# Regulation of phenotypic switching and heterogeneity in *Photorhabdus luminescens* cell populations

**Dissertation**

zur Erlangung des Doktorgrades

der Naturwissenschaften

(Dr. rer. nat.)

der Fakultät für Biologie

der Ludwig-Maximilians-Universität München


vorgelegt von

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| Datum der Abgabe: | 17.11.2016 |
| Datum der mündlichen Prüfung: | 12.12.2016 |

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Munich, 17.11.2016 Angela Langer

# Contents

# Nomenclature

Gene products are numbered in a way that the first methionine of the wild-type protein is designated “1” in the amino acid sequence (if present: independently of the N-terminal affinity tag).

N-terminal and C-terminal affinity tags are marked in genes and proteins corresponding to their position (e.g. 6His-HexA or HexA-6His).

Deletions of genes are marked by “Δ”. Unless otherwise noted, nucleotide positions indicate the distance from the transcriptional start site (+1).

The two phenotypic forms of *Photorhabdus luminescens* TT01 are called primary and secondary cells and can also be presented as TT01-1° and TT01-2° or 1° and 2°.

# Abbreviations

| | |
|---|---|
| aa | amino acid |
| AHL | acyl homoserine lactone |
| AQ | anthraquinone |
| ATP | adenosine-5’-triphosphate |
| AU | arbitrary unit |
| bp | base pairs |
| cfu | colony forming units |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| DTT | 1,4-Dithiothreitol |
| IJ | infective juvenile |
| JHEH | juvenile hormone epoxide hydrolase |
| EMSA | electrophoretic mobility shift assay |
| EYFP | enhanced yellow fluorescent protein |
| n-His tag | affinity tag composed of n histidine residues |
| HexA | hyperproduction of exoenzymes |
| HTH | helix-turn-helix |
| IJ | Infective Juvenile |
| LB | lysogeny broth |
| LrhA | LysR homolog A |
| mRNA | messenger RNA |
| OHHL | N-3-oxo-hexanoyl homoserine lactone |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| QS | quorum sensing |
| Pcf | *Photorhabdus* clumping factor |
| PPYs | photopyrones |
| PpyS | Photopyrone synthase |
| RNA | ribonucleic acid |

| | |
|---|---|
| RNase | ribonuclease |
| SPR | Surface Plasmon Resonance |
| ST | stilbene |
| sRNA | small RNA |
| TCA | tricarboxylic acid |
| wHTH | winged helix-turn-helix |

# Publications and Manuscripts presented in this thesis

## Chapter 2:

Glaeser, A. [1] and Heermann, R. [1] (2015). A novel tool for stable genomic reporter gene integration to analyze heterogeneity in *Photorhabdus luminescens* at the single-cell level. *BioTechniques* 59 (2):74-81.

## Chapter 3:

Heinrich, A.K.*[2], Glaeser, A.* [1], Tobias, N.J. [2], Heermann, R.[# 1], Bode, H.B[#2] (2016). Heterogeneous regulation of bacterial natural product biosynthesis via a novel transcription factor. *Heliyon* 2(11): e00197. eCollection 2016.

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## Chapter 4:

Glaeser, A. [1], Moldovana A. [1], Harmath, C. [1], Joyce S.A. [3], Clarke D.J.[3]. Heermann, R. [1] (2016). HexA is a versatile regulator involved in the control of phenotypic heterogeneity of *Photorhabdus luminescens*. *PLoS ONE*: submitted.

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# Contributions to publications and manuscripts presented in this thesis

## Chapter 2:

Angela Glaeser and Ralf Heermann designed the experiments. Angela Glaeser carried out all the experiments. Ralf Heermann coordinated the experiments. Angela Glaeser and Ralf Heermann wrote the manuscript.

## Chapter 3:

Angela Glaeser, Antje K. Heinrich, Nick J. Tobias, Ralf Heermann and Helge B. Bode designed the experiments. Antje K. Heinrich constructed the deletion of *antJ*, performed the DNA protein pull down assay and the EMSA and analyzed the production of anthraquinones and other secondary metabolites via HPLC-UV/ MS analysis. Antje K. Heinrich performed the RNA sequencing and Antje K. Heinrich and Nick J. Tobias analyzed the data. Antje K. Heinrich and Nick J. Tobias performed the phylogenetic analysis. Angela Glaeser created the integration reporter strains, performed the promoter activity analysis in *P. luminescens* and *E. coli*, fluorescence microscopy and qRT-PCR. Ralf Heermann performed Surface Plasmon Resonance spectroscopy. Angela Glaeser, Antje K. Heinrich, Nick J. Tobias, Ralf Heermann and Helge B. Bode analyzed the data. Angela Glaeser, Antje K. Heinrich, Ralf Heermann and Helge B. Bode wrote the paper.

## Chapter 4:

Angela Glaeser performed the generation of the reporter strains, reporter gene analyses, bioluminescence measurements, fluorescence microscopy, overproduction and purification of HexA, and fluorescence-based thermal stability assays. Adriana Moldovan performed the promoter activity assay of $P_{pcfA}$ in *Sh. oneidensis* and analysed the promoter activity with the truncated promoter versions in *E. coli*. David J. Clarke and Susan A. Joyce provided the strains *P. luminescens* TT01-2° and TT01-1°Δ*hexA*. Cristian Harmath performed 2D-PAGE and Ralf Heermann performed the Surface Plasmon Resonance spectroscopy. Angela Glaeser and Ralf Heermann designed the experiments, analyzed the data and wrote the paper.

We hereby confirm the above statements:

…… ……

Angela Langer geb. Glaeser PD Dr. Ralf Heermann

……

Antje K. Heinrich

## Summary

*Photorhabdus luminescens* is a bioluminescent, Gram-negative bacterium with a highly complex life cycle, which involves a mutualistic interaction with nematodes and pathogenicity towards insects. *P. luminescens* exists in two phenotypically different forms, the primary (1°) and the secondary (2°) cells. After prolonged cultivation individual 1° cells convert to 2° cells, which lack numerous 1°-specific phenotypes such as pigmentation, bioluminescence and the capability to support growth and development of the nematodes. The regulator HexA was found to be involved in the control of the phenotypic switching process as a deletion of *hexA* in 2° cells of *P. temperata* partially restored 1°-specific features. However, the molecular mechanism how phenotypic switching is regulated in *P. luminescens* remained unclear.

Phenotypic heterogeneity describes the observation that individual cells can differ from each other with respect to gene expression within a genetically homogeneous population and under similar environmental conditions. In this thesis, the regulation of phenotypic heterogeneity in *P. luminescens* was investigated. As first step, a genetic tool was established to integrate DNA fragments into the chromosome of *Photorhabdus*, which allowed monitoring of gene expression in *P. luminescens* at the single-cell level. One predominant specific phenotype of 1° cells is pigmentation, which is mediated by the production of anthraquinones (AQs). Investigation of the regulation of AQ production at the single-cell level demonstrated a heterogeneous activation of the corresponding *antABCDEFHI* operon in *P. luminescens* 1° cells. Thereby, a novel type of transcriptional regulator, AntJ, proved to heterogeneously activate AQ biosynthesis in 1° cells. The native levels of AntJ within the cell are important for the heterogeneity since a simple gene duplication of *antJ* led to a more homogeneous activation of the *ant* operon. In contrast, the activity of the AQ promoter was found to be basally but homogeneously active in 2° cells, although AntJ levels in 1° and 2° cells were comparable. Thus, the importance of a potential ligand binding to AntJ controlling activity of the transcriptional activator and therefore heterogeneity is discussed.

Another predominant phenotype that is characteristic for 1° cells is bioluminescence due to production of bacterial luciferase. Investigation of the regulation of the corresponding *luxCDABE* operon identified HexA as a regulator of bioluminescence in *P. luminescens*. Compared to 1° cells, enhanced transcriptional and translation levels of HexA were detected

in 2° cells. Moreover, bioluminescence was observed to be indirectly regulated by HexA. Thereby, most likely sRNAs are involved in this regulation process, as enhanced transcription of *hfq*, encoding the RNA chaperone Hfq, was observed in 2° cells. In contrast, expression of the *pcfABCDEF* operon that mediates cell clumping was found to be directly regulated by HexA and identified as another 1°-specific feature. Thus, HexA could be revealed as a DNA-binding protein with the *pcf* operon being described as its first direct target. This identified HexA as versatile regulator that both directly and indirectly controls expression of various 1°- and 2°-specific genes in *P. luminescens*.

Finally, the socio-biological aspect of the two phenotypic cell types was investigated. Competition assays revealed a growth advantage of 1° cells in the exponential growth phase, whereas 2° cells overgrew the 1° cells in the stationary phase, regardless of the initial ratio of the two cell types. This gives first insights that phenotypic switching of *P. luminescens* is driven by nutrient limitation as well as general stress, and might therefore ensure survival of the bacterial population.

# Zusammenfassung

*Photorhabdus luminescens* ist ein biolumineszentes, Gram-negatives Bakterium, welches sich durch einen komplexen Lebenszyklus auszeichnet. Dieser besteht sowohl aus einer symbiotischen Phase mit Nematoden, als auch aus einem insektenpathogenen Stadium.
Darüber hinaus kommt *P. luminescens* in zwei phänotypisch unterschiedlichen Zellformen vor, den Primär- und den Sekundärzellen, wobei einzelne Sekundärzellen nach Langzeitkultivierung von Primärzellen entstehen. Diesen Sekundärzellen fehlen zahlreiche primär-spezifische phänotypische Merkmale, wie beispielsweise Pigmentierung, Biolumineszenz sowie die Fähigkeit, das Wachstum und die Entwicklung der Nematoden zu unterstützen.
In früheren Studien konnte gezeigt werden, dass eine Deletion des Gens *hexA* in Sekundärzellen von *P. temperata* zur Ausprägung einiger primär-spezifischer Merkmalen führt. Dadurch konnte eine Beteiligung des Proteins HexA an der Regulation des phänotypischen Phasenwechsels gezeigt werden. Der regulatorische Mechanismus, der hinter dem phänotypischen Phasenwechsel in *P. luminescens* steckt, blieb jedoch unklar.

Unterscheiden sich einzelne, genetisch identische Zellen hinsichtlich ihrer Genexpression unter ähnlichen Umweltbedingungen, so wird dies als phänotypische Heterogenität bezeichnet. Um die Untersuchung phänotypischer Heterogenität in *P. luminescens* zu ermöglichen, wurde zunächst ein genetisches Werkzeug zur Integration von DNA-Fragmenten in das Chromosom von *P. luminescens* etabliert. Dadurch wird die Expressionsanalyse ausgewählter Gene in *P. luminescens* mit Hilfe eines Reportergens auf Einzelzellebene ermöglicht.
Die durch Anthraquinon (AQ)-Produktion bedingte Pigmentierung einer *P. luminescens* Kultur stellt einen vorherrschenden Phänotyp dar, der hinsichtlich phänotypischer Heterogenität auf Einzelzellebene untersucht wurde. Dabei konnte eine heterogene Aktivierung des entsprechenden *antABCDEFGHI* Operons in Primärzellen von *P. luminescens* aufgedeckt werden. Des Weiteren wurde ein neuartiger Regulator namens AntJ identifiziert, der für die heterogene Aktivierung der AQ-Biosynthese in Primärzellen verantwortlich ist. Die natürlicherweise vorliegende Konzentration von AntJ in der Zelle spielt dabei eine wichtige Rolle, da bereits eine Verdopplung der Kopienzahl in einer homogeneren Aktivierung des *ant*-Operons resultierte. Es konnte zudem gezeigt werden, dass in Sekundärzellen eine basale aber homogene Aktivierung des AQ-Promotors vorliegt, wobei

die Konzentrationen von AntJ in Primär- und Sekundärzellen vergleichbar sind. Somit wird die Bedeutung eines potenziellen Liganden von AntJ diskutiert.
Ein weiteres charakteristisches Merkmal für Primärzellen ist ihre Biolumineszenz, die durch bakterielle Luciferase hervorgerufen wird. Anhand von Untersuchungen des entsprechenden Operons *luxCDABE* konnte dabei das Protein HexA als Regulator in *P. luminescens* identifiziert werden. Verglichen mit Primärzellen wurde eine erhöhte Transkription und Translation von *hexA* in Sekundärzellen nachgewiesen. Darüber hinaus wurde beobachtet, dass die Biolumineszenz indirekt durch HexA reguliert wird. Da die Transkription des RNA-Chaperon kodierenden Gens *hfq* in Sekundärzellen erhöht war, gilt die Beteiligung kleiner RNAs als wahrscheinlich.
Im Gegensatz dazu konnte das *pcfABCDEF* Operon als erstes direkt reguliertes Ziel von HexA identifiziert und HexA als ein DNA-bindendes Protein nachgewiesen werden. Dieses *pcf* Operon ist für das Phänomen der Zellverklumpung verantwortlich, welches als weiteres primär-spezifisches Merkmal charakterisiert werden konnte.
Somit konnte HexA als vielseitiger Regulator beschrieben werden, der sowohl direkt als auch indirekt die Expression verschiedener primär- und sekundärspezifischer Gene kontrolliert.

Schließlich wurden die sozio-biologischen Aspekte der zwei Zellformen untersucht. Kompetitive Wachstumsversuche deckten einen Wachstumsvorteil von Primärzellen in der exponentiellen Phase auf. In der stationären Phase überwuchsen allerdings die Sekundärzellen, unabhängig von dem ursprünglich eingesetzten Verhältnis der zwei Zellformen, stets die Primärzellen. Diese Erkenntnisse deuten darauf hin, dass phänotypischer Phasenwechsel von *P. luminescens* durch Nährstoffmangel sowie Stress im Allgemeinen bedingt wird und daher für das Überleben der bakteriellen Population von Bedeutung sein könnte.

# 1 Introduction

## 1.1 The genus *Photorhabdus*

The genera *Photorhabdus* and *Xenorhabdus* comprise Gram-negative bacteria that belong to the family of Enterobacteriaceae and live in symbiotic mutualism with entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema*, respectively. In 1979, a newly isolated bacterium that was associated with *Heterorhabditis* nematodes was first named *Xenorhabdus luminescens*, because of its ability to glow. In 1993, due to big differences in respect to DNA relatedness and phenotypic characteristics, the new genus *Photorhabdus* was proposed and the bacterium was renamed as *Photorhabudus luminescens* (Thomas & Poinar, 1979; Boemare *et al.*, 1993).

The genus *Photorhabdus* was subdivided into three different species. Besides *P. luminescens* the two other nowadays known *Photorhabdus* species are *P. temperata* and *P. asymbiotica* (Fischer-Le Saux *et al.*, 1999). *P. temperata* and *P. luminescens* share the same complex life cycle with nematodes of the Heterorhabditidiae family as symbiosis partners and insect larvae e.g. *Galleria mellonella* or *Manduca sexta* as pathogenic targets (Akhurst, 1980). *P. asymbiotica* is able to associate with nematodes as well but besides being pathogenic against insects it additionally is known to be responsible for local infections of human soft tissue (Gerrard *et al.*, 2006, Gerrard *et al.*, 2004).

### 1.1.1 Life cycle of *Photorhabdus luminescens*

*Photorhabdus* species colonize the gut of the infective juvenile (IJ) stage of soil-dwelling *Heterorhabditis* nematodes (Figure 1-1). The IJs represent a non-feeding stage of the nematodes that actively seek out for insect prey. Upon the appearance of an insect larva, they infect it by entering through mouth, anus or spiracles or slicing the cuticle via a dorsal tooth-like appendage (Kaya & Gaugler, 1993; Bedding & Molyneux, 1982). Once inside the larva, the IJs enter the hemolymph where they regurgitate the *Photorhabdus* bacteria from their gut (Ciche & Ensign, 2003). The bacteria start to proliferate exponentially and reach cell densities of up to $10^9$ cfu/ insect within 48 hours (Watson *et al.*, 2005). The bacteria use several strategies to overcome the immune response of the insect. Via the small molecule rhabduscin, *Photorhabdus luminescens* effectively inhibits the enzyme phenol oxidase which plays a central role in invertebrate immunity and it also contains a type three secretion system, which

together with the effector protein LopT prevents the phagocytic uptake by insect macrophage cells, called hemocytes (Crawford *et al.*, 2012; Brugirard-Ricaud *et al.*, 2005).

In order to kill the insect, the bacteria secrete a wide variety of virulence factors, like the metalloprotease PrtA or the Tc (Toxin complex) and Mcf (Makes caterpillar floppy) toxins (Daborn *et al.*, 2001; Daborn *et al.*, 2002). Tc toxins consist of high molecular weight proteins that confer oral toxicity (Waterfield *et al.*, 2001). The Mcf toxin causes apoptosis in the midgut epithelium and hemocytes and leads to a rapid loss of body turgor of the larvae (Daborn *et al.*, 2002).

Additionally, *Photorhabdus* produces several antibiotics that keep other bacteria from invading the carcass (Akhurst, 1982). The produced hydroxystilbene derivative 3,5-dihydroxy-4-isopropylstilbene confers antimicrobial activity against Gram-positive bacteria, whereas the produced carbapenem, a β-lactam antibiotic, is mainly effective against Gram-negative bacteria (Derzelle *et al.*, 2002). Secretion of a wide range of extracellular hydrolytic enzymes of *Photorhabdus* causes a conversion of all the internal organs and tissues into biomass, which serves as food source for bacteria and nematodes (ffrench-Constant *et al.*, 2003).

Once the IJs have entered the hemolymph, they recover and develop into self-fertile hermaphrodites. This step is predominantly achieved via signals in the insect hemolymph. Subsequently, the adult hermaphrodite lays 200 to 300 eggs, which undergo four larval stages, called L1-L4, and then develop into female or male adult nematodes. The presence of *Photorhabdus* is an obligate requirement for this process, as the bacteria provide nutrients and/or signals, which are essential for the development and reproduction of the nematodes (Han & Ehlers, 2000). ExbD, a component of the ExbB-ExbD-TonB complex plays a major role in the uptake of small molecules like siderophores. The deletion of *exbD* results in a mutualism-deficient phenotype, which could be rescued via the addition of iron. Thus, the resulting lower levels of iron within the mutant bacteria seem to be responsible for the incapability of symbiosis (Watson *et al.*, 2005). Furthermore, the gene *ngrA*, encoding a phosphopantetheinyl transferase, is responsible for siderophore and antibiotic production and essential for symbiosis (Ciche *et al.*, 2001).

After two to three generations of nematode development, when all nutrients are depleted, IJs are formed, their guts get colonized by *Photorhabdus* and the nematode-bacteria complexes evade the cadaver (ffrench-Constant *et al.*, 2003). Bennett & Clarke (2005) identified the *pgbPE* operon to be required for pathogenicity as well as for the colonization of

*Photorhabdus* in the gut of the IJs. This was the first example for a genetic overlap of symbiosis and pathogenicity.

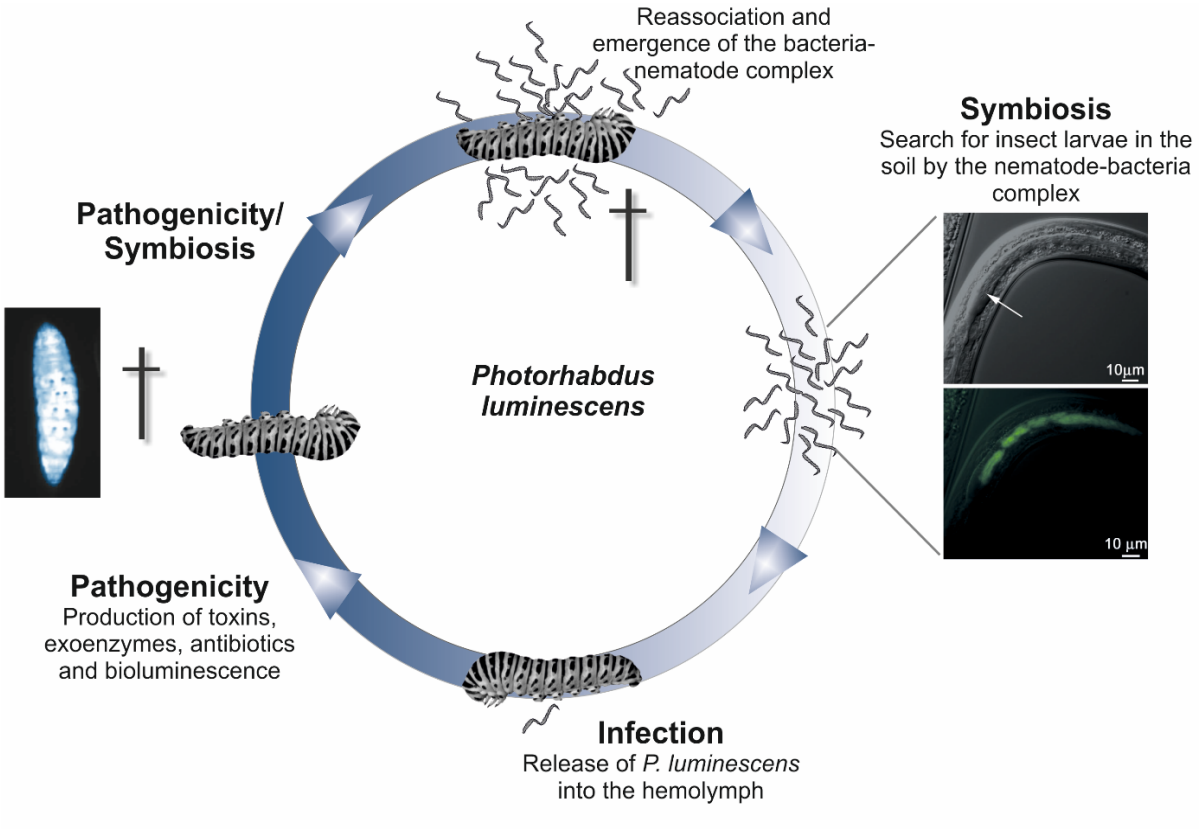


**Figure 1-1: Lifecycle of *Photorhabdus luminescens.*** At the beginning of the life cycle, the bacteria are associated within the gut of heterorhabditid nematodes, which search for insect larvae in the soil. Once the nematodes invade an insect larva, the bacteria are regurgitated into the hemolymph of the insect, rapidly reproduce and produce toxins, exoenzmyes, antibiotics and bioluminescence. After the death of the larva, the cadaver serves as a nutrient source for the nematodes and bacteria. Upon limitation of the nutrients, the nematodes and bacteria reassociate and emerge from the insect carcass (Waterfield *et al.*, 2009). Picture in the right panel: GFP-labelled *P. luminescens* cells in the intestine of *H. bacteriophora* (Ciche & Ensign, 2003). Picture in the left panel: infected, bioluminescent insect larva (Jannis Brehm, LMU).

### 1.1.2 Quorum sensing in *Photorhabdus* species

In order to adapt to changes in their environment, bacteria need to sense their environmental conditions. Furthermore, a coordination of their behavior as a bacterial group is achieved via communication. If the decision of a behavior is made due to the correlation of the population density via the use of small signaling molecules it is referred to as quorum sensing (QS) (Waters & Bassler, 2005). QS circuits are often involved in the regulation of bioluminescence, biofilm formation, motility, antibiotic biosynthesis, sporulation and the production of virulence factors (Eberl, 1999; Bassler & Losick, 2006). The first known example for QS is the Gram-negative bacterium *Vibrio fischeri*, a marine bacterium living in

squid, whose ability to produce bioluminescence is QS dependent (Nealson & Hastings, 1979). In *V. fischeri* the signaling molecule, also known as autoinducer (AI), is an N-acyl homoserine lactone (AHL), which is synthesized via a LuxI-type synthase and sensed by the cytoplasmic receptor LuxR. As soon as the concentration of the AHLs exceeds a certain threshold, they bind to LuxR proteins and the LuxR-AHL complexes activate transcription of the *luxICDABE* operon resulting in the production of light (Engebrecht *et al.*, 1983; Engebrecht & Silverman, 1984; Waters & Bassler, 2005).

According to their chemical properties, AIs can be quite diverse. Gram-negative bacteria often use AHLs as signaling molecules, which maintain their specificity by variation in size and composition of their acyl chains (Whitehead *et al.*, 2001). Gram-positive bacteria, like *Staphylococcus*, use small peptides for signaling, which are sensed via transmembrane receptors belonging to a two-component signal transduction module (TCS) and thereby activate an intracellular response pathway (Lyon & Novick, 2004). Additionally, a cell-cell communication system that can be found in Gram-positive as well as Gram-negative bacteria involves the signaling molecule AI-2, which is produced by the synthase LuxS and facilitates interspecies communication (Winzer *et al.*, 2002).

The genomes of all three *Photorhabdus* species do not encode any LuxI synthase and are therefore not capable of producing AHLs. Nevertheless, they possess an extraordinary high number of LuxR proteins, which are referred to as LuxR orphans or solos (Heermann & Fuchs, 2008; Subramoni & Venturi, 2009; Brameyer *et al.*, 2014). A phylogenetic analysis revealed that besides genomes that exclusively contain complete QS circuits, some bacteria, like *Photorhabdus* species, have at least one LuxR homolog but no LuxI synthase. In addition, some organisms, like *Pseudomonas aeruginosa*, harbor classical LuxR/LuxI systems and additionally several LuxR solos, which lack a cognate LuxI synthase. Those LuxR solos are assumed to be involved in cell-cell and/or inter-kingdom communication and often play a role in virulence as described for *P. aeruginosa* or *Agrobacterium tumefaciens* (Case *et al.*, 2008).

*P. luminescens* harbors three different types of LuxR solos, whereas all of them share a DNA-binding domain with a helix-turn-helix motif, the “HTH LuxR” motif, at the C-terminus. The major differences can be found in their signal binding domains at the N-terminus: Two LuxR solos, called SdiA and PluR, contain an AI binding site (AHL-domain), 35 possess a PAS4-domain at the N-terminus and three LuxR solos harbor a yet unidentified binding site (Heermann & Fuchs, 2008; Brameyer *et al.*, 2014).

When further investigating the LuxR solos with the AHL-domains, it was suggested that SdiA senses exogenously produced AHLs, as this is the case for the SdiA homolog of *Salmonella*

*enterica* (Michael *et al.*, 2001). However, it could be found that PluR, the second LuxR solo of this group, senses endogenously produced signaling molecules, the so-called photopyrones (PPYs). A ketosynthase-like protein PpyS produces these α-pyrones. When PluR senses PPYs, it directly activates the promoter of the *pcfABCDEF (Photorhabdus* clumping factor) operon, which in turn leads to cell clumping. The Pcf molecule that mediates cell clumping has not yet been identified, but an effect on virulence could be observed. This is the first example of a LuxR solo recognizing a non-AHL signal and therefore the PpyS-PluR system represents a new type of cell-cell communication (Brachmann *et al.*, 2013; Figure 1-2).

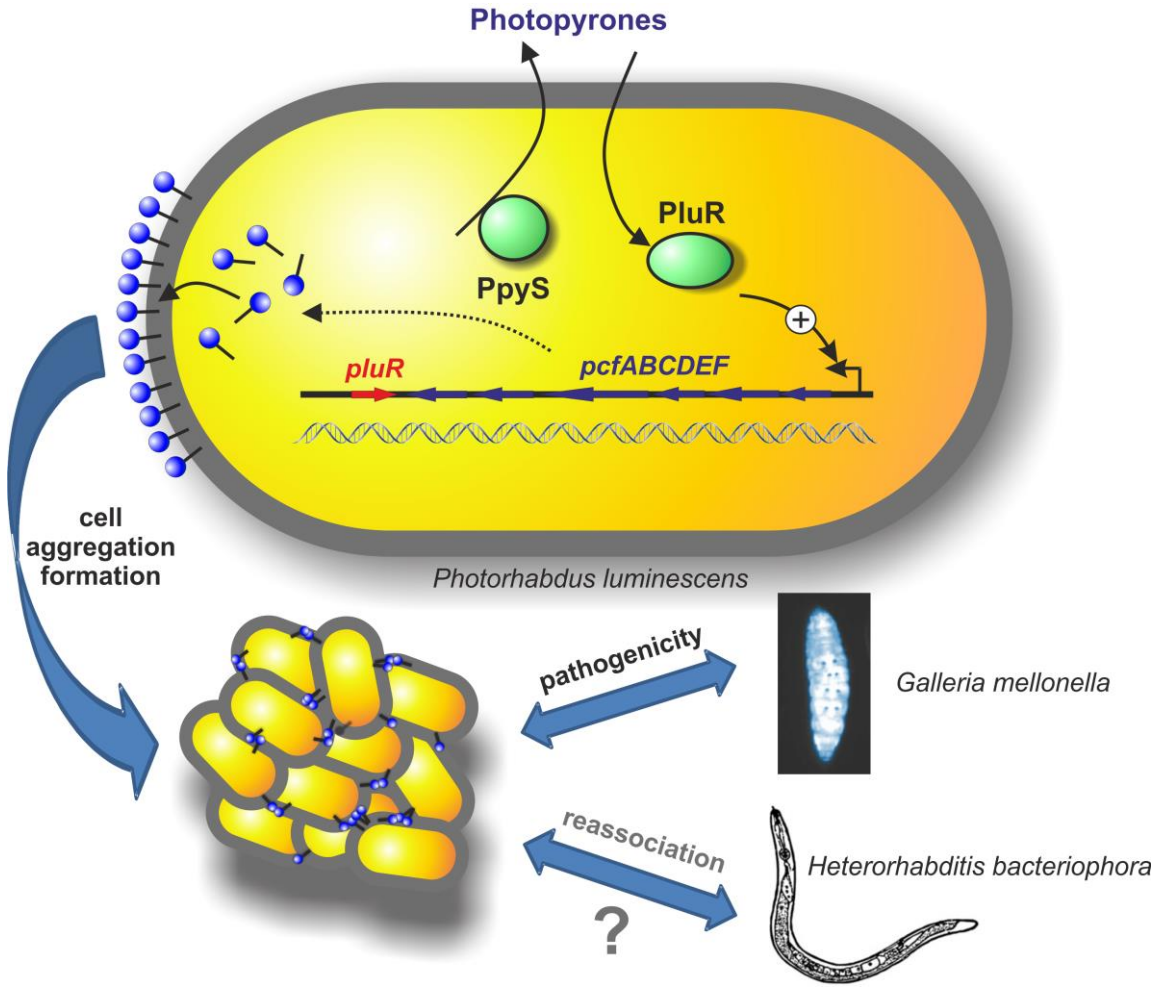


**Figure 1-2: Model of PpyS-PluR signaling in *P. luminescens*.** The pyrone synthase PpyS produces photopyrones that are sensed via the LuxR-type receptor PluR. PluR then activates transcription of the *pcfABCDEF* operon, which leads to the formation of cell clumps. The cell clumps contribute to virulence against insect larvae e.g *Galleria mellonella* and might also play a role in the reassociation with the nematodes. Figure modified after (Brachmann *et al.*, 2013).

*P. asymbiotica* contains the PluR homolog called PauR and the *pcfABCDEF* operon but no PPY synthase. Instead of sensing PPYs, different signaling molecules, called

dialkylresorcinols could be identified which are produced by the *darABC* operon and are in turn sensed by PauR (Brameyer *et al.,* 2015). However, it is not known if PluR or PauR are the only regulators of the *pcfABCDEF* operon and if cell clumping is exclusively considered to be a virulence factor or if it might also contribute to symbiosis.

### 1.1.3 Secondary metabolism in *Photorhabdus* species

*P. luminescens* is known for its ability to produce a wide range of secondary metabolites. It was revealed that 6.5 % of its 5.5. Mb large genome encodes genes, predicted to be involved in the production of secondary metabolites (Duchaud *et al.,* 2003; Bode, 2009). As already mentioned above, *P. luminescens* produces several kinds of toxins, like Tc and Mcf, and antibiotics, such as carbapenem and stilbene (Daborn *et al.,* 2001; Daborn *et al.,* 2002; Derzelle *et al.,* 2002). Stilbenes are polyketide molecules that usually occur in plants in response to stress and infection. *Photorhabdus* is the only non-plant organism known to produce stilbene and its production was found to be significantly different from that observed in plants (Williams *et al.,* 2005; Joyce *et al.,* 2008). Besides its antibiotic activity against Gram-positive bacteria and fungi, stilbene furthermore plays a role in the inhibition of the insect`s immune system component phenol oxidase and is necessary for nematode development (Eleftherianos *et al.,* 2007; Joyce *et al.,* 2008). Interestingly, genes involved in the stilbene biosynthesis are not clustered indicating a complex regulation (Bode, 2009). *Photorhabdus* is capable of producing many more natural products such as the GameXPeptides, whose biological functions remain unknown even though a role in immune suppression is suggested (Nollmann & Dauth *et al.,* 2015; Mulley *et al.,* 2015). The recently identified phurealipids are assumed to contribute to bacterial virulence by inhibiting juvenile hormone epoxide hydrolase (JHEH) of the insect and thereby preventing its growth and development (Nollmann & Heinrich *et al.,* 2015). Another mechanism of insect immune suppression is mediated via the production of glidobactin, which is reported to be a potent proteasome inhibitor (Dudnik *et al.,* 2013; Figure 1-3).

The characteristic brownish pigmentation of *P. luminescens* is a result of the so called anthraquinones (AQs) (Richardson *et al.,* 1988). The respective operon *antABCDEFGHI* could be identified to encode the enzymes of AQ biosynthesis. The genes *antD*, *antE* and *antF* encode for the minimal polyketide synthase II. Additionally, several modifying enzymes, including a ketoreductase (AntA), two cyclases (AntH and AntC), a phosphopantetheinyl transferase (AntB), a coenzyme A ligase (AntG) and a hydroxylase/ peptidase (AntI) are also encoded by the *ant* gene cluster (Brachmann *et al.,* 2007).

*Photorhabdus* is the only known Gram-negative producer of AQs, which usually occur only in plants, fungi and Streptomyces. Furthermore, until now only one additional Gram-negative bacterium, called *Stigmatella aurantiaca*, is known to possess a type II polyketide synthase, which produces aurachin antibodies (Sandmann *et al.*, 2007)).

AQs are known to display weak antimicrobial activity and they serve as deterrent against birds and scavenger insects in order to protect the food source, the dead insect cadaver (Hilker & Köpf, 1994; Gulcu *et al.*, 2012). Recently, anthraquinones from *Photorhabdus temperata* were shown to be lethal to mosquito larvae and might therefore provide a potentially useful biopesticide (Ahn *et al.*, 2013).

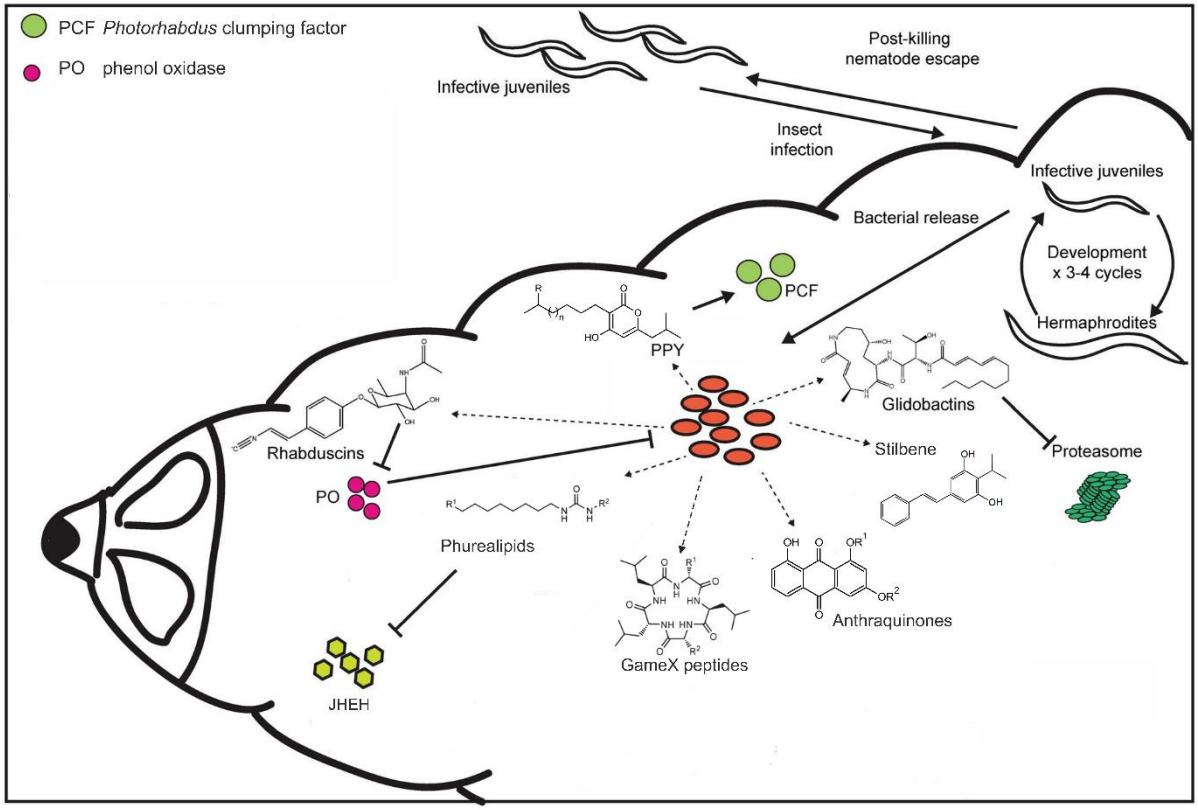


**Figure 1-3: Schematic summary of secondary metabolites produced by *Photorhabdus* and their predicted functions.** After infection, the Infective juveniles undergo several rounds of development while the insect is killed. The bacteria (labelled in red) release numerous compounds (dashed arrows) that serve as virulence factors and/or affect the insect`s immune response. JHEH: juvenile hormone epoxide hydrolase PPY: photopyrone. Modified after (Tobias *et al.*, 2016).

## 1.2 Phenotypic heterogeneity

In order to enable a diversity of different phenotypes within a bacterial population, which can adapt differentially to changing environmental conditions, a well-known strategy is the occurrence of genetic modifications such as genetic rearrangements or DNA modification, e.g. via DNA methylation (Smits *et al.*, 2006). Hence, another approach, which has mostly

remained unknown, exists among genetically identical individuals and is referred to as phenotypic heterogeneity. Thereby, within a genetically identical population and without environmental variation single cells differ from each other in terms of gene expression and therefore in different phenotypic traits (Elowitz *et al.,* 2002). For analysis of phenotypic variation within a population, single-cell technologies like fluorescence microscopy or flow cytometry have to be applied using fluorescence proteins serving as gene expression reporters (Brehm-Stecher & Johnson, 2004). In the last couple of years, the awareness of non-genetic individuality has increased and this phenomenon is often associated with persistence (Balaban *et al.,* 2004), bacterial competence (Smits *et al.,* 2005) or spore formation (Veening *et al.,* 2005).

### 1.2.1 Different types of phenotypic heterogeneity

In biological systems, random fluctuations in biochemical reactions occur and are referred to as noise, which is a key determinant of phenotypic variation. In particular, if molecular systems within a cell are based on molecules in small numbers, they are susceptible to noise (Elowitz *et al.,* 2002). The lactose utilization in *Escherichia coli* was the first example showing that only individual cells initiate the expression of the respective gene(s) among an isogenic population (Novick & Weiner, 1957). *E. coli* cells segregated into two sub-populations, if they were exposed to near-threshold concentration of the inducer. This phenomenon is called bistability, which is defined as the formation of two distinct subpopulations via the stochastic fluctuations in the cellular components that determine cellular states (Veening & Smits *et al.,* 2008).

The *lac* operon comprises the three genes *lacZ, lacY* and *lacA,* encoding a β-galactosidase, a lactose permease and a transacetylase, respectively. This operon is negatively regulated by the repressor LacI, which in turn is inhibited by allolactose. The lactose permease LacY enables the uptake of lactose, which leads to accumulation of allolactose within the cell via the β-galactosidase LacZ. The allolactose binds to the intracellular operon repressor LacI and leads to its inhibition. A positive feedback occurs as the *lacY* expression is increased and the intracellular concentration of the inducer increases (Jacob & Monod, 1961; Ozbudak *et al.,* 2004).

The bistability of the *lac* operon was observed with a so-called gratuitous inducer, which is a compound that binds to and inactivates the repressor, but is not metabolized by the induced

enzymes. Thereby, the requirement for the internal synthesis of allolactose by LacZ is bypassed and the bistability is a result from the LacY activity (Ozbudak *et al.,* 2004).

A common feature of bistability is hysteresis ((Ninfa & Mayo, 2004). Hysteresis refers to the situation in which the transition from one state to the other requires an induction or relief of induction greater than that for the reverse transition (Smits *et al.,* 2006).

The hysteretic behavior is a result of the abundance and stability of the lactose permease LacY. The concentration of inducer that is required to trigger the stimulatory loop is high, when little permease is present. However, when the level of permease is high, which usually is the case at an already induced state, the cells need little inducer to maintain high levels of *lac* expression (Elowitz *et al.,* 2002; Ozbudak *et al.,* 2002).

One explanation why phenotypic variation occurs is bet-hedging. Under challenging conditions, the existence of offspring with variable phenotypes makes it more likely that one offspring benefits under a given situation. This can also be referred to as risk-spreading strategy, as not every individual cell will be optimally suited for the future environment, but the overall fitness of the genotype increases because some individuals will be properly adapted (Cohen, 1966; Veening & Smits *et al.,* 2008). Sporulation in *B. subtilis* displays a typical example for bet-hedging as some cells sporulate and others utilize alternative metabolites to continue growth. The advantage of spores lies in the resistance to various environmental conditions, whereas the remaining vegetative cells could rapidly resume growth upon influx of new nutrients (Veening & Stewart *et al.,* 2008).

Another important purpose of phenotypic variation, which is fundamentally different from bet hedging, is the division of labor. In this case one cell type expresses a behavior that is beneficial for a second cell type in the same microenvironment, without the first one getting a direct benefit in return. Typically, certain metabolites or other products are produced that get secreted and are thus accessible for the whole population (Ackermann, 2015). One example is the synthesis of the protease subtilisin E of *Bacillus subtilis* that is produced upon nutrient-limitation. Subtilisin E is secreted and degrades proteins outside the cell. The resulting products can be consumed by all cells in the microenvironment, whether they contribute to its secretion or not. It was observed that only a minority of the cells within a population express the gene encoding subtilisin E. This suggests that only some individuals are involved in the production and secretion of a potentially costly product but provides benefits to all individuals in the community (Veening & Igoshin *et al.,* 2008).

### 1.2.2 Phenotypic heterogeneity in *P. luminescens*

*P. luminescens* exists in two phenotypic different variants, the primary (1°) and the secondary (2°) form. The 2° cells appear after prolonged cultivation of a population exclusively consisting of the 1° form. Until today the switch has been observed unidirectional in *Photorhabdus*, exclusively occurring from the 1° to the 2° form, whereas in *Xenorhabdus* an infrequent reversion has been reported (Forst & Clarke, 2002). This might result from a lacking signal for *P. luminescens* that induces switching from 2° to 1° under laboratory conditions. The characteristic features of *P. luminescens* such as pigmentation due to anthraquinone production, bioluminescence as well as the production of crystalline inclusion proteins, proteases and antibiotics are absent in the 2° form (Akhurst, 1980, Boemare & Akhurst, 1988; Richardson *et al.*, 1988; You *et al.*, 2006). If cell clumping also occurs in 2° cells or is exclusively considered as a 1°-specific feature remains to be determined (Brachmann *et al.*, 2013). Remarkably, besides being also pathogenic towards insects, 2° cells are not capable of supporting growth and development of the nematodes (Han & Ehlers, 2001; Figure 1-4). Due to the inefficiency to fulfill the symbiotic part of the life cycle, it is assumed that the 2° cells might be better adapted for a free life in the soil (Smigielski *et al.*, 1994). For agricultural industry, the nematode-bacteria complexes are cultivated in liquid media and then spread onto fields to prevent crop failure. The pre-incubation with the bacterial symbiont is essential for the nematode`s development and reproduction. Thus, phenotypic switching is one of the major reasons for process failure in industrial mass production (Han & Ehlers, 2001).


| Phenotype | Primary cells (1°) | Secondary cells (2°) |
|---|---|---|
| Bioluminescence | +++ | + |
| Clumping | + | ? |
| Protease production | +++ | - |
| Pigmentation | +++ | - |
| Crystal proteins | + | - |
| Pathogenicity | +++ | +++ |
| Symbiosis | +++ | - |

**Figure 1-4: Differences in the phenotypes of the *P. luminescens* primary (1°) and the secondary (2°) form.** In 2° cells the bioluminescence is diminished and pigmentation, protease and crystal protein production as well as symbiosis are absent. Cell clumping occurs in 1° cells and it is unknown if it is also present in 2° cells. 1° and 2° cells are pathogenic towards insects, whereas only the insect cadavers that were infected with 1° cells get pigmented. The pictures of the pigmented and non-pigmented culture were taken by Antje K. Heinrich (Goethe-Universität Frankfurt). The table was modified after (ffrench-Constant *et al.,* 2003).

Despite the occurrence of the two phenotypically different 1° and 2° cells, a small-colony variant, which is also referred to as M-form is known to initiate mutualism by colonizing the IJs. This M-form is formed via a single reversible promoter inversion of the *mad* fimbrial locus, which enables the adherence to the nematode intestine (Somvanshi *et al.,* 2012). Cells stochastically express *mad* and thereby a highly mutable contingency locus is indicated (Somvanshi *et al.,* 2012; Moxon *et al.,* 1994).

Comparative genomic studies between the 1° and 2° cells via a combination of macrorestriction and DNA microarray experiments have not revealed any differences between the 1° and the 2° form in the past, suggesting that the occurrence of the two phenotypic variants is due to true phenotypic and not genotypic heterogeneity (Gaudriault *et al.,* 2008). However, the precise mechanism of phenotypic switching remains unknown. Proteomic analysis revealed the drastic down-regulation of membrane-associated and secreted proteins and the up-regulation of proteins that are involved in oxidative stress, energy metabolism and translation in 2° cells. The transport and binding of iron was negatively affected and a down-regulation of molecular chaperones could be observed. Furthermore, H-NS was up-regulated in 2° cells, suggesting a potential role in phenotypic switching (Turlin *et al.,* 2006). As the 2°

variant can be isolated after extended cultivation under laboratory conditions, a response to environmental or metabolic stress is suggested (Joyce *et al.*, 2006). Low osmolarity triggered phenotypic switching in some strains of *P. luminescens* (Krasomil-Osterfeld, 1995).

The tricarboxylic cycle seems to be important for the secondary metabolism and for the interaction of the bacteria-nematode complex, as a deletion of the gene encoding malate dehydrogenase results in the loss of 1°-specific features, like stilbene, anthraquinone and light production. The respective mutant is still virulent against insects but unable to support symbiosis (Lango & Clarke, 2010).

The two-component system AstS/AstR is involved in the adaption to the stationary phase and has been identified to control timing of phenotypic switching in *P. luminescens*. If *astR* is deleted, the cells undergo phenotypic switching earlier than the wild-type. It was observed that the universal stress protein UspA is positively regulated via AstRS revealing that this regulation cascade might prevent the cells from general stress and therefore delay switching (Derzelle *et al.*, 2004).

After experiencing periods of starvation, 2° cells can adapt to the addition of nutrients much faster than 1° cells and start to grow after 2 to 4 hours, whereas 1° cells need 14 hours to recommence growth. Hence, 2° cells are thought to be more efficient in the uptake of nutrients. Activity of the major respiratory enzymes and levels of the transmembrane proton motive force were found to be increased in 2° compared to 1° cells (Smigielski *et al.*, 1994). These results support the idea that 2° cells are better adjusted to a symbiosis-independent life in the soil than the 1° cells.

The LysR-type regulator HexA has been identified to play an important role for the 2° phenotype and will be discussed below (Joyce & Clarke, 2003). The purpose of phenotypic switching is still unknown and it is also still not understood if and how one phase can control the respective other one to create a certain balance between the two phases.

## 1.3 LysR-type transcriptional regulator (LTTRs)

The LysR-type transcriptional regulators (LTTRs) were first described by (Hennikoff *et al.*, 1988) and comprise the largest family of prokaryotic transcription factors (Pareja *et al.*, 2006). The group is named after LysR, the transcriptional activator of *lysA*, which encodes a diaminopimelate decarboxylase and catalyses the decarboxylation of diaminopimelate to produce lysine in *E. coli* (Stragier *et al.*, 1983). LTTRs are ubiquitous amongst bacteria and the majority of identified LTTRs can be found in proteobacteria of the α and γ subdivision, whereas a smaller amount of the known LTTRs belong the β class and the Gram-positive

bacteria (Schell, 1993; Reen *et al.*, 2013). Functional orthologues of LTTRs can also be found in archaea and eukaryotes (Stec *et al.*, 2006; Perez-Rueda & Collado-Vides, 2001; Sun & Klein, 2004).

It is known that members of the LysR-type family cannot only activate but also repress transcription of either single genes or operons by affecting the efficiency of transcription initiation (Maddocks & Oyston, 2008). Many respective target genes of LTTRs are divergently transcribed but can also be located elsewhere in the genome and most of these regulators show negative autoregulation (Schell, 1993). Nevertheless, some members, such as LrhA in *E. coli*, are found to be positively autoregulated (Lehnen *et al.*, 2002). LTTRs are known to be involved in the regulation of a diverse set of features, like metabolism, virulence, motility, oxidative stress responses or QS (Picossi *et al.*, 2007; Russell *et al.*, 2004; Lehnen *et al.*, 2002; Chiang & Schellhorn, 2012; Kim *et al.*, 2004).

### 1.3.1 Structure and function of LTTRs

LTTR monomers comprise approximately 300 to 350 amino acids and contain an N-terminal winged helix-turn-helix (wHTH) DNA-binding domain and a C-terminal co-factor-binding domain, separated by a short linker helix (Schell, 1993; Figure 1-5). The amino acids 20 to 80 within the wHTH domain are highly conserved, whereas the C-terminal domain shows little conservation. The HTH motif comprises a three-helical bundle with an open conformation, with the second and the third helix interacting with DNA. Thereby the third helix is inserted into the major grove of the DNA double helix. The winged helix variety possesses a β-pleated-sheet hairpin between the second and the third helix (Huffman & Brennan, 2002; Brennan, 1993). The C-terminal domain comprises two α/β subdomains, regulatory domain 1 and 2, which contain an interdomain cleft in between to potentially accommodate a co-inducer (Stec *et al.*, 2006).

It is suggested that the LTTRs are functionally active in their tetrameric form, with multiple binding sites on the DNA of the target promoter region. Some LTTRs are also known to form homooctamers (Sainsbury *et al.*, 2009). A high affinity repressor binding site (RBS), which is located at -80 to -50 relative to the transcriptional start site of the target gene is necessary for anchoring the LTTR onto the DNA and contains the so-called LTTR box, which consists of the consensus sequence T-$N_{11}$-A (Schell, 1993). Many LTTRs are known to be negatively autoregulated and it is suggested, that the RBS site is necessary for autoregulation in a co-inducer-independent manner. Moreover, an activation binding site (ABS), which binds the

LTTR with less affinity, is located near the -35 regulatory region of the target gene and is needed to confer interaction with the RNA polymerase. Thereby, one LTTR dimer of the tetramer binds at the RBS and the second LTTR dimer at the ABS site and causes the DNA to bend (Tropel & van der Meer 2004).

A conformational change upon ligand binding then prompts the LTTR to move a variable number of base pairs from a more proximal ABS subsite (ABS`) to a more distal ABS subsite (ABS``), which causes the relaxation of the DNA bending angle and enables transcription of the target gene(s). This is referred to as the so-called `sliding dimer` hypothesis, which could recently be confirmed by analysis of the LTTR DntR (Porrua *et al.*, 2007; Lerche *et al.*, 2016). Thus, it is considered that the compact *apo*-configuration of the LTTR represses transcription, whereas its expanded *holo*-conformation promotes it.

### 1.3.2 HexA in *Photorhabdus* and other organisms

In *Photorhabdus* species HexA is a member of the LTTR family and known to be involved in phenotypic switching (Joyce & Clarke, 2003). HexA is a homolog of LrhA (LysR homolog A) in *E. coli*, which is responsible for the negative regulation of flagella, motility and chemotaxis. LrhA is known to positively autoregulate expression of its own gene (Lehnen *et al.*, 2002). HexA from the plant pathogen *Erwinia carotovora* represses the expression of flagellar genes as well as the genes encoding the exoenzyme virulence factors pectate lyase, cellulase and protease (Harris *et al.*, 1998). It could be shown that HexA from *E. carotovora* negatively regulates the transcription of the regulatory RNA *rsmB*, thereby inhibiting the synthesis of the QS molecule OHHL (N-[3-oxohexanoyl]-L-homoserine lactone). Furthermore, HexA decreases the levels of the stationary phase sigma factor RpoS (Mukherjee *et al.*, 2000). The destabilizing effect on RpoS via LrhA has extensively been studied in *E. coli*. The repression of *rpoS* translation by LrhA is dependent on the small chaperone Hfq. Furthermore, LrhA putatively controls one or more small RNAs but the precise regulation mechanism is still not known (Gibson & Silhavy, 1999; Peterson *et al.*, 2006). The synthesis of *lrhA* itself is repressed via the RcsCDB phoshorelay system, a cell envelope stress-sensing pathway (Peterson *et al.*, 2006).

In 2° cells of *P. temperata*, primary-specific features such as bioluminescence, crystal protein production and pigmentation could be restored upon deletion of *hexA*. Moreover, this *hexA* mutant regained the ability to support nematode growth and development. However, the deletion of *hexA* in 2° cells caused a severe attenuation of pathogenicity, which claims that

symbiosis and pathogenicity have to be temporally regulated. Via northern blot analysis an enhanced transcription level of *hexA* could be found in 2° cells of *P. temperata* (Joyce & Clarke, 2003). Therefore, it is speculated that HexA is responsible for the repression of 1° specific features in 2° cells (Figure 1-5). How these differences in *hexA* expression between 1° and 2° cells are achieved and how HexA is able to regulate such a high number of phenotypic features is not known.

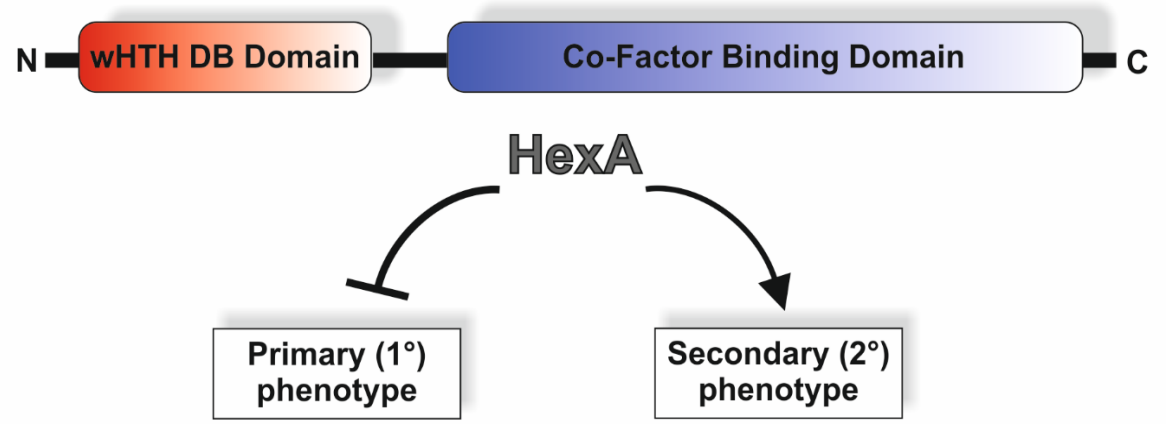


**Figure 1-5 Structure and function of HexA in *Photorhabdus*.** HexA belongs to the LysR-type transcriptional regulators and consists of a winged N-terminal helix-turn-helix DNA-binding domain and a C-terminal co-factor binding domain. HexA is known to be involved in the repression of 1°-specific features and supports the 2° phenotype.

## 1.4 Scope of this thesis

The investigation of phenotypic heterogeneity requires the use of single-cell analytical techniques, which were not available for *P. luminescens*. For that purpose, molecular tools had to be established that allow the integration of reporter genes as single copy into the chromosome of *P. luminescens* for the analysis of promoter activities and translation at the single and population level.

Pigmentation, mediated by anthraquinone (AQ) biosynthesis, is one of the most predominant phenotypic features in which *P. luminescens* 2° cells differ from 1° cells. Therefore, the regulation mechanism of AQ production should be investigated at the population as well as at the single-cell level with respect to phenotypic heterogeneity.

Furthermore, the regulatory mechanism of HexA, which is known to be involved in the regulation of phenotypic switching in *P. temperata*, should be investigated in *P. luminescens*. It should be resolved if HexA binds DNA and how the regulator promotes the repression of 1°-specific features in 2° cells in *P. luminescens*. Bioluminescence, one prominent characteristic of 1° cells, should be studied as a potential target of HexA. Cell clumping

mediated by quorum sensing is another characteristic phenotypic feature in *P. luminescens*. It should be elucidated if clumping is also different in 1° and 2° cells and therefore another predominant heterogenic phenotype. Furthermore, the influence of HexA on the regulation of cell clumping had to be investigated.

Finally, the socio-biological function of phenotypic switching for the *P. luminescens* population should preliminarily be examined. For that purpose, growth competition assays between 1° and 2° cells should enlighten whether the two cell forms influence each other in growth or respond differently to nutrient availability or oxidative stress.

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# 2 A novel tool for stable genomic reporter gene integration to analyze heterogeneity in *Photorhabdus luminescens* at the single-cell level

Reproduced from BioTechniques (2015) Glaeser, A. and Heermann, R. A novel tool for stable genomic reporter gene integration to analyze heterogeneity in *Photorhabdus luminescens* at the single-cell level. 59 (2):74-81.
doi: 10.2144/000114317

**Abstract**

Determination of reporter gene activity at the single-cell level is a prerequisite for analyzing heterogeneous gene expression in bacteria. The insect pathogenic enteric bacterium *Photorhabdus luminescens* is an excellent organism in which to study heterogeneity since it exists in two phenotypically different forms, called the primary and secondary variant. A tool for generating stable genomic integrations of reporter genes has been lacking for these bacteria, and this has hampered the acquisition of reliable data sets for promoter activities at the single-cell level. We therefore generated a plasmid tool named pPINT-mCherry for the easy and stable introduction of gene fragments upstream of an *mCherry* reporter gene followed by stable integration of the plasmid into the *P. luminescens* genome at the *rpmE/glmS* intergenic region. We demonstrate that the genomic integration of reporter genes for single-cell analysis is necessary in *P. luminescens* since plasmid-borne reporter genes mimic heterogeneity and are therefore not applicable in these bacteria, in contrast to their use in single-cell analysis in other bacteria like *Escherichia coli*.

Full-text article:

www.biotechniques.com/multimedia/archive/00250/BTN_A_000114317_O_250483a.pdf

# 3 Heterogeneous regulation of bacterial natural biosynthesis via a novel transcription factor

Reproduced from Heliyon (2016) Heinrich, A.K., Glaeser, A., Tobias, N.J., Heermann, R., Bode, H.B. Heterogeneous regulation of bacterial natural product biosynthesis via a novel transcription factor. 2(11): e00197. eCollection 2016.
doi: 10.1016/j.heliyon.2016.e00197

**Abstract**

Biological diversity arises among genetically equal subpopulations in the same environment, a phenomenon called phenotypic heterogeneity. The life cycle of the enteric bacterium *Photorhabdus luminescens* involves a symbiotic interaction with nematodes as well as a pathogenic association with insect larvae. *P. luminescens* exists in two distinct phenotypic forms designated as primary (1°) and secondary (2°). In contrast to 1° cells, 2° cells are non-pigmented due to the absence of natural compounds, especially anthraquinones (AQs). We identified a novel type of transcriptional regulator, AntJ, which activates expression of the *antA-I* operon responsible for AQ production. AntJ heterogeneously activates the AQ production in single *P. luminescens* 1° cells, and blocks AQ production in 2° cells. AntJ contains a proposed ligand-binding WYL-domain, which is widespread among bacteria. AntJ is one of the rare examples of regulators that mediate heterogeneous gene expression by altering activity rather than copy number in single cells.

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# 4 HexA is a versatile regulator involved in the control of phenotypic heterogeneity of *Photorhabdus luminescens*

# HexA is a versatile regulator involved in the control of phenotypic heterogeneity of *Photorhabdus luminescens*

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## Abstract

Phenotypic heterogeneity in microbial communities enables genetically identical organisms to behave differently even under the same environmental conditions. *Photorhabdus luminescens*, a bioluminescent Gram-negative bacterium, contains a complex life cycle, which involves a symbiotic interaction with nematodes as well as a pathogenic association with insect larvae. *P. luminescens* exists in two distinct phenotypic cell types, designated as the primary (1°) and secondary (2°) cells. 1° cells are bioluminescent, pigmented and can support nematode growth and development. Individual 1° cells undergo phenotypic switching after prolonged cultivation and convert to 2° cells, which lack the 1° specific phenotypes. The LysR-type regulator HexA has been described as major regulator of this switching process. Here we show that HexA controls phenotypic heterogeneity in a versatile way, directly and indirectly. Expression of *hexA* is enhanced in 2° cells, and the corresponding regulator inhibits 1° specific traits in 2° cells. HexA does not directly affect bioluminescence, a predominant 1° specific phenotype. Since the respective *luxCDABE* operon is repressed at the post-transcriptional level and transcriptional levels of *hfq* are also enhanced in 2° cells, small regulatory RNAs are presumably involved that are under control of HexA. Another phenotypic trait that is specific for 1° cells is quorum sensing mediated cell clumping. The corresponding *pcfABCDEF* operon could be identified as the first direct target of HexA, since the regulator binds to the *pcfA* promoter region and thereby blocks expression of the target operon. In summary, our data show that HexA fulfills the task as repressor of 1° specific features in 2° cells in a versatile way and gives first insights into the complexity of regulating phenotypic heterogeneity in *Photorhabdus* bacteria.

## Introduction

*Photorhabdus luminescens* is a Gram-negative soil bacterium, which lives in symbiosis with soil nematodes of the genus *Heterorhabditis bacteriophora* and is in turn highly pathogenic against insect larvae [1]. The bacteria colonize the upper gut of the nematodes in its infective juvenile (IJ) stage, which search for insect larvae in the soil. Upon encountering its prey, the nematode enters the hemocoel and releases the bacteria into the insect´s hemolymph by regurgitation [2]. Then, the bacteria produce a huge set of different toxins that effectively kills the insect within 48 hours. Furthermore, the bacteria produce several exoenzymes to convert the cadaver into a rich nutrient soup that is used by the bacteria as well as the nematodes for growth and reproduction. At this stage, the bacteria are bioluminescent and the insect cadaver begins to glow. Antibiotics are produced to defend the cadaver from other bacteria or fungi. When the cadaver is depleted, nematodes in the IJ stage re-associate with the bacteria and search for another insect prey in the soil [3];[4].

*P. luminescens* exists in two phenotypically different cell types, designated as primary (1°) and secondary cells (2°). The 1° cells are able to associate with the nematodes and show the characteristic features like bioluminescence, pigmentation, production of exoenzymes and antibiotics. The 2° cells lack all these phenotypes [3];[5]. In the past, the production of anthraquinones was found to be responsible for the pigmentation of 1° cells. The synthesis of anthraquinones is accomplished via the operon *antABCDEFGHI,* which encodes a type II polyketide synthase and several modifying enzymes [6]. It was recently found, that the regulator AntJ is required for the heterogeneous activation of the expression of *antABCDEFGHI* in 1° cells, whereas only a basal but homogeneous activation was observed in 2° cells. Artificial overproduction of AntJ leads to anthraquininone production in the usually non-pigmented secondary cells, showing that the non-pigmentation is due to a tight regulation of gene expression rather than the result of a special metabolic condition in 2° cells [7].

Despite the fact that 2° cells are also pathogenic towards insect larvae, they are unable to support nematode growth and development. Therefore, it is assumed that 2° cells are better adapted to a free life in the soil as they cannot use the nematodes as a shuttle to reach their prey, like 1° cells do [8]. Furthermore, a huge set of metabolic enzymes were

found to be up-regulated in 2° cells, which lends support to the idea that 2° cells have adapted to use the limited nutrients that are present in the soil [9].

Recently, it was found that cell clumping in *P. luminescens* is mediated via a novel communication system. Thereby, so-called photopyrones are produced via the photopyrone synthase PpyS. The photopyrones are recognized by the LuxR solo PluR, which then activates the promoter of the *pcfABCDEF* (*Photorhabdus* clumping factor, PCF) operon. Expression of the *pcf* operon leads to the formation of cell clumps, which is important for the virulence of the bacteria [10]. Whether PCF-mediated cell clumping is another 1° phenotypic feature is unclear.

In the past, the transcriptional regulator HexA has been identified to play an important role in the occurrence of the two phenotypic cell types [11]. HexA belongs to the family of LysR transcriptional regulators (LTTRs) with an N-terminal DNA-binding helix-turn-helix motif and a C-terminal co-inducer-binding domain of yet unknown function. LTTRs belong to the most abundant type of transcriptional regulators of prokaryotes and activators as well as repressors are included in this group [12]. It was found that expression of *hexA* is enhanced in *Photorhabdus temperata* 2° cells. Interruption of *hexA* in 2° cells resulted in a bright phenotype of the normally non-bioluminescent 2° cells. Additionally, the 2° Δ*hexA::Tn5* mutant exhibited several other 1° specific phenotypes, revealing that HexA might act as a repressor of 1° specific phenotypes in the 2° cells [11]. The exact mechanism how HexA can control the various phenotypes remained unclear and DNA-binding of HexA has never been shown. Here we describe that HexA acts as a versatile regulator, which controls gene expression directly at the transcriptional level, and indirectly at the post-transcriptional level and give first insights into the complexity of the regulation of phenotypic heterogeneity in *P. luminescens*.

## Material and Methods

### Materials

Strains used in this study are listed in Supplementary Table S2, plasmids are listed in Supplementary Table S3, and primers are listed in Supplementary Table S4. PCRs were performed using Q5 Polymerase and OneTaq Polymerase from New England Biolabs (Frankfurt, Germany). Restriction enzymes and T4 DNA ligase were also purchased from New England Biolabs. Plasmid isolations were performed using the HiYield Plasmid Mini Kit and DNA fragments were purified via the HiYield PCR DNA Fragment Extraction Kit (Süd-Laborbedarf, Gauting, Germany). Genomic DNA was isolated using the Ultra-Clean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, USA). Sequencing was performed in the Genomics Service Unit of the LMU Biocenter.

### Bacterial strains and growth conditions

*P. luminescens* and *Sh. oneidensis* were cultivated aerobically at 30°C and *E. coli* was grown aerobically at 37° in lysogenic broth (LB) (10g NaCl, 10 g/l tryptone, 5 g/l yeast extract) on a rotary shaker. For preparation of agar plates, 1.5% (w/v) agar was added to the medium. If necessary, the medium was supplemented with 50 µg/ml kanamycin, 15 µg/ml gentamicin, 20 µg/ml chloramphenicol, or 100 µg/ml ampicillin. When *E. coli* strain ST18 was cultivated, the medium was supplemented with 50 µg/ml 5-aminolevulinic acid. Pre-cultures were grown overnight and inoculated at an $OD_{600}$ of 0.05 in fresh medium. For induction of the *lac* and the *ara* promoters, different concentrations of IPTG (0.2 mM or 2 mM) and arabinose [0.02% (w/v) or 0.2% (w/v)] were added, respectively unless otherwise stated.

### Generation of the plasmids

For generation of the plasmid pPINT-$P_{hexA}$-*hexA*-*mCherry*, $P_{hexA}$ and *hexA* were amplified using the primers PhexA-BamHI_fwd and hexA-XmaI_rev using genomic DNA of *P. luminescens* TT01 as template. The PCR product was then inserted into the plasmid pPINT-*mCherry* via the restriction sites BamHI and XmaI.

The promoter of *hfq* was amplified by PCR with the primers Phfq-NheI_fwd and Phfq-BamHI_rev using *P. luminescens* TT01 genomic DNA as template. The PCR product was cloned into pPINT-*mCherry* using the restriction enzymes NheI and BamHI. For

investigating $P_{pcfA}$ activity, a PCR with the primers PpcfA-NheI_fwd and PpcfA-XmaI_rev and genomic DNA of *P. luminescens* TT01 as template was performed in order to amplify the promoter of *pcfA* with subsequent restriction of the PCR product and pPINT-mCherry with NheI and XmaI. The resulting plasmids pPINT-$P_{hfq}$-*mCherry* and pPINT-$P_{pcfA}$-*mCherry* were sequenced using the primers check-mcherry-ins_fwd and check-mcherry-ins_rev.

Plasmid pBAD24_$P_{ara}$_*pluR*_$P_{lac}$_*hexA* for *pluR* and *hexA* expression in *E. coli* was generated by amplifying the *lacI* gene and the *lac* promoter from the pACYC-Duet1 as template using the primers Plac(h)_fwd and lacI-SalI-rev. The *hexA* gene was amplified from *P. luminescens* TT01 genomic DNA template using the primer pair hexA_fwd and hexA-PstI_rev. Subsequently, an overlap PCR was performed by using the primers hexA-PstI_rev and lacI_SalI_rev. The overlap PCR product was ligated into vector pBAD24-*pluR* using restriction enzymes SalI and PstI. Correctness of the resulting plasmid was verified by sequencing using the primers check-Plac-hexA_fwd, pBAD24seq_fwd and hexA_fwd, respectively.

The gene *hexA* was amplified in order to create pBAD24-*hexA* by using the primers hexA-EcoRI_fwd and hexA-NdeI_rev. Subsequently, the PCR product and the plasmid pBAD24-yehU were cut with EcoRI and NdeI and ligated. Correctness of the resulting plasmid was checked by sequencing using the primers pBAD24seq_fwd and pBAD24seq_rev.

In order to generate pBAD24-$P_{lac}$-pluR-$P_{ara}$-*hexA*, $P_{lac}$-*pluR* was amplified with the primers Plac-PluR_fwd and PluR-PstI_rev using pCOLA-*ppyS*-His-*pluR* as a template. The gene *lacI* was amplified using the primers lacI_fwd and lacI-SalI_rev using template pCOLA-*ppyS*-His-*pluR*. An overlap-PCR of *lacI* and $P_{lac}$-*pluR* was performed with the primers PluR-PstI_rev and lacI-SalI_rev and inserted into the plasmid pBAD24-hexA via the restriction enzymes PstI and SalI and subsequent ligation. Correct insertion of the DNA fragment was checked by sequencing using the primers pBAD24seq_fwd and pBAD24seq_rev.

Plasmid pACYC_*hexA* was generated by amplifying the *hexA* gene using the primer pair hexA-NcoI_fwd and hexA-SacI_rev and *P. luminescens* TT01 genomic DNA as template. The PCR product was then inserted into the vector pACYC via NcoI and SacI restriction

sites. The correctness of the plasmid was checked by sequencing using the primers check-pACYC_fwd and check-pACYC_rev.

The plasmid pACYC-$P_{lac}$-*hexA*_$P_{ara}$-*pluR* was generated in order to place *hexA* under the control of the inducible *lac* promoter and *pluR* under the control of the inducible arabinose promoter for $P_{pcfA}$ reporter gene analyses in *Shewanella oneidensis*. Thereby, *araC* and *pluR*, which is under control of the *ara* promoter, were amplified with the primers araCPluR-fwd and pluR-XhoI_rev using pBAD24-*pluR* as template. After restriction with NdeI and XhoI, the PCR product was ligated into equally treated vector pACYC_*hexA*. The correct insertion of the DNA fragment was checked by sequencing using primers pACYC_check_rev and pBAD24seq_fwd.

The plasmid pBBR-$P_{lac}$-*lux* was created by amplification of $P_{lac}$ from plasmid pEYFP as template with the primers Plac-NheI_fwd and Plac-BamHI_rev, and subsequent ligation via the restriction sites NheI and BamHI and ligation. Correctness of the plasmid was verified by sequencing using the primer check-pBBR-Plac-fwd.

**Generation of the *P. luminescens* Δ*hexA* strain**

The *hexA* gene was deleted in the *P. luminescens* 1° cells using a previously described method [13]. Briefly, the upstream and downstream genomic regions surrounding *hexA* (*plu3090*) were amplified by PCR using the primers FA_hexA_fwd/ FA_hexA_rev and FB_hexA_fwd/ FB_hexA_rev and the amplicon was cloned into pDS132, resulting in pDS-*hexA*. This plasmid was conjugated from *E. coli* S17-1λpir into 1° cells and exconjugants were selected as $Rif^R$ $Cm^R$ colonies. The pDS132 plasmid contains the *sacB* gene and, after growth in LB broth (with no selection), putative mutants were identified by screening for $Rif^R$ $Suc^R$ $Cm^S$ colonies. The deletion of *hexA* was confirmed by PCR and DNA sequencing.

**Competent cells and transformations**

*E. coli* cells were made chemically competent and transformed as described elsewhere [14]. Electrocompetent *E. coli* cells were prepared by cultivation of the cells in LB medium at 37°C up to an $OD_{600nm}$ of 1. Cells were then harvested and washed three times with ice-cold 10 % (v/v) glycerol and subsequent centrifugation steps (1 min, 16 000 rpm, 4°C). Finally, cells were resuspended in 1/150 of the starting volume in 10% (v/v) glycerol. A

similar procedure was used for the preparation of electrocompetent *Sh. oneidensis*, except that all washing steps were performed with ice-cold sorbitol (1M). Electrocompetent cells were shortly incubated with 50-100 ng of plasmid DNA and then electroporation was performed in 0.2 cm cuvettes, using a pulse of 2.5 kV for 4-6 msec for *E. coli* cells and a pulse of 1.8 kV for 4-6 msec for *Sh. oneidensis* cells. The cells were then resuspended in 1 mL LB medium and incubated for 45-60 minutes at 37°C (*E. coli)* or 30°C (*Sh. oneidensis)* under constant shaking, plated on LB agar supplemented with the appropriate antibiotics and the plates were incubated at 37°C or 30°C overnight.

**Integration of reporter genes into the *P. luminescens* genome**

For the integration of the different promoter-*mCherry* fusions as well as the *hexA-mCherry* fusion into the genome of *P. luminescens*, the donor strain *E. coli* ST18 [15], which requires the addition of 5-aminolevulinic acid for growth, was first transformed with the respective plasmids. Then, the conjugative plasmid transfer was achieved via the filter mating method [15]. For that purpose, the donor as well as the recipient strain was cultivated in LB medium at 30° or 37°, respectively, and grown up to an $OD_{600}$ of 0.8-1 in LB medium, which was supplemented with the respective additives if required. The donor strain was washed three times with LB medium and then mixed with the recipient strain in a ratio of 1:5 in a final volume of 1/10 of the donor`s initial volume. The mixed cells were then dropped onto a nitrocellulose filter, which had been positioned into the middle of an LB agar plate. The plate was incubated at 30°C over night, the cells were scratched from the filter and suspended in 500 µl LB medium before they were spread onto LB agar plates containing the appropriate antibiotics. The plates were then incubated for two days at 30°C. Single colonies were picked, cultivated in LB medium at 30° and the genomic DNA was isolated. Then, the correct insertion of the plasmids into the genome was checked via PCR and sequencing using primers check-rpmE_fwd, oriT_fwd, gmR-pNPTS_fwd, check-mcherry-ins_rev, check-glmS_rev, and the genomic DNA of the clones as template.

**Promoter activity analyses**

For promoter activity assays in *E. coli* Δ*lrhA and Sh. oneidensis,* cells were inoculated in single wells of microtiter plates with an $OD_{600}$ of 0.05 and aerobically grown at 37°C or 30°C, respectively, in a Tecan Infinite F500 plate reader (Tecan, Salzburg) for 8 hours.

The $OD_{600}$ and luminescence were measured every 10 minutes. Different concentrations of arabinose and/or IPTG were added to induce *pluR* and/or *hexA* expression after the cells reached an $OD_{600}$ of 0.2. Data are reported as relative light units (RLU) in counts per second per milliliter per $OD_{600}$.

For reporter activity assays in *P. luminescens*, the $OD_{600}$ was set to 0.05 and the cells were aerobically grown at 30°C in microtiter plates in a Tecan Infinite F500 system (Tecan, Salzburg). Cells density was determined at $OD_{600}$ and the fluorescence intensity of mCherry was measured (560 nm excitation, 612 nm emission, 20 nm bandwidth). The data were reported as relative fluorescence units (RFU) and then normalized with the optical density ($OD_{600}$) of the respective culture.

**Fluorescence microscopy**

In order to investigate promoter activities in *P. luminescens* at the single cell level, a fluorescence microscope (Leica, Bensheim) with an excitation wavelength of 546 nm and a 605 nm emission filter with 75 nm bandwidth was used to detect fluorescence of mCherry fluorophore. The respective liquid culture was set to an OD of 0.05 and at appropriate time points, 5 to 15 µl of the culture were dropped onto agarose pads [0.5% (w/v) agarose in PBS buffer, pH 7.4 on a microscope slide]. When cell clumping was investigated, no agar pads were used but the culture was directly dropped onto a microscope slide.

**Two-dimensional gel electrophoresis and protein identification via MALDI-TOF**

*P. luminescens* TT01-1° and *P. luminescens* TT01-1° Δ*hexA* were cultivated aerobically in 200 ml CASO medium at 30°C. After cultivation for 48 hours, cells were incubated with 1 mg/ml (w/v) chloramphenicol to inhibit protein translation and harvested (4.500 x g for 10 min at 4°C). The cells were washed with buffer [100 mM Tris-HCl, pH 7.5, 0.1 mg/ml (w/v) chloramphenicol], and the cell pellets were stored at -80°C until use. After re-suspending the cells in disruption buffer [10 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$; 50 µg/mL (w/v) RNase; 50 µg/mL (w/v) DNase; 100 µg/mL (w/v) lysozyme; 1.39 mM PMSF], they were disrupted by sonification (three times 30 sec pulse interrupted by a 30 sec pause). Cell debris was removed by centrifugation (10 min at 16.100 x g at 4°C). A total protein amount of 350 µg was lyophilized overnight, solubilized in rehydration buffer [8 M urea; 2

M thiourea; 2% (w/v) CHAPS; 1.25% (w/v) IPG buffer pH4-7 l (GE Healthcare, München); 28.4 mM DTT; 0.05% (w/v) bromophenol blue] and separated on IPG stripes (GE Healthcare, München) at 19,650 Vh. The strips were then equilibrated in buffer [50 mM Tris-HCl pH 6.8; 6 M urea; 30% (v/v) glycerol; 4% (w/v) SDS; 18.2 mM DTT] twice for 15 min. Protein separation in the second dimension was performed by SDS-PAGE [16] using 1 mm gels of the size 16 cm × 20 cm with 13% (w/v) acrylamide in the separation gel and 7% (w/v) in the spacer gel at 15 °C with 5 mA overnight. Gels were stained for 1 h in Coomassie blue solution [40% (v/v) ethanol; 10% (v/v) acetic acid; 0.2% (w/v) Coomassie brilliant blue R250], destained for 1 h with destaining solution [40% (v/v) ethanol; 10% (v/v) acetate], and the entire complete destaining was performed with 10% (v/v) acetic acid (Weber K, 1969). The gels were scanned and analyzed via the PDQuest software (Bio-Rad, München) in triplicates, comparing the wild-type TT01-1°proteome with the proteome of TT01-1°Δ*hexA*. Proteins with altered production pattern were analyzed by MALDI-TOF. For that purpose, protein spots were cut out of the gels, washed with deionized water (4 times for 30 min at 37°C while shaking at 850 rpm) and incubated in 50% (v/v) acetonitrile twice for 15 min at 37°C. Proteins were then digested via the addition of trypsin in 40 mM ammonium bicarbonate (Promega, Mannheim) overnight at 37°C and samples were desalted using ZipTip µ-C18 columns (Millipore, Eschborn), and then directly eluted with 1 µl matrix [saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% (v/v) acetonitrile and 0.6% (v/v) trifluoroacetic acid]. The samples were analyzed in a Voyager DE STR MALDI-TOF system (Applied Biosystems, Foster City, USA) using the reverse modus in the range of 700–3.500 Da. Peptide masses were calibrated using known masses of autolysis peptide of trypsin. Proteins were identified via their masses using the search engine MASCOT on http://www.matrixscience.com/ [17].

**Purification of HexA-6His**

HexA-6His was purified using $Ni^{2+}$ affinity chromatography. As first step, *E. coli* BL21 cells harboring plasmid pBAD24-*hexA* were cultivated up to an $OD_{600}$ of 0.5 and gene expression was induced by adding 0.1% (w/v) L-arabinose. After 4 h of aerobic cultivation at 30°C, cells were harvested and disrupted using a Cell disruptor (Constant Cell Disruption Systems, Northants, UK) at 1.35 kbar and 4°C in lysis buffer [50 mM Tris-HCl

(pH 7.5), 10% glycerol (v/v), 10 mM $MgCl_2$, 0.5 mM PMSF, 1 mM DTT and 10 ng/mL DNase I]. Subsequently, HexA was eluted using elution buffer B [50 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 200 mM NaCl, 250 mM imidazole, 2 mM β-mercaptoethanol]. Purification technique was in principle carried out as described for the response regulator KdpE before (Heermann *et al.,* 2003). The equilibration and washing steps were performed in buffer E [50 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 200 mM NaCl, 2 mM β-mercaptoethanol, 10 mM imidazole] and 250 mM imidazole (final concentration) was added for the elution of the protein from the column. Purified HexA was subjected to SDS-PAGE and the bands of eight lanes (approximately 100 µg protein) corresponding to HexA were cut out of the gel and used to generate αHexA rabbit IgG antiserum at BioGenes GmbH (Berlin, Germany).

**Surface plasmon resonance (SPR) spectroscopy**

SPR assays were performed in a Biacore T200 (GE Healthcare, München) using carboxymethyl dextran sensor chips pre-coated with streptavidin (XanTec SAD500L, XanTec Bioanalytics GmbH, Düsseldorf). Biotinylated fragments of the *pcfA* promoter were generated by amplification of the $P_{pcfA}$ region using chromosomal DNA of *P. luminescens* as template with the biotinylated primer PpcfA-Btn_fwd and PpcfA _rev. As a negative control a *sacB* fragment was amplified using the biotinylated primer sacB-Btn_fwd and sacB_rev. Before immobilizing the DNA fragment, the chip was equilibrated by three injections using 1 M NaCl/50 mM NaOH at a flow rate of 10 µl/min. 10 nM of the respective double-stranded biotinylated DNA fragment was injected using a contact time of 420 seconds and a flow rate of 10 µl/min. 1 M NaCl/50 mM NaOH/50% (v/v) isopropanol was injected as a final wash step. Approximately 300 RU of the DNA fragment was captured onto flow cell 2 or 4, respectively, of the chip. HexA was diluted in HBS-EP+ buffer [10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% (v/v) Surfactant P20] and passed over flow cells 1 to 4 in different concentrations (0 nM, 125 nM, 250 nM, 500 nM, 1000 nM and 2000 nM) using a contact time of 180 sec followed by a 240 dissociation time before the next cycle started. The experiments were carried out at 25°C at a flow rate of 30 µl/min. After each cycle, regeneration of the surface was achieved by injection of 2.5 M NaCl for 60 sec at 30 µl/min flow rate. Sensorgrams were recorded using the Biacore T200 Control software 2.0 and analyzed with the Biacore T200 Evaluation software 2.0

(GE Healthcare, München). The surface of flow cell 1 was used to obtain blank sensorgrams for subtraction of bulk refractive index background. The referenced sensorgrams were normalized to a baseline of 0. The 1:1 binding algorithm was used for calculation of the binding affinity. Peaks in the sensorgrams at the beginning and the end of the injection emerged from the runtime difference between the flow cells of each chip. Experiments were performed in the Bioanalytics core facility of the LMU München.

**Fluorescence-based thermal stability assay**

The fluorescence-based thermal stability assays were performed as described before [18]. Purified HexA-6His was used at a final concentration of 0.72 µM. Various buffers containing different concentrations of glycerol, β-mercaptoethanol and urea were tested. The iQ5 real-time PCR detection system (BioRad, München) with a temperature gradient of 1°C/ min from 5°C to 95°C was used. Results were obtained from three independently performed experiments.

## Results

***P. luminescens* 2° cells contain increased levels of HexA**

As a first step to get more insights into the mechanism of HexA to act as a regulator of phenotypic heterogeneity of *P. luminescens*, we investigated HexA levels in 1° and 2° cells. In previous studies, via Northern Blot analyses it was shown that the *hexA* mRNA levels in different *Photorhabdus* species are enhanced in 2° compared to 1° cells [11]. To further investigate HexA levels in *P. luminescens*, we determined the promoter activity of *hexA* using a strain carrying a chromosomal $P_{hexA}$-*mCherry* fusion [19]. The fluorescence intensities were up to 1.4 fold higher in 2° cells compared to 1° cells. The fluorescence was comparable in a 1° *hexA* deletion strain and 1° wild-type cells, revealing that $P_{hexA}$ activity is not under autoregulation of HexA (Fig 1A). As a next step, we generated a chromosomal translational fusion of $P_{hexA}$-*hexA-mCherry*. Fluorescence intensities were approximately 2 fold higher in 2° compared to 1° cells, revealing that not only *hexA* transcription, but also HexA protein levels are enhanced in 2° cells (Fig 1B). Moreover, $P_{hexA}$-*mCherry* as well as $P_{hexA}$-*hexA-mCherry* mediated fluorescence was homogeneously distributed 1° as well as in 2° cells at the single cell level (data not shown).

Therefore, our data confirms previous reports describing enhanced HexA levels in *P. luminescens* 2° cells.

**Influence of HexA on the 1° specific phenotype bioluminescence**

One of the most striking phenotypic differences between 1° and 2° cells of *P. luminescens* is bioluminescence, which is predominantly present in 1° cells. Since the studies with the $P_{hexA}$-*mCherry* and $P_{hexA}$-*hexA-mCherry* reporters revealed a putative involvement of HexA in 1° specific phenotypes, we investigated a potential influence of HexA on the bioluminescence by comparing light production of a 1°, 2° and 1°Δ*hexA* population in dependence of the growth phase. We observed that the bioluminescence is significantly increased in the 1°Δ*hexA* population, especially when the cells enter the stationary growth phase (Fig. 2A). It was hypothesized before that HexA might act as repressor for bioluminescence in 1° cells [11]. As expected, the light production is only weakly detectable in the 2° population in all growth phases. When introducing only one additional copy of *hexA* that is under control of its native promoter the bioluminescence drastically decreased in the 1° cells. Furthermore, introduction of an additional *hexA* gene completely abolished bioluminescence in the 2° population. In the stationary growth phase, enhanced HexA levels led to a decrease in bioluminescence of 86% in the 1° population. Complementation of *hexA* in the 1°Δ*hexA* population reduced the production of light to its native 1° level. In summary, *hexA* gene dosage and the resulting level of HexA is crucial for the different levels of light production in 1° and 2° cells.

We then investigated the influence of HexA on the expression of the corresponding *luxCDABE* operon. For that purpose, we fused the $P_{luxC}$ promoter to *mCherry* and integrated a single copy of the reporter into the chromosome of *P. luminescens* 1°, 2° and 1°Δ*hexA*. However, the fluorescence intensities of all three reporter strains and therefore the $P_{luxC}$ activity was comparable at the population level, even though the respective bioluminescence phenotype was completely different (Fig 2B). Furthermore, the $P_{luxC}$ activity was homogeneously distributed in all three reporter strains at the single cell level (Fig 2C). Thus, HexA does not regulate the expression of the *luxCDABE* operon directly, and therefore the effect of HexA on light production must be at the post-transcriptional level.

**Hfq is involved in HexA regulation**

Regulation of gene expression at the post-transcriptional level is often controlled by small regulatory RNAs (sRNAs) mediated by the RNA chaperon Hfq (see [20] for overview). Therefore, we investigated if Hfq is also involved in the regulation of phenotypic heterogeneity of *P. luminescens.* We first introduced a chromosomal copy of $P_{hfq}$-*mCherry* in 1°, 2° and 1°Δ*hexA* cells and investigated the fluorescence intensities and therefore $P_{hfq}$ activities of all reporter strains at different growth phases. The 1°Δ*hexA* population showed $P_{hfq}$ activities comparable to the 1° population, except for the early exponential growth phase where it was slightly enhanced. However, the $P_{hfq}$ activity was up to 3.4 fold enhanced in the 2° compared to the 1° population (Fig 3A). The promoter activity of $P_{hfq}$ was homogeneously distributed in all three reporter strains (Fig 3B). This reveals that Hfq and therefore presumably regulation via sRNAs may be involved in the regulation of phenotypic heterogeneity in *P. luminescens*. However, as we could not detect significantly different $P_{hfq}$ activity upon deletion of *hexA* in 1° cells, we suggest that expression of *hexA* itself is regulated via Hfq and thus is located downstream of the Hfq regulation cascade.

**Influence of HexA on the primary specific feature cell clumping**

In order to investigate the full regulatory influence of HexA we compared the proteomes of the exponential and stationary growth phase of 1° and 1°Δ*hexA* cells via 2D-PAGE*,* and identified 22 proteins that were differentially produced in the *hexA* deletion strain (Suppl. Fig S1, Suppl. Table S1). These included proteins that are involved in metabolism, antibiotic and toxin production, cell adhesion, 3 proteins of yet unknown function and one regulator (PAS4-LuxR receptor, Plu2016). Furthermore, elevated levels of the 3 proteins PcfA, PcfB and PcfC were found in the Δ*hexA* strain, which are encoded by the *pcfABCDEF* (*Photorhabdus* clumping factor) operon, responsible for the formation of cell clumps in *Photorhabdus* and regulated by the novel PpyS/PluR quorum sensing system [10]. To determine if HexA is directly involved in regulation of *pcfABCDEF* expression, and therefore cell clumping, we integrated a $P_{pcfA}$-*mCherry* reporter into the genomes of *P. luminescens* 1°, 2° and 1°Δ*hexA* and then analyzed fluorescence intensities as well as cell clumping at different growth phases via microscopy. Cell clumps of 1° cells were visible at the beginning of the stationary growth phase (48 h of incubation). The 1°Δ*hexA* strain already formed cell clumps in the exponential growth phase (24 h of incubation).

However, the 2° cells did not form cell clumps, even after seven days of incubation (Fig 4A, Suppl. Fig S2). Furthermore, the activity of $P_{pcfA}$ was maximal in the 1°Δ*hexA* mutant, moderate in 1° cells and totally absent in 2° cells (Fig 4B). Therefore, these results strongly suggest that HexA acts as a repressor of the *pcf* operon.

**Binding of HexA to the $P_{pcfA}$ promoter**

To investigate whether HexA directly or indirectly acts on *pcfABCDEF*, we determined $P_{pcfA}$ activity in *E. coli* as a heterologous system. Since *E. coli* contains a HexA homolog called LrhA, an *E. coli* Δ*lrhA* strain was used and the expression of the *luxCDABE* operon was put under the control of the *pcfA* promoter. $P_{pcfA}$ activity can be induced by over-expression of *pluR* since PluR is a direct activator for *pcfABCDEF* expression [10]. For that reason, the reporter strain was further equipped with *pluR*, which is under control of the arabinose inducible promoter $P_{ara}$. The *hexA* gene was set under control of the promoter $P_{lac}$ and its expression could therefore be achieved via the addition of IPTG. Thus, the addition of arabinose led to a signal-independent activation of $P_{pcfA}$ by simple overproduction of PluR. We observed that with increasing concentrations of IPTG and thus with increasing levels of HexA, the activity of $P_{pcfA}$ decreased to 61% (Fig 5A). These results strongly suggest that HexA represses *pcfABCDEF* expression. Since *E. coli* contains the LysR-type regulator and HexA homolog LrhA, the repression in this heterologous system might still be indirect although LrhA is absent. In order to further exclude this hypothesis, we also tested the similar $P_{pcfA}$ reporter assay in *Shewanella oneidensis*, which lacks any homolog of HexA. Similar to the observations in the *E. coli* reporter strain, HexA overproduction also led to a decrease of $P_{pcfA}$ activity, which dropped to 49% compared to the conditions where HexA was not overproduced (Fig 5A). To make sure that the results were not due to the differences in the chosen inducible promoters $P_{ara}$ and $P_{lac}$, the promoters were swapped, putting *pluR* expression under the control of $P_{lac}$ and *hexA* expression under the control of $P_{ara}$. Comparable results as described above were obtained (Suppl. Fig S3). To exclude potential effects of HexA on the *luxCDABE* operon, a reporter assay with $P_{lac}$-*luxCDABE* was performed in *E. coli* Δ*lrhA*. No inhibiting effect due to HexA overproduction was detected (Suppl. Fig S4). In summary, these data support the idea that HexA directly controls the activity of $P_{pcfA}$.

The binding site of PluR within the $P_{pcfA}$ region was identified (Sophie Brameyer and Ralf Heermann, LMU, unpublished information). We were now interested to find out whether the binding site of HexA is located up- or downstream of the PluR binding site. Therefore, we tested the activity of different truncated *pcfA* promoter constructs for inhibition by HexA using the above-described heterologous *E. coli* Δ*lrhA* $P_{pcfA}$-*luxCDABE* reporter system. When the promoter upstream of the PluR binding site was deleted, the HexA-mediated repression could still be observed as $P_{pcfA}$ activity was reduced by 50% upon HexA overproduction (Fig 5B). Using a $P_{pcfA}$-construct truncated close to the PluR binding site, a reduced activation via PluR was measured (data not shown). Nevertheless, compared to the respective maximum induction of this construct, a HexA-mediated repression of 80% was observed (Fig 5B). Thus, it is assumed that HexA binds downstream of the PluR binding site. However, this could not be investigated further via this assay since any additional truncation would destroy the PluR DNA-binding site and $P_{pcfA}$ could not be activated any more.

To prove direct binding of HexA to the *pcfA* promoter region, we performed surface plasmon resonance (SPR) spectroscopy. As a first step, a HexA-6His variant was overproduced and purified using Ni-NTA affinity chromatography. The purification of the correct protein was verified using αHexA specific antibodies. Then, optimal buffer conditions were screened via a fluorescence-based thermal stability assay. Gel filtration experiments as well as dynamic light scattering assays showed that HexA is primarily present as a tetramer (Suppl. Fig S5). For the SPR assays, a 400 bp biotinylated DNA fragment comprising the *pcfA* promoter region was captured onto a streptavidin pre-coated sensor chip. As a control, a 400 bp DNA fragment of the *sacB* gene from *Bacillus subtilis* was used. Subsequently, different concentrations of purified HexA were injected and passed over the chip surface. As it can be seen in Fig 6, HexA specifically bound to the *pcfA* promoter DNA with an overall affinity of 1.3 µM, which was calculated by the 1:1 binding algorithm. However, the sensorgram shape does not reveal a true 1:1 binding, since even at higher HexA concentrations the sensorgrams do not reach saturation. Due to the low association ($k_a$=1300 M*s) and very high dissociation ($k_d$=0.002/s) it appears likely that a potential HexA ligand is absent and this ligand might influence the DNA-binding affinity of HexA. However, the addition of several primary metabolites (amino acids, sugars, compounds of the tricarboxylic acid cycle) and also secondary metabolites

(cinnamic acid) did not influence the DNA-binding activity of HexA. However, although a putative ligand of HexA that mediates DNA-binding activity remains elusive, we have shown for the first time that HexA is a DNA-binding protein. Moreover, HexA exerts its regulation on phenotypic variation using a combination of direct (e.g. *pcf* operon) and indirect (e.g. *lux* operon) mechanisms.

**Discussion**

The LysR-type regulator HexA is a major regulator in control of phenotypic heterogeneity of *P. luminescens*. It has been proposed before that HexA might act as a global repressor of 1° specific genes in *P. temperata* [11], but it was unclear how HexA could fulfill the function as a direct regulator of such a huge subset of genes and operons that are specifically expressed in 1° and not in 2° cells. Here we show for the first time that HexA is a regulator that can bind DNA and therefore acts as direct repressor of 1° specific genes like cell clumping in *P. luminescens*. Moreover, HexA can also indirectly influence expression of 1° specific genes like the *lux* operon. It is likely that sRNAs are involved in this process since the promoter activity of *hfq*, encoding for the RNA chaperone Hfq, is enhanced in 2° cells. We could confirm increased transcriptional levels of *hexA* in *P. luminescens* 2° cells, supporting the assumption that HexA acts as repressor of 1° specific features. In the early exponential phase, the transcriptional as well as the translational fusions of *hexA* with *mCherry* were comparable between 1° and 2° cells, and the biggest differences were detectable when the cells entered the stationary growth phase. These results support the fact that HexA mainly regulates the phenotypic switching process in the stationary phase since the 1° specific features typically occur in the post-exponential growth phase. Furthermore, the translational fusions of $P_{hexA}$-*hexA-mCherry* clearly showed that the 2° cells have higher HexA protein levels than the 1° cells. The values of the translational reporter were even differing more drastically between the both cell types in comparison with the transcriptional fusions ($P_{hexA}$-*mCherry*). This suggests that the HexA protein level is not only regulated at the transcriptional level but also by post-transcriptional differences, e.g. less degradation of mature proteins leading to a longer half-life of HexA in 2° cells. This is in accordance with the identification of a protease inhibitor in 2° cells [21]. *E. coli* contains a homologous regulator of HexA, which is called LrhA and known to be involved in the repression of flagella, motility and

chemotaxis genes [22]. In contrast to HexA of *P. luminescens,* LrhA of *E. coli* positively auto-regulates expression of its own gene [22]. However, since we could not identify any auto-regulation of *hexA*, the regulation mechanism that leads to enhanced transcription of *hexA* in 2° cells remains unclear.

How does HexA indirectly influence expression of 1° specific genes? We investigated the 1° specific feature bioluminescence and could show, that the light production is dependent on HexA in *P. luminescens*. The deletion of *hexA* led to the brightest phenotype and complementation of *hexA* restored the native levels of bioluminescence. In contrast, promoter activity of the *luxCDABE* operon showed no significant differences in 1°, 2° and 1°Δ*hexA* cells, which revealed a post-transcriptional regulation of the *luxCDABE* expression. In *E. carotovora* as well as in *E. coli*, it was found that HexA or LrhA, respectively, control the levels of RpoS and therefore influence the expression of hundreds of stationary-phase genes [23];[24]. Furthermore, in *E. coli* it could be found that LrhA represses the sRNA RprA, which is an activator of RpoS translation. Another unidentified sRNA is regulated by LrhA, influencing RpoS translation in an RprA-independent manner [25]. The absence of the stationary-phase specific features in 2° cells of *P. luminescens* might also be explained with reduced RpoS levels caused by enhanced HexA levels. However, as LrhA in *E. coli* is known to regulate sRNAs it seems even more likely that HexA in *P. luminescens* also influences the expression of sRNAs to control 1° specific gene expression. This is supported by the fact that that the promoter activity of *hfq* is enhanced in 2° cells. Hfq is an RNA chaperone that facilitates RNA-RNA interactions involved in post-transcriptional regulation [26]. Hfq is known to be required for the stability and/or interactions with the target mRNA of numerous *E. coli* sRNAs [27]. It is known that several sRNAs *e.g*. DsrA. OxyS and RprA are involved in regulation of *rpoS* translation. Interestingly, in turn the association with Hfq is needed for the function of these sRNAs. Furthermore, it is described that LrhA-dependent repression of *rpoS* translation is also dependent on the RNA chaperone Hfq in *E. coli* [25]. We could not detect any differences of *hfq* promoter activity upon deletion of *hexA* in 1° cells. Therefore, we conclude that Hfq influences *hexA* expression and not *vice versa*. This is supported by the finding that a deletion of *hfq* in *P. luminescens* 1° cells caused a drastic increase in *hexA* expression [28]. Furthermore, nearly no production of secondary metabolites and the inability to

support symbiosis with nematodes was observed for 1°Δ*hfq*. A double deletion of *hfq* and *hexA* restored secondary metabolite production as well as the recovery of infective juveniles to nematodes [28]. However, we observed enhanced HexA levels and increased promoter activity of *hfq* in 2° cells. Since Hfq is known to be autoregulated at the translational level in *E. coli* [29], it could also be possible that translation of *hfq* or protein activity is somehow diminished or impaired in *P. luminescens* 2° cells.

We found that HexA directly regulates expression of the *pcfABCDEF* operon, which induces cells clumping. The promoter activity of *pcfA* was repressed in 2° cells and enhanced upon deletion of *hexA* in 1° cells, suggesting that HexA represses *pcfABCDEF* expression. Direct binding to the *pcfA* promoter region was verified by SPR spectroscopy. The *pcf* operon is under positive control of the PpyS/PluR quorum sensing system in *P. luminescens* [10]. Thereby, *P. luminescens* does not communicate via acyl-homoserine-lactones (AHLs) like many other Gram-negative bacteria, but by photopyrones (PPYs), which are produced by the photopyrone synthase PpyS and sensed by the LuxR-type receptor PluR. We found that the formation of cell clumps is impaired and the promoter activity of the *pcf* operon is repressed in 2° cells. A link of HexA to quorum sensing has also been found in the plant-pathogenic bacterium *Erwinia carotovora* ssp. *carotovora*, where HexA negatively regulates the production of the quorum sensing signal OHHL [N-(3-oxo-hexanoyl)-L-homoserine lactone] besides the exoenzyme production, and thereby influencing virulence [30];[31];[24]. In the plant-pathogenic bacterium *Pantoea stewartii* it was discovered that a high concentration of AHLs leads to a deactivation of *lrhA* (*hexA* homologue) expression directly via the LuxR transcriptional regulator EsaR, which in turn is important for virulence against corn plants [32];[33]. Thereby, a feedback loop of HexA on the quorum sensing network downstream of transcriptional regulator EsaR has been proposed. Since cell-clumping has also been proposed to contribute to the high pathogenicity of *P. luminescens* [10];[34], it is likely that HexA also indirectly influences pathogenicity of 2° cells. However, since pathogenicity of 2° cells has not been found to be attenuated in *P. temperata* [11] but 2° cells are not symbiotic any more, cell clumping might not only be important for pathogenicity, but also for symbiosis. This is supported by the fact that a Δ*pluR* deletion strain showed reduced reassociation with the nematodes (DJC and RH, unpublished results). Thereby, cell clumping might facilitate uptake of the bacteria by the nematodes.

HexA contains a C-terminal domain that is proposed to bind a putative, yet unidentified, molecule. SPR analyses and the respective association and dissociation rates with an overall affinity of 1.3 µM revealed that DNA-binding of HexA is not as stable as observed for other DNA-binding proteins. Therefore, the binding of a metabolite that might modulate binding affinity is likely. Since the metabolic state of the cell was proposed to somehow influence phenotypic switching [35], binding of a primary metabolite to HexA would be conceivable. Binding of metabolites to LysR-type regulators have been shown before. One example is ArgP of *E. coli*, which directly activates the transcription of the lysine transporter gene *lysP* and binds lysine to modulate DNA-binding affinity [36]. Since *P. luminescens* produces a huge set of secondary metabolites, whereby most of them are only present in 1° cells, binding of a compound of the primary metabolism to HexA might be likely. The LysR-type receptor RovM of *Yersinia pseudotuberculosis* regulates expression of the temperature-dependent virulence regulator gene *rovA* [37]. The secondary structure of RovM revealed that it binds small inducer molecules to modulate DNA-binding affinity [38]. We have preliminary tested different primary as well as secondary metabolites and their effect to modulate DNA-binding of HexA (data not shown). However, no putative ligand of HexA could yet be identified and therefore remains elusive.

Taken together we conclude that HexA is a versatile transcriptional regulator that directly but also indirectly represses 1° specific features in 2° cells of *P. luminescens* to regulate expression of a huge subset of different genes. However, it is unclear which role HexA plays in 1 ° cells, where it occurs at low levels. If HexA itself or its activity is regulated via other regulator(s), ligands or the involvement of Hfq remains to be determined (Fig 7).

**Author contributions**

AG performed the generation of the reporter strains, reporter gene analyses, bioluminescence measurements, fluorescence microscopy, overproduction and purification of HexA, and fluorescence-based thermal stability assays. AM performed the promoter activity assay of $P_{pcfA}$ in *Sh. oneidensis* and analysed the promoter activity with the truncated promoter versions in *E. coli*. CH performed 2D-PAGE and RH performed the SPR experiments. DJC and SAJ generated strains *P. luminescens* TT01-2° and TT01-1°*ΔhexA*. AG and RH designed the experiments, analyzed the data and wrote the paper.

**Acknowledgments**

This work was supported by the Deutsche Forschungsgemeinschaft (SPP1617, HE-5247/5-1). We are grateful to Dr. Sophie Brameyer (LMU München, Germany) for providing the *E. coli* Δ*lrhA* strain and the plasmids listed in the supplementary information. SPR assays were performed in the Bioanalytics core facility of the LMU Biocenter. The authors declare no conflicts of interest.

**Figure Legends**

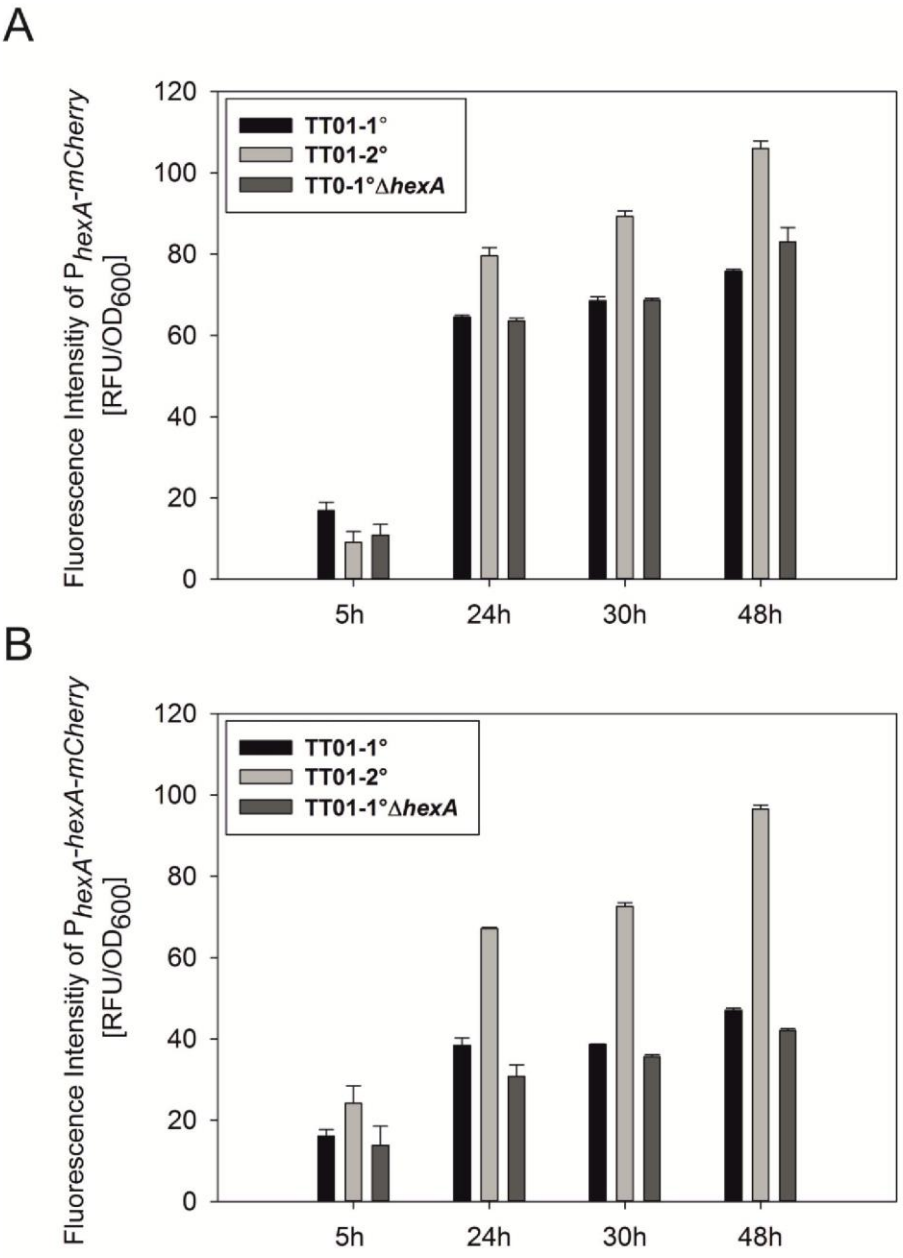


**Fig. 1: Transcriptional and translational levels of *hexA* expression.** The transcriptional level, by fusing the promoter of *hexA* to *mCherry* (A), as well as the translational level, by generating protein hybrids of HexA with mCherry under the control

of the *hexA* promoter (B), were investigated. The reporter constructs $P_{hexA}$-*mCherry* and $P_{hexA}$-*hexA-mCherry*, respectively, were integrated into the chromosome and fluorescence intensities were measured after 5 h (early exponential phase), 24 h (mid-exponential phase), 30 h (stationary phase), and 48 h (late stationary phase) in the *P. luminescens* strains TT01-1°, TT01-2°, TT01-1°Δ*hexA*. Error bars represent standard deviation of three independently performed experiments.

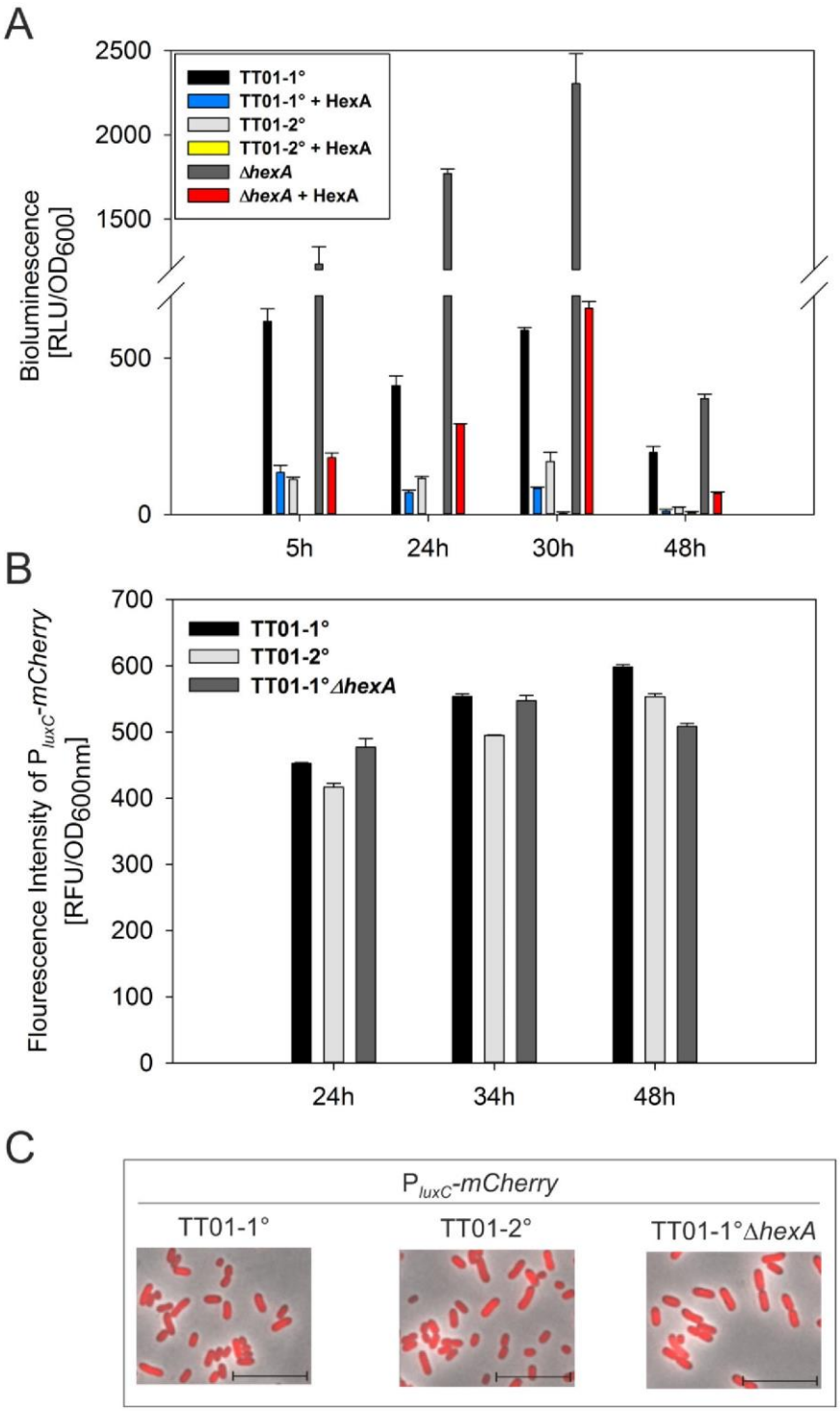


**Fig. 2: Effect of HexA on the bioluminescence of *P. luminescens*.** Bioluminescence in *P. luminescens* TT01-1°, TT01-2° and TT01-1°Δ*hexA* under native conditions and with

enhanced levels of HexA. Error bars represent standard deviation of three independently performed experiments (A). Promoter activity of *luxCDABE* at the population level. The respective reporter construct $P_{luxC}$-*mCherry* was integrated into the chromosome and fluorescence intensities were measured after 5 h (early exponential phase), 24 h (mid-exponential phase), 30 h (stationary phase) and 48 h (late stationary phase) in the strains TT01-1°, TT01-2°, TT01-1°-Δ*hexA* (B). $P_{luxC}$ activity in strains TT01-1°, TT01-2° and TT01-1°Δ*hexA* at the single cell level. The scale depicts 10 µM. Representative images from one of three independently performed experiments are shown (C).

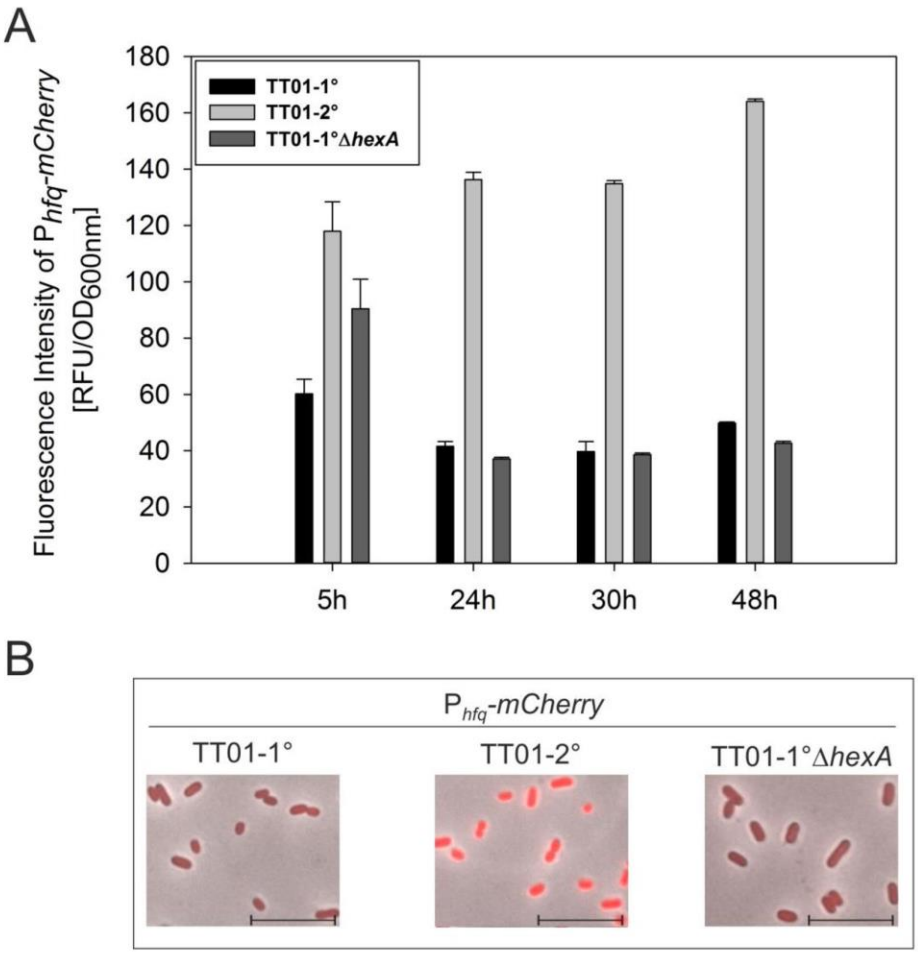


**Fig. 3: Transcriptional levels of *hfq* in *P. luminescens* TT01-1°, TT01-2° and TT01-1°Δ*hexA***. Promoter activity of *hfq* at the population level. The respective reporter construct $P_{hfq}$-*mCherry* was integrated into the chromosome and fluorescence intensities were measured after 5 h (early exponential phase), 24 h (mid-exponential phase), 30 h

(stationary phase) and 48 h (late stationary phase) in the strains TT01-1°, TT01-2°, TT01-1°-Δ*hexA*. Error bars represent standard deviation of three independently performed experiments (A). $P_{hfq}$ activity in TT01-1°, TT01-2° and TT01-1°Δ*hexA* at the single cell level. The scale depicts 10 µM. Representative images from one of three independently performed experiments are shown (B).

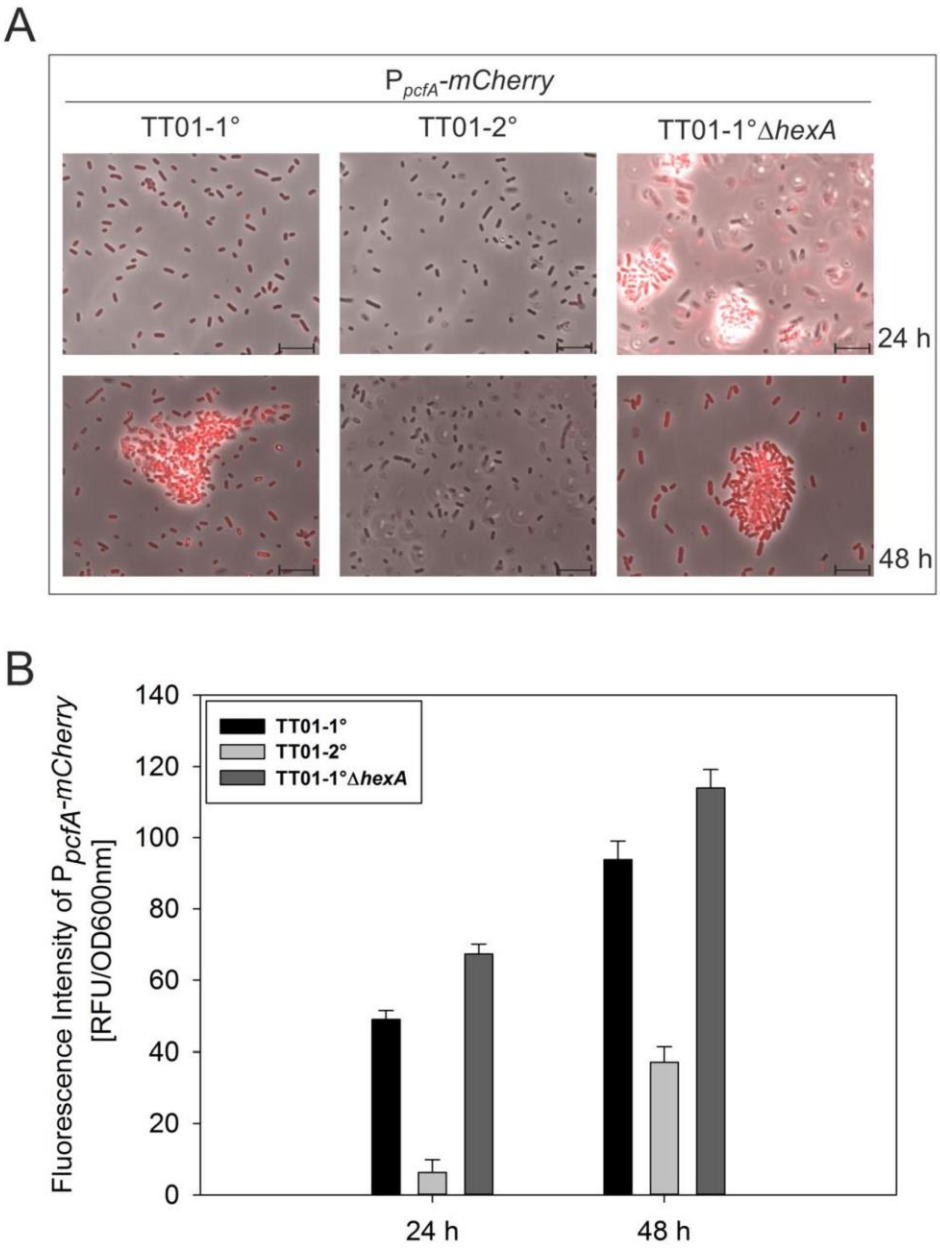

**Fig. 4: Cell clumping in *P. luminescens* TT01-1°, TT01-2° and TT01-1°*ΔhexA*.** $P_{pcfA}$-*mCherry* activity and cell clumping in TT01-1°, TT01-2° and TT01-1°Δ*hexA*. The scale depicts 10 µM. Representative images from one of three independently performed experiments are shown (A). Promoter activity of *pcfABCDEF* at the population level in TT01-1°, TT01-2° and TT01-1°Δ*hexA*. Error bars represent standard deviation of three independently performed experiments (B).

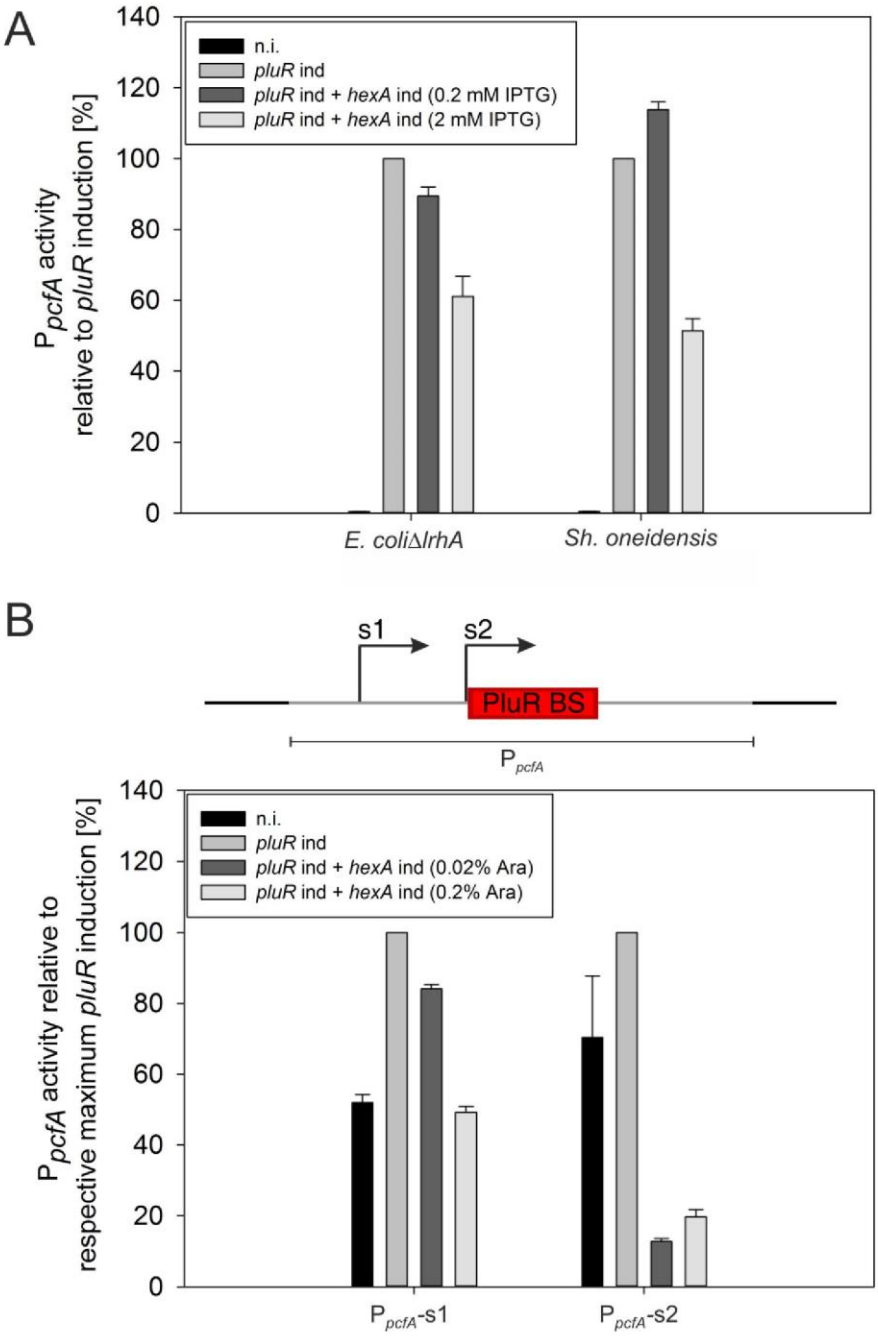


**Fig. 5: Effect of HexA on the $P_{pcfA}$ activity in the heterologous systems of *E. coli* Δ*lrhA* and *Sh. oneidensis*.** *E. coli ΔlrhA and Sh. oneidensis* were transformed with

plasmids pBAD24-P*ara*-*pluR*_P*lac*-*hexA* and pACYC-P*lac*-*hexA*_P*ara*-*pluR*, respectively, in combination with plasmid pBBR-P*pcfA*-*lux*. In *E. coli* Δ*lrhA* the *pluR* expression was achieved via the addition of 0.1% (w/v) arabinose and in *Sh. oneidensis* 0.02% (w/v) arabinose was added for *pluR* expression. The values were measured as relative light units [RLU] divided by $OD_{600nm}$ (A). In the upper panel the promoter region of *pcfA* with the PluR binding site (PluR BS) is depicted. In *E. coli* Δ*lrhA* two different truncations s1 and s2 of the *pcfA* promoter were tested. Thereby, *pluR* induction was achieved via the addition of 1 mM IPTG and *hexA* expression was induced via 0.02% (w/v) or 0.2% arabinose (w/v) on plasmid pBAD24-P*lac*-*pluR*_P*ara*-*hexA* (B). The figures represent three biological replicates; n.i.: non-induced, ind: induced; All values are given in percentage, relative to the respective maximum *pluR* induction.

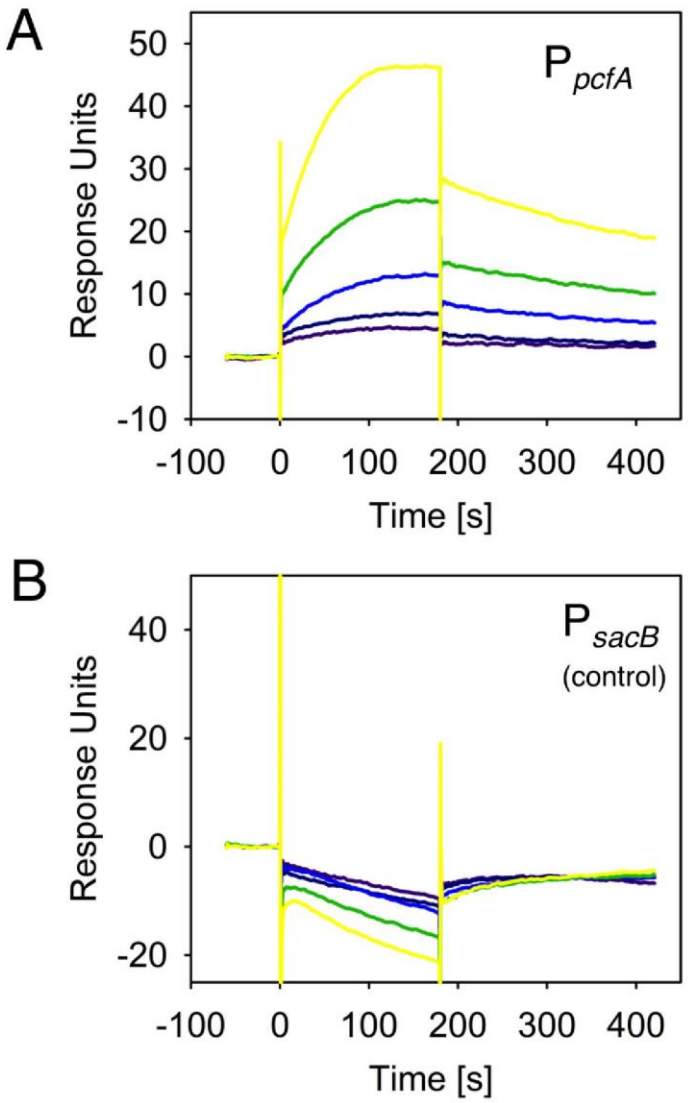


**Fig. 6: Direct binding of HexA to the *pcfA* promoter region.** The biotinylated $P_{pcfA}$ DNA-fragment was captured onto a streptavidin-coated (SA) sensor-chip. Different concentrations of His-tagged HexA (125 nM: purple line; 250 nM: dark blue line; 500 nM: light blue line; 1000 nM: green line; 2000 nM: yellow line) were passed over the chip. An overall affinity of $K_D$ 1.3 µM was determined, the association and dissociation rates were determined as $k_a$=1300 M*s and $k_d$=0.002 1/s, respectively. As a negative control for unspecific binding, the SA chip was coated with a *sacB* DNA fragment.

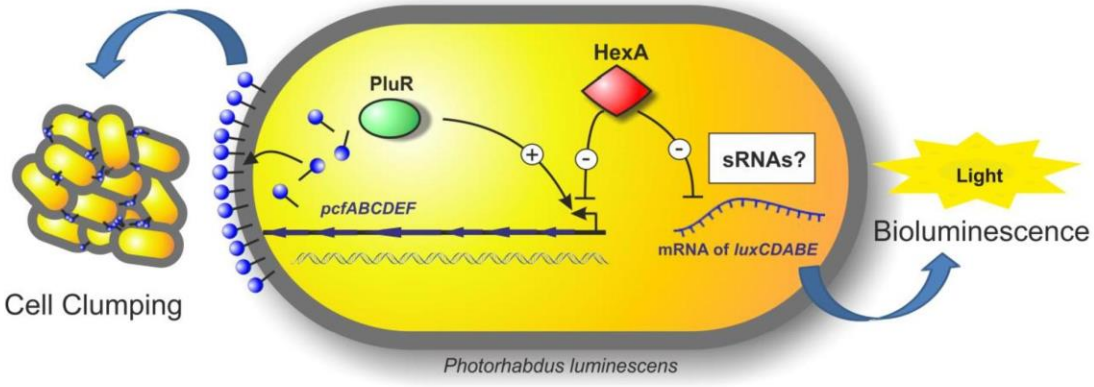


**Fig. 7: Model of the versatile role of HexA controlling 1° and 2° specific phenotypes in *P. luminescens.*** HexA directly represses the promoter of the *pcfABCDEF* operon, which is responsible for the formation of cell clumps, and indirectly represses the translation of *luxCDABE,* presumably via sRNAs, and thereby diminishes light production.

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# 5 Social biological relevance of the two phenotypic cell forms for the population of *Photorhabdus luminescens*

## 5.1 Introduction

*Photorhabdus luminescens* is an ideal bacterium to study phenotypic heterogeneity as it exhibits two different phenotypic forms, the primary (1°) and secondary (2°) cells. *P. luminescens* is symbiotically associated with insect pathogenic nematodes of the family Heterorhabditidae and can be found within the gut of the nematode in its infective juvenile (IJ) stage, when it actively seeks out for insect larvae e.g. *Galleria mellonella* in the soil. Upon invasion of the nematode into the larva, the bacteria get released into the hemolymph of the insect, replicate rapidly and start to produce toxins, exoenzymes and antibiotics. This causes the death of the larva, enables the supply of nutrients and prevents other bacteria from invading (Ciche & Ensign, 2003; Daborn *et al.*, 2001). The bacteria and the nematodes feed upon the nutrients and the presence of the bacteria is mandatory for growth and development of the nematodes. When all the nutrients are depleted the bacteria reassociate with the nematode and the nematode-bacteria-complex leaves the cadaver to search for new insect prey (Han & Ehlers, 2000; ffrench-Constant *et al.*, 2003).

Prolonged cultivation of the 1° form, which is considered as the wild-type, leads to a conversion of individual cells into the 2° form. The 2° form lacks characteristic 1°-specific features of *P. luminescens*, like the production of crystal proteins, antibiotics and proteases and pigmentation (Akhurst, 1980; Boemare & Akhurst, 1988; You *et al.*, 2006). Additionally, besides still being capable of killing insect larvae, the 2° cells are no longer able to live in mutualistic interaction with the nematodes (Han & Ehlers, 2001).

The pigmentation is a result of so called anthraquinones, which are produced via a polyketide synthase II and several modifying enzymes, encoded by the *antABCDEFGHI* operon (Richardson *et al.*, 1988, Brachmann *et al.*, 2007). Recently, it has been found, that the regulator AntJ activates the expression of this operon and artificial overproduction of AntJ could lead to the production of anthraquinones in the usually non-pigmented 2° form. This is a clear indication that the non-pigmentation and presumably also the lack of other primary-specific phenotypes in the 2° cells are mediated via regulation rather than due to metabolic conditions that prevents their production (Heinrich *et al.*, 2016).

HexA, which is a member of the LysR transcriptional regulators, is supposed to play an important role in phenotypic switching as a deletion of *hexA* in the 2° cells of *P. temperata* led to the restoration of many distinct 1°-specific features (Joyce & Clarke, 2003). Until now,

this switch has only been observed unidirectional, from the 1° to the 2° form. However, as an infrequent reversion of the switch has been observed in the closely related species *Xenorhabdus*, the form might be trapped in this state under laboratory conditions possibly due to the lack of a certain signal (Forst & Clarke, 2002). The biological role of the different phases remains unknown but an advantage of the 2° cell at a free-living stage is suggested. The nematode-bacteria-complexes are of use in the agricultural industry to prevent crop failure and as the occurrence of the phenotypic switching causes loss of efficacy it is important to understand the reason for phenotypic heterogeneity in *Photorhabdus* species (Han & Ehlers, 2001).

## 5.2 Material and Methods

### 5.2.1 Materials

Strains used in this study are listed in Table 5-1, plasmids are listed in Table 5-2 and primers are listed in Table 5-3.

PCR was performed using Q5 Polymerase and OneTaq Polymerase from New England Biolabs (Frankfurt, Germany). Restriction enzymes and T4 DNA ligase were also taken from New England Biolabs. Plasmid isolations were performed using the HiYield Plasmid Mini Kit and DNA fragments were purified via the HiYield PCR DNA Fragment Extraction Kit (Süd-Laborbedarf, Gauting, Germany). Genomic DNA was isolated using the Ultra-Clean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, USA). Sequencing was performed in the Genomics Service Unit of the LMU Munich.

**Table 5-1 Bacterial Strains**

| Bacterial Strain | Genotype | Reference |
|---|---|---|
| *P. luminescens* subsp. laumondi TT01-1° | Wild-type 1° variant | (Duchaud *et al.*, 2003) |
| *P. luminescens* subsp. laumondi TT01-2° | Wild-type 2° variant | Lab collection, Dr. David Clarke, University College Cork |
| *P. luminescens* TT01-1°-Gent$^{R}$ | TT01-1° harboring $P_{less}$-*mCherry* reporter integrated at the *rpmE*/*glmS* | (Glaeser & Heermann, 2015) |

| | | |
|---|---|---|
| | site, $Kan^R$, $Gent^R$ | |
| *P. luminescens* TT01-2° - $Cam^R$ | TT01-2° harboring $Cam^R$ cassette integrated at the *rpmE*/*glmS* site, $Kan^R$, $Cam^R$ | This study |
| *E. coli* Dh5α λ*pir* | *recA1, gyrA (lacIZYA-argF) (80d lac [lacZ] M15) pir RK6* | (Miller & Mekalanos, 1988) |
| *E. coli* ST18 | *E. coli* S17 *λpir* Δ*hemA* | (Thoma & Schobert, 2009) |

**Table 5-2 Plasmids**

| **Plasmid** | **Genotype** | **Reference** |
|---|---|---|
| pPINT | $Km^R$, *rpmE* and *glmS* site of *P. luminescens* in PNPTS-138-R6KT | (Glaeser & Heermann, 2015) |
| pPINT-*mCherry* | $Km^R$, $Gm^R$ and *mCherry* in pPINT | (Glaeser & Heermann, 2015) |
| pPINT-$Cm^R$ | $Km^R$, $Cm^R$ in pPINT | This study |
| pBAD33 | $Cm^R$, arabinose inducible pBAD promoter, p15A origin | (Guzman *et al.*, 1995) |

**Table 5-3 Oligonucleotides**

| **Primer name** | **Sequence (5`-3`)** |
|---|---|
| CmR-PstI_fwd | GCTCTGCAGAGCCAGTATACACTCCGC |
| CmR-EagI_rev | GCGCGGCCGATTACGCCCCGCCCTGCCACTC |
| check-CmR_fwd | CTGGTTTCATAATTTCGCC |
| check pNPTS-FA FB_rev | GTAAAACGACGGCCAGTCC |
| check-rpmE_fwd | CTCCCAAATAAAGTTTAGG |
| check-glmS_rev | GTACGTGAATCTGATTTTG |
| oriT_fwd | CAGGGTTATGCAGCGGAAA |

## 5.2.2 Bacterial strains and growth conditions

*P. luminescens* was cultivated aerobically at 30°C and *E. coli* was grown aerobically at 37° in lysogenic broth (LB) (10g NaCl, 10 g/l tryptone, 5 g/l yeast extract) on a rotary shaker. For

preparation of agar plates, 1.5% agar was added to the medium. If necessary, the medium was supplemented with 50 µg/ml kanamycin, 15 µg/ml gentamicin or 20 µg/ml chloramphenicol. When *E. coli* ST18 was cultivated, 50 µg/ml 5-aminolevulinic acid was added. Pre-cultures were grown overnight and inoculated at an $OD_{600}$ of 0.05 in fresh medium.

### 5.2.3 Plasmid generation

A PCR with the primers CmR-PstI_fwd and CmR-EagI_rev was performed to amplify the chloramphenicol resistance cassette using the template pBAD33. After subsequent restriction of the insert and the plasmid pPINT with the enzymes PstI and EagI, the ligation was performed. The correct insertion of the DNA fragment into the vector backbone was checked via sequencing with the primers check-CmR_fwd and check pNPTS-FA FB_rev.

### 5.2.4 Competent cells and transformations

*E. coli* cells were made chemically competent and transformed as described elsewhere (Inoue *et al.,* 1990).

### 5.2.5 Integration of reporter genes into the *P. luminescens* genome

For the integration of the chloramphenicol resistance cassette into the genome of *P. luminescens* TT01-2°, the donor strain *E. coli* ST18 (Thoma & Schobert, 2009), which requires the addition of 5-aminolevulinic acid for growth, was first transformed with pPINT-CmR. The conjugative plasmid transfer was achieved via the filter mating method (Thoma & Schobert, 2009). Therefore, the donor as well as the recipient strain were cultivated up to an $OD_{600}$ of 0.8-1 in LB medium, which was supplemented with the respective additives if required. The donor strain was washed in LB medium for 3 times and subsequently mixed with the recipient strain in a ratio of 1:5 in a final volume of 1/10 of the donor`s initial volume. Cells were mixed and dropped onto a nitrocellulose filter, which had been positioned onto an LB agar plate. After the incubation at 30°C over night, the cells were resuspended in 500 µl LB and spread onto LB agar plates containing chloramphenicol and incubated for two days at 30°C. Genomic DNA of single colonies was used as a template to check for chromosomal integration of the plasmid via PCR (check-rpmE_fwd, check-glmS_rev, oriT_fwd).

### 5.2.6 Growth analysis of 1° and 2° cells

Pre-cultures of the two strains *P. luminescens* TT01-1°-$Gm^R$ and TT01-2°-$Cm^R$ were grown in LB over night at 30°C. At the next day, 200 ml of each culture was inoculated at an $OD_{600}$ of 0.05. In order to obtain different ratios of TT01-1°-$Gm^R$ and TT01-2°-$Cm^R$, different volumes of these two cultures were mixed to reach the final volume of 50 ml as seen in Table 5-4.

**Table 5-4 Protocol for obtaining different ratios of *P. luminescens* TT01-1° and TT01-2°**

| **Proportion of TT01-1° [%]** | **TT01-1°-$Gm^R$** | **TT01-2°-$Cm^R$** |
|---|---|---|
| **5%** | 2.5 ml | 47.5 ml |
| **25%** | 12.5 | 37.5 ml |
| **50%** | 25 ml | 25 ml |
| **75%** | 37.5 ml | 12.5 ml |
| **95%** | 47.5 ml | 2.5 ml |
| **control 0%** | 0 | 50 ml |
| **control 100%** | 50 ml | 0 ml |

The mixed cultures were grown at 30°C while shaking and samples were taken in order to measure growth and bioluminescence. Furthermore, the cultures were plated on $LB_{Km}$ plates with serial dilutions to obtain individual colonies. At least 50 colonies for each condition were striked on $LB_{Gm}$, $LB_{Cm}$ and $LB_{Km}$ plates. The bioluminescence and the pigmentation of the colonies were checked.

To test the composition of TT01-1°-$Gm^R$ and TT01-2°-$Cm^R$ under stress conditions, 25 ml of the respective medium were inoculated with an initial $OD_{600}$ of 0.05 of each strain and then mixed. For nutrient limitation, the media was tenfold diluted. The oxidative stress was induced upon the addition of a final concentration of 0.3% (v/v) hydrogen peroxide.

## 5.3 Results

### 5.3.1 2° cells outcompete 1° cells in the stationary phase

In order to investigate, if one phenotypic cell type of *P. luminescens* influences the respective other one with respect to growth, 1° and 2° cells were mixed at different ratios and analyzed if one cell type is able to outgrow or outcompete the respective other one. As a first step we

made sure that the different cell types showed comparable growth behavior when cultivated separately (Figure 5-1).

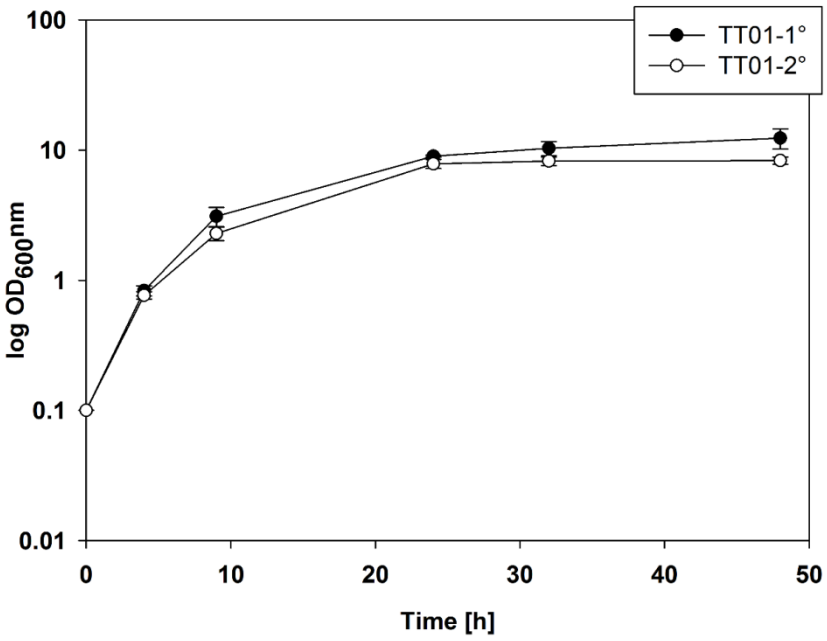


**Figure 5-1: Growth behavior of *P. luminescens* TT01-1° and TT01-2°.** Depicted is the growth of *P. luminescens* TT01-1° and TT01-2° for 48 hours. The growth is given in optical density at 600 nm on a logarithmic scale. The standard deviation was calculated from three independently performed experiments.

The 1° cells were tagged with a gentamicin resistance cassette and the 2° cells with a chloramphenicol resistance cassette. Additionally, both engineered strains are kanamycin resistant. The two cultures were set to an $OD_{600}$ of 0.05 separately and then mixed at different ratios. The cultures were plated every day and single colonies were isolated and transferred to check for gentamicin or chloramphenicol resistance. In Figure 5-2, the initial composition of the cultures at day 0 is indicated. When the 1° and 2° cells were initially mixed in a 1:1 ratio (50%), the culture was composed of 94% 1° cells and only 6% 2° cells after one day of cultivation, which reflects the exponential growth phase. After two days, reflecting the stationary growth phase, the situation was completely converted and at an initial 1:1 mixture, only 8% of 1° cells were present and 92% of 2° cells were detected. After 3 and 4 days of cultivation, only in the initial 95% 1° mixture, the 1° cells were still well represented with 60% after 3 days and 49% after 4 days. When analyzing the initial 95% composition after 5 days of growth, 1° cells only contributed with 4% to the overall population. However, they still grew in the control sample, which exclusively consists of 1° cells.

It is important to mention that none of the cell types converted to the respective other one during the experiment. The isolated colonies were investigated according to their phenotypic

characteristics like pigmentation and bioluminescence and as their initial phenotypic state was marked via different antibiotic cassettes we could observe that none of the cells performed a phenotypic switch.

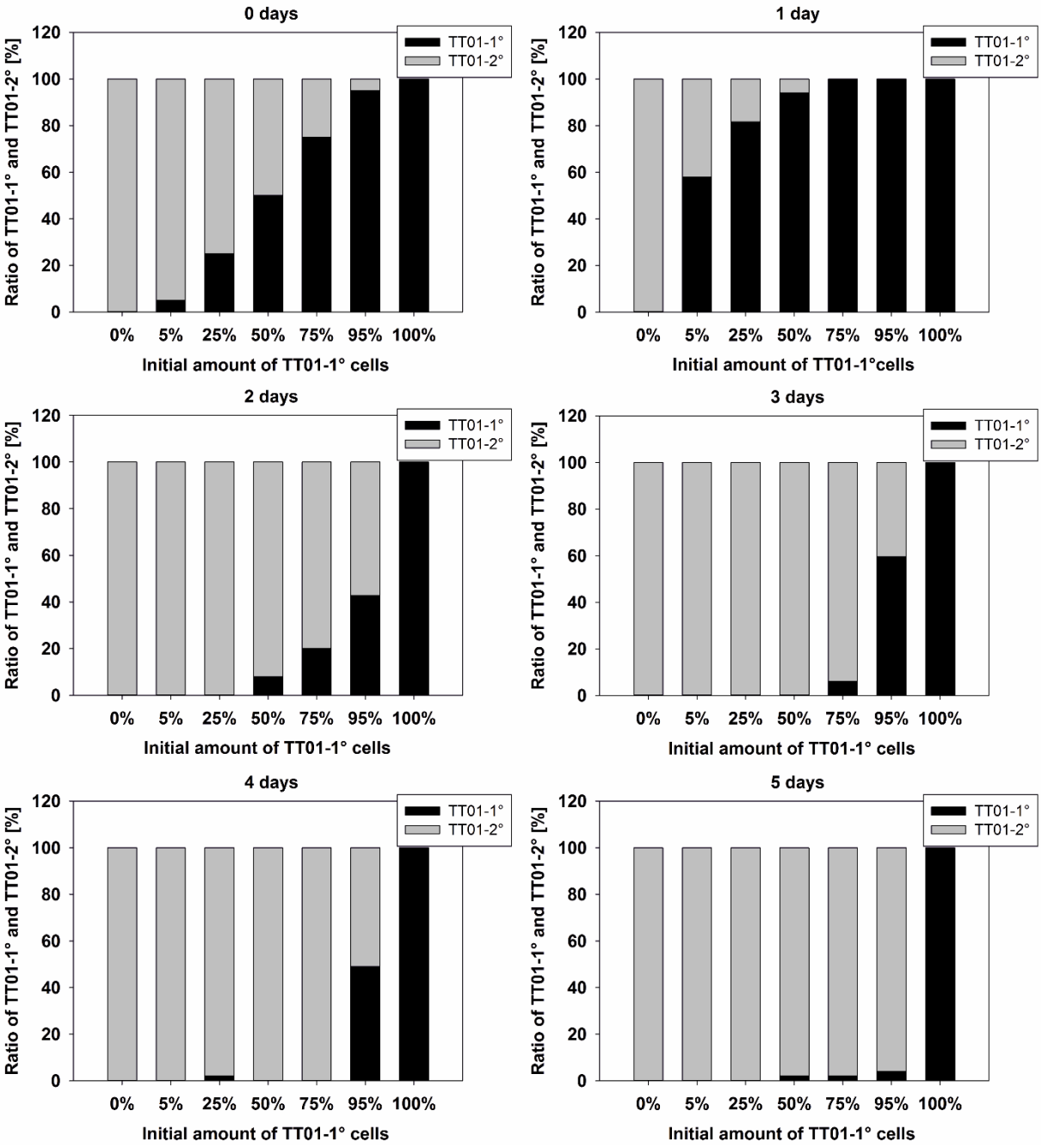


**Figure 5-2: Growth competition assays of *P. luminescens* cultures consisting of different 1° and 2° cell ratios**. Tagged 1°-$Gm^R$ and 2°-$Cm^R$ cells were mixed at different ratios and the cultures were plated after one, two, three, four and five days of growth. Single clones were then checked for their antibiotic resistances on plates containing gentamicin, chloramphenicol and kanamycin. The results represent one characteristic of three independent biological experiments.

### 5.3.2 Influence of stress conditions on the growth behavior in a growth competition assay of 1° and 2° cells

In order to investigate why 1° cells seem to be better adapted in the exponential phase, whereas 2° cells clearly show an advantage in the stationary phase, we were interested how nutrient availability as well as oxidative stress influence the growth advantages of 1° and 2° cells, respectively.

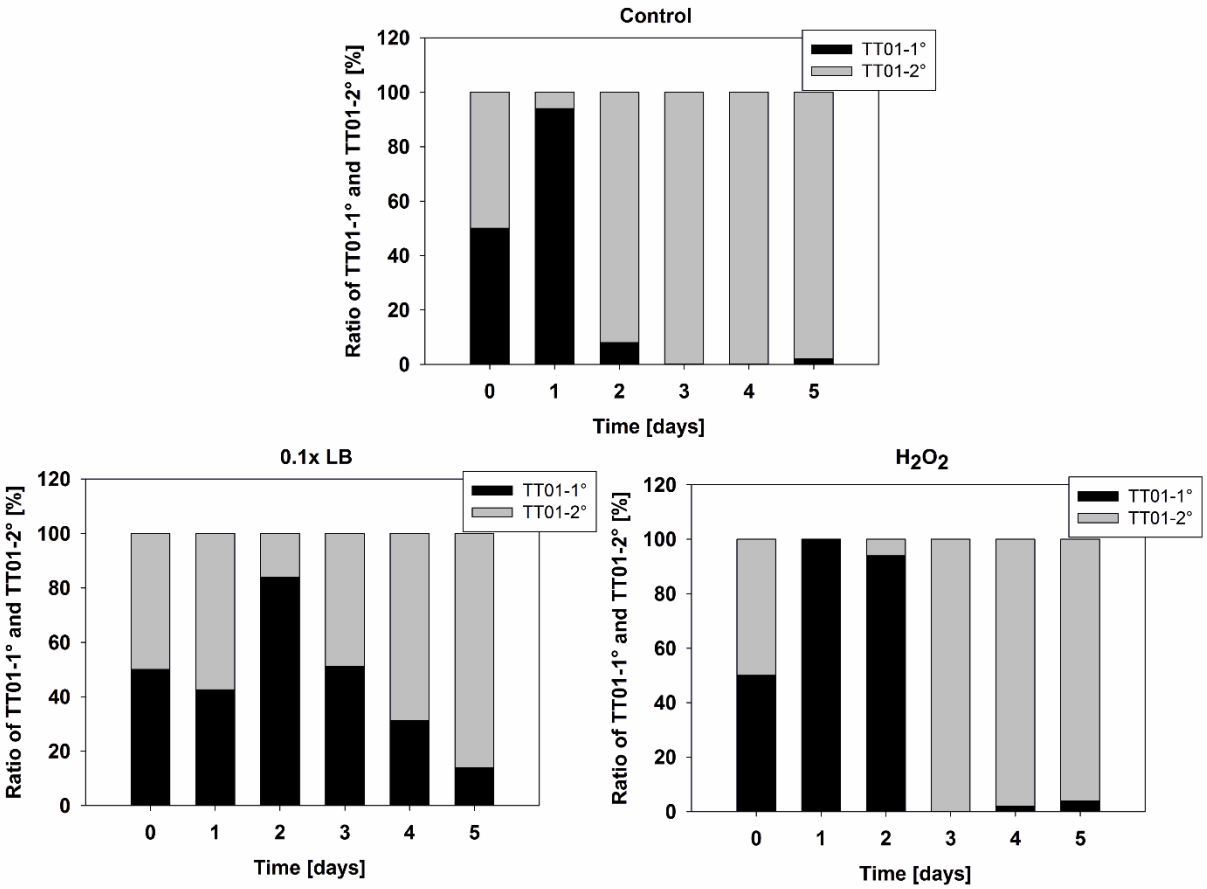


**Figure 5-3: Influence of nutrient limitation and oxidative stress on *P. luminescens* cultures consisting of equal 1° and 2° cell ratios.** Tagged 1°-$Gm^R$ and 2°-$Cm^R$ cells were mixed at ratio 1:1 (50%) and inoculated in the different media: LB as control, tenfold diluted LB and LB containing 0.3% (v/v) $H_2O_2$. The cultures were plated after one, two, three, four and five days of growth. Single clones were then checked for their antibiotic resistances on plates containing gentamicin, chloramphenicol and kanamycin. The results represent one characteristic of three independent biological experiments.

The medium was diluted tenfold in order to observe if nutrient limitation as well as a resulting low osmolarity influence the growth advantages of 1° and 2° cells at the different growth phases. Furthermore, hydrogen peroxide was added to see a potential influence via oxidative stress. If the tenfold diluted complex medium was taken, the overgrowing effect of 1° cells in the exponential phase was delayed and only occurred on the second day. This might result from the slower growth due to a lack of nutrients. Hence, following the composition after

three, four and five days, a decrease of growing 1° cells could be observed in the cultures (51 %, 31 % and 14 %, respectively) comparable to the effect seen in the control (Figure 5-3). Upon addition of hydrogen peroxide, the majority of the culture did not only consist of 1° cells in the exponential growth phase but also in the early stationary phase after two days with 94%. Under oxidative stress it took three days until the 2° cells overgrow the 1° cells (Figure 5-3). In conclusion, a preferred growth of 1° cells can be seen in the exponential growth phase and a growth advantage in the stationary growth phase can be observed for 2° cells. Under nutrient limitation or oxidative stress no significant influence but simply a delay with respect to the observed phenomena can be detected.

## 5.4 Discussion

In 1980, the occurrence of two phenotypic cell types, referred to as primary (1°) and secondary (2°), within the populations of *Photorhabdus* and *Xenorhabdus* species were reported (Akhurst, 1980). The 2° cells arise after prolonged cultivation in a culture exclusively consisting of 1° cells. It was found that these two forms differ in various morphological and physiological traits. *P. luminescens* 2° cells show diminished levels of bioluminescence, a lack of antibiotic production and pigmentation and even though they are also pathogenic against insects, they are no longer able to support the symbiosis with nematodes (Boemare & Akhurst, 1988). As the usefulness of nematodes for the control of insect pests is entirely dependent on their bacterial symbiont, a phenotypic switching of 1° to 2° cells severely decreases the efficiency of the nematode-bacteria complex as agricultural weapon against insects (Han & Ehlers, 2001). Therefore, it is of major interest to elucidate the mechanism and role of the 2° variant. In this work, we were interested to get first insights into the socio-biological aspect of occurrence of the 2° cells in *P. luminescens* cell populations. We were wondering if one cell type influences the respective other one in growth at different growth phases. By tagging the phases with different antibiotic cassettes, we were able to trace back their origin upon co-cultivation. When taking different ratios of 1° and 2° cells (5%, 25%, 50%, 75%, 95%), we were surprised to observe that the 1° cells overgrew the 2° cells after 24 hours of cultivation, regardless of the initial composition (Figure 5-2). Thus, the 1° cells show a clear growth advantage in the exponential phase. However, when cultivating the cell types separately, no significant differences in the growth rates were observed (Figure 5-1). Interestingly, after two days, when the cells have reached the stationary phase, the phenomenon was completely reversed and the 2° cells overgrew the 1° ones, even if the culture initially consisted of only 5% 2° cells. This phenomenon also retained after three, four

and five days. Thus, after starvation, 2° cells seem to be able to regain growth much faster than 1° cells and outcompete them. These results are underlined by the observation that 2° cells recommenced growth 10 to 12 hours earlier than 1° cells after periods of starvation. It was assumed that a more efficient nutrient uptake is present in the 2° cells (Smigielski *et al.,* 1994). This is in accordance with the observed upregulation of the universal stress protein UspA in the 2° form (Turlin *et al.,* 2006). UspA is known to play an important role in the recovery of *E. coli* following starvation of nutrients (Siegele, 2005).

However, upon nutrient limitation in combination with hypoosmotic stress, the previously observed phenomenon was delayed, probably due to the slower growth of the cells (Figure 5-3). Therefore, the nutrients that are left in a tenfold diluted complex medium can still be consumed by 1° cells in the exponential phase and lead to enhanced growth in comparison to 2° cells.

UspA is also required in the defense against superoxide-generating agent, however, the addition of hydrogen peroxide to the mixed 1°: 2° culture did not trigger a growth advantage of 2° cells in the exponential growth phase (Figure 5-3). Moreover, in the presence of hydrogen peroxide, the 2° cells only overgrew the 1° cells after 3 days, one day later as in the control.

The obtained results of delayed outgrowth of 2° cells upon co-cultivation with 1° cells under stress conditions favor the theory of a specific adaption of 2° cells upon entry into the stationary growth phase. 2° cells do not produce a wide variety of the secondary metabolites and are not able to assist nematode growth and development, which supports the fact that mutualism is dependent on the production of secondary metabolites (Han & Ehlers, 2001; Lango & Clarke, 2010). As secondary metabolites are typically produced during the stationary growth phase and the synthesis is very cost-intensive, these cells might be able to save energy and are therefore better adapted to starvation conditions.

The occurrence of 1° and 2° cells might be a typical form of bet-hedging, having one sub-population that can withstand nutrient limitation better than the other one. After depletion of all the nutrients in the insect cadaver, the 1° cells reassociate with the nematodes and are taken to a new insect larva, which again provides a nutrient-rich environment. Meanwhile, the 2° cells stay in the soil and can quickly recommence growth upon availability of nutrients.

Bet-hedging is a typical phenomenon of phenotypic heterogeneity. Under challenging environmental conditions, the production of subpopulations with variable phenotypes enhances the chance that at least one will be adapted under the given situation (Cohen, 1966). Bacterial persistence is one of the most prominent examples of a bacterial bet-hedging

strategy. Thereby, persister cells enter a transient growth arrest state that can survive antibiotic treatment. Once they start to grow those cells are antibiotic-sensitive whereas a small subpopulation consists of persisters. The switch from normal growth to persisters and *vice versa* is due to stochastic and epigenetic events (Balaban *et al.,* 2004, Veening *et al.,* 2008). So far, elevated levels of the regulator HexA have been found in the 2° cells of *P. luminescens* (Glaeser *et al.,* 2016) Therefore, stochastic events might lead to the repression of 1°-specific features by enhancing the copies of HexA within the cell. Until now, not much is known about the regulation of *hexA* expression, yet Hfq was recently found to influence the expression of *hexA* (Tobias *et al.,* 2016; Glaeser *et al.,* 2016). However, as the phenotypic switching of *P. luminescens* is a rather rare event that only occurs after prolonged cultivation and needs at least seven days, this might prevent the population from exclusively becoming 2°-like under native conditions, which would be fatal for the bacteria´s life cycle.

In summary, this work gives a first insight into the differences of 1° and 2° cells of *P. luminescens* in growth upon co-cultivation. It remains to be determined what exactly causes the growth advantage of the 2° cells in the stationary growth phase and various stress conditions have to be tested to shed more light onto this observation.

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# 6 Concluding Discussion

*Photorhabdus luminescens* is an ideal organism to study phenotypic heterogeneity as it occurs in two distinct phenotypic forms, the primary (1°) and secondary (2°) cells. So far, it has been elucidated that 1°-specific features like pigmentation, bioluminescence and symbiosis are absent in the 2° form and that the regulator HexA somehow plays a role in the occurrence of the 2° phenotype (Boemare & Akhurst, 1988; Joyce & Clarke, 2003). In this work, the regulation of phenotypic heterogeneity in *P. luminescens* was investigated. As a first step, a single-cell analysis tool was established, using a fluorescent reporter system to elucidate transcription and/or translation of genes of interest (Chapter 2). In the course of this work, the regulator AntJ of the anthraquinone (AQ) biosynthesis cluster, which is responsible for the 1°-specific phenotype pigmentation, could be identified. AntJ was found to be heterogeneously activated within a population consisting of 1° cells, whereas basal homogeneous activation with comparable AntJ levels could be determined for 2° cells, suggesting the role of a putative ligand binding to AntJ (Chapter 3). Similar promoter activity of *luxCDABE* in 1° and 2° cells revealed that the regulator HexA indirectly represses light production in 2° cells. However, the *pcf* operon mediating cell clumping in 1° cells was found to be directly repressed by HexA, revealing that HexA is a versatile regulator of phenotypic switching (Chapter 4). Finally, this work provides first insights into the socio-biological relationship of 1° and 2° cells and reveals that 2° cells are better adapted to starvation conditions in the stationary growth phase than 1° cell. Thus, they might be better prepared to a life in the soil, independently from the nematode partner (Chapter 5).

## 6.1 Heterogeneity in the regulation of secondary metabolism

Secondary metabolites are products, which are not essential for survival but likely confer evolutionary advantage to the producer organism and include communication molecules, nutrient transport compounds or competitive weapons (O'Connor, 2015). Nowadays, secondary metabolites are widespread in industrial and pharmaceutical applications. One famous source of medical compounds is found within *Streptomyces* species, which are known to produce antibiotics (e.g. daptomycin), but also immunosuppressants (e.g. rapamycin), antifungals (e.g. amphotericin B), anticancers (e.g., doxorubicin), and antiparasitics (e.g. ivermectin) (Hwang *et al.*, 2014).

Biosynthesis enzyme complexes like polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS), whose products often have functions as antibiotics, pigments,

siderophores or toxins are known to be the largest enzymes in nature and can comprise sizes in the megadalton range (Fischbach & Walsh, 2006; Wang *et al.*, 2014).
6.5% of the *P. luminescens* genome contains genes, which are predicted to play a role in secondary metabolism and include at least 23 biosynthesis gene clusters (Duchaud *et al.*, 2003; Bode, 2009). The best characterized natural products are the stilbene-based antibiotic 3,5 dihydroxy-4-isopropylstilbene, bioluminescence and the anthraquinone (AQ) pigment (Nealson & Hastings, 1979; Joyce *et al.*, 2008; Brachmann *et al.*, 2007).

The *antABCDEFGHI* operon encodes a type II polyketide synthase and several modifying enzymes and has been identified to be responsible for the production of the secondary metabolites AQs (Brachmann *et al.*, 2007).
The newly identified transcriptional regulator AntJ is responsible for the direct activation of the AQ operon and the AntJ DNA-binding site could be determined consisting of the two redundant sequences AATGCT, which are separated by a 28 bp long spacer (Chapter 3, Figure 1, 2 and 3). Upon substitution of the spacer region, AntJ could no longer activate the promoter of the AQ operon suggesting an additional binding sequence within this region (Chapter 3, Figure 3).
Besides AntJ, the LysR-type regulator Plu2548 and the LuxR like protein Plu0919 bind to the promoter of *antA* but a deletion of these two genes did not alter the pigmentation (Chapter 3, Figure 1). Plu0919 contains a PAS4 domain, which shows homology to the PAS3 domain of insects (Brameyer *et al.*, 2014; Heermann & Fuchs, 2008). PAS3 domains in *Drosophila melanogaster* are known as insect juvenile hormone receptors and Plu0919 might therefore recognize insect-specific signals (Dubrovsky, 2005). Thus, this regulator might only have an influence on the AQ production in the insect host contributing to a more complex regulation of pigment production.
In the absence of the global regulator HfdR, *P. luminescens* is not only incapable of realizing the transmission of its symbiont but is also less pigmented due to the down-regulation of the *antABCDEFGHI* operon (Easom & Clarke, 2012). As HfdR was not co-eluted with $P_{antA}$ in the DNA affinity chromatography assay, an indirect regulation is suggested (Chapter 3, Figure 1).

To study phenotypic heterogeneity, techniques to discriminate gene activity at the single-cell level within a population are required. Therefore, a suitable tool is the use of fluorescent reporters, which can be analyzed by fluorescence microscopy or flow cytometry.

Furthermore, the destiny of a single cell can be tracked via time-lapse microscopy (Smits *et al.*, 2006). The use of plasmid-based reporter systems is often preferred as it is timesaving and due to multiple copies of the fluorescent reporter, higher signal intensity can be achieved for weak gene expression. However, upon transformation of a plasmid in *P. luminescens*, the plasmid-encoded gene was only expressed in part of the population and an unequal division of the plasmid was postulated (O'Neill *et al.*, 2002). Therefore, plasmids do not seem to be useful for single-cell analysis. In this thesis, a novel integration tool for *Photorhabdus* was established, in order to chromosomally encode genes and/ or reporters. Thereby, only one copy of the desired reporter gene is inserted and it could be shown that the use of plasmids falsifies the results as the reporters are not being expressed in every single cell despite being controlled by homogenously active promoters (Chapter 2, Figure 2 and Figure 4).

Via this novel tool a heterogeneous activation pattern of the *antACDEFGHI* operon could be detected in 1° cells of *P. luminescens* (Chapter 2, Figure 2.

AntJ is a member of the WYL-domain transcription factors with a truncated WYL-domain at the C-terminus, which is predicted to contain a putative ligand-binding domain, whose ligand remains unknown. Even though the native levels of AntJ drive the heterogeneous activation of the AQ biosynthesis cluster in 1° cells, *antJ* expression was not observed to be heterogeneously distributed within a population (Chapter 3, Figure 4 and Figure 5).

This is why it is postulated that not only the low levels of the regulator itself but in particular the native levels of a potential ligand binding to AntJ contribute to the heterogeneity of AQ production. In comparison, 2° cells are not capable of AQ production and the AQ operon was found be activated in a basal, homogeneous manner, whereas the AntJ levels are comparable between 1° and 2° cells (Chapter 3, Figure 4 and 5, Figure 6-1). Only drastic overproduction of AntJ could force 2° cells to produce AQs (Chapter 2, Figure 2). Thus, an activating ligand might be absent or an inhibiting ligand might be over-represented in the 2° cells, causing the incapability of AQ production. Which one of the two types of ligand is actually responsible has yet to be determined. Additionally, the role of a putative yet unidentified repressor of the *ant* operon cannot be excluded.

The primary metabolism significantly influences secondary metabolism as it provides the precursor metabolites and reducing equivalents (Rokem *et al.*, 2007). Acetyl-CoA, generated from central carbon metabolism, and malonyl-CoA are the starter units for AQ synthesis and the biochemistry underlying the biosynthesis of polyketides is closely related to the synthesis of fatty acids (Brachmann *et al.*, 2007). The production of AQ and stilbene (ST) increase

upon addition of L-proline (Crawford *et al.,* 2010). As L-proline is present in high concentrations in the hemolymph of the insects, L-proline might act as host-specific signal (Wyatt, 1961). The activity of proline dehydrogenase (encoded by *putA*) is responsible for the increased ST production upon L-proline addition by converting proline to glutamate and generating NADH. Glutamate can then be assimilated through the tricarboxylic acid (TCA) cycle (Crawford *et al.,* 2010). Upon deletion of the *mdh* gene, encoding the malate dehydrogenase of the TCA cycle, *P. luminescens* is no longer capable of stilbene, AQ and light production and even though virulence remains unaffected, the symbiosis with the nematodes is impaired (Lango & Clarke, 2010). Therefore, the TCA cycle is required for the transition from the pathogenic to the mutualistic phase of *Photorhabdus* and its production of secondary metabolites. The secondary metabolite ST has not only been stated as antibiotic but was also found to be important for the support of nematode growth and development (Joyce *et al.,* 2008). The phenylalanine-ammonium lyase StlA is responsible for the non-oxidative deamination of phenylalanine resulting in the production of cinnamic acid (Williams *et al.,* 2005). Mutants, which are incapable of ST production due to a deletion in the *stlA* gene, show severely increased AQ pigmentation, whereas the addition of cinnamic acid could restore native AQ production (personal communication with Helge Bode, Goethe-University Frankfurt, Frankfurt). Thus, a link between ST and AQ production and presumably other secondary metabolites might be possible. A ST precursor might therefore be the yet unidentified ligand of AntJ. This mechanism would ideally fit into the model of division of labor, to make sure that one cell does not have to produce all potential secondary metabolites. However, to date neither phenylalanine nor cinnamic acid could be identified as the ligand of AntJ (Angela Glaeser and Ralf Heermann, unpublished).

As *Photorhabdus* produces a large number of secondary metabolites, the synthesis of all secondary metabolites by a single cell would cost a large proportion of its resources. It seems more favorable, if the tasks are split within a population, meaning that sub-populations produce different secondary metabolites to share the costs and benefits. This division of labor is a popular strategy of heterogeneity and can be observed in biofilm formation of *Bacillus subtilis*. The exopolysaccharide operons *epsA-O* and *yqxM* are negatively regulated via the repressor SinR. Repression by SinR is relieved upon binding of the small antagonist SinI (Branda *et al.,* 2006). It was found that even in biofilm-inducing media, only a small minority of cells (2%) express *sinI*. Furthermore, the heterogeneous gene expression of *sinI* results from the phosphorylation state of the transcriptional regulator Spo0A, which causes the expression of biofilm matrix only in a small subpopulation (Chu *et al.,* 2006). It could be

demonstrated in the past that the matrix components can be shared and distributed throughout the community and therefore a typical division of labor is displayed, which is advantageous for the bacterial population (Hammer & Bassler, 2003; Chu *et al.,* 2006).

Interestingly, once Spo0A gets phosphorylated (Spo0A~P) is does not only activate *sinI* but also *spo0A* itself. Biofilm genes are induced at low levels of Spo0A~P, whereas genes for spore formation have weak binding sites and only get activated when Spo0A~P accumulates at higher levels (Fujita *et al.,* 2005). In the past a bimodal switch for Spo0A~P was postulated (Veening *et al.,* 2005). However, it could be shown that the extent of sporulation is driven by broadly heterogeneous levels of Spo0A~P, due to limiting the phosphate flux through the phosporelay, which serves as noise generator (Chastanet *et al.,* 2010). By variation in the rates of accumulation of Spo0A~P in *B. subtilis*, the cells exhibit a variety of phenotypes such as biofilm formation, cannibalism and sporulation and are therefore well adapted to different environmental conditions.

In conclusion, the heterogeneous production of AQs shows a typical example for division of labor. AQs exhibit antimicrobial activity and are also supposed to act as deterrents by repelling scavenger insects and birds due to their reddish color (Gulcu *et al.,* 2012; Brachmann *et al.,* 2007; Pankewitz & Hilker, 2008). As the AQs are secreted out of the cell, they are advantageous for the whole population and promote the survival of the whole population, even though only a sub-population is involved in their production and has to bear the production costs. If in return the non-producing 1° cells synthesize other important secondary metabolites remains to be determined.

## 6.2 The regulatory network of phenotypic switching

Even though the 2° cells display pathogenicity against insects, they are incapable of forming a symbiosis with nematodes (Boemare & Akhurst, 1988). As a result, the 2° form is no longer capable of participating in the complex lifecycle of *Photorhabdus* and cannot leave the cadaver with the nematodes. Therefore, it might be condemned to a free life in the soil (Smigielski *et al.,* 1994). The efficiency of nematode-bacteria complexes, which are used in the agricultural industry to prevent insect-caused crop failure, is drastically diminished by the occurrence of 2° cells (Akhurst, 1993). Thus, one has to understand the switching process and the role of the two different cell forms.

So far, it has remained unknown how the absence of such a huge amount of phenotypic features in the 2° cells is regulated. In *P. temperata*, the regulator HexA was found to be

important for the suppression of primary-specific features in 2° cells. Upon disruption, 2° cells restore features such as production of light, antibiotics and crystal proteins (Joyce & Clarke, 2003). However, a deletion of *hexA* decreases virulence significantly, suggesting that bacteria cannot simultaneously express all the genes required for symbiosis and pathogenicity.

### 6.2.1 Regulation of *hexA* expression

In this work, enhanced transcriptional and translational levels of *hexA* in 2° cells of *P. luminescens* could be observed and overproduction of HexA in 1° cells resulted in a loss of 1°-specific features like bioluminescence (Chapter 4, Figure 1 and 2). Therefore, the expression of *hexA* must somehow be regulated. Unlike the homolog LrhA in *E. coli*, HexA in *P. luminescens* was not found to be autoregulated (Lehnen *et al.*, 2002; Chapter 4, Figure 1).

The Rcs phosphorelay system, a cell envelope stress-sensing pathway, represses the synthesis of *lrhA* in *E. coli* and it would be of interest to elucidate if this is also true for HexA from *P. luminescens* as genes with homologies to *rcsC, rcsB, rcsD, rcsA* and *rscF* are also present in *P. luminescens* (Peterson *et al.*, 2006; Huang *et al.*, 2006).

The alarmone (p)ppGpp, which is synthesized by RelA and SpoT, is important for the transition from pathogenicity to mutualism in *P. luminescens* (Bager *et al.*, 2016). It is suggested that (p)ppGpp could be positively regulated by the TCA cycle, while negatively regulating HexA. This is in accordance with the findings that a Δ*mdh* strain resembles the phenotype when (p)ppGpp is absent due to deletion of *spoT* and *relA* (Lango & Clarke, 2010; Bager *et al.*, 2016). Therefore, it is proposed that (p)ppGpp accumulates upon nutrient-limitation in the dead insect and then leads to the production of secondary metabolites and initiation of nematode development presumably via inhibition of HexA.

A drastic up-regulation of *hexA* was observed upon deletion of the RNA chaperone encoded gene *hfq* in 1° cells and it is speculated that Hfq influences *hexA* expression and not *vice versa*, as no differences in *hfq* promoter activity was detected upon deletion of *hexA* in 1° cells (Tobias *et al.*, 2016; Chapter 4, Figure 3). An additional deletion of *hexA* in the Δ*hfq* mutant could restore the production of secondary metabolites and symbiosis with the nematodes (Tobias *et al.*, 2016). However, in this work enhanced promoter activity of *hfq* was detected in 2° cells, which contain elevated levels of HexA (Chapter 4, Figure 3). Since Hfq in *E. coli* is known to be autoregulated at the translational level it might be possible that the translation of *hfq* or protein activity is somehow diminished or impaired in 2° cells (Vecerek *et al.*, 2005). Hfq facilitates RNA-RNA interactions, which are involved in post-

transcriptional regulation and is known to be required for the stability and/or interactions with the target mRNA of numerous *E. coli* sRNAS (Møller *et al.*, 2002; Valentin-Hansen *et al.*, 2004). With the observation that RelA stimulates Hfq multimerization to enhance sRNA binding, a link between (p)ppGpp and Hfq can be drawn (Argaman *et al.*, 2012).

### 6.2.2 Regulation of primary-specific features via HexA

Upon investigating targets of HexA, an indirect repression of the bioluminescence operon, encoding the genes *luxCDABE*, has been found (Chapter 4, Figure 2, Figure 6-1). *Photorhabdus* is the only terrestrial bacterium known to produce light and it is speculated that light production might attract other insects (Clarke, 2014). The luciferase, consisting of LuxA and LuxB sequentially binds $FMNH_2$, $O_2$ and aliphatic aldehyde (RCHO) that are converted to an aliphatic acid, FMN, and water and then those compounds are released with the concomitant production of light (Meighen, 1991). Therefore, bioluminescence might also be useful to remove oxygen and/ or to burn reducing power (Clarke, 2014). A regulation mechanism of the *luxCDABE* operon at the post-transcriptional level via small RNAs is suggested and could verify enhanced promoter activity of the small RNA chaperone Hfq in 2° cells, which show enhanced levels of HexA (Chapter 4, Figure 3). Besides Hfq being involved in the regulation of HexA itself as seen above, it might also have an important function to regulate HexA-mediated gene expression. HexA homologs, like LrhA from *E. coli*, are known to regulate sRNAs (Peterson *et al.*, 2006).

HexA from *E. carotovora* and LrhA from *E. coli* were both found to reduce the levels of RpoS and therefore influence the repression of hundreds of stationary-phase genes (Gibson & Silhavy, 1999; Mukherjee *et al.*, 2000). Thereby, LrhA represses the sRNA RrpA, which is an activator of RpoS translation. Another yet unidentified sRNA influences RpoS translation in an RprA-independent manner and is also known to be regulated by LrhA. Additionally, the LrhA mediated repression of RpoS translation was found to be dependent on the RNA chaperone Hfq (Peterson *et al.*, 2006). These findings suggest that the absence of certain stationary-phase features in 2° cells of *P. luminescens* might result from reduced RpoS translation due to enhanced levels of HexA, which mediate the activity of certain small RNAs.

The novel quorum sensing system PpyS/PluR was discovered to activate the operon *pcfABCDEF (Photorhabdus* clumping factor), resulting in the formation of cell clumps. The photopyrone synthase PpyS produces photopyrones (PPYs), which are then sensed via the

LuxR solo PluR and activate the *pcfA* promoter. So far, it is known that the operon encodes proteins of the amino acid metabolism and one putative efflux transporter and that *pcf*-induced cell clumping is also functional in *E. coli*. However, the exact nature of the PCF is still unknown (Brachmann *et al.*, 2013).

A proteome analysis identified the three proteins PcfA, PcfB and PcfC, which are encoded by the *pcfABCDEF* operon, to be more abundant in the absence of HexA (Chapter 4, Fig. S1). A deeper investigation led to the identification the *pcfA* promoter as a direct target of HexA (Chapter 4, Figure 5 and 6). Cell clumps were not visible in the 2° cells and the responsive operon is repressed (Chapter 4, Figure 4, Figure 6-1). Thus, cell clumps might not only contribute to the pathogenicity of *P. luminescens* (Brachmann *et al.*, 2013) but they could also be important for the symbiosis by facilitating the uptake of the bacteria by the nematodes. This is in accordance with the observation that a Δ*pluR* strain showed reduced reassociation with the nematodes (personal communication with Dr. David Clarke, University College Cork, Ireland).

Even though the communication via AHLs is widespread among Gram-negative bacteria, a LuxI synthase, which is responsible for the production of AHLs, is missing in *Photorhabdu*s species (Heermann & Fuchs, 2008). Therefore, it has long remained unknown if the bacteria are able to communicate with each other or if they can only eavesdrop via their LuxR solos, recognizing signaling molecules by other organisms. The PpyS/PluR system is the first known QS system in *P. luminescens* (Brachmann *et al.*, 2013). HexA homologs in other organisms are known for being involved in QS. One example is the HexA homolog LrhA in the plant-pathogenic bacterium *Pantoea stewartii*, which is repressed upon accumulation of AHLs via the LuxR regulator EsaR (Ramachandran *et al.*, 2014; Kernell Burke *et al.*, 2015). Furthermore, HexA of *Erwinia carotovora* represses the production of the signaling molecule N-3-oxo-hexanoyl homoserine lactones (OHHLs) (Harris *et al.*, 1998). As *Photorhabdus* is neither capable of producing AHL or derivatives nor contains a homolog of EsaR, HexA might play a role in the regulation via AHL signaling molecules from other bacteria. However, as HexA plays a major role in the mutualism with nematodes this is rather unlikely. It seems more likely that HexA itself regulates QS in *P. luminescens*. This can be explained as a numerous amount of secondary metabolites is repressed via HexA and 2° cells produce e.g. PPYs in lower amounts compared to the 1° cells (Kontnik *et al.*, 2010; Supplemental Material Chapter 3, Figure S8). Why HexA might control cell clumping via two different mechanisms, on the one hand via decrease of the signaling molecule PPY and on the other hand via direct repression of the promoter remains to be determined and might be a form of fine-tuning.

Interaction of LTTRs is known to occur at two dissimilar sites with the promoter region. The repressor binding site (RBS) contains the LTTR consensus binding motif T-$N_{11}$-A within an approximately 15 bp interrupted dyad symmetry region and can even vary in both base pair composition and length and is usually centered near position -65 relative to the transcriptional start of the promoter. The active binding site (ABS) does not contain this motif and often overlaps the -35 box (Parsek *et al.*, 1994; Tropel & van der Meer, 2004).

As the consensus sequence of the RBS is not very specific, it is hard to conclude at which position the HexA binding site might be positioned upstream of *pcfA*. According to the heterologous assays in *E. coli*, it can be concluded that the binding site of HexA lies within 142 bps upstream of the *pcfA* gene and is positioned downstream of the putative binding site of the activator PluR (Chapter 4, Figure 5).

LTTRs contain an N-terminal winged helix-turn-helix DNA binding domain and a co-factor binding domain at the C-terminus. Even though most LTTRs are transcriptional activators, HexA and its homologs in other organisms are known to be repressors. RovM is a HexA homolog in *Yersinia pseudotuberculosis* and represses the virulence activator gene *rovA* by binding to the promoter region. It is suggested that the *rovA* promoter geometry structurally alters to hinder proper recognition by the RNA polymerase or prevents the transition from a closed transcription initiation complex to an open form of this complex (Heroven & Dersch, 2006). It is well known, that most LTTRs interact with small specific signal molecules that are typically metabolites, catabolites or intermediates of a biochemical pathway via their co-inducer binding site (Schell, 1993). The LysR regulator CatM of *Acinetobacter calcoaceticus* activates enzymes for benzoate degradation and represses genes that are used to degrade alternative aromatic carbon sources and is induced by cis-cis-muconate, an intermediate product of benzoate degradation (Brzostowicz *et al.*, 2003). Until now, a ligand of HexA and its homolog in other organisms has not yet been identified and according to *in vitro* analysis of the binding affinity of HexA towards the promoter region of the *pcfABCDEF* operon, a potential ligand is missing under these conditions (Chapter 4, Figure 6).

### 6.2.3 Potential role of the two phenotypic forms for a *P. luminescens* population

Until now genomic analysis have not led to the identification of any gene rearrangements or mutations within the genome of the 2° cells, underlining the hypothesis that phase switching in *P. luminescens* is considered as phenotypic heterogeneity (Gaudriault *et al.*, 2008; personal

communication with Maria-Antonia Zamora-Lagos, Max-Planck-Institute for Biochemistry, Martinsried). As 2° cells can only be isolated after an extended period of cultivation, phenotypic switching has been assumed to be a response to environmental or metabolic stress (Joyce *et al.*, 2006). Upon co-cultivation of 1° and 2° cells at different ratios, 1° cells show a growth advantage in the exponential phase whereas 2° cells overgrow the 1° cells in the stationary phase (Chapter 5, Figure 5-2). Separate cultivation of the two cell forms showed no significant differences in respect to growth behavior (Chapter 5, Figure 5-1). Previously, it was observed that 2° cells recommenced growth 10 to 12 hours earlier than 1° cells after an extended time of starvation and it was concluded that 2° cells contain a more efficient nutrient uptake (Smigielski *et al.*, 1994). Additionally, the universal stress protein UspA, which is necessary for recovery of *E. coli* after nutrient starvation, was upregulated in the 2° cells (Siegele, 2005; Turlin *et al.*, 2006).

However, the exposure to nutrient limitation in combination with hypoosomotic stress simply causes a delay until the 2° cells gain ascendancy (Chapter 5, Figure 5-3). This might be explained by the slowed growth and thereby delayed entry into the stationary phase. The few nutrients that are available can apparently be consumed by 1° cells and lead to enhanced growth of 1° cells in the exponential phase.

As UspA is also required to protect the cell from superoxide-generating agents, the mixed cultures were exposed to oxidative stress (Chapter 5, Figure 5-3). However, upon the addition of hydrogen peroxide, the 2° cells were only able to overgrow the 1° cells one day later as in the control. Thereby, 2° cells seem to be specifically well adapted to the stationary growth phase, in contrast to the 1° cells. As 2° cells do not produce most of the cost-intensive secondary metabolites and do not support nematodes in their growth and development, they might be able to save energy and are subsequently better adapted to starvation periods (Han & Ehlers, 2001; Lango & Clarke, 2010). Therefore, 2° cells are assumed to be better prepared for a life in the soil without the nematode partner.

The two-component regulatory system AstRS, which is involved in adaptation of *P. luminescens* cells to the stationary phase and starvation survival, controls the timing of phenotypic switching as a mutation induces an earlier transition to the 2° phenotype (Derzelle *et al.*, 2004). Furthermore, UspA was found to be downregulated upon deletion of *astR* (Derzelle *et al.*, 2004). Therefore, the AstS/AsrR signal transduction pathway is thought to prevent or delay phenotypic switching by protecting the cells from stress (Joyce *et al.*, 2006).

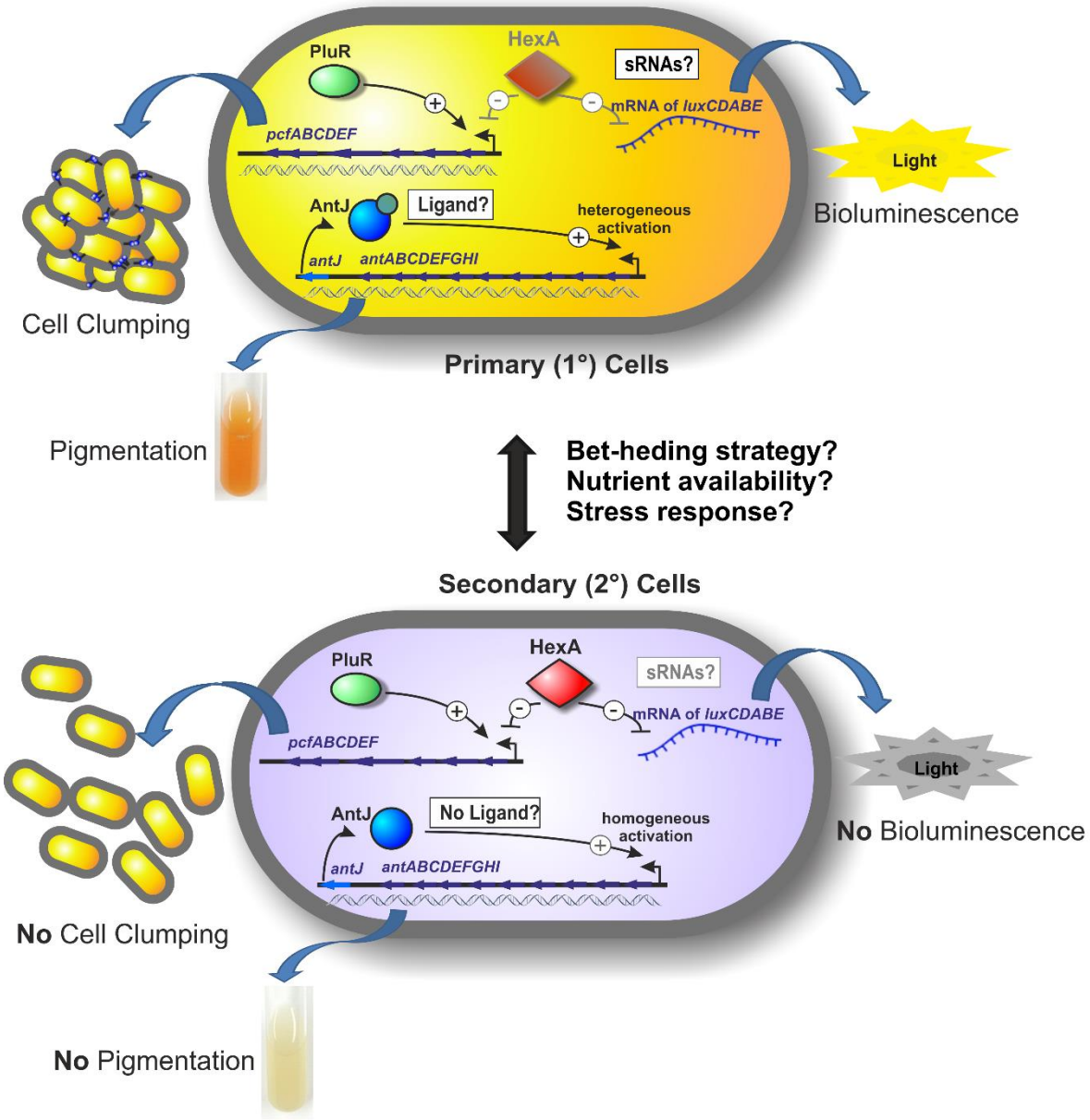


**Figure 6-1 Model of the regulation of the 1°- and 2°- specific phenotype in *P. luminescens*.** Low HexA levels in 1° cells lead to cell clumping via expression of the *pcfABCDEF* operon and translation of the *luxCDABE* mRNA leads to light production. The heterogeneous activation of the *antABCDEFGHI* operon might be mediated via the ligand-bound AntJ. The enhanced HexA levels in 2° cells prevent the formation of cell clumps via repression of the *pcfA* promoter activity and diminish bioluminescence via impaired translation of the *luxCDABE* mRNA presumably via small RNAs. A basal homogeneous $P_{antA}$ activity might result from a missing ligand for AntJ and causes non-pigmentation (see text for details).

The phenomenon of phenotypic variation is often found to provide the bacteria with a mechanism of bet hedging, in order to enhance the fitness of the population in a particular environment (Veening *et al.*, 2008). The co-occurrence of 1° and 2° cells might be a typical example for bet-hedging (Figure 6-1). Only a small proportion of the bacteria will colonize

the gut of the nematode in its IJ stage, when leaving the depleted insect cadaver. The vast majority will be eaten by the nematode or be left within the insect cadaver (Joyce *et al.*, 2011). Therefore, the establishment of 2° cells enables those cells to prepare for life in the absence of the nematodes by switching off the symbiosis genes. If one population splits up into sub-populations with different phenotypes, the chance increases that at least one sub-population will be adapted under challenging environmental conditions (Cohen, 1966).

One sub-population, the 1° cells, reassociates with the nematode and is brought to a new insect larva, which provides a nutrient-rich environment. The 2° cells as second sub-population stay in the soil after nutrient depletion of the insect cadaver and are able to withstand nutrient limitation better and can rapidly recommence growth upon nutrient supply. This hypothesis can be supported by the observation of increased levels of proteins, like Lrp, chaperonins and iron-scavenging proteins, in 2° cells that could confer an advantage in the nutrient limiting and stressful conditions, which generally apply in the soil (Turlin *et al.*, 2006). However, neither 1° nor 2° cells have been isolated from the soil yet, even though *Photorhabdus* was shown to survive in soil under laboratory conditions (Smigielski *et al.*, 1994; Bleakley & Chen, 1999).

A typical example for bet-hedging is the occurrence of bacterial persister cells, which enter a transient growth arrest state and can therefore survive antibiotic treatment. The switch from normally growing cells to the persister state is induced via stochastic and epigenetic events (Balaban *et al.*, 2004; Veening *et al.*, 2008). Thus, the elevated HexA levels in 2° cells might be a result of stochastic distribution.

One might therefore speculate that (p)ppGpp and Hfq levels are enhanced in 1° cells causing only basal levels of HexA and thus raising the abundance of small RNAs that might activate the respective operons. In 2° cells diminished levels of (p)ppGpp and thereby less Hfq might be present leading to enhanced HexA levels and inhibition of the target genes via direct repression or due to missing activating of respective genes via small RNAs.

## 6.3 Outlook

It is of major interest to elucidate what exactly causes the switching and if signaling molecules are involved. The exact purpose of the 2° cells and if this is indeed a bet-hedging strategy that serves as benefit for both phenotypic forms and ensures survival of the whole community still needs to be unraveled. Therefore, the identification of 2° cells in the soil under rather native conditions might help to understand this phenomenon. Until now a reverse

switch from 2° to 1° cells has never been observed, which could be a result of the experimental setup under laboratory conditions. Thus, several conditions could be tested to induce a reverse switching e.g. under native conditions in the life cycle of *P. luminescens* in combination with its two hosts.

As the reason for heterogeneity of anthraquinone production presumably is due to the existence of a ligand that binds to the WYL-domain of AntJ, the identification of this putative ligand and whether it is acting as activating or inhibiting ligand is of major interest. Is the ligand an intermediate of another secondary metabolite or a precursor of anthraquinones such as acetyl-CoA or malonyl-CoA? This could either be tested via Surface Plasmon Resonance (SPR) Spectroscopy or a fluorescence-based thermal stability assay. The latter enables the screening of a great number of candidates and is based on the principle that the thermal stability of a protein enhances upon ligand binding due to conformational changes (Boivin *et al.*, 2013). Furthermore, the spacer region of the AntJ binding motif, which was seen to be required for $P_{antA}$ activation, should be investigated further, presumably via the previously reported heterologous luciferase-based assay in *E. coli*.

The question remains how the elevated HexA levels in 2° cells are achieved. Do the (p)ppGpp levels differ in the two cell forms and regulate *hexA* expression? Therefore, *relA* and *spoT* expression and activity could be followed via a fluorescence-based approach. Furthermore, the expression of *hexA* could be followed in a mutant lacking *relA* and *spoT*.
The putative ligand of HexA is still unknown and a crystallization approach might solve this question if the ligand gets co-crystallized. Moreover, potential candidates might also be tested via SPR or a fluorescence-based thermal stability assay. Furthermore, the role of Hfq and sRNAs in the occurrence of the 2° cells should be determined. Therefore, a high-throughput sequencing of small RNAs might help in the discovery of small RNAs, which are involved in phenotypic switching (Liu & Camilli, 2011).

In order to gain more insights into the socio-biological aspects of the two cell forms, the growth advantage of 2° cells in the stationary phase should be observed further. Thus, it is of interest to see if mixed cultures, consisting of 1° and 2° cells in a 1:1 ratio, behave the same way when they are injected into the insect. Does the whole population exclusively consist of 2° cells upon re-isolation of the bacteria after infection and death of the insect or is a different result obtained?

Finally, the understanding of the complex regulation of phenotypic heterogeneity in *P. luminescens* might help to unravel global principles of heterogeneous behavior that could be transferred to other clinical and biotechnological microorganisms.

## 6.4 References of Concluding Discussion

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## Supplemental Material Chapter 2

# A novel tool for stable genomic reporter gene integration to analyze heterogeneity in *Photorhabdus luminescens* at single-cell level

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# Supplemental Material Chapter 3

## SUPPLEMENTARY CONTENT

### Heterogeneous regulation of bacterial natural product biosynthesis via a novel transcription factor

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# Supplemental Material Chapter 4

## Supporting Information

## HexA is a versatile regulator involved in the control of phenotypic heterogeneity of *Photorhabdus luminescens*

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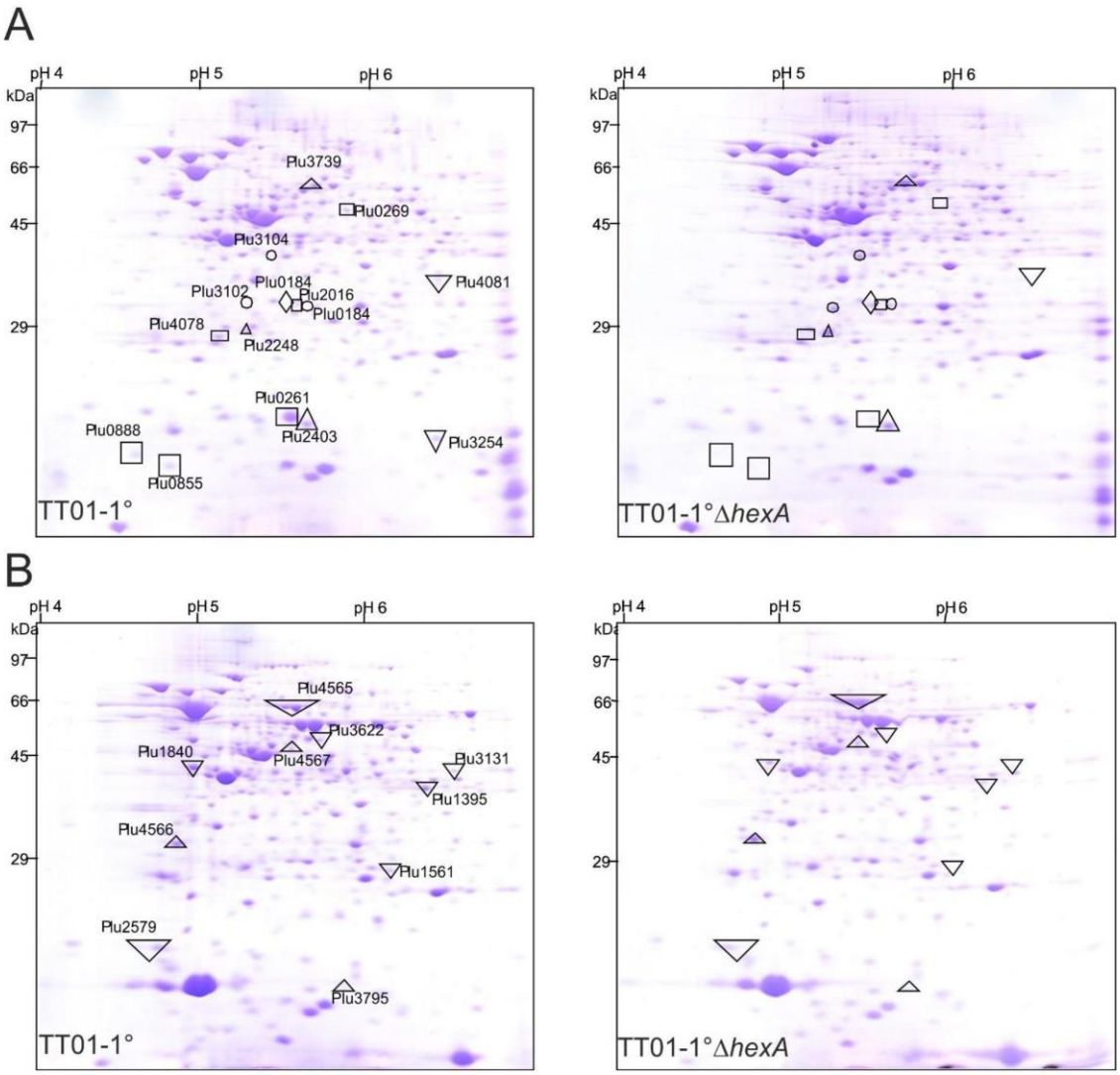


**Fig. S1: Proteome analysis of *P. luminescens* TT01-1° and TT01-1°Δ*hexA*.** Cells were cultivated and harvested in exponential (A) and in the stationary phase (B). Cytosolic proteins were extracted and then subjected to 2D-PAGE. Gels were scanned, and compared for protein spots of different sizes. Proteins with enhanced production (△), with reduced production (▽) or overproduced (◊) in the Δ*hexA* mutant and proteins that were completely absent in the Δ*hexA* mutant (□) or in the wildtype (○) were analyzed via MALDI-TOF.

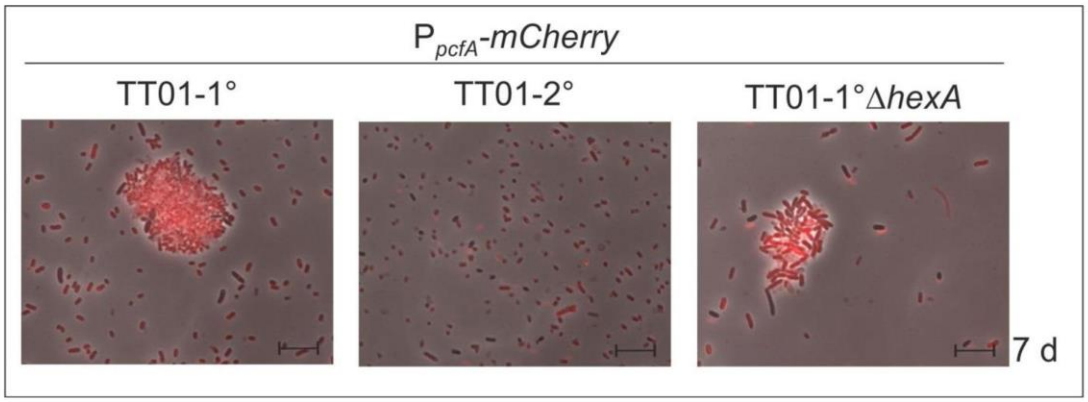


**Fig. S2: Cell clumping in *P. luminescens* TT01-1°, TT01-2° and TT01-1°*ΔhexA* after 7 days.** $P_{pcfA}$ activity and cell clumping in TT01-1°, TT01-2° and TT01-1°Δ*hexA*. The scale depicts 10 µM. Representative images from one of three independently performed experiments are shown.

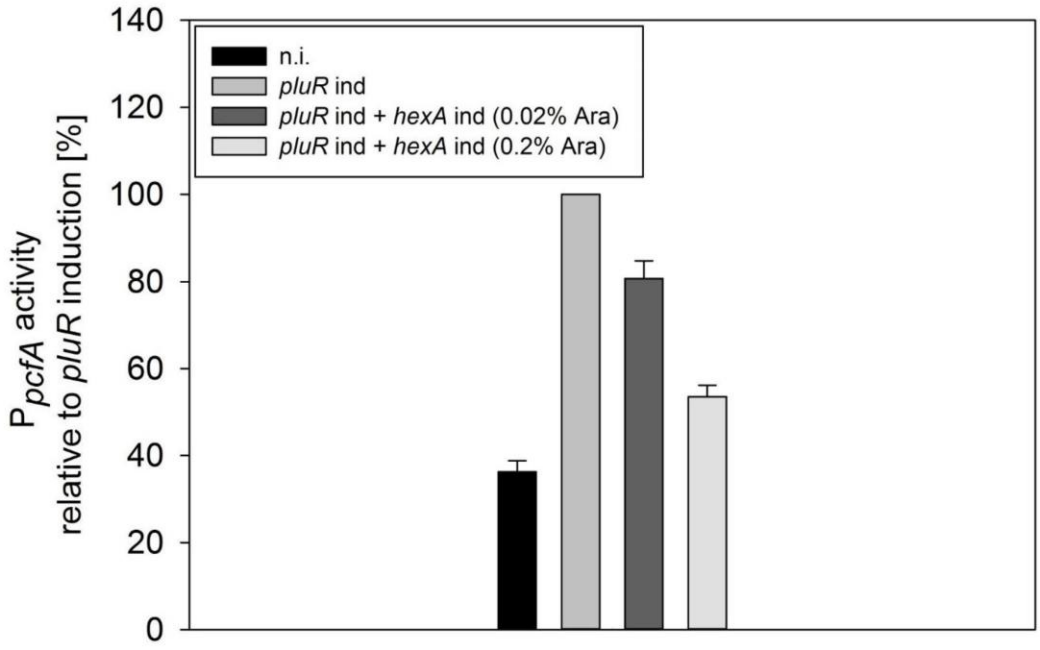


**Fig. S3: Effect of HexA on the $P_{pcfA}$ activity in the heterologous systems of *E. coli* Δ*lrhA*.** In *E. coli* Δ*lrhA* the constructs pBAD24-$P_{lac}$-*pluR*_$P_{ara}$-*hexA* and pBBR-$P_{pcfA}$-*lux* were tested. The expression of *pluR* was achieved via the addition of 1 mM IPTG and *hexA* expression was induced via the addition of 0.02 and 0.2% arabinose (Ara). The figure represents three biological replicates. All values are given in percentage, relative to the maximum *pluR* induction. The values were measured as Relative Light Unit [RLU] divided by $OD_{600nm}$.

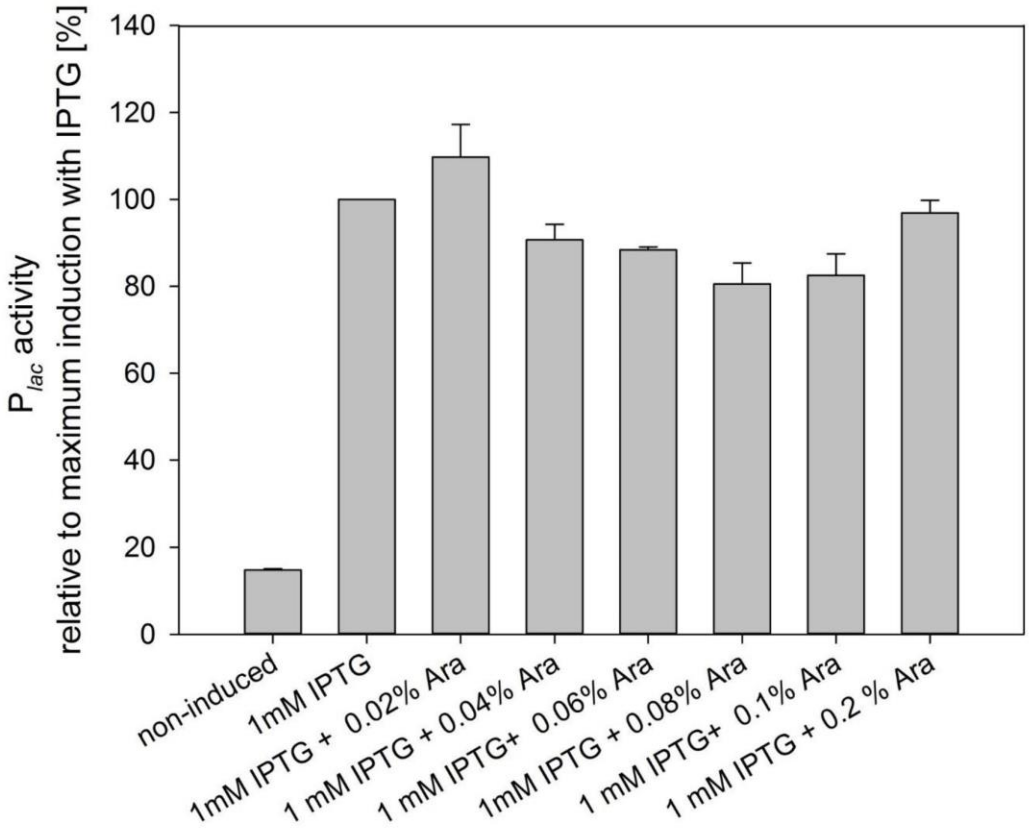


**Fig. S4: Investigation of an effect of HexA on the *lac* promoter and the *luxCDABE* operon**. The constructs pBAD24-$P_{lac}$-*pluR*-$P_{ara}$-*hexA* and pBBR-$P_{lac}$-*lux* were tested in *E. coli*Δ*lrhA* and 1 mM IPTG was added. Expression of *hexA* was induced via the addition of 0.02-0.2% arabinose (Ara). The graph corresponds to measurements performed 3 hours after induction. The figures represent three biological replicates. All values are expressed in percentages, relative to the values of the *pluR* maximum induction upon addition of 1 mM IPTG.

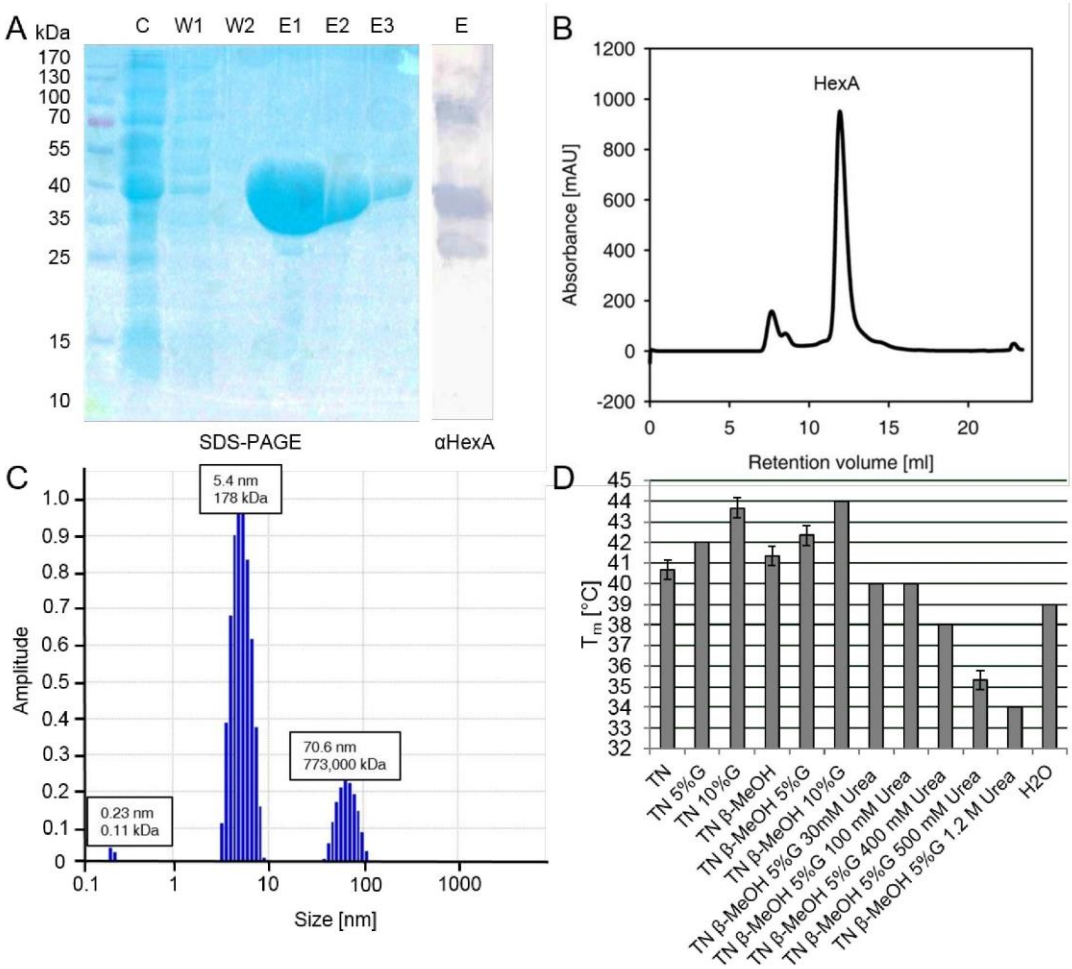


**Fig. S5: Purification and biochemical investigation of HexA-6His.** Purification of HexA via Ni-NTA affinity chromatography. Left panel shows a Coomassie blue stained SDS gel; right panel shows a Western blot with αHexA antiserum. C=cytosolic fraction; W1=washing fraction 1; W2=washing fraction 2; E1=elution fraction 1; E2=elution fraction 2; E3=elution fraction 3; E=pooled elution fraction (A). Gel filtration of purified HexA-6His (E) using Superdex 200 column (B). Size and molecular weight determination of "HexA" peak fraction (gel filtration) using Dynamic Light Scattering (DLS) (C). Stability measurement of HexA-6His in different buffers using a fluorescence-based thermal stability assay. Tm=melting temperature, TN=50mM Tris/HCl pH 7.5, 200 mM NaCl; G=glycerol; β-MeOH = 2 mM β-mercaptoethanol (D).

**Table S1: Proteins with altered production in the proteome of TT01-1°Δ*hexA* compared to TT01-1°.** Differences in the cytosolic proteome were detected in the exponential (EX) and stationary (STAT) growth phase.

| Protein | Putative function | Growth phase | Δ*hexA*/ wild-type |
|---|---|---|---|
| Plu0184 (CpmC) | Role in Carbapenem biosynthesis | | +4.2 |
| Plu0261 | Similiarities with type 1 fimbrial protein precursor | EX | n.d. in Δ*hexA* |
| Plu0269 | Unknown, hypothetical secreted protein | EX | n.d. in Δ*hexA* |
| Plu0885 | Pyocin S3 protein, „killer protein“ | EX | n.d. in Δ*hexA* |
| Plu0888 | Colicin/Pyocin protein, „killer protein“ | EX | n.d. in Δ*hexA* |
| Plu1395 | Cystein Synthase A | STAT | -1.7 |
| Plu1561 | $Ca^{2+}$-dependent cell adhesion molecule | STAT | -2.6 |
| Plu1840 | unknown | STAT | -1.6 |
| Plu2016 | PAS4-LuxR regulator | EX | n.d. in Δ*hexA* |
| Plu2248 | Carbonic anhydrase | EX | +4.0 |
| Plu3102 | methyltransferase | EX | n.d. in WT |
| Plu3104 | unknown | EX | n.d. in WT |
| Plu3110 (ArgM) | Succinylornithine transaminase | STAT | -1.4 |
| Plu3254 | Hcp family T6SS protein CtsH1 | EX | -2.7 |
| Plu3622 (AceF) | dihydrolipoamide acetyltransferase; pyruvate dehydrogenase subunit E2 | STAT | -2.2 |
| Plu3739 (AldB) | Aldehyde Dehydrogenase B | EXP | +1.8 |
| Plu3795 | unknown | STAT | +2.4 |
| Plu4078 | Dimethylmenaquinone methyltransferase | EXP | n.d. in Δ*hexA* |
| Plu4081 | Putative aldolase | | -3.2 |

| | | | |
|---|---|---|---|
| Plu4565 (PcfA) | Cysteine synthase | STAT | +2.0 |
| Plu4567 (PcfB) | Ariginosuccinate synthase | STAT | +2.0 |
| Plu4566 (PcfC) | Glycine amidino transferase | STAT | +2.1 |

**Table S2: Bacterial Strains.**

| Bacterial Strain | Genotype | Reference |
|---|---|---|
| *P. luminescens* subsp. laumondi TT01-1° | Wild-type 1° variant | [1] |
| *P. luminescens* subsp. laumondi TT01-2° | Wild type 2° variant | Lab collection, Dr. David Clarke, University College Cork |
| *P. luminescens* TT01-1° Δ*hexA* | Wild-type 1° variant containing a deletion of *hexA (plu3090)* | Lab collection, Dr. David Clarke, University College Cork |
| *P. luminescens* TT01-1° $P_{hexA}$-*mCherry* | TT01-1° harboring $P_{hexA}$-*mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | [2] |
| *P. luminescens* TT01-2° $P_{hexA}$-*mCherry* | TT01-2° harboring $P_{hexA}$-*mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | This study |
| *P. luminescens* TT01-1° Δ*hexA* $P_{hexA}$-*mCherry* | TT01-1° Δ*hexA* harboring $P_{hexA}$-*mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | This study |
| *P. luminescens* TT01-1° $P_{hexA}$-*hexA-mCherry* | TT01-1° harboring $P_{hexA}$-*hexA-mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | This study |
| *P. luminescens* TT01-2° $P_{hexA}$-*hexA-mCherry* | TT01-2° harboring $P_{hexA}$-*hexA-mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | This study |

| | | |
|---|---|---|
| *P. luminescens* TT01-1° Δ*hexA* $P_{hexA}$-*hexA-mCherry* | TT01-1° Δ*hexA* harboring $P_{hexA}$-*hexA-mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | This study |
| *P. luminescens* TT01-1° $P_{luxC}$-*mCherry* | TT01-1° harboring $P_{luxC}$-*mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | [2] |
| *P. luminescens* TT01-2° $P_{luxC}$-*mCherry* | TT01-2° harboring $P_{luxC}$-*mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | This study |
| *P. luminescens* TT01-1° Δ*hexA* $P_{luxC}$-*mCherry* | TT01-1° Δ*hexA* harboring $P_{luxC}$-*mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | This study |
| *P. luminescens* TT01-1° $P_{hfq}$-*mCherry* | TT01-1° harboring $P_{hfq}$-*mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | This study |
| *P. luminescens* TT01-2° $P_{hfq}$-*mCherry* | TT01-2° harboring $P_{hfq}$-*mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | This study |
| *P. luminescens* TT01-1° Δ*hexA* $P_{hfq}$-*mCherry* | TT01-1° Δ*hexA* harboring $P_{hfq}$-*mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | This study |
| *P. luminescens* TT01-1° $P_{pcfA}$-*mCherry* | TT01-1° harboring $P_{pcfA}$-*mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | This study |
| *P. luminescens* TT01-2° $P_{pcfA}$-*mCherry* | TT01-2° harboring $P_{pcfA}$-*mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | This study |
| *P. luminescens* TT01-1° Δ*hexA* $P_{pcfA}$-*mCherry* | TT01-1° *ΔhexA* harboring $P_{pcfA}$-*mCherry* reporter integrated at the *rpmE*/*glmS* site, $Kan^R$, $Gent^R$ | This study |
| *E. coli* Dh5α λ*pir* | *recA1, gyrA (lacIZYA-argF) (80d lac [lacZ] M15) pir RK6* | [3] |
| *E. coli* S17-1 λpir | $Tp^R$ $Sm^R$ *recA*, *thi*, *pro*, *hsdR*-M+RP4: 2-Tc:Mu: Km Tn7 λpir | Biomedal S.L. Sevilla, Spain |
| *E. coli* ST18 | *E. coli* S17 *λpir ΔhemA* | [4] |

| | | |
|---|---|---|
| *E. coli* BL21 (DE3) Star | $F^-$ *ompT* *hsd*$S_B$ ($r_B^-$ $m_B^-$) *gal dcm rne*131 (DE3) | Invitrogen |
| *E. coli* JW2284 | $Kan^R$, BW25113 *lrhA::npt* | [5] |
| *E. coli ΔlrhA* | Removal of the *npt* cassette in *E. coli* JW2284 by P1 transduction | Dr. Sophie Brameyer, unpublished |
| *Sh. oneidensis* MR1 S79 | Wild type isolate | [6] |

**Table S3: Plasmids.**

| Plasmid | Genotype | Reference |
|---|---|---|
| pPINT-*mCherry* | $Km^R$, $Gm^R$ and *mCherry* in pPINT | [2] |
| pPINT-$P_{hexA}$-*mCherry* | $Km^R$, $Gm^R$, *hexA* (*plu3090*) promoter upstream of *mCherry* | [2] |
| pPINT-$P_{hexA}$-*hexA-mCherry* | $Km^R$, $Gm^R$, *hexA* promoter upstream of *hexA (plu3090)-mCherry* | This study |
| pPINT-$P_{luxC}$-*mCherry* | $Km^R$, $Gm^R$, *luxC (plu2079)* promoter upstream of *mCherry* | [2] |
| pPINT-$P_{hfq}$-*mCherry* | $Km^R$, $Gm^R$, *hfq (plu4581)* promoter upstream of *mCherry* | This study |
| pPINT-$P_{pcfA}$-*mCherry* | $Km^R$, $Gm^R$, *pcfA (plu4568)* promoter upstream of *mCherry* | This study |
| pBAD24-*pluR* | $Ap^R$, *pluR* (*plu4562*) in pBAD24 | [7] |
| pBAD24-*yehU* | $Ap^R$, *yehU*-6His in pBAD24 with a C-terminal HisTag | [8] |
| pBAD24-*hexA* | $Ap^R$, *hexA*-6His (*plu3090*) in pBAD24 with a C-terminal HisTag | This study |

| | | |
|---|---|---|
| pCOLA-*ppyS*-His-*pluR* | $Km^R$, *ppyS (plu4844)* and 6His-*pluR* (*plu4562*) in pCOLA, IPTG inducible | Dr. Sophie Brameyer, unpublished |
| pBAD24-$P_{ara}$-*pluR*_$P_{lac}$-*hexA* | $Ap^R$, *pluR* (*plu4562*) under the control of an arabinose inducible promoter, *hexA* (*plu3090*) under the control of an IPTG inducible promoter | This study |
| pBAD24-$P_{lac}$-*pluR*_$P_{ara}$-*hexA* | $Ap^R$, *pluR* (*plu4562*) under the control of an IPTG inducible promoter, *hexA* (*plu3090*) under control of an arabinose inducible promoter | This study |
| pBBR1-$P_{pcfA}$-*lux* | $Gm^R$, l*uxCDABE* under the control of the *pcfA* (*plu4568*) promoter | [7] |
| pBBR1-$P_{pcfA}$-s1-*lux* | $Gm^R$, *luxCDABE* under the control of the truncated promoter construct $P_{pcfA}$-S1 | Dr. Sophie Brameyer, unpublished |
| pBBR-$P_{pcfA}$-s2-*lux* | $Gm^R$, *luxCDABE* under the control of the truncated promoter construct $P_{pcfA}$-S2 | Dr. Sophie Brameyer, unpublished |
| pBBR-$P_{lac}$-*lux* | $Gm^R$, *luxCDABE* under the control of the *lac* promoter | This study |
| pACYC-Duet1 | $Cm^R$, Expression vector, IPTG inducible | Novagen® |
| pACYC-*hexA* | $Cm^R$, *hexA* (*plu3090*) in pACYC-Duet1 | This study |
| pACYC-$P_{lac}$-*hexA*_$P_{ara}$-*pluR* | $Cm^R$, *pluR* (*plu4562*) under the control of an arabinose inducible promoter, | This study |

| | | |
|---|---|---|
| | *hexA* (*plu3090*) under the control of an IPTG inducible promoter | |
| pEYFP | $Ap^R$, *lac*-promoter upstream of *eYFP* | Takara-Clonetech, Saint-Germain-en-Laye, France) |
| pD132 | $Cm^R$, ori R6K, oriT RK2, *sacB* | [9] |
| pDS-*hexA* | Flanking regions of *hexA* (*plu3090*) in pD132 | This study |

**Table S4: Oligonucleotides.**

| Primer name | Sequence (5`-3`) |
|---|---|
| PhexA-BamHI_fwd | GCTGGATCCTCTTACCTTATCTTGGTAAA |
| hexA-XmaI _rev | GCTCCCGGGCTCATCAATAATATCGTCATCATCA |
| Phfq-NheI_fwd | GCGGCTAGCTCACTGAACTGACTACATTG |
| Phfq-BamHI_rev | GCTGGATCCTCTATATTTTCCTTATTTTGTT |
| PpcfA-NheI_fwd | AATGGAGCTAGCAGCAGAATTCGGGTTAGTTATCTATGC |
| PpcfA-XmaI_rev | ACTAAGCCCGGGACCAGCTTTATCCCTTATGTC |
| check-mcherry_ins_fwd | CTGGTTTCATAATTTCGCC |
| check-mcherry-ins_rev | GGCCTTCCTTCTCCTTCAC |
| check-rpmE_fwd | CTCCCAAATAAAGTTTAGG |
| check-glmS_rev | GTACGTGAATCTGATTTTG |
| oriT_fwd | CAGGGTTATGCAGCGGAAA |
| gmRpNPTS_fwd | GATAAGCTGTCAAACATGAGAGTAGCGTATGCGCTCAC |
| Plac(h)_fwd | ATTGCATTTATCATGGTATATCTCCTTATTAAA |
| PlacI-SalI_rev | GCTGTCGACTCACTGCCCGCTTTCCAGTC |
| hexA_fwd | ATGATAAATGCAAATCGTC |

| | |
|---|---|
| hexA-PstI-rev | GCTCTGCAGTTACTCATCAATAATATCG |
| pBAD24_seq_fwd | GCCGTCACTGCGTCTTTTACTGG |
| pBAD24_seq_rev | CGCTACGGCGTTTCACTTCTG |
| hexA-EcoRI_fwd | GCTGAATTCATGATAAATGCAAATCGTCC |
| hexA-NdeI_rev | GCGCATATGCTCATCAATAATATCGTCATCATC |
| Plac-PluR_fwd | TCTTCAAAGCTTGCGGCCGCATAATG |
| PluR-PstI_rev | GCGCTGCAGGTTATATGATTAGATTATATGCTATTGC |
| lacI_fwd | CAAGCTTTGAAGATCGAATGGCGCAAAACCTT |
| lacI-SalI_rev | GCTGTCGACTCACTGCCCGCTTTCCAGTC |
| check-PlachexA_fwd | CTACCAGAGAAGTTGAAGT |
| hexA-NcoI_fwd | GCTCCATGGATGATAAATGCAAATCGTCC |
| hexA-SalI_rev | GCGGTCGACTTACTCATCAATAATATC |
| check-pACYC_fwd | ATTCACCACCCTGAATTGA |
| check-pACYC_rev | CTAGTTATTGCTCAGCGGT |
| araCPluR_fwd | GCGCATATGACTCCGTCAAGCCGTCAA |
| pluR-XhoI_rev | TAGCCCTCGAGCTGTGATGATGATGATGATGATGATGATG |
| | ACGACCTTCGATATGGCCGCTTATATGATTAGATTATATGC |
| PpcfA-Btn_fwd | TATTTGTCTTTATAATGATAAT |
| PpcfA_rev | ACCAGCTTTATCCCTTATGTC |
| sacB-Btn_fwd | GCAGAAGTTTTTGACTTTCTTG |
| sacB_rev | ACATCTGACGGAAAAATCCGT |
| Plac-NheI_fwd | GCGGCTAGCGCGCAACGCAATTAATGTG |
| Plac-BamHI_rev | CGCGGATCCAGCTGTTTCCTGTGTGAAA |
| check-pBBR-Plac_fwd | CCGTCGTATTAAAGAGGGG |
| FA_hexA_fwd | GAATTGTTGTTGTTTTTTA |
| FA_hexA_rev | CATTGTTTATTCATCACTTT |

| | |
|---|---|
| FB_hexA_fwd | TAATATCTGAAACACTTCTC |
| FB_hexA_rev | AATCAATGATTGATGGAGTG |

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# Acknowledgments

First of all, I would like to express my sincere gratitude to PD Dr. Ralf Heermann for giving me the opportunity to work in his group with the exciting bacterium *Photorhabdus luminescens* and for having the chance to study this very interesting but also challenging topic of phenotypic heterogeneity. Furthermore, I would like to thank him for his guidance, support and advice throughout the years.

I am very thankful to my thesis committee, especially to Prof. Dr. Marc Bramkamp for being the second examiner of my thesis.

Moreover, I would like to thank Prof. Dr. Helge B. Bode and his group from the Goethe-University in Frankfurt for the fruitful collaboration. In particular, I want to mention Antje Heinrich as it was a pleasure to work with her on the AntJ project.

I am deeply grateful to the whole *Photorhabdus* group. I want to thank Dr. Sophie Brameyer for providing a lot of help and scientific discussion as the advanced "senior PhD student". Of course; I also want to thank the other members Jannis Brehm and Simone Eckstein who always spread such a great mood and who have become more than just colleagues.

I want to thank all the former and actual members of the groups of Prof. Dr. K. Jung and Prof. Dr. H. Jung. In my opinion the friendly atmosphere in these groups is very exceptional and I have always felt extremely comfortable. Moreover, I am thankful for many scientific and non-scientific discussions. Especially, I want to mention Dr. Luitpold Fried, Dr. Matthias Reiger, Dr. Susanne Bracher, Dr. Jürgen Lassak, Ralf Krafczyk, Wolfram Volkwein, Bruno Pinheiro, Miriam Pfab, Korinna Burdack, Sabine Scheu, Yang Wang, Kim Laus, Laura Lindenthal, Anna Haslberger and Agata Rhomberg. Of course, I would also like to thank my former students Jasmin Kuhn, Martin Grafe, Kate McGraphery, Raphaela Schloßnikel and Adriana Moldovan.

Moreover, I have to deeply thank my office girls Nicola Lorenz (Schlori aka Schniecki aka Schnicola) and Hannah Schramke (Schrumpi aka Ekmarsch) as well as the associate office member Laure Plener (Lord P aka Schlaure). We had so much fun together and besides being the best colleagues I could ever imagine you also became true friends.

I sincerely thank my husband Alexander, who is always there for me and has never stopped encouraging me. And of course, I want to thank my family and especially my parents for their confidence in me and their never-ending support. Thank you for always believing in me.