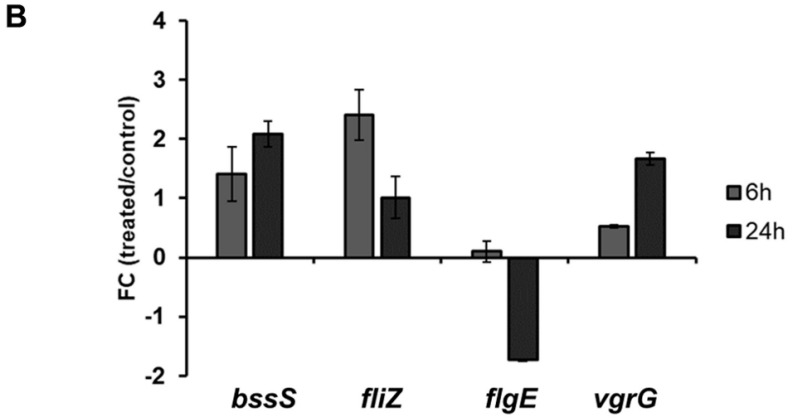

FIG 2 (A) DEGs of *P. luminescens* 2° cells in response to pea root exudates. Subset of *P. luminescens* 2° DEGs that showed modulated expression in response to root exudates from *Pisum sativum* variant *Arvica*. The first column represents the different gene classes. The second column shows the relative gene expression level ($-1 \leq \log_2$ fold change ≥ 1; $P \leq 0.05$) of *P. luminescens* 2° cells cultivated with root exudates in comparison to that of those cultivated in the absence of the exudates. The third column

(Continued on next page)

Nicotiana benthamiana induced by this phytopathogen (31). In *Pseudomonas fluorescens* Pf29Arp, T6SS genes were expressed when the bacterium was located on healthy roots, which further increased on fungus-infected roots (32), suggesting not only an interbacterial competition role in *P. fluorescens* but also a possible root signal involved in the modulation of the T6SS in *P. luminescens*. Indeed, *P. luminescens* 2° T6SS genes showed a complex modulation pattern in response to the root exudates, which is not unusual since root exudates include a complex mixture of metabolites, small molecular signals, and inhibitory compounds (33, 34) (Fig. 2a; see also Table S2 for the root exudate composition).

The comparative transcriptome analysis further highlighted genes that are supposed to be implicated in microbe-plant interaction and colonization, such as the biofilm formation regulator BssS (*PluDJC_09560*), a putative chitinase (*PluDJC_11885*), an iron ABC transporter permease (*PluDJC_14950*) that was upregulated, and the xenobiotic transporter (*PluDJC_20195*), which was downregulated (Fig. 2a; see also Table S1). *PluDJC_09560* is homologous to *yceP* (*bssS*) in *E. coli* K-12, a biofilm formation regulator, which regulates several genes involved in catabolite repression, stress responses, regulation of quorum sensing (QS), and putative stationary-phase signal(s). Moreover, it has been reported that YceP is implicated in the regulation of indole synthesis as well as its uptake and secretion together with YliH (35). Indole is involved in interkingdom signaling between bacteria and plants, and it acts as a potent plant growth modulator as reported for *Arabidopsis thaliana* (36). This suggests a possible similar function of BssS (PluDJC_09560) in *P. luminescens* 2° cells, besides regulation of stress response and QS, by regulating secretion of indole, which is used as a remote messenger to manipulate plant growth and development.

Chitinases are very useful in agriculture as biocontrol agents against phytopathogenic fungi due to their ability to hydrolyze the chitinous fungal cell wall (37). The transcriptome analysis presented here shows a chitinase-encoding gene (*PluDJC_11885*) upregulated in the presence of plant root exudates, hypothesizing that *P. luminescens* 2° cells secrete a chitinolytic enzyme in the rhizosphere environment, a characteristic behavior observed for PGPR such as *Pseudomonas* and *Bacillus* spp. (38, 39).

Iron ABC transporters are involved in siderophore-dependent iron uptake pathways, and they were highlighted as important plant root colonization genes in *Pseudomonas simiae* and *P. putida* (40). Iron is a highly insoluble important micronutrient required by microbes and plants in the rhizosphere. The production of iron-binding ligands and transporters ensures advantages over other microorganisms, e.g., phytopathogens (41). In fact, microorganisms producing siderophores restrict the growth of deleterious microorganisms by limiting iron availability and at the same time promoting plant growth (42). For instance, the expression of genes encoding iron binding and transporter activity was modulated in the presence of the root exudates in *P. luminescens* 2° cells. Particularly, *PluDJC_14950*, encoding a putative iron ABC transporter permease, was positively regulated. PluDJC_14950 is homologous to the cation ABC transporter ATP-binding protein PP_3802 of *P. putida*, which was found to be important for root colonization (40). This suggests that plant root exudates might influence the siderophore activity in *P. luminescens* 2° cells, which could be a survival strategy of the bacteria in plant root environments.

In the rhizosphere, rhizodeposits (exudates released from the root cap cells) and root exudates shape the microbial population, a process important for the defense against plant-pathogenic fungi, bacteria, nematodes, and viruses (43). Some microor-

FIG 2 Legend (Continued)
describes the gene names and their putative function. Red represents upregulation, whereas green denotes downregulation of gene expression. (B) Real-time qPCR considering selected *P. luminescens* 2° DEGs to confirm the RNA-seq data analysis. The plot shows the fold change (FC) (*P. luminescens* 2° cells in LB with pea plant root exudates ["treated"]/*P. luminescens* 2° cells in LB ["control"]) expression level of the following selected genes of interest: *PluDJC_09560* (*bssS*), *PluDJC_10165* (*fliZ*), *PluDJC_09965* (*flgE*), and *PluDJC_04230* (*vgrG*). The analysis was performed at 6 h (gray) and 24 h (dark gray) postinoculation of the cells. Error bars represent standard deviation of at least three independently performed biological experiments.

ganisms have developed strategies to increase their capacity to resist antimicrobial rhizodeposits and xenobiotic compounds released by the roots and heavy metals in the soil. For instance, *Sinorhizobium meliloti* can degrade rhizopine, a compound toxic for microorganisms and found in nitrogen-fixing nodules (44). Bcr/CflA xenobiotic antiporters are also involved in heavy metal resistance and copper homeostasis (45). We found the Bcr/CflA major facilitator superfamily members to be downregulated in *P. luminescens* 2° cells in the presence of root exudates. This indicates a putative capacity of *P. luminescens* 2° cells to modulate mechanisms to cope with xenobiotic compounds released by the roots or with heavy metals present in the soil, thus providing a selective advantage over other bacteria to survive in the rhizosphere, especially in the presence of heavy metals.

The comparative transcriptome analysis successfully identified candidate genes that are involved in the interaction of *P. luminescens* 2° cells with plant roots. We could also validate the results of the RNA-seq analysis via real-time quantitative PCR (RT-qPCR) using selected DEGs (Fig. 2b). Nevertheless, it is important to consider not only the advantages but also the limitations of our approach. One limitation is that the transcriptome profiling was performed from cultures grown in exudate-supplemented complex medium. Therefore, it is possible that exudate effects might be inhibited or overrun by medium components and, consequently, not affecting gene expression anymore. Furthermore, a putative dilution of several exudate molecules in the growth medium could lead to a loss of induction or repression of the bacterial gene expression. In the future, we will therefore consider different root exudate fractions to identify the signal molecule(s) that is important for the *P. luminescens*-plant root interaction. Finally, it will be necessary to analyze the molecular mechanism(s) behind the interaction between this insect pathogen and plants.

Evaluation of phenotypic traits important for the *P. luminescens*-rhizosphere interaction. (i) Chitin degradation and fungal growth inhibition activities. The comparative transcriptome analysis highlighted *PluDJC_11885* encoding a putative chitinase as the most upregulated gene in response to the plant root exudates. This result suggested that *P. luminescens* 2° cells could have chitin degradation activity in the presence of plant root exudates. For that reason, we further investigated the ability of *P. luminescens* to degrade chitin and inoculated the two phenotypic forms of *P. luminescens* and *P. simiae* WCS417, a PGPR that is already characterized (46), on chitin agar plates. The chitin degradation activity of *P. luminescens* 2° cells cultivated in the presence of root exudates was significantly higher ($P \leq 0.05$) than that in their absence (Fig. 3a and b). Although *P. luminescens* 1° cells also exhibited chitinase activity, the activity was not significatively influenced by the root exudates. The increasing chitin degradation activity of *P. luminescens* 2° cells in the presence of root exudates was in line with what was observed for *P. simiae* (Fig. 3a and b). We then tested the capacity of *P. luminescens* to inhibit fungal growth, considering *P. luminescens* 1° and 2° cells as well as *P. simiae* cells cultivated with or without root exudates against *Fusarium graminearum* strain HM6PIS. *P. luminescens* 2° cells were able to inhibit the growth of *F. graminearum* HM6PIS in the presence of the root exudates, a behavior also observed for *P. simiae* (Fig. 3c). In contrast, *P. luminescens* 1° cells did not show any fungal growth-inhibitory effect. This result is in line with our initial hypothesis that *P. luminescens* 2° cells could be more adapted to a free-soil lifestyle, subsequently interact with plant roots, and protect them from pathogenic fungi. However, this fungal growth-inhibitory effect observed for *P. luminescens* 2° cells is not only due to the chitinase-related gene *PLUDJC_11885*, since this inhibitory effect could also be observed for its Δ*PLUDJC_11885* mutant (see Fig. S2 in the supplemental material). Therefore, chitinase activity could be involved in a more complex fungal growth-inhibitory pathway which is not yet clear. Finally, RT-qPCR was performed to confirm the chitinase expression pattern observed during RNA-seq analysis. In this analysis, we also considered *P. luminescens* 1° cells to test whether the corresponding gene *PluDJC_11885* is also influenced by the presence of root exudates in this phenotypic variant. Gene expression analysis showed a positive effect of the root exudates on *P.*

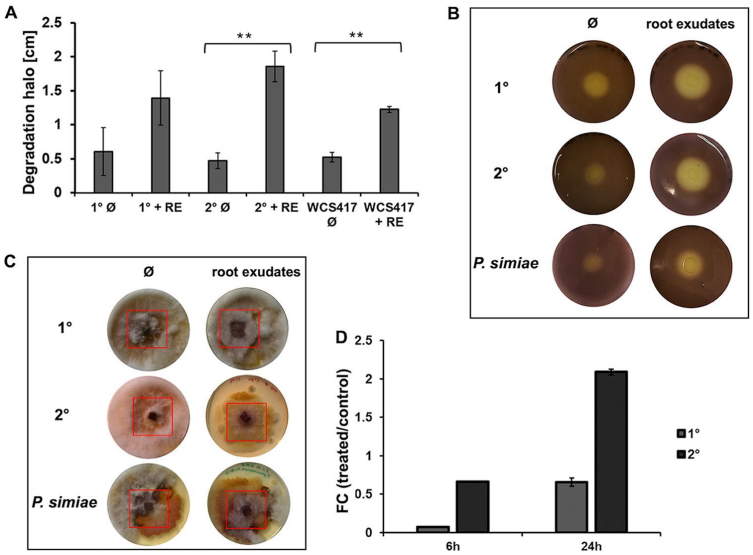

FIG 3 Plant root exudates influence the chitin degradation capacity and fungal growth inhibition of *P. luminescens* 2° cells. (A) Chitin degradation halos (shown in panel B) in centimeters obtained during the chitin degradation assay. The plot shows the degradation halo (Ø) measured with ImageJ represented by the average and the standard deviation of three biological replicates (**, $P \leq 0.05$). RE, root exudates. (B) Chitin degradation halo of *P. luminescens* 1° cells (1°), *P. luminescens* 2° cells (2°), and *P. simiae* WCS417 cultivated with or without root exudates. (C) Fungal growth inhibition assay using phytopathogenic *Fusarium graminearum* HM6PIS performed on YMG agar plates. *P. luminescens* 1° cells, *P. luminescens* 2° cells, and *P. simiae* WCS417, cultivated with and without plant root exudates, were placed around HM6PIS (red square) and incubated for 14 days at 26°C. (D) Expression level (fold change) of the chitinase-encoding gene *(PluDJC_11885)* in *P. luminescens* 1° and 2° cells using real-time qPCR analysis. *P. luminescens* 1° and 2° cells were cultivated in LB medium with root exudates (treated) or without (control) and collected at 6 h and 24 h postinoculation. Error bars represent standard deviation of at least three independently performed biological experiments. All pictures represent one characteristic of at least three independently performed experiments with similar outcomes.

luminescens 2° cells at 24 h postinoculation (4-fold upregulation), whereas in 1° cells, expression of this gene was only slightly influenced by the root exudates (Fig. 3d).

(ii) Chemotaxis and swimming assay. Chemotaxis is an important feature in PGPR. It allows bacterial movement toward the root surface, and it has been identified to be one of the first colonization steps (47). For that reason, we tested chemotaxis through swimming activity of *P. luminescens*, considering both cell variants in the presence of root exudates, using tryptone LB (without yeast extract) or M9 (to exclude any effect of LB compounds) soft agar plates. We could show that *P. luminescens* 2° cells chemotactically responded to the portion of the root exudates extracted with MetOH (Fig. 4a and b). A similar behavior was observed for *P. simiae* (Fig. 4a and b). Moreover, for a higher concentration of root exudates extracted with MetOH (5% to 10%), both *P. luminescens* 2° and *P. simiae* cells showed a similar swarming pattern in LB and M9 (Fig. 4b and d). Neither *P. luminescens* 1° cells nor *P. luminescens* mutants (with inactivation of the two chemotaxis receptor genes *PluDJC_09715* and *PluDJC_09720* or flagellin [Δ*fliC* used as negative control] were inactivated) (Fig. 4c) showed any chemotaxis activity. In summary, these results highlight the capacity of *P. luminescens* 2° cells to chemotactically respond to attractants or repellents in plant root exudates and focus the attention on chemotaxis receptors PluDJC_09715 and/or PluDJC_09720. These receptors are homologous to Tar (type II methyl-accepting chemotaxis protein) and Tsr (MCP-I) of *E. coli*, respectively. Tsr and Tar are involved in chemotaxis activity toward serine, maltose, and aspartate (48), compounds released by plant roots in the rhizosphere (49) and that are also present in the pea root exudates used here (see Table S2).

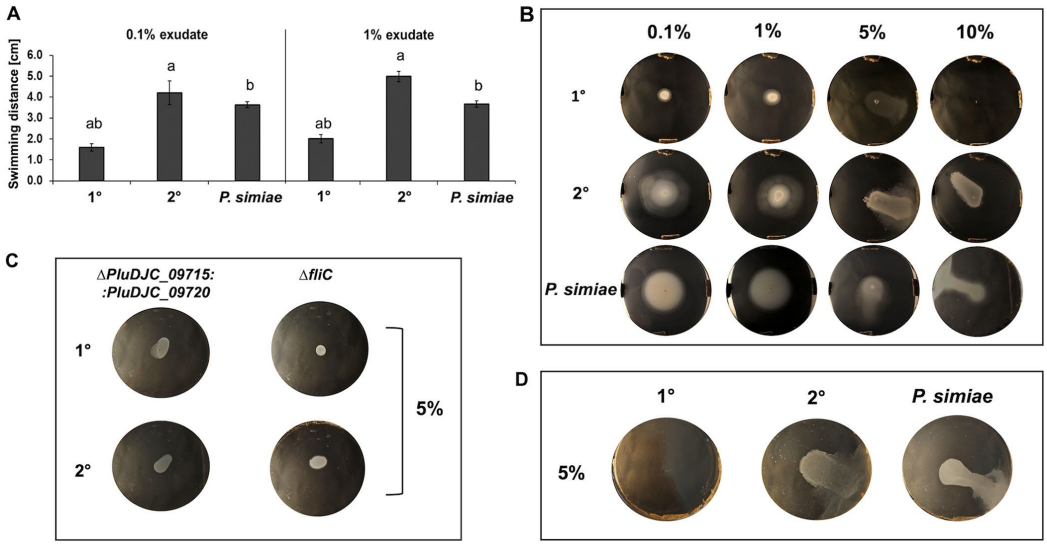

FIG 4 Chemotaxis, swimming, and swarming. The chemotaxis assays were performed in LB swimming agar plates using the MetOH-extracted fraction of pea root exudates. (A) Quantification of the swimming assays shown in panel B using 0.1% and 1% pea plant root exudates. The plots show the swimming halo measured with ImageJ represented by the average and the standard deviation of four biological replicates (different lowercase letters between the bars indicate a *P* value of ≤0.05). (B) Chemotaxis assays of *P. luminescens* 1° and 2° cells as well as *P. simiae* WCS417 using 0.1%, 1%, 5%, and 10% of plant root exudates, respectively. *P. luminescens* 2° cells and *P. simiae* show swimming behavior at a concentration of 0.1% and 1% of plant root exudates, while at ≥5%, they showed swarming behavior. (C) Chemotaxis assays using *P. luminescens* 1° and 2° chemotaxis receptor Δ*PluDJC_09715* Δ*PluDJC_09720* (double deletion) and Δ*fliC* (negative control) mutants. (D) Swarming assays on M9 minimal medium with 5% of plant root exudates to exclude LB compounds to be responsible for swarming. All images represent one characteristic of four independently performed experiments with similar outcomes.

(iii) Lateral root formation induction and root colonization. Root hairs (RH) and later roots (LR) are important root traits that facilitate plant anchorage and water and mineral scavenging. Beneficial microorganisms can induce alteration in root morphology, enhancing LR and RH formation as demonstrated for *Pseudomonas* species rhizobacteria (46). To get insights on the root development effect caused by *P. luminescens*, we analyzed the developmental responses of *A. thaliana* Col-0 to *P. luminescens* 1° and 2° cells considering also *P. simiae* WCS417 for comparison, since the effect of this microorganism on root development was already established. After 8 days of cocultivation, we observed a reduction of ~20% of the primary roots exposed to *P. luminescens* 2° cells compared to that of the negative control. This result was similar for the seedlings exposed to *P. simiae*. In contrast, primary roots exposed to *P. luminescens* 1° cells showed only a small reduction (Fig. 5a and b). A similar root development was observed considering the same experiment using a split plate (Fig. 5b). In this experiment, the bacteria were placed only in one side of the plate, and the root development was analyzed. In cases of whether similar plant root development can be observed in both sides of the split plate, involvement of bacterial volatile organic compounds (VOCs) in this mechanism can be concluded, which was the case for *P. luminescens* 2° cells as well as for *P. simiae*.

In summary, these results indicate that plant roots reduced primary root elongation in response to *P. luminescens* 2° cells, an effect that might be due to the inhibition of cell expansion as has also been reported for *P. simiae* (46). Finally, we investigated the capacity of *P. luminescens* 2° cells to colonize plant roots. For that purpose, *Arabidopsis* roots were colonized with *P. luminescens* 1° and 2° cells as well as *P. simiae* as a positive control. This analysis showed a similar colonization pattern for *P. luminescens* 2° cells and *P. simiae*, highlighting the capacity of *P. luminescens* 2° cells to specifically colonize

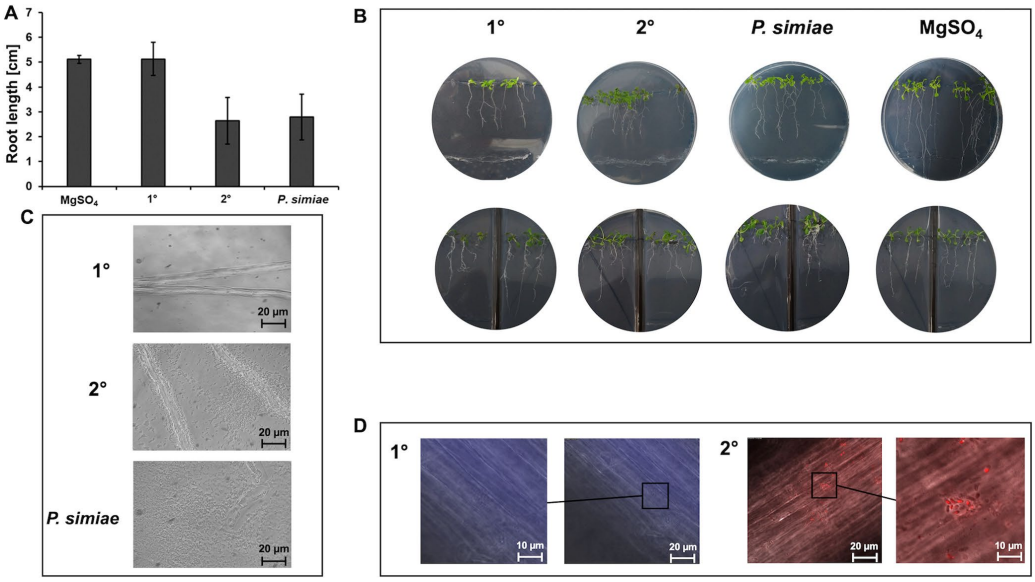

FIG 5 Bacterium-plant cocultivation assays and VOC tests on *Arabidopsis thaliana* Col-0 seedlings. (A) *P. luminescens* 1° and 2° cells as well as *P. simiae* WCS417 were spotted at a 5-cm distance from 4-day-old *A. thaliana* Col-0 seedlings on MS agar plates and cultivated for 8 days at 24°C (plates are shown in panel B, top). The root lengths were measured using ImageJ. Error bars represent standard deviation of at least three independently performed experiments. For the split agar assays (B, bottom), the left side of the MS agar plates contained only the seedlings, while on the right side, the respective bacteria were spotted at the bottom of the plate to test whether VOCs produced by bacteria have an influence on plant root length and development. (C) Phase contrast microscopy of *A. thaliana* roots colonized with *P. luminescens* 1° and 2° cells as well as *P. simiae* WCS417 (positive control). (D) Fluorescence microscopy of *A. thaliana* roots with attached *P. luminescens* 1° cells tagged with mTFP and *P. luminescens* 2° cells tagged with mCherry. All pictures show one representative of at least three independently performed experiments.

the plant roots, features that were not observed for *P. luminescens* 1° cells (Fig. 5c). Following this observation, we then investigated the capacity of *P. luminescens* 1° and 2° cells to attach to the *Arabidopsis* roots. For that purpose, *P. luminescens* 1° cells tagged with monomeric teal fluorescent protein (mTFP) and *P. luminescens* 2° cells tagged with mCherry were exposed to *Arabidopsis* roots. Then, the roots were washed and analyzed by fluorescence microscopy using the appropriate fluorescence channels (Fig. 5d). *P. luminescens* 2° cells were found attached to the *Arabidopsis* root surface, whereas for 1° cells, we could not detect bacterial cells attached to the roots. The exact mechanisms of *P. luminescens* 2° cells that influence root development, alteration, and attachment remain to be clarified. Further analyses must be performed to understand how *P. luminescens* 2° cells and their volatile compounds can influence plant root development and their role in triggering plant ISR. For instance, some PGPR can influence auxin transport and signaling by influencing the ethylene and jasmonic acid pathways (50), thus suggesting a possible signaling mechanism of *P. luminescens* 2° cells to interact with plant roots and trigger their ISR.

In conclusion, in this study, we could show that *P. luminescens* 2° cells have an alternative lifestyle in the soil in the absence of their nematode partners and away from infecting insects (Fig. 6). The bacteria can specifically respond to and interact with plant roots after undergoing the phenotypic switch from 1° to 2° cells and being left by the nematode partner in soil after the insect infection cycle. In this context, it seems that the plant can benefit from this interaction since the bacteria promote root development and can defend the plant from phytopathogens. Since 2° cells are still pathogenic toward insects, it can be assumed that they also protect the plant from insect predators. Whether and when the bacteria can reenter their pathogenic life cycle through a

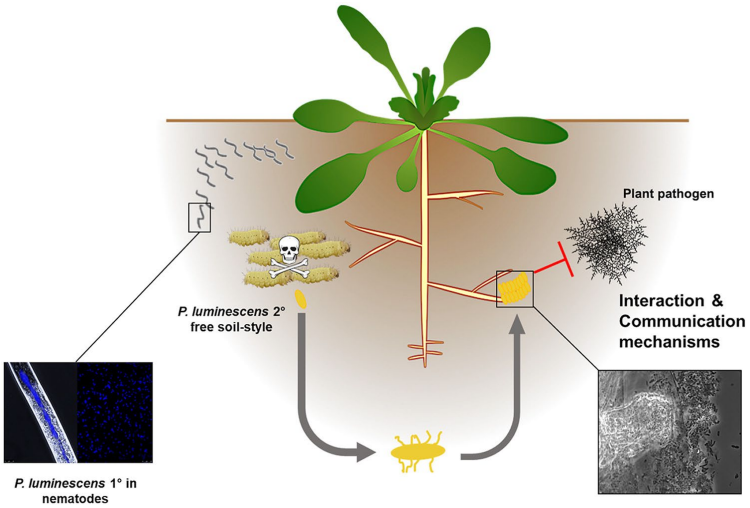

FIG 6 Model of the *P. luminescens* 2° cell alternative life cycle in the rhizosphere. During the insect infection cycle, single *P. luminescens* cells undergo phenotypic switching from the 1° to the 2° phenotype. The 2° cells cannot reassociate with the nematode symbiosis partner and are left behind in the soil when the nematodes leave the depleted insect cadaver. Then, the 2° cells chemotactically respond to plant root exudates and specifically colonize and attach to the roots. The metabolism of the bacteria adapts to plant-derived nutrients, and the cells protect the plants from phytopathogens. Since 2° cells are still pathogenic against insects, it can be assumed that they also protect the plant roots from insect predators.

possible reswitch from 2° to 1° remain unclear. Overall, this work broadens our understanding of both beneficial and insect-pathogenic bacterial responses to a host plant and might help to improve sustainable agricultural techniques using EPNs in the future.

MATERIALS AND METHODS

Bacterial and fungal strains. For this study, 1° and 2° cells of *P. luminescens* strain DJC were used (51). *Pseudomonas simiae* WCS417 (Utrecht University, The Netherlands) was used as a positive control strain for plant interactions. *Fusarium graminearum* HM6PIS (Institute of Biotechnology and Drug Research [IBWF], Kaiserslautern, Germany) was used for fungal growth inhibition activity assays. *P. luminescens* 1° and 2° Δ*PluDJC_09715* Δ*PluDJC_09720* (double mutant), Δ*PluDJC_fliC*, and Δ*PluDJC_11885* in-frame deletion mutants were obtained through conjugation and homologous recombination. For that purpose, the upstream and downstream fragments (500 bp) of the desired regions were cloned into the pNPTS138-R6KT suicide vector using appropriate primers (listed in Table 1), and conjugation was performed as previously described (52). *P. luminescens* 1° and 2° cells tagged with mTFP and mCherry under the control from an exogenous P_{tac} promoter were obtained using the method described earlier (53).

The bacteria were aerobically cultivated at 30°C in LB medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl), M9 minimal medium (33.7 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.55 mM NaCl, 9.35 mM NH_4Cl, 1 mM $MgSO_4$, 100 μM $CaCl_2$, 0.2% [wt/vol] glucose), or YMG medium (1% [wt/vol] malt extract, 0.4% [wt/vol] yeast extract, 1% [wt/vol] glucose, pH 5.5), respectively.

Root exudate collection. Root exudates were collected from *Pisum sativum* variant *Arvica* (Bayerische Futtersaatbau, Ismaning, Germany) grown in controlled conditions (2 weeks incubation at 24°C; 16 h light/8 h dark regime). Then, 75 plants were collected and washed of vermiculite residues, and roots were put into vessels containing 250 ml of sterile distilled H_2O or methanol (MetOH) with continuous shaking for 12 to 14 h to ensure the extraction of most root exudate substances and signaling molecules. The root exudate solutions were filter sterilized, lyophilized (H_2O portion), and stored at −20°C in the dark until use.

Transcriptome profiling and RNA-seq analysis. The influence of root exudates collected from *Pisum sativum* variant *Arvica* on the transcriptome of *P. luminescens* 2° cells was investigated by using RNA-seq. *P. luminescens* 2° cultures were cultivated in 50 ml LB medium supplemented with 3% (vol/vol) root exudates (treated) or in LB medium without root exudates (control). The pea root exudates used were collected from the same batch. The cultures were aerobically grown under shaking at 30°C, and the

TABLE 1 List of primers used in this study

Primer name	Sequence	Characteristic
Up*09715*_fw_BamHI	CCTAGGATCCTATCGAAATACTGAAAGTACAGGAG	*PluDJC_09715 PluDJC_09720* deletion double mutant
Up*09715*_rv_ovl	CGTCAGTAGATCTTAAACATGTTTTCCCTTTTTACAATAG	*PluDJC_09715 PluDJC_09720* deletion double mutant
Down*09720*_fw_ovl	GATCTACTGACGTCAGACTCACTGAGGCCAGATG	*PluDJC_09715 PluDJC_09720* deletion double mutant
Down*09720*_rv_EagI	CGTTCGGCCGCATCCAGTCGATAAACCCCTTTG	*PluDJC_09715 PluDJC_09720* deletion double mutant
Up*fliC*_fw_BamHI	ACGGGATCCGGCAACGAATGCATCATG	*fliC* deletion mutant
Up*fliC*_rv_ovl	CCCTAGCTGAGCGATTAACGTGCCATAGTTAGAGTTCC	*fliC* deletion mutant
Down*fliC*_fw_ovl	GGAACTCTAACTATGGCACGTTAATCGCTCAGCTAGGG	*fliC* deletion mutant
Down*fliC*_rv_EagI	ACTCGGCCGCAATCACGGCTCCTTAAC	*fliC* deletion mutant
Up*11885*_fw_BamHI	GAGGGATCCCCATATATAACCTCTCCTGA	*PluDJC_11885* deletion mutant
Up*11885*_rv_ovl	CCTGAGCTTGACATAAATCACCTCGACTAG	*PluDJC_11885* deletion mutant
Down*11885*_fw_ovl	AAGCTCAGGCATAATTAATTAAGCCAAGCCAC	*PluDJC_11885* deletion mutant
Down*11885*_rv_EagI	TGACGGCCGGTTGGAATTTCACTGCGCAG	*PluDJC_11885* deletion mutant
*rpoD*qPCR_fwDJC	CGGAAGATATCGTCGATTCCGA	Housekeeping, *PluDJC_19710*
*rpoD*qPCR_rvDJC	TGTCGTTAGCGGTTTCTGCT	Housekeeping, *PluDJC_19710*
*chitin*qPCR_fwDJC	GGTCGCAATATGACGGTCG	Chitinase for qPCR, *PluDJC_11885*
*chitin*qPCR_revDJC	GGCAAATAATGGCGCTTGCT	Chitinase for qPCR, *PluDJC_11885*
*vgrG*qPCR_fwDJC	ACAGCTTTATCGCCTGACGTT	*vgrG* for qPCR, *PluDJC_04230*
*vgrG*qPCR_rvDJC	GTCCGTTCGGTGATGCCATT	*vgrG* for qPCR, *PluDJC_04230*
*flgE*qPCR_fwDJC	AGGTGGGACTGGGGGTAAAA	*flgE* for qPCR, *PluDJC_09965*
*flgE*qPCR_rvDJC	ACCGCCTTGCATACGGAAAA	*flgE* for qPCR, *PluDJC_09965*
*bssS*regqPCR_fw	TTTGCAATGTCAGTTGTCAACCA	*bssS* for qPCR, *PluDJC_09560*
*bssS*regqPCR_rv	AACGCATCCTGTTGTAGGCT	*bssS* for qPCR, *PluDJC_09560*
*fliZ*qPCR_fw	TTGTCACAAAGCTCTTGACCGT	*fliZ* for qPCR, *PluDJC_10165*
*fliZ*qPCR_rv	TGCAAAAACGACATAACGCGA	*fliZ* for qPCR, *PluDJC_10165*

cells were harvested after 6 h (exponential growth phase), when the culture reached an optical density at 600 nm (OD_{600}) of 0.8 to 1, and 24 h (stationary growth phase; OD_{600} of 8 to 10). In total, three independent biological replicates were sampled for every condition considered, and the total RNA was isolated using AquaPhenol-chloroform-isoamyl alcohol as described previously (16). Successively, 5 μg of treated RNA was subjected to rRNA depletion using the RiboMinus kit (Invitrogen), and 150 ng of depleted RNA was processed using NEBNext Ultra II directional RNA library prep kit for Illumina (New England BioLabs [NEB]) according to the protocol of the distributor. Finally, a total concentration of 4 mM from the obtained library was sequenced on a MiSeq sequencer (Illumina; 2 × 75 bp paired-end sequencing, v3 chemistry) (Genomics Core Facility, LMU München). Raw reads were trimmed, mapped to the reference genome (*P. luminescens* DJC; GenBank accession number NZ_CP024900.1), and differentially expressed genes (DEGs = $-1 \leq \log_2$ fold change ≥ 1; $P \leq 0.05$) were identified. The function of the DEGs and gene ontology (GO) were extracted from UniProt (https://www.uniprot.org) and NCBI (https://www.ncbi.nlm.nih.gov).

Validation of the transcriptome profiling experiment was carried out by RT-qPCR on selected candidate genes identified from the RNA-seq experiment. The cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) followed by qPCR using specifically designed primers (Table 1) and GoTaq qPCR master mix (Promega). The gene designated as *rpoD* (*PluDJC_19710*) was used as a reference. The relative expression values of the target genes and the standard error (SE) were calculated using the Pfaffl and Simon equations, respectively (54, 55). The primer efficiencies were calculated with the LingRegPCR program (http://LinRegPCR.nl).

HPLC-DAD analysis. High-performance liquid chromatography with diode array detector (HPLC-DAD) analysis of *P. sativum* plant root exudates was performed on a Shimadzu LC 20A Prominence system (Shimadzu, Griesheim, Germany) equipped with two LC-20AD pumps, a DGU-20A degassing unit, a SIL 20AC autosampler, a CBM-20A controller, and a CTO-20AC column oven. Separations were performed using an analysis reversed-phase C_{18} column (Waters SunFire C_{18}; particle size, 5 μm; 4.6 by 250 mm) at 20°C. A linear gradient starting from 99% 0.1% (vol/vol) trifluoroacetic acid and 1% (vol/vol) acetonitrile to 100% (vol/vol) acetonitrile in 20 min and then maintaining 100% (vol/vol) acetonitrile for 3 min and an additional equilibration time of 7 min was used at a flow rate of 1 ml/min. Injection volume of sample solution was 20 μl. A Shimadzu SPD-M20A diode array detector was used from 200 to 800 nm to record the spectra and detect separated metabolites at 210 nm, 250 nm, 300 nm, 350 nm, and 400 nm. Data and spectra were analyzed using the LabSolutions 5.54 software (Shimadzu, Griesheim, Germany).

Chitin degradation activity assays. The chitin degradation activity assay was performed in chitin agar plates (0.01% [wt/vol] peptone, 0.025% [wt/vol] KCl, 0.2% [wt/vol] K_2HPO_4, 0.025% [wt/vol] $MgSO_4$, 1% [wt/vol] colloidal chitin) (56). Overnight cultures of *P. luminescens* 1° and 2° cells and *P. simiae* WCS417 cultivated in LB medium with or without 3% (vol/vol) root exudates were adjusted to an OD_{600} of 0.1 (10^7 CFU/ml), and 50 μl was spotted in the center of the chitin agar plate, which was then incubated for 5 days at 30°C. The resulting halo diameter was measured using ImageJ (https://imagej.nih.gov/ij/), and statistical significance was evaluated through *t* test. Three biological independent replicates were performed.

Fungal growth inhibition assays. For fungal growth inhibition assays, agar plugs harboring actively growing *F. graminearum* HM6PIS were placed into the middle of YMG agar plates. Then, *P. luminescens*

1° and 2° cells and *P. simiae* WCS417 were cultivated overnight in LB medium with or without 3% (vol/vol) root exudates at 30°C. Cultures were adjusted to an OD_{600} of 2. Then, four 50-μl aliquots were spotted and square connected around the fungal plug. The plates were further incubated at 26°C, and fungal growth was observed over 14 days. The experiment was repeated three times.

Chemotaxis and swimming assays. Chemotaxis and swimming assays were performed using soft agar plates containing 0.3% (wt/vol) agar, 1% (wt/vol) tryptone, and 0.3% (wt/vol) NaCl or M9 soft agar plates (M9 medium supplemented with 0.3% [wt/vol] agar) with different concentrations of root exudates or without (control). Overnight cultures of *P. luminescens* 1° and 2° cells and the respective Δ*PluDJC_09715* Δ*PluDJC_09720* double mutant aerobically grown at 30°C were washed with 10 mM $MgSO_4$ and adjusted to an OD_{600} of 0.1 (10^8 CFU/ml). Then, 10 μl of the cell suspensions were spotted in the center of the agar plates and incubated for 24 h at 30°C. The resulting swimming halo diameter was measured using ImageJ (https://imagej.nih.gov/ij/), and statistical significance was evaluated through *t* test. Four independent biological replicates for each considered condition were performed.

Bacterium-plant cocultivation assays and microscopy. Bacterium-plant cocultivation and VOC assays on 4-day-old *Arabidopsis thaliana* Col-0 seedlings cultivated in MS agar (0.4% [wt/vol] MS basal salt mixture, 3% [wt/vol] sucrose, 0.8% [wt/vol] agar) at 24°C with a 16 h light/8 h dark regime were performed as reported previously (46). Briefly, *P. luminescens* 1° and 2° cells and *P. simiae* WCS417 were grown in LB medium at 30°C overnight. Cells were collected by centrifugation (5 min at 5,000 rpm), washed with 10 mM $MgSO_4$, and adjusted to an OD_{600} of 0.002 (10^5 CFU/ml). Then, 240 μl of the bacterial suspension (or 10 mM $MgSO_4$ as control) was spotted at a 5-cm distance of the seedlings. For experiments involving bacterial VOCs, 120 μl of the culture was spotted in one side of the split plate. For experiments involving root colonization, 120 μl (OD_{600} = 0.02) of the previously considered bacteria or *P. luminescens* 1° and 2° cell culture tagged with mTFP and mCherry, respectively, was spotted onto the root tip. For established colonization capacity, after 2 days, the roots were observed by phase-contrast microscopy (Leica; magnification, ×40). For root attachment assays, after 2 days, Col-0 roots were thoroughly washed and then analyzed by fluorescence microscopy (Leica DMi8 fluorescence imaging system) using 4′,6-diamidino-2-phenylindole (DAPI) and Texas Red fluorescence filter to verify the presence of *P. luminescens* cells attached on the roots. The experiments were performed three times.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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We declare no conflict of interest.

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4. The insect pathogen *Photorhabdus luminescens* protects plants from phytopathogenic *Fusarium graminearum* via chitin degradation

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The insect pathogen *Photorhabdus luminescens* protects plants from phytopathogenic *Fusarium graminearum* via chitin degradation

Nazzareno Dominelli[1], Fabio Platz[1], Ralf Heermann[1*]

[1]Johannes-Gutenberg-University Mainz, Institute of Molecular Physiology (imP), Biocenter II, Microbiology and Biotechnology, Hanns-Dieter-Hüsch-Weg 17, 55128 Mainz, Germany
*corresponding author (heermann@uni-mainz.de)

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Abstract

Phytopathogens represent a big agricultural challenge. The use of chemical pesticides is harmful for the environment, for animals and humans. Therefore, new sustainable and biological alternatives are urgently needed. The insect pathogenic bacterium *Photorhabdus luminescens*, already used in combination with entomopathogenic nematodes (EPNs) as biocontrol agent, is characterized by two different phenotypic cell forms called primary (1°) and secondary (2°). The 1° cells are symbiotic with EPNs and are used for biocontrol, the 2° cells are unable to undergo symbiosis with EPNs and remain in the soil after insect infection and specifically interact with plant roots. A previous RNAseq analysis showed that genes encoding exochitinase Chi2A and chitin binding protein CBP are highly upregulated in 2° cells exposed to plant root exudates. Here, we investigate the Chi2A and CBP function and demonstrate that both are necessary for *P. luminescens* 2° cells for inhibiting growth of phytopathogenic *Fusarium graminearum*. We provide evidence that Chi2A digests chitin and thereby inhibits fungal growth. Furthermore, we showed that 2° cells specifically colonize fungal hyphae as one of the first mechanism to protect plants from fungal phytopathogens. Finally, soil pot bioassays proved plant protection from *F. graminearum* by 2° cells, whereas Chi2A/CPB were essential for this process. This work gives molecular insights in a new applicability of *P. luminescens* as plant-growth promoting and protecting organism in agriculture.

Importance

The enteric enterobacterium *Photorhabdus luminescens* is already used as bioinsecticide since it is highly pathogenic towards abroad range of insects. However, the bacteria exist in two phenotypic different cell types called 1° and 2° cells. Whereas only 1° cells are symbiotic with their nematode partner to infect insects, 2° cells were shown to remain in the soil after an insect infection cycle. It was demonstrated that the 2° cells specifically interact with plant roots. Here we show that the bacteria are beneficial for the plants by protecting them from phytopathogenic fungi. A specific colonization of the fungus mycelium as well as chitin degrading activity mediated by the chitin binding protein CBP and the chitinase Chi2A is essential for this process. Our data give evidence for a novel future applicability of *P. luminescens* as plant growth promoting organism and biopesticide.

Introduction

Plants encounter different types of challenges as they usually are susceptible to environmental stress, pests, and diseases. This represents a food security issue regarding a growing population that must be fed since it impacts the crop yields as well as food production. The agricultural major losses derive from weeds, animal pests and from phytopathogens such as bacteria, fungi, and viruses (1). In the past, an excessive use of chemical pesticides improved the crop yields leading to higher food security but also to environmental damages and toxicity against non-target organisms such as pollinators and humans (2, 3) affecting the soil microbiome in its composition as well as the plant´s metabolism and growth (4). Hence, biological pesticides are considered as alternatives. Since they are less harmful than chemical pesticides, they are becoming an emerging branch in biotechnological research. In the last decades biological control agents such as beneficial plant growth promoting rhizobacteria (PGPRs) and entomopathogenic nematodes (EPNs) arose as new sustainable agricultural techniques to protect plants from pests. For EPNs such as *Steinernematidae* and *Heterohabditidae* biocontrol activity against insect pests is well known, moreover, an effect on plant pathogens and indirect improvement of soil quality has been suggested (5, 6). EPNs live in symbiosis with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, which are the main agents involved in insect killing. *Photorhabdus luminescens* occurs in two phenotypically different cell variants. The pigmented primary cell form (1°) lives in symbiosis with the nematodes and, in contrast, the non-pigmented 2° cell form cannot undergo symbiosis with EPNs, so that the bacteria are left in soil after the insect infection cycle (7, 8). Both cell forms are genetically identical (9) (N.D. and R.H., unpublished data), but differ in various phenotypic traits such as bioluminescence, production of secondary metabolites, cell clumping, and biofilm formation, phenotypes that are only present in 1° cells and absent from 2° cells (9–11). Within the big spectra of secondary metabolites produced only by *P. luminescens* 1° cells, an isopropylstilbene (IPS) was observed to have fungicidal effects (12, 13). In recent studies on the reaction and response of *P. luminescens* 2° cells to plant root exudates (PRE), we previously showed that this cell form also can inhibit fungal growth in presence of PRE, speculating that they might also be used as biocontrol agent in plant protection (14). Since 2° cells almost produce very limited spectrum of secondary metabolites, another mechanism to combat phytopathogenic fungi is likely. A previously conducted comparative transcriptome

analysis between 2° cells and 2° cells exposed to PRE revealed chitinase and chitin binding protein (CBP) related genes to be highly upregulated in 2° cells in presence of PRE, thus suggesting their involvement in antifungal activity in order to protect the host plant (14). The fungal cell wall mainly consists of chitin, a heterogenous polymer of β–1,6-linked *N*-acetylglucosamine linked to glucan (15), making the cell wall of phytopathogenic fungi a perfect target for bacterial chitinases. Therefore, chitinases are useful enzymes for biocontrol application in agriculture, since these glycosyl hydrolases catalyze the first step of chitin degradation (16, 17).

For PGPRs like *Pseudomonas fluorescence* chitinase activity has already been demonstrated. The bacteria are capable to reduce fungal growth and germination of phytopathogenic *Aspergillus flavus* (18). Moreover, chitinases derived from *S. marcescens* displayed antifungal activity, pointing out the use of chitin degrading bacteria as biocontrol alternative (19).

Here we investigate the capacity of *P. luminescens* Chi2A (PluDJC_11885) to hydrolyze chitin and inhibit fungal growth of phytopathogenic *F. graminearum*. Furthermore, we examined the ability of *P. luminescens* 2° cells not only to colonize fungal hyphae as first step of competition mechanism, but we also investigated bacterial plant protecting ability in pot bioassays. However, even if chitinases are involved in degradation of chitin and inhibition of fungal growth, respectively, the activity of chitin binding proteins CBPs is still essential for chitinase activity. Overall, this work gives deeper insights about the role of *P. luminescens* 2° in soil, demonstrating a novel applicability of these bacteria in agriculture as plant growth promoting organism and biopesticide.

Material and Methods

Bioinformatic analysis of chitinase encoding genes in *P. luminescens*

The genome of *P. luminescens* harbors three genes, that code for putative exochitinases: PluDJC_11885 (Chi2A), PluDJC_12975 (Chi2B) and PluDJC_12990 (Chi2C). To determine the domain of all three proteins, HMMER a bioinformatic tool using profile hidden Markov Models (https://www.ebi.ac.uk/Tools/hmmer/) was exploited (20). Moreover, multiple sequence alignment was performed using Clustal Omega (21) to compare *P. luminescens* chitinases with described ChiB (WP_016926761.1) of *Serratia marcescens* and Chi2 (WP_064513229.1) of *Yersinia*

entomophaga, both, GH18 protein members with chitinolytic activity and modelling of tertiary protein structure was performed using Phyre2 (22).

Bacterial and fungi strains

In this study antifungal activity of *P. luminescens* DJC 1° and 2° wildtype and the respective mutants were analyzed (23). Deletion mutants of *PluDJC_11885* (*chi2A*), *PluDJC_12460* (*cbp*) were obtained through *in-frame* deletion via double homologues recombination. For that purpose, 500 bp fragments up- and downstream of the desired gene were cloned into pNPTS138-R6KT suicide vector using primer pairs FA *cbp* fw EcoRI + FA *cbp* re ovl FB for flank A and FB *cbp* fw ovl FA + FB *cbp* re EagI for deletion of *cbp* as well as FA *chi2A* fw EcoRI + FA *chi2A* re ovl FB primers for flank A and FB *chi2A* fw ovl FA and FB *chi2A* re EagI for flank B for deletion of *chi2A* (Table 1) fused and inserted into pNPTS138-R6KT using the respective restriction sites. Obtained vectors were transferred into *P. luminescens* cells via conjugation (24, 25). Complementation of the respective mutants occurred by integration of exogenous and constitutive *tac* promoter fused with the respective genes, P_{tac}-*chi2A* and P_{tac}-*cbp,* respectively, into a pPINT. For amplification of the *tac* promoter P*tac* fw PstI + P*tac* re ovl were used, whereas with primer pairs *cbp* fw ovl Pt*ac* + *cbp* re EagI and *chi2A* fw ovl Pt*ac* + *chi2A* re EagI the respective genes were obtained (Table 1) and inserted into pPINT vector using the respective restriction sites. Gene complementation was obtained, as *glmS-rpmE* site of pPINT vector integrates into the respective *P. luminescens* mutant genome (26). These integration vectors were additionally transferred into *P. luminescens* WT cells to overexpress the respective genes. Furthermore, fluorescently labeled *P. luminescens* cells were obtained by mCherry tagging under the control of the exogenous *tac* promoter as previously described (26). *E. coli* BL21 (pLysS) strains were used to heterologeously express *chi2A* or *cbp.* For that purpose, pBAD24-6xHis-*chi2A,* in which the *chi2A* expression is under the control of the inducible *ara* promoter (27) and pET16-*cbp*, in which the *cbp* expression is under the control of a *tac* promoter, were generated using *chi2A*-N6xHis fw XmaI_pBAD24 + *chi2A* re XbaI_pBAD24 and *cbp* fw NdeI_pET16b + *cbp* re XhoI_pET16b, respectively (Table 1). Expression vectors were then transferred into chemical competent *E. coli* cells via transformation. This study includes phytopathogenic *Fusarium graminearum* strain (Institute of Biotechnology and Drug Research, IBWF, Mainz, Germany), an isolate from tomato plant, a phytopathogenic fungus usually

causing head blight or scab on wheat (28) or tomato plants (29) to determine antifungal activity of *P. luminescens*. Bacterial cultures were inoculated into LB medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.5% [w/v] NaCl) supplemented with or without the respective antibiotics and aerobically cultivated at 30°C for *P. luminescens* and or 37°C for *E. coli,* respectively. Fungi were cultivated on YMG agar plates (1% [w/v] malt extract, 0.4% [w/v] yeast extract, 1% [w/v] glucose, pH 5.5) at 26°C. Three biological independent replicates were performed. If designated kanamycin was added to the media with a final concentration of 60 µg/ml and carbenicillin with 100 µg/ml.

Fungal growth inhibition assay

Fungal growth inhibition assays to determine influence of *P. luminescens* 1° and 2° lacking chitinase (Chi2A) and chitin binding protein (CBP) towards *F. graminearum* were performed as previously described (14). Furthermore, *P. luminescens* 1° and 2° wild type and the respective deletion mutant strains transformed with pPINT-P*tac*-*chi2A* or pPINT-P*tac*-*cbp,* overexpressing *chi2A* or *cbp*, were obtained to check whether the wildtype behavior of the deletion mutants with respect to the antifungal growth inhibition can be restored and/or antifungal activity can be enhanced.

E. coli BL21 (pLysS)::pBAD24-6xHis-*chi2A* and pET16-*cbp*, respectively, were also tested to determine whether antifungal effect observed in *P. luminesces* is solely caused by Chi2A and/or Cbp.

For these tests, a fungal agar plug with a diameter of 0.9 mm was placed in the middle of a YMG agar plate. Then, an overnight culture of bacterial strains was adjusted to an OD_{600} of 2.0 and four spots of 50 µl each were dropped around the fungal plug, forming the corners of a square and connected alongside. Plates were then incubated at RT and observed over a period of 14 d. *P. luminescens* and *E. coli* cells carrying a vector for expression of *chi2A* and *cbp*, respectively, were cultivated overnight in LB with the respective antibiotics, then the following day the OD_{600} was adjusted to 0.1 and the bacteria were then cultivated until reaching an OD_{600} of 0.4 and induced with 1 mM IPTG for vectors with *tac* promoter or 0.5% (v/v) *L*-arabinose for vectors with *ara* promoter. Induction of gene expression occurred at 30°C until bacteria reached an OD_{600} 0f 2.0. Here, fungal growth inhibition assay was performed on YMG agar plates (for experiments performed with *E. coli* cells glucose was replaced with 1% glycerol [v/v] in YMG agar) supplemented with the respective antibiotic and inductor. Negative controls were provided by strains carrying the empty vector.

Plant protection activity of *P. luminescens*

To investigate biocontrol activity of *P. luminescens in planta* assays with beef tomato *Solanum lycopersicum* (Magic Garden Seeds; https://www.magicgardenseeds.de) and *F. graminearum* were performed. For that purpose, the surface of *S. lycopersicum* seeds was sterilized for 30 min with 50% (v/v) Chlorix, 20% EtOH (v/v) and washed 3 times with sterile H_2O, and 2-3 seeds were sown on sterile vermiculite in one pot (in total 5 seeds per replicate). Overnight cultures of *P. luminescens* 2° wildtype, Δ*chi2A*, and Δ*cbp* cells as well as 1° cells (control) were adjusted to an OD_{600} of 0.5, washed and resuspended in MS-medium [0.4% (w/v) MS basal salt mixture, 1% (w/v) glucose]. Afterwards, 1 ml of bacterial cell suspension (containing approximately ~$5*10^8$ cells) was sprayed using a vaporizer on the vermiculite surface containing the *S. lycopsersicum* seeds and incubated under controlled conditions (40 days, at 25°C, 16 h light/8 h dark). Control seeds were sprayed with MS-medium without bacteria. For plant infection *F. graminearum* was cultivated in YMG medium [1% (w/v) malt extract, 0.4% (w/v) yeast extract, 1% (w/v) glucose, pH 5.5] for 5 days and 1 ml of a 1:10-diltuion (in MS-medium) was pipetted along the tomato plant stem towards the roots. Then, the pots with the plants were packed into plastic bags to keep high humidity to allow the phytopathogen to grow. After 24 h 1 ml of the bacterial cell suspension of an OD_{600} of 0.5 (in MS-medium) was sprayed along the stem at the same site where the fungus was applied. After 5 days the plants were analyzed for growth and photographically documented. Three independent biological replicates including up to 5 plants each were performed. The experiment was approached using a completely randomized design, meaning that from a selection of 30 seeds, 5 were selected and each seed was treated with the five respective bacterial strains or MS-medium as control, so that 5 plants (distributed over two pots) were treated with similar conditions. This experiment was repeated independently in the same way on different days with different bacterial and fungal cell cultures. The amount of healthy (H) and sick (S) plants was evaluated as 1 and 0, respectively, and the experimental blocks were plotted in a table calculating percentage of survival. Plants that were withered or displayed lesions at the application site, where the fungus was applied, were evaluated as sick (S).

Heterologous overexpression of *chi2A* and purification of the respective protein

Heterologous expression of *chi2A* was carried out in *E. coli* BL21 pLysS strain carrying the recombinant vector pBAD24-6xHis-*chi2A*. For that purpose, *chi2A* was amplified via PCR from *P. luminescens* DJC gDNA using *chi2A*-N6xHis fw XmaI pBAD24 + *chi2A* re XbaI pBAD24 *(*Table 1) and inserted downstream of P_{ara} into the pBAD24 expression vector using restriction sites XmaI and XbaI (carbenicillin resistance) (27) 6xHis codon was N-terminally added to the gene via PCR. An overnight culture of *E. coli* BL21::pBAD24-6xHis-*chi2A* cells inoculated in 1 l of LB medium supplemented with the respective antibiotic at an OD_{600} = 0.1. The culture was then incubated at 37°C and 150 rpm until reaching an OD_{600} = 0.4. Then, gene expression was induced via P_{ara} by addition of 0.5% (v/v) *L*-arabinose and bacteria were further aerobically cultivated at 30°C and 150 rpm for 4 h. The bacteria were then harvested by centrifugation for 30 min at 4,500 rpm at 4°C and the pellet was resuspended in phosphate lysis buffer (50 mM NaH_2PO_2, 300 mM NaCl, 20 mM Imidazole, 0.5 mM PMSF, 2 mM DTT, pH 7.4). Bacterial cells were disrupted using a French press running three cycles at 1.35 kBar, and cell debris was removed by centrifugation for 15 min at 4,500 rpm and 4°C. Separation of bacterial cytosol (supernatant) from the membrane fraction (pellet) was obtained by ultracentrifugation at 45,000 rpm for 1 h at 4°C. As Chi2A is a soluble exoenzyme, the cytosolic fraction was kept for further purification using Äkta™ Pure system (Cytiva).

Ni^{2+}-NTA affinity chromatography

Purification of 6xHis-tag labeled chitinase Chi2A was performed using 1 ml HisTrap™ FF Crude (Cytiva) columns for FPLC in the Äkta™ Pure system (Cytiva) with an attached sample pump S9H (Cytiva) and fraction collector F9-C (Cytiva) module. The cytosolic sample was applied onto the previously equilibrated column and the flowthrough was kept using a fraction collector followed by two washing steps with phosphate buffer [50 mM NaH_2PO_2, 300 mM NaCl, 10% glycerol (v/v), pH 7.4], one with 20 mM Imidazole (buffer A) and the other with 500 mM Imidazole (buffer B) in a 98%:2% ratio. With that unspecific binding was decreased on the column. Elution was obtained with increasing concentration of imidazole starting with a buffer A and B ratio of 98%:2% to final ratio of 70%:30% in a total volume of 10 ml removing Chi2A from the column. Purity of the elution fraction was determined via SDS-PAGE analysis (Fig. S1) (30). Furthermore, Western blot analysis for immunodetection of the protein using

rabbit-anti-His antibody (rabbit monoclonal, clone RM146, Sigma Aldrich) and anti-rabbit antibody [anti-rabbit IgG (whole molecule) – alkaline phosphatase antibody produced in goat, Sigma Aldrich] was performed (Fig. S1).

For *in vivo* chitinase tests of Chi2A on fungi, the cytosolic fraction was incubated for 1 h at 4°C with Ni^{2+}-NTA-Agarose beads (Qiagen, Hilden) under gentle shaking. Then, the beads-protein solution was loaded onto a column and the flowthrough was collected followed by two washing steps with phosphate washing buffer (40 mM Imidazole). Protein elution from the beads occurred using a 5 mM phosphate elution buffer containing 50 mM EDTA, instead of imidazole, to reduce side effects on fungi when applying the purified protein.

Nano Differential Scanning Fluorimetry (nanoDSF)

NanoDSF, a modified version of differential scanning fluorimetry, is a label free technique using the intrinsic fluorescence of the aromatic amino acids tryptophane and tyrosine to determine protein folding and stability (31, 32). To determine the stability of the purified Chi2A over time, Prometheus NT.48 (NanoTemper) was used with standard capillaries (NanoTemper). For that purpose, the protein sample was set up to a concentration of 0.3 mg/ml for every measurement and loaded into capillaries which were then heated with a laser inside the machine leading to unfolding of the intact protein and therefore provides accessibility of the intrinsic aromatic amino acids. The increasing fluorescence was then measured at 320 nm and 350 nm, determining T_m as point where half of the protein is unfolded. The measurement was performed from 20°C to 90°C with a temperature raise of 1.5°C/min. Chi2A stability was measured over a period of 14 d of storage at 4°C.

Chitinolytic activity of Chi2A

A chitinolytic activity of *P. luminescens* Chi2A has not been experimentally demonstrated so far. Therefore, chitin degrading capability of the purified Chi2A was tested by spotting 20 µl of 0.3 mg/ml protein solution in the middle of colloidal chitin agar plates [0.02% (w/v) peptone, 0.05% (w/v) KCl, 0.1% (w/v) K_2HPO_4, 0.5% (w/v) $MgSO_4$, 0.5% (w/v) dried colloidal chitin, 1.5% agar (pH 7.0)] and incubated for 5 d. Colloidal chitin was prepared according to a modified protocol described earlier (14). The plates were then stained using iodine-potassium iodide solution (Lugol's iodine;

Carl Roth, Karlsruhe). Briefly, the plates were covered with Lugol's iodine solution, incubated for 30 min, and washed with H_2O. Degradation of chitin became visible as unstained halo around the spot of application. Further, the purified protein was tested on inhibition of fungal growth on phytopathogenic *F. graminearum*. For that purpose, a fungal agar plug with a diameter of 0.9 mm was placed in the middle of a YMG agar plate and was then incubated for 3 d at 26°C, 20 µl of purified protein (0.3 mg/ml) were spotted right next to the fungal plug. The same amount of elution buffer was spotted on the other side of the plug as control. Additionally, fungal spore germination in soft agar plates was analyzed in presence of Chi2A. One layer of YMG agar (10 ml) was poured in a petri dish and sterile pipet tips were set upside down on top of the solidified agar. After that, minimal medium soft agar [0.00025% (v/v) biotin, 1x nitrate salts, 0.001% (v/v) thiamine, 0.001% (v/v) X trace elements, 0.8% agar] containing *F. graminearum* spores was poured on top of the YMG agar. After solidification pipet tips were removed in order to obtain wells in which 50 µl Chi2A or buffer were pipetted. Fungal growth and spore germination was monitored over time.

Fungal hyphae colonization assays

To investigate whether *P. luminescens* colonize hyphae of *F. graminearum*, the organisms were co-cultivated and analyzed using fluorescence microscopy as described elsewhere (32). For that purpose, fluorescently labeled *P. luminescens* cells (carrying a chromosomal P_{tac}-*mCherry* promoter fusion and constitutively expressing mCherry) were used to determine colonization of fungal hyphae. A plug of YMG agar [1.5% (w/v) agar; 60 µg/ml kanamycine] was poured onto an object slide, on which a piece of *F. graminearum* (Ø 2 mm) was placed. *P. luminescens* 1°::pPINT-P_{tac}-*mCherry* and 2°::pPINT-P_{tac}-*mCherry* overnight cultures were adjusted to an OD_{600} of 1.0 and 1 µl of the cell suspension was spotted onto the *F. graminearum* plug. The object slide was then placed into a sterile petri dish with wet filter paper and sealed with parafilm, and then incubated at 26°C. The object slides were analyzed for the presence of the bacteria on the fungal hyphae after 24 h and 48 h using a Leica DMi8 microscope using bright field and fluorescence microscopy. A filter for Texas red fluorescent dye with an excitation between 540-580 nm and an emission of 592-668 nm was used to observe mCherry tagged cells. Images were recorded and postprocessed using the "Leica LAS-X" software.

Insect pathogenicity assays

Pathogenicity towards insects was studied to investigate, whether deletion of *chi2A* and *cbp* has an influence on pathogenicity of *P. luminescens*. For that purpose, overnight cultures of the respective *P. luminescens* strains were adjusted to an OD_{600} of 1.0 (~$1*10^9$ cells) and further serial dilutions were prepared. Right before injection, fifth instar larvae of *Galleria mellonella* were numbed by placing them on ice and superficially sterilized with 80% (v/v) ethanol. Infection was performed by injecting 20-2.000 cells into the last leg segment of the *G. mellonella* larvae. For each experiment, five larvae were infected and incubated at 30°C. LB was used as negative and *P. luminescens* 1° and 2° wild type strains as positive controls. Pathogenicity and red pigmentation of dead larvae were analyzed after 24, 48 and 72 h. Three biological replicates were performed.

Results and Discussion

***P. luminescens* harbors three GH18 family exochitinases with a conserved catalytic site**

A bioinformatic analysis revealed that the *P. luminescens* DJC genome harbors three genes coding for putative chitinases (*chi2A*, *chi2B*, *chi2C*) belonging to the glycoside hydrolase family 18 (GH18) (Fig. 1A). GH18 chitinases are widely distributed in nature. They catalyze the degradation of β-1,4-glycosidic bonds in polysaccharides like chitin (34, 35). Both, *chi2B* and *chi2C*, form a cluster together with TC-toxin related genes (Fig. 1B), which was defined as pathogenicity island and found to be important for insect pathogenicity of *P. luminescens* and *Yersinia entomophaga* (36). The Chi2A encoding gene is situated in a different locus and was not speculated to be involved in insect pathogenicity before. Indeed, the promoters of *chi2A* and *stlA* are located within the same genomic region in opposite direction and therefore expression of both genes might be differentially regulated (Fig. 1B). StlA is a phenylalanine ammonia-lyase involved in the production of the fungicidal stilbene, that is produced by *P. luminescens* 1° cells (37), suggesting the involvement of GH18 family chitinase Chi2A in another inhibitory process towards fungi. It seems that both *stlA* and *chi2A* might be differently regulated in both phenotypic cell forms of *P. luminescens* as *stlA* is only upregulated in 1° cells (11) and *chi2A* in 2° cells, especially in the presence of PRE (14). PHYRE2 predictions of all three putative chitinases reveals different putative protein tertiary structures (Fig. 1C), and further comparison of the HMM logos with the chitinase of *S.*

marcescens and *Y. entomophaga* (38) reveals a triosephosphate isomerase (*D*xDxE) conserved motif (39) in the catalytic domain of all three GH18 of *P. luminescen*s (Fig. 1D). This conserved motif is described to catalyze the hydrolytic reaction in chitin degradation (34, 35). In particular, the first aspartate in the motif (in italics) is described to be essential for catalytic activity (39), which is also present in all three chitinase encoding genes of *P. luminescens* and therefore suggesting chitinolytic activity for all of them.

Chi2A and CBP are involved in antifungal activity of *P. luminescens* 2° cells

PGPRs such as *Pseudomonas fluorescens* were described to inhibit growth of phytopathogenic fungi (40). Also, for a chitinase isolated from *S. marcescens* antifungal activity was observed (19). In *P. luminescens* comparative transcriptome analysis revealed two genes, *chi2A* and *cbp*, to be highly upregulated in presence of PRE. Moreover, 2° cells displayed a highly fungicidal activity against phytopathogenic *F. graminearum* (14). To determine whether Chi2A and/or CBP are involved in chitinolytic activity of *P. luminescens* 2° cells, *chi2A* and *cbp* deletion mutants were generated, and fungal growth inhibition assays were performed. Deletion of *chi2A* as well as *cbp* led to a total loss of antifungal activity in *P. luminescens* 2° cells (Fig. 2A) indicating that chitinase Chi2A is involved in antifungal activity of 2° cells. Furthermore, when lacking the chitin binding protein CBP, 2° cells were not able to degrade the fungal hyphae, suggesting an important role of CBP in antifungal activity of *P. luminescens.* Usually, chitinase genes harbor a CBP domain essential for enzymatic activity, which is not the case for *P. luminescens* Chi2A. Indeed, also in *Vibrio spec.* it was shown that so called truncated chitinases without a CBP domain were still able to degrade chitin. However, these truncated chitinases were not able to directly bind the polymer (41), making CBP an essential part in the first step for the enzymatic activity of chitinases. Other studies confirmed a CPBs role in chitin degradation (42). Here, the loss of antifungal activity of *cbp* deletion mutant of *P. luminescens* 2° points out the synergistic work between Chi2A and CPB in the bacterial antifungal activity. Additionally, *P. luminescens chi2A* and *cbp* deletion mutant strains were complemented by using integration vector pPINT with P_{tac} upstream of the respective genes. Our experiment showed that the complementation (*P. luminescens* 2° Δ*chi2A* + pPINT-P_{tac}-*chi2A* and Δ*cbp* + pPINT-P_{tac}-*cbp*) restored the wildtype phenotype, by inhibiting fungal growth (Fig. 2B) indicating that Chi2A and CPB are responsible for the

P. luminescens 2° antifungal activity. Additionally, *in vivo* experiments revealed antifungal activity of Chi2A since both *E. coli* and *P. luminescens* 1° cells that express *chi2A* inhibited the growth of *F. graminearum* (Fig. 2C and 2D). However, the effect observed for *E. coli* BL21 expressing *chi2A* was weaker than for *P. luminescens*. This might be due to the fact that *E. coli* does not produce a CBP triggering the chitinolytic activity of Chi2A. Moreover, an inhibitory effect on fungal growth when overexpressing *cbp* could be observed, indicating that *P. luminescens* CBP is also involved in chitin degradation as it was described for CBPs of *Vibrio spec.* (42), so that CBP could bind the chitin mediating better access to Chi2A, which then degrades the polymer.
These data indicate that Chi2A and CBP of *P. luminescens* are both involved in antifungal activity of *P. luminescens* 2° cells. Hence, Chi2A is the main actor in hydrolyzing fungal chitin, while the CBP is essential in binding the chitin giving chitinase more accessibility to fully degrade the fungal cell wall.

Chi2A is a stable exoenzyme with chitinolytic activity inhibiting fungal growth

To further determine chitin digesting activity of Chi2A, chitinolytic activity of purified Chi2A was performed. For that purpose, heterologous overexpression of *chi2A* and protein purification was performed (Fig. S1). First, purified Chi2A was applied on agar plates containing colloidal chitin. Chitin was successfully degraded, showing a halo around the application site after staining with Lugol's iodine solution compared to the buffer control (Fig. 3B) thus suggesting that Chi2A hydrolyses chitin. After 7 d of storage at 4°C the protein still exhibited chitinolytic activity (Fig. 3B). Furthermore, the protein also inhibited the growth of *F. graminearum* after 14 d storage: the fungal hyphae was degenerated at the application site, inhibiting hyphae production as well as spore germination (Fig. 3C). In summary, these data confirm chitinolytic activity of Chi2A, showing inhibitory effects against fungi. Additionally, nanoDSF measurements showed that the protein started to unfold at 46°C, designated as the onset point (T_{ON}). The inflection point at 54°C indicated the moment, where half of the protein appeared unfolded and is equal to T_M, (measured for all the samples also after 14 d) confirming high stability of the protein and long-term activity of Chi2A (Fig. 3A). The temperature tolerance observed here, and the long-term storage capability are optimal characteristics for an exoenzyme thus indicating a high potential to Chi2A to be used in innovative agriculture application.

Chitinase Chi2A is not involved in insect pathogenicity

The catalytic domain of all three chitinases in *P. luminescens* reveals putative degrading activity against chitin. Additionally, *chi2A* was highly upregulated in the presence of PRE in *P. luminescens* 2° cells (17) and contrary to the others, the corresponding gene is not located adjacent to a pathogenicity island responsible for insect killing, indicating that it is not involved in this biological process. Therefore, we performed insect pathogenicity assays with *P. luminescens* 1° and 2° Δ*chi2A* and Δ*cbp*, respectively, to determine whether the respective proteins are involved in pathogenicity against the larvae. Deletion of *chi2A* did not affect virulence of *P. luminescens* against *G. mellonella* since 1° and 2° (mutants and WT) cells killed the larvae effectively within 24-48 h (Fig. 4). These results reveal that Chi2A is a third chitinase of *P. luminescens* playing an important role in other pathogenic processes different from those described for the other two exochitinases. Furthermore, we could demonstrate that bacteria lacking *cbp* have an impaired pathogenicity towards insects. While 20-2.000 *P. luminescens* WT cells usually kill the insects within 24 h, for the *cbp* deletion mutant pathogenicity was delayed since the cells needed 48 h to kill all the larvae. Furthermore, only 20 *P. luminescens* Δ*cbp* cells were not sufficient to exhibit pathogenicity against *G. mellonella* (Fig. 4). Therefore, we can conclude that CBP must be indirectly involved in exhibiting full pathogenicity against the insects but, however, it is not essential. Therefore, it could be possible that an interplay between CBP and the Chi2B and Chi2C chitinases is important for insect killing by accelerating insect tissue digestion.

P. luminescens* 2° cells colonize hyphae of phytopathogenic *F. graminearum

P. luminescens 2° cells inhibited the growth of phytopathogenic *F. graminearum* with direct contact (14). For *Pseudomonas fluorescens*, colonization of fungal hyphae of plant pathogenic *Fusarium oxysporum* was described (33). Therefore, *F. graminearum* was co-cultivated with fluorescently labelled *P. luminescens* cells to detect whether the bacteria directly colonize the fungal hyphae. Indeed, the bacterial cells surrounded the hyphae in large cell clumps indicating direct hyphae colonization by contact, which might be a first step of competition and antifungal activity displayed by *P. luminescens* 2° cells (Fig. 3D). Moreover, colonization occurred especially in regions with freshly

grown mycelia. Therefore, we conclude that *P. luminescens* 2° cells can directly colonize the hyphae of *F. graminearum*. For *P. luminescens* 1° cells instead, which are not able to inhibit fungal growth upon direct contact (14), no attachment to fungal hyphae was observed (Fig. 2D), suggesting an alternative, phenotypic dependent way of *P. luminescens* 1° to inhibit fungal growth. Therefore, we suggest two fungal growth inhibition strategies: 1° cells produce metabolites with fungicidal activity (12, 43), while 2° cells react to fungi with chitinolytic enzymatic activity after direct contact, whereby colonization of the bacteria on the fungal hyphae are essential.

***P. luminescens* 2° cells protect plants from *F. graminearum* plant infection**

We showed that *P. luminescens* 2° cells specifically exhibit chitinase activity and thereby degrade the fungal cell wall inhibiting their growth. In order to determine whether *P. luminescens* can protect plants from fungal infection, we performed *in planta* experiments. For that purpose, *S. lycopersicum* seeds were treated with the respective *P. luminescens* strain prior germination and after sowing them on vermiculite in pots. The plants were grown for 40 d, whereupon phytopathogenic *F. graminearum* was applied along the plants' stem and treated again with the respective bacteria. Plants displaying lesions, which were not able to further grow upon fungal colonization were designated as sick (S), whereas plants not affected by *F. graminearum* and displaying no lesions were designated as healthy (H) (Fig. 2B). Application of 2° wildtype cells on the plants showed an effective inhibition of *F. graminearum* infection as 93% ($P < 0.01$) of the plants were healthy upon treatment. Indeed, the fungus was not able to colonize and the plants were able to grow healthy, suggesting that 2° cells protect them from fungal infection. This was not observed for the control plants treated with MS-medium or 1° wildtype cells (Fig. 5). In both control groups *F. graminearum* formed hyphae along the application site colonizing the plant leading to lesions thereby weaking the plants. Indeed, the plant survival rate was at 0% ($P < 0.01$) for both, thus suggesting a specific biocontrol activity restricted to *P. luminescens* 2° cells. Furthermore, *P. luminescens* 2° Δ*chi2A* and Δ*cbp* strains, which already showed reduced fungal growth inhibitory activity, were also tested for plant protecting ability. Indeed, both mutants could not successfully protect the plants from *F. graminearum* infection since only 7% ($P < 0.01$) and 13% ($P < 0.05$) of the plants treated with 2° Δ*chi2A* or Δ*cbp*, respectively, were protected from *F. graminearum* colonization and remained healthy (Fig. 5). To sum up, these data further suggest

chitinolytic activity of *P. luminescens* 2° as an essential weapon to inhibit fungal growth to protect plants, assigning 2° cells as effective biocontrol agent in agriculture.

Conclusion

It is important to further investigate microorganisms with a potential biocontrol capability that efficiently protect plants from disease especially in agriculture but do not affect the soil borne microbiome. In this study, we could demonstrate the ability of *P. luminescens* 2° cells to function as biocontrol agent against phytopathogenic fungi since the bacteria effectively prevented plant infection by *F. graminearum*. It seems that *P. luminescens* uses different strategies to effectively inhibit fungal growth based on their phenotypic appearance. While only *P. luminescens* 1° cells are known to produce secondary metabolites, of which some with fungicidal activity (e.g., IPS), here we could show that 2° cells instead need a direct contact to affect the fungi. As a first step *P. luminescens* 2° cells colonize the fungal hyphae, then chitin binding protein CBP binds chitin in the cell wall of phytopathogenic *F. graminearum,* where chitinase Chi2A putatively degrades the cell wall, thus inhibiting fungal plant infection. This process might be enhanced by PRE that are putatively sensed by a receptor regulating *chi2A* and *cbp* expression (Fig. 6). For *P. luminescens* Chi2A not only chitinolytic, but also fungicidal activity was observed, which is necessary for plant host protection, advising a role to Chi2A of *P. luminescens* 2° cells in the use as biocontrol active compound in agriculture for plant protection. Furthermore, CBP is very essential for chitinolytic activity: both, Chi2A and CBP of *P. luminescens* are synergistically employed and might be used to achieve a maximum degradation of phytopathogenic fungi by the microorganisms. CBP also synergistically acts with Chi2B and Chi2C and seems to play a role also in insect pathogenicity putatively enhancing activity of the involved chitinases (Fig. 6). Furthermore, plant seed germination and plant growth are generally not affected by *P. luminescens*. Since 2° cells could prevent fungal infection on plants, we suggest the use of *P. luminescens* 2° cells in biocontrol as pre-treatment of plant seeds as well as on growing plants to prevent the fungal colonization in the future.

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Conflict of Interest

The authors declare no conflict of interest.

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Tables

Table 1: List of primers used in this study

Primer name	Sequence 5' ➔ 3'
FA *cbp* fw EcoRI	TGAGAATTCGCGCTATTTCAAGCAATGGT
FA *cbp* re **ovl** FB	CCTGAGCTTTACATAATAGCGTCCTCCAC
FB *cbp* fw **ovl** FA	AAGCTCAGGCCTGCTTGATATTTGGTACA
FB *cbp* re EagI	CATCGGCCGCTAGTGCAACAAGCAGCAGA
FA *chi2A* fw BamHI	GAGGGATCCCCATATATAACCTCTCCTGA
FA *chi2A* re **ovl** FB	CCTGAGCTTGACATAAATCACCTCGACTAG
FB *chi2A* fw ovl FA	AAGCTCAGGCATAATTAATTAAGCCAAGCCAC
FB *chi2A* re EagI	TGACGGCCGGTTGGAATTTCACTGCGCAG
P*tac* fw PstI	GAGCTGCAGTCGATGGTGTCAACGTAAAT
P*tac* re ovl gene	AAGCTCAGGCCACACATTATACGAGCCGA
cbp fw ovl Pt*ac*	CCTGAGCTTATGTATAAACATAAAGTGAAAGTG
cbp re EagI	TGACGGCCGTCAAGCAGGGCTAATTGTTG
chi2A fw ovl Pt*ac*	CCTGAGCTTATGTCAAAAATAATCCAGACAG
chi2A re EagI	GAGCGGCCGTTATGCAATTTTTACCCAAGG
cbp fw NdeI_pET16b	GAGCATATGATGTATAAACATAAAGTG
cbp re XhoI__pET16b	TAGCTCGAGTCAAGCAGGGCTAATTGTTG
chi2A-**N6xHis** fw XmaI_pBAD24	GCGCCCGGGATG**CATCATCACCACCACCAT**TCAA AAATAATCCAGACAGA
chi2A re XbaI_pBAD24	GAGCGGCCGTTATGCAATTTTTACCCAAGG

Figures

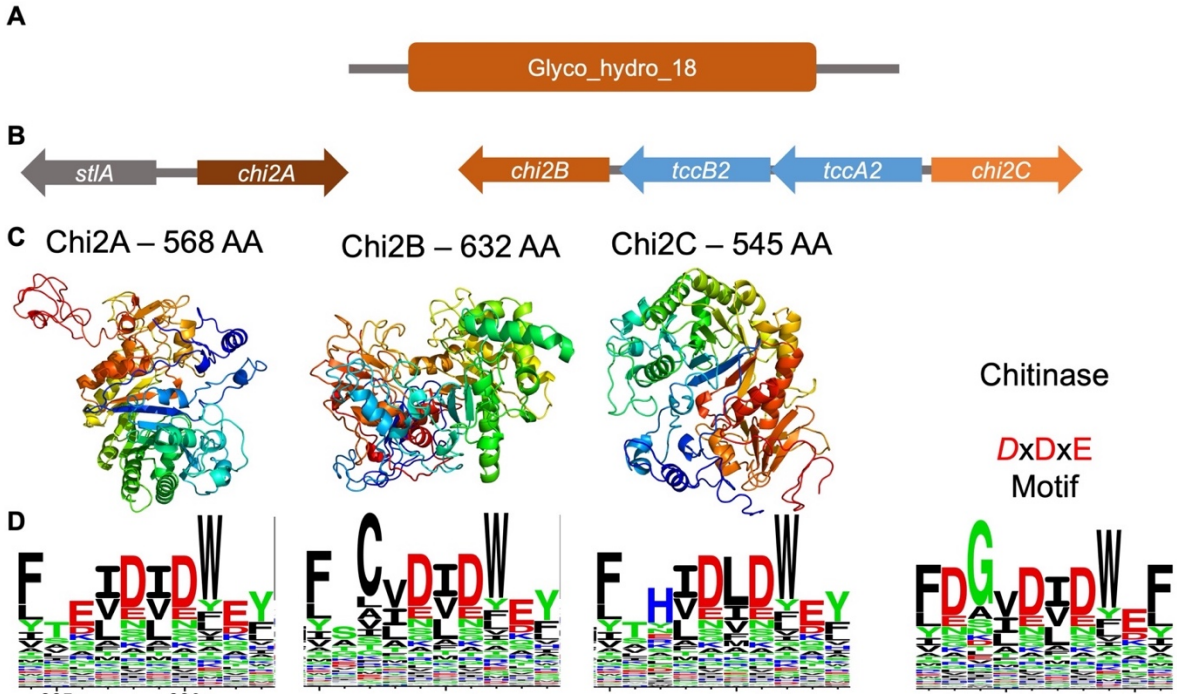


Figure 1: *P. luminescens* harbours three genes encoding exochitinases. Protein sequence analysis of chitinases Chi2A, B and C. (A) Pfam database reveals a glycoside hydrolase family 18 domain, usually found in enzymes involved in chitin degradation. (B) Gene loci encoding the chitinases. On the right *chi2b*, *chi2c* form a gene cluster with Tc-toxin encoding genes *tccA2*, *tccB2* and on the left *chi2A* located in a different gene locus downstream to *stlA*. (C) Protein tertiary structure prediction of all three chitinases Chi2A, B and C of *P. luminescens* was performed by Phyre2 (22). (D) Profile HMM logos created via HMMER using HmmerWeb version 2.41.2 [20] highlighting the triosephosphate isomerase motif *D*xDxE found in all three chitinases Chi2A, B and C. On the right: in particular the first aspartate (in italics) is highly conserved also among different microorganisms as indicated by the *Serratia marcescens* chitinase HMM logo (on the right side).

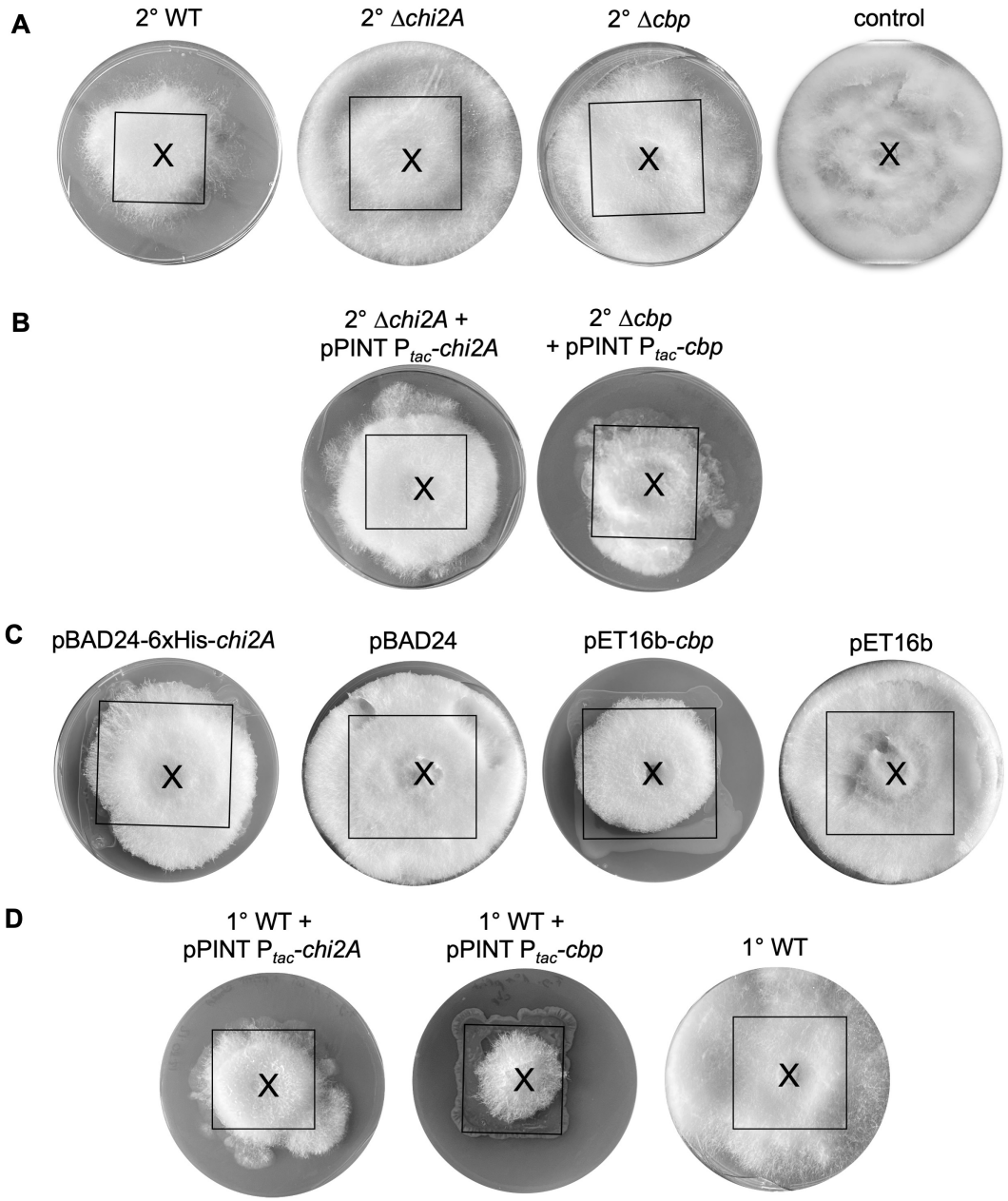


Figure 2: Fungal growth assays using *P. luminescens* and *E. coli* expressing *chi2A* or *cbp* against phytopathogenic *F. graminearum*. *P. luminescens* wildtype (WT), *chi2A* and *cbp* deletion mutant strains and *E. coli* BL21 strains expressing *chi2A* or *cbp* were tested for antifungal activity against *F. graminearum*. (A) Effect of *chi2A* and *cbp* deletion on antifungal activity of *P. luminescens* 2°. (B) Complementation of *chi2A* and *cbp* using integration vector pPINT restored WT phenotype in the respective mutants in 2° cells. (C) Antifungal activity of *E. coli* BL21 (DE3) cells heterologously expressing *chi2A* and *cbp*. Anti-fungal activity was not observed for *E. coli* controls with the empty expression vectors pBAD24 and pET16b. (D) *P. luminescens* 1° WT cells overexpressing *chi2A* and *cbp*, respectively, using the respective pPINT integration vectors. X: application site of the fungal agar plug; square: application area of the respective *P. luminescens* strains surrounding the fungus. The pictures represent data of least three independently performed experiments with similar outcome.

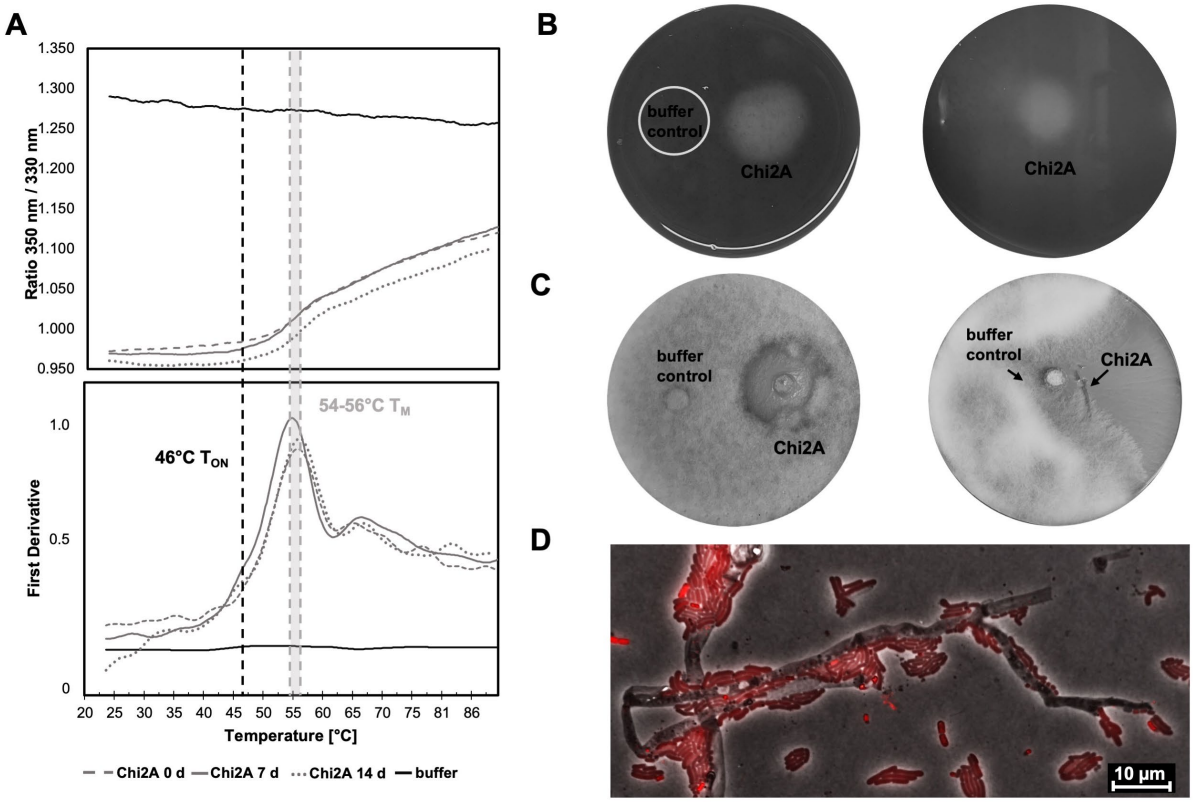


Figure 3: Chitinase Chi2A is a stable exoenzyme degrading chitin and fungal cell wall. Stability and activity of purified Chi2A. (A) nanoDSF analysis of Chi2A shows protein stability over the measured time points of 0, 7 and 14d. The upper graph represents emission ratio of intrinsic tryptophane (iTrp) fluorescence at 350 and 330 nm. The lower graph represents the 1st derivative of the ratio curve. The maximum peak (T_M in light grey) represents the melting temperature of the protein, where half of the protein is denatured, ranging between 54°C and 56°C. The black dotted line shows the onset temperature (T_{ON} 46°C) where the protein starts to unfold. (B) Chitinolytic activity of *P. luminescens* Chi2A tested on chitin agar plates. On the left panel chitin degradation was performed with freshly purified protein (halo ~5.8 cm; ± 0.3 cm $P \leq 0.05$), on the right panel chitin degradation was determined with 7d old (degradation halo ~3.9 cm ± 0.2 cm, $P \leq 0.05$) purified protein. Buffer control: elution buffer without protein was used. (C) Fungal degradation activity of Chi2A on *F. graminearum*. On the left panel, an agar well diffusion assay with *F. graminearum* spores in soft agar is shown. Wells were filled up with buffer and Chi2A and fungal growth was analysed and growth inhibition zone was measured (~5 cm ± 0.3 cm; $P \leq 0.05$). On the right panel Chi2A and buffer were directly spotted on a YMG agar plates with an agar plug containing *F. graminearum* and growth was monitored. All data shown represent one characteristic of at least three independently performed experiments. (D) Fluorescence microscopy of *P. luminescens* 2° cells tagged with *mCherry* co-cultivated with *F. graminearum* on sterile object slides with YMG agar pads. 2° cells (in red) colonize hyphae of phytopathogenic *F. graminearum.* The data in (A) and pictures in (B)-(D) represent data of least three independently performed experiments with similar outcome.

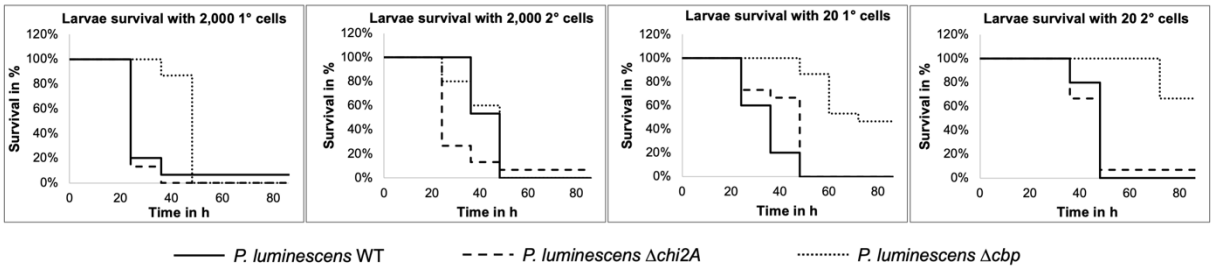
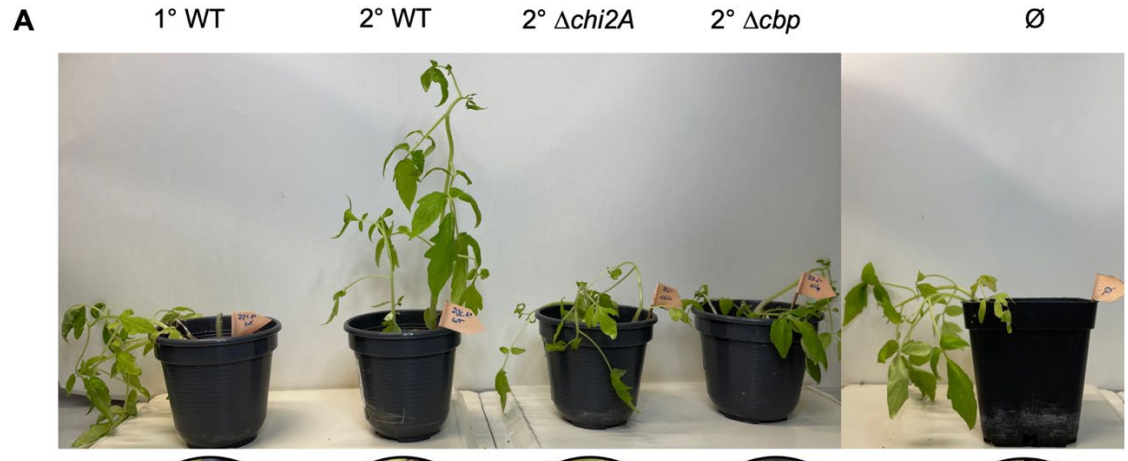


Figure 4: Insect pathogenicity of *P. luminescens*. Insect pathogenicity of *P. luminescens* 1° and 2° wild type (WT) and Δ*chi2A* and Δ*cbp* deletion mutants against *Galleria mellonella*. For each strain, 20 to 2.000 cells were injected into insect larvae and mortality was recorded at different timepoints after injection. The graphs show the larvae survival rate toward time calculated with the log rank method (44) ($P \leq 0.01$). The plots represent the average survival of at least 15 insect larvae.



	1° WT		2° WT		2° Δ*chi2A*		2°Δ*cbp*		Ø	
	S	H	S	H	S	H	S	H	S	H
Trial 1	5	0	0	5	5	0	5	0	5	0
Trial 2	5	0	1	4	5	0	4	1	5	0
Trial 3	5	0	0	5	4	1	4	1	5	0
Total amount	15	0	1	14	14	1	14	1	15	0
Survival rate	0%*		93%		7%*		13%**		0%*	

* $P < 0.01$; ** $P < 0.05$

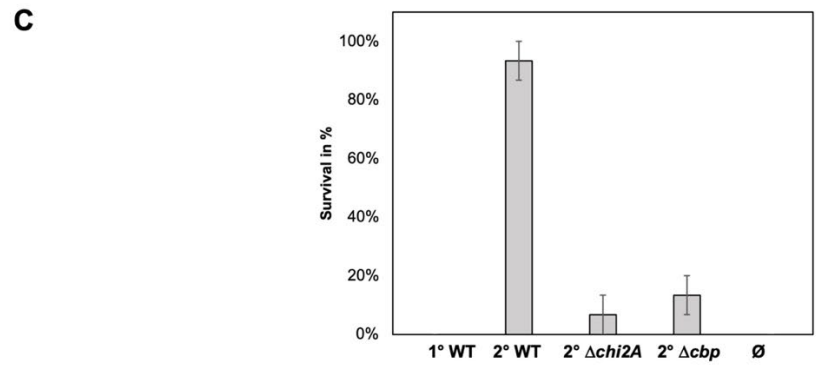


Figure 5: Plant protection by *P. luminescens*. Plant protecting ability of *P. luminescens* 1° and 2° wildtype (WT), 2° Δ*chi2A*, and 2° Δ*cbp* towards *S. lycopersicum* against *phytopathogenic F. graminearum.* 2° wildtype cells effectively prevent fungal colonization and infection. Ø: negative control (addition MS-medium without bacteria). At the lower panel lesions at the stem of *S. lycopersicum* caused by *F. graminearum* are shown, which was not observed for plants treated with *P. luminescens* 2°. The pictures shown are representative of least three independently performed experiments with similar outcome. (B) Evaluation of single plant protection experiment trials displaying the number of sick (S), and healthy (H) plants within a trial containing in total 5 grown plants for each bacterial strain treatment. The total amount of survived plants is represented by the sum of sick plants counted as 0 and healthy plants counted as 1. The survival rate (in percentage) indicates the total number of plants survived (showing no lesions) after infection with *F. graminearum* and treatment with different *P. luminescens* cells (* indicates $P < 0.01$, and ** indicates $P < 0.05$). (C) Graphic representation of the plant survival rate. Error bars represent standard deviation of the three independently performed experiments.

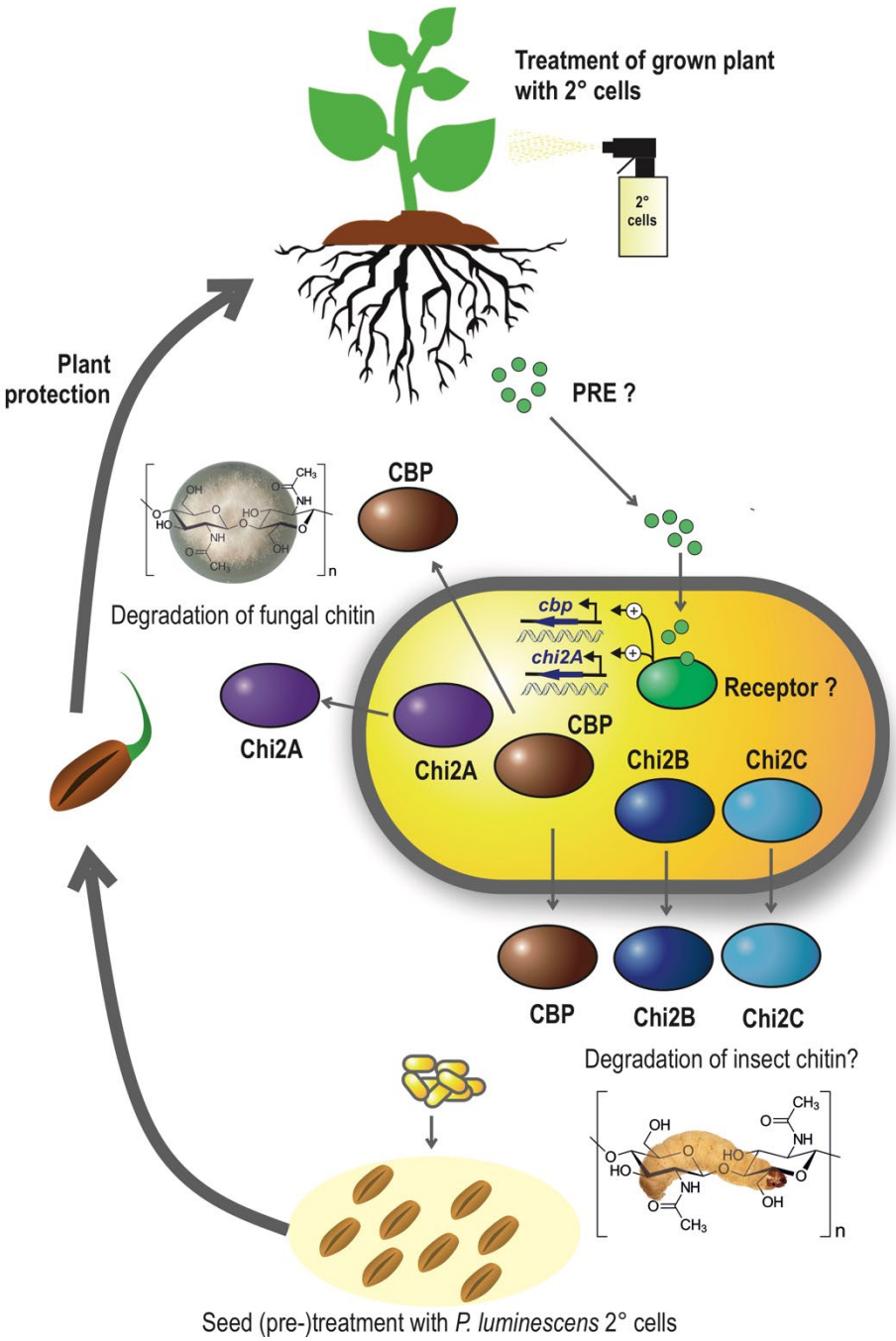


Figure 6: **Model of the putative *P. luminescens* chitinases mode-of-action.** *P. luminescens* 2° use Chi2B and Chi2C to degrade insect tissue in their infective life cycle. As this cell form does not reassociate with nematodes the bacteria are left in the soil. A putative plant-derived signal (PRE) might be sensed by a receptor regulating the transcription of genes coding for chitinase Chi2A and chitin binding protein CBP. *P. luminescens* 2° Chi2A and CBP act together to inhibit the growth of phytopathogenic fungi (e.g.: *F. graminearum*) and consequently protecting the plant from their infection. Seeds treated with *P. luminescens* 2° cells before germination as well as after fungal colonization are more resistant towards the infection.

5. SdiA mediated interkingdom communication of *Photorhabdus luminescens* with plants and its role in biofilm formation and motility

Dominelli N[1]*, Regaiolo A*, and Heermann R[1] (2022). SdiA mediated interkingdom communication of *Photorhabdus luminescens* with plants and its role in biofilm formation and motility.

* Authors contributed equally.

Johannes-Gutenberg-University Mainz, Institute of Molecular Physiology (imP), Biocenter II, Microbiology and Biotechnology, Hanns-Dieter-Hüsch-Weg 17, 55128 Mainz, Germany

Abstract

In bacteria, group coordinated behavior is often mediated via quorum sensing (QS). In inter- and intra-species communication bacteria use small diffusible molecules to modulate expression of different genes as biofilm formation or virulence, phenotypes that are important to orchestrate the interaction with the host or their persistence in a specific environment. The canonical QS systems of Gram-negative bacteria signal use *N*-acyl homoserine lactones (AHLs) that are produced by LuxI-type autoinducer synthases for communication. AHLs are sensed by LuxR-type receptors, which act as transcriptional regulators controlling the expression of specific target genes. Many bacteria harbor LuxR type receptors lacking a cognate LuxI-type synthase, which are therefore designated as LuxR solos. *Photorhabdus luminescens* harbors 40 LuxR solos, of which only two contain a putative AHL-signal binding domain: i) PluR, for which photopyrones and not AHLs were identified as endogenous QS signal molecule, and ii) one SdiA-like receptor, for which neither a respective signaling molecule nor specific target genes have been identified yet. In this work, we show that SdiA of *P. luminescens* is involved in regulation of motility and biofilm formation. Using surface plasmon resonance spectroscopy, we demonstrate that SdiA acts as bidirectional regulator of transcription binding within the promoter region of its own gene as well as the adjacent *PluDJC_01670* (*aidA*) gene with high affinity. SdiA showed binding ability towards diverse AHLs but also to plant derived signals. Therefore, we suggest that SdiA is a main player in interkingdom signaling (IKS) in *Photorhabdus*-plant interaction.

5.1 Introduction

Like humans or animals, bacteria can communicate with each other to coordinate group-coordinated behavior. Bacterial communication employs small diffusible signaling molecules in a process designated as quorum sensing (QS) in which the group-coordinated behavior is dependent on population density or quorum (Waters and Bassler, 2005). The most common and well-studied QS-based communication in bacteria is the canonical LuxI/LuxR-type communication in Gram-negative bacteria, where acylated homoserine lactones (AHLs) are used for communication, which are produced by the autoinducer synthase LuxI and sensed by cognate LuxR-type receptor when exceeding a minimal threshold concentration. These receptors usually consist of a N-terminal signal binding domain (SBD), which binds the AHLs, and a C-terminal

helix-turn-helix (HTH) DNA-binding domain (DBD), which binds DNA and thereby modulates the transcription of the respective target genes (Choi and Greenberg, 1991; Hanzelka and Greenberg, 1995; Marchler-Bauer et al., 2012). Once AHLs bind to the LuxR, the receptor undergoes a conformational change so that the protein exhibits higher affinity towards target promoters, therefore constantly affecting expression of the respective genes. Furthermore, a positive feedback loop occurs as transcription of the cognate *luxI* is also regulated by LuxR upon signal binding, leading to excessive production of AHLs, for which reason these signaling molecules are designated as autoinducers (Fuqua et al., 1994, 1996, 2001; Waters and Bassler, 2005). Many proteobacteria harbor LuxR type receptors, however, some of them are lacking a cognate LuxI synthase, necessary for the synthesis of the autoinducer molecule. Such LuxR homologs are designated as LuxR orphans or solos and are widespread among proteobacteria (Case et al., 2008; Patankar and González, 2009; Subramoni and Venturi, 2009). Many enterobacteria such as *Escherichia, Salmonella* or *Yersinia* and plant associated bacteria like *Pseudomonas*, *Xanthomonas* or *Agrobacterium* contain many LuxR solos (Case et al., 2008). Some of them belong to non-AHL producing bacteria and can sense exogenous AHLs or hormone-like signal molecules produced by bacteria or eukaryotes (Subramoni and Venturi, 2009). SdiA is a LuxR solo transcriptional regulator found in *E. coli* and *Salmonella enterica* harboring an AHL-signal binding domain but lacking a cognate LuxI synthase. For that reason, SdiA was suggested to bind exogenic AHLs that are produced by neighboring bacteria (Michael et al., 2001). Recent docking studies revealed ability of SdiA to bind long chain AHLs with high affinity (Almeida et al., 2016).

SdiA-homologs are also found in enteric *Photorhabdus* species i.e., among the 40 LuxR solos found in insect pathogenic *P. luminescens* two of them, PluR and SdiA, have a typical AHL signal binding domain (Heermann and Fuchs, 2008; Brameyer et al., 2014). However, for PluR recent studies revealed a modification in the SBD which leads to perception of endogenous α-pyrones (photopyrones) instead of AHLs representing a novel cell-cell communication circuit (Brachmann et al., 2013), whereas for SdiA in *P. luminescens* no signal molecule has been identified, yet. So far it is known that the SdiA-SBD of *P. luminescens* shares high homology with known SdiA sequences and contains the conserved amino acid motif (WYDPWG) necessary for AHL-binding (Brameyer et al., 2014). For similar LuxR-type receptors, which are widely distributed in plant associated bacteria like *Pseudomonades,* a possible sensing of

AHL-like signal molecules produced by plant hosts was suggested (Bais et al., 2006; Bez et al., 2021). To shed light on the role of SdiA in *P. luminescens* and its possible role as interkingdom receptor, we investigated the effect of SdiA on specific phenotypes like motility or biofilm formation and the binding capacity of SdiA to different AHLs as well as plant root exudate HPLC fractions. We also identify genes regulated by SdiA highlighting the receptor-self modulation and the control of a neighboring gene *aidA*, putatively involved in *Photorhabdus*-plant interaction.

5.2 Material and Methods

Bioinformatic analysis

Among the 40 LuxR solos found in the genomes of *P. luminescens* of which two contain an AHL-like signal binding domain, we considered SdiA-like LuxR solo PluDJC_01675 which was already highlighted in a previous analysis (Brameyer et al., 2014). Multiple sequence alignment of SdiA with several other AHL-LuxR solos was performed using Clustal Omega (Madeira et al., 2019) to identify the signal binding site and the DNA binding site motives. Furthermore, the putative tertiary protein structure was predicted using SWISS-MODEL (Guex et al., 2009; Bertoni et al., 2017; Bienert et al., 2017; Waterhouse et al., 2018; Studer et al., 2020). Lastly, the BLAST tool was used to search homologous of *aidA* (gene upstream of *sdiA*) in other organisms.

Bacterial Strains and creation of deletion mutants

In this study *Photorhabdus luminescens* spp. *laumondii* DJC 1° and 2° wildtype and respective mutants were used (Zamora-Lagos et al., 2018). Deletion mutants of *PluDJC_01675* (*sdiA*) were obtained through *in-frame* deletion via double homologous recombination. For that purpose, ~500 bp fragments up- and downstream of the desired gene were cloned into pNPTS138-R6KT (Lassak et al., 2010) suicide vector using primer pairs FA_*sdiA*_fwd_EagI (catCGGCCGATGAATATTAATCGACCATATGCC) + FA_*sdiA*_rev_**ovl**_FB (**CCTGAGCTT**TCAGCACAGGCCGGAAATTTAGAAC) for flank A and FB_*sdiA*_fwd_**ovl**_FB (**AAGCTCAGG**CCAGGCAATAGCTAAAGCTG) + FB_*sdiA*_rev_SalI (cctGTCGACCCCAAGCTCTGGAAGAATTCCCAT) for flank B for deletion of *sdiA*. Both flanks were fused and inserted into pNPTS138-R6KT using respective restriction sites. Finally, the obtained vector was transferred into *P.*

luminescens 1° and 2° cells via conjugation (Thoma and Schobert, 2009; Lassak et al., 2010). *E. coli* BL21 (pLysS) strain was used to heterologous express *sdiA*. For that purpose, recombinant pBAD24-N-6xHis-*sdiA* vector, in which *sdiA* expression is under control of the inducible arabinose (*ara*) promoter (Guzman et al., 1995) was generated using *sdiA*-N-**6xHis**_fwd_XmaI (gcgCCCGGGATG**CATCATCACCACCACCAT**AATATTAATCGACCATATGCCTTA) + *sdiA*_rev_XbaI (gctTCTAGATTATATATAGCCAAGTAATACAGCTT) and inserted into pBAD24 using respective restriction sites. Bacterial cultures were inoculated into LB medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 1% [w/v] NaCl) supplemented with or without the respective antibiotics an aerobically cultivated at 30°C or 37°C for *P. luminescens* and *E. coli*, respectively. If designated kanamycin was added with a final concentration of 60 µg/ml and carbenicillin with 100 µg/ml.

Motility and Biofilm assays

To test whether LuxR solo SdiA is involved in modulation of motility or biofilm formation in *P. luminescens*, respective swimming, twitching and biofilm assays with Δ*sdiA* deletion mutant in *P. luminescens* and the isogenic wild type were performed. For that purpose, *P. luminescens* overnight cultures were prepared and OD_{600} was adjusted for the respective assay. For swimming motility 10 µl of an overnight culture with an OD_{600} = 0.1 was spotted in the center of swimming agar plates (0.3% [w/v] agar, 1% [w/v] tryptone, and 0.3% [w/v] NaCl) and incubated for 24 h at 30°C. The resulting swimming halo diameter was measured using ImageJ (https://imagej.nih.gov/ij/). For twitching motility 10 µl of an overnight culture with an OD_{600} = 0.1 was spotted between the twitching agar (2% [w/v] agar, 1% [w/v] tryptone, and 0.3% [w/v] NaCl) and the petri dish by stabbing the pipette tip through the agar. Plates were incubated for 24 h at 30°C. Then, the agar was removed from the petri dish, the plates were quickly washed with water, and after drying stained with 1% (w/v) crystal violet and incubated for 30 min. Afterwards the plate was washed twice and dried overnight. The following day, twitching motility on the surface became visible as bacteria attached on the surface of the petri dish were stained by crystal violet. For quantification of biofilm production, biofilm assays were performed (Christensen et al., 1985; O'Toole and Kolter, 1998; O'Toole, 2011; Zamora-Lagos et al., 2018). For that, overnight cultures of *P. luminescens* were adjusted to an OD_{600} of 0.5 in LB and 135 µl per well of the bacterial

suspension was pipetted into transparent polystyrene 96-well microtiter plates and incubated for 24 h and 72 h at 30° under static condition and supplemented with or without PRE to analyze the effect of plant derived signals on biofilm formation of *P. luminescens*. After incubation the liquid phase of the culture and therefore planktonic cells were removed by gently washing twice the microtiter plates in a water tub and air-dried for at least 5 min. For staining the biofilm, 135 µl 1% (w/v) crystal violet solution was added to each well and incubated for 30 min at RT. Microtiter plates were then washed twice gently to remove excessive crystal violet and subsequently air-dried overnight at RT. Biofilm quantification occurred by resuspending the bound crystal violet with 135 µl 30% (v/v) acetic acid and monitoring the absorbance at 575 nm using Tecan Spark plate reader.

Extraction of plant root exudates (PRE)

Plant root exudates were collected similar as described in (Regaiolo et al., 2020). Briefly, *Pisum sativum* variant *Arvica* (Bayrische Futtersaatbau, Ismaning, Germany) were grown at 24°C; 16 h light/8 h dark for 2 weeks in vermiculite. 75 plants were collected, washed, and put into vessels with 250 ml sterile ddH_2O (for hydrophilic compounds) or methanol (for lipophilic compounds) under continuous shaking for 16 h. The solutions were then sterilized by filtration and stored at 4°C in the dark until use.

Heterologous overexpression of *sdiA* and protein purification

Heterologous expression of *sdiA* was carried out in *E. coli* BL21 pLysS strain carrying the recombinant vector pBAD24-N-6xHis-*sdiA*. 6xHis codon was N-terminally added to the gene via PCR and the resulting construct was inserted downstream of the P_{ara} in the expression vector pBAD24. For protein production an overnight culture of *E. coli* BL21::BAD24-N-6xHis-*sdiA* cells was prepared and 1 l of LB medium supplemented with the respective antibiotic was inoculated at an OD_{600} = 0.1 and incubated at 37°C at 150 rpm. Once the OD_{600} of the cells reached 0.4, gene expression was induced by adding 0.1% (v/v) *L*-arabinose to the culture and bacteria were further aerobically cultivated at 30°C for 3 h. Cells were then harvested by centrifugation for 30 min at 4,500 rpm at 4°C, whereupon the pellet was resuspended in Tris lysis buffer (50 mM Tris/HCl, 300 mM NaCl, 5 mM imidazole, 0.5 mM PMSF, 2 mM DTT, pH 7.5). Then the cells were lysed using a French press running three cycles at 1.35 kBar. Cell debris were removed by centrifugation at 4,500 rpm at 4°C for 15 min. Afterwards, the

cytosolic fraction was recovered via ultracentrifugation at 45,000 rpm and 4°C for 45 min. Then, the cytosolic fraction was incubated under gentle shaking at 4°C with Ni^{2+}-NTA-Agarose beads (Qiagen) for purification. After 1 h of incubation, the bead-protein solution was loaded onto a column, then the beads were washed twice using 15 ml Tris-washing buffer (50 mM Tris/HCL pH 7.5, 10% glycerol, 300 mM NaCl, 40 mM Imidazole). SdiA was eluted using Tris-elution buffer (50 mM Tris/HCL pH 7.5, 10% glycerol, 300 mM NaCl, 250 mM Imidazole), where 6x 500 µl of the proteins were collected. To check for successful SdiA production, SDS-PAGE according to (Laemmli, 1970) and Western Blot analyses using rabbit-anti-His antibody (rabbit monoclonal, clone RM146, Sigma Aldrich) and anti-rabbit antibody (anti-rabbit lgG (whole molecule) – alkaline phosphatase antibody produced in goat, Sigma Aldrich) were performed.

Investigating SdiA stability throughout Nano Differential Scanning Fluorimetry (nanoDSF)

NanoDSF is a microscale label free approach for rapid and easy detection of protein stability using the intrinsic aromatic amino acids (AA) tryptophane and tyrosine to determine protein folding and stability (Niesen et al., 2007; Alexander et al., 2014). Here, we analyzed stability of 6His-SdiA after purification and we tested the influence of several compounds such as AHLs (10 nM C_4-AHL, 10 nM C_{12}-AHL), 3.3% (v/v) PRE and their respective HPLC-separated fractions on protein stability. For that, the protein sample was adjusted to a concentration of 0.3 mg/ml and loaded into capillaries which were placed into Prometheus NT.48 (NanoTemper https://nanotempertech.com/prometheus/) device for the nanoDSF measurements. The measurements were performed in a temperature range between 20°C and 90°C with a temperature slope of 1.5°C/min. The resulting data were analyzed using the PR.ThermControl software (https://nanotempertech.com/prometheus-pr-thermcontrol-software/).

Investigating DNA-binding capacity of SdiA through Surface plasmon resonance (SPR) spectroscopy

SPR analysis allows real-time detection of different types of biomolecular interactions, where bindings, specificities, kinetics, and affinities can be determined. Here, we performed SPR analysis using Biacore T200 (Cytiva, Freiburg) and precoated SA sensor chips (Xantec Bioanalytics GmbH, Düsseldorf), where streptavidin is covalently

attached to a carboxymethyldextran matrix on a surface. To test whether SdiA binds to selected promoter regions, respective DNA fragments were 5'biotinylated and amplified via PCR from *P. luminescens* DJC gDNA. To achieve ~180 bp fragments of each, [Btn]-P_{sdiA}, [Btn]-P_{aidA}, [Btn]-P_{fliE}, respective primer pairs [Btn]-P_{sdiA} fwd + P_{sdiA} rev, [Btn]-P_{aidA} fwd + P_{aidA} rev and [Btn]-P_{fliE} fwd + P_{fliE} rev were used (Table 1). Chip equilibration occurred by injection of 90 µl 1 M NaCl/50 mM NaOH at a flow rate of 10 µl/min for three times. Then, 40 nM of the respective biotinylated promoters were injected with a contact time of 420 s at a flow rate of 10 µl/min and immobilized on the SA chip. The first out of four flow cells of the chip was kept free and used as blank for subtraction of bulk refractive index background for data evaluation.

For analyzing binding kinetics, different concentrations (1.5625 nM, 3.125 nM, 6.25 nM, 12.5 nM, 2 x 25 nM, 50 nM, 100 nM, 200 nM) at a final volume of 150 µl for each dilution of SdiA were prepared in HPS-EP+ buffer [0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) Tween-20, filtered and degassed]. Additionally, SdiA binding properties to the different promoters were tested under the influence of PRE and the respective controls. The run started with an injection time of 180 followed by a dissociation time of 420 s at flow rate of 30 µl/min. Between every cycle the chip surface was regenerated by first injecting 2.5 M NaCl for 30 s at a flow of 60 µl/min followed by 0.5% (w/v) SDS for 30 s at a flow of 60 µl/min. The resulting sensorgrams were recorded using the Biacore T200 3.2 control software and analyzed with the Biacore T200 3.2 evaluation software to determine the binding affinity (K_D) as well as association (k_a) and dissociation rates (k_d) of SdiA to the tested promoters setting a 1:1 binding algorithm.

Table 1: Oligonucleotides used for amplification of biotinylated DNA for SPR analysis

Primer Name	Sequence 5'➔ 3'
[Btn]-P_{sdiA} fwd	[Btn]-GATTATTAGGATTTCAATCCTATTGATAT
P_{sdiA} rev	TCAATGTCCTCTTGAAAATTAAG
[Btn]-P_{aidA} fwd	[Btn]-GACACCTCTTTACATATTTAAACTATT
P_{aidA} rev	CTATATGAAGCAATACCTAATAAATATATG
[Btn]-P_{fliE} fwd	[Btn]-GTCATTATTCGCTGTTCACTC
P_{fliE} rev	AAAAACCTCGTGTTAAACCAC

Preparative High-performance liquid chromatography (HPLC)

To determine putative plant derived signal molecules binding to SdiA PRE (20 mg/ml in acetonitrile) were fractionized into 48-well plates via preparative HPLC on an Agilent LC system using LiChrospher 100 RP18 (125 x 4 mm, 5µm) column at 40°C. A linear gradient starting from 1% (v/v) acetonitrile to 99% (v/v) acetonitrile in 25 min and then maintaining 100% (v/v) acetonitrile for 3 min was used at a flowrate of 1 ml/min. Injection volume of the sample was 20 µl/run. Plates were dried to remove residual acetonitrile and stored at -20°C until further use.

5.3 Results and Discussion

Bioinformatic analysis of SdiA

SdiA is a LuxR family transcriptional regulator containing a N-terminal AHL signal binding domain (SBD) and exists in Gram-negative bacteria like *E. coli* (Ahmer, 2004), and is also found in entomopathogenic *P. luminescens* (Brameyer et al., 2014). Although SdiA of *P. luminescens* harbors the six conserved amino acid (AA) WYDPWG-motif (**Fig. 1A**) (Brameyer et al., 2014) essential for binding AHLs, a respective signaling molecule has not been identified yet. Before starting different experimental approaches with SdiA, protein sequence was analyzed and compared to other AHL-LuxR solos occurring in different bacteria. Indeed, the SBD of SdiA harbors in total 10 AA important for binding AHLs. Throughout the clustal alignment analysis we observed the conserved WYDPWG-motif present in all analyzed AHL-LuxR receptors shaping the basic structure of the ligand-binding pocket (**Fig. 1A and B** [marked in cyan]). However, the other 4 AAs in the essential part of the SBD vary between the different LuxRs (**Fig. 1B** [highlighted in orange]), very likely resulting in an altered shape of the ligand-binding pocket, putatively making the specificity towards different signaling molecules (Covaceuszach et al., 2013; Bez et al., 2021). These variations also occur between the SdiAs of different organisms, i.e., at position (3) (**Fig. 1B**) Tyr73 of SdiA in *P. luminescens* is substituted with Phe76 in *E. coli.* Interestingly, variations in these regions in LuxRs of plant associated bacteria were reported, whereupon these differences suggested specificity towards different molecules including plant-derived compounds (González et al., 2013; Coutinho et al., 2018; Bez et al., 2021). *P. luminescens* harbors a gene upstream of *sdiA* coding for

PluDJC_01670 (AidA), a protein containing a PixA domain, similar to AidA2, found in plant pathogenic *Ralstonia solanacearum*. Interestingly, *R. solanacearum* harbors two AidA coding genes (here named *aidA2* and *aidA1*), both located upstream of *solR*, which codes for an AHL-LuxR strongly regulating expression of both *aidA* genes (Flavier et al., 1997; Meng et al., 2015). Although the orientation of *aidA* and *sdiA* in *P. luminescens* differs from the *aidA-solR* cluster in *R. solanacearum*, we assumed that *aidA* expression is also regulated by SdiA in *P. luminescens* as the genes share a 310 bp long intergenic promoter region (**Fig. 1C**). This intergenic region contains putative *lux*-box like motifs (data not shown), which are known to be important for DNA-binding of LuxR-type receptors (Antunes et al., 2008). Furthermore, BLAST analysis revealed similarity of about 27% between AidA of both bacteria. Therefore, it is obvious that AidA could be somehow involved in host colonization of *P. luminescens*, similar as described for *R. solanacearum* (Meng et al., 2015). Interestingly, similar *sdiA-aidA* clusters are found in entomopathogenic *P. temperata* and human pathogenic *P. asymbiotica* (named *sdiA-aidA* cluster 2) with a 516 bp and 502 bp long intergenic promoter region, respectively. Upstream of this cluster, *P. asmybiotica* harbors another *sdiA-aidA* cluster (designated as cluster 1) harboring 5 *aidA* genes (*aidA1_1* – *aidA1_5*) (Fig. 1C).

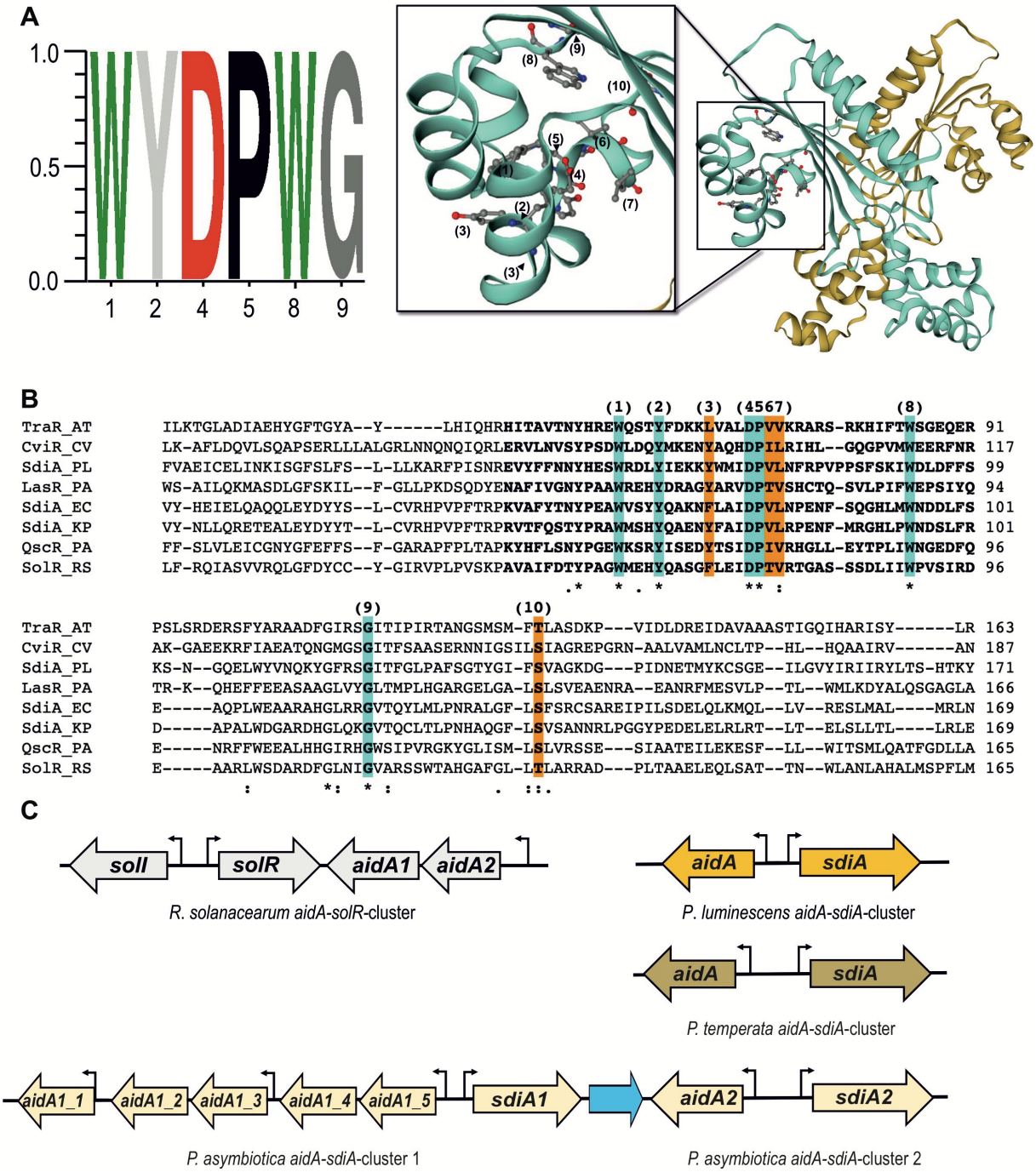


Figure 1: Protein sequence analysis of AHL-LuxR solo SdiA (PluDJC01675) of *P. luminescens*. (A) Left panel: Sequence logo motif of the six conserved amino acids (AAs) found in AHL-LuxR receptors created with WebLogo3 (Crooks et al., 2004). Right panel: SdiA tertiary structure predicted with SWISS-MODEL (Guex et al., 2009; Bertoni et al., 2017; Bienert et al., 2017; Waterhouse et al., 2018; Studer et al., 2020), pointing out the signal binding-pocket of SdiA. The numbers 1, 2, 4, 5, 8 and 9 below the logo indicate the position of the six conserved amino acids (WYDPWG) in the SdiA model that are essential for AHL binding. (B) Structure-based multiple sequence alignment of the protein sequences of the signal binding domains (SBD) of AHL-LuxRs TraR (B9K461) from *Agrobacterium tumefaciens* (AT), CviR (Q7NQP7) from *Chromobacterium violacaeum* (CV), SdiA (Q7N9K5) from *P. luminescens* (PL), LasR (P25084) from *Pseudomonas aeruginosa* (PA), SdiA (P07026) from *E. coli* (EC), SdiA (A0A0H3GS53) from *Klebsiella pneumoniae* (KP), QscR (G3XD77) from *P. aeruginosa* (PA), and SolR (P58590) from *Ralstonia solanacearum* (RS). The six conserved AAs are highlighted in cyan. In orange the variable AAs essential for AHL binding of the proteins are highlighted. The numbers 1 to 10 indicate all AAs essential for AHL binding of *P. luminescens* SdiA in the SWISS-MODEL displayed above. (C) Genetic

loci of *luxR-aidA* cluster identified in the plant pathogen *R. solanacearum*, *P. luminescens, P. temperata* and human pathogenic *P. asmybiotica.* The latter one harbors two *sdiA-adiA* clusters, whereas cluster 1 contains *5 aidA coding genes. The blue arrow indicates an unknown gene (PAU_RS01255) between both sdiA-aidA* clusters in *P. asymbiotica.*

Influence of *sdiA* deletion on motility and biofilm formation

For bacterial host colonization and virulence not only biofilms but also motility through swimming and twitching are essential. Especially twitching motility, which is a movement driven by pilus extension, attachment, and retraction on viscous or solid surfaces, plays a major role in pathogenesis (Mattick, 2002; Hall-Stoodley et al., 2004). Indeed, for plant pathogenic *R. solanacearum* both motility strategies are critical for plant colonization and expression of full virulence (Tans-Kersten et al., 2001; Corral et al., 2020). For *P. luminescens* it is known that 2° cells are highly motile compared to 1° cells and react to PRE, which was suggested to be an important trait for plant colonization, although only swimming capacity was considered (Eckstein et al., 2019; Regaiolo et al., 2020). Therefore, we first performed twitching motility assays and could show that 2° cells display significantly higher twitching ability on solid petri dish surface compared to 1° cells (**Fig. 2B**), an important trait that 2° cells might use to move in the rhizosphere.

Generally, biofilm formation and motility can be regulated among others by LuxR mediated QS as it was demonstrated for the LuxR solo SdiA of non-AHL producing enteric bacteria like *Escherichia, Salmonella,* or *Klebsiella,* influence gene expression associated with virulence factors such as biofilm formation or motility (Ahmer et al., 1998; Kanamaru et al., 2000; Antunes et al., 2010; Sharma et al., 2010; Tavio et al., 2010; Culler et al., 2018), which is not necessarily mediated upon signal binding (Lindsay and Ahmer, 2005; Dyszel et al., 2010; Hughes et al., 2010; Sharma et al., 2010; Shimada et al., 2014; Nguyen et al., 2015). For that purpose, we analyzed the influence of *sdiA* deletion on swimming and twitching motility as well as biofilm formation of *P. luminescens*. Remarkably, *P. luminescens* 2° Δ*sdiA* deletion mutant showed a totally impaired swimming as well as twitching capacity in comparison to the 2° WT (**Fig.2 A and B**). A similar behavior has already been observed for other bacteria like *Vibrio* and plant pathogenic *Acidovora*, when deleting the respective *luxR*-like genes (Yang and Defoirdt, 2015; Wang et al., 2016). Furthermore, Yang and Defoirdt as well as Wang and colleagues also reported increased biofilm formation upon *luxR*

deletion, which was also described for pathogenic *Klebsiella* lacking *sdiA* (Pacheco et al., 2021), an effect that we also observed for *P. luminescens* Δ*sdiA*. Indeed, the mutants displayed an increased biofilm formation of about 67% compared to the WT in 2° cells, which was significant only after 24 h of incubation (**Fig. 2C, left panel**). Therefore, from our data we could hypothesize a signal independent regulatory role of *P. luminescens* SdiA positively regulating motility, whereas biofilm formation is repressed. It is likely that SdiA plays a role in regulating a switch between a sessile and a motile lifestyle in *P. luminescens* 2°. Interestingly, when deleting *sdiA P. luminescens* displayed similar phenotypes in respect to motility and biofilm formation similar to as it was observed when supplementing the wildtypes with PRE. While motility of *P. luminescens* decreased upon exposition towards PRE (Regaiolo et al., 2020), biofilm formation increased of about 54% (**Fig. 2C, right panel**). However, this effect was only visible after 72 h of incubation. Therefore, from our finding we further propose that the regulatory role of SdiA of biofilm and motility only functions in absence of signaling molecules. This regulation might be affected in presence of PRE, which is in accordance with the finding that LuxR receptor EsaR in *Pantoea* was only active in absence of the respective signaling molecule (Tsai and Winans, 2010). Furthermore, researchers also revealed for *E. coli* SdiA that primarily alteration of the protein sequence had more impact on biofilm formation, rather than binding a signal molecule. However, a plant derived indole derivative led to altered biofilm formation after binding SdiA indicating a negative effect on the regulatory role of the receptor upon signal binding (Lee et al., 2007, 2009). Therefore, we could further assume that SdiA is involved in interkingdom signaling (IKS) communication in *P. luminescens* with plants. It is likely, that SdiA in its native conformation represses biofilm formation in 2° cells, while the receptor acts as activator for motility genes. This regulation is then changed once 2° cells are in close proximity to the plants, which are sensed via SdiA. Thereby, motility is reduced, while biofilm is putatively induced to colonize the new plant host.

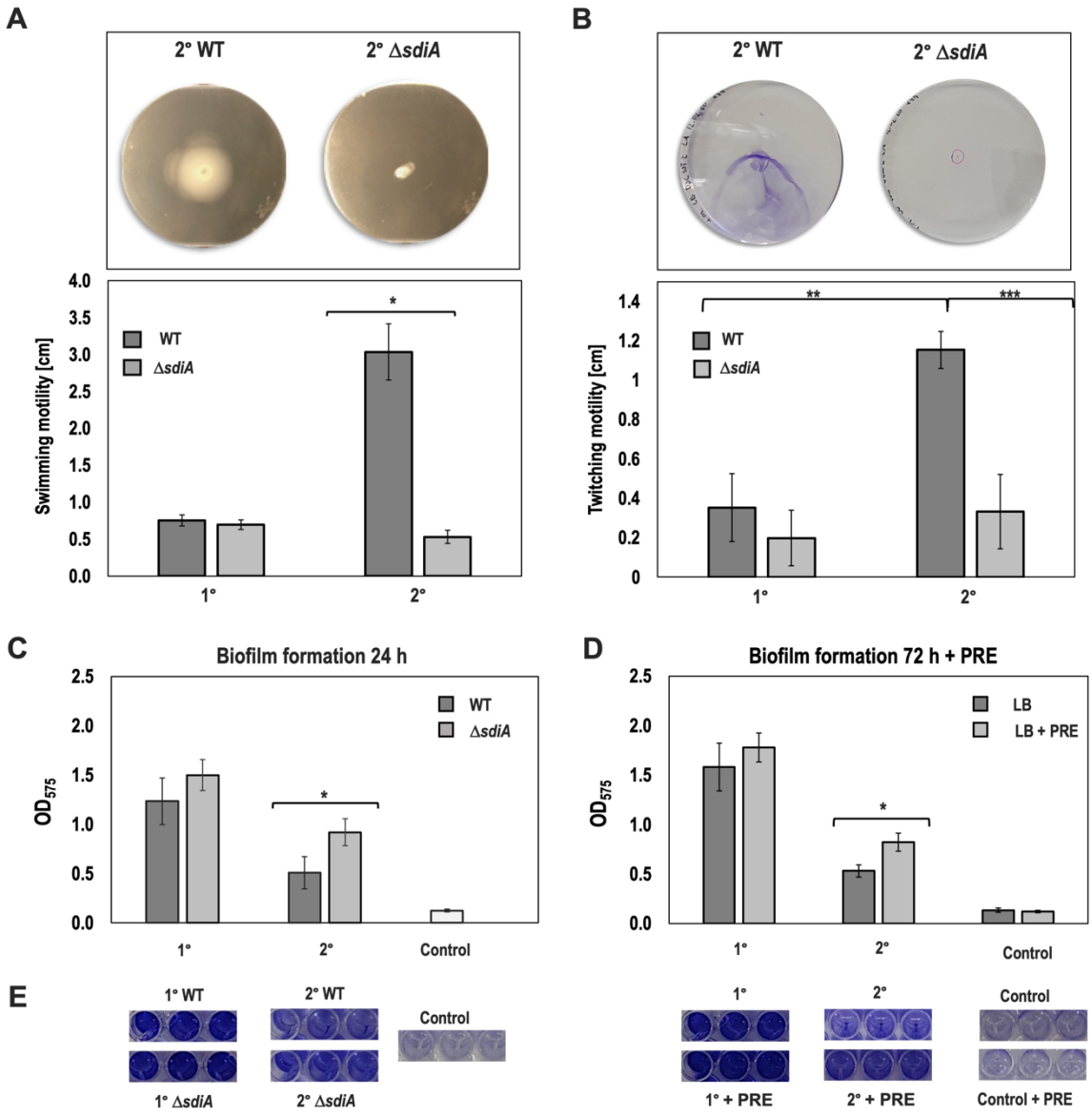


Figure 2: Influence SdiA on motility, twitching and biofilm formation of *P. luminescens*. (A) Swimming motility: deletion of *sdiA* in 2° cells led to significantly impaired motility compared to the wildtype (WT), effect not observed for non-motile 1° cells (*,*P ≤ 0.05*). (B) Twitching motility: at the top crystal violet staining of twitched cells adhering on the surface. 2° cells display significantly higher twitching ability compared to the wildtype (WT) effect that is also impaired in the 2° Δ*sdiA* mutant (*, *P ≤ 0.05*). The pictures represent one characteristic of at least three independently performed experiments with similar outcomes. (C) Biofilm formation: crystal violet stained biofilm was quantified at 575 nm. Usually, 1° cells produce significantly more biofilm compared to 2° cells. However, deletion of *sdiA* led only in 2° cells to a significantly increased biofilm formation of about 67% (*, *P ≤ 0.05*) after 24 h. (D) Biofilm formation of *P. luminescens* 1° and 2° upon 3.3% (v/v) PRE. Significant difference occurs only after 72 h of incubation with an increased biofilm of about 54% for 2° cells (*, *P ≤ 0.05*). The error bars represent standard deviation of at least three biological replicates. (E) Biofilm formation. Crystal violet staining of *P. luminescens* DJC. Left panel: 1° and 2° WT and Δ*sdiA*; right panel: 1° and 2° WT supplemented with 3.3% PRE. The pictures represent one characteristic of at least three independently performed experiments with similar outcomes.

Fractions of PRE and AHLs influence protein stability indicating putative signals for the LuxR solo SdiA

Similar phenotypes with respect to biofilm formation and motility were observed in *P. luminescens* 2° in presence and absence of PRE and the *sdiA* deletion. Therefore, it might be possible that a putative signal sensed by SdiA is present in PRE. To test this hypothesis, we performed nano differential scanning fluorimetry (nanoDSF) analyses measuring protein stability of purified SdiA in presence and absence of PRE. Furthermore, nanoDSF was used i) to exclude possible buffer derived denaturing of SdiA during protein purification and ii) to test the protein stability upon PRE as well as AHLs, since signal binding to LuxR receptors promotes conformational changes influencing protein stability (Whitehead et al., 2001). The measurement showed high thermostability of SdiA with an onset point (T_{ON}) in average of about 44°C, where the protein started to unfold. Furthermore, the inflection point in average at 53.3°C indicates the moment, where half of the protein appears in unfolded state and is equal to T_M (**Fig 3A**). Control measurement supplementing SdiA with the respective solvent that was used to dissolve the AHLs showed a SdiA T_M decreased to 46.1°C (**Fig. 3A, right panel**). After establishing a good protein stability, we tested the influence of several compounds such as short chain C_4-AHL, long chain C_{12}-AHL as well as PRE and respective HPLC-fractions on the protein folding properties in order to identify possible signal molecules recognized by this receptor. A putative ligand binding to LuxR-type receptors leading to conformational changes (Whitehead et al., 2001) becomes visible as temperature shift on the protein folding temperature (T_M) which can be measured. We could observe that both, C_4- and C_{12}-AHL, influenced SdiA folding temperature (C_4-ΔT_M= -3.9°C and C_{12}-ΔT_M= +1.6°C), however, upon binding of C_{12}-AHL SdiA increased in stability, appearing as a 'right-shifted' curve with higher T_M (47.7°C). With C_4-AHL SdiA appeared less stable, indicated by the 'left-shifted' curve with lower T_M (42.2°C) when compared to the control protein (**Fig. 3A, right panel**). These data indicate a lower selectivity of SdiA towards different AHLs which is likely dependent on the lengths of the acyl chain. Probably, the signal-binding pocket of the receptor appears in different conformational states, upon signal binding, putatively influencing DNA-binding properties of SdiA. Similarly, for *E. coli* SdiA it was shown that at least derivatives of an AHL were also capable to act as folding switch autoinducers for SdiA (Yao et al., 2006). Indeed, our data strengthens the hypothesis, that SdiA

signal independent regulatory role is disturbed by signal binding, since we could observe unstable conformations of the protein upon putative signal binding.

Additionally, it is known that plants produce molecules mimicking AHLs, which are sensed by e.g. *Pseudomonas* (Bais et al., 2006; Bez et al., 2021). For that reason and to gain insights whether SdiA might bind a plant-derived compound, we tested PRE as full mixture and subsequently as well as PRE fractions previously separated through HPLC. Indeed, our data indicated a yet unknown compound in PRE that binds SdiA, as the protein stability was affected, i.e., fractions B1, C3 and D7 were decreasing the SdiA folding temperature (lower T_M), which was visible as a 'left-shifted' curve compared to the SdiA-control (**Fig. 3A, left and middle panel**). Accordingly, we conclude that PRE contain putative signal molecules, which are recognized by SdiA of *P. luminescens*. However, the chemical nature of this plant-derived signal has still to be elucidated. At this point various molecules produced by plants could act as signal for SdiA. Interestingly, different studies showed that plants secret a variety of derived molecules that bind to LuxR regulators. These molecules can either be bacterial AHL-mimicking compounds (Teplitski et al., 2000), or small molecules that are recognized by a subgroup of LuxR solos (Patel et al., 2013). Therefore, we suggest that SdiA putatively senses an AHL-like molecule produced by plants. However, some other possible candidates of signal molecules binding SdiA are glycerol and respective derivatives, which bound to SdiA of EHEC in absence of AHLs (Nguyen et al., 2015). Both molecules have also been found to be present in PRE (Regaiolo et al., 2020), indicating that *P. luminescens* SdiA could also detect those molecules as signals. Furthermore, it has been shown before that plant-derived indole compounds influenced SdiA-regulated gene expression in *E. coli* (Lee et al., 2007). Interestingly, in recent studies plant derived ethanolamine derivatives were shown to bind LuxR a regulator from *Pseudomonas* GM79 that subsequently led to expression of different genes (Coutinho et al., 2018). Therefore, all these plant-derived signals are promising candidates as signal sensed by *P. luminescens* SdiA.

Taken together, we suggest a new IKS communication circuit in *P. luminescens*, where SdiA is involved in communication with plants, whereupon SdiA gene modulation might be affected by signal binding due to conformational changes. Our findings suggest that it is likely that SdiA undergoes a conformational change, indicated by the temperature shifts, upon binding short chain AHL and PRE. Subsequently, dimerization of the protein is putatively impaired, resulting in reduced DNA-binding affinity, similar as

observed for EsaR of *Pantoea stewartii*, where AHLs blocked DNA binding capacity (Castang et al., 2006; Cui et al., 2005; Minogue et al., 2002; 2005). Hence, since *P. luminescens* displayed similar phenotypes when deleting *sdiA* or after exposure to PRE, we suggest that PRE either affect SidA signal binding or include a signal sensed by SdiA. However, it still has to be determined, which plant derived compound binds to SdiA, and whether SdiA-DNA-binding capacity is really affected upon signal binding.

SdiA binds P_{sdiA} and P_{aidA} with high affinity

It is well known that LuxR transcriptional regulators undergo conformational changes upon signal binding enabling the C-terminal HTH domain to bind their target gene promoter within the *lux* box controlling gene expression (Devine et al., 1989; Stevens and Greenberg, 1997). Usually, LuxR receptors also regulate transcription of the cognate *luxI* gene, which is designated as autoinduction (Fuqua et al., 1994). However, since SdiA is a LuxR solo lacking a cognate LuxI synthase, we hypothesized that SdiA regulates its own promoter region. To validate this idea, and also the hypothesis that SdiA regulation is signal independent we first analyzed the binding affinity and binding kinetics of SdiA to its respective promoter (P_{sdiA}) via Surface Plasmon Resonance (SPR) spectrometry without addition of a putative ligand. Since located in the same intergenic region, we also analyzed the binding of SdiA towards the promoter of the neighboring gene *aidA* (P_{aidA}). The gene *aidA* is located upstream of *sdiA* and codes in the opposite orientation, thus sharing a 310 bp long promoter containing intergenic region, both harboring possible *lux*-box-like motifs. Furthermore, we also analyzed binding of SdiA to P_{fliE} as negative control. Within P_{fliE} no *lux*-box-like motif was found, assuming that the promoter sequence cannot be bound by the SdiA-HTH-domain (**Fig. 3B**). Remarkably, SdiA showed high affinity binding towards its own promoter (K_D = 27.4 nM) with high association and high dissociation rate (P_{sdiA} - k_a = 1.12E+05 1/Ms, k_d = 3.07E-03 1/s). Interestingly, SdiA bound with a ~3.5x higher association rate to the promoter of *aidA*, however, the protein-DNA complex remained stable with an extreme low dissociation rate K_D = 4.36 nM (P_{aidA} - k_a = 3.92E+05 1/Ms, k_d = 1.71E-03 1/s) (**Fig. 3B**). This indicates a very strong interaction of SdiA with P_{aidA}, proving our hypothesis that SdiA regulates expression of *adiA* similar to as it was described for SolR LuxR of *Ralstonia* which positively regulates expression of *aidA* genes required for virulence (Meng et al 2015). Additionally, for the SdiA-P_{aidA} interaction we could

observe a 1:2 binding stoichiometry indicated by reaching the double R_{max} value, which is in accordance with the occurrence of two putative LuxR binding boxes in the respective region. In contrast for P_{sdiA} with only one putative LuxR binding site, we observed a 1:1 binding stoichiometry. According to the different binding kinetics and stoichiometry, we conclude that the 310 bp long intergenic region between *aidA* and *sdiA* acts a bidirectional promoter. Thus, SdiA is capable of bidirectional stimulation of transcription of these two oppositely oriented genes similar as it was already described for LuxR of *Vibrio fischeri* stimulating expression of the *lux* operons (Shadel and Baldwin, 1991).

In order to elucidate whether DNA-binding ability of SdiA is affected in presence of putative signaling molecules the binding properties of SdiA towards P_{sdiA} and P_{aidA} was investigated in the presence of PRE. Indeed, binding affinity towards both promoters was reduced, while association of SdiA towards P_{sdiA} remained similar, dissociation from the promoter occurred with a K_D = 109 nM faster in presence of PRE (P_{sdiA} - k_a = 1.84E+05 1/Ms, k_d = 2.00E-02 1/s) (**Fig. 3B bottom left**). Similarly, a faster dissociation of SdiA from P_{aidA} occurred upon PRE indicated by a K_D = 13.3 nM, while here also association towards P_{aidA} was affected (P_{aidA} - k_a = 8.23E+04 1/Ms, k_d = 1.10E-3 1/s) (**Fig. 3B, bottom middle panel**). Our data further support the hypothesis that a plant derived signaling molecule binds to SdiA and subsequently influences its DNA-binding property (**Fig. 3B**). Although, the mechanism behind is still unknown, and considering the role of *aidA* in *R. solanacearum* and its regulation mediated by SolR, our data further propose a putative role of *aidA* in *P. luminescens* in bacterial-plant interaction as it is i) regulated by the LuxR solo coding neighboring *sdiA*, similar to *R.* solanacearum (**Fig. 1B**), and ii) which regulation via SdiA is influenced by the presence of PRE.

The exact mechanism behind this regulation, the role of *aidA* in *Photorhabdus*-plant interaction as well as the chemical nature of the plant derived signaling molecule binding to SdiA are still under study. Hence, further insights into the effect of the putative mentioned signal molecules on SdiA DNA-binding property should be gained, to understand the regulatory hierarchy of SdiA upon plant signals in *P. luminescens*.

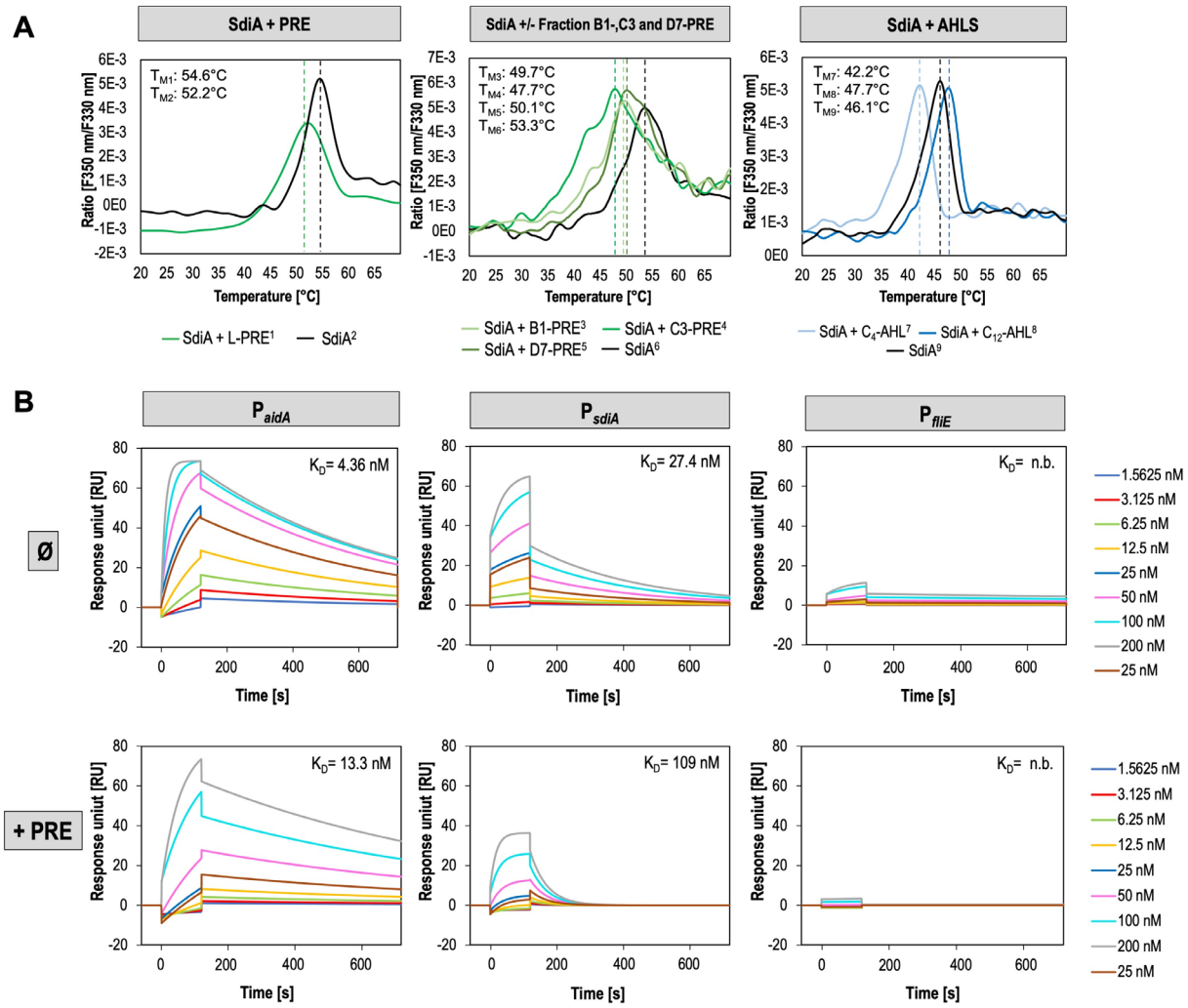


Figure 3: Influence of putative signaling molecules, SdiA purification and binding kinetics of SdiA to different promoters. (A) nanoDSF analyses of SdiA with supplementation of lipophilic plant root exudates (L-PRE, left panel), the selected respective fractions (B1, C3, D7, middle panel) and 10 nM of C_4- or C_{12}-AHLs (right panel). The graphs represent the 1st derivative of the measured ratio of intrinsic tryptophane fluorescence at 350 and 330 nm of the protein. The maximum peak represents the melting temperature T_M, where half of the protein is denatured. T_M values are indicated by the dotted lines colored respectively to the curve. In all measurements the black line shows the control protein measured with solvent, when necessary. Left shift of the graphs indicates different protein conformation upon molecule binding. Left panel: PRE influence SdiA stability upon putative signal molecule binding with ΔT_M -2.4°C. Middle panel: putative signaling molecule found in PRE-fractions indicated by ΔT_M -3.8°C (B1, light green), ΔT_M -5.8°C (C3, green), and ΔT_M -3.4°C (D7 dark green). Right panel: AHLs putatively bind to SdiA with different modes of action indicated by ΔT_M -3.9°C (C4-AHL, light blue) and ΔT_M +1.6°C (C12-AHL, blue), showing increased stability of the protein. (B) Binding kinetics of SdiA to the promoters P_{aidA}, P_{sdiA}, and P_{fliE} (negative control). The biotinylated DNA fragments were immobilized onto a SA sensor chip and various concentrations of SdiA (1.5625-200 nM) were injected without (top panel indicated by Ø) and with addition of PRE. The graphs show high affinity binding of SdiA to P_{aidA} and P_{sdiA} and K_D values indicate the binding affinities; n.b. = no binding. All graphs and sensograms represent one characteristic measurement of at least three independently performed experiments with similar outcome.

Conclusion

Recent research showed potential biocontrol ability of *P. luminescens* 2° cells, indeed the bacteria can colonize plant roots protecting them from phytopathogenic fungi infection. As 2° cells do not re-associate with nematodes and colonize plant roots, it was important to understand and to determine how the bacteria sense the rhizosphere environment. For that purpose, we indicate the LuxR solo SdiA harboring an AHL-like SBD as a putative IKS communication receptor between *P. luminescens* and plants. First, in this work we could demonstrate a regulatory role between SdiA motility as well as biofilm formation regulating a putative switch between a sessile and motile lifestyle (**Fig. 4**). Nevertheless, how and which respective genes are regulated is unknown and must be determined. Based on our findings we propose that SdiA regulates expression of the target genes in a signal-independent manner, which is then impaired upon signal binding (**Fig. 4**). Indeed, in this work we could demonstrate, that SdiA protein conformation is affected by short-chain AHLs and PRE and respective fractions indicating i) plant derived molecule as signal for SdiA and ii) an influence of signal binding on the regulatory role of SdiA. Although the nature of the plant derived compound must be determined yet, the data suggest a SdiA mediated interkingdom communication of *P. luminescens* with plants as DNA binding was also influenced upon PRE. We also identified that SdiA acts as bidirectional regulator for the intergenic region of *sdiA* and *adiA* since it binds both promoters with high affinity. Lastly, for AidA of *P. luminescens*, of which homologs are also found in other plant associated bacteria and the expression of the respective gene is regulated by a LuxR-type receptor, we suggest that AidA is important for *P. luminescens* plant colonization with its regulation mediated by SdiA and impacted upon PRE (**Fig. 4**).

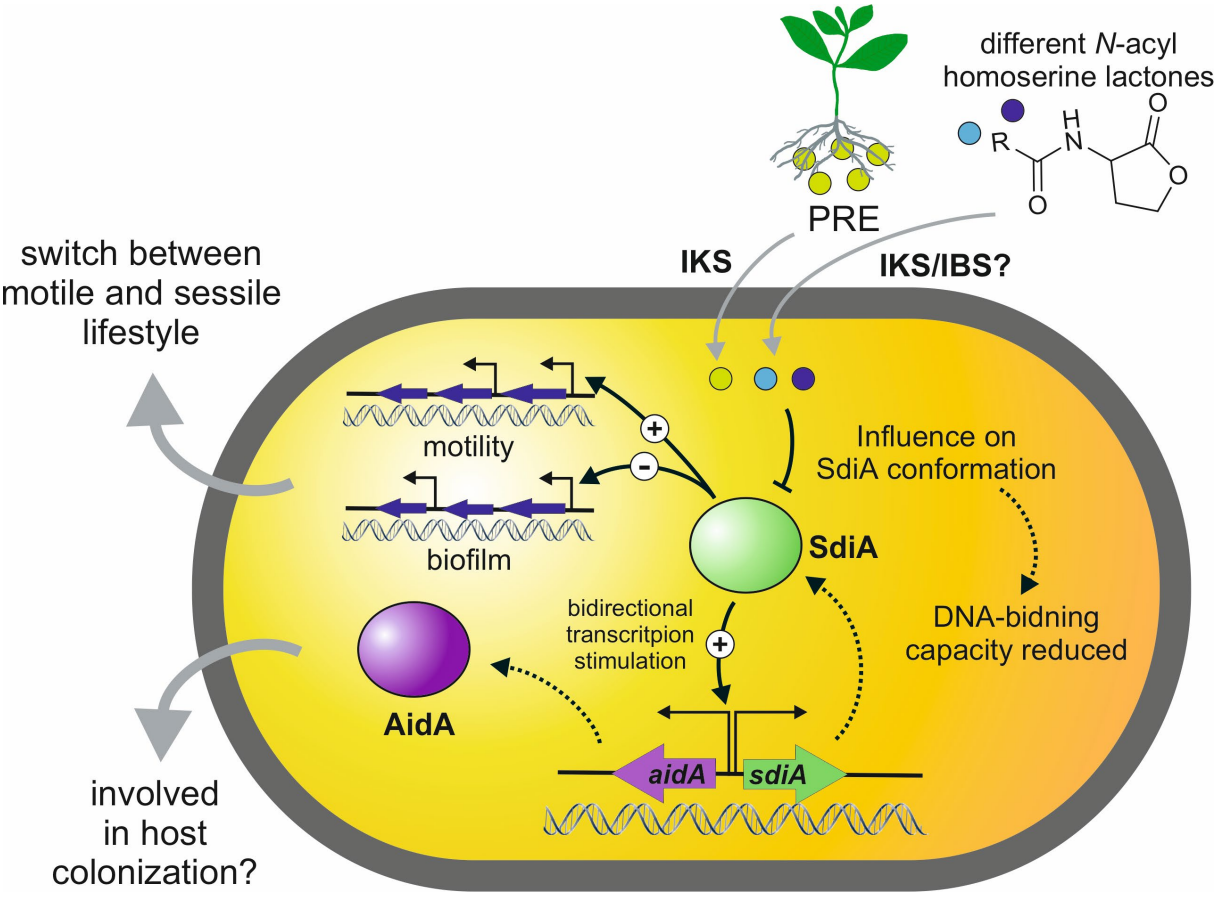


Figure 4: Model of the putative role of AHL-LuxR solo SdiA of *P. luminescens*. The LuxR type receptor SdiA (PluDJC_016750) regulates the expression of several genes in absence of a signaling molecule, thereby modulating a switch of *P. luminescens* cells between a sessile and motile lifestyle. SdiA is involved in interkingdom signaling (IKS) communication with plants. Upon sensing a plant-derived signal, the SdiA undergoes a conformational change resulting in reduced DNA-binding affinity. SdiA acts as bidirectional regulator of transcription, binding within the intergenic promoter region of *sdiA* and *aidA* genes. AidA might be therefore involved in SdiA mediated host colonization. However, SdiA reacts also to different AHLs, thereby it might also be involved in interspecies bacterial signaling (IBS) communication.

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6. High throughput sequencing analysis reveals genomic similarity in phenotypic heterogeneous *Photorhabdus luminescens* cell population

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High throughput sequencing analysis reveals genomic similarity in phenotypic heterogeneous *Photorhabdus luminescens* cell populations

Nazzareno Dominelli[1], Heidi Yoko Jäger[2], Angela Langer[3], Andreas Brachmann[4], Ralf Heermann[1*]

[1]Johannes-Gutenberg-University Mainz, Institute of Molecular Physiology (imP), Biocenter II, Microbiology and Biotechnology, Hanns-Dieter-Hüsch-Weg 17, 55128 Mainz, Germany
[2] Institute for Mummy Studies, EURAC Research, Viale Druso 1, 39100 Bolzano, Italy
[3]Ludwig-Maximillians-University München, Biocenter, Microbiology, Großhaderner Str. 2-4, 82152 Martinsried, Germany
[4]Ludwig-Maximillians-University München, Biocenter, Genetics, Großhaderner Str. 2-4, 82152 Martinsried, Germany

*****corresponding author (heermann@uni-mainz.de)

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Abstract

Purpose

Phenotypic heterogeneity occurs in many bacterial populations: single cells of the same species display different phenotypes, despite being genetically identical. The Gram-negative entomopathogenic bacterium *Photorhabdus luminescens* is an excellent example to investigate bacterial phenotypic heterogeneity. Its dualistic life cycle includes a symbiotic stage interacting with entomopathogenic nematodes (EPNs) and a pathogenic stage killing insect larvae. *P. luminescens* appears in two phenotypically different cell forms: the primary (1°) and the secondary (2°) cell variant. While 1° cells are bioluminescent, pigmented and produce a huge set of secondary metabolites, 2° cells lack all these phenotypes. The main difference between both phenotypic variants is that only 1° cells can undergo symbiosis with EPNs, a phenotype that is absent from 2° cells. Recent comparative transcriptome analysis revealed that genes mediating 1° cell-specific traits are modulated differently in 2° cells. Although it was previously suggested that heterogeneity in *P. luminescens* cells cultures is not genetically mediated by e.g., larger rearrangements in the genome, the genetic similarity of both cell variants has not clearly been demonstrated yet.

Methods

Here, we analyzed the genomes of both 1° and 2° cells by genome sequencing of each six single 1° and 2° clones that emerged from a single 1° clone after prolonged growth. Using different bioinformatics tools, the sequence data were analyzed for clustered point mutations or genetic rearrangements with respect to the respective phenotypic variant.

Result

We demonstrate that isolated clones of 2° cells that switched from the 1° cell state do not display any noticeable mutation and do not genetically differ from 1° cells.

Conclusion

In summary, we show that the phenotypic differences in *P. luminescens* cell cultures are obviously not caused by mutations or genetic rearrangements in the genome but truly emerge from phenotypic heterogeneity.

Findings

Bacteria constantly encounter different environmental stress conditions, whereupon they have evolved different survival strategies to cope with these challenges. Besides altering the expression of single genes, one of these adaptation strategies are genetic modifications such as occurrence of DNA methylation or genomic rearrangements to evolve a different phenotype for adaptation (Smits et al. 2006). Phenotypic heterogeneity instead is another strategy, e.g., for bet-hedging to ensure survival of a bacterial population describing the appearance of different phenotypic cells within a genetically identical population (Avery 2006; Davidson and Surette 2008; Grote et al. 2015), resulting in phase variation mostly correlated with altered gene expressions (Elowitz et al. 2002; van der Woude 2011; Davis and Isberg 2016). Examples of this adapting phenotypic heterogeneity are persister cells (Balaban et al. 2004) as well as the occurrence of competence or sporulation of the Gram-positive bacterium *Bacillus subtilis* (Veening et al. 2005; Smits et al. 2006) Phenotypic heterogeneity also occurs in the entomopathogenic bacterium *Photorhabdus luminescens*, which exists in two phenotypically different cell forms, the endosymbiotic primary (1°) cells and the free-living secondary (2°) cells. In its dualistic life cycle the 1° cell variant colonizes entomopathogenic nematodes (EPNs), which invade insect larvae in the soil. Once inside, the EPNs release the bacteria into the haemocoel, where *P. luminescens* produces a huge set of toxins to effectively kill the larvae (Forst et al. 1997). During the infective cycle (also after prolonged cultivation in the laboratory) up to 50% of 1° cells switch to the 2° cell variant. The 1° cells exhibit different phenotypes such as biofilm formation, pigmentation, bioluminescence and the production of secondary metabolites, characteristics that are absent from 2° cells (Akhurst 1980; Forst et al. 1997; Joyce and Clarke 2003; Eckstein and Heermann 2019). Moreover, 2° cells can neither reassociate with EPNs nor support their growth and development anymore and therefore remain in the soil when the EPNs have left the depleted insect cadaver. Recent studies indicated a new fate of these 2° cells in soil, as this cell variant reacts to and interacts with plant roots (Regaiolo et al. 2020). Furthermore, comparative transcriptome analysis revealed that genes responsible for 1° cell-specific phenotypes are downregulated in 2° cells (Eckstein et al. 2019). Although the exact regulation mechanism of this phenotypic switching in *P. luminescens* is yet unknown, some studies showed, that transcriptional regulators play an important role during this event. One of these regulators is HexA, a member of the LysR-type transcriptional regulator

family. HexA is involved in the phenotypic switching process of *P. luminescens*, by directly and indirectly repressing expression of 1° cell specific genes (Joyce and Clarke 2003; Langer et al. 2017). Moreover, the RNA chaperone Hfq regulates expression of *hexA* mediating higher copy numbers of HexA in 2° cells, suggesting that Hfq is also involved as global regulator in the regulation of phenotypic heterogeneity in *P. luminescens* cell populations (Neubacher et al. 2020). Furthermore, XRE-like transcriptional regulators were shown to also control phenotypic heterogeneity in *P. luminescens*. Indeed, deletion of *xreR1* in 1° and *xreR2* in 2° cells and insertion of extra copies of *xreR1* in 2° cells and *xreR2* in 1° cells led to the opposite phenotype in both cell forms (Eckstein et al. 2021). The two-component system AstS/AstR was found to control timing of phenotypic switching in *P. luminescens*, since deletion of *astR* led to faster switching of 1° cells compared to the wildtype (Derzelle et al. 2004).

Phenotypic switching in *P. luminescens* has previously been referred to as phase variation (Akhurst and Boemare 1988). However, this phenomenon has been suggested to be different from classical bacterial phase variations as both cell forms were suspected to be genetically homogeneous (Forst et al. 1997). Classical phase variation involves reversible genetic events, occurs at significant frequency and is almost reversible. Larger DNA rearrangements or modifications, genetic instability, or the loss of plasmids were excluded in *P. luminescens* 2° cell formation suggesting that the differences between 1° and 2° cells are caused by phenotypic and not genetic heterogeneity in *P. luminescens* (Akhurst et al. 1992; Forst et al. 1997; Hu and Webster 1998; Forst and Clarke 2002). However, none of the previous studies could provide evidence that heterogeneity in *P. luminescens* cell populations is due to true phenotypic heterogeneity. For that reason, we analysed and compared genomes of both *P. luminescens* subs. *laumondii* strain DJC 1° and 2° (Zamora-Lagos et al. 2018) [later reclassified as *P. laumondii*, (Machado et al. 2018)] to prove whether the different characteristics of *P. luminescens* 1° and 2° derives from phenotypic and not from genotypic heterogeneity.

The experimental workflow is schematically presented in Figure 1. First, 1° cells were aerobically cultivated at 30°C by shaking at 200 rpm over 11 days in LB broth [1% NaCl (w/v); 1% tryptone (w/v); 0.5% yeast (w/v)] and streaked on LB agar plates. Upon the phenotypic appearance of red pigmentation and bioluminescence six single colonies of each cell variant were picked, bioluminescent and pigmented colonies as representatives for the 1°, and dark non-pigmented colonies as representatives for the

2° variant. Then, the genomic DNA was extracted from over-night cultures using genomic DNA extraction kit (Südlaborbedarf, Gauting, Germany) according to the manufacturers protocol, and high throughput sequencing (HTS) analysis was performed including the laboratory strains of *P. luminescens* 1° and 2°. For that, library preparation of 50 ng gDNA was performed using Nextra Library Prep Kit (Illumina) according to the manufacturers protocol. Libraries were quality controlled with DNA High Sensitivity DNA Kit on Bioanalyzer (Agilent) and quantified on Qubit 2.0 Fluorometer (ThermoFisher Scientific with ds HS Assay Kit). Genome sequencing was performed in the Genomics Service Unit (LMU Biocenter, Munich) on Illumina MiSeq with v2 chemistry (2x 250 bp paired-end sequencing). Resulted HTS data were processed using different bioinformatics tools. First, reads were aligned and mapped against the *Photorhabdus luminescens* subs. *laumondii* DJC reference genome (GenBank: CP024900.1) using Bowtie 2 (v 2.3.5) and then quality filtered with SAMtools (v. 1.13), allowing alignments with mapping quality >30 (Li et al. 2009; Langmead and Salzberg 2012). After that, read duplicates were marked via Picard tool (v. 2.21.4) (http://broadinstitute.github.io/picard/) to avoid distort genome coverage. Qualimap 2 was used for multi-samples quality control of HTS data (Okonechnikov et al. 2015). Pairwise comparison of the genomes was performed using VarScan 2 (v. 2.4.2), where variants with a base and mapping quality >30 were called and filtered for single nucleotide polymorphisms (SNPs) with a coverage of >20x (Koboldt et al. 2012). The resulting data were manually inspected for informative SNPs. The sequence data were uploaded at the NCBI sequence read archive under BioProjects PRJNA812858 for 1° clones and PRJNA812795 for 2° clones (https://www.ncbi.nlm.nih.gov). For the laboratory strains of *P. luminescens* 1° (DJC 1°) and 2° (DJC 2°), both with a coverage of >90% no SNPs were detected when comparing to reference genome, whereas the mean GC-content of the mapped reads ranged between 42-47% (Table S1). During library preparation, PCR is one of the principal sources leading to GC-content bias in HTS. Indeed, diverse base composition bias in the G and C bases emerge during library preparation upon PCR (Dohm et al. 2008, Aird et al. 2011, Benjamin and Speed, 2012). With a coverage of 100% both variants are genetically identical confirming that the different appearances are due to phenotypic heterogeneity upon different gene expression. However, the sequencing data displayed various SNPs occurring in 1° cells as well as in 2° cells only after prolonged cultivation. In average with a mean rate of 95% all sequenced samples displayed high coverage of the reference genome

(Table S1). Generally, we observed only a few mutations among all tested samples, which is in accordance with the observation that *P. luminescens* has lowest mutation rate among bacteria (Pan et al. 2021). Nevertheless, all these mutations are not consistent, as all the *P. luminescens* 1° as well as 2° samples displayed mutations in different loci (Table 1). In all tested samples 1-4 SNPs for 1° cells and 1-6 SNPs for 2° cells were detected. Spontaneous gene mutation during replication, presumably also after prolonged cultivation, is not a rare phenomenon, their occurrence was explained to keep a balance between effects of deleterious mutation rate and metabolic costs (Drake et al. 1998; Denamur and Matic 2006). Generally, the mutation rate in bacteria was described to range between $1*10^{-6}$ and $1*10^{-8}$ base substitutions per nucleotide per generation (Westra et al. 2017). For example, in *E. coli* a deleterious mutation rated of $2\text{-}8*10^{-4}$ and a beneficial mutation rated of $2*10^{-9}$ per genome per replication has been calculated (Kibota and Lyncht 1996; Boe et al. 2000; Imhof and Schlotterer 2001), whereas recent studies reported a low mutation rate with a low base-substitution rate of $5.94*10^{-11}$ per nucleotide site per cell division in *P. luminescens* (Pan et al. 2021). Most of the genes affected from this spontaneous mutation in our analysis code for phage tail fibers in 1° as well as 2° cells. These genes are: *PluDJC_00175* (phage tail collar domain), *PluDJC_15370* (phage tail domain), *PluDJC_15455* (phage tail fiber repeat and collar domain). The latter one occurred to have the most mutations in both cell variants and in some cases point mutations led to a base pair exchange not affecting the amino acid sequence. Moreover, in three from the six switched clones (2° cells) we observed single point mutations in different regions of *rPOD* (*PluDJC_19710*). However, this mutation was not observed in the 2° cell control genome and the other switched variants. Further genes or promoters displaying mutations are listed in Table 1. Additionally, some of the genes that displayed a mutation in the genome analysis have been further checked on mutations. For that purpose, single 1° and 2° colonies were again picked and aerobically cultivated for 48 h at 30°C, and genomic DNA was extracted using genomic DNA extraction kit (Süd-Laborbedarf, Gauting) according to the manufacturers protocol. Primers (Table 2) were designed to amplify the respective genes, sequenced, and aligned to the reference genome. Although no mutations were found for *hexA* and *hfq* in HTS analysis, primers for both including the promoters of both respective genes were designed to exclude potential mutations in the promoter regions affecting gene expression. Sequencing data revealed no mutations in the *rpoD*, *PluDJC_00175,* and *hex* and *hfq* promoter

regions neither in 1° nor in 2° cells. For *PluDJC_15370* and *PluDJC_15455* (only few) mutations for both cell variants were detected. These mutations were located in different parts of the respective genes as pointed out in the HTS data, but they were not equally distributed throughout the cell variations and were not found in all switched samples. Even though, these mutations are inconsistent and do not lead to genotypic heterogeneity, considering that no phenotypic cell variations have been observed, further work should be investigated to understand the higher mutation rate in *P. luminescens* phage related genes (e.g., *PluDJC_15370* and *PluDJC_15455*). Mutations in loci coding for phage subunits as well as loci involved in immunity against phages are known to have a faster mutation rate compared to point mutations in random genomic regions (Bikard and Marraffini 2012).

Taken together our data prove evidence that variations in *P. luminescens* subs. *laumondii* DJC cell population is truly based on phenotypic heterogeneity. The identified mutations after long term cultivation are due to spontaneous mutations that are randomly distributed on different genes and not always located in the same genetic area, so that genetic modifications or genomic rearrangements are not involved in phenotypic heterogeneity, i.e. phenotypic switching from 1° to 2° cells in *P. luminescens* cell populations.

TABLES

Table 1: Point mutations in different gene loci of *P. luminescens* 1° and 2° clones identified by HTS. Sample ID 1 indicates 1° cells and 2 indicates 2° cells of *P. luminescens*. A-F indicate the 6 different replicates of each tested phenotypic variant. Info indicates the sum of coverage with the reference genome (n1), the number of MATCHES with the covered region (n2), and the number of occurring SNPs within n1 (n3) represented as n1:n2:n3.

Position of the mutation	Base exchange	AA change	Sample ID	Info	Gene name
30026	G > A	Ala >Val	1B	33:22:11	*PluDJC_00175*
30026	G > A	Ala >Val	2E	36:26:10	*PluDJC_00175*
30038	A > G	Met > Thr	1B	33:25:8	*PluDJC_00175*
30038	A > G	Met > Thr	2E	37:26:11	*PluDJC_00175*
287646	T > C	-	2E	24:17:7	$P_{Plu\_DJC01390}$
609596	C > A	Ala > Glu	1A	43:0:43	*PluDJC_15370*
609596	C > A	Ala > Glu	1F	22:15:7	*PluDJC_15370*
609596	C > A	Ala > Glu	2C	29:21:9	*PluDJC_15370*
3433543	C > T	Ser > Asn	1E	34:27:7	*PluDJC_15455*
3433543	C > T	Ser > Asn	2D	29:21:7	*PluDJC_15455*
3433563	C > T	Ala > Ala	2D	30:23:7	*PluDJC_15455*
3433570	T > C	Glu > Ala	1B	29:22:7	*PluDJC_15455*
3433570	T > C	Glu > Ala	2D	28:20:8	*PluDJC_15455*
3433581	T > C	Thr > Thr	1E	31:24:7	*PluDJC_15455*
3433581	T > C	Thr > Thr	2D	30:22:8	*PluDJC_15455*
3433581	T > C	Thr > Thr	2A	30:23:7	*PluDJC_15455*
3433584	T > C	Leu > Leu	1E	27:20:7	*PluDJC_15455*
3433584	T > C	Leu > Leu	2D	31:23:8	*PluDJC_15455*
4487898	A > G	Glu > Gly	2A	40:40:0	*PluDJC_19710* (*rpoD*)
4487898	A > G	Glu > Gly	2B	37:37:0	*PluDJC_19710* (*rpoD*)
4487898	A > G	Glu > Gly	2F	23:23:0	*PluDJC_19710* (*rpoD*)
4513459	C > A	-	2D	62:62:0	P_{PluDJC_19885}
4841989	C > T	Gly > Arg	2C	45:45:0	*PluDJC_21265*

Table 2: Primers used for amplification of the respective genes carrying point mutations identified by HTS analysis. Primers were used for gene amplification and further sequencing.

Primer name	Sequence (5´→3´)	Gene
PluDJC_00175 fwd	cccaatattgcggtttctgg	*PluDJC_00175* (phage tail collar domain)
PluDJC_00175 rev	ctccatatgtaaccctgtc	
PluDJC_15370 fwd	ccagcacactgcttcaacac	*PluDJC_15370* (phage tail collar domain)
PluDJC_15370 rev	cccttgaatgaggtgctgca	
PluDJC_15455 fwd	gcatggtagattgtcagcca	*PluDJC_15455* (phage tail collar and fiber domain)
PluDJC_15455 rev	acctatggggataacggt	
rpoD fwd	ctataagtgggcagcggcaa	*rpoD* (*PluDJC_19710*)
rpoD rev	tcaccggatggaaaacgac	
hexA fwd	cgaggagctaatacctcctt	P_{hexA} + *hexA* (*PluDJC_15800*)
hexA+Prom rev	ttctttgacgtgagtcag	
hfq fwd	cgttcaaacaaaggtgcgac	P_{hfq} +*hfq* (*PluDJC_22705*)
Hfq+Prom rev	ccagagcaagctttaagcac	

FIGURES

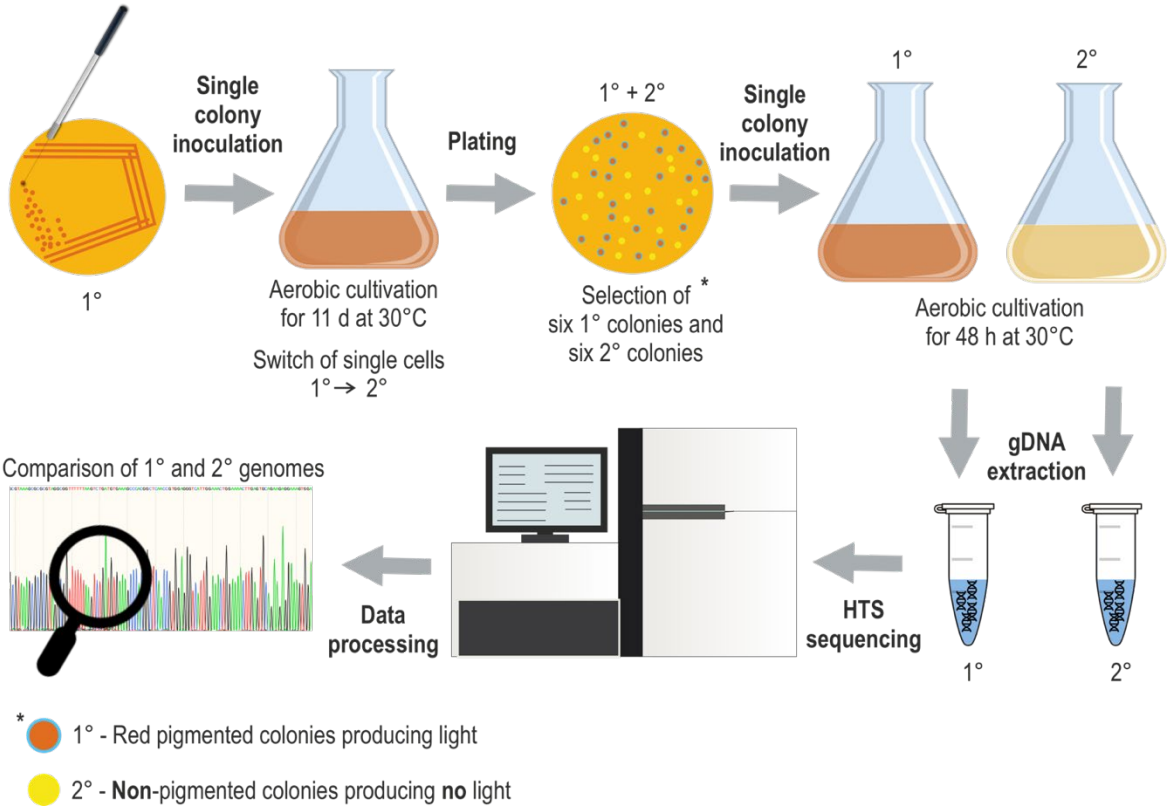

Figure 1: Schematic presentation of the experimental workflow for genomic comparison between *P. luminescens* 1° and 2° variants. In brief, *P. luminescens* 1° cells were cultivated for 11 days so that a large proportion of single cells were switched to the 2° phenotype, and then plated on LB agar. Then, six single colonies of each variant, 1° and 2°, were picked, cultivated and genomic DNA was isolated, analysed by HTS, and resulted DNA sequence data was mapped against the *P. luminescens* ssp. *laumondii* DJC reference genome.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article or are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

N.D. performed the PCR and sequencing analyses of single genes including point mutations. H.Y.J. has performed the bioinformatics analyses and genome comparisons. A.L. performed the phenotypic switching assays and isolated the DNA from primary and secondary cell clones for genome sequencing. A.B. performed high throughput sequencing analyses. R.H. coordinated the project. N.D. and R.H wrote the manuscript. All authors reviewed the manuscript.

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7. “Small Talk” – Die stille Kommunikation der Bakterien

Dominelli N and Heermann R (2020). “Small Talk” – Die stille Kommunikation der Bakterien. Biol Unserer Zeit **50:6,** 414-423. https://doi.org/10.1002/biuz.202010720

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Die stille Kommunikation der Bakterien

„Small Talk“

NAZZARENO DOMINELLI | RALF HEERMANN

***Photorhabdus luminescens* auf einer Platte mit Kulturmedium. Das „Z“ stellt den Bakterienrasen dar. Vorne links im Bild sind zwei Insektenlarven zu sehen.**

Der Austausch von Informationen spielt nicht nur bei höheren Organismen eine wichtige biologische Rolle. Auch Bakterien können miteinander kommunizieren. So tauschen sie Botschaften sowohl untereinander, als auch mit ihren eukaryotischen Wirten wie Pflanzen, Tieren und sogar mit uns Menschen aus. Die Entschlüsselung des molekularen Mechanismus dieses „Small Talks“ spielt in der aktuellen mikrobiologischen Forschung eine zentrale Rolle, da sie Basis für die Entwicklung neuer Medikamente gegen Infektionskrankheiten sein könnte.

Bakterien sind überall – in der Luft, im Boden, in Seen und Meeren, auf Pflanzen und Tieren, sowie auf und in uns Menschen. Sie sind uns in ihrer Anzahl und Vielfalt weitaus überlegen. In der Natur leben Bakterien aber nicht als Einzelgänger, sondern meistens organisiert in Gemeinschaften. Für das Leben der Bakterien in solchen Gemeinschaften ist es von zentraler Bedeutung, dass die einzelnen Zellen miteinander kommunizieren und sich „absprechen“. Dafür haben Bakterien spezifische Kommunikationswege, ja sogar unterschiedliche „Sprachen“ entwickelt. Diese Sprachen sind, anders als bei uns Menschen, aber nicht von verbaler, sondern von „stiller“ Natur. Bakterien nutzen zur Kommunikation kleine diffusionsfähige Moleküle, die sie kontinuierlich in ihre Umgebung abgeben. Bei einer steigenden Zellzahl steigt somit auch die Konzentration dieser Sprachmoleküle, so dass die Gemeinschaft die Anzahl ihrer Artgenossen erkennen und entsprechend darauf reagieren kann. Wenn ein bestimmtes ▸„Quorum“, also eine Anzahl an Zellen in der Umgebung und somit eine bestimmte Konzentration an Sprachmolekülen, erreicht ist, aktivieren die einzelnen Zellen bestimmte Transkriptionsregulatoren und damit Phänotypen, die für ein Leben in der Gemeinschaft wichtig sind. Dieser Prozess wird daher auch als „Quorum sensing“ (QS) bezeichnet (Abbildung 1). Unter Kontrolle des QS stehen beispielweise solche Phänotypen, die für eine symbiotische, aber auch für eine pathogene Lebensweise der Bakterien entscheidend sind. Neben der Kommunikation untereinander gibt es neue Erkenntnisse darüber, dass Bakterien auch mit ihren Wirtsorganismen wie Tieren, Pflanzen und uns Menschen kommunizieren. In den letzten Jahren wurden zahlreiche molekulare Mechanismen der bakteriellen Kommunikation aufgeklärt, es wurden viele neue bakterielle „Sprachen“ und „Dialekte“ entdeckt.

Prototypen der bakteriellen Kommunikation

Die bakterielle Kommunikation ist zuerst beim ▸Gram-negativen Bakterium *Vibrio fischeri* aufgeklärt worden [1]. Diese Bakterien kolonisieren die Leuchtorgane von Tintenfischen und betreiben ▸Biolumineszenz – eine Eigenschaft, die unter Kontrolle des QS steht. Die Synthese des Sprachmoleküls, ein Acyl-Homoserinlakton (AHL), wird durch das Enzym LuxI katalysiert. Acyl-Homoserinlaktone bestehen aus einem Laktonring, welcher mit einer Fettsäureseitenkette modifiziert ist. Die hydrophoben AHL sind membrangängig und reichern sich so in der Umgebung der Zellen an. Nach Erreichen einer bestimmten Schwellenkonzentration nehmen die Bakterien AHL über einen im Zytoplasma lokalisierten Rezeptor, LuxR, wahr. Nach Interaktion mit den AHL binden LuxR-Rezeptoren an spezifische Bereiche der DNA und aktivieren oder reprimieren dadurch die Expression der stromabwärts lokalisierten Gene. Im Fall von *V. fischeri* gehört dazu

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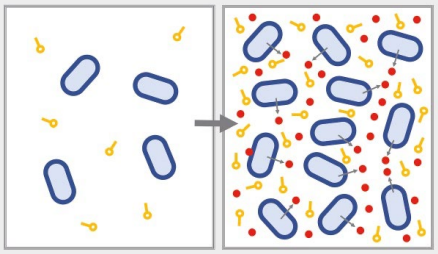
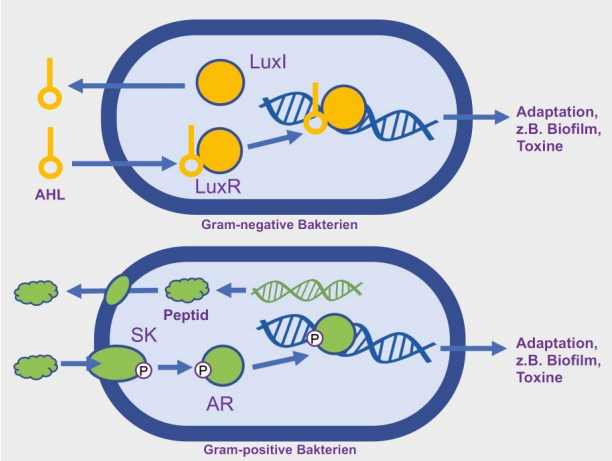
ABB. 1 | „QUORUM SENSING“ BEI BAKTERIEN



Die Zellen (blau) produzieren kontinuierlich „Sprachmoleküle“, hier in orange dargestellt. Ist die Zellzahl gering (links), so ist auch die Konzentration dieser Moleküle in der Umgebung gering. Steigt die Zellzahl (rechts), so steigt auch die Konzentration der „Sprachmoleküle“ in der Umgebung, welche zusätzlich durch eine Autoinduktion weiter erhöht wird. Die hohe Konzentration dieser Botenstoffe wird von den Bakterien wahrgenommen. Als Reaktion darauf beginnen sie ihren Phänotyp auf gruppenkoordiniertes Verhalten umzustellen (rote Kreise). Dies können beispielsweise die Produktion von Toxinen oder von Faktoren für die Bildung eines Biofilms sein.

ABB. 2 | PROTOTYPEN DER BAKTERIELLEN KOMMUNIKATION



Bei Gram-negativen Bakterien erfolgt die Kommunikation in der Regel über LuxI/LuxR-Systeme, die kleine diffusionsfähige Moleküle wie AHL (orange, oben) als Signalmoleküle verwenden. Gram-positive Bakterien verwenden stattdessen Zweikomponentensysteme, die Peptide (grün, unten) als „Sprachmoleküle“ verwenden. SK: Sensorkinase, AR: Antwortregulator. Details siehe Text.

auch das *lux*- ▸ Operon, welches das Enzym Luziferase kodiert. Wenn in der Umgebung eine hohe Dichte an Artgenossen erreicht ist, beginnen die Zellen deshalb, Licht zu produzieren. Da auch *luxI*, das für das Syntheseenzym der AHL kodiert, zu den QS-kontrollierten Genen gehört, kommt es zu einer sogenannten Autoinduktion, die eine weitere Steigerung der AHL-Synthese zur Folge hat. Acyl-Homoserinlaktone werden deshalb auch als Autoinduktoren bezeichnet. Das LuxI/LuxR-QS-System ist prototypisch für viele Gram-negative Bakterien (Abbildung 2). Während *V. fischeri* mit C6-AHL, einem AHL, dessen Laktonring mit einer sechs C-Atome langen hydrophoben Seitenkette modifiziert ist, kommuniziert, nutzen andere Gram-negative Bakterien unterschiedliche AHL, die z. B. in der Länge der Seitenkette variieren (Tabelle 1).

Aufgrund ihrer dickeren Zellwand nutzen ▸ Gram-positive Bakterien andere Komponenten zum QS (Abbildung 2). Dabei basiert das prototypische QS-System auf einem konventionellen bakteriellen ▸ Zweikomponentensystem, welches aus einer membranständigen Sensorkinase und einem im Zytoplasma lokalisierten Antwortregulator besteht. Gram-positive Bakterien nutzen kleine Peptide zur Kommunikation, die aktiv über ein Exportsystem aus der Zelle sezerniert werden [1]. Dabei erfolgt meist eine Modifikation und damit eine Aktivierung des Signalpeptids. Nach Erreichen einer Schwellenwertkonzentration bindet die Sensorkinase das Peptid, was zu einer ▸ Autophosphorylierung des Rezeptors führt. Diese Phosphorylgruppe wird umgehend auf den zugehörigen Antwortregulator übertragen, welcher daraufhin an die DNA bindet und die Expression der entsprechenden Zielgene reguliert. Unter diesen befindet sich oft auch das Gen, welches für das Signalpeptid kodiert, so dass auch bei Gram-positiven Bakterien eine Autoinduktion des QS erfolgt. In Abwesenheit des Signals hat die Sensorkinase gegenüber dem Antwortregulator Phosphataseaktivität. Indem sie den Antwortregulator dephosphoryliert, ist sie also in der Lage, die ▸ Signaltransduktionskaskade wieder abzuschalten. Aufgrund der chemischen Vielfalt von Peptiden haben auch die verschiedenen Gram-positiven Bakterien unterschiedliche „Sprachen“ entwickelt (Tabelle 2).

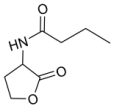
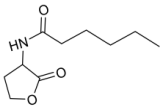
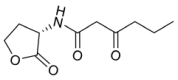
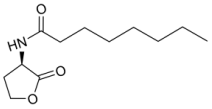
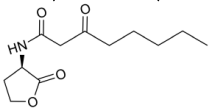
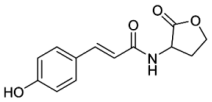
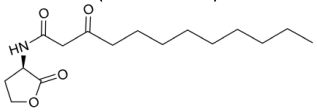
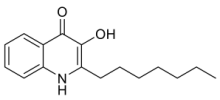
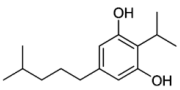
Die unterschiedlichen „Sprachen“ von Gram-negativen Bakterien

Die Kommunikation ähnelt bei fast allen Bakterien den oben beschriebenen Prototypen. Die Signalmoleküle bzw. Autoinduktoren unterscheiden sich aber in der Grundstruktur, wodurch eine artspezifische Kommunikation gewährleistet wird. Es können aber auch chemische

IN KÜRZE

- *Bakterien kommunizieren **über kleine Moleküle** miteinander und mit ihren eukaryotischen Wirten, um sich in ihren Lebensgemeinschaften „abzusprechen“ und anzupassen.*
- *Die bakterielle Kommunikation ist fast immer Grundvoraussetzung für Pathogenität, so dass die bakteriellen „Sprachen“ und „Dialekte“ vielversprechende **Wirkorte für neue Medikamente** gegen Infektionskrankheiten sind.*

TAB 1. BAKTERIELLE KOMMUNIKATION UND DIE ZUGEHÖRIGEN SENSORSYSTEME UND SPRACHMOLEKÜLE BEI GRAM-NEGATIVEN BAKTERIEN

Spezies	QS-System	Sprachmoleküle
		N-Butyryl-AHL (C4-AHL) 
Pseudomonas aeruginosa *Aeromonas hydrohila* *Aeromonas salmonicida*	RhlI/RhlR AhyI/AhyR AsaI/AsaR	
		N-Hexanoyl-AHL (C6-AHL) 
Chromobacterium violaceum *Yersinia enterocolitica* *Rhizobium legominosarum* *Ralstonia solanacearum*	CviI/CviR YenI/YenR RhiI/RhiR SolI/SolR	
		N-3-oxo-Hexanoyl-AHL (3-oxo-C6-AHL) 
Yersinia enterocolitica *Erwinia agglomerans* *Erwinia chrysanthemi*	YenI/YenR EsaI/EsaR ExpI/ExpR	
		N-Oktanoyl-AHL (C8-AHL) 
Ralstonia solanacearum	SolI/SolR	
		N-3-oxo-Oktanoyl-AHL (3-oxo-C8-AHL) 
Enterobacter agglomerans *Erwinia carotovora*	EagI/EagR ExpI/ExpR, CarI/CarR	
		p-Cumaroyl-AHL 
Rhodopseudomonas palustris	RpaI/RpaR	
		N-3-oxo-Dodekanoyl-AHL (3-oxo-C12-AHL) 
Pseudomonas aeruginosa	LasI/LasR	
		3,4-Dihydroxy-2-Heptylquinolin (PQS) 
Pseudomonas aeruginosa	PqsABCD/PqsR	
		Dialkylresorcinol (DAR) 2-Isopropyl-5-(2-Metyhlpentyl)-Resorzinol 
Photorhabdus asymbiotica	DarA/DarB/DarC/PauR	

Modifikationen der „Sprachmoleküle“ auftreten, wie beispielsweise unterschiedliche Längen in der hydrophoben Seitenkette von Acyl-Homoserinlaktonen, welche dann auch von anderen Bakterienarten mit geringerer Spezifität „verstanden“ werden können. Der Laktonring der AHL wäre vergleichbar mit dem Sprachstamm, die unterschiedlich langen Seitenketten (C4-C18) wiederum mit einem Dialekt. So sind bei verschiedenen Gram-negativen Bakterien unterschiedliche „Sprachen“ und „Dialekte“ identifiziert worden (Tabelle 1) [2, 3]. Einzelne Bakterienarten können auch mit mehreren Dialekten parallel kommunizieren.

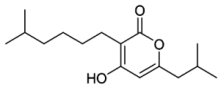
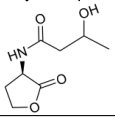
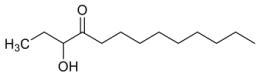
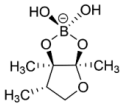
Besonders gut ist die bakterielle Kommunikation bei *Pseudomonas aeruginosa* verstanden [4]. Die Bakterien lösen nosokomiale Infektionen aus und können insbesondere für Mukoviszidosepatienten problematisch sein. Zur Steuerung der ▸ Pathogenität besitzen sie zwei AHL-QS-Systeme, LasI/LasR und RhlI/RhlR (Abbildung 3). Das Las-System nutzt das langkettige 3-oxo-C12-AHL zur Kommunikation, das Rhl-System das kurzkettige C4-AHL. Das Gen, das die LuxI-Autoinduktorsynthase RhlI kodiert, ist unter Kontrolle des Las-Systems. Somit kann die Expression verschiedener Sets von Genen nicht nur in Abhängigkeit der Zelldichte, sondern auch zeitabhängig kontrolliert werden. Die Expression der frühen Pathogenitätsgene wie z. B. solche, die für Adhäsionsfaktoren kodieren, steht unter Kontrolle des Las-Systems. Erst wenn diese exprimiert werden, wird auch die Expression von Genen der späten Infektionsphase, beispielsweise solchen für die ▸ Toxinproduktion, angeschaltet. Die Bakterien kommunizieren also in verschiedenen Infektionsphasen mit unterschiedlichen „Dialekten“. Zusätzlich besitzt *P. aeruginosa* einen dritten LuxR-Rezeptor, QscR, welcher keine zugehörige LuxI-Synthase besitzt. Solche LuxR-Rezeptoren werden auch als LuxR-Solos bezeichnet und sind unter Gram-negativen Bakterien weit verbreitet [5]. QscR kann das von LasI gebildete 3-oxo-C12-AHL binden,

und somit die Expression von weiteren Virulenzgenen induzieren. Vermutlich wird hier die Genexpression auch durch fremde, von anderen Bakterienarten gebildete AHL moduliert. *P. aeruginosa* kommuniziert jedoch nicht nur mit AHL, die Bakterien sind sozusagen „multilingual". Sie besitzen ein weiteres QS-System, PqsABCD/PqsR, welches das Quinolon PQS zur Kommunikation nutzt (Tabelle 1). Das Signalmolekül wird von den Enzymen PqsABCD synthetisiert, und von dem LysR-Rezeptor PqsR sensiert. Durch die Integration einer weiteren „Sprache" in die Kontrolle der Pathogenitätsgene wird deutlich, wie vielschichtig und komplex bakterielle Kommunikation sein kann.

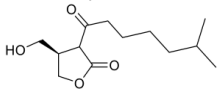
Bakterien integrieren aber nicht nur eine zeitliche Abfolge ihres Handelns über ihre Kommunikation, sondern können diese auch auf einen bestimmten Ort festlegen. So kommuniziert *Rhodopseudomonas palustris* mit einem QS-System, RpaI/RpaR, und nutzt ein Aryl-AHL zur Kommunikation (Abbildung 3) [6]. Die Autoinduktorsynthase dieses Systems, RpaI, nutzt das von Pflanzen produzierte Cumarin zur Produktion von *p*-Cumaroyl-AHL, welches vom LuxR-Rezeptor RpaR sensiert wird. Damit wird die Rpa-abhängige Kommunikation zur Bestimmung der Zelldichte nur in Gang gesetzt, wenn sich die Bakterien auf Pflanzen befinden, so dass die Expression von Genen, die für die Pflanzeninteraktion wichtig sind, gleichzeitig von der Zellzahl und von der Lokalisation der Bakterien abhängig ist.

Inzwischen sind zusätzlich zu den AHL und PQS weitere bakterielle „Sprachen" entdeckt worden. So besitzt beispielsweise das insektenpathogene Gram-negative Bakterium *Photorhabdus luminescens* 40 LuxR-Solos, aber keine LuxI-Synthase und kommuniziert daher nicht über AHL [7]. Der LuxR-Solo PluR kommuniziert über α-Pyrone, auch Photopyrone (PPY) genannt, welche von der Pyronsynthase PpyS synthetisiert werden, und steuert damit die Zellverklumpung, welche eine wichtige Rolle bei der Pathogenität der Bakterien im Wirt spielt (Abbildung 3) [8]. Das nahverwandte humanpathogene Bakterium *Photorhabdus asymbiotica* besitzt einen zu PluR homologen Rezeptor, PauR, jedoch kein PpyS-Homolog. Die Bakterien kommunizieren mit einer anderen chemischen Sprache, den Dialkyresorzinolen (DAR), welche genauso wie die Photopyrone in verschiedenen Derivaten synthetisiert werden und daher in verschiedenen „Dialekten" vorkommen [9].

FORTSETZUNG VON TABELLE 1

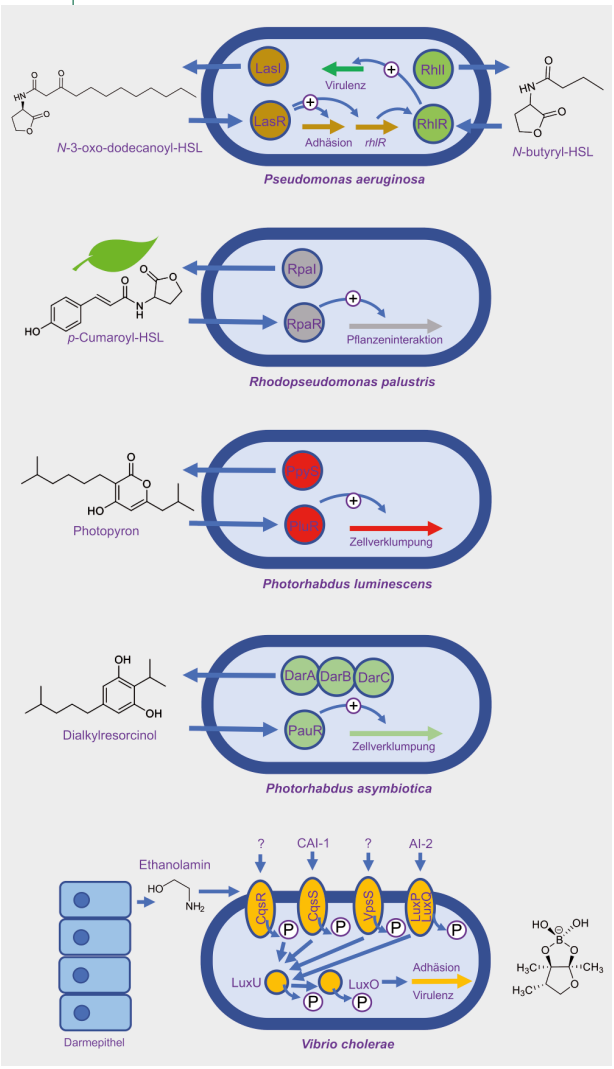
Photorhabdus luminescens	PpyS/PluR	Photopyron (PPY) 3-(5-Methylhexyl)-4-Hydroxy-6-(2-Methylpropyl)-Pyran-2-on 
Vibrio harveyi	LuxM/LuxN/LuxU/LuxO	HAI-1 3-oxo-Butanoyl-AHL (3-oxo-C4-AHL) 
Vibrio cholerae	CqsS/LuxU/LuxO	Cholera Autoinduktor-1 (CAI-1) 3-Hydroxytridekan-4-on 
Vibrio cholerae	LuxP/LuxQ/LuxU/LuxO	Autoinduktor-2 (AI-2) (1R,2S,5S)-7,7-Dihydroxy-1,2,5-Trimethyl-4,6,8-Trioxa-7-Boranuidbicyclo[3.3.0]oktan (Furanosylboratdiesther) 

TAB 2. BAKTERIELLE KOMMUNIKATION UND DIE ZUGEHÖRIGEN SENSORSYSTEME UND SPRACHMOLEKÜLE BEI GRAM-POSITIVEN BAKTERIEN

Spezies	QS-System	Signalmoleküle	
Staphylococcus aureus	AgrC/AgrA	Autoinduktorpeptid	
		AIP-I	MIFD*C*TSY
		AIP-II	FLSS*C*ANVG
		AIP-III	LLFD*C*NI
Bacillus subtilis	ComP/ComA	ComX	ADPITRQ+WGD
Bacillus cereus	Opp/RapB/RapC	CSF	ERGMT
Bacillus thuringiensis			
Enterococcus faecalis	FsrC/FsrA	GBAP	*MWQGFINP*SNQ
Streptomyces griseus	AfsA/ArpA	γ-Butyrolakton 2-Isocapryloyl-3R-Hydroxymethyl-gamma-Butyrolakton 	

* zeigt die Zyklisierungsstelle und + die Modifikationsstelle im jeweiligen Peptid an.

ABB. 3 | VERSCHIEDENE KOMMUNIKATIONSSYSTEME BEI GRAM-NEGATIVEN BAKTERIEN



Dargestellt sind die Quorum-Sensing-Systeme und die entsprechenden Sprachmoleküle am Beispiel von *P. aeruginosa*, *R. palustris*, *P. luminescens*, *P. asymbiotica* und *V. cholerae*. LasI/LasR (gold), RhlI/RhlR (grün) und RpaI/RpaR (grau) sind homolog zum prototypischen LuxI/LuxR-System, die Kommunikation erfolgt über AHL bzw. Cumaroyl-AHL. PluR (rot) und PauR (hellgrün) sind LuxR-Homologe, die Kommunikation erfolgt aber nicht über AHL, sondern über PPY bzw. DAR, welche durch die Enzyme PpyS (rot) bzw. DarABC (hellgrün) gebildet werden. CqsR, CqsS, VpsS und LuxP/LuxQ sind Sensorkinasen (orange). Zusammen mit LuxU (Histidinphosphotransferprotein) und LuxO (Antwortregulator) entspricht hier die Kommunikation eher dem prototypischen System von Gram-positiven Bakterien. Die Sprachmoleküle sind aber (wahrscheinlich) keine Peptide.

DAR werden über einen Biosyntheseweg gebildet, der aus den Enzymen DarABC besteht. Da viele humanpathogene Bakterien neben den zu *luxI/luxR* homologen Genen auch Homologe von *darB* sowie LuxR-Solos besitzen, ist davon auszugehen, dass diese auch über DAR kommunizieren können. Der Erwerb einer DAR-abhängigen Kommunikation wird daher als entscheidender evolutiver Schritt für die Entwicklung von einer Insekten- zu einer Humanpathogenität angesehen.

Ein Paradebeispiel für bakterielle Kommunikation ist der Gram-negative Choleraerreger *Vibrio cholerae*. Wie auch in dem nah verwandten Bakterium *V. harveyi*, weisen die Bakterien eine Art Mischform der prototypischen QS-Systeme auf, vereinen also Teile der Kommunikation von Gram-negativen und Gram-positiven Bakterien (Abbildung 3). Die Kommunikation erfolgt über vier verschiedene Systeme [10]. Die Rezeptoren dieser Systeme sind Sensorkinasen, welche eigentlich von Gram-positiven Bakterien zur Kommunikation genutzt werden. In Abwesenheit des jeweiligen Sprachmoleküls besitzen die vier membrangebundenen Sensorkinasen CqsR, CqsS, VqsS und LuxP/LuxQ Kinaseaktivität, was zu einer dauerhaften Phosphorylierung des Phosphotransferproteins LuxU führt. Dieser überträgt die Phosphorylgruppe dann auf den Antwortregulator LuxO. Über die Expression von Genen, die für kleine RNAs kodieren, steht die Bildung verschiedener Adhäsions- und Virulenzfaktoren unter indirekter Kontrolle von LuxO. Nach Bindung des jeweiligen Autoinduktors bei hoher Zelldichte aktivieren die Sensorkinasen dagegen ihre Phosphataseaktivität gegenüber LuxU und damit LuxO, was dann zur Produktion der Adhäsions- und Virulenzfaktoren führt. Für *V. cholerae* sind bisher nur zwei der zugehörigen Sprachmoleküle bekannt: der Cholera-Autoinduktor-1 (CAI-1) als Signal für CqsS sowie der durch das periplasmatische Bindeprotein LuxP sensierte Autoinduktor-2 (AI-2), der in seiner an LuxP gebundenen Form als Signal für LuxQ dient. Die chemische Natur der Autoinduktoren, welche die Sensorkinasen CqsR und VpsS aktivieren, ist bisher nicht bekannt. Allerdings kann Ethanolamin, eine Verbindung, die in hoher Konzentration im Darm von Säugetieren vorkommt, die Signaltransduktion über CqsR aktivieren. Man geht davon aus, dass dieser Mechanismus für eine erfolgreiche Darmbesiedlung durch *V. cholerae* wichtig ist, wenn das QS durch andere Moleküle oder Faktoren im Wirt blockiert ist.

Das nah verwandte marine Bakterium *Vibrio harveyi* hat drei anstatt vier Sensorkinasen für die Kommunikation: LuxPQ, CqsS und LuxN. Letzteres sensiert das kurzkettige C4-AHL, und kommt damit dem prototypischen System von Gram-negativen nahe. Der Grund für die parallel laufenden QS-Kaskaden ist vermutlich, dass sich so die Expression der Zielgene besser modulieren und feinabstimmen lässt. In *V. harveyi* wurde gezeigt, dass die drei Autoinduktoren hier als biologische „Timer“ fungieren, die zu unterschiedlichen Zeiten im Wachstum der Bakterien auftreten und für die Kommunikation genutzt werden [11].

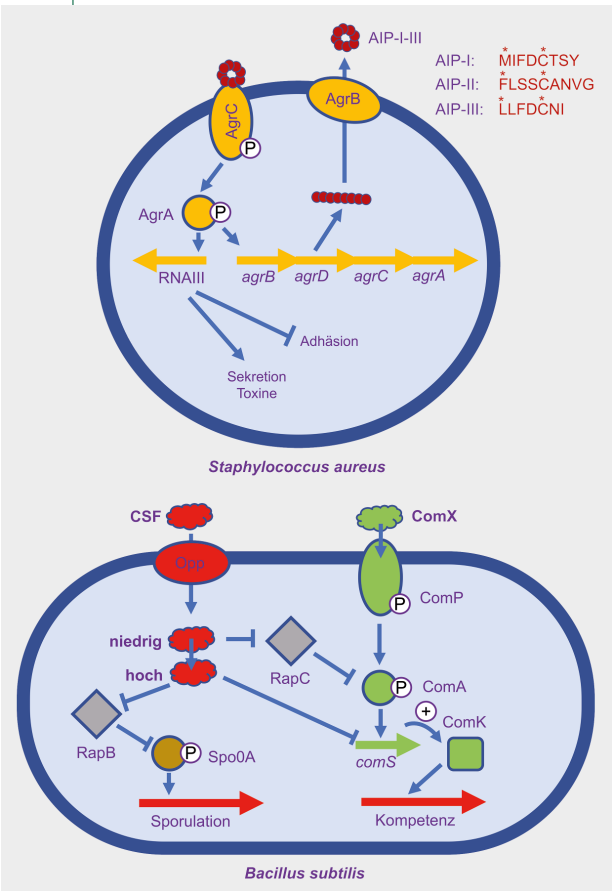
Außerdem können in einer homogenen *V. harveyi*-Zellpopulation die einzelnen Bakterien unterschiedliche „Sprachen" verwenden und damit ihre Genexpression modulieren – ein Prozess der als phänotypische Heterogenität bezeichnet wird [12].

Die unterschiedlichen „Sprachen" von Gram-positiven Bakterien

Gram-positive Bakterien nutzen Peptide zur Kommunikation [4]. Diese sind nicht membrangängig und müssen deswegen außerhalb der Zytoplasmamembran detektiert werden. Für diesen Zweck machen sich Gram-positive Bakterien die unter allen Bakterien weitverbreiteten Zweikomponentensysteme für die Kommunikation zu Nutze. Da das Signal auf der Außenseite der Cytoplasmamembran wahrgenommen wird, kann der Rezeptor nicht selbst die Expression der Zielgene kontrollieren. Stattdessen muss das Signal ins Zellinnere übertragen werden, was im Gegensatz zu Gram-negativen Bakterien mit Ausnahme von *V. cholerae* und *V. harveyi* (s. o.) über eine Phosphorylierungskaskade geschieht. Verschiedene bekannte QS-Peptide und deren zugehörige Systeme sind in Tabelle 2 aufgeführt.

Das QS bei *Staphylococcus aureus* ist eines der am besten untersuchten Kommunikationssysteme von Gram-positiven Bakterien (Abbildung 4) [1]. *S. aureus* ist nosokomial infektiös und kann verschiedene Krankheiten wie Lungenentzündung, Endokarditis, Osteomyelitis und Wundinfektionen hervorrufen. Zur Infektion des Wirtes nutzen die Bakterien eine biphasische Virulenzstrategie: bei niedriger Zelldichte produzieren sie Kolonisierungs- und Adhäsionsfaktoren, während diese bei hoher Zelldichte reprimiert werden. Stattdessen werden dann die Produktion und Sekretion von Toxinen und Proteasen aktiviert, was vermutlich für die weitere Verbreitung der Zellen wichtig ist. Dieser Wechsel in der Genexpression steht unter Kontrolle des Agr-QS-Systems, welches aus der Sensorkinase AgrC und dem Antwortregulator ArgA besteht. Die Bakterien kommunizieren über das zyklische Peptid AIP (Autoinduktorpeptid), welches durch den Transporter ArgB exportiert und dabei durch Anfügen eines Thiolaktonrings modifiziert wird. Die Bindung von AIP an AgrC aktiviert die Agr-Phosphorylierungskaskade und führt zur Expression einer kleinen RNA (RNAIII), welche die Produktion von Zelladhäsionsfaktoren inhibiert und die der Toxine sowie die Proteasesekretion induziert. Phosphoryliertes AgrA induziert außerdem die Expression des *agrBDCA*-Operons, wodurch es zu einer weiteren Erhöhung der AIP-Konzentration kommt. Somit wird gewährleistet, dass die gesamte Population ihr Verhalten bei hoher Zelldichte verändert. Erstaunlich ist, dass die vier bekannten Gruppen von *S. aureus* anhand ihrer spezifischen AIP diagnostiziert werden können. Darüber hinaus führt die Bindung eines nicht zugehörigen AIP zur Inaktivierung von AgrC. Dies bedeutet, dass sich die jeweiligen *S. aureus*-Gruppen mit ihren AIP-„Dialekten" im Wettstreit befinden. Die Gruppe, die sozusagen das erste „Wort" hat, besiedelt den Wirt, die anderen kommen nicht zum Zuge. Da jede *S. aureus*-Gruppe an unterschiedliche Nischen im Wirt angepasst ist, kommen sich diese in der Natur allerdings nur selten in die Quere. Es wird angenommen, dass die Entwicklung von neuen bakteriellen Sprachen auch im Zusammenhang mit der Entstehung neuer Bakterienarten stehen könnte.

ABB. 4 | VERSCHIEDENE KOMMUNIKATIONSSYSTEME BEI GRAM-POSITIVEN BAKTERIEN



Dargestellt sind die Kommunikationssysteme bei *S. aureus* und *B. subtilis*. AgrC/AgrA (orange) und ComP/ComA (grün) sind Zweikomponentensysteme, bei denen die Bindung des jeweiligen Sprachpeptids eine Autophoshorylierung des Antwortregulators hervorruft und damit die Expression der Zielgene aktiviert. Spo0A (gold) ist der Antwortregulator des Spo-Systems, durch welches Stress wie Nährstoffmangel sensiert wird. Aus Übersichtsgründen ist nur der Antwortregulator Spo0A gezeigt. AIP = Autoinduktorpeptid; CSF = Kompetenz- und Sporulationsfaktor. Die Phosphatasen RapC und RapB (grau) können die Antwortregulatoren Spo0A bzw. ComA dephosphorylieren. ComS und ComA sind Transkriptionsfaktoren. Opp (rot): Transporter für CSF.

Ein weiteres sehr gut untersuchtes Kommunikationssystem bei Gram-positiven Bakterien ist das des Bodenbakteriums *Bacillus subtilis* (Abbildung 4) [1]. Die Bakterien sind in der Lage, unter ungünstigen Bedingungen ▸ Endosporen zu bilden, einer Dauerform, in der die Bakterien besonders widerstandsfähig sind und Perioden von Nahrungsmangel oder Trockenheit überdauern können. Um den Sporulationssprozess erfolgreich abschließen zu können, benötigen die Bakterien Energie, welche von Artgenossen bereitgestellt wird, die sich für die Gruppe „opfern“. Ein alternativer Prozess, um Mangelsituationen zu überstehen, ist der Erwerb von Kompetenz, bei dem die Bakterien extrazelluläre DNA aufnehmen und so neue Eigenschaften z. B. für die Verwertung alternativer Nährstoffe gewinnen. Deshalb werden bei der Entscheidung zwischen Endosporenbildung und Kompetenzerwerb auch zwei Signale integriert und kommuniziert: zum einen Stress wie Nahrungsmangel, zum anderen die Zelldichte, die letztendlich zu einer Entscheidung der einzelnen Zelle in die jeweils eine oder andere Richtung führt. *B. subtilis* hat für diesen Entscheidungsprozess die zwei Peptidautoinduktoren ComX und den Kompetenz- und Sporulationsfaktor CSF etabliert. Beide werden durch Transportsysteme aus der Zelle ausgeschleust und häufen sich bei hoher Zelldichte in der Umgebung an.

Das Zweikomponentensystem ComP/ComA sensiert das ComX-Peptid. Bei hoher Zelldichte liegt ComA phosphoryliert vor (ComA-P), was dazu führt, dass die Expression des *comS*-Gens erfolgt. Das Protein ComS erhöht wiederum die Konzentration von ComK, einem weiteren Transkriptionsfaktor, der die Expression der Kompetenzgene stimuliert. Die Konzentration des zweiten Autoinduktors CSF wird dagegen intrazellulär gemessen, denn es wird durch ein Transportsystem wieder in die Zelle aufgenommen. Bei niedriger intrazellulärer Konzentration inhibiert CSF die ComA-spezifische Phosphatase RapC, was zu einer Erhöhung der ComA-P Konzentration führt, und damit die Entscheidung der Zelle in Richtung Kompetenz drängt. Bei hoher CSF-Konzentration wird hingegen die Kompetenz unterdrückt und die Expression der Sporulationsgene stimuliert. Dies lässt sich dadurch erklären, dass unter diesen Umständen die Kinaseaktivität von ComS inhibiert wird, so dass die Expression der Kompetenzgene nicht mehr induziert wird und sich die Entscheidung in Richtung Sporulation verschiebt. Außerdem inhibiert CSF die Phosphatase RapB, die einen weiteren Antwortregulator, Spo0B, dephosphorylieren und damit inhibieren kann. Spo0B-P induziert die Expression der Sporulationsgene und ist Teil eines weiteren Zweikomponentensystems, durch welches andere Signale wie z. B. Nährstoffmangel in die Steuerung der Sporulationsgene integriert werden. Die Kommunikation von *B. subtilis* ist ein weiteres sehr gutes Beispiel dafür, wie komplex und vielschichtig bakterielle Kommunikation sein kann, und auf wie vielen Ebenen neben der Zelldichte weitere Signale in die Entscheidungen von Bakterien einfließen können.

Das ▸ „Esperanto“ der Bakterien

Neben der Unterhaltung mit ihren Artgenossen haben viele Bakterien eine artübergreifende Kommunikation entwickelt [1]. Dieses „Esperanto“ beruht auf einem Furanosylboratdiester als Sprachmolekül, welches auch als Autoinduktor-2 (AI-2) bezeichnet wird. Wie bereits oben beschrieben wurde die Kommunikation über AI-2 zuerst bei *V. harveyi* und *V. fischeri* gefunden. Das Signalmolekül wird über das Enzym LuxS gebildet, welches Teil des Methylstoffwechsels in vielen Bakterien ist. Das *luxS*-Gen findet sich in etwa der Hälfte der bisher bekannten bakteriellen Genome, was darauf hindeutet, dass sehr viele Bakterien über AI-2 mit benachbarten Zellen der eigenen Art, aber auch mit nicht verwandten Bakterien kommunizieren. In Vibrionen wird AI-2 von dem periplasmatischen Protein LuxP gebunden und dieses interagiert dann mit der Sensorkinase LuxQ. Letztere aktiviert die Phosphorylierungskaskade, die über die Proteine LuxU und LuxO verläuft, welche als Teile der spezifischen Kommunikationssysteme gruppenkoordiniertes Verhalten auslösen (Abbildung 3). In *Escherichia coli* und *Salmonella*-Arten wird AI-2 anders als in Vibrionen sensiert. Hier bindet das periplasmatische Protein LsrB das AI-2 Signal, wird daraufhin über ein ABC-Transportsystem in die Zelle gebracht, durch die Kinase LsrK phosphoryliert und interagiert dann mit einem globalen Transkriptionsregulator, LsrR, welcher die Expression verschiedener Gene moduliert. Andere Bakterien wie *Helicobacter pylori*, infektiöse Besiedler des menschlichen Magen-Darm-Traktes, sensieren AI-2 durch Chemorezeptoren, welche unterschiedliche Umweltsignale und Nährstoffe wahrnehmen, um Schwimm- und Schwarmverhalten anzupassen. Aber auch wir Menschen mischen bei diesem „Small Talk“ der Bakterien mit: So produziert unser Darm ein dem AI-2 ähnliches Molekül, wenn das Darmepithel geschädigt ist. Es wird vermutet, dass unser Darm dadurch die Bakterien unseres eigenen Mikrobioms zur Hilfe „ruft“, damit diese die geschädigten Stellen besiedeln und so an der Reparatur der Epithelzellen mitwirken, indem sie diese vor Eindringlingen schützen [13].

Die Kommunikation zwischen Bakterien und ihren Wirten

Bakterien sind nicht nur in der Lage, untereinander über verschiedene „Sprachen“ und „Dialekte“ zu kommunizieren, sondern besitzen darüber hinaus auch die Fähigkeit, die chemische Sprache ihrer Wirte zu verstehen. Man bezeichnet diesen Vorgang auch als „Inter-kingdom-signaling“ (IKS). Dabei nehmen die Bakterien hormonähnliche Signalmoleküle wahr und können ihr Verhalten an den jeweiligen Wirt spezifisch anpassen [14]. Als eines der ersten wurde das IKS von enterohämorrhagischen *Escherichia coli* (EHEC) – Erreger schwerer blutiger Durchfallerkrankungen beim Menschen – beschrieben. Bei der Infektion des Wirtes werden die Hormone Epinephrin und Norepinephrin, sowie ein drittes unbekanntes Autoinduktor-

molekül (AI-3) vom Zweikomponentensystem QseC/QseB wahrgenommen. Daraufhin wird ein weiteres Zweikomponentensystem, QseE/QseF, aktiviert, welches die Expression der Virulenzgene stimuliert. Somit „belauschen" die Bakterien sozusagen die chemische Kommunikation des Wirtes und können sich anhand dieser Signale orientieren.

Im Insektenpathogen *P. luminescens* besitzen die meisten der 40 LuxR-Solos eine Signalbindedomäne, die darauf hindeutet, dass es sich bei dem dazugehörigen Signalmolekül um eines eukaryotischer Herkunft handelt [7]. Konkret ähnelt die Bindedomäne der eines Regulatorproteins in Fruchtfliegen, das ein Insektenjuvenilhormon bindet und die Entwicklung der Fliegen steuert. Es wird daher angenommen, dass diese LuxR-Solos von *P. luminescens* Insektenhormone erkennt, und damit die Expression spezifischer Virulenzgene steuert. Die chemische Struktur dieses Signalmoleküls ist aber bisher unbekannt. Auch pflanzenassoziierte Bakterien wie *Pseudomonas* und *Xanthomonas* besitzen LuxR-Solos, über die sie die Pflanze als Wirt erkennen. Bei dem von der Pflanze produzierten Signalmolekül handelt es sich um HEHEAA, *N*-(2-Hydroxyethyl)-2-(2-Hydroxyethylamino), das endophytische Pseudomonaden über den LuxR-Solo PipR wahrnehmen [15]. Daneben gibt es weitere pflanzliche Signalmoleküle, die als Signal für LuxR-Solos dienen, deren chemische Natur aber noch unbekannt ist. So erkennen Pflanzenpathogene der Gattung *Xanthomonas* ein noch unbekanntes Signalmolekül über den LuxR-Solo OryR und steuern dadurch die Expression von Genen, die für die Besiedlung der Pflanze wichtig sind [5]. OryR besitzt eine Signalbindedomäne, die denen der AHL-Rezeptoren ähnelt, so dass sich vermuten lässt, dass das von der Pflanze produzierte Molekül eine ähnliche chemische Natur oder Größe aufweist [16].

Die Erforschung der Kommunikation zwischen Bakterien und ihren Wirten steckt quasi noch in ihren Kinderschuhen. Man weiß, dass es diese Kommunikation gibt und dass viele pathogene Bakterien diese für die erfolgreiche Besiedlung des Wirts und damit für ihre Virulenz nutzen. Obwohl über den molekularen Mechanismus und die Sprachen des Inter-kingdom-signaling nur wenig bekannt ist, bieten diese Systeme neben den QS-Systemen vielversprechende Angriffspunkte für neue spezifische Medikamente gegen bakterielle Infektionskrankheiten.

Bakterien „mundtot" oder „taub" machen

Wer zuerst kommt, mahlt zuerst. Frei nach diesem Motto kommunizieren Bakterien nicht nur untereinander, sondern greifen auch in die Kommunikation anderer Bakterien

GLOSSAR

Autophosphorylierung: *Ein Prozess, bei dem eine Kinase als ihr eigenes Substrat dient und sich selber phosphoryliert.*

Biofilm: *Eine Lebensgemeinschaft von Bakterien einer oder mehrerer Arten, die in einer schleimartigen Matrix aus extrazellulären Polymeren – den Exopolysacchariden – leben. Im Biofilm finden Stoffaustausch und Nährstoffaufnahme statt. Durch Bildung eines Biofilms sind Mikroorganismen extrem widerstandsfähig gegenüber äußeren Einflüssen und können Oberflächen wie Kunststoffe, Zähne und Wasserleitungen besiedeln.*

Bioluminezenz: *Ausstrahlung von sichtbarem Licht durch Mikroorganismen. Diese wird durch Oxidation bestimmter Stoffe durch das Enzym Luziferase begünstigt.*

Endosporen: *Viele Gram-positive Bakterien der Gattung* Bacillus *und* Clostridium *bilden aufgrund von Nährstoffmangel Dauerformen aus, die äußerst widerstandsfähig gegenüber Hitze, Trockenheit und chemischen Agenzien sind. Eine ungleiche Zellteilung führt zu einer Sporenbildung im Inneren der Mutterzelle, welche dann durch Lyse freigesetzt wird. Bei günstigen Bedingungen keimt die Spore wieder zu einer vegetativen Zelle aus. Die Bakterien können so mehrere Jahre überstehen, ohne nennenswerten Stoffwechsel betreiben zu müssen.*

Esperanto: *Esperanto ist eine Plansprache, die 1887 von dem Augenarzt Ludwik Lejzer Zamenhof veröffentlicht wurde und als Weltsprache gedacht war. Sie ist die am weitesten verbreitete Plansprache.*

Gram-positiv/Gram-negativ: *Bakterien können grob nach einer Färbemethode des dänischen Bakteriologen Hans Christian Gram (1853–1938) in zwei große Gruppen eingeteilt werden. Die Eigenschaft, Bakterien mit dieser Methode zu färben, geht auf die Dicke ihrer Zellwand zurück. Gram-positive Bakterien besitzen eine dicke Zellwand und werden so durch diese Technik violett gefärbt. Gram-negative Bakterien haben eine viel dünnere Zellwand und erscheinen nach Entfärbung und Gegenfärbung rot.*

Operon: *Die Gesamtheit mehrerer gemeinsam regulierter Gene auf der DNA, die verschiedene Funktionen kodieren. Ein Operon besteht aus einem Operator, der die regulatorischen Proteine bindet, sowie aus einem Promotor, mehreren Genen und einem Terminator.*

Pathogenität: *Die Fähigkeit eines Mikroorganismus in einem Wirt eine Krankheit hervorzurufen.*

Quorum: *Unter Quorum versteht man die Anzahl der Stimmen und damit die Beschlussfähigkeit einer Gruppe. Der Begriff geht auf das antike Rom zurück und fand dort bereits im Senat Anwendung.*

Signaltransduktion: *Die Umwandlung eines externen Signals oder Reizes in eine intrazelluläre Antwort. Mikroorganismen müssen direkt auf Umweltreize reagieren und nehmen verschiedene chemische sowie physikalische Reize aus ihrer Umwelt wahr. Signaltransduktion spielt auch bei der Kommunikation zwischen Mikroorganismen eine wichtige Rolle.*

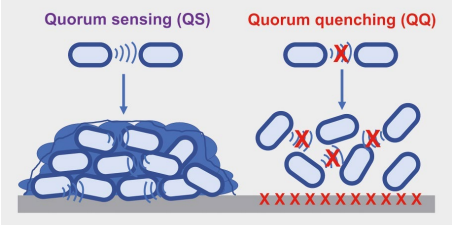
Toxin: *Substanzen, die von Organismen produziert werden und schädlich bzw. tödlich für andere Organismen sind. Toxine greifen in den essentiellen Stoffwechsel der Wirtszelle ein und können z. B. die Proteinbiosynthese inhibieren oder Kanäle und Rezeptoren des Wirtes blockieren.*

Zweikomponentensystem: *Ein bakterieller Signaltransduktionsmechanismus zur Weiterleitung von Informationen von außen in die Zelle oder auch innerhalb der Zelle. Solche Systeme bestehen meist aus zwei Proteinkomponenten; einem meist membrangebundenen Rezeptor, und einem zytoplasmatischen Protein, das als Transkriptionsregulator fungiert. Der Rezeptor ist eine Histidinkinase (Sensorkinase), die sich nach Wahrnehmung eines bestimmten Reizes an einem Histidin autophosphoryliert und anschließend die Phosphorylgruppe auf ein Aspartat des Antwortregulators überträgt, der dann mit der DNA interagiert und so die Expression der Zielgene beeinflusst.*

ein, um sich in bestimmten Habitaten oder Wirten Vorteile gegenüber Konkurrenten zu verschaffen. Dieser Vorgang wird als „Quorum quenching“ (QQ) bezeichnet [17]. Auch verschiedene Wirte nutzen die Möglichkeit, Bakterien „mundtot“ oder „taub“ zu machen, indem sie deren Signalmoleküle durch spezifische Enzyme spalten oder deren Rezeptoren durch sogenannte Antagonisten blockieren. Zwei bekannte Beispiele für QQ-Enzyme sind die Laktonase und Acylase, Enzyme, die den Laktonring bzw. die Amidbindung von AHL spalten und somit das Sprachmolekül inaktivieren. Diese Enzyme findet man auch in *P. luminescens*, das dadurch bei der Besiedlung seiner Wirte die Kommunikation anderer Bakterien blockiert [7]. Auch Pflanzen produzieren Laktonasen und Acylasen, um sich vor dem Befall mit schädlichen Bakterien zu schützten, in dem sie diese „mundtot“ machen. Auf der anderen Seite sind aber auch Beispiele für die Bildung von Antagonisten bekannt, welche LuxR-Rezeptoren blockieren und die Bakterien damit „taub“ machen. So produziert Knoblauch die schwefelhaltige Verbindung Ajoen, welche an die LuxR-Rezeptoren LasR und RhlR von *P. aeruginosa* bindet und diese blockiert [18]. Das scheint auch Mukoviszidosepatienten helfen zu können, denn eine aerosolische Verabreichung von Ajoen-Extrakten führte bei ihnen zu einer geringeren Besiedlung des Lungenepithels durch *P. aeruginosa*. Auch in *P. luminescens* finden sich neue Naturstoffe, die QQ-Wirkung auf verschiedene Bakterien haben und daher von hohem biotechnologischem Interesse sind.

Multiresistente Krankheitserreger wie Methicillin-resistente *S. aureus* (MRSA) oder Vancomycin-resistente *Enterococcus spec.* (VRA) sind Auslöser nosokomialer Infektionen, die mit klassischen Antibiotika nicht mehr behandelbar sind. Die Virulenz dieser Bakterien steht unter Kontrolle des Quorum sensing, so dass QQ-Naturstoffe vielversprechende Wirkstoffe und Alternativen zu klassischen Antibiotika gegen Infektionskrankheiten darstellen.

ABB. 5 | EINFLUSS VON „QUORUM SENSING“ UND „QUORUM QUENCHING“ AUF DIE BIOFILMBILDUNG



Die Kommunikation (blaue Wellen) durch „Quorum sensing“ (QS) führt bei vielen Bakterien zur Produktion einer Schleimschicht, die die Biofilmbildung ermöglicht (links). Unterbindet man diese Kommunikation (QQ, rote Kreuze) z. B. durch enzymatische Spaltung der „Sprachmoleküle“ oder durch Blockieren des Rezeptors durch Antagonisten, produzieren die Bakterien keinen Biofilm mehr (rechts).

Im Gegensatz zu Antibiotika wirken QQ-Naturstoffe viel spezifischer, da sie nur die Kommunikation einer bestimmten Gruppe von Bakterien blockieren. Außerdem werden die Bakterien nicht wie durch herkömmliche Antibiotika abgetötet, was der Bildung möglicher Resistenzen entgegenwirkt. Aber nicht nur im medizinischen, sondern auch in verschiedenen industriellen Bereichen könnten QQ-Wirkstoffe Anwendung finden. In diversen Bereichen wie Trinkwasser-führenden Systemen oder an Schiffsrümpfen ist die Besiedlung der Oberfläche mit bakteriellen ▸ Biofilmen problematisch und führt zu hohen finanziellen Schäden. Ein Biofilm von nur einem Zehntel Millimeter Dicke verringert durch einen erhöhten Reibungswiderstand die Geschwindigkeit eines Tankers um bis zu 15 Prozent, was zu erhöhtem Treibstoffbedarf und daher zu einer zusätzlichen Belastung der Umwelt führt. Diese Biofilme werden zurzeit mit aufwendigen mechanischen und chemischen Mitteln entfernt. Da die Schiffe dafür auf Trockendocks gebracht werden müssen, kommt es zu Ausfällen und enormen Kosten. Die angewandte Forschung fokussiert momentan darauf, neue QQ-Wirkstoffe zu identifizieren, welche nicht biozid wirken, sondern die Bakterien lediglich „mundtot“ oder „taub“ machen, um diese in medizinische und biotechnologische Anwendung zu bringen (Abbildung 5). Diese neuen QQ-Wirkstoffe könnten dann in Oberflächen wie Implantaten, Schläuchen oder Schiffslacken eingebracht werden, um die Besiedlung mit Bakterien und damit die Biofilmbildung zu unterbinden.

Ausblick

Als vor etwa 40 Jahren die bakterielle Kommunikation entdeckt wurde, haben Mikrobiologen sicher noch keine Vorstellung davon gehabt, wie viele unterschiedliche bakterielle Sprachen und Dialekte existieren könnten. Und auch heute stehen wir noch am Anfang ihrer Erforschung. Durch moderne Sequenzierungsmethoden ist es jedoch möglich, bakterielle Genome innerhalb sehr kurzer Zeit zu entschlüsseln und bioinformatisch zu analysieren. Dies führt nahezu täglich zu einem rasanten Anstieg von Informationen und zu Hinweisen auf neue Kommunikationsmöglichkeiten der Bakterien untereinander und mit ihren Wirten. Es bleibt daher spannend, welche Kommunikationswege es für Bakterien neben den bereits bekannten noch gibt, und wie diese in Zukunft als Wirkorte für die Entwicklung von neuen, dringend benötigten Wirkstoffen genutzt werden könnten.

Zusammenfassung

Bakterien kommunizieren über kleine diffusionsfähige Moleküle, ein Prozess, den Mikrobiologen als „Quorum sensing“ bezeichnen. Die Sprachmoleküle werden von den Bakterien in die Umgebung abgegeben und dann von den Artgenossen über spezifische Rezeptoren sensiert. Somit kann sich die Gemeinschaft absprechen und bestimmte Phänotypen an die Zellzahl, das Quorum, anpassen. Durch die verschiedenen chemischen Strukturen und Modifikatio-

nen dieser Sprachmoleküle haben Bakterien unterschiedliche Sprachen und Dialekte entwickelt, die ihnen zusätzlich auch Informationen über Zeit und Ort geben können. Darüber hinaus sind Bakterien in der Lage, mit ihren Wirten wie Tieren, Pflanzen und sogar uns Menschen „Small Talk" zu betreiben. Da bei pathogenen Bakterien die Kommunikation fast immer Voraussetzung für die Infektion der Wirte ist, bieten die molekularen Komponenten der bakteriellen Sprache potenzielle Wirkorte für neue Medikamente zur Bekämpfung von Infektionskrankheiten.

Summary

The silent communication of bacteria

Bacteria communicate via small diffusible molecules, a process that microbiologists refer to as quorum sensing. These language molecules are released by the bacteria in the environment and are then sensed by their neighbours via specific receptors. Thus, the community can arrange and adapt specific phenotypes in dependence on the cell count termed quorum. Due to the different structures and modifications of the communication molecules bacteria have evolved different languages and dialects, which can in addition give information about time and venue. Moreover, bacteria have small talk with their hosts such as animals, plants and yet humans. Since communication is a prerequisite for the infection of hosts by pathogenic bacteria, the molecular components of the bacterial communication are promising candidates as targets for badly needed new antimicrobial drugs.

Schlagworte:

Quorum sensing, Quorum quenching, Bakterielle Kommunikation, Inter-kingdom-signaling

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Die Autoren

Nazzareno Dominelli, Jahrgang 1994, Studium der Biologie am Biozentrum der Ludwig-Maximilians-Universität München (2018). Anschließend Beginn der Promotion an der LMU München im Fach Mikrobiologie. Seit 2019 Promotionsstudent im Bereich Mikrobiologie an der Johannes-Gutenberg-Universität Mainz und Mitglied der Gutenberg-Akademie als Nachwuchswissenschaftler. Forschungsschwerpunkt: „Inter-kingdom signaling" zwischen Photorhabdus luminescens *und seinen eukaryotischen Wirten.*

Ralf Heermann, Jahrgang 1972, Studium der Biologie und Promotion im Fach Mikrobiologie (2001) an der Universität Osnabrück. Anschließend Postdoktorand an der Technischen Universität Darmstadt (bis 2004), danach Wissenschaftlicher Assistent und Habilitation im Fach Mikrobiologie (2010) am Biozentrum der Ludwig-Maximilians-Universität München. Anschließend Akademischer (Ober-)Rat, Arbeitsgruppenleiter und Leiter der Serviceeinheit Bioanalytik an der LMU. Seit 2018 Universitätsprofessor für Mikrobiologie an der Johannes-Gutenberg-Universität Mainz. Forschungsschwerpunkte sind molekulare Mechanismen der Signaltransduktion und der bakteriellen Kommunikation in insektenpathogenen Bakterien.

Korrespondenz:
Prof. Dr. Ralf Heermann
Johannes-Gutenberg-Universität Mainz
Biozentrum II
Institut für Molekulare Physiologie, Mikrobiologie und Weinforschung
Hanns-Dieter-Hüsch-Weg 17
55128 Mainz
E-Mail: heermann@uni-mainz.de

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8. Freund oder Feind? – Die zwei Gesichter von *Photorhabdus luminescens*

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Bakterielle Genregulation

Freund oder Feind? – Die zwei Gesichter von *Photorhabdus luminescens*

NAZZARENO DOMINELLI, RALF HEERMANN
INSTITUT FÜR MOLEKULARE PHYSIOLOGIE, MIKROBIOLOGIE UND WEINFORSCHUNG, UNIVERSITÄT MAINZ

***Photorhabdus luminescens* is an enteric bacterium with two faces: on the one hand these bacteria live in symbiosis with soil nematodes, on the other hand they are highly pathogenic for insects. The population is also phenotypically heterogeneous: one part lives in symbiosis with nematodes, the other part symbiotically interacts with plants. Cell-cell communication, inter-kingdom signaling, and other gene regulation processes are behind the complex decision of being friend or foe.**

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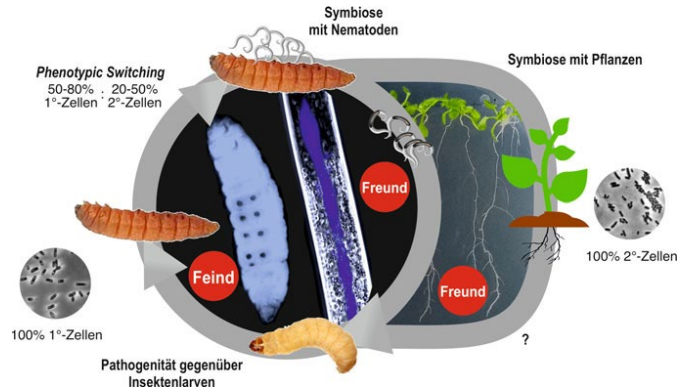
Es passiert in der dunklen Erde, im Wald, in Ackerböden, im Sand der Küste und in unseren Vorgärten: Winzige Fadenwürmer (Nematoden) mit dem Namen *Heterorhabditis bacteriophora* suchen nach Opfern. Um erfolgreich zu töten, tragen sie eine gefährliche Waffe in ihrem Vorderdarm: insektenpathogene Bakterien der Art *Photorhabdus luminescens*. So sind die Nematoden bestens gerüstet, um ahnungslose Insektenlarven zu erlegen. Ist das Opfer ausgemacht, geht alles blitzschnell: Die Nematoden bohren ein Loch in die Haut des Insekts und schlüpfen ins Innere. Dort angekommen, würgen sie die Bakterien hinaus und lassen sie ihre Arbeit verrichten.



▲ **Abb. 1:** Die Lebenszyklen von *Photorhabdus luminescens*. 1°- und 2°-Zellen sind hochpathogen gegenüber Insektenlarven. Ausschließlich die 1°-Zellen leben in Symbiose mit den Nematoden (Freund). Letztere infizieren Insektenlarven mit 1°-Zellen und töten sie durch die bakteriell produzierten Toxine (Feind) effizient und schnell. Nachdem der Kadaver des Opfers durch die Bakterien verstoffwechselt ist, wechseln 20–50 Prozent der 1°-Zellen zum 2°-Phänotyp. Während die restlichen 1°-Zellen wieder von den Nematoden aufgenommen werden (Freund), interagieren 2°-Zellen symbiotisch mit Pflanzenwurzeln und verteidigen diese vor dem Befall durch phytopathogene Pilze (Freund). Ob die 2°-Zellen wieder zu 1°-Zellen konvertieren können, ist bisher unklar.

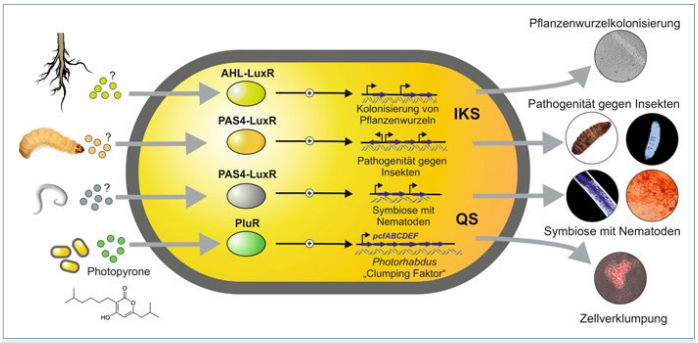
P. luminescens ist ein echter Killer, ausgestattet mit einem großen Arsenal verschiedener Toxine und Sekundärmetabolite, die das Opfer innerhalb kurzer Zeit töten. In der Folge bilden die Bakterien das Enzym Luziferase, sodass das tote Insekt luminesziert – der „leuchtende Tod" hat zugeschlagen.

Danach produzieren die Bakterien diverse Exoenzyme, die den Kadaver des Opfers zu einer nährstoffreichen „Suppe" zersetzen, die nun sowohl die Bakterien als auch die Nematoden nutzen können. Die Bakterien unterstützen zudem ihre Symbiosepartner in Fortpflanzung und Entwicklung durch die Produktion noch unbekannter Sekundärmetabolite. Durch die Produktion von Antibiotika und Fungiziden verteidigt *Photorhabdus* sein neues Habitat vor dem Befall durch andere Bodenmikroorganismen. Sind die Nährstoffe aufgebraucht, nehmen die Nematoden die Bakterien wieder auf, verlassen das aufgezehrte Insekt und bringen ihre Biowaffe in ein neues Opfer (**Abb. 1**).

Freund oder Feind – bakterielle phänotypische Heterogenität

Doch *P. luminescens* ist außerhalb der Nematoden nicht allen Eukaryoten gegenüber feindlich gestimmt. So profitieren Pflanzen von einer Interaktion mit den Bakterien [1]. Die Gram-negativen Enterobakterien kommen in zwei verschiedenen phänotypischen Formen vor, den primären (1°) und sekundären (2°) Zellen [2]. Genetisch sind beide Zellformen identisch, unterscheiden sich aber in verschiedenen phänotypischen Eigenschaften. Nur die 1°-Zellen produzieren Antibiotika, Pigmente und sind biolumineszent. Einer der wichtigsten Unterschiede der beiden Zellformen ist jedoch, dass ausschließlich 1°-Zellen mit den Nematoden eine Verbindung eingehen können. Während des Infektionszyklus vollziehen einzelne

1°-Zellen einen Phasenwechsel, sodass nach etwa 28 Tagen fast die Hälfte der Population zum 2°-Phänotyp gewechselt ist. Die 2°-Zellen verbleiben im Boden, wenn die neue Generation der Nematoden den aufgezehrten Insektenkadaver verlassen hat. Die 2°-Zellen sind beweglicher als 1°-Zellen und werden von Pflanzenwurzelexsudaten angelockt [3]. Sie gehen eine spezifische Interaktion mit den Wurzeln ein und produzieren hier eine noch unbekannte Substanz, die die Pflanzen vor dem Befall phytopathogener Pilze schützt [1]. Weiterhin bewirkt die Kolonisierung der Bakterien eine Verzweigung des Wurzelwachstums, was für die Pflanze wachstumsfördernd ist [1]. Da die 2°-Zellen ebenfalls insektenpathogen sind, könnten sie die Pflanzenwurzeln auch vor Fraß durch Insektenlarven schützen. Die Rückkehr vom 2°- zum 1°-Phänotyp ist wahrscheinlich, aber bisher noch nie beobachtet worden.



▲ **Abb. 2:** Bakterielle Kommunikation. Über eine Vielfalt von LuxR-Rezeptoren mit unterschiedlichen Signalbindedomänen (PluR, PAS4, AHL) können *Photorhabdus luminescens*-Zellen sowohl untereinander als auch mit ihren eukaryotischen Wirten kommunizieren. Dabei wird der LuxR-Rezeptor PluR für die Kommunikation der Bakterien untereinander genutzt, während die PAS4-LuxR-Rezeptoren wahrscheinlich beim *inter-kingdom signaling* mit den eukaryotischen Wirten (Insekten und Nematoden) eine Rolle spielen. Dem AHL-LuxR-Rezeptor SdiA wird eine Funktion bei der Kommunikation mit den Pflanzen zugeschrieben. Die chemische Natur des zugehörigen Signalmoleküls ist ausschließlich für PluR bekannt. QS: Quorum sensing; IKS: *inter-kingdom signaling*.

Bakterielle Kommunikation

Um zwischen Freund und Feind zu unterscheiden, müssen Bakterien kommunizieren. Sie können sowohl mit ihren Artgenossen als auch mit anderen Bakterienarten oder ihren eukaryotischen Wirten über kleine Moleküle Informationen austauschen. In Gram-negativen Bakterien erfolgt diese Kommunikation oft über LuxR-Rezeptoren [4]. *P. luminescens* besitzt 40 verschiedene solcher LuxR-Rezeptoren, mehr als jede andere bisher entdeckte Bakterienart [5]. Untereinander kommunizieren *P. luminescens*-1°-Zellen mit α-Pyronen, auch Photopyrone (PPY) genannt, die von der Photopyronsynthase PpyS synthetisiert werden. Der LuxR-Rezeptor PluR sensiert diese und steuert somit die Verklumpung der Zellen und damit die Virulenz (**Abb. 2**, [6]). Wie 2°-Zellen untereinander kommunizieren, ist unklar. Die nahverwandte Art *P. asymbiotica*, die beim Menschen Hautinfektionen verursacht, nutzt keine PPY zur Kommunikation, sondern Dialkyresorzinole (DAR) [7]. Die Änderung der Kommunikationsweise von PPY zu DAR wird als entscheidender evolutiver Schritt von Insekten- zu Humanpathogenität gesehen.

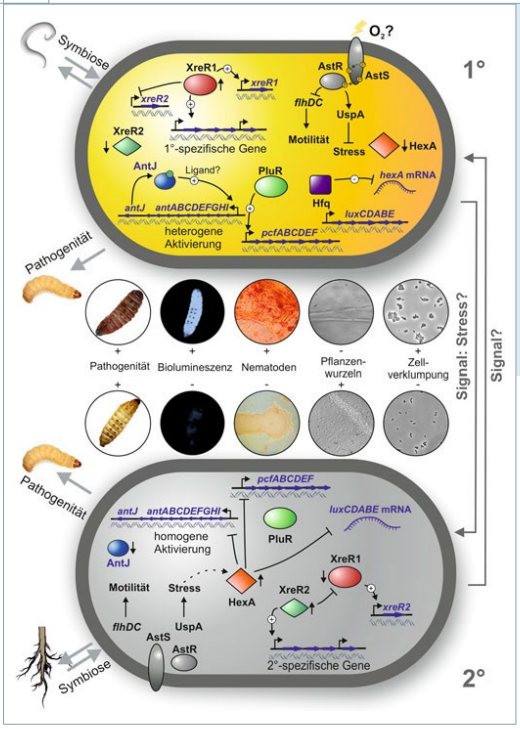
Insgesamt 35 der LuxR-Rezeptoren in *P. luminescens* besitzen eine PAS4-Signalbindedomäne, die sie wahrscheinlich für die Kommunikation mit eukaryotischen Wirten nutzen [5]. Erste Ergebnisse zeigen, dass die Bakterien über einen dieser PAS4-LuxR-Rezeptoren Signalmoleküle aus Wachsmottenlarven wahrnehmen (unveröffentlichte Daten). Andere der PAS4-LuxR-Solos könnten sie auch für die Erkennung der Nematoden nutzen (**Abb. 2**). *P. luminescens* besitzt außerdem einen LuxR-Solo, der eine Acyl-Homoserinlakton(AHL)-Bindedomäne besitzt und homolog zu SdiA ist [5]. Da einige pflanzenpathogene Bakterien über SdiA pflanzliche Signalmoleküle und keine AHL wahrnehmen, könnte bei *P. luminescens* SdiA ebenfalls für die Kommunikation zwischen Pflanze und 2°-Zellen wichtig sein (**Abb. 2**). Insgesamt deutet die hohe Anzahl der LuxR-Rezeptoren in *P. luminescens* darauf hin, dass die Bakterien echte Kommunikationstalente sind und viele „Sprachen" nutzen, um Freund und Feind zu unterscheiden.

Regulation von Freundschaft und Feindschaft

Die Entscheidung für das *phenotypic switching* von 1° zu 2° muss gut in der Population abgestimmt sein. Einer der Hauptregulatoren des *switching* ist der LysR-ähnliche Regulator HexA (**Abb. 3**), ein Repressor für 1°-spezifische Gene [8]. So ist die Konzentration von HexA in 2°-Zellen im Vergleich zu 1°-Zellen erhöht. HexA reguliert die Expression einiger Zielgene direkt und anderer indirekt über kleine regulatorische RNAs [8]. Die Expression von *hexA* selbst unterliegt der Kontrolle des RNA-Chaperons Hfq, sodass die Steuerung der HexA-Kopienzahl posttranskriptional reguliert wird [9]. Die zeitliche Koordination des *switching*-Prozesses wird über das Sensorkinase/Antwortregulatorsystem AstS/AstR reguliert (**Abb. 3**, [3]). Welchen Reiz die Sensorkinase wahrnimmt, um den Antwortregulator AstR zu phosphorylieren, ist unklar. Da das universelle Stressprotein UspA unter Kontrolle von AstS/AstR steht, wird vermutet, dass globaler Stress zum Einleiten des *switching* führt [3].

Die Pigmentierung in 1°-Zellen wird durch Anthraquinone (AQ) hervorgerufen, welche die Kolonien der Bakterien sowie auch die toten Insektenlarven rot erscheinen lassen [10]. Der AQ-Biosyntheseweg wird vom *antABCDEFGHI*-Operon codiert, dessen Expression unter Kontrolle des Regulators AntJ steht (**Abb. 3**). Die Kopienzahl von AntJ ist in einzelnen 1° Zellen erhöht und damit heterogen verteilt [11]. Weiterhin spielen bei der Aktivierung des *phenotypic switching* zwei Regulatoren der XRE-Familie eine besondere Rolle – XreR1 und XreR2 (**Abb. 3**, [12]). Die Kopienzahl von XreR1 ist in 1°-, die von XreR2 in 2°-Zellen erhöht. Das Regulatorpaar scheint durch positive Autoregulation einen epigenetischen *switch* zu konstituieren, indem XreR1 die Expression von *xreR2* reprimiert und XreR2 die Expression seines eigenen Gens durch direkte Wechselwirkung mit XreR1 steigert [12].

Wir sind erst am Anfang zu verstehen, wie komplex die Regulation der phänotypischen Heterogenität in *P. luminescens* ist. Es ist unklar, welche Signale zum Auslösen des *switching* führen und was letztendlich die heterogene Aktivierung einzelner regulatorischer Gene bewirkt.



◀ **Abb. 3:** Regulation phänotypischer Heterogenität in *Photorhabdus luminescens*. Das *phenotypic switching* von 1° zu 2° und die daraus resultierenden Phänotypen (siehe Bildmitte) werden durch ein komplexes Zusammenspiel unterschiedlicher Regulatoren kontrolliert. Dabei spielt der Regulator HexA eine zentrale Rolle. Dieser ist in 2°-Zellen in hohen Konzentrationen vorhanden und inaktiviert 1°-spezifische Gene, die wichtig für Pigmentierung, Bioluminezenz, Zellverklumpung und die Nematoden-Interaktion sind. Außerdem spielen zwei XRE-Regulatoren eine wichtige Rolle beim Wechsel von 1° zu 2°. Während XreR1 in 1°-Zellen in hohen Konzentrationen vorhanden ist und 1°- spezifische Gene aktiviert, wird dieser in 2°-Zellen durch direkte Wechselwirkung mit XreR2 reprimiert. Dabei werden 2°-spezifische Gene in 2°-Zellen aktiviert, die für die Kolonisierung von Pflanzenwurzeln essenziell sind. Das Sensorkinase/Antwortregulatorsystem AstS/AstR kontrolliert dabei das zeitliche Einleiten des *switching*-Prozesses. Das globale Signal für den Phasenwechsel von einzelnen Zellen in einer Population sowie die Signale für die einzelnen am Prozess beteiligten Regulatoren sind nicht bekannt.

Vielfältige Anwendung in der Biotechnologie

P. luminescens ist nicht nur ein exzellenter Modellorganismus, um bakterielle Kommunikation, Pathogenität oder phänotypische Heterogenität zu studieren. Die Bakterien sind auch vielseitig biotechnologisch nutzbar. So werden die entomopathogenen Nematoden bereits als Bioinsektizide für die Bekämpfung von Schädlingen eingesetzt. Weiterhin sind die Bakterien Reservoir für eine Vielzahl neuer Naturstoffe, die als Wirkstoffe in Biotechnologie und Medizin zum Einsatz kommen könnten. 2°-Zellen könnten zudem zukünftig als Biostimulanzien zur Ertragssteigerung in der Agrarbiotechnologie dienen.

P. luminescens – Freund oder Feind? Eine Frage, die ganz klar vom Blickwinkel des Betrachters abhängt. Aus Sicht der Insekten sind die Bakterien sicherlich ein todbringender Feind, aber aus Sicht der Nematoden und der Pflanzen eher ein Freund. Und für uns Menschen? Aufgrund ihrer vielseitigen biotechnologischen Anwendungsmöglichkeiten ist *P. luminescens* für uns sicherlich ein sehr guter Freund.

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Korrespondenzadresse:
Prof. Dr. Ralf Heermann
Johannes Gutenberg-Universität Mainz
Institut für Molekulare Physiologie
Mikrobiologie und Weinforschung
Biozentrum II
Hanns-Dieter-Hüsch-Weg 17
D-55128 Mainz
heermann@uni-mainz.de

AUTOREN

Ralf Heermann
Studium der Biologie und Promotion im Fach Mikrobiologie. 2001–2004 Postdoc, Universität Osnabrück und TU Darmstadt. 2004–2010 Wissenschaftlicher Assistent und Habilitation im Fach Mikrobiologie der LMU München. 2010–2018 Akademischer (Ober-)Rat und Arbeitsgruppenleiter an der LMU. Seit 2018 Universitätsprofessor für Mikrobiologie an der Universität Mainz und seit 2021 Assoziierter Professor am Institut für Biotechnologie und Wirkstoff-Forschung (IBWF) in Mainz.

Nazzareno Dominelli
2012–2014 Studium Lehramt Biologie und Chemie an der LMU München. **2014–2018** Bachelor- und Masterstudium der Biologie am Biozentrum der LMU. **2018–2019** Promotionsstudent im Fach Mikrobiologie an der LMU und seit **2019** an der Universität Mainz. Mitglied der Gutenberg-Akademie als Nachwuchswissenschaftler.

9. Concluding discussion

The use of biocontrol agents harmless to the environment, pollinators, animals, or humans becomes more and more important since the chemical pesticide employment is constantly decreasing due to safety concerns. Entomopathogenic nematodes (EPNs) are an excellent example of such biocontrol agents (Lacey and Georgis, 2012; Shehata et al., 2020). EPNs live in symbiosis with entomopathogenic bacteria of the genus *Photorhabdus* or *Xenorhabdus* and are able to kill a wide range of insect pests. A closer look into the lifecycle of *Photorhabdus luminescens* reveals that during the pathogenic part of the lifecycle the bacteria undergo a phenotypic switch from a nematode symbiotic and pigmented 1° cell variant into a non-symbiotic, not pigmented 2° cell variant (Akhurst and Boemare, 1988). Although, both cell variants differ in many phenotypic traits, they were advised to be genetically equal. Neither DNA rearrangements or modifications, nor genetic instability, or loss of plasmids were found in 2° cells, thus this phenomenon was designated as phenotypic heterogeneity (Akhurst et al., 1992; Forst et al., 1997; Hu and Webster, 2000; Daborn et al., 2001; Forst and Clarke, 2002). The 2° cell variant does not reassociate with EPNs for which reason in the past it was already suggested that the bacteria remain in soil (Smigielski et al., 1994; Turlin et al., 2006), but the fate of *P. luminescens* 2° cells is still unclear. In a first step a comparative transcriptome analysis comparing 1° and 2° cells was performed, to understand, which genes are involved in regulation of phenotypic heterogeneity in *P. luminescens*. Furthermore, to get insights into the fate of 2° cells in the rhizosphere, another RNA-Seq analysis including plant root exudates (PRE) was performed. Lastly, high throughput sequencing (HTS) of both, 1° and 2° cells, should provide evidence about genomic similarity of both cell variants. e. Subsequently, further studies were performed including plant root colonization and behavior towards phytopathogenic fungi to elucidate the ecological role of 2° cells in the rhizosphere. Finally, a possible LuxR-mediated interkingdom communication towards plant hosts or microorganisms living in the same biological niche mediated by SdiA was considered. In the course of this work, comparative transcriptome analysis could confirm mediation of phenotypic heterogeneity in *P. luminescens* on transcriptional level. Moreover, first indications of the fate of 2° cells in the rhizosphere were obtained, suggesting a putative applications as novel biocontrol agent. *P. luminescens* 2° not only reacts to plants and their exudates, but also interacts with them and protects them from phytopathogenic fungi via chitinase activity. Comparison of HTS data give evidence of

genomic similarity of 1° and 2° cells. Finally, this work gives first indications that LuxR solo SdiA not only binds AHLs that are probably released by microorganisms living in the rhizosphere niche together with *P. luminescens,* but it could sense plant derived signals thus mediating *P. luminescens*-host interaction and shedding light on a new IKS communication circuit in *P. luminescens*.

9.1 The adaptation of *P. luminescens* 2° cells in the rhizosphere and its potential as biocontrol agent

When *P. luminescens* 2° is left in the soil after an insect infection cycle, the cells experience a drastic change in nutrient availability because of the environmental switch from a nutrient-rich insect to a nutrient-poor environment – the soil. In the past it has already been shown that *P. luminescens* 2° cells are able to faster overcome periods of starvation compared to 1° cells (Smigielski et al., 1994). Indeed, 2D-PAGE proteome analysis revealed proteins involved in stress response, metabolism, translation and the binding of iron, amino acids, or sugar to be differently regulated in 2° variant (Turlin et al., 2006), which is in accordance with our transcriptome analysis showing genes involved in starvation, amino acid (AA) transport or the metabolism of alternative nutrient sources to be highly upregulated in 2° cells (Eckstein et al., 2019, chapter 2, Table S1). These positively modulated genes indicated a fast adaptation of 2° cells in different carbon and nitrogen sources utilization. As an example, these are genes involved in different pathways such as those encoding the AST-pathway (*astABDE*) or an AA-permease (*PluDJC_15875*), which are induced by AA and under nitrogen limitation (Schneider et al., 1998; Easom and Clarke, 2012). Moreover, genes involved in the metabolism of hydroxyphenylacetate (HPA), *PluDJC_04995* [*hpaC*]*, PluDJC_05000* [*hpaB*]*, PluDJC_05035* [*hpaX*]*, PluDJC_05040* [*hpaI*], which is often found in the soil as byproduct of degraded plant material (Díaz et al., 2001), are also upregulated in 2° cells (Eckstein et al., 2019, **Chapter 2, Table S1**). For *E. coli* Díaz and colleagues showed that it converts hydroxyphenylacetate and metabolize the products to achieve succinate and pyruvate, which then can be further metabolized by the cells (Díaz et al., 2001). Therefore, 2° cells undergo metabolic reorganization to overcome starving conditions in the soil by rearranging its metabolism using amino acids or sugars secreted by plant roots in the rhizosphere (Badri and Vivanco, 2009) as alternative nitrogen source and hydroxyphenylacetate as carbon source.

Once emerging from the insect and the previously rich nutrient source, nutrients become immediately limited for 2° cells in the soil. Therefore, they first need to migrate into the rhizosphere, hence using several strategies such as flagella or pilus driven twitching to actively navigate when undergoing starvation. Starvation-dependent motility is well known from plant beneficial bacteria like *Bacillus* and *Pseudomonas* (Mattick, 2002; Jarrell and McBride, 2008; Aroney et al., 2021) or plant pathogenic *R. solanacearum* (Tanns-Kersten et al., 2001; Corral et al., 2020). Bacterial swimming towards nutrient-rich areas is mostly driven by chemotaxis systems that respond to attractant stimuli upon ligand binding to chemoreceptors called methyl accepting chemotaxis protein (MCPs). These activate a signal transduction cascade thus stimulating different motility events which promote bacterial directional movement towards the nutrient rich source (Kearns, 2010).

The comparative transcriptome analysis between 1° and 2° cells revealed upregulation of DEGs involved in motility in 2° cells, e.g. the *fliC* or *flhDC*-operon, and chemotaxis (MCPs, methyl-accepting proteins: *PluDJC_09715* and *PluDJC_09720*). These results could be confirmed by different chemotaxis assays that proved increased swimming, twitching and chemotaxis capacity of 2° cells thus making motility a 2°-specific feature (Eckstein et al., 2019, **Chapter 2, Table 2 & Fig. 4; Chapter 5, Fig. 2B**). Indeed, when deleting *fliC* and both MCPs in 2° cells (Regaiolo et al., 2020, **Chapter 3, fig. 4C**), motility is totally abolished, which is in agreement with a behavior that was observed when regulation of phenotypic heterogeneity via AstS/AstR was investigated. *P. luminescens* 1° Δ*astR* mutants not only displayed a faster switching ability compared to the wildtype, but these cells also exhibited enhanced motility compared to the 1° parental wildtype (Derzelle et al., 2004).

In contrast, motility was described to be a 1°-specific feature in close related strains of *Xenorhabdus nematophila* and *P. temperata*, (Hodgson et al., 2003), which was reported to be positively regulated by LrhA, a LysR-type transcriptional regulator (Richards et al., 2008). A homolog of this regulator, HexA, is also found in *P. luminescens* and the corresponding gene was found to be highly upregulated in 2° cells (Eckstein et al., 2019, **Chapter 2, Table S1**). HexA is believed to act as master repressor of 1°-specific traits in 2° cells (Joyce and Clarke, 2003). It can be suggested that HexA affects motility in *P. luminescens* 1°, since for HexA of *E. coli* it is known to be involved in repression of motility and chemotaxis gene (Lehnen et al., 2002). Finally,

this supports the assumption that 2° cells are adapted to a free-soil lifestyle by boosting flagella motility to reach areas where more nutrients are available.

Interestingly, for plant associated bacteria (PAB) like *Pseudomonas* motility and chemotaxis is an essential trait in the rhizosphere, e.g., *Pseudomonas fluorescens* is highly motile and chemotactically active in the rhizosphere (van Elsas et al., 1991; Worrall and Roughley, 1991) with FliC and FlhDC hypermotility mediators as well as MCPs playing an essential role in plant root colonization (Lugtenberg et al., 2001; Berg and Smalla, 2009; Redondo-Nieto et al., 2012; Barahona et al., 2016; Cole et al., 2017). The motility and chemotaxis related genes that are highly upregulated in 2° cells show similarities to those in *Pseudomonas* thus hypothesizing that *P. luminescens* 2° cells could interact with plant roots. It could be verified that 2° cells reacted to plant root exudates (PRE) and interacted with plant roots, colonizing them as novel hosts. These data indicate that this cell variant use nutrients produced by plants thus adapting to an alternative lifestyle (Eckstein et al., 2019, **Chapter 2 Fig. 6**; Regaiolo et al., 2020, **Chapter 3, Fig. 4A & B**). Furthermore, a positive effect of 2° cells on plant development was observed. The bacteria displayed a beneficial influence on plant root growth by promoting root hair (RH) and lateral root (LR) formation, a phenotype that was not observed for 1° cells. A similar effect was also observed when the bacteria and plants were spatially separated on agar plates, indicating the production of volatile organic compounds (VOCs) only by *P. luminescens* 2° cells (Regaiolo et al., 2020, **Chapter 3, Fig. 5**). Moreover, a beneficial microbe-plant interaction induces alteration in plant root morphology, which are prompted by plant growth promoting rhizobacteria (PGPRs) like bacteria of the genus *Pseudomonas* that induce RH and LR development 6/28/2022 3:34:00 PM. Therefore, a high potential role of *P. luminescens* 2° to act as putative plant growth promoting bacterium can be suggested.

However, at this point it was important to understand how this *Photorhabdus*-plant interaction occurs. Generally, the rhizosphere is characterized by PRE acting as attractants and therefore triggering microbes-plant interaction and microbial rhizosphere persistence (Morrissey et al., 2004). During a microbe-plant interaction both organisms influence each others behavior. For example, chemotaxis of different *Pseudomonas* strains is increased at a certain distance to the plant roots where concentration of PRE is low, whereas chemotaxis decreased at root proximity where the PRE concentration is increased, whereupon a set of genes coding for the flagellar machinery is downregulated (Mark et al., 2005; López-Farfán et al., 2019). For that

purpose, it was tested how 2° cells react to PRE performing a RNAseq analysis using 2° cells cultures supplemented with PRE, to determine *P. luminescens* 2° transcription profile that can trigger plant interaction. The analysis identified 741 DEGs of which 233 were positively and 508 were negatively modulated indicating a 2° cells transcriptional reprogramming in presence of PRE (Regaiolo et al., 2020, **Chapter 3, Fig. 1**). Indeed, similar to as observed for *Pseudomonas* strains, *P. luminescens* 2° chemotaxis and motility decreased with increasing concentration of PRE (Regaiolo et al., 2020, **Chapter 3, Fig. 4A & B**) (**Fig. 9-1**). Remarkably, all the respective motility genes were downregulated in 2° cells in the presence of PRE. Furthermore, *fliZ*, a gene coding for a global regulatory protein, which is known to be involved in regulation of motility in *X. nematophila*, was upregulated upon addition of PRE in *P. luminescens* 2° cells (Jubelin et al., 2013; Regaiolo et al., 2020, **Chapter 3, Fig. 2A**). It was observed that FliZ positively influences bacterial adhesion (Jubelin et al., 2013), suggesting that 2° cells use FliZ at root proximity to trigger bacterial adhesion and consequently biofilm formation, a primary trait important for plant root colonization as observed in *Pseudomonas* (Dunne, 2002; Spiers and Rainey, 2005; Huang et al., 2007; Koza et al., 2009; Barahona et al., 2010; Mann and Wozniak, 2012; Martin et al., 2016). Increased PRE concentration (up to 10% [v/v]) led to less motility of *P. luminescens* 2° cells, while in contrast the same concentration triggered 70% more biofilm formation. Additionally, the related DEG, *PluDJC_09560* (biofilm formation regulator BssS), which is involved in regulating genes belonging to catabolite repression, stress responses, regulation of QS, and putative stationary phase signal classes (Domka et al., 2006), was upregulated in presence of PRE (Regaiolo et al., 2020, **Chapter 3, Fig. 2A**). Concomitantly with increased motility and plant roots colonization, *P. luminescens* 2° cells must also adjust their metabolism and react to different stresses in order to persist in the rhizosphere. In general, PREs alter the expression of genes involved in diverse metabolic processes, transports, regulation, and stress response in rhizobacteria (Mavrodi et al., 2021). The data suggest that not only an adaptation of *P. luminescens* 2° to a free soil-lifestyle occurs, but also a reprogramming in its metabolism upon PRE. Genes involved in different metabolic processes like gluconeogenesis (*PluDJC_05875*), protein transport and carbohydrate utilization were differently expressed (Regaiolo et al., 2020, **Chapter 3, Fig. 1**). Moreover, also several universal and oxidative stress-response genes, like *uspB* (*PluDJC_00675*), and *PluDJC_11030*, or *PluDJC_17730,* were upregulated in 2° cells further confirming the capacity of this

variant to adapt to the rhizosphere. Furthermore, besides starvation soil living bacteria encounter several other stress situations including permanently changing temperatures in the rhizosphere. Remarkably, it could be shown that 2° cells display higher temperature tolerance compared to 1° cells. Although, for *P. luminescens* it was described that the bacteria do not survive temperatures exceeding 35°C (Fischer-Le Saux et al., 1999), 2° cells were still able to replicate at 37°C and were additionally more tolerant to cold temperatures, while 1° cells died (Eckstein et al., 2019, **Chapter 2, Fig. 7**). In conclusion, 2°-specific features focus on sensing and utilizing different nutrients deriving from plants, adapting to starvation and different temperatures, and putatively moving towards new nutrient sources. Therefore, an additional lifecycle of *P. luminescens* 2° cells could be discovered, where 2° cells adapt to a free-soil lifestyle (**Fig.- 9-1**), assigning plant growth promoting, and plant roots colonization abilities as 2°-specific features, therefore shedding light to the phenotypic heterogeneity in *P. luminescens* cell populations (**Fig. 9-3**). The data suggest a model in which 2° cells move towards the plant roots upon a PRE concentration gradient and once reaching a certain root-proximity, the motility decreases, and biofilm formation is induced, indicating an adaption of 2° cells to the new plant host (**Fig. 9-1**). However, response of the plant towards the bacterial colonization as well as the nature of the produced VOCs leading to plant-growth promotion have to be determined yet.

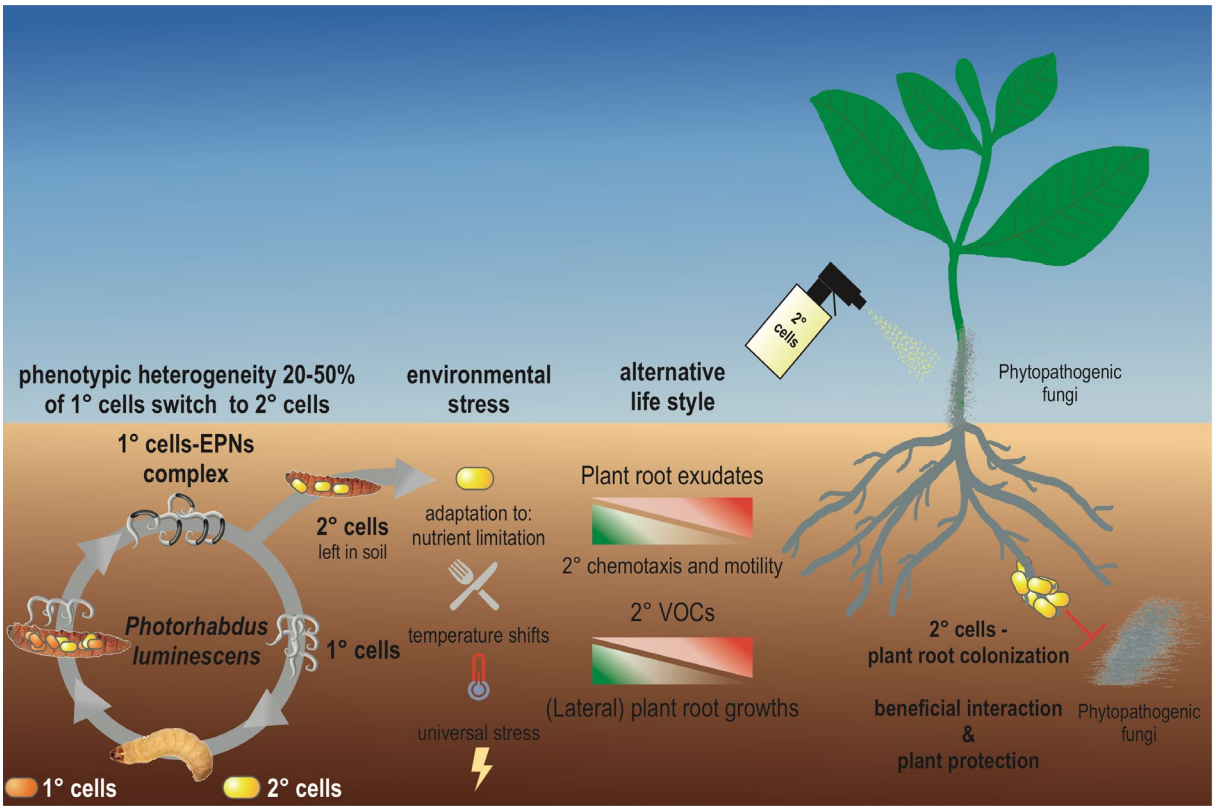


Figure 9-1: Model for the extended lifecycle of *P. luminescens* and the fate of the 2° cells. During the infective part of the life cycle up to 50% of *P. luminescens* 1° cells switch to 2° cells, which are unable to reassociate with nematodes (EPNs) and are therefore left in soil after an insect infection cycle. The 2° cells need to adapt to different environmental stress e.g., nutrient limitation, temperature stress and others. In contrast to 1° cells, *P. luminescens* 2° cells are highly motile and chemotactically react to PRE, a behavior that is reduced at plant root proximity, where the 2° cells organize in biofilm as well as root adherence finally colonizing the plant roots. 2° cells putatively produce volatile organic compounds (VOCs) that have a plant beneficial effect and further protect plants from phytopathogenic fungi.

Biocontrol agents are considered as new sustainable agricultural technique to prevent reduced crop yields due to pests and phytopathogens, without harming the environment, animals, humans, and the rhizosphere. The use of plant growth promoting rhizobacteria (PGPRs) and EPNs have shown to improve pest management and enhance the plant biomass production while increasing plant resistance to diseases (Zhang et al., 2016). The use of *P. luminescens* as biocontrol agent is already exploited in combination with EPNs, whereas the bacteria are the agent with a direct toxic effect on insect pests. Furthermore, the use of EPNs displayed an improvement of the soil quality, since they were able to increase soil oxygenation and plant nutrient uptake (Lacey and Georgis, 2012; Pieterse et al., 2014). Although the biocontrol role can be assigned only to *P. luminescens* 1° cells, as only this cell variant lives in symbiosis with EPNs (Akhurst and Boemare, 1988). A beneficial role for 2° cells that

are left behind in the soil interacting with plant roots was not considered so far. Hence a closer look at their biocontrol capacity became necessary.

The comparative transcriptome analysis performed with 2° cells treated with and without PRE highlighted two genes that were highly expressed in the presence of PRE: *PluDJC_12460* (*cbp*) and *PluDJC_11885* (*chi2A*), both putatively involved in chitin degradation activity (Regaiolo et al., 2020, **Chapter 3, Fig. 2A**; Dominelli et al., 2022, **Chapter 4**). Chitinases are glycosyl hydrolases present in many organisms from prokaryotes to eukaryotes. Their size ranges from 20 to 90 kDa, (Shahidi and Abuzaytoun, 2005; Bhattacharya et al., 2007) and their sub-classification is based on their N-terminal domain, the isoelectric point, pH, localization, signal peptide, and the inducer. Bacteria contain chitinases of the class II with an exo-action, which are known to be induced in presence of pathogens like fungi (Patil et al., 2000; Hamid et al., 2013). In the past, exochitinases have already received attention as they play an important role in biocontrol of fungal phytopathogens and insects (Mendonsa et al., 1996; Mathivanan et al., 1998). Several soil bacteria produce chitinases (Hamid et al., 2013), including PGPRs like *Pseudomonas fluorescens*, which are capable to reduce fungal growth and spore germination upon chitinase activity (Akocak et al., 2015). This effect was also observed in *Serratia*, whereupon chitin degradation by these bacteria were suggested as biocontrol alternative (Zarei et al., 2011). *P. luminescens* harbors three class II chitinases, of which two - Chi2B and Chi2C - were already described to play a pathogenic role towards insects, a role that can be excluded for Chi2A (Hurst et al., 2011, Dominelli et al., 2022, **Chapter 4 Fig. 1 & 4A**). *P. luminescens* chitinases (Chi2A, Chi2B and Chi2C) showed homology in their catalytic domains with a conserved DxDxE motif similar to the chitinase found in *Serratia*. Interestingly, only Chi2A, which biological role has not been described yet, is induced by an unknown plant-derived signal, whereupon 2° cells display antifungal activity towards phytopathogenic *Fusarium graminearum* (Regaiolo et al., 2020, **Chapter 3, Fig. 3C**). It could be demonstrated that antifungal activity of *P. luminescens* 2° cells derives from Chi2A as the purified enzyme showed colloidal chitin digestion and directly reduced fungal growth. Additionally, Chi2A showed high stability over long-term storage, (confirmed by nanoDSF analysis), which is a promising characteristic for the protein to be used in biocontrol management. Moreover, *P. luminescens* 2° cells lacking *chi2A* lost their ability to reduce fungal hyphae development (a phenotype restored via complementation) as well as plant protective activity of 2° cells against *F. graminearum*

(Dominelli et al., 2022, **Chapter 4, Fig. 5A**). Even though solely Chi2A can degrade (fungal) chitin, chitin binding protein (Cbp) activity is somehow essential for *P. luminescens* to function as biocontrol agent against phytopathogenic fungi, as bacteria lacking *cbp* also lost their growth inhibitory activity towards *F. graminearum* (Dominelli et al., 2022, **Chapter 4, Fig. 2**). During the fungal growth inhibitory process of 2° cells, the bacteria are attracted by the fungi as they actively attached to their hyphae. 2° cells could use this strategy as a first mechanism to protect the plant against phytopathogenic fungi. It is likely that right after attaching to fungal surface, the bacteria release Chi2A and Cbp and therefore initiate hydrolysis of the fungal cell wall. A similar effect was also observed in plant protection assays, where fungal hyphae could not develop and colonize plants. Indeed, a two-step treatment with *P. luminescens* 2° cells (pre-treatment of the seeds and treatment of the grown plant fully protected the plants from *F. graminearum* infection, assigning 2° cells a plant protecting ability (**Fig. 9-1**). This effect was abolished in *chi2A* and *cbp* deletion mutants (Dominelli et al., 2022, **Chapter 4, Fig. 4B**).

In conclusion, not only EPNs carrying 1° cells but also free-living 2° cells are applicable as biocontrol agent in agriculture, latter one as biofungicide due to their chitinolytic activity. Moreover, attachment to plant roots as well as to fungal hyphae and chitinase activity can be described as 2°-specific feature which is absent from 1° cells (**Fig. 9-3**). The 2° cells sense PRE in the rhizosphere thus inducing expression of several genes important for plant root colonization and plant protection (Dominelli et al 2022, **Chapter 4, Fig. 5**). Finally, a biological role for the Chi2A of *P. luminescens* could be demonstrated for the first time.

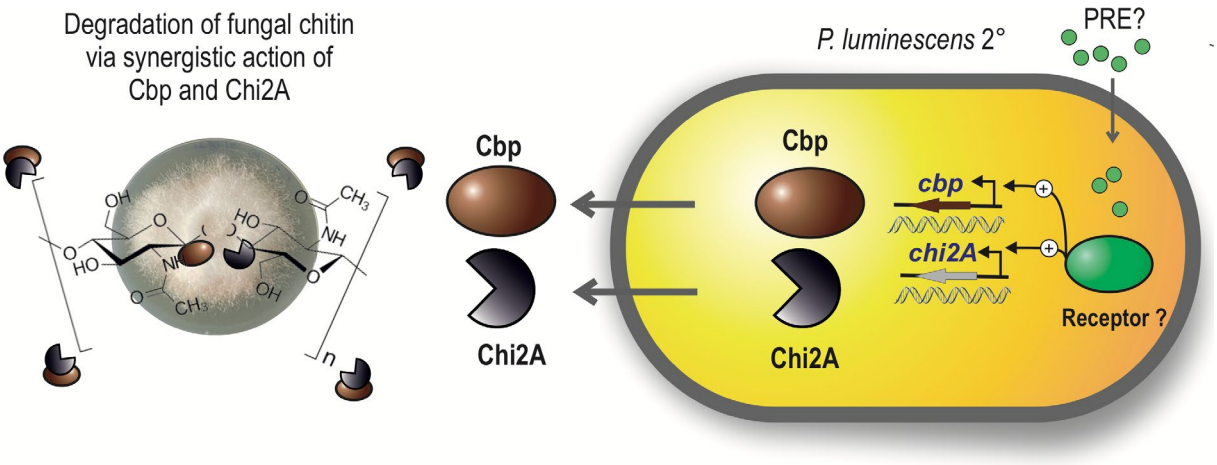


Figure 9-2: Model of the putative synergistic mode of action of Chi2A and Cbp in *P. luminescens* 2°. *P. luminescens* 2° senses plant signals that lead to an upregulated expression of *chi2A* and *cbp*.

The Cbp and Chi2A enzyme are released and synergistically act together to efficiently degrade fungal chitin.

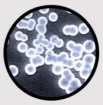
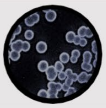
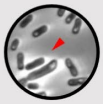
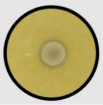
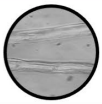
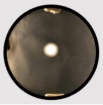
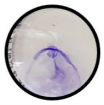
Phenotypes	1° cells		2° cells	
Bioluminescence	+++		+	
Cell clumping (Pcf)	+		-	
Protease production	+++		+++	
Pigmentation	+++		-	
Crystal proteins	+		-	
Pathogenicity	+++		+++	
Biofilm production	+++		+	
Symbiosis with nematodes	+++		-	
Antibiotic production	+		-	
Interaction with plant roots	-		+	
Swimming motility	+		+++	
Twitching	-		+++	
Chitinolytic activity against fungi	-		+++	

Figure 9-3: Extended list of phenotypic differences between *P. luminescens* 1° and 2°. The 2° cells lack most of the 1°-specific phenotypes. In this thesis further phenotypic traits could be assigned, i.e., the interaction with plant roots, swimming, and twitching motility, and chitinolytic antifungal activity as 2°-specific phenotypes, phenotypes that are absent from 1° cells. Moreover, protease production was also observed for 2° cells, whereas antibiotic production and biofilm formation were exclusively found as 1°-specific feature.

9.2 *Photorhabdus* – plant interkingdom communication

Interkingdom communication describes the cell-cell communication between eukaryotic hosts with bacteria or vice versa and is discussed as prerequisite either for symbiotic as well as pathogenic interactions between pro- and eukaryotes. LuxR-type receptors are involved in bacterial cell-cell communication, for which reason so called LuxR solos have been speculated before to be involved in interkingdom signaling (Patankar and González, 2009) *P. luminescens* harbors 40 LuxR solos which are classified into three types according to their N-terminal signal binding domain (SBD): i) AHL-binding domain (two LuxR solos), ii) PAS4-binding domain (35 LuxR solos), iii) unknown binding domain (three LuxR solos) (Brameyer et al., 2014). Some LuxR solos in different bacteria showed the capacity to mediate interkingdom signaling communication (IKS). In a previous work two PAS4-LuxR solos in *P. luminescens* namely PikR1/PikR2 were shown to bind eukaryotic signaling molecules to sense the host species and to adapt pathogenicity, and therefore act as IKS receptors. Indeed, PikR1 and PikR2, reacted to stearic- and palmitic acid, fatty acids derived from insects, which were found in lyophilic fractions of *G. mellonella* insect homogenate (Brehm, 2021).

LuxR solos from plant associated bacteria (PABs) are also involved in IKS (González and Venturi, 2013; Venturi and Fuqua, 2013) and harbor AHL-SBD with differences in the conserved residue, e.g. in TraR of *A. tumefaciens* W_{57} and Y_{61} are substituted with methionine (M) and tryptophane (W), respectively (Ferluga and Venturi, 2009; Venturi and Fuqua, 2013). Remarkably, in *P. luminescens* two AHL-LuxR-type regulators are present: PluR, the receptor responsible for QS via so called photopyrones (Brachmann et al., 2013; Brameyer et al., 2014), and SdiA for which the signal molecule is still unknown. Since *P. luminescens* SdiA harbors the conserved amino acid WYDPWG-motif specific for AHL-binding, the bacteria might sense exogenous AHLs produced from other bacteria or eukaryotic organisms present in the same niche of *P. luminescens* (Brameyer et al., 2014). Interestingly, SdiA homologs in PABs were involved in sensing plant derived signals, thus acting as IKS and important for plant colonization and interaction (Mosquito et al., 2020; Bez et al., 2021). It could be demonstrated that *P. luminescens* 2° can colonize the plant root, but how the bacteria sense the plants, and whether it relies on IKS was still an open question. To elucidate this hypothesis, the putative role of *P. luminescens*' SdiA in IKS towards plant roots

was investigated. For the LuxR solo SdiA, found in different non-AHL producing enteric bacterial genera like *Escherichia, Klebsiella,* or *Salmonella*, implication of the receptor in regulating virulence factors like antibiotic resistance, biofilm formation and motility was reported (Ahmer et al., 1998; Kanamaru et al., 2000; Antunes et al., 2010; Sharma et al., 2010; Tavio et al., 2010; Culler et al., 2018). Upon AHL binding SdiA is supposed to act as transcriptional regulator for several genes involved in metabolism, motility, and virulence (Kim et al., 2014). Indeed, the expression of bacterial virulence genes, biofilm formation and motility modulated via LuxR receptors can also be triggered by plant signals (Anetzberger et al., 2009; Venturi and Fuqua, 2013; Yang and Defoirdt, 2015). However, researchers pointed out that SdiA mediated regulation of motility and biofilm formation was not necessarily directly tethered to signal binding (Lindsay and Ahmer, 2005; Dyszel et al., 2010; Hughes et al., 2010; Sharma et al., 2010; Shimada et al., 2014; Nguyen et al., 2015). In EHEC strains lacking *sdiA* biofilm formation as well as motility was highly increased (Culler et al., 2018), whereas, for SdiA of *Klebsiella* lack of the respective gene led to increased biofilm formation and altered expression of fimbriae coding genes revealing a repressive role of SdiA in bacterial cell adherence (Pacheco et al., 2021). Similarly, for *P. luminescens* SdiA modulation of motility and biofilm formation could be observed. *P. luminescens* lacking *sdiA* displayed impaired motility suggesting SdiA as positive regulator for *P. luminescens* motility. Whereas SdiA represses biofilm formation (**Chapter 5, Fig. 2**), usually a 1°-specific feature (**Fig. 9-3**) (Eckstein et al., 2019, **Chapter 2**), as deletion of *sdiA* in both cell variants led to an increased biofilm formation especially in 2° cells (**Chapter 5, Fig. 2**). Therefore, these data propose that SdiA evolved to regulate a switch between a sessile and motile lifestyle in *P. luminescens*. Thereby, biofilm formation and motility are regulated via SdiA in a signal-independent manner, similar to as it was described for *E. coli*, where mutating the sensor rather than changing the signal, already led to enhanced biofilm formation (Lee et al., 2007). Furthermore, *E. coli* SdiA responded to extracellular plant derived interspecies indole with decreased biofilm production (Lee et al., 2007, 2009). This suggests that SdiA regulation of biofilm formation is influenced by specific signals dependent from the environment. Indole-3-acetic acid (IAA) is a hormone important for many physiological processes in the plant (Lazar, 2003) and is likely involved in IKS communication. Interestingly, supplementation with PRE led to alteration in motility, which decreased (Regaiolo et al., 2020, **Chapter 3, Fig. 4A & B**), and biofilm formation, which increased (**Chapter 5, Fig. 2D**) in *P. luminescens*, thus

proposing that SdiA-mediated biofilm formation might also be influenced by compounds like indole or derivatives produced by plants. Subsequently, altered SdiA DNA-binding affinity can be suspected once 2° cells are in the rhizosphere and in proximity of plant roots. Therefore, SdiA might be involved in IKS with plants, sensing and binding a plant derived signal, leading to altered regulation of the already modulated target genes involved in plant host colonization. Consequently, the ability of the LuxR-type receptor, SdiA; to recognize exogenous signals from plant roots was determined. So far, for SdiA it is known that it harbors an AHL-SBD (Brameyer et al., 2014) and docking analysis of *E. coli* SdiA only suggested high affinity binding towards long chained AHLs (Almeida et al., 2016), which was also shown for LoxR, a SdiA homolog found in endophytic *Kosakonia* (Mosquito et al., 2020).

For that purpose, nanoDSF analysis with purified SdiA of *P. luminescens* was performed using C_4-AHL and C_{12}-AHL as well as PRE to determine changes in protein stability upon a putative signal binding. SdiA displayed increased stability in presence of long chain AHL, whereas it was less stable upon short chain AHL, indicating different SdiA folding conformations (**Chapter 5, Fig. 3A**). It can therefore be hypothesized that SdiA might be inactivated in presence of short chain AHLs, similar to those described for members of the LuxR family, like EsaR-like proteins that only display DNA-binding affinity in absence of AHLs (Tsai and Winans, 2010; Minogue et al., 2002, 2005; Cui et al., 2005; Castang et al., 2006). Reduced DNA-binding was putatively due to impaired dimerization of the proteins through change in their conformation, resulting in reduced DNA-binding affinity of the DBD (Tsai and Winans, 2010). A similar effect could be observed for *P. luminescens* SdiA in the presence of PRE: three PRE-HPLC fractions mediated a change in SdiA folding, putatively upon signal binding to the SBD (**Chapter 5, Fig. 3A**), suggesting that i) activity of SdiA is blocked by a yet unknown plant compound and ii) subsequently, SdiA is involved in IKS with plants similar to AHL-LuxR solos found in various plant associated bacteria (PABs). Plants produce various molecules that are secreted into the rhizosphere and might be involved in the IKS communication with bacteria. Studies reported different molecules that can bind *E. coli* SdiA homolog, such as glycerol (Nguyen et al., 2015), present in PRE (Regaiolo et al., 2020, **Chapter 2, Table S2**), or the plant hormone indole (Lazar, 2003; Lee et al., 2006). Teplitski and colleagues discovered AHLs mimicking molecules in PRE that interfere with bacterial QS and e.g., interacted with LuxR-receptors like CviR from *Chromobacterium violaceum* or LasR from *Pseudomonas aeruginosa* (Teplitski et al.,

2000, 2004). Furthermore, some LuxR solos responsed to plant derived low molecular weight compounds, which are found in different PABs and are important for plant host recognition. Representatives of these QS-related LuxR solos are OryR and XccR from plant pathogenic *Xanthomonas campestris* and *Xanthomonas* oryzae, respectively, or PipR and PsoR from plant beneficial *Pseudomonas* GM79 and *Pseuodmonas fluorescens*, respectively (Ferluga et al., 2007; Ferluga and Venturi, 2009; Patankar and González, 2009; Subramoni and Venturi, 2009; Coutinho et al., 2018; Mosquito et al., 2020). It is likely that the chemical nature of these plant compounds is diverse, as PipR from root endophytic *Pseudomonas,* responded to ethanolamine derivatives (Coutinho et al., 2018), while OryR responded to a yet unknown plant compound (Zhang et al., 2007). These PAB *luxR* solo genes are found adjacent to the virulence-associated proline iminopeptidase coding *pip* gene, which e.g., is indispensable for *Xanthomonas* virulence after sensing the plant host via OryR or orthologous XccR (Ferluga et al., 2007; Zhang et al., 2007; Chatnaparat et al., 2012). Interestingly, *P. luminescens* harbors a homologous *pip* gene (*PluDJC_14120*) also coding for a proline iminopeptidase and might also be important in plant host colonization. Therewith, *P. luminescens* might yield free proline and adapt to the new environment in the rhizosphere when colonizing plant roots. Indeed, it is already known that *P. luminescens* switches from the nematode symbiotic to the pathogenic lifestyle upon sensing L-proline in insects' hemolymph. Therefore, *P. luminescens* cells released from nematodes into the insects adapt to the new host environment (Crawford et al., 2010, Waterfield, 2013).

Besides binding signaling molecules, LuxR regulators also act as transcriptional factors since they have a C-terminal HTH DNA binding domain (DBD) (Shadel et al., 1990; Slock et al., 1990; Choi and Greenberg, 1991, 1992; Fuqua et al., 1994). In the canonical QS system LuxR-type regulators control transcription of specific genes by binding the respective promoters at a conserved site called *lux* box (Devine et al., 1989; Stevens and Greenberg, 1997). Although all LuxR receptors share a low homology grade, there are specific conserved AA that can be found in both domains throughout all LuxR receptors. According to Fuqua et al., 1996 three conserved AA - E178, L182, and G188 - are present in the DBD of LuxR, critical for DNA binding properties of the protein (Whitehead et al., 2001; Zhang et al., 2002) and are also found in the DBD of SdiA in *P. luminescens*. In presence of the respective signaling molecule LuxR receptors change their conformation and subsequently activate or repress the

expression of target genes. Adjacent to *sdiA*, *P. luminescens* harbors a gene named *PluDJC_01670*, coding for a PixA inclusion body protein, which function is still unclear. Although suggested, PixA is not involved in virulence and colonization towards insect hosts in *Xenorhabdus* nor in *Photorhabdus* strains (Goetsch et al., 2006). However, *PluDJC_01670* sequence shows homology to *aidA* of the PAB *Ralstonia solanacearum*, which leds to the assumption that PixA might be involved in *Photorhabdus*-plant interaction. For this purpose, *PluDJC_01670*/*pixA* was re-named into *aidA*. Indeed, in *R. solanacearum* AidA plays an important role in temperature dependent virulence against plants. Like in *P. luminescens* the respective gene *aidA* is located adjacent to *luxR*-like gene, *solR,* which codes for a LuxR regulator that strongly influences *aidA* expression in *R. solanacearum* (Flavier et al., 1997; Meng et al., 2015). Therefore, DNA binding properties of SdiA were further analyzed in absence and presence of putative signal molecules via SPR. Thereby, the intergenic region between *sdiA* and adjacent *aidA* were considered as region that putatively contains the promoters, both harboring a *lux*-box-like motif in the shared promoter region (**Chapter 5**). Indeed, SdiA binds its own promoter in absence of a signal molecule with high affinity. However, the fast dissociation indicated a fast and accurate autoregulatory mechanism of SdiA independent of a signal molecule (**Chapter 5, Fig. 3B**), suggesting an intrinsic mechanism of QS modulation, similar to those described for EsaR of *Pantoea,* where EsaR regulates its own expression via signal-independent repression, whereas a de-repression occurred upon signal binding (Minogue et al., 2002). Moreover, two binding events with high affinity and stability for SdiA of *P. luminescens* towards the promoter of *aidA* in absence of a signal molecule could be identified, indicating that this 310 bp long intergenic region between *aidA* and *sdiA* could act as bidirectional promoter. Therefore, these data propose that SdiA is capable of bidirectional stimulation of transcription of these two oppositely oriented genes, similar to LuxR of *Vibrio fischeri* towards the *lux* genes (Shadel and Baldwin, 1991). The DNA binding property of SdiA towards both promoters was reduced upon PRE (**Chapter 5, Fig. 3B**), confirming the hypothesis that SdiA modulate genes signal independent and DNA-binding ability is impaired upon signal binding as mentioned above. Usually, AHLs binding to LuxR receptors leads to, among other things, dimerization of the proteins, subsequently initiating DNA-binding (Nasser and Reverchon, 2007). In contrast, it might that dimerization of N-terminal SBD of SdiA is inhibited or occurs differently upon signal binding, which in turn might inhibit

dimerization and activity of the C-terminal DBD. Whether SdiA in its native conformation regulates expression of *sdiA* itself or the expression of *aidA* in a positive or negative way is yet unknown. So far, due to the findings that *aidA* is regulated by SdiA, which was altered upon PRE, like it was reported for plant pathogenic *R. solanacearum* (Flavier et al., 1997), a role of AidA of *P. luminescens* in plant host colonization can be concluded. Generally, AidA could be involved in SdiA-mediated host colonization in *Photorhabdus* spec. Indeed, also *Photorhabdus temperata* and *Photorhabdus asymbiotica* harbor the *sdiA-aidA* gene cluster which might be essential for eukaryotic host colonization (**Chapter 5, Fig. 1**).

Taken together, a novel IKS communication circuit in *P. luminescens* involving LuxR solos with plants could be determined. It is in accordance with the unidirectional IKS circuit that has been described to be evolved from canonical AHL-QS systems where the LuxR solos no longer respond to endogenously produced AHLs, but to plant signals (González and Venturi, 2013; Venturi and Fuqua, 2013). Furthermore, for SdiA regulator an accurate signal independent (auto-) regulatory mechanism in *P. luminescens* is suggested, changing its mode of action upon signal binding, likely due to conformational changes Whether genes are positively or negatively regulated by SdiA still has to be investigated. It might be that LuxR solos such as SdiA can already activate the expression of genes in a signal-independent manner, which are then impaired upon signal binding to adapt to their new environment or host. Furthermore, these data give evidence of a plant derived compound binding to SdiA of *P. luminescens*. Although the chemical nature is still unknown, on the basis that SdiA reacted to AHLs, a putative AHL-mimicking substance produced by the plant might be involved in SdiA-IKS. Upon the observed correlation between SdiA and PRE, in respect to motility and biofilm phenotypes, it can be assumed that SdiA putatively modulates the expression of different genes, which emerged to be differently expressed in 2° cells in the presence of PRE. The expression of genes like *chi2A* or *cbp* that are involved in biocontrol of 2° cells might be very likely modulated by SdiA (**Fig. 9-4**). Lastly, a putative novel role to AidA (previously named PixA) in the plant host colonization of *Photorhabdus,* since involvement in insect host virulence and nematode colonization was excluded (Goetsch et al., 2006)

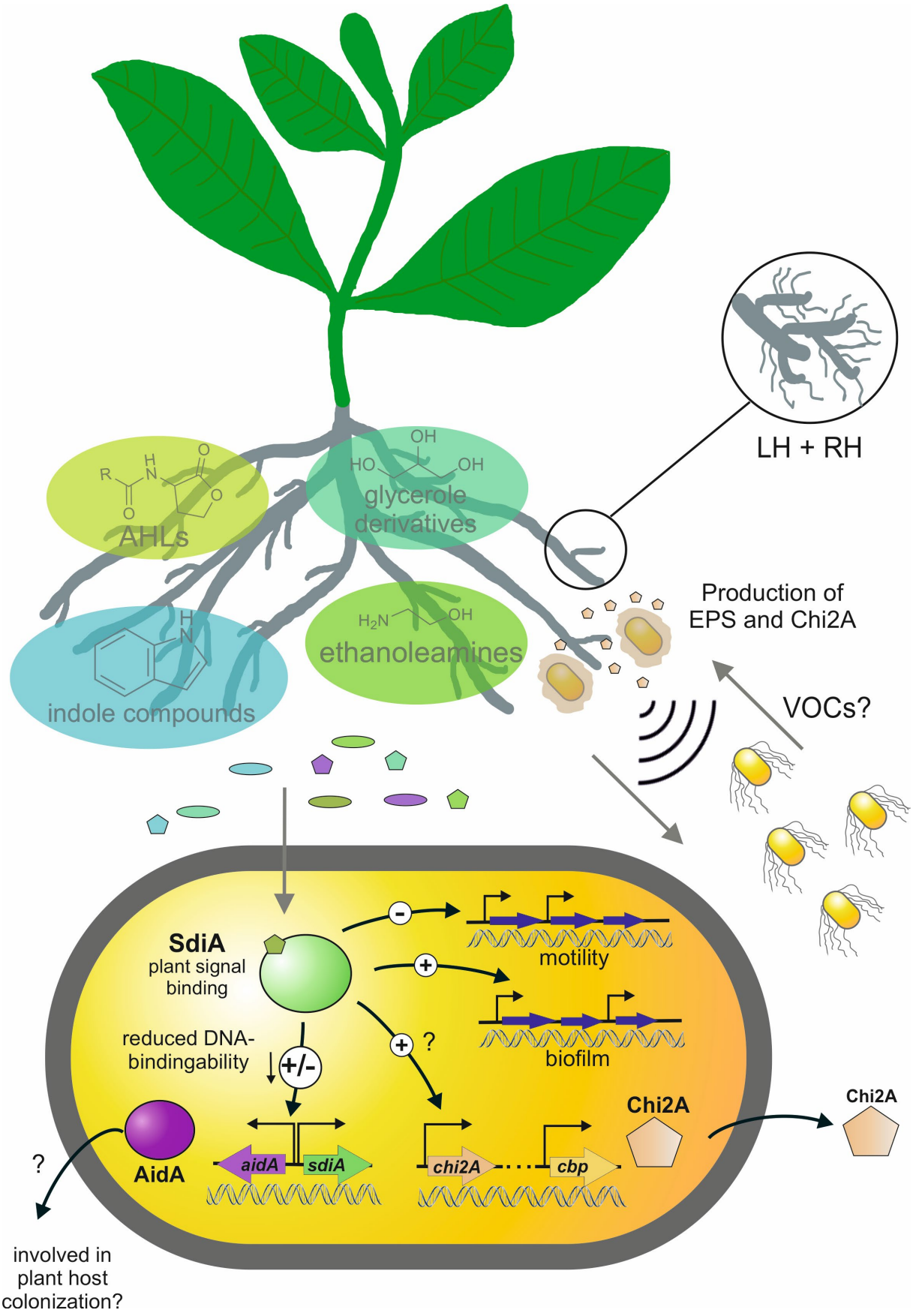


Figure 9-4: Influence of plant signal binding on SdiA regulation and the effect on *P. luminescens* 2° behavior in the rhizosphere. Plants produce different compounds like derivatives of indole, glycerol, or ethylamine, as well as AHLs that are sensed by different LuxR regulators. The LuxR solo SdiA of *P.*

luminescens is a regulator of, among putative other phenotypes, motility and biofilm formation. In absence of a putative ligand SdiA positively modulates motility, while expression of biofilm formation is inhibited. Once a plant derived signal binds to SdiA, motility of *P. luminescens* 2° is impaired, while the bacteria start to produce an extracellular polymeric substance (EPS) matrix resulting in biofilm. Furthermore, the expression of genes involved in biocontrol activity, like *chi2A* or *cbp* might also be modulated by SdiA upon signal binding, since these genes are upregulated in 2° cells in presence of PRE. DNA-binding affinity of SdiA towards the bidirectional promoter between *aidA* and *sdiA* is reduced upon PRE, indicating a role of AidA in plant host colonization. The plants profit from this interaction with 2° cells since the development of lateral roots (LR) and root hairs (RH) is increased and they are protected from phytopathogenic fungi.

9.3 *P. luminescens* 1° and 2° cells - different phenotypes despite the same genome

The occurrence of two distinct phenotypes in a genetical homogenous population is referred to as phenotypic heterogeneity (Avery, 2006; Davidson and Surette, 2008; Grote et al., 2015), resulting in a phase variation correlated with altered gene expression (Elowitz et al., 2002; van der Woude, 2011; Davis and Isberg, 2016), a phenomenon also observed for entomopathogenic *P. luminescens.* In its dualistic lifecycle up to 50% of the 1° cells switch into the 2° cell variant lacking several 1°-specific phenotypes such as symbiosis with nematodes, biofilm formation, pigmentation, bioluminescence, and the production of secondary metabolites (Akhurst, 1980; Forst et al., 1997; Joyce and Clarke, 2003; Eckstein and Heermann, 2019). So far, larger DNA rearrangements or modifications, genetic instability, or the loss of plasmids could be excluded in the switching process to *P. luminescens* 2° cells, thus suggesting that the differences between 1° and 2° cells could be caused by phenotypic and not genetic heterogeneity, but no evident data was provided, yet (Akhurst et al., 1992; Forst et al., 1997; Hu and Webster, 1998; Forst and Clarke, 2002). To confirm this hypothesis, the genomes of *P. luminescens* subs. *laumondii* strain DJC 1° cells was compared with those of 2° cells (Zamora-Lagos et al., 2018) [later reclassified as *P. laumondii*, (Machado et al., 2018)]. HTS genome comparison of both laboratory strains as well as switched 2° cells (resulted from prolonged cultivation of 1° in the laboratory), showed no evident point mutations or DNA rearrangements in the genome, moreover, all indicated SNPs were not equally distributed and were inconsistent. Inconsistent SNPs were also observed in 1° cells after prolonged cultivation (Dominelli et al., 2022b, **Chapter 6**), which is a common phenomenon occurring in nature: bacteria replicating over a long time undergo several stress conditions, such as nutrient limitation leading to the occurrence of spontaneous mutations with a rate ranging

between $1*10^{-6}$ and $1*10^{-8}$ base substitutions per nucleotide per generation and is supposed to create a balance between effects of deleterious mutation rate and metabolic costs (Drake et al., 1998; Denamur and Matic, 2006; Westra et al., 2017). As in all tested replicates point mutations were unequally distributed and not located in the same genetic region, it can be concluded that *P. luminescens* 1° and 2° cells are genetically identical and the different phenotypic traits result from true phenotypic and not genotypic heterogeneity (Dominelli et al., 2022b, **Chapter 6, Table 1**). For that reason, the different traits displayed by 1° and 2° cells are a result of a complex regulation on transcriptional or posttranscriptional level as it could partially be addressed by performing comparative transcriptome analysis of 1° and 2° cells, revealing different expression patterns in 672 genes. Indeed, the analysis could confirm that all the genes involved in 1°-specific traits such as *luxCDABE* for bioluminescence, *antABCDEFGHI* for pigmentation, *cipA/cipB* for crystal inclusion proteins, *pcfABCDEF* for cell clumping, *stlA* for antibiotics are downregulated in 2° cells thus providing evidence that the difference between 1° and 2° cells is due to regulation at transcriptional level (Eckstein et al., 2019, **Chapter 2, Table 1 & Fig. 2**).

Furthermore, in different organisms non-genetic phenotypic heterogeneity in response to quorum sensing (QS), whereupon two distinct sub-population evolved, was observed (Anetzberger et al., 2009; Pradhan and Chatterjee, 2014; Cárcamo-Oyarce et al., 2015; Bauer et al., 2017). The transcriptome analysis revealed that 13 out of the 40 LuxR solos in *P. luminescens* were differently expressed, indicating a possible QS dependent regulation of phenotypic heterogeneity in *P. luminescens*. Genes coding for PluR and PpyS (pyrone synthase) are downregulated in 2° indicating that the PluR/PpyS QS is used for cell-cell-communication only in 1° cells (Eckstein et al., 2019, Chapter 2). This observation is in accordance with the regulatory role of PluR/PpyS on the expression of *pcf* operon, encoding the cell clumping factor PCF, which is a 1°-specific trait (Brachmann et al., 2013). However, 12 further genes coding for LuxR solos were upregulated in 2° cells. These are i) one operon consisting of 8 genes, *PluDJC_10415-PluDJC_10460*, and two single genes, *PluDJC_04850* and *PluDJC_18380*, all coding for PAS4-LuxR solos, which putatively are involved in IKS (Brameyer et al., 2014; Eckstein et al., 2019, **Chapter 2, Table S1**) and ii) two more LuxR solos, *PluDJC_09555* and *PluDJC_21150* with yet undefined SBDs (Brameyer et al., 2014). For the unidentified SBD in PluDJC_21150 via SMART (http://smart.embl-heidelberg.de) a GAF domain in the HTH-DNA binding site could be identified

(Dominelli and Heermann, unpublished), which is an ubiquitous signal motif acting as a cGMP receptor and it is involved in perception of stimuli and signal transduction (Ho et al., 2000). This suggests a QS-dependent putative role of cyclic nucleotides in phenotypic heterogeneity regulation in *P. luminescens*. In conclusion, it could be confirmed that 1° and 2° cells are genetically identical, and the occurrence of phenotypic heterogeneity could be caused by differential modulation of the transcriptome; and we pointed out that both cell variants use distinct QS mechanisms for a cell-cell-communication, however, whether they are involved in regulation of phenotypic heterogeneity, must be clarified.

9.4 Outlook

In this work novel insights into the fate of *P. luminescens* 2° cells in the soil and the rhizosphere as well as a novel bio applicability in agriculture could be identified. It opened several questions regarding biotechnological as well as infection biological aspects. It is of great importance to deeper understand i) the ecological meaning behind the strategy of a pathogenic bacterium to remain in soil after killing its host, and ii) the biotechnological applicability of 2° cells in the rhizosphere in agriculture regulatory mechanism behind communication between *P. luminescens* and plants.

A major open question is the ecological meaning behind the alternative lifestyle *P. luminescens* undergoes after killing the insect host and switching to 2° cells. Regarding infection biological aspects, it is of great importance to understand whether this phenotypic switching is an alternative strategy pathogenic bacteria undergo to persist or to find another host. This can be of great relevance to study survival strategies of human, animal, or plant pathogenic bacteria after leaving their hosts. Therefore, *P. luminescens* might serve as good model organism to study such alternative lifestyles enteric pathogens undergo. However, for deeper understanding this strategy, the reason why 1° cells switch to 2° and undergo an alternative lifestyle must still be examined, and the switching responsible signal molecule needs to be determined.

To deeper elucidate the biotechnological aspects the range and limits of applicability of 2° cells as biocontrol agent must be determined. Therefore, further work should be investigated to test which phytopathogenic fungi are affected by *P. luminescens* 2°, and whether 2° cells antifungal activity is only restricted on chitinous fungi. Moreover, the range of plant hosts colonized by 2° cells should be determined. Another biotechnological relevant approach would be to elucidate whether 2° cells produce

volatile organic compounds (VOCs) which have a positive effect on plant root formation. For that, different approaches according to Rering et al., 2020 could be followed using methods like solid-phase microextraction (SPME) or solid-phase extraction (SPE) for volatile collection, which can then be either applied on plants or further analyzed via gas-chromatography-mass spectrometry for detection.

However, another open point is the IKS of 2° cells with plants that must be uncovered. Which is the plant derived signaling molecule that is finally sensed by SdiA? For that, the chemical nature of the PRE-fractions that bound to SdiA should be determined. There plant molecules can be analyzed via mass spectrometry to gain insights into the molecular weight and further the respective HPLC-UV-spectra can be compared to known QS-signaling molecules with similar masses. Applying these HPLC-fractions in bio-reporter assays using *Chromobacterium violaceum* CV026 strain (McClean et al 1997, Cha et al 1998), or *Agrobacterium tumefaciens* A136 (pCF218) (pMV26) (Sokol et al., 2003; Chambers et al., 2005) could detect presence of AHLs. Moreover, effect of further potential signals, such as indole or glycerol, on SdiA protein stability should be analyzed.

Additionally, proteome analysis of *sdiA* deletion mutants would give an overall insight into the regulatory role of SdiA, giving indications how genes and which proteins in respect to e.g., biofilm and motility are modulated. Subsequently, fluorescent based reporter assays using the respective promoters harboring a *lux*-box like motif of e.g., flagellin coding *fliC* or biofilm regulator *bssS*, but also the promoters of *aidA* and *sdiA,* which already showed binding affinity towards SdiA, should also be taken in consideration. In these *in vivo* studies the reporters would also help to further investigate the HPLC fractions from PRE, but also the effect of AHLs and further compounds like indole.

Moreover, the proteome analysis would also give insights into the effect of SdiA on AidA. Nevertheless, to understand the role of AidA in plant host colonization, respective deletion mutants in *P. luminescens* 1° and 2° should be generated and plant colonization assays can be performed. Additionally, the *lux*-box like motifs of the tested promoters that are bound by SdiA, should be comparatively analyzed for conserved nucleotides sequence to define the specific DNA-recognition motif for SdiA. Thus, these nucleotides can be deleted or substituted, and further surface plasmon resonance spectrometry (SPR), or fluorescent-based reporter assays should be performed.

9.5 References of discussion

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Curriculum vitae

Personal Data

Name	Nazzareno Dominelli
Date of Birth	17.04.1994
Place of Birth	Munich, Germany
Nationality	Italian

Education

Since 02.2019	Doctoral Student in the group of Prof. Dr. Ralf Heermann in the institute of molecular physiology, microbiology and biotechnology, *Johannes Gutenberg-Universität, Mainz* (Translocation of the laboratory from Munich to Mainz)
09.2018-02.2019	Doctoral Student in the group of Prof. Dr. Ralf Heermann in the microbiology department, biology *Ludwigs-Maximilians-Universität München*
10.2016-06.2018	Master of Science, Biology *Ludwigs-Maximilians-Universität München* Masters' thesis: "Left outside alone – Adaptation of *Photorhabdus luminescens* 2° cells to the rhizosphere environment." Department of microbiology in the group of PD Dr. Ralf Heermann. (Grade 1.0)
10.2014-09.2016	Bachelor of Science, Biology *Ludwigs-Maximilians-Universität München* Bachelors' thesis: "Die Rolle von Phytochrom A in diversen Ökotypen von *Arabidopsis thaliana*." Department of molecular plant science in the Leister group (PD. Dr. Cordelia Bolle; Grade 1.3)
10.2012-09.2014	Lehramtstudium (teacher training), Biology & Chemistry *Ludwigs-Maximilians-Universität München* (4 semesters; switch to B.Sc. Biology)

Teaching experiences at the Ludwigs-Maximilians-Universität München

06.2017-02.2019	Trainer for the electure "biomolecular interactions"
04.2017	Tutor for the basic course animal physiology

02.2017-03.2017	Tutor for the basic course microbiology I
02.2016-03.2016	Tutor for the basic course microbiology I
04.2016-05.2016	Tutor for the basic course animal physiology
04.2015-05.2015	Tutor for the basic course animal physiology
03.2015	Tutor for the basic course microbiology I

Teaching experiences at the Johannes-Gutenberg-Universität Mainz

Since 2022	Practical course and seminar molecular bioinformatics (Fernstudium)
Since 2021	Lecture series molecular microbiology (bachelor biology and molecular biology)
Since 2021	Practical basic course microbiology (bachelor biology, biotechnology, and molecular biology)
Since 2020	Soft skills seminar (bachelor biology and molecular biology)
Since 2019	Lecture, seminar, and practical course biomolecular interactions (master biology and biotechnology)

Awards and promotions

01.03.-31.05.2021	Research grants for doctoral students for a stay abroad (DAAD) - guest researchers at the International Center for Genetic Engineering and Biotechnology (ICGEB) in Triest (IT) with Dr. Vittorio Venturi
Since 04.2020	Junior member of the Gutenberg Academy at the Johannes-Gutenberg-Universität Mainz

Conferences

18.03-19.03.2021	Annual Conference of the Association for General and Applied Microbiology - VAAM (online): „Best of VAAM"; Talk: Learning languages – first insights into *Photorhabdus luminescens* – plant interkingdom signaling via the LuxR solo SdiA
01.09.-06.09.2019	EMBO Workshop – Bacterial Network (BacNet19) in Sant Feliu de Guíxols, Girona (ES); Poster*:* First insights in the effect of plant root exudates on insect pathogenic *Photorhabdus luminescens* 2° cells and a putative adaptation to the rhizosphere environment
27.03.-29.03.2019	SPP1617 International Conference II "Phenotypic heterogeneity and sociobiology of bacterial population" in

	Hohenkammer, Bayern (DE); Poster*:* Phenotypic heterogeneity in insect pathogenic *Photorhabdus luminescens* – specific sensing of external signals gives first insights for a fate of 2° cells in the soil
17.03.-20.03.2019	Annual Conference of the Association for General and Applied Microbiology - VAAM in Mainz, Rheinland-Pfalz (DE); Poster*:* Strategy of insect pathogenic *Photorhabdus luminescens* 2° cells for adaptation to the rhizosphere environment
23.09.-25.09.2018	"How microorganisms view their world" in Marburg, Hessen, (DE); Poster: Left outside alone – Adaptation of insect pathogenic *Photorhabdus luminescens* 2° cells to the rhizosphere environment
17.09-19.09.2018	VAAM Summerschool „Mechansims of Generegulation" in Tutzing, Bayern (DE); Poster: Left outside alone – The fate of insect pathogenic *Photorhabdus luminescens* 2° cells in the rhizosphere environment

Work experience

09.2015-06.2018	*Ludwigs-Maximilians-Universität München* Assistant for teaching evaluations of the biology faculty (Dr. Frank Landgraf); Creating and evaluating evaluation form for the biology department
07.2016-08.2016	*Ludwigs-Maximilians-Universität München* Assistant in the laboratory for molecular plant science (PD Dr. Cordelia Bolle) Heterologous expression of plant genes in *E. coli* and purification of proteins for antibody production
01.2013-06.2018	Hagebaumarkt & Gartencenter München, Eching (DE)
12.2011-12.2012	Ihle GmbH & Co. KG Landbäckerei München
05.2010-10.2011	Obi Heimwerkmarkt München, Eching (DE)