# Phenotypic heterogeneity – Towards the sociobiology of insect pathogenic *Photorhabdus luminescens* and the decision of being different

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Simone Eckstein

# Content

# Nomenclature

The two phenotypic cell forms of *Photorhabdus luminescens* DJC are called primary and secondary cells and are also termed as DJC-1° and DJC-2° or 1° and 2°.

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

N-terminal and C-terminal affinity tags are marked in genes and proteins corresponding to their position (e.g. $His_{10}$-XreR2 or SUMO-$His_6$-XreR2).

Figures and tables are numbered according to the chapter they belong to (e.g. Figure 2 of Chapter 6 = Fig. 6-2 or Table 1 in Chapter 4 = Tab. 4-1).

# Abbreviations

| | |
|---|---|
| aa | amino acid |
| AHL | acyl homoserine lactone |
| AQ | anthraquinone |
| bp | base pairs |
| CFU | colony forming units |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| DTT | 1,4-Dithiothreitol |
| n-His tag | affinity tag composed of n histidine residues |
| HTH | helix-turn-helix |
| IJ | infective juvenile |
| Km | kanamycin |
| LB | lysogeny broth |
| LrhA | LysR homolog A |
| mRNA | messenger RNA |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| Pcf | *Photorhabdus* clumping factor |
| PpyS | photopyrone synthase |
| QS | quorum sensing |
| RNA | ribonucleic acid |
| Suc | succrose |
| SPR | surface plasmon resonance |
| sRNA | small RNA |
| Rif | rifampicin |
| XRE | xenobiotic response element |

# Publications and Manuscripts presented in this thesis

**Chapter 2:**

Zamora-Lagos MA*[3], Eckstein S*[2], Langer A[2], Gazanis A[2], Pfeiffer F[3], Habermann B#[3,4], Heermann R#[2]. Phenotypic and genomic comparison of *Photorhabdus luminescens* subsp. *laumondii* TT01 and a widely used rifampicin-resistant *Photorhabdus luminescens* laboratory strain. *BMC Genomics* (2018) 19:854

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**Chapter 3:**

Eckstein S[1,2], Dominelli N[1], Brachmann A[5], Heermann R[1]. Phenotypic heterogeneity of insect pathogenic *Photorhabdus luminescens* - insights into the fate of secondary cells. *Appl Env Microbiol* (2019) AEM.01910-19

**Chapter 4:**

Eckstein S[1,2], Seidel M[2], Brehm J[1], Heermann R[1]. Two novel XRE-transcriptional regulators play a major role in regulation of phenotypic heterogeneity in *Photorhabdus luminescens* cell populations. *Manuscript*

**Chapter 5:**

Eckstein S[1,2], Heermann R[1]. Regulation of phenotypic switching and heterogeneity in *Photorhabdus luminescens* cell populations. *J Mol Biol.* (2019) pii: S0022-2836(19)30211-6. *Review*

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# Contributions to publications and manuscripts presented in this thesis

**Chapter 2:**

S.E., A.G. and A.L. designed the biological experiments. A.G. performed the biofilm-assay. A.L. extracted and prepared the genomic DNA of *P. luminescens* TT01 and DJC. S.E. performed all experiments for phenotypic comparison and analyzed the data. M.-A.Z-L. and F.P. designed and performed bioinformatic analyses and annotated the genome. R H. supervised the biological studies and B.H. supervised the bioinformatics studies. The paper was written by R. H., S. E., F. P., and B. H.

**Chapter 3:**

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

**Chapter 4:**

S.E. and R.H. designed the experiments. S.E. created the deletion, insertion and complementation strains of *xreR2* and *xreR1*. S.E. overproduced and purified XreR2 and XreR1. M.S. constructed the *ccdB*-like and *ccdA*-like deletion as well as *ccdB*-like insertion strains and measured bacterial growth. S.E. performed the whole experiment with *E. coli* overexpressing *ccdB*-like. M.S. and S.E. performed and analyzed MST analysis with XreR2 and XreR1. J.B. and S.E. conducted SPR analysis. R.H. and S.E wrote the manuscript.

**Chapter 5:**

S.E. and R.H. wrote the review.

We hereby confirm the above statements:

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Maria-Antonia Zamora-Lagos

# Summary

*Photorhabdus luminescens* are Gram-negative bacteria that live in symbiosis with soil nematodes and are simultaneously highly pathogenic towards insects. The bacteria exist in two phenotypically different forms, designated as primary (1°) and secondary (2°) cells. After prolonged cultivation up to 50% of 1° convert into 2° cells. An important difference between the two phenotypic forms is that 2° cells are unable to live in symbiosis with nematodes, and therefore are believed to remain in the soil after a successful infection cycle. Furthermore, as a 100% switching frequency would be fatal for the bacteria's life cycle the switching process has to be tightly controlled. Therefore, the fate of 2° cells in soil as well as the regulation mechanism of phenotypic heterogeneity were the main focuses of this work.

The *P. luminescens* subsp. *laumondii* TT01 strain as well as its rifampicin resistant mutant ($TT01^{Rif}$) are the most common *P. luminescens* strains used in scientific research. However, the genome of $TT01^{Rif}$ has never been sequenced and referring to it as only TT01 in literature causes difficulties in clear assignment. As a first step of this work, both strains were compared genetically as well as phenotypically. Thereby, the $TT01^{Rif}$ strain could be identified as an independent isolate rather than a TT01 mutant and was therefore renamed into *P. luminescens* subsp. *laumondii* DJC.

The new DJC reference genome enabled comparative transcriptome analysis of *P. luminescens* DJC 1° and 2°. Thereby, mediation of 1°-specific features such as e.g. bioluminescence, antibiotic production or pigmentation at transcriptional level could be proven as the respective genes were found to be down-regulated in 2° cells.

Furthermore, we found initial evidence for 2° cells being adapted to an alternative environment. Metabolic changes and increased motility as well as chemotactic activity of 2° cells towards molecules presumably derived from the rhizosphere suggest an adaptation to alternative nutrients. Additionally, by up-regulation of several genes

involved in stress resistance including starvation-related genes and modification of the LPS via changed O-antigen synthesis, 2° cells seem to be well-prepared for a live outside the host(s). Moreover, the *P. luminescens*-specific quorum sensing system PpyS/PluR was found to be down-regulated in 2° cells indicating an alternative way of cell-cell communication and putatively inter-kingdom signaling.

Finally, two novel XRE-like transcriptional regulators, XreR1 and XreR2, could be identified, which play an important role in phenotypic switching of *P. luminescens*. Both inserting additional copies of *xreR2* and deleting *xreR1* in 1° cells, respectively, induced the 2° phenotype. In contrast, deletion of *xreR2* or insertion of extra copies of *xreR1* in 2° cells led to the 1° phenotype. The exact mode of action of both proteins still remains unclear. However, *xreR2* appears to be directly repressed by XreR1 while *xreR1* seems not to be under the control of XreR2. As XreR1 and XreR2 were also shown to interact with each other evidence is given that those two regulators constitute an epigenetic switch whereby XreR2 induces and maintains the 2° phenotype.

Lastly, XreR2 was shown to bind to the promoter region of an operon encoding a putative toxin-antitoxin system (TAS), CcdAB-like, which was also up-regulated in 2° cells. Since the putative toxin is C-terminally truncated its toxic effect is presumably abolished indicating another function of the system. TAS in general are known to be involved in the process of persister cell formation in other bacteria. Thus, the putative role of CcdAB-like in phenotypic switching of *P. luminescens* DJC is discussed.

In conclusion, the compiled data provides evidence that 2° cells of *P. luminescens* are better adapted to a life outside the host(s), presumably feeding from plant root exudates. Furthermore, two novel transcriptional regulators, XreR1 and XreR2, could be identified. These regulators were found to play a major role in the process of phenotypic switching and initial insights about their molecular mechanisms were gained.

# Zusammenfassung

*Photorhabdus luminescens* sind Gram-negative Bakterien, die in Symbiose mit Bodennematoden leben und gleichzeitig hoch pathogen gegenüber Insekten sind. Die Bakterien existieren in zwei phänotypisch unterschiedlichen Formen, die als primäre und sekundäre Zellen bezeichnet werden. Nach längerer Kultivierung entstehen aus bis zu 50% der Primärzellen, Sekundärzellen. Ein wichtiger Unterschied zwischen den beiden phänotypischen Formen besteht darin, dass Sekundärzellen nicht in der Lage sind, in Symbiose mit Nematoden zu leben, und daher nach einem erfolgreichen Infektionszyklus vermutlich im Boden verbleiben. Da eine 100-prozentige Konvertierung in Sekundärzellen den Lebenszyklus der Bakterien zum Erliegen brächte, muss diese streng reguliert sein. Daher wurde in dieser Arbeit das Schicksal der Sekundärzellen sowie die regulatorischen Abläufe die zu phänotypischer Heterogenität führen näher untersucht.

Der Stamm *P. luminescens* subsp. *laumondii* TT01 sowie seine Rifampicin-resistente Mutante ($TT01^{Rif}$) sind die in der wissenschaftlichen Forschung am häufigsten verwendeten *P. luminescens Stämme*. Die Tatsache, dass das $TT01^{Rif}$ Genom bisher nicht sequenziert wurde, beide Stämme jedoch in der Literatur als TT01 bezeichnet werden führt häufig zu Schwierigkeiten eindeutiger Zuordnung. Daher wurden als erster Schritt dieser Arbeit beide Stämme sowohl genetisch als auch phänotypisch verglichen. Dadurch konnte der $TT01^{Rif}$-Stamm als unabhängiges Isolat und nicht als TT01-Mutante identifiziert werden und aufgrund dessen in *P. luminescens* subsp. *laumondii* DJC umbenannt.

Mit dem korrekten DJC Genom konnte dann eine vergleichende Tanskriptomanalyse von *P. luminescens* DJC Primär- und Sekundärzellen durchgeführt werden welche beweisen

konnte, dass primär-spezifische Merkmale wie z.B. Biolumineszenz, Pigmentierung, Antibiotikasynthese und Zellverklumpung auf Transkriptionsebene vermittelt werden.

Darüber hinaus wurden erste Hinweise gefunden, dass Sekundärzellen an eine alternative Umgebung adaptiert sind. Stoffwechselveränderungen und erhöhte Motilität sowie chemotaktische Aktivität von Sekundärzellen gegenüber Molekülen, die vermutlich aus der Rhizosphäre stammen, legen eine Anpassung an alternative Nährstoffe nahe. Darüber hinaus scheinen Sekundärzellen durch Hochregulierung mehrerer Gene, die an Stressresistenz beteiligt sind. einschließlich Gene für Nährstoffdefizit, und Modifikation des LPS durch veränderte O-Antigen-Synthese gut auf ein Leben außerhalb des Wirts vorbereitet zu sein. Außerdem wurde festgestellt, dass während das *P. luminescens*-spezifische Quorum-Sensing-System PpyS/PluR in Sekundärzellen herunterreguliert ist, mehrere andere LuxR solos hochreguliert, was auf eine alternative Art der Zell-Zell-Kommunikation oder gar Inter-kingdom Signaling hinweist.

Schließlich konnten zwei neuartige Transkriptionsregulatoren der XRE-Familie, XreR1 und XreR2, identifiziert werden, die eine wichtige Rolle beim phänotypischen Phasenwechsel von *P. luminescens* DJC spielen. Das Einfügen zusätzlicher Kopien von *xreR2* oder das Deletieren von *xreR1* in Primärzellen reichten aus, um den sekundären Phänotyp zu induzieren. Im Gegensatz dazu führte die Deletion von *xreR2* bzw. die Insertion zusätzlicher Kopien von *xreR1* in Sekundärzellen zum primären Phänotyp. Der genaue Wirkmechanismus beider Proteine verbleibt noch aufzuklären. Die Bindung an ihre jeweiligen Promotorregionen lässt allerdings eine positive Auto-Regulation beider Proteine vermuten. XreR1 zeigte zusätzlich eine Bindung an den *xreR2*-Promotor. Außerdem konnte ein erhöhtes *xreR2* Level in dem Δ*xreR1* Stamm nachgewiesen werden. Diese zwei Ergebnisse lassen auf eine Inhibierung der *xreR2*-Expression schließen. Bei hohem *xreR1*-Spiegel könnte so der primäre Phänotyp aufrechterhalten werden. XreR2 zeigte zwar keine Bindung an $P_{xreR1}$, dafür aber an die Promotorregion

eines mutmaßlichen Toxin-Antitoxin-Systems (TAS), CcdAB-like, welches auch in Sekundärzellen hochreguliert war. Die Funktion dieses Systems konnte in dieser Arbeit nicht gelöst werden. Da das mutmaßliche Toxin allerdings C-terminal verkürzt ist wodurch die toxische Wirkung vermutlich aufgehoben wurde, und bekannt ist, dass TAS am Prozess der Bildung persistierender Zellen in anderen Bakterien beteiligt ist wird die mutmaßliche Rolle von CcdAB-like beim phänotypischen Phasenwechsel von *P. luminescens* DJC diskutiert.

Zusammenfassend zeigen die Daten dieser Arbeit, dass *P. luminescens* Sekundärzellen besser an ein Leben außerhalb des Wirts angepasst sind und sich vermutlich von Pflanzenwurzelexsudaten ernähren. Darüber hinaus konnten zwei neue Transkriptionsregulatoren, XreR1 und XreR2, identifiziert und erste Einblicke in ihre molekularen Mechanismen gewonnen werden

# 1 Introduction

## 1.1 The genus *Photorhabdus*

In 1979 a new bacterium associated with *Heterorhabditis* nematodes was discovered. It was specified as a member of the *Xenorhabdus* genus, which belongs to the family of Enterobacteriaceae and comprises Gram-negative bacteria that usually live in mutualistic symbiosis with nematodes of the genus *Steinernema*. Because of its ability to produce light it was termed *Xenorhabdus luminescens*. However, some bacteria of the *Xenorhabdus* genus differed a lot in their phenotypic characteristics and had big mismatches regarding their DNA. Therefore, in 1993 the genus *Photorhabdus* was invented and the bioluminescent bacterium was renamed as *Photorhabdus luminescens* (Thomas & Poinar, 1979; Boemare et *al*., 1993).

Based on biomolecular analyses the genus has been divided into three species. Besides *P. luminescens*, the two other species are *P. temperata* and *P. asymbiotica* (Fischer-Le Saux et *al*., 1999). Recently, genomes of 11 new isolates as well already described strains were sequenced. Thereby, 14 new *Photorhabdus* subspecies were identified and a re-organization of the taxonomy by raising several subspecies to species level was proposed (Machado et *al*., 2018).

However, all *Photorhabdus* species share the same complex life cycle. They live in a symbiotic mutualism with nematodes of the *Heterorhabditidiae* family and are highly pathogenic towards insect larvae such as e.g. *Galleria mellonella* or *Manduca sexta* (Akhurst, 1980). *P. asymbiotica* is furthermore the only *Photorhabdus* species that is additionally able to interact with human soft tissue causing skin infections (Gerrard et *al*., 2004, Gerrard et *al*., 2006).

The most common *P. luminescens* strain used in scientific research is *P. luminescens* subsp. *laumondii* TT01. Here, usually the laboratory strain described as spontaneously rifampicin resistant mutant ($TT01^{Rif}$) is used (Bennett & Clarke, 2005). Although both strains differ in some phenotypic traits, they are both commonly referred to as TT01 in literature causing difficulties in assignment (Bager et *al*., 2016; Engel et *al*., 2017; Langer et *al*., 2017). However, as the $TT01^{Rif}$ strain has not been sequenced yet, no proper comparison for clear distinction of TT01 and $TT01^{Rif}$ is available so far.

### 1.1.1 The life cycle of *Photorhabdus luminescens*

*Photorhabdus* species colonize the upper gut of soil-living *Heterorhabditis* nematodes that are in the nonfeeding infective juvenile (IJ) stage (Fig. 1-1). In this stage the IJs actively seek out for insect prey to infect them by invading into the haemocoel. To do so, the nematodes enter insect larvae by either entering through mouth, anus or spiracles or by slicing the cuticle via a dorsal tooth-like appendage (Kaya & Gaugler, 1993; Bedding & Molyneux, 1982). Once inside the larvae, the *Photorhabdus* bacteria are regurgitated from the gut of the nematodes into the hemolymph of the insect (Ciche & Ensign, 2003) where they start to proliferate exponentially, reaching cell densities of up to $10^9$ colony forming units (CFU) per insect within 48 hours (Watson et *al.,* 2005). Upon release into the hemolymph the bacteria are exposed to the fast-acting innate immune system of the insect (Hoffmann & Hoffmann,1990). To overcome this immune response *Photorhabdus luminescens* developed different approaches. First, the bacteria inhibit the central enzyme, phenol oxidase, in the invertebrate immune system via the secretion of the small molecule rhabduscin. Furthermore, they manage to prevent being taken up phagocyticly by the insect macrophage cells, via a type three secretion system (TTSS) in combination with the effector protein LopT (Crawford et *al.,* 2012; Brugirard-Ricaud et *al.,* 2005). To kill the insect host the bacteria begin to produce a wide variety of virulence factors, such as the Makes caterpillars floppy (Mfc) toxins and Toxin complexes (Tc's) or

the metalloprotease PrtA (Daborn et *al.,* 2001; Daborn et *al.,* 2002). The Mcf toxins owe their naming to the fact that they cause apoptosis in the midgut epithelium and hemocytes and thereby lead to a rapid loss of the insect's body turgor (Daborn et *al.,* 2002). In contrast, the Tc toxins display oral toxicity as it consists of proteins with high molecular weight (Waterfield et *al.,* 2001). Additionally, by secreting various lipases and proteases, *Photorhabdus* bioconverts the insect body into a rich food source, which is used for growth by the bacteria as well as by the nematodes (ffrench-Constant et *al.,* 2003). At this point, the bacteria are switching back to the symbiotic lifestyle again and are support the growth of the nematodes.

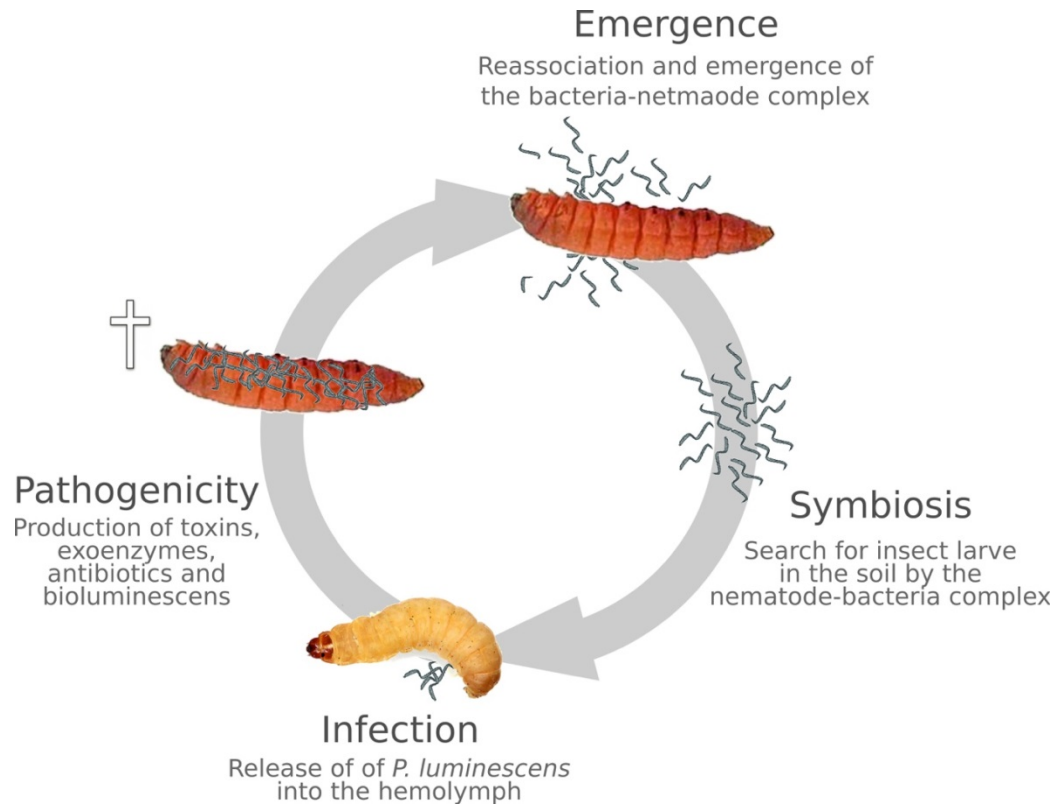


**Figure 1-1: Life cycle of *Photorhabdus luminescens*.** At the beginning of the life cycle, the bacteria colonize the upper gut heterorhabditid nematodes, which search for insect larvae in the soil. The nematodes infect an insect larva by invading into the hemocoel and regurgitate the bacteria into the hemolymph of the insect. Once inside, the bacteria start to rapidly grow 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. When all nutrients are depleted the nematodes and bacteria re-associate and emerge from the insect carcass (Waterfield et *al.,* 2009).

Although the exact mechanism is still unknown, it seems likely that *Photorhabdus* provides essential nutrients that are required for efficient nematode proliferation (Han & Ehlers, 2000). To defend the carcass against other bacteria *Photorhabdus* produces several structurally different antibiotics (Akhurst, 1982). To assert themselves against Gram-positive bacteria they produce e.g. a stilbene antibiotic (3,5-dihydroxy-4-isopropylstilbene). On the other hand, the chemical nature of another compound was solved: carbapenem, a β-lactam antibiotic, shows antimicrobial activity against some Gram-positive but especially against Gram-negative bacteria (Derzelle et *al.,* 2002).

When all nutrients of the larva are depleted, IJs are formed, *Photorhabdus* and the nematodes re-associate and emerge from the cadaver (ffrench-Constant et *al.,* 2003). Two or three generations of nematodes develop during infection. In one larva infected with a single IJ >100,000 new IJs develop within 2 to 3 weeks, underlining the high efficiency of *Photorhabdus-Heterorhabditis* interaction (Forst et *al*., 1997; Clarke, 2008; Waterfield et *al*., 2009).

## 1.2 Phenotypic heterogeneity

To persist against the selection pressure, bacterial populations have to develop different phenotypes that differ in their ability to adapt to changing environmental conditions. Here, well-established strategies are e.g. genetic rearrangements or DNA modifications, e.g. via DNA methylation (Smits et *al.,* 2006).

However, under evolutionary pressure, many bacteria evolved another strategy that results in a fitness benefit termed as phenotypic heterogeneity. Thereby, single cells of a genetically identical population in one microenvironment differ in their phenotypic traits only by exhibiting alterations in gene expression levels (Elowitz et *al.,* 2002).

One key determinant of phenotypic heterogeneity is the stochastic variation of biochemical reactions in a biological system, referred to as noise. According to the ‘finite

number effect', noise is predicted to have the highest impact when the number of involved molecules is small (Veening et *al.,* 2008).

During the last decades research on this "nongenetic" variations increased rapidly. Phenotypic heterogeneity is widely spread among Gram-negative as well as Gram-positive bacteria. Nowadays well-known examples are antibiotic resistance and persister cell formation, sporulation, bacterial competence and quorum sensing (QS)-mediated processes (Grote et *al*., 2015).

QS-dependent bioluminescence of the marine bacterium *Vibrio fischerii* was one of the first reports for a heterogeneous QS response. Unlike the theory of homogenous light production at high cell density, individual cells differed not only in their onset of bioluminescence but also in its intensity (Perez & Hagen, 2010).

Another well-studied phenotypically different system is the formation of persister cells. This phenomenon describes the conversion of a small fraction within an initial homogenous population upon antibiotic treatment. The respective fraction enters a distinct physiological state in which they can persist against the antibiotic activity (Helaine et *al*., 2014). Here, toxin-antitoxin systems are thought to play an important role (Schuster & Bertram, 2013).

One explanation of phenotypic variation is the so-called bet-hedging or risk-spreading strategy. Thereby, regardless of the environmental conditions some single individuals are properly adapted to certain impacts while the majority of the population is not. This results in an increased overall fitness of the genotype although not every cell is optimally suited (Cohen, 1966; Veening et *al.,* 2008) and allows organisms to persist fluctuating environmental conditions with no need to 'sense and respond'. Persister cell formation, described above, is one of the best-documented examples for bet-hedging. Furthermore, sporulation bistability of *B. subtilis* also forms a good example. Under nutrient limiting conditions some cells utilize alternative metabolites for growth while some cells start to

form spores. While the spore forming cells are able to resist various environmental conditions the remaining vegetative cells can easily resume growth when new nutrients are provided (Veening et *al.,* 2008).

Bet-hedging is a well-studied strategy to benefit of phenotypic heterogeneity. However, another important strategy which is fundamentally different from bet-hedging is division of labor. Hereby, the benefit is mostly asymmetrical, meaning that one cell type expresses a behavior from which the second cell type in the same microenvironment benefits from without getting a direct benefit in return (Ackermann, 2015). One example is the expression of the type three secretion system 1 (*tss*-1) of *Salmonella enterica* subsp. *enterica* serovar Typhimurium. Here the subpopulation *tss*-1 ON invades the human gut tissue and causes inflammation but grows slowly and rarely survives. In contrast, the ttss-1 OFF subpopulation benefits from the inflammation caused by tss-1 ON cells and grows quickly (Ackermann, 2015).

Well-established methods for analyzing phenotypic heterogeneity within a population at single-cell levels are e.g. fluorescence microscopy or flow cytometry using fluorescent reporter strains (Brehm-Stecher & Johnson, 2004). Furthermore, microfluidic devices and cell traps are often used for data evaluation and single-cell tracking (Probst et *al.*, 2013a; Probst et *al.,* 2013b).

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

*P. luminescens* exists in two phenotypically different cell forms designated as primary (1°) and secondary (2°) cells. Initially, phenotypic switching of *P. luminescens* has been referred to as phase variation (Boemare & Akhurst, 1988). However, comparative genomic studies are available which confirm that the differences between 1° and 2° cells are really due to phenotypic and not genotypic heterogeneity as macrorestriction and DNA microarray experiments did not reveal any differences (Gaudriault et *al*., 2008). At

the beginning of the bacteria's life cycle the population exclusively consists of 1° cells but during the infection of the insect larva some of the bacterial cells switch and turn into 2° cells. When nutrients are depleted, and the bacteria-nematode association is formed again, up to 50 % of the cells have converted into 2° cells. After one successful infection, only 1° cells re-associate with the nematodes and emerge from the cadaver to search for a new prey (Fig. 1-2A). This phenotypic switch can also be observed after prolonged cultivation under laboratory conditions (Fig. 1-2B).

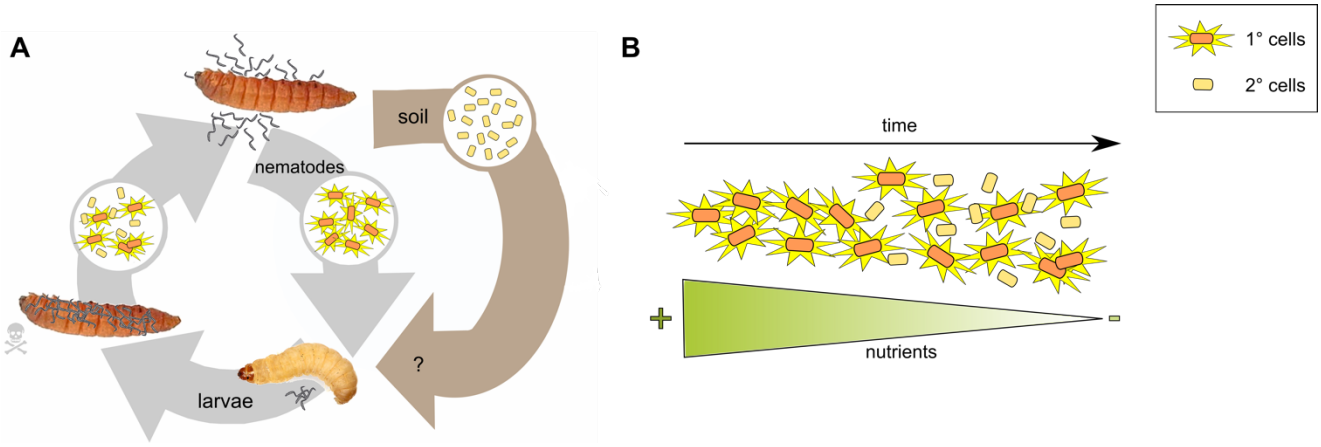


**Figure 1-2: Model of phenotypic switching process of *P. luminescens in vivo* and *in vitro*.** A) The nematodes are colonized by a 100% population of primary (1°) cells. During the infection of the larvae some of the 1° cells switch and convert into secondary (2°) cells. As only 1° cells are able to re-associate with the nematodes, 2° cells are left behind in soil after the nutrients of the larvae are depleted. B) The phenotypic switch also appears under laboratory conditions. Here, after prolonged cultivation 2° cells can be observed. Among several phenotypic differences 2° cells lack the red pigmentation shown by 1° cells as well as the production of light (depicted as yellow flashes).

The two cell forms not only differ in their cell morphology as 1° cells are long-shaped rods while 2° cells are smaller short rods (Wang, et *al.,* 2006), 1° cells also exhibit several other characteristics that are absent or diminished in 2° cells. Among these, most apparent is the lack of bioluminescence as well as the production of antibiotics, proteases and crystalline inclusion proteins CipA and CipB in 2° cells. Furthermore, 1° cells are red pigmented while 2° cells are non-colored (Akhurst, 1980, Boemare & Akhurst, 1988; Richardson *et al.,* 1988; You et *al.,* 2006). Recently, the production of the

cell clumping factor PcfA was discovered to also be a 1°-specific feature (Langer et *al.*, 2017). Remarkably, while both cell forms are equally pathogenic towards insect larvae 2° cells lost their ability to support growth and development of the nematodes and therefore cannot live in mutualistic symbiosis anymore (Han & Ehlers, 2001; Fig. 1-3).

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

**Figure 1-3: Phenotypic differences of the primary (1°) and secondary (2°) cell form of *P. luminescens.*** In contrast to 1° cells, 2° cells only slightly produce bioluminescence. Furthermore, they are non-pigmentation and do not produce any proteases, antibiotics or crystal proteins anymore. Also, cell clumping only occurs in 1° not in 2° cells. 1° and 2° cells are equally pathogenic towards insects, but 2° cells are not capable to live in symbiosis with the nematodes anymore. The table was modified after ffrench-Constant et *al.,* 2003 and Langer et *al*., 2017.

To the current state of knowledge phenotypic switching in *Photorhabdus* only appears unidirectional, occurring from the 1° to the 2° cell form. However, for the closely related genus *Xenorhabdus* infrequent reversion of the switching process has been reported (Forst & Clarke, 2002). Therefore, the switch back from 2° to 1° cells in *P. luminescens*

might be induced by specific environmental conditions or the presence of a specific signal absent under laboratory conditions.

In addition to 1° and 2° cells two heterogenous colony forms of *P. luminescens* are described designated as M- and P-forms. The small colony variant referred to as M-form got its naming as it initiates mutualism by colonizing the IJs gut. In contrast, the larg-colony variant, the P-form, causes pathogenicity. The switch between the two forms is controlled by a single promoter inversion of the *mad* fimbrial locus and therefore no true phenotypic heterogeneity (Somvanshi et *al.,* 2012, Moxon et *al.,* 1994). In 1° and 2° cells both directions of the $P_{mad}$ promoter are found indicating no difference between the two cell forms (Eckstein & Heermann, 2019).

The sociobiological aspects and thus the advantages of the whole cell population to exist in two different cell forms are still unknown. Furthermore, how 1° cells decide to become 2° also remains unclear.

### 1.2.2 Phase-specific features of *P. luminescens* 1° cells

One of the most apparent differences between the two phenotypic cell forms of *P. luminescens* is the reddish brown pigmentation of 1° cells. This coloring is caused by the production of so called anthraquinones (AQs) (Richardson *et al.,* 1988). AQ production usually occurs in plants, fungi and Streptomyces. Until today, *Photorhabdus* is the only known Gram-negative bacterium which produces AQs. Beside its weak antimicrobial activity, AQs are supposed to deter birds or scavenger insects in order to protect the nutrient source (Hilker & Köpf, 1994; Gulcu et *al*., 2012). In 2007, the respective operon *antABCDEFGHI* was identified to encode a type II polyketide synthase and several modifying enzymes responsible for AQ biosynthesis (Brachmann *et al.,* 2007). The current model supposes ligand-dependent activation of AntJ by a specific, yet unknown,

metabolite only present in 1° cells which leads to heterogenous activation of the $P_{antA}$ promoter and thereby to AQ production (Heinrich et *al.*, 2016).

In contrast to 2° cells, 1° cells produce antibiotics such as e.g. stilbenes (Derzelle *et al.,* 2002). Stilbenes are polyketide molecules that are usually produced by plants upon infection or under various stress conditions. Beside plants, *Photorhabdus* is the only organism known to produce stilbene so far. However, the production was found to significantly differ from that observed in plants (Williams *et al.,* 2005; Joyce *et al.,* 2008). As it was found that genes involved in stilbene synthesis are not clustered a complex regulation is supposed (Bode 2009). Stilbenes not only exhibit antimicrobial activity against fungi and Gram-positive bacteria but also play a role in overcoming the insect's immune system by suppressing the phenol oxidase. Furthermore, stilbene is also necessary for nematode development (Eleftherianos *et al.,* 2007; Joyce *et al.,* 2008) which is again only supported by 1° cells.

Another 1°-specific feature is the high bioluminescence. The biochemistry and physiological regulation of bioluminescence in *P. luminescens* has been well studied. The bacterial luminescence results from a typical luciferase reaction. The respective *lux* operon comprises five genes *luxCDABE*. The *luxC*, *luxD* and *luxE* genes code for enzymes for the faddy acid reductase complex which produces the long-chain aldehyde substrate. The two subunits of the luciferase are encoded by l*uxA* and *luxB* (Forst, 1997). However, the reason and need of this bacterial light production is still unclear.

Recently, production of the *Photorhabdus* clumping factor, PcfA, has been found to be also 1°-specific. Expression of the *pcfABCDEF* operon is directly activated by the LuxR solo PluR. This LuxR solo is part of the novel PluR/PpyS QS system present in *P. luminescens*. It recognizes photopyrones (PPYs) which are synthesized by the ketosynthase-like protein PpyS (Brachmann et *al*., 2013).

The exact mechanism of how *P. luminescens* supports the nematode's growth and development is not clear. However, the crystal inclusion bodies CipA and CipB were reported to be essential as single deletions of each *cip* gene led to an abolishment of nematode development. (Bintrim & Ensign, 1998; You et *al*., 2006). This fits into theory as both, nematode support as well as crystal inclusion body formation is 1°-specific.

Both phenotypically heterogeneous cell forms of *P. luminescens* are genetically identical. However, whether the phenotypic traits are mediated at transcriptional level or regulated post-transcriptionally has not been described yet.

### 1.2.3 The role of *P. luminescens* 2° cells

2° cells of lack many characteristics important for the life cycle of *P. luminescens*. It is still not known which function they fulfill and what happens to them after the 1° cells re-associated with the nematodes to re-enter the life cycle.

The current theory suggests an adaption of 2° cells to a nematode-independent life in soil (Smigielski et *al*., 1994). This idea is supported by several findings: It was shown that upon adding nutrients after periods of starvation, 2° cells recovered faster and restarted growth 2 to 4 hours while it took about 14 hours until 1° cells grew again. Thus, 2° cells seem to be more efficient in the uptake of nutrients than 1° cells. Furthermore, proteome analysis revealed an up-regulation of metabolic enzymes. Here, higher levels of major respiratory enzymes as well as an up-regulation of the transmembrane proton motive force were found (Smigielski et *al*., 1994; Turlin et *al*., 2006). However, if 2° cells truly have developed methods to persist in soil has not been proven yet and it also still remains unclear what genes might be involved. Finally, so far nothing is known about the biology of 2° cells being somehow able to become 1° again or if they might have found another way to re-enter the life cycle.

Thus, the sociobiological aspects to exist in two different cell forms and therefore the advantages for the whole cell population are still unclear. Furthermore, the bacteria-nematode complex is used as bio-insecticide to prevent crop failure and thereby spread onto agricultural fields. Therefore, knowledge about putative interactions with soil living organisms or plants would be of great interest but neither of it has been investigated yet.

## 1.3 Regulation of phenotypic switching in *P. luminescens*

Since 2° cells of *P. luminescens* are not known to have the capability to re-enter the nematodes after one complete cycle of insect infection, phenotypic switching of the whole cell population would lead to a breakdown of the bacteria's life cycle. Therefore, the switching process has to be tightly controlled. Since the 2° variant also occurs after prolonged cultivation under laboratory conditions, a response to metabolic or environmental stress is suggested (Joyce et *al.*, 2006). Low osmolarity triggered phenotypic switching in some strains of *P. luminescens* (Krasomil-Osterfeld, 1995). The complex regulatory network has not been solved yet. However, to the current state of knowledge at least two pathways are suggested to be involved in controlling phenotypic switching: a HexA-dependent pathway and an $O_2$-dependent pathway via the AstS/AstR system. Although they both seem to be activated by global stress factors, no direct connection between the two regulation pathways is known so far (Joyce et *al*., 2006).

### 1.3.1 HexA – a master repressor of 1°-specific features

The LysR-type transcriptional regulator (LTTR) HexA has been identified as a mastor regulator of phenotypic heterogeneity of *P. luminescens* (Joyce & Clarke 2003). In *E. coli*, LrhA, to which HexA is homologous, is responsible for the negative regulation of flagella, motility and chemotaxis. Since deletion of the *hexA* gene in 2° cells was sufficient to restore the 1°-specific phenotype including the ability to support growth and development of the nematodes it is assumed to act as master repressor of 1°-specific

features (Joyce & Clarke 2003). Furthermore, overexpression of *hexA* in 1° cells led to the 2° phenotype indicating that high levels of HexA are needed to maintain the 2° form (Joyce et *al*., 2006). Notably, virulence against insect larvae was weakened in the 2° Δ*hexA* strain, implying an involvement of HexA in pathogenicity of the bacteria (Joyce & Clarke 2003). As LrhA is known to positively autoregulate expression of its own gene (Lehnen et *al.,* 2002) a positive feedback loop causing *hexA* expression seems very likely. Like all LTTRs HexA consists of a N-terminal DNA-binding domain and a C-terminal co-factor binding domain separated by a short linker region (Schell, 1993). Until now no substrate ligand has been identified yet. However, recent studies identified the *pcfABCDEF* operon, which is responsible for production of the 1°-specific cell clumping factor, as the first direct target of negative regulation by HexA, (Langer et *al.*, 2017). Bioluminescence is also affected in the *hexA* mutant. However, the respective *luxCDABE* operon is not directly targeted by HexA but seems to be repressed at the post-transcriptional level (Langer et *al.*, 2017). In *E. coli* LrhA acts via regulation of translation of the alternative sigma factor RpoS, the chaperone Hfq and small RNAs (Peterson et *al*., 2006). Thus, HexA might also comprises complex regulatory functions including small RNAs (Joyce & Clarke 2003; Peterson et *al*., 2006). In summary, HexA directly and indirectly fulfills the task as repressor of 1°-specific features in 2°cells. However, the complete regulatory mechanism still remains elusive.

### 1.3.2 The AstS/R system – a timer of phenotypic switching

It has been shown that the AstS/AstR system of *P. luminescens* which is homologous to the Rcs phosphorelay system of *E. coli*, controls timing of phenotypic switching. In *P. luminescens* cells that lack the response regulator AstR phenotypic switching was premature by 7 days compared to the respective wild-type strain. Proteome analysis of the Δ*astR* strain revealed positive regulation of the gene encoding UspA, the universal stress protein by AstS/AstR (Derzelle et *al*. 2004). As such proteins are induced during

stress situations like oxidative or osmotic stress it is suggested that the AstS/AstR pathway protects the cell from stress and therefore delays phenotypic switching (Joyce et *al*., 2006). This in turn supports the theory of global stress as major signal to induce the switching process.

In *Photorhabdus* HexA seems to have a different regulatory mode of action as in contrast to *E. coli*, where the *hexA* homolog *lrhA* is under control of Rcs, *hexA* is not regulated by AstS/AstR (Derzelle et *al*. 2004). Furthermore, HexA does not control motility (Joyce & Clarke, 2003) while flagella formation is directly repressed by LrhA in *E. coli* (Gibson & Silhavy, 1999). In *Photorhabdus* the functional AstS/AstR system represses flagella formation (Derzelle et *al*., 2004) as the Δ*astR* mutant was shown to be hypermotile. However, this was only true under anaerobic conditions (Hodgson et *al*., 2003).

Thus, only little is known about how *P. luminescens* cells decide to become different and the molecular mechanisms behind.

## 1.4 Scope of the thesis

The phenomenon of phenotypic heterogeneity in *P. luminescens* cell populations is still puzzling. So far, no specific function of 2° cells could be determined and the exact regulatory processes of the switching remain elusive. Therefore, to understand the purpose of the complex life cycle of *Photorhabdus*, it is of main interest to shed led onto those queries.

As both *P. luminescens* subsp. *laumondii* TT01 as well as TT01$^{Rif}$ are easy to access, the first step should be to perform a genomic and phenotypic comparison in order to eliminate ambiguities in naming of the two strains. Furthermore, insights about genes involved in phenotypic heterogeneity of *P. luminescens* cell populations might be provided.

The investigation of genes involved in phenotypic switching as well as putative functions of 2° cells requires a detailed knowledge of genes differentially expressed in both cell forms. For that purpose, comparative transcriptome analysis (RNA-Seq) should be performed. Analysis of the resulting genes according to their function and fold change yield might reveal insights into the fate of 2° cells as well as provide information about genes involved in regulation of phenotypic switching. Furthermore, RNA-Seq data could clarify if the 1°-specific traits are mediated at transcriptional level

The current theory purposes an adaption of 2° cells to a free-living state in soil. Here, it would be crucial for the cells to be more resistant against nutrient limitation. Browsing comparative transcriptomics data could reveal an up-regulation of the respective genes in 2° cells. Furthermore, 2° cells would have to adapt to different nutrients compared to those provided by the larvae, which are not always available in close proximity. Therefore, increased steady-state motility of 2° cells as well as chemotactic response towards alternative nutrients should be investigated. As the majority of compounds present in the rhizosphere is derived by plants, the response of 2° cells to root exudates should be examined.

To enlighten the regulation process of phenotypic heterogeneity regulatory genes with highly different expression levels in 1° and 2° cells, respectively, should be selected and their putative effect on phenotypic switching should be investigated. Therefore, deletion as well as overexpressing strains should be generated and the most predominant phenotypes of 1° and 2° cells should be analyzed. If an effect can be observed, the properties of the regulators should be examined by determining their respective superfamily, domains and structural properties. Furthermore, screening for interaction partners and DNA targets might provide insights into the mode of action and regulatory functions of the proteins.

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## 1.5 References of Introduction

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# 2 Phenotypic and genomic comparison of *Photorhabdus luminescens* subsp. *laumondii* TT01 and a widely used rifampicin-resistant *Photorhabdus luminescens* laboratory strain

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# Phenotypic and genomic comparison of *Photorhabdus luminescens* subsp. *laumondii* TT01 and a widely used rifampicin-resistant *Photorhabdus luminescens* laboratory strain

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**Abstract**

**Background:** *Photorhabdus luminescens* is an enteric bacterium, which lives in mutualistic association with soil nematodes and is highly pathogenic for a broad spectrum of insects. A complete genome sequence for the type strain *P. luminescens* subsp. *laumondii* TT01, which was originally isolated in Trinidad and Tobago, has been described earlier. Subsequently, a rifampicin resistant *P. luminescens* strain has been generated with superior possibilities for experimental characterization. This strain, which is widely used in research, was described as a spontaneous rifampicin resistant mutant of TT01 and is known as TT01-Rif$^R$.

**Results:** Unexpectedly, upon phenotypic comparison between the rifampicin resistant strain and its presumed parent TT01, major differences were found with respect to bioluminescence, pigmentation, biofilm formation, haemolysis as well as growth. Therefore, we renamed the strain TT01-Rif$^R$ to DJC. To unravel the genomic basis of the observed differences, we generated a complete genome sequence for strain DJC using the PacBio long read technology. As strain DJC was supposed to be a spontaneous mutant, only few sequence differences were expected. In order to distinguish these from potential sequencing errors in the published TT01 genome, we re-sequenced a derivative of strain TT01 in parallel, also using the PacBio technology. The two TT01 genomes differed at only 30 positions. In contrast, the genome of strain DJC varied extensively from TT01, showing 13,000 point mutations, 330 frameshifts, and 220 strain-specific regions with a total length of more than 300 kb in each of the compared genomes.

**Conclusions:** According to the major phenotypic and genotypic differences, the rifampicin resistant *P. luminescens* strain, now named strain DJC, has to be considered as an independent isolate rather than a derivative of strain TT01. Strains TT01 and DJC both belong to *P. luminescens* subsp. *laumondii*.

## Background

*Photorhabdus spp.* are pathogenic enteric bacteria that maintain a mutualistic interaction with heterorhabditid nematodes and can infect a wide variety of insect species. To date, three *Photorhabdus* species are known: *P. luminescens*, *P. temperata*, and *P. asymbiotica* [1]. Whereas the first two species are highly pathogenic toward insects, *P. asymbiotica* is additionally associated with severe soft-tissue and systemic infections in humans, and is considered as an emerging threat [2]. Commonly, the bacteria colonize the gut of the infective juvenile stage of *Heterorhabditis spp.* nematodes. Upon entering insect larvae, the nematodes inject the bacteria by regurgitation into the insect's hemocoel. Once inside the insect, the bacteria replicate rapidly and quickly establish a lethal septicaemia in the host by production of a broad range of different toxins that kill the insect within 48 h. Bioconversion of the insect's body by *Photorhabdus spp.* produces a rich food source for the bacteria as well as for the nematodes. Nematode reproduction is

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supported by the bacteria, probably by providing essential nutrients that are required for efficient nematode proliferation [3]. Furthermore, the bacteria produce several secondary metabolites like antibiotics to defend the insect cadaver from invasion by other microorganisms. *P. luminescens* glows because of bacterial luciferase production. When the insect cadaver is depleted, the nematodes and bacteria re-associate and emerge from the carcass in search for a new insect host (see [4, 5] for review).

*P. luminescens* subsp. *laumondii* strain TT01 (DSM 15139) was originally isolated from *Heterorhabditis bacteriophora* nematodes in Trinidad and Tobago [6]. Since strain TT01 was difficult to access for genetic manipulation methods, a rifampicin resistant strain was isolated by the group of David J. Clarke (University College Cork, Ireland) by growing strain TT01 in the presence of the antibiotic [7]. This strain showed enhanced suitability for genetic manipulation due to the resistance marker, and was described as a spontaneous rifampicin resistant mutant of strain TT01 (TT01-Rif$^R$) [7]. In the scientific literature, authors working with either TT01-Rif$^R$ or the original TT01 strain commonly refer only to TT01, making this assignment highly ambiguous [8–10].

Here we performed a phenotypic comparison between *P. luminescens* strains TT01 and TT01-Rif$^R$. Since both strains differed in many phenotypic traits, we performed detailed genomic analysis, generating a finalized complete genome sequence based on the PacBio long read approach [11]. We compared the genomes of the two strains in detail and report extensive sequence differences, indicating that TT01-Rif$^R$ is an independent isolate from type strain TT01. Therefore, we renamed TT01-Rif$^R$ to DJC.

## Results

### Phenotypic comparison of *P. luminescens* strains TT01 and DJC

As a first step to investigate the differences between *P. luminescens* TT01 and DJC we started by comparing some of the most important phenotypes of *Photorhabdus spp.* like growth rate, pigmentation, bioluminescence, insect pathogenicity and nematode support.

*Growth behaviour. P. luminescens* strains TT01 and DJC showed differences in growth behaviour. The growth rate in the exponential growth phase was higher for strain TT01 ($\mu = 0.39$/h) compared to DJC ($\mu = 0.16$/h). Furthermore, in LB broth strain TT01 ($OD_{600} = 21$) reached higher cell densities compared to strain DJC ($OD_{600} = 16$) in the stationary growth phase ($t > 90$ h) (Fig. 1a). The maximal cell density remained constant over a long period (up to 170 h) and no cell lysis was observed neither for strain TT01 nor strain DJC.

*Pigmentation.* Pigment production of both cultures was different after 48 h of cultivation. Whereas the medium containing strain TT01 became dark yellow, the one inoculated with strain DJC turned orange, revealing that both *P. luminescens* strains have differences in secondary metabolite production and/or the regulation of the corresponding genes (Fig. 1b).

*Pathogenicity and bioluminescence.* We next analyzed pathogenicity against *Galleria mellonella* wax moth larvae of both *P. luminescens* strains. For that purpose, *G. mellonella* larvae were infected with either 200 or 200,000 cells, respectively, of *P. luminescens* strain TT01 or DJC. However, we could not observe major differences in pathogenicity between the two strains: 100% of the larvae died within 48 h after infection either with strain TT01 or DJC, respectively. Approximately 1/3 of the larvae even died after 24 h for both strains at the higher bacterial load (Fig. 1c). Furthermore, *G. mellonella* larvae killed by either TT01 or DJC both turned red due to anthraquinone production and were both positive for bioluminescence (Fig. 1c). Additionally, light production of populations of both strains was quantified in liquid culture. Here we observed that bioluminescence of *P. luminescens* strain TT01 was significantly higher compared to strain DJC ($p$-value < 0.001), especially at the time point of growth when the cells entered the stationary growth phase (Fig. 1d).

*Nematode symbiosis.* To investigate the symbiotic capacity of both *P. luminescens* strains, we tested whether the bacteria were able to support nematode development. For that purpose, infective juveniles (IJs) of *Heterorhabditis bacteriophora* were added to lipid agar plates containing either *P. luminescens* strain TT01 or DJC, respectively. After 8 days of incubation, the number of hermaphrodites that developed from the IJs were counted. No significant differences between *P. luminescens* strain TT01 and DJC were observed (Fig. 1e).

*Rifampicin resistance.* Strains DJC and TT01 were tested for rifampicin resistance, and only strain DJC was found to be resistant (Fig. 2a). Furthermore, we tested both strains for their ability to produce exoproteinases, their ability to perform haemolysis and for antibiotic production (Fig. 2a). To compare proteolytic activity, we spotted *P. luminescens* strain TT01 and DJC, respectively, on Ca-caseinate agar plates. Both strains showed comparable protein degradation (Fig. 2a). Furthermore, we plated both strains on sheep blood agar plates and LB agar plates to investigate haemolysis and antibiotic production, respectively. Surprisingly, *P. luminescens* DJC showed a significantly higher haemolytic activity ($p$-value < 0.001) as well as antibiotic production ($p$-value < 0.05) compared to strain TT01 (Fig. 2a).

*Biofilm formation.* Finally, we analysed both strains for their ability to form biofilms. Both strains were incubated under gentle movement in cavities of 96 well plates to allow them to attach to the surface, before the medium was gently removed. The remaining cells that organized in a biofilm were re-suspended and quantified by crystal violet staining. Remarkably, strain DJC showed a significantly

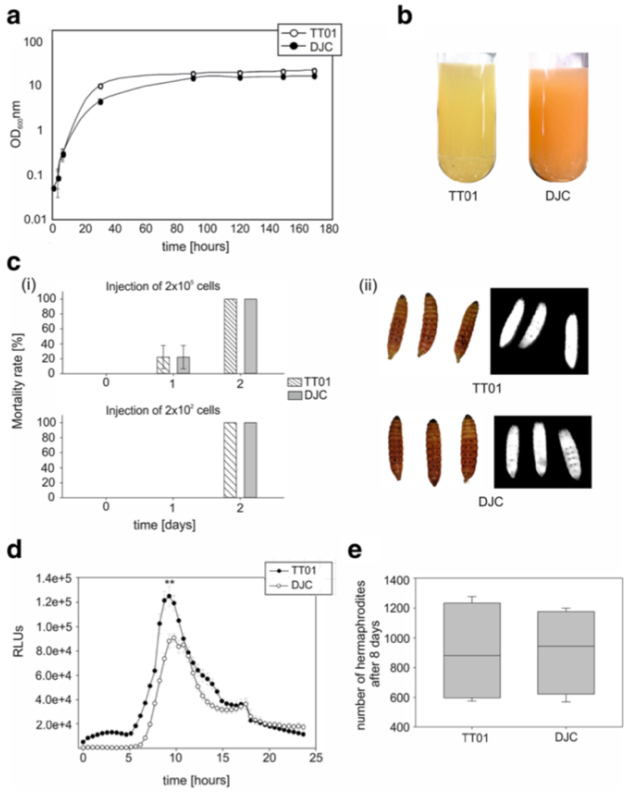


**Fig. 1** Growth, pigmentation, pathogenicity, symbiosis and luminescence of *P. luminescens* strains TT01 and DJC. **a** Growth curve of *P. luminescens* TT01 and DJC cultivated in LB broth at 30 °C for 7 days. **b** Pigmentation of a liquid culture of *P. luminescens* strain TT01 and DJC after 48 h of growth in LB broth at 30 °C. **c** Insect pathogenicity. Mortality rate of *Galleria mellonella* larvae after injection of 200,000 or 200 cells, respectively of *P. luminescens* strain TT01 or DJC (i). Pigmentation (left panel) and bioluminescence (right panel) of dead larvae 48 h after being infected by either *P. luminescens* strain TT01 or DJC (ii). **d** Quantification of bioluminescence of *P. luminescens* strain TT01 and DJC cultivated in LB broth. RLUs = Relative Light Units. **e** Symbiosis assays. Number of *Heterorhabditis bacteriophora* hermaphrodites 8 days after addition of nematodes to the respective *P. luminescens* strain as read-out for the ability to support nematode development. The asterisks (**) indicate statistically significant differences with a *p*-value smaller than 0.001. Error bars represent standard error of three independently performed experiments.

higher ability for biofilm production ($p$-value < 0.001) compared to TT01 (Fig. 2b). Summarizing, we found that strain DJC not only differs from strain TT01 in resistance against rifampicin, but also in many other phenotypes that are important for the *P. luminescens* life cycle, such as bioluminescence, haemolysis, antibiotic production, and biofilm formation, revealing that both strains are more different from each other than initially thought. To investigate these differences further, we decided to compare the two *P. luminescens* strains at genome level.

### Genome sequencing and assembly for *P. luminescens* strains TT01 and DJC

The genomes of *P. luminescens* strain DJC and of a variant of strain TT01 were sequenced using the long read PacBio technology with at least 180-fold coverage. This allowed us to assemble the sequences in one step into a single contig representing the final complete circular genome with high sequence reliability. The reconstructed TT01 wild-type sequence was used for all subsequent analyses (see Methods section). Further on, we refer to this genome sequence as TT01m. The overall characteristics of the genomes are shown in Table 1.

### The newly sequenced *P. luminescens* TT01m genome sequence is highly similar to the previously published TT01 genome sequence

We first attempted to estimate the divergence between the two versions of the strain TT01 genome. We only found 30 differences between the published

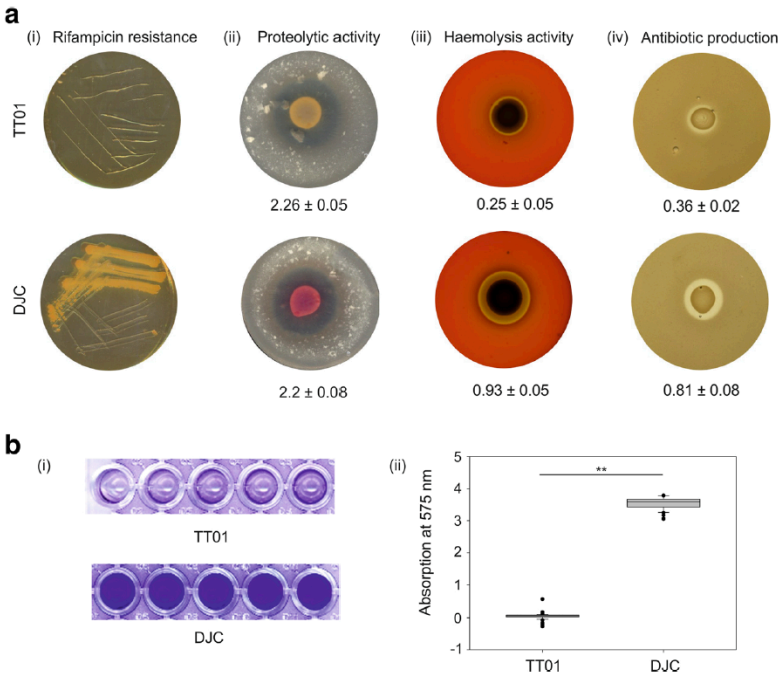


**Fig. 2** Rifampicin resistance, proteolytic and haemolytic activity, production of antibiotics and biofilm formation of *P. luminescens* strains TT01 and DJC. **a** Growth and extracellular activities of *P. luminescens* strain TT01 (upper panel) and strain DJC (lower panel). (i) Resistance towards rifampicin (50 μg/ml) after 48 h of incubation at 30 °C. (ii) Proteolytic activity on Ca-caseinate plates after 48 h of incubation at 30 °C. (iii) Secreted haemolytic activity of *P. luminescens* TT01 and DJC on sheep red blood agar plates after 4 days of incubation at 30 °C. (iv) Antibiotic effect on *B. subtilis* agar plates after 48 h of incubation at 30 °C. **b** Biofilm formation. Crystal violet staining of *P. luminescens* strain TT01 and DJC grown in LB broth and cultivated for 72 h under gentle shaking (150 rpm) at 30 °C. The planktonic cells were removed and the sessile cells, i.e. biofilm, was stained violet. The stained plates are shown on the left panel (i), whereby the quantification of the staining is shown right (ii). The asterisks (**) indicate statistically significant differences with a *p*-value smaller than 0.001. Error bars and values represent standard error of three independently performed experiments

TT01 and our newly sequenced TT01m genome (see Additional file 1: Table S1), confirming the overall high reliability for both sequencing efforts. Observed differences included point mutations, one-base indels, copy number variations, genome inversions, and two long indels.

*Coding regions affected by genomic differences between* P. luminescens *TT01m and TT01.* We found 14 protein-coding genes that are affected by the 30 differences between the genomes. In the published TT01 genome, 2 mutations are synonymous and 5 non-synonymous, 3 mutations result in aberrant termini, 2 proteins are split, with N-

**Table 1** General characteristics of the sequenced *P. luminescens* genomes

| | *P. luminescens* DJC | *P. luminescens* TT01m | *P. luminescens* TT01 |
|---|---|---|---|
| Reference | This paper | This paper | [6] |
| Accession | CP024900 | CP024901 | BX470251 (refseq:NC_005126) |
| Length (bp) | 5,536,539 | 5,687,677 | 5,688,987 |
| Protein-coding genes | 4841 | 4943 | 4839 |
| Pseudogenes | 329 | 351 | 157 |
| Genome coverage | 194-fold | 182-fold | 7-fold |

The type of data is indicated in the 1st column. The data are shown for the newly sequenced *P. luminescens* genomes DJC and TT01m. For comparison, data are also provided for the published version of the strain TT01 genome. Data were taken from [6]. Disrupted genes (pseudogenes) may be annotated as multiple independent genes, especially if targeted by a mobile genetic element. Such genes may not have been rated to be pseudogenes in [6]

and C-terminal parts annotated as independent proteins and 2 proteins are affected in multiple ways. One point mutation is located in an rRNA gene. Our new genome sequence consolidates disrupted genes in the published TT01 genome, which points to a higher reliability of the sequence we have obtained (Additional file 1: Table S1).

*Copy number variations of tandem repeats.* There were 4 differences between the genomes due to tandem repeats of 8–16 bases. In some of these, two distinct repeats are tandem-repeated directly adjacent to each other. Some tandem repeats exist in many copies (up to 47); and some show copy number differences also in the *P. luminescens* DJC genome (see below).

*Large genome inversions.* We encountered two large inversions (3.4 and 5.8 kb), one of which was associated with a frameshift difference. In both cases, the inverted region is bounded by a long inverted repeat (35 and 84 bp) and is located in a prophage region.

*Large indels.* We found two large indels, one additional region in each *P. luminescens* TT01 genome version. In both cases, the observed indel is due to a highly conserved repeat, which we refer to as phage-related repeat A (PhRepA). The originally reported genome sequence for TT01 lacks the 2nd of 3 tandem copies at 4.23 Mb, while the TT01m sequence lacks the 2nd of 4 tandem copies at 4.35 Mb (Fig. 3a).

### *P. luminescens* strain DJC is an independent isolate rather than a spontaneous mutation of strain TT01

Next, we compared the sequences of the newly obtained *P. luminescens* TT01m genome with that of strain DJC. This revealed many more differences than expected given the reported genealogy, i.e. that strain DJC was a spontaneous $Rif^R$ mutant [7].

We performed a detailed comparison between the two genomes based on MAFFT pairwise alignments [12] (see Methods for details). In brief, our method splits the genomes in "matching segments" (matchSEGs), most of which have less than 1% sequence difference and in "divergent segments" (divSEGs), which are either indels or regions of higher sequence divergence. The genome switches between these two types of segments.

*Matching genome segments.* The genomes were split into a total of 225 matchSEGs. These cover the majority of both genomes, 91.5% for the *P. luminescens* TT01m genome and 94.0% for strain DJC. They have an overall sequence identity of 99.7% (Additional file 1: Table S2). The majority of the matchSEGs (178 in total) cover 5.02 Mbp and have less than 1% sequence divergence (99.8% cumulative sequence identity). The residual 47 matchSEGs cover 180 kb and have more than 1% sequence difference, with 97.8% cumulative sequence identity. These are generally shorter, but only 5 are longer than 10 kb. Overall, we detected 12,967 point mutations and 333 frameshifts in the 225 matching genome segments.

*Divergent genome segments*. The strain-specific sequences (divergent segments, divSEGs) sum up to 333,729 bp for *P. luminescens* strain DJC or 6% of the DJC genome and 484,908 bp for strain TT01m (8.5% of the genome).

DivSEGs were separated into four categories according to the following characteristics (Additional file 1: Figure S1, Additional file 1: Table S2): (a) indels: an indel is continuous in one genome and has an insertion in the other so that the extra sequence can be pinpointed to an exact position. There are 83 insertions in TT01m and 68 insertions in strain DJC. (b) approximate inserts: these are inserts which can only be positioned with an error tolerance of up to 10 bp due to unaligned bases in the other genome. We encountered 13 approximate inserts, 8 in the DJC and 5 in the TT01m genome. (c) replacements: there are 47 replacements that have dissimilar sequences in both strains, located at an equivalent position with 1-base resolution. These are either completely unrelated or homologous and may reach more than 90%, but less than 95% sequence identity. (d) copy number variations of tandem repeats (7–12 bp), where the number of copies differs from 10 to 49 copies.

The majority of the inserted sequences (indels and approximate inserts) are mobile genetic elements, which are described in more detail below. The remainder of the inserted sequences and the replacement sequences frequently represent genome-internal duplications (flagged InternallyRepeated in Additional file 1: Table S2). A total of 10 long insertions in either genome TT01m or DJC are prophages. Several of the larger indels or replacements represent copies of the closely related repeat PhRepA. A small number of strain-specific sequences were found to be unrelated to the other genome, having either no or only a partial BLASTn hit. On several of these, a mobile genetic element was present as a passenger along with other sequences.

Both genomes contain 6 CRISPR arrays with two variants of the repeat (GTKCACTGCCGTACAGGCAGCT TAGAAA, whereas K can be G or T). In each of the CRISPR arrays, at least some of the spacers differ (see Additional file 1: Table S2). At the end of the 2nd array, the TT01m genome has a deletion, which truncates the *cas1* gene. Some spacers occurring in one strain match to strain-specific sequences of the other strain.

In summary, the extensive differences in the two genomes make it likely that strain DJC represents an independent isolate rather than a mutant or a derivative of strain TT01. These findings support our assignment of a new strain name (DJC) instead of the original one ($TT01\text{-}Rif^R$). We used our genome alignment to look at differences in protein-coding genes, prophages, as well as mobile elements between the two strains in more detail.

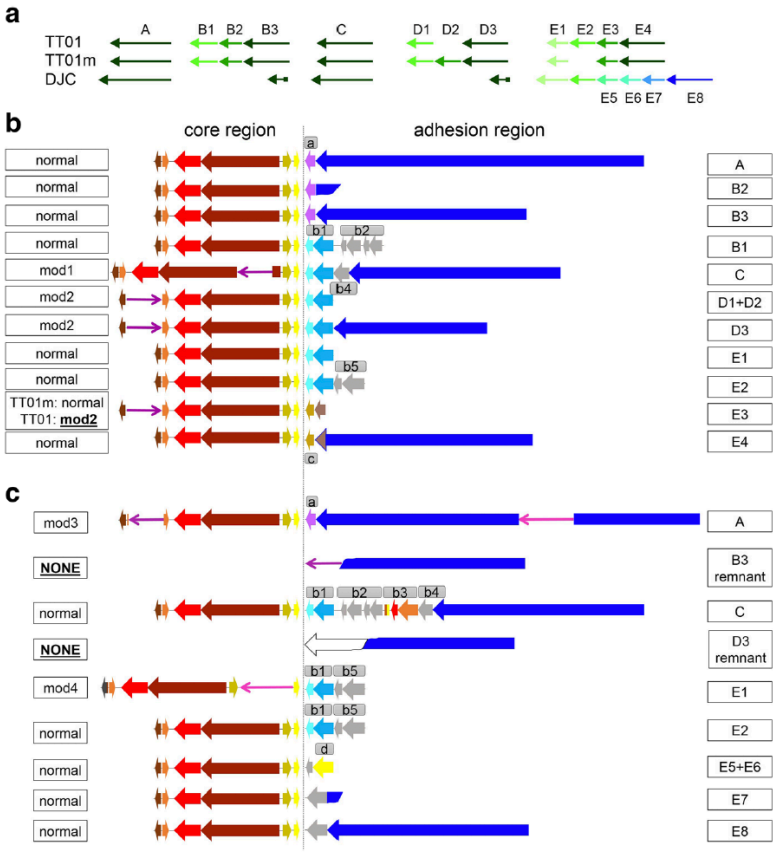


**Fig. 3** Presence of phage-related repeat PhRepA in the *P. luminescens* TT01, TT01m and DJC genome. **a** The PhRepA genes are organized in five clusters (A, B, C, D, E). Homologous clusters are drawn in similar color. (**b**, **c**) Detailed view of the prophage-related regions PhRepA in the *P. luminescens* TT01/TT01m (**b**) and DJC (**c**) genome. The different clusters are named with letters already used in the overview. The PhRepA regions can be subdivided into two parts, the "core region" (left of the vertical line) and the adhesion region (to the right). Normal = normal composition and presence of core genes; mod = modifications of the normal composition and presence of core genes. Homologous genes are displayed in similar colors. See text for details

*Taxonomic analyses*. We compared the sets of 16S rRNA genes between TT01m and DJC. Each genome has 7 operons. When the 16S rRNAs encoded within in the TT01m genome are compared to detect polymorphisms, there are up to 10 base differences. When comparing the TT01 and DJC genomes, the 7 rRNA operons are found at equivalent positions, so that position-correlated 16S rRNA sequences can be compared. We found that 4 are identical and 2 differ by only a single base. The 7th operon is the one with the highest number of polymorphic bases in TT01m and shows 9 base differences to the 16S rRNA sequence of strain DJC. However, the DJC sequence differs by only a single base from that of another 16S rRNA, likely an effect of sequence harmonization by genome-internal translocation. We also analysed 4 conserved genes which have been proposed as taxonomic markers (*recA*, *gyrB*, *dnaN*, *gltX*) [13]. They show up to 5 point mutations, of which up to 4 are non-silent. Strain DJC shown an ANIb value of 99.49, based on 94% of its genome. From these data it can be concluded that both strains share a common taxonomic position at the subspecies level.

## Comparison of the protein-coding genes between the *P. luminescens* genomes TT01, TT01m and DJC

### *Comparing the protein-coding genes between the two versions of the* P. luminescens *strain TT01 genome*

We correlated the ORFs sets of the two versions of the *P. luminescens* TT01 genome, which reflect genome annotation

inconsistencies rather than genome sequence differences, with just a few exceptions. If discrepancies pointed to a problem in the newly sequenced genome, we applied manual curation to improve the annotation. The main purpose of this comparison was to provide the community with a full mapping of the established ORF codes (plu numbers) with the ORF codes as assigned by the PGAP pipeline (PluTT01m numbers). The data, which also contain the mapped codes for the DJC strain (PluDJC numbers), are provided as Additional file 2: Table S3b, and a detailed legend is provided with a sample table as Additional file 1: Table S3a.

### *Comparing the protein-coding genes between the* P. luminescens *strain TT01m and strain DJC*

We correlated the ORFs sets initially predicted by the PGAP annotation pipeline for the genomes DJC and TT01m. With all genome regions assigned into matchSEGs and divSEGs and the MAFFT alignments for each segment, we could compute positional correlations and use these data for ORF mapping (for details see Methods). For cases of perfect mapping, where both termini were assigned to equivalent positions in the two genomes and were located in the same segment, and to which identical protein names had been given, we accepted the automatic annotation. All other ORFs were subjected to manual curation.

We were interested in differences between the two strains with respect to the set of their protein-coding genes. We thus extracted strain-specific protein coding genes (Additional file 1: Table S4) and those that were disrupted in one strain (pseudogene) and regular in the other (Additional file 1: Table S5). To focus on genes of higher relevance for *P. luminescens*, various gene categories were excluded, such as transposases, ORFs on the PhRepA repeat or phage-related proteins. We also excluded strain-specific genes with a close homolog of at least 75% protein sequence identity in the other strain and disrupted strain-specific genes. In total, strain DJC encodes 155 proteins that are not encoded in the TT01m genome, while 244 proteins that are found in TT01m are not present in DJC. The majority, 104 unique to DJC and 136 unique to TT01m, were annotated as hypothetical and could not be assigned a function. Both strains have sets of unique DNA-binding, DNA-modifying, restriction and DNA-replication enzymes, transcription factors, different types of toxin-antitoxin systems, as well as a set of unique proteins containing conserved domains of unknown function (DUF). However, the strain-specific proteins cannot be directly attributed to the observed phenotypic differences.

Furthermore, there are 31 and 32 disrupted genes in DJC and TT01m, respectively, which encode full-length proteins in the other strain (Additional file 1: Table S5).

Interestingly, both strains have two homologous CRISPR/CAS systems. One of the Cas3 helicases is disrupted in DJC. Most likely, prophage targeting resulted in two fragments of Cas3. In summary, both *P. luminescens* strains differ in presence or absence of a large number of genes, the majority encoding proteins of yet unknown function.

*Investigating rifampicin resistance in the DJC genome.* Rifampicin (Rif) is an antibiotic that inhibits the bacterial transcription machinery by interacting with the β-subunit of the RNA polymerase, which is encoded by *rpoB*. Mutations in *rpoB* can lead to resistance to rifampicin [14]. We investigated the genomic locus of *rpoB* in strain DJC ($Rif^R$), as rifampicin resistance is the distinguishing characteristic reported for this strain. The genome of *P. luminescens* strain DJC shows 9 point mutations compared to the TT01m genome, which are located within the *rpoB* gene. While 7 mutations are silent, 2 point mutations cause amino acid replacements H526Y and E995G in the RpoB protein. It is noteworthy to mention that the H526Y replacement is located within the rifampicin-resistance hotspot 1 described for *E. coli* [15].

### Prophages and phage-related repeat PhRepA in *P. luminescens*

Many of the large-scale divergences between the genomes of *P. luminescens* TT01, TT01m, and DJC seemed phage-related. Therefore, we performed an extensive analysis of prophages. We used PhiSpy [16, 17], as well as Prophinder from the ACLAME web server [18] (see Methods for details) to predict prophages (Additional file 1: Table S6). We found considerable differences in the predictions, even if the same method was applied to near-identical genomes. If the predictions from the two programs were overlapping, we combined them as "prophage region".

*The majority of long indels are integrated prophages.* We encountered a total of 12 long insertions (> 10 kb), 7 in the TT01m genome (up to 79 kb) and 5 in the DJC genome (up to 35 kb). Of these, 10 were assigned to be prophages according to PhiSpy and ProPhinder. An indel with 26 kb in TT01m corresponds to PhRepA copy D. An indel with 12.7 kb in DJC is unlinked to prophages. The longest sequence in the replace category of divSEGs is a 57 kb region predicted to be a prophage in the DJC genome. An unrelated 5.7 kb sequence is at the equivalent position in the TT01m genome.

*Prophage integration in coding sequences.* We observed three cases where a prophage might have targeted a protein-coding gene. The gene fragments were located more than 25 kb apart and the intervening sequences were part of predicted prophages. Coding sequence disruption is not uncommon as revealed by the bioinformatics prediction and analysis of 36,000 prophages [17]. As mentioned above, one prophage has targeted the *cas3f* gene in strain DJC. One prophage in each of the strains seems to have integrated into a pre-existing

prophage, leading to a prophage conglomerate. Such conglomerates may explain the heterogeneity of the prophage prediction results from the two programs. In strain DJC, a prophage has integrated into a holin gene, in the TT01m genome, a prophage was found integrated into a restriction methylase.

*Prophages with internal inversions.* Two prophages contain an inverted region when comparing the newly sequenced TT01m genome to the published TT01 genome. The first inversion is specific to the TT01m genome while both, the published TT01 and the strain DJC genome contain this segment in the same orientation. The second inversion occurs only in the published TT01 genome while the TT01m and DJC genomes have this segment in the same orientation. However, within the same prophage region, part of the sequence is inverted in the DJC genome, whereas both versions of *P. luminescens* TT01 contain the segment in the same orientation. An additional 0.9 kb inversion in strain DJC differs from both TT01 genomes. This region however is not predicted to be a prophage.

*The phage-related repeat A region.* One prophage covers a repeat, which is a patchwork of highly conserved but also of highly diverse sequences among the analysed strains. We have named these sequences the phage-related repeat A (PhRepA) region, since some of them are in regions assigned to be prophages (Additional file 1: Table S7). The two large indels between the two versions of TT01 represent extra copies of this repeat, one in each genome (Fig. 3a). In general, there are 10 copies present in each of the TT01 genomes. In strain DJC, there are 8 copies of which 4 correspond to those of TT01m/TT01 while the other 4 are specific for strain DJC. The copies of PhRepA in the analysed *P. luminescens* genomes TT01, TT01m and DJC are schematically drawn in Fig. 3a and listed in Additional file 1: Table S7. As it can be seen, the PhRepA repeat has a tendency to form tandem duplications. Only two elements are singlets (copies A and C in both, TT01 and DJC). The other copies occur as tandem duplicates with 2 to 6 copies within each cluster (copies B, D, and E). In those clusters, the terminal copy is complete while the other copies are truncated. Many strain differences are due to heterogeneity in these clusters of tandem duplications. Two long indels between TT01 and TT01m are copies of PhRepA. Many of the PhRepA copies differ between strains TT01 and DJC: there are six tandem copies in strain DJC but only the first two correspond to the four copies found in strain TT01 in cluster E (Fig. 3a). DJC contains only remnants of clusters B and D.

Theoretically, the observed additional copies of PhRepA could represent genome assembly errors rather than biological differences. However, we consider misassembly of the TT01m genome as unlikely. Though PhRepA elements have extremely high similarity over several kb, the PacBio long read technology was shown to efficiently cope with duplications of that size [19].

PhRepA consists of two subregions that we refer to as the "core region", which is complete in all copies and encodes 6 genes, and the "adhesion region", which is rather diverse between different copies of PhRepA and is affected by truncation. The overview of the "core" and "adhesion" regions present in the TT01/TT01m genomes is displayed in Fig. 3b, copies and organization of these regions in the DJC genome is shown in Fig. 3c and details are described in Additional file 1: Text S1 and listed in Additional file 1: Tables S7 and S8.

The core region codes for a central gene pair, one gene containing a DNA primase (IPR13264 and IPR034151) and the other an integrase/recombinase (IPR011010) domain. This gene pair is highly conserved among all copies of PhRepA. Adjacent to the integrase is a short gene coding for a DNA-binding protein with a Cro/C1-type HTH domain (IPR001387), which is not well conserved among PhRepA copies. Located next to the gene encoding the DNA-binding protein is a gene coding for a protein with a SymE-like toxin domain (IPR014944), which also occurs in several distinct subtypes.

The adhesion region of complete PhRepA elements, which can either be singlets or terminal copies of clusters, codes for a long protein (2135–4582 amino acids) with adhesion-related domains. These include several copies of pectin lyase fold domains (IPR012334) and of hemagglutinin repeats (IPR025157). Between this gene and the core region is a rather variable set of 1 to 7 genes. Adjacent tandem-duplicated copies of the PhRepA repeat have a tendency to share the same gene set and may contain an adhesion protein remnant as a truncated gene. All genes encoded on the different copies are schematically drawn in Fig. 3b (TT01 and TT01m) and Fig. 3c (DJC) and are described in Additional file 1: Text S1 and listed in Additional file 1: Tables S7 and S8.

### Mobile genetic elements in the *P. luminescens* TT01/TT01m and DJC genomes

We performed a detailed transposon analysis of the *P. luminescens* DJC and both TT01 genomes. According to ISFinder, there are 22 distinct transposons present in *P. luminescens* [20], some of which have been submitted in the course of this study. Some of these have a high number of copies (up to ~20 complete copies). We also identified a few types of MITEs.

*Transposons identified in the three* P. luminescens *genomes.* Many insertions in the indels and approximate inserts represent mobile genetic elements. They commonly include a target site duplication (TSD). The relative frequency of individual transposon classes is shown in Additional file 1: Figure S2.

The transposons with the highest mobility are related to IS630. These belong to the IS630/Tc1/mariner superfamily which is found in both, prokaryotes and eukaryotes [21–23]. Although this class of transposons has been preferentially analysed in plants, such elements have also been identified in nematodes. We categorized IS630-type elements from *P. luminescens* as CCC-type (ISPlu3, ISPlu8, ISPlu19) and as AATAA-type (ISPlu10, ISPlu16), according to characteristic sequences at or very close to the beginning of the element (Fig. 5).

*MITEs identified in the three* P. luminescens *genomes.* MITEs are mobile genetic elements, which are too short to carry a transposase gene. However, they have inverted terminal repeats related to other transposons and thus are mobilized *in trans* by the corresponding transposase [24]. During our analysis, we identified 6 new MITE types and submitted these to ISFinder.

The most frequent repeat with 552 complete copies in the TT01 genome and a typical length of 123 bp is MITEPlu5. Of the 552 complete copies, 467 have a length of 123 bp and were used to compute a sequence logo (Fig. 4a), and subsequently a consensus sequence. Given the obvious high sequence conservation, it is remarkable that only a few of these elements are truly identical to each other and that none of the copies matches exactly to the consensus sequence. This MITE seems to be highly mobile, as 47 of these elements represent indels between the TT01m and the DJC genome. A related element has been described as an ERIC sequence [25] and is reported in ISFinder as MITEEc1. MITEPlu5 shows an extremely strong secondary structure when analysed by RNAfold [26] (Fig. 4b) as also previously reported for MITEYpe1 [27]. We analysed this element in more detail (Fig. 5). We detected sequence similarities between MITEPlu5 and a subset of the IS630-type transposons with marked conservation of a CCC trinucleotide close to the terminus as found for ISPlu3, ISPlu8 and ISPlu19. For an extended description of this element see Additional file 1: Text S2.

We consider MITEPlu5 as non-coding. However, some of the copies lack stop codons in some frames. This has resulted in protein coding gene annotation by the PGAP pipeline [45]. We have retained these ORFs but have assigned the protein name "pseudocoding frame MITEPlu5" as a warning for annotation robots.

Our observations suggest that MITEs and potentially other transposable elements can lead to mis-annotations by the PGAP pipeline. Short ORFs consisting largely of MITEPlu5 and only few bases from adjacent unique genome sequence (< 100 bp) were mis-annotated to have specific protein names. The ORFs were annotated as "riboflavin synthase", "chorismate lyase", "addiction toxin module relE", "SprT family protein", "pirin family protein". We performed BLASTx comparisons against the UniProt and NCBI nr databases to validate that the genome-derived section does not support the mis-assigned protein name. In several cases, identical mis-annotations have been made for both genomes. To avoid mis-annotation in the future, we suggest that automated annotation robots should be optimized to deal with such situations.

### Differentiation between *P. luminescens* strain TT01 and DJC via PCR

The knowledge that *P. luminescens* DJC and TT01 are two independent strains and the fact that scientists working with either $Rif^R$ or the Rif sensitive strain refer to each of them as TT01 prompted us to design primer pairs for easy distinction between DJC and TT01 (Additional file 1: Table S9). We chose five gene regions where the same pair of primers can be used, but the PCR product length differs by at least 400 bp (Table 2; Fig. 6).

The DJC strain was sent to the Clarke laboratory in July 2000 by the laboratory of Dr. Noel Boemare (Université de Montpellier). However, it is standard to send

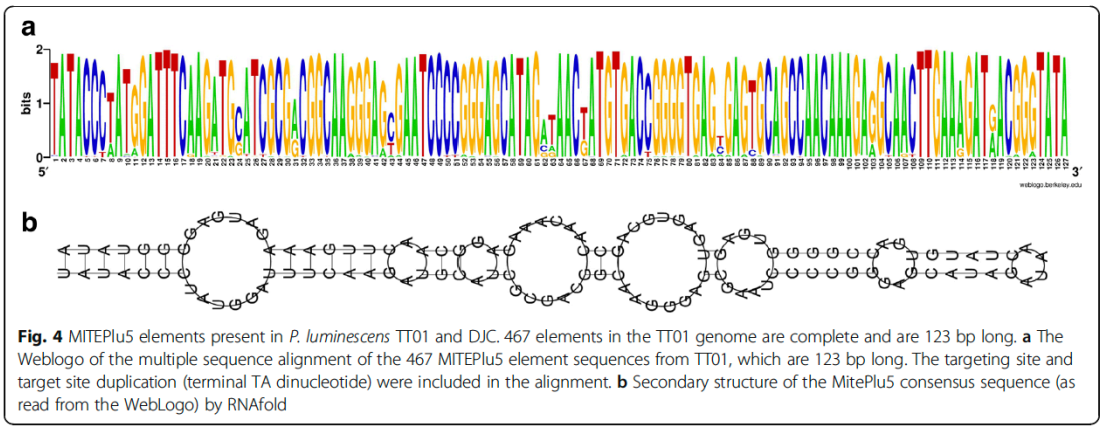


**Fig. 4** MITEPlu5 elements present in *P. luminescens* TT01 and DJC. 467 elements in the TT01 genome are complete and are 123 bp long. **a** The Weblogo of the multiple sequence alignment of the 467 MITEPlu5 element sequences from TT01, which are 123 bp long. The targeting site and target site duplication (terminal TA dinucleotide) were included in the alignment. **b** Secondary structure of the MitePlu5 consensus sequence (as read from the WebLogo) by RNAfold

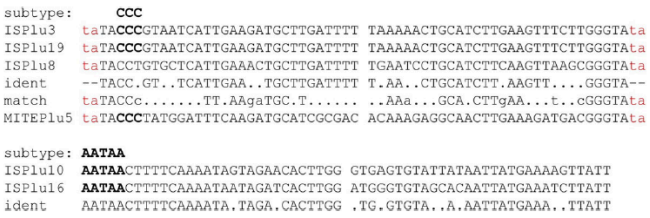


**Fig. 5** Terminal regions of IS630-type transposons in *P. luminescens* TT01 and DJC. The terminal 30 bp from IS630-type (left 5′ end, right 3′ end) including the targeting site (ta dinucleotide) and target site duplication (also ta, both in lowercase red) are shown. The MITEPlu5 sequence was read from the WebLogo (Fig. 4a). MITEPlu5 shares similarity to the CCC subtype of IS630-type transposons. Ident: bases that are identical for the transposons of the corresponding subtype are shown. Dots: differing bases. Hyphens: the targeting site and target site duplication are not part of the element. Match: bases that match between MITEPlu5 and the transposons. In the match line, uppercase: full match, lowercase: match to one of the transposons. Conservation of targeting site/target site duplication is indicated by lowercase red letters

the *Heterorhabditis bacteriophora* nematodes carrying the bacteria rather than the isolated *Photorhabdus luminescens* strains, so that detection of phenotypic differences between TT01 type strain and another isolate is impossible. With the PCR reactions using the primers mentioned here it was demonstrated that the original frozen stock of the DJC parent strain (prepared in August 2000) produces the same profile as the $Rif^R$ derivative and a distinct profile from TT01 (Dr David Clarke, data not shown). This suggests that the divergence between strain TT01 and DJC predates the arrival of this isolate in the Clarke laboratory. Although most likely being independent isolates, both strains interact specifically with *Heterorhabditis bacteriophora* nematodes.

## Discussion

We aimed to clarify the ambiguous designation of *P. luminescens* TT01. Until now *P. luminescens* strain DJC was known as a $Rif^R$ derivative of strain TT01 (TT01-$Rif^R$) [7]. However, we found major phenotypic as well as genomic differences between both strains. Our data in fact suggest that strain DJC is an independent *P. luminescens* isolate.

The $Rif^R$ phenotype of strain DJC is an advantage in experiments where selection is required, such as genetic manipulations or strain checking. Rifampicin inhibits the bacterial transcription machinery by interacting with the *rpoB* gene. Among the two non-silent point mutations in *rpoB* in strain DJC, one (H526Y) locates within the rifampicin-resistance hotspot 1 described for *E. coli* [15]. It has been shown earlier for *P. luminescens* strain LN2 that a rifampicin resistance-causing mutation in the *rpoB* gene leading to amino acid replacement P564L developed nematocidal activity to axenic nematodes of *Heterorhabditis bacteriophora* H06 [28]. Moreover, the rifampicin resistant *P. luminescens* LN2 even supported nematode growth and development of the animals, which are normally non-compatible with the bacteria. It is assumed that at least seven putative proteins including DsbA, HlpA, RhlE, RplC, NamB, and two hypothetical proteins of unknown function were probably involved in the nematocidal activity of rifampicin resistant *P. luminescens* LN2 cells against H06 nematodes [28]. It is further assumed that altered expression of the corresponding genes is responsible for this phenotype. Here we found genomic differences concerning genes that encode putative secretion factors, regulators and genes encoding proteins of unknown function between *P. luminescens* strain DJC and TT01. However, although not checked for nematocidal activity, we found no difference in nematode symbiosis between *P. luminescens* strain DJC and TT01.

However, phenotypically both *P. luminescens* strains differed in pigmentation. The red colour of strain DJC is caused by the production of several anthraquinones [29]. The biosynthesis pathway is encoded in the *antABCDEFGHIJ* operon, which is present in both *P. luminescens* strains. The regulation of the *ant* operon has been investigated in strain DJC (earlier described as TT01-$Rif^R$), and there is positive regulation of a novel type of regulator named AntJ [30]. However, a set of other proteins has been

**Table 2** Characteristics of gene regions used for PCR diagnostics to distinguish between *P. luminescens* strain TT01 and DJC

| | Gene region | Putative function | TT01 | DJC |
|---|---|---|---|---|
| Candidate 1 | Parts of *plu4513–4514* | N/A | 969 | 1443 |
| Candidate 2 | Parts of *plu2222* | Probable membrane protein | 829 | 1217 |
| Candidate 3 | Parts of *plu2649–2651* | Hypothetical secreted protein | 1264 | 695 |
| Candidate 4 | Parts of *plu2372–2373* | N/A | 1199 | 487 |
| Candidate 5 | *plu1790/* insert | N/A | 547 | 1952 |

The length of the amplified DNA using the primers presented in Additional file 1: Table S9 are listed in the right two columns (respective for strain TT01 and DJC)

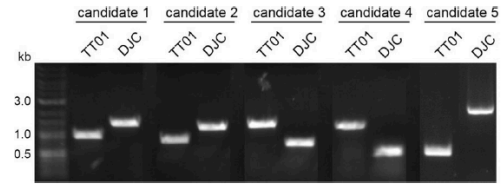


**Fig. 6** Polymerase chain reaction for *P. luminescens* strain diagnostics. In total, five candidate genetic regions were chosen, which differ in length in *P. luminescens* strain TT01 and DJC, if amplified with the same primer pair. The primer pairs chosen for the PCR reactions are listed in Additional file 1: Table S9 and the characteristics of the five candidate genes and the exact specific sizes in the DJC and TT01 genome are listed in Table 2

found to bind to the $P_{antA}$ promoter, which might act as further repressors to tightly control anthraquinone production under different life styles of *P. luminescens* [30]. We found that both strains produced similar anthraquinone levels in insect larvae, because both turned red after infection with the bacteria. Consequently, the *ant* operon required for strain pigmentation must be regulated differently in the two strains, for instance by the presence of different inducer signals and an altered gene regulation.

Since *P. luminescens* strain DJC was initially described as a spontaneous mutant of strain TT01, the number of genome sequence differences towards strain TT01 was expected to be relatively small, and in the magnitude of a direct offspring of strain TT01, TT01m, which we have sequenced. We expected its genome sequence to be identical to the type strain except for the altered genome region, as well as a low number of anticipated genome sequencing errors. The number of sequence differences between the TT01 and TT01m genomes was only 30 and thus very low. Most differences were ambiguous with respect to distinguishing the correct and incorrect sequence version. Yet, our newly obtained *P. luminescens* TT01m genome sequence has resolved a number of obvious frameshift errors while none have been newly introduced. The encountered inversions within prophages may have occurred during strain manipulation. It is, however, also possible that there is heterogeneity within the population, which is either fixed by single cell cloning or even by random selection of one variant during genome assembly.

The observed number of differences between *P. luminescens* TT01m and DJC genomes is significantly higher, with thousands of point mutations, hundreds of frameshifts, indels, replacements, inversions and differences in transposable elements. We identified several genes and therefore proteins that are absent in either of the strains. Both strains mainly differ in the number of proteins of unknown function and those containing conserved protein domains of unknown function, which makes it difficult to correlate these with the different phenotypic traits of strains TT01 and DJC. However, as several regulatory proteins are different in both strains, also the expression of several genes that are present in both strains might be differentially regulated and mediate the different phenotypes. Furthermore, we identified several types of phage-related repeats that are present in different copy numbers in both strains. *P. luminescens* DJC lacks several clusters (B1, B2, B3-truncated, D1, D2, D3-truncated, E3, E4), but also has several repeats that are not present in strain TT01/TT01m (E5, E6, E7, E8). Since each of the repeats also contains adhesion elements, the presence or differential expression of these genes compared to strain TT01 might be involved in the higher ability of strain DJC to organize in biofilms.

It has been suggested earlier that temperate phages may play an important role in the evolution and genomic diversification of bacterial pathogens [31]. Many bacterial genomes contain a range of intact and remnant prophage elements, and important bacterial traits like bacteriocins are discussed to be phage-derived [32, 33]. Furthermore, phage-related sequences have more frequently been observed in pathogenic than in non-pathogenic bacteria, and the acquisition of prophages can also be associated with changes in pathogen virulence [34–36]. Although we have not observed major differences in pathogenicity against insects between both *P. luminescens* strains, bacterial biofilm formation is frequently known to be a virulence factor. Temperate phages have recently been observed to be involved not only in bacterial biofilm formation for the human pathogen *Pseudomonas aeruginosa*, but are also described as major drivers of host cell evolution [37].

Another interesting feature was the high number of MITEPlu5 elements that we identified in both *P. luminescens* genomes. We were astonished to find approximately 450 complete copies per genome. We were also intrigued by their similarity to eukaryotic transposable elements. Whether the MITEPlu5 elements play a role in host pathogenicity or phenotypic heterogeneity is unclear. Their similarity to eukaryotic transposable elements could point to a possible function in interacting with their hosts: in the ciliate *Tetrahymena*, an RNAi-related mechanism produces small noncoding RNAs that induce heterochromatin formation, which is followed by DNA elimination. Therefore, many transposon-related sequences are removed from the somatic macronucleus of ciliates during sexual reproduction [38]. For that reason, it is conceivable that the *P. luminescens* derived MITEPlu5 elements interfere with transposable elements in the eukaryotic host cells and thus block their life cycle. However, another possibility is that these MITEPlu5 elements or their respective RNA play a role in phenotypic heterogeneity of the bacteria and control phenotypic switching. *P. luminescens* is known to exist in two phenotypically different variants that are called primary (1°) and secondary (2°), whereby both differ in a large number of

phenotypic but not genotypic traits. 2° cells develop from 1° cells during prolonged cultivation. However, both cells are comparably pathogenic towards insects, while 2° cells lack the ability to support nematode reproduction and development [39]. It has been recently discussed that, besides the activity of different transcription factors, the presence of non-coding RNAs might play a major role in the expression of 1° and 2° specific genes [8]. The protozoon *Euplotes crassus* uses transposon-like elements for precise transcriptional regulation: the Tec*1* and Tec*2* transposon-like element families are excised from the genome during a discrete time period of macronuclear development. With approximately 30,000 copies, these elements are also unusually abundant. *P. luminescens* might employ a similar mechanism during phenotypic switching. However, larger genome rearrangements have never been observed during the *P. luminescens* life cycle and phenotypic switching. Interestingly though, sequence similarity between Tec transposon-like elements and the previously described Tc*1*-IS*630* family of transposases has been observed, which includes ORFs from bacterial, nematode and insect transposons [40]. Our findings also indicate sequence-similarity of MITEPlu5 with a subtype of IS630-type transposons.

## Conclusion

Based on phenotypic and molecular comparison, we conclude that the genome sequence of *P. luminescens* strain DJC is much more divergent to TT01 than previously anticipated. With approximately 13,000 point mutations, 330 frameshifts, and 220 strain-specific regions, covering more than 300,000 bp, this strain is certainly an independent *P. luminescens* isolate. Since both *P. luminescens* strains equally interact with *H. bacteriophora* TT01 nematodes, it would appear that originally there must have been several stocks of "TT01" nematodes with the different bacterial loads. In accordance with David J. Clarke, who had originally isolated the TT01-Rif$^R$ strain, the name was changed to DJC.

## Methods

### Materials

Primers used in this study are listed in Additional file 1: Table S9. PCR was performed using Q5 Polymerase or OneTaq Polymerase from New England Biolabs (Frankfurt, Germany). Restriction enzymes and T4 DNA ligase were also purchased from New England Biolabs. Genomic DNA was isolated using the Ultra-Clean Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA). All other chemicals or reagents were analytical grade and obtained from commercial sources.

### Bacterial strains and growth conditions

*P. luminescens* subsp. *laumondii* TT01-Rif$^R$ was obtained from the lab of David J. Clarke (University College Cork, Ireland). *P. luminescens* subsp. *laumondii* TT01 (DSM 15139) was obtained from the Deutsche Sammlung für Mikroorgansimen und Zellkulturen (DSMZ, Braunschweig, Germany). Both *P. luminescens* strains were cultivated aerobically in LB medium [1% (*w/v*) NaCl; 1% (*w/v*) tryptone; 0.5% *(w/v)* yeast extract] or CASO complex medium [5% *(w/v)* NaCl; 1.5% (*w/v*) peptone from casein; 0.5% (*w/v*) peptone from soymeal] at 30 °C. For preparation of agar plates, 1.5% (*w/v*) agar was added to the respective medium. For growth of *P. luminescens* DJC (TT01-Rif$^R$), the medium was supplemented with 50 μg/ml rifampicin (Sigma Aldrich, Deisenhofen). *Bacillus subtilis* was obtained from the strain collection of Dr. Marc Bramkamp (LMU München, Germany) and cultivated in LB medium at 30 °C. Luminescence measurements were performed by cultivation of *P. luminescens* in Corning black 96-well plates with transparent bottom (Fisher Scientific, Schwerte), and optical density as well as luminescence was recorded using an Infinite-500 reader (Tecan, Salzburg).

### Caseinate bioassays

For caseinate bioassays, the bacteria were grown over night at 30 °C in LB medium. Then, an aliquot of 30 μl ($OD_{600}$ = 1.0) was dropped onto the middle of a caseinate agar [0.5% (*w/v*) NaCl; 0.5% (*w/v*) meat extract; 0.25% (*w/v*) casein; 0.015% (*w/v*) $Ca(OH)_2$; 0.005% (*w/v*) $CaCl_2$; 1.35% (*w/v*) agar], and the plates were incubated for 2 d at 30 °C.

### Haemolysis bioassays

For haemolysis bioassays, the bacteria were grown over night at 30 °C in LB medium. Then, an aliquot of 30 μl ($OD_{600}$ = 1.0) was dropped onto the middle of a haemolysis agar [0.5% (*w/v*) NaCl; 1.0% (*w/v*) meat extract; 1.0% (*w/v*) peptone; 0.5% (*v/v*) sheep blood; 1% (*w/v*) agar; pH 7.5]. The plates were incubated for 4 d at 30 °C.

### Antibiotic bioassays

For testing antibiotic activity, we used soft agar plates supplemented with *Bacillus subtilis* as test strain. For that purpose, an overnight culture of *B. subtilis* of an $OD_{600}$ = 2–3 in 1:100 dilution was added to liquid hand-warm soft LB agar with 0.8% (*w/v*) agar. After the plates were polymerized, an aliquot of 30 μl ($OD_{600}$ = 1.0) of the respective *P. luminescens* culture was dropped onto the middle of the agar plate and incubated for 2 d at 30 °C.

### Symbiosis bioassays

An aliquot of 50 μl of the respective *P. luminescens* overnight culture diluted to an $OD_{600}$ of 1.0 was spread in a Z pattern onto the surface of a lipid agar plate [1% (*v*/v) corn syrup; 0.5% (*w/v*) yeast extract; 5% (*v/v*) cod liver; 2% (*w/v*) $MgCl_2 \times 6\ H_2O$; 2.5% (*w/v*) Difco nutrient agar (Becton Dickinson, Heidelberg)] using an inoculating loop. The plates were incubated at 30 °C for 3 days before

adding 50 surface sterilized infective juvenile nematodes (IJs) to the bacterial biomass. Nematodes were surface-sterilized by washing in a solution [0.4% (*w/v*)] of hyamine (Sigma-Aldrich, Deisenhofen)]. The plates were kept at room temperature. Nematode recovery was assessed 7–8 days after addition of IJs by counting the number of hermaphrodites on the lipid agar plate.

#### Pathogenicity bioassays

Fifth instar larvae of *Galleria mellonella* (reared in our lab) were incubated on ice for 10 min to reduce movements and surface sterilized in a 70% (*v/v*) ethanol bath followed by a bath of sterile water. Larvae were infected with the respective *P. luminescens* strain by injection of 10 μl cell suspension containing approximately 200 or 200,000 cells subcutaneously using a sterilized micro syringe (Hamilton 1702 RN, 25 μl), and incubated at 25 °C. Mortality rate was determined by counting dead and alive animals at several time points. At the day of larval death, luminescence was monitored using a Chemiluminescence Imager (Peqlab, Erlangen) using 5 min exposure time.

#### Biofilm assays

For quantification of bacterial biofilm production, a modification of a published protocol was used [41–43]. *P. luminescens* was cultivated in LB medium over night at 30 °C. Then, the cultures were diluted in CASO medium in a volume of 125 μl per well of a 96-well polystyrene micotiter plate (Sarstedt, Nümbrecht) at a final $OD_{600}$ of 0.5. The microtiter plate was then incubated for 72 h under gentle shaking (150 rpm) at 30 °C. Then, the liquid phase of the culture was removed by turning the plate. The planktonic cells were removed by gently submerging the plate two times in a water tub. After drying for 5 min, 125 μl of 1% (*w/v*) crystal violet (Merck, Darmstadt) was added to the wells. After 15 min incubation at room temperature, unbound crystal violet was removed by gently submerging the plate for two times in water. The plate was then dried over-night at room temperature. For quantification, 125 μl of 30% (*v/v*) acetic acid (Sigma-Aldrich, Deisenhofen) was added to each well to solubilize the crystal violet from the biofilm. After 15 min of incubation at room temperature, absorbance was quantified in a plate reader (Tecan, Salzburg) at 575 nm.

#### Polymerase chain reaction (PCR)

To differentiate between *P. luminescens* strain TT01 and DJC five PCR reactions were performed amplifying DNA fragments of different length for the respective strain using identical primer pairs (Additional file 1: Table S9). First, genomic DNA from *P. luminescens* strains TT01 and DJC was isolated using the Ultra-Clean Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA). PCRs were performed using OneTaq polymerase from New England Biolabs (Frankfurt, Germany) according to the manufacturer's instructions. Oligonucleotides were purchased from Sigma-Aldrich/Merck KGaA (Darmstadt, Germany).

#### Genome sequencing and assembly

Fresh cultures from *P. luminescens* subsp. *laumondii* strains DJC and a TT01 variant, in which one genome region was replaced by an antibiotic cassette, were grown in LB medium at 30 °C and harvested at exponential growth phase ($OD_{600}$ of 2–3) by centrifugation. Genome sequencing, including DNA extraction, long-read library preparation, sequencing on a PacBio RSII sequencer, and genome assembly was performed at the Max-Planck Genome Center Cologne (http://mpgc.mpipz.mpg.de). For genome assembly, the SMRTanalysis pipeline (PacificBiosciences) was used to run HGAP (DAGCON-based hierarchical genome assembly process, RS_HGAP_assembly.2 version 2.3.0) following the steps pre-assembly, de novo assembly with the Celera assembler and final polishing with Quiver. For strain DJC, data originated from 2 SMRT cells, resulting in 300,000 raw reads. After filtering, 154,151 reads with an average length of 9770 bp (1.51 GBp total) were assembled into the chromosome, which was obtained as one contig with an average 194-fold coverage. For the TT01 mutant, data originated from 1 SMRT cell, resulting in 150,000 raw reads. After filtering, 89,346 reads with an average length of 17,496 bp (1.56 GBp total) were assembled into the chromosome, which was obtained as one contig with an average 182-fold coverage. For both genomes, the assembly resulted in a single contig with redundant termini, indicating circularization. The sequences were trimmed and the point of ring opening was shifted in order to match that of the published TT01 genome sequence [6]. A deviating region in the original assembly was converted to the TT01 wildtype sequence (positions 1,700,480–1,708,758), using Sanger sequencing data obtained for the wildtype strain. The Sanger sequencing results indicated identity to the corresponding region in the published TT01 genome.

#### Genome sequence validation

In a parallel project, mutant analysis was performed by Illumina sequencing of clonal variants (AL/MA-ZL/FP/RH/BH, unpublished). The Illumina reads were mapped as described elsewhere [19]. The *P. luminescens* DJC genome was used as a reference in this comparison. Besides allowing the detection of a small number of mutations in clonal variants, this analysis also verified the correctness of the strain DJC reference genome for the bulk of the reads.

### Genome comparison

For comparison of closely related genome sequences we had developed a custom tool during the analysis of *Haloquadratum walsbyi* [44]. This tool, here referred to as "mapper", proved useful to compare the re-sequenced TT01m genome to the originally published genome sequence of strain TT01 [6].

In brief, the mapper tool splits the input sequences into an alternate set of "runs", defined as subsequences that are completely identical, and "connectors", which are the divergent sequences that occur between runs. During comparison of the two TT01 genome sequence versions, nearly all of the sequences were found in runs. All encountered differences are listed in Additional file 1: Table S1. Point mutations, one-base indels and few-base differences were taken directly from the mapper output. More complex differences (inversions and long indels) were taken from BLAST analyses as the mapper tool is not capable to delineate exact coordinates.

When using the mapper tool to compare the *P. luminescens* TT01m genome to that of strain DJC, the longest region of complete sequence identity (run) was only 55 kb, indicating extensive dissimilarity. Therefore, a different strategy was applied for genome comparison, which is based on sequence alignments using MAFFT [12]. Overall, the genomes were largely co-linear but toggled between (a) "matching segments" (matchSEGs) with ca 99% sequence identity and (b) "divergent segments" (divSEGs) which were either indels or regions of increased sequence divergence.

Three passes of sequence comparison were performed. In the 1st pass, the genomes were compared in chunks of 200 kb. For each chunk, a suitable start position was selected and the subsequent sequence block of 200 kb was aligned. The beginning of the last "matching sequence" segment was selected as start position for the next chunk. Beginning at the 5′ end of both sequences, this allowed us to completely traverse both chromosomes. In the 2nd pass, individual segments of matching sequence were extracted, based on visual inspection of the aligned 200 kb chunks from the 1st pass. The segment under analysis was extended if no indel longer than 100 bp was detected or no significant increase in sequence dissimilarity was encountered. In such a case, the matchSEG was considered to have terminated. matchSEG boundaries were trimmed such that they terminated at the end with a matching base. For each matchSEG, the sequences were re-aligned with MAFFT. The resulting data were then subjected to script-based computational checking and computation of statistical data. In the 3rd pass, problems identified by the checking script were resolved. matchSEGs were split if the MAFFT alignments contained indels longer than 100 bp. matchSEGs were fused if they were separated by less than 100 bp in both genomes. All matchSEGs having more than 1% sequence divergence were visually inspected. The corresponding region could represent either a valid matchSEG with increased dissimilarity. Alternatively, it could have been misclassified as a matchSEG but actually represents a conserved but strain-specific sequence. In areas of uncertainty, we attempted to minimize matchSEGs with high divergence; at the same time, we tried not to split the genome into an unnecessarily high number of short matchSEGs.

For matchSEGs, sequence similarity statistics were computed from the MAFFT alignments by a custom script. Each position was classified to be a "match" (m), a "mismatch" (mm), a "gap open" (go) or a "gap extension" (ge) position. Gap extension positions were excluded from subsequent computations. Therefore, sequence difference is calculated as "mm + go/ m + mm + go".

matchSEGs are separated by divergent segments (divSEGs). These were classified into categories and tagged by content as detailed in the text and in the legend to Additional file 1: Table S2. After finalization of the analysis, it was ensured that each genome position is classified exactly once, either as part of a matchSEG or part of a divSEG. All MAFFT alignments were confirmed to represent the specified genome region. We ensured that each matchSEG starts and ends with a matching base. The complete list of matchSEGs and divSEGs is provided in Additional file 1: Table S2.

"Pairwise position correlation data" were computed for the *P. luminescens* DJC and TT01m genomes. Each genome position was classified into one of three categories: (i) "mapped" to a position in the other genome; these positions are within a matchSEG and the positional correlation is computed from the MAFFT alignment; (ii) "gap": a position in a matchSEG, is located opposite to a gap in the other genome in the MAFFT alignment; (iii) "strain-specific"; these positions are within a divSEG.

### Genome annotation

An automatic annotation was generated using the NCBI PGAP pipeline upon GenBank submission [45]. The annotation was only partially subjected to further curation (see below).

To support the annotation process, the proteome from strain TT01 was downloaded from UniProt (UP000002514, release 2017_10), as well as from GenBank (accession BX470251) [6].

### Correlation of the theoretical proteomes of the two genome sequences of *P. luminescens* TT01

Using a set of custom scripts combined with manual inspection, the (curated) theoretical proteome of the *P. luminescens* TT01m genome was compared to the published proteome of strain TT01 as extracted from

GenBank (accession BX470251). Because genome sequence differences are minor (Additional file 1: Table S1), a "pairwise position correlation" could be easily computed as a tool for ORF correlation.

We attempted to correlate each protein-coding gene [hereafter referred to as open reading frame (ORF)] from the *P. luminescens* TT01m genome version to an ORF from the TT01 genome version. All ORFs, which traverse any of the sequence differences between the TT01 and TT01m genomes (Additional file 1: Table S1) were excluded from automatic analysis and were correlated manually. Automatic ORF matching was based on the detection of corresponding C-terminal positions. The protein sequences of the correlated ORFs must be identical in case of a consistent start codon assignment, given that the genomes are identical except for 30 differences. For inconsistent start codon assignments, the C-terminal fragments must be identical for the length of the shorter ORF if the assigned start codon is an ATG. If the shorter sequence has GTG or TTG assigned as a start codon, the internal Val or Leu of the longer sequence was converted to Met prior to sequence comparison. About 89% of both proteomes could be automatically mapped by this procedure. The remainder of the proteomes was subjected to manual correlation, mainly using the BLAST suite of program [46]. A significant fraction of the ORFs which cannot be automatically mapped were either (a) disrupted and hence pseudogenes or invalidly considered to be disrupted; (b) missing gene calls in the published TT01 genome; (c) not mappable due to missing gene calls by the PGAP annotation pipeline. Such ORFs were post-predicted, except for few short fragments of disrupted genes; (d) spurious ORFs: several ORFs in TT01 were rated to be spurious, i.e. ORFs which are unlikely to be protein-coding genes (for usage of this term see [47]). Such spurious ORFs are typically not predicted by the PGAP pipeline, are short, and have no or extremely few BLAST hits in the UniProt database (as analysed in January 2018). It should be noted that disrupted genes may be annotated as a single ORF in one strain, but a set of two or three ORFs in the other strain.

An exhaustive list with all correlated and non-correlated ORF codes (locus tags) is provided for the genomes from *P. luminescens* strain TT01 (plu numbers), TT01m (PluTT01m numbers) and DJC (PluDJC numbers) as Additional file 2: Table S3b; Additional file 1: Table S3a.

### Correlation of the theoretical *P. luminescens* TT01 and DJC proteomes

The theoretical proteomes predicted for the *P. luminescens* DJC and TT01m genomes by the PGAP pipeline were compared in detail, using custom PERL scripts.

Again, we attempted to correlate each protein-coding gene from one strain to an ORF from the other strain. The mapping was based on positional correlation, using the "pairwise position correlation data" (see above). We first tried to correlate ORFs by their C-terminal positions. For ORFs, which could be correlated by C-terminal position, we checked if the N-terminal position can be correlated as well. For ORFs which could not be correlated by their C-terminal position, we attempted correlation by their N-terminal position. It should be noted that this algorithm allows a correlation only if at least one of the termini is within a matchSEG (see above). When correlation was successful and both termini were within the same matchSEG, the ORF was classified as perfectly correlated. Such perfectly correlated ORFs were excluded from subsequent manual curation unless their protein names differed or they were disrupted genes according to the PGAP pipeline. All ORFs that did not show such a perfect correlation were subjected to manual curation (see below).

Manual curation triggered various annotation updates (e.g. improvement of the protein name or start codon reassignment). Also, some disrupted genes (i.e. pseudogenes) were initially annotated as regular by PGAP and vice versa. Finally, some of the annotated genes were found to be "spurious ORFs".

For manual curation, ORFs were subjected to BLAST analyses [46]. BLASTp comparisons were made against the theoretical proteomes from the two *P. luminescens* strains DJC and TT01m, as well as the UniProt proteome of strain TT01. BLASTx comparisons were carried out against the DJC, TT01m and TT01 genomes. Protein-coding genes, which were regular in one strain but disrupted in the other (Additional file 1: Table S5) were identified and validated by BLASTx analyses. For some ORFs, positional mapping had failed but BLASTp analysis allowed to identify the correlation. The analysis allowed us to identify missing gene calls, if ORFs initially seemed strain-specific but showed strong BLASTx matches. Such ORFs were post-predicted and correlated manually. Other ORFs were validated to be strain-specific (Additional file 1: Table S4) by BLASTp and BLASTx analyses. Some ORFs predicted in only one strain were rated to be spurious when they were short, a corresponding gene would have been disrupted in the other genome, and there were no or extremely few BLAST hits in UniProt.

### Additional bioinformatics tools

As general tools, MUMMER and the BLAST suite of programs were used for genome comparisons [46]. For ORF post-prediction, we used the Translate Tool from the Expasy Server (https://www.expasy.org). We analysed the *P. luminescens* TT01 and DJC genomes for CRISPRs encoding genes using the CRISPRFinder web server (http://crispr.i2bc.paris-saclay.fr) [48]. Prophages were analysed for all three strains by PhiSpy (http://edwards.sdsu.edu/PhiSpy) [16, 17] and for the newly sequenced *P. luminescens* strains

by Prophinder (http://aclame.ulb.ac.be/Tools/Prophinder) [18]. Prophages for the published sequence of TT01 were found pre-computed on the ACLAME web server [18]. Phage-related repeat PhRepA was analysed using the BLAST suite, including BLASTx comparison against the UniProt database. RNA secondary structures were predicted using the RNAfold webserver from the ViennaRNA Web Services (http://rna.tbi.univie.ac.at) [26]. For ANIb computations (based on BLASTn analyses) we used JSpeciesWS (http://jspecies.ribohost.com/jspeciesws) [49].

### Transposon analysis

Transposons were identified by BLASTn and BLASTx comparison to the ISFinder database [20, 24] by a described procedure [19]. Identified transposons were collected in an in-house database and were used for a subsequent iterative transposon analyses using BLAST. Few additional transposons were identified and submitted to ISFinder. In several cases, our analyses showed that the boundaries of the transposons in ISFinder needed to be shifted. This information was forwarded to ISFinder. In addition to canonical transposons, we identified several MITEs (Miniature Inverted-Terminal-repeat Elements), which were submitted to and accepted by ISFinder for their recently introduced MITE subsection.

### Note added in proof

*Photorhabdus luminescens* subsp. *laumondii* has been recently suggested to be renamed as *Photorhabdus laumondii* (Machado et al 2018 https://doi.org/10.1099/ijsem.0.002820) [50].

## Additional files

**Additional file 1:** Text S1. Phage-related repeat PhRepA in *P. luminescens*. Text S2. **Mobile genetic elements in the *P. luminescens* TT01/TT01m and DJC genomes**. **Table S1.** Differences between the newly sequenced *P. luminescens* genome TT01m and the originally published genome TT01. **Table S2.** Genome comparison between *P. luminescens* strain DJC and TT01 (as represented by the newly sequenced genome TT01m). **Table S3a.** Mapping of gene codes between the TT01, TT01m and DJC genomes. **Table S4.** Strain-specific protein-coding genes. **Table S5.** Genes which are disrupted in only one *P. luminescens* strain. **Table S6.** Prophages as predicted by PhiSpy and Prophinder. **Table S7.** Copies of phage-related Repeat A (PhRepA) in the three *P. luminescens* strains. **Table S8.** Genes and intergenic regions on PhRepA in the element copies. **Table S9.** Oligonucleotides used for *P. luminescens* strain. **Figure S1.** Relative contribution of divergence region classes between *P. luminescens* TT01 and DJC. **Figure S2.** Relative frequency of transposons and other mobile genetic elements in indels and approximate inserts. (PDF 2620 kb)

**Additional file 2: Table S3b.** Mapping of gene codes between the TT01, TT01m and DJC genomes. (XLSX 191 kb)

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#### Availability of data and materials

The nucleotide sequence accession numbers are CP024900 (*P. luminescens* strain DJC) and CP024901 (TT01m, re-sequenced strain TT01). The PacBio reads of strain DJC were submitted to the sequence read archive (SRA) under accession SRR7001683. *P. luminescens* strain DJC has been disposed to the DSMZ strain collection under DSM number 107756 (Braunschweig, Germany).

#### Authors contributions

SE, AG and AL performed biological experiments. MA-ZL and FP performed bioinformatic analyses and annotated the genome. RH supervised the biological studies and BH supervised the bioinformatics studies. The manuscript was written by RH, SE, FP, and BH. All authors participated in the discussion, read and approved the final manuscript.

#### Ethics approval and consent to participate

Research did not involve human subjects, human material, or human data. *Photorhabdus luminescens* subsp. laumondii TT01 (DSM number 15139) has been obtained from the Deutsche Stammsammlung für Zellkulturen und Mikroorganismen (DSMZ). *Photorhabdus luminescens* subsp. laumondii DJC (new DSM number 107756) was originally obtained from the laboratory of Dr. David Clarke (University College Cork, Ireland).

#### Consent for publication

Not required.

#### Competing interests

The authors declare that they have no competing interests.

## Publisher's Note



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# 3 Phenotypic heterogeneity of insect pathogenic *Photorhabdus luminescens* - insights into the fate of secondary cells

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

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

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

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

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

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

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

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

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

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

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

## RESULTS AND DISCUSSION

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

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

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

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

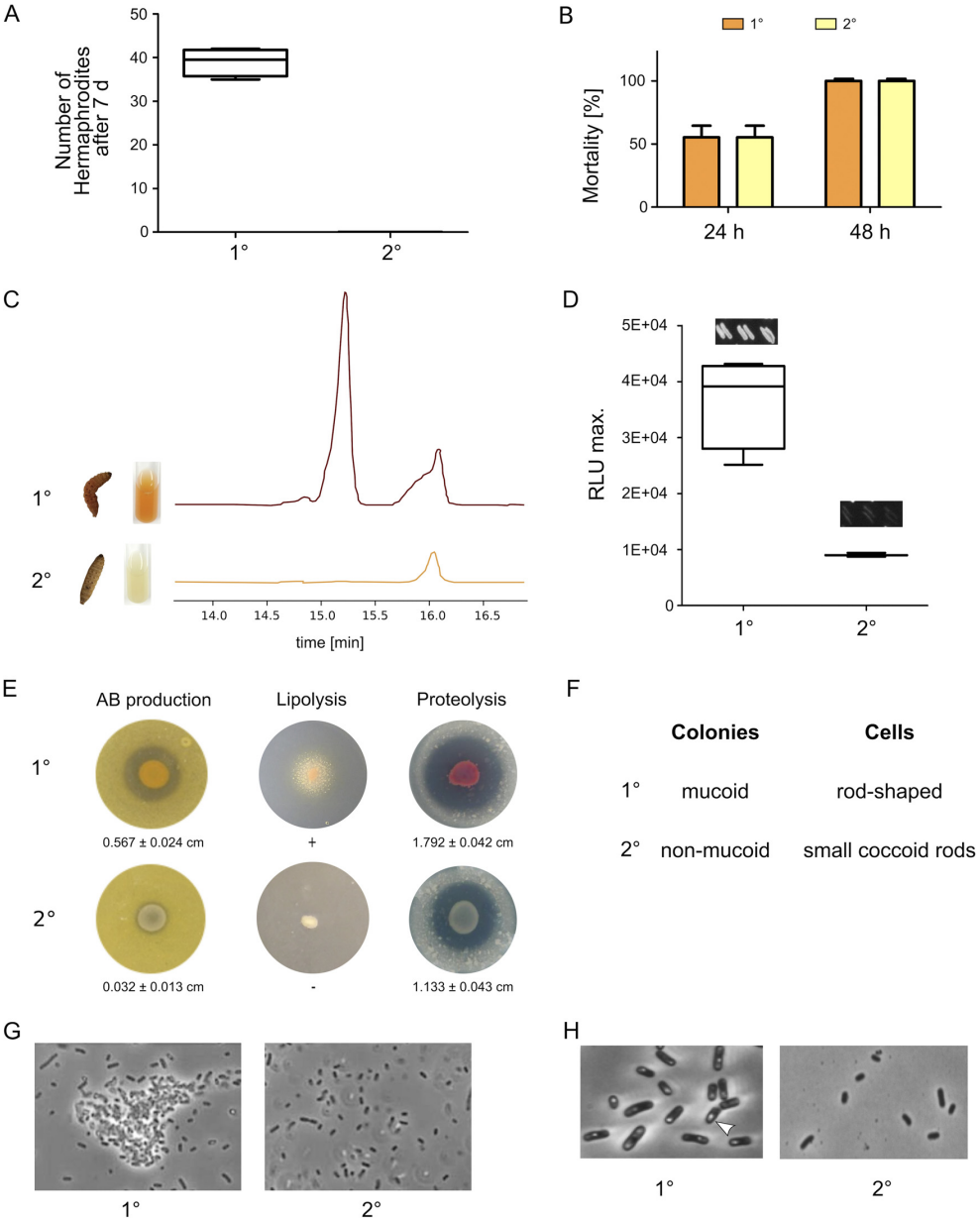


**FIG 1** Phenotypic comparison of *P. luminescens* DJC 1° and 2° cells. (A) Nematode bioassay. Fifty axenic *Heterorhabditis bacteriophora* IJs were spotted on 1° or 2° cells grown on lipid agar plates. After 7 days the number of developed hermaphrodites was counted. (B) Pathogenicity assay. Approximately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

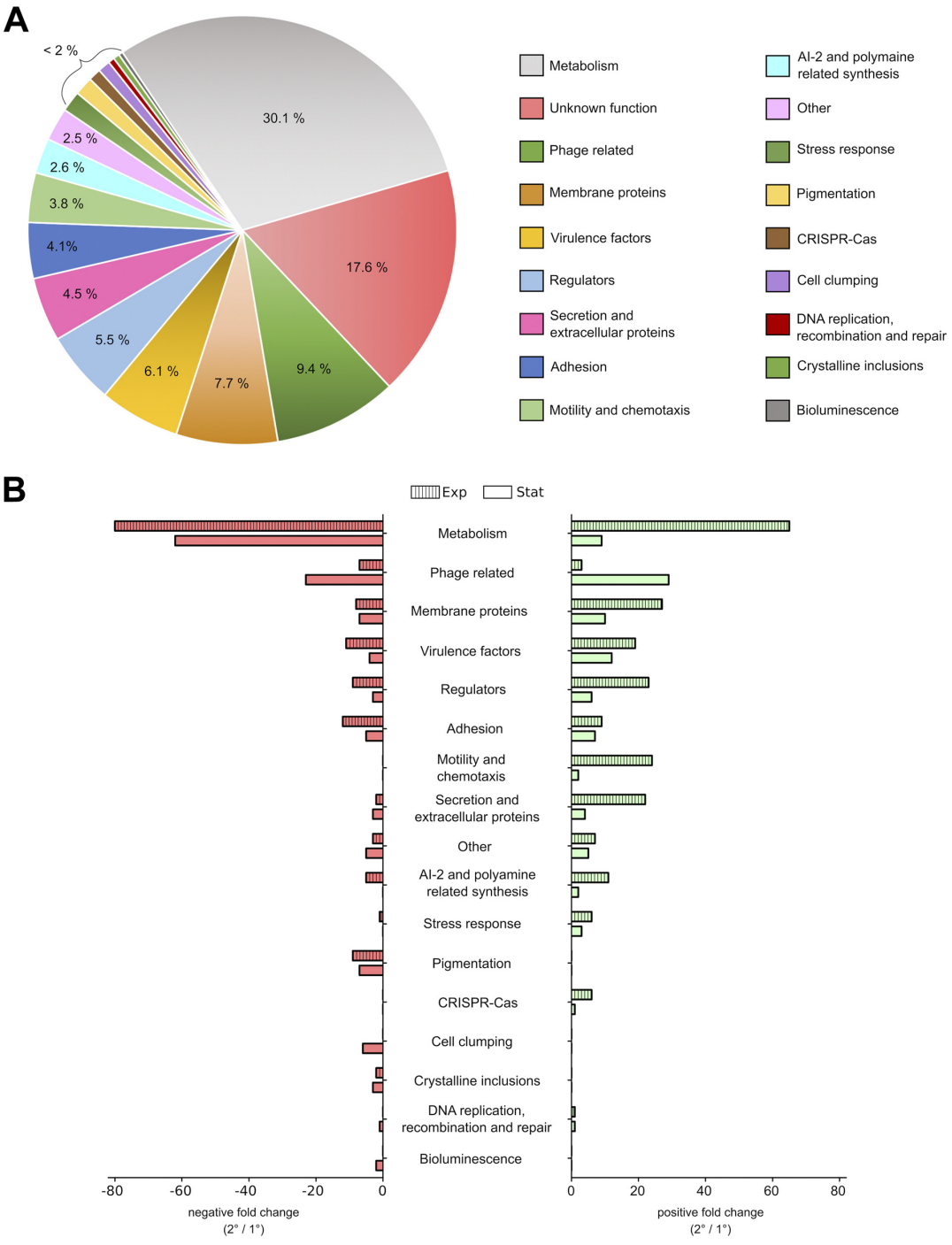


**FIG 2** Overview of genes differentially expressed in 2° cells compared to levels in 1° cells. (A) Division of the 638 DEGs according to their functionality into 19 subgroups. (B) Negative (red) and positive (green) FCs of all DEGs obtained at the exponential (Exp; striped bars) as well as stationary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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

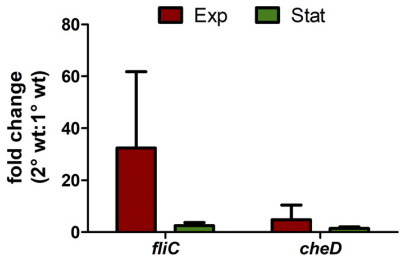


**FIG 3** RT-qPCR data on *fliC* and *cheD* displaying higher transcription in 2° than in 1° cells. RT-qPCR revealed a higher level of transcription of *fliC* and *cheD* in 2° cells than in 1° cells either in the exponential growth phase (red) or in the stationary phase (green); the fold change is significantly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

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

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

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

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

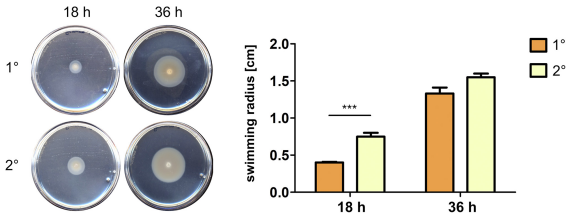


**FIG 4** Enhanced swimming motility of 2° cells in comparison to that of 1° cells. Upon spotting $5 \times 10^6$ 1° or 2° cells onto semisolid swimming agar plates, 2° cells showed significantly increased swimming activity compared to that of 1° cells after 18 h of incubation. Error bars represent standard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

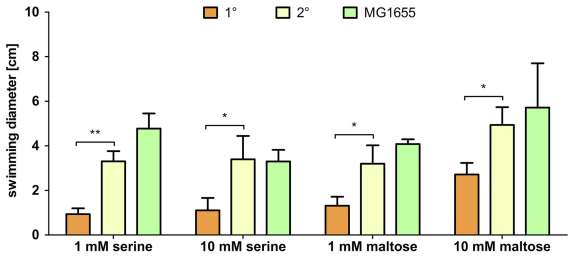


**FIG 5** Swimming diameters after addition of different putative attractants. Attractant-dependent motility of *P. luminescens* DJC 1° and 2° cells and *E. coli* MG1655 cells, as indicated, was determined. Error bars represent standard deviations of at least three independently performed experiments.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

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

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

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

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

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

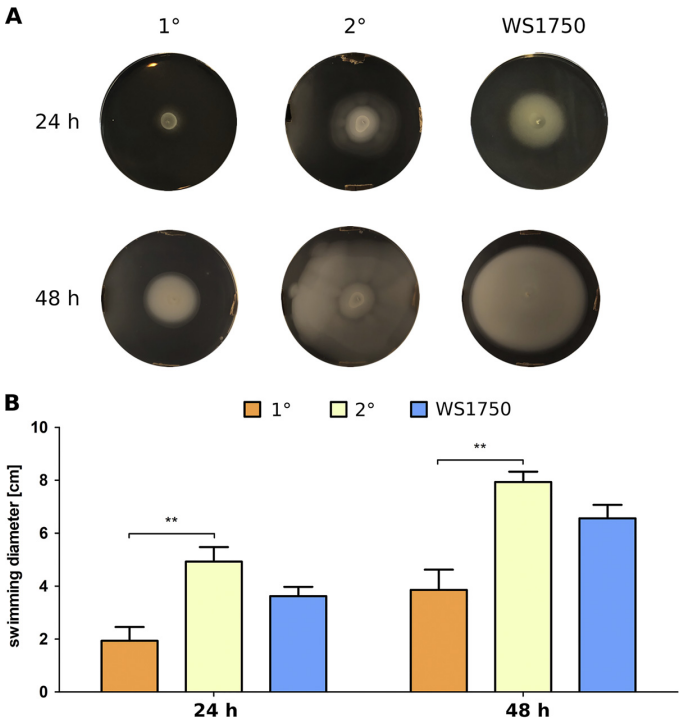


**FIG 6** Effects of plant root exudates on swimming motility of *P. luminescens* 1° and 2° cells. On plates containing MeOH-Ex, 2° cells showed a significantly stronger response in terms of increased swimming activity than 1° cells. The recorded swimming diameters were even bigger than those observed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

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

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

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

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

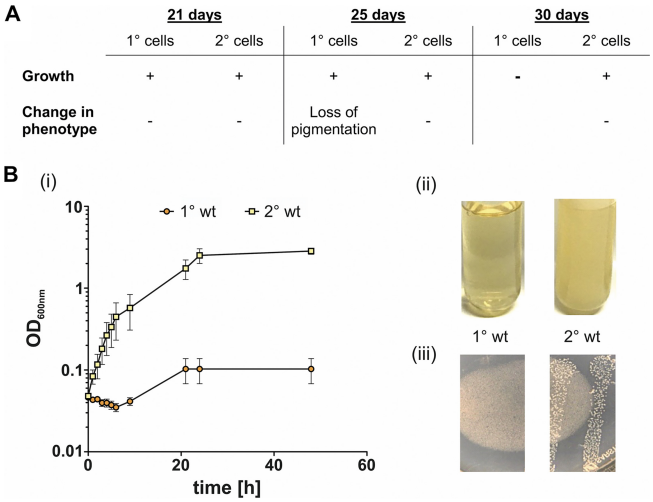


**FIG 7** Growth and phenotype of 1° and 2° cells at high and low temperatures. (A) 1° cells do not recover growth after being incubated for 30 days at 4°C and already show loss of pigmentation after 25 days at 4°C. In contrast, 2° cells restart growth after 30 days of exposure to cold and are not affected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

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

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

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

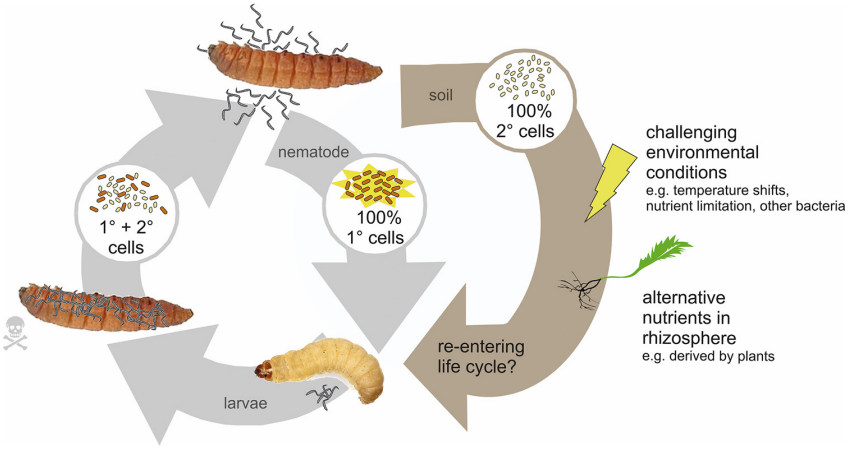


**FIG 8** Model of extended life cycle of 2° cells in soil. As only 1° cells are able to reassociate with the nematodes and emerge from the cadaver, 2° cells are left behind in the soil. Based on our transcriptome data, it seems likely that 2° cells are better adapted to free living in soil and thereby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

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

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

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *E. coli* strains MG1655 and DH5$\alpha$ $\lambda$*pir* were used in this study. They were routinely grown at 37°C in LB medium [1% (wt/vol) NaCl, 1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract]. If necessary, 50 $\mu$g/ml antibiotic was added into the medium. *P. luminescens* DJC (2) 1° and 2° cells were obtained from the lab of David Clarke (University College Cork, Ireland) and were cultivated aerobically in either LB medium or CASO medium (0.5% [wt/vol] NaCl, 0.5% [wt/vol] peptone from soy, 1.5% [wt/vol] tryptone) at 30°C. If necessary, the growth medium was supplemented with 50 $\mu$g/ml rifampin (Sigma-Aldrich). For preparation of agar plates, 1.5% (wt/vol) agar was added to the respective medium.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

## SUPPLEMENTAL MATERIAL

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

**SUPPLEMENTAL FILE 1**, PDF file, 0.8 MB.

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

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# 4 Two novel XRE-transcriptional regulators play a major role in regulation of phenotypic heterogeneity in *Photorhabdus luminescens* cell populations

# Two novel XRE-transcriptional regulators play a major role in regulation of phenotypic heterogeneity in *Photorhabdus luminescens* cell populations

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## Abstract

The insect pathogenic bacterium *Photorhabdus luminescens* exists in two phenotypically different forms, designated as primary (1°) and secondary (2°) cells. Upon yet unknown environmental stimuli as well as global stress conditions phenotypic switching of up to 50% of 1° to 2° cells is initiated. Among others, an important difference between the phenotypic forms is that 2° cells are unable to live in symbiosis with nematodes, and therefore are not able to re-associate with the nematodes. As a 100 % switching of 1° to 2° cells of the whole population would lead to a break-down of the bacteria's life cycle the switching process must be tightly controlled. However, the regulation mechanism of phenotypic switching is still puzzling. Here we describe two novel transcriptional regulators, XreR1 and XreR2, that play a major role in the switching process. Deletion of *xreR1* in 1° or *xreR2* in 2° cells as well as insertion of extra copies of *xreR1* into 2° or x*reR2* into 1° cells, respectively, was sufficient to induce the respective other phenotype. Furthermore, both regulators specifically bind to different promoter regions putatively fulfilling a positive auto-regulation. We found initial evidence that XreR1 and XreR2 constitute an epigenetic switch whereby XreR1 represses *xreR2* expression and XreR2 self-reinforces its own gene by binding to XreR1. However, how expression of both transcriptional regulators is regulated still remains elusive.

## Introduction

*Photorhabdus luminescens* subsp. *laumondii* DJC is a Gram-negative, entomopathogenic bacterium of the family of *Enterobacteriaceae* (Forst et *al*., 1997, Zamosa & Eckstein et *al.,* 2018). *P. luminescens* harbors a complex dualistic life cycle including two hosts. Initially, the bacteria live in mutualistic symbiosis with infective juvenile (IJ) *Heterorhabditidae* nematodes colonizing their upper gut. These nematodes invade insect larvae such as *Galleria mellonella* where *P. luminescens* is released into the hemolymph and switches to its pathogenic part killing the insects (Forst et *al*.,1997).

The bacteria exist in two phenotypically different cell forms referred to as the primary (1°) and the secondary (2°) cells (Akhurst, 1980). These two cell forms are easy to distinguish as 1° cells exhibit specific phenotypic features that are absent in 2° cells. These properties include the biosynthesis of antibiotics or production of anthraquinones which results in reddish-brown pigmentation as well as bioluminescence, or the formation of crystalline inclusion proteins and cell clumps (Akhurst, 1980, Heinrich et *al*., 2016, You et *al*., 2006, Langer et *al*., 2017, Eckstein et *al.,* 2019). Importantly, while both cell forms are equally pathogenic towards insects, 2° cells are not able to re-associate with the nematodes after depletion of nutrients derived by the insect host (Han & Ehlers, 2001, Eckstein et *al.,* 2019). Since phenotypic switching also takes place after prolonged cultivation under laboratory conditions, a response to metabolic or environmental stress is suggested (Joyce et *al*., 2006). So far, the switch has only been observed unidirectional occurring from 1° to 2° cells suggesting that a key signal which is missing under laboratory conditions (Forst & Clarke, 2002).

Since 2° cells of *P. luminescens* are not known to have the capability to re-associate with the nematodes after one cycle of insect infection phenotypic switching of the whole cell population would lead to a breakdown of the bacteria's life cycle. Therefore, the switching process has to be tightly controlled. To the current state of knowledge at least two pathways are suggested to be involved in controlling phenotypic switching: a HexA-dependent pathway and an $O_2$-dependent pathway via the AstS/AstR system. HexA ist a LysR-type transcriptional regulator which has been shown to suppress 1°-specific features in a versatile way, directly or indirectly (Joyce & Clarke 2003; Langer et *al.*, 2017). In contrast, AstS/AstR reacts to global stresses and was shown to delay phenotypic switching. Although they both seem to be activated by global stress factors, no direct connection between the two regulation pathways is known so far (Joyce et *al*., 2006). However, the complex regulatory network has not been fully understood yet. The nematode-bacteria complexes are used in agricultural industry where they are cultivated

in liquid media and then spread onto fields to prevent crop failure caused by insects. Hereby, the nematodes are pre-incubated with the bacterial symbiont as they essentially support their development and reproduction. Thus, phenotypic switching is one of the major reasons for process failure in industrial mass production (Han & Ehlers, 2001) and therefore the regulatory mechanism needs to be elucidated.

Recently, comparative transcriptome analysis of 1° and 2° cells was performed. Thus, in total up about 640 genes were found to be differentially expressed in 2° cells. Among these some predicted regulators with yet unknown function were either highly up- or down-regulated in 2° cells (Eckstein et *al*., 2019). In this study demonstrate that two of these transcriptional regulators, *PluDJC_21235* (XRE-transcriptional Regulator up-regulated in 2° cells, ***xreR2***) and *PluDJC_21265* (XRE-transcriptional Regulator up-regulated in 1° cells, ***xreR1***) play an important role in the phenotypic switching process in *P. luminescens* DJC cell populations. Here we show that XreR1 and XreR2 play an important role in the control of phenotypic switching in *P. luminescens* as deletion or insertion of either *xreR2* or *xrerR1* in 1° as well as 2° cells, respectively, was sufficient to induce the respective other phenotype. Furthermore, we could prove a DNA-binding function as we identified promoter regions to that both, XreR2 and XreR1 specifically bind to. Lastly, we found first evidence that XreR1 and XreR2 constitute an epigenetic switch whereby the 2° phenotype is maintained by high *xreR2* levels.

## Results

**Effect of Δ*xreR2* and Δ*xreR1* on *P. luminescens* 1° and 2° cells** The high differences in expression of *xreR1* and *xreR2* in 2° compared to 1° cells indicate an importance of those two transcriptional regulators in the process of phenotypic switching of *P. luminescens*. After we confirmed an up-regulation of *xreR2* in 2° cells and higher transcription of *xreR1* in 1° cells via RT-qPCR (Fig. 4-1), we attempted to analyze the putative impacts of *xreR2* or *xreR1* on phase variation in *P. luminescens* cell populations.

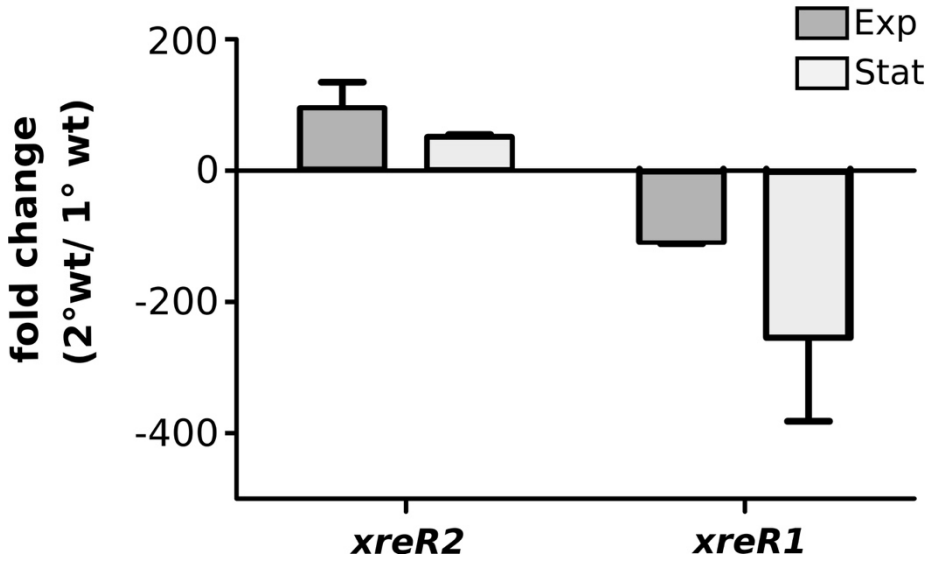


**Figure 4-1 Validation of gene expression levels via qRT-PCR** To analyze gene expression levels of *xreR1* and *xreR2* in 1° as well as 2° cells qRT-PCR was performed. Therefore, RNA was collected from the respective *P. luminescens* strains during exponential (Exp, dark grey bars) as well as stationary (Stat, light grey bars) growth phase via P/C/I extraction and gene expression was depicted comparatively (fold change) from 2° to 1° cells. Error bars represent standard deviation of three independently performed experiments.

To do so, we deleted *xreR1* and *xreR2* in the respective cell form. As *xreR2* was higher expressed in 2° cells we generated 2° cells lacking this gene (2° Δ*xreR2*) as well as 1° cells lacking *xreR1* (1° Δ*xreR1*) since this gene was higher expressed in 1° cells. The two cell forms of *P. luminescens* are easily to distinguish as they differ in many

phenotypic traits (Fig. 4-2A). Hereby, we observed red pigmentation of 2° Δ*xreR2* cells which usually is a 1°-specific feature and a loss of pigmentation in the 1° Δ*xreR1* strain (Fig. 4-2B).

Upon these findings we went on analyzing other 1°-specific traits. And indeed, we observed the 1°-specific phenotype in the 2° Δ*xreR2* strain as it not only started to produce pigments but also light as well as antibiotics. Additionally, 2° Δ*xreR2* cells formed mucoid colonies on agar-plates, which is also specific for 1° cells. In contrast, cells of the 1° Δ*xreR1* strain were not bioluminescent anymore and stopped to produce antibiotics as well forming mucoid colonies and thereby exhibited the 2°-specific phenotypes (Fig. 4-2B). Therefore, both deletion strains exhibit the phenotype of the respective other cell form, in the most predominant phenotypes of 1° and 2° cells (Tab. 4-1).

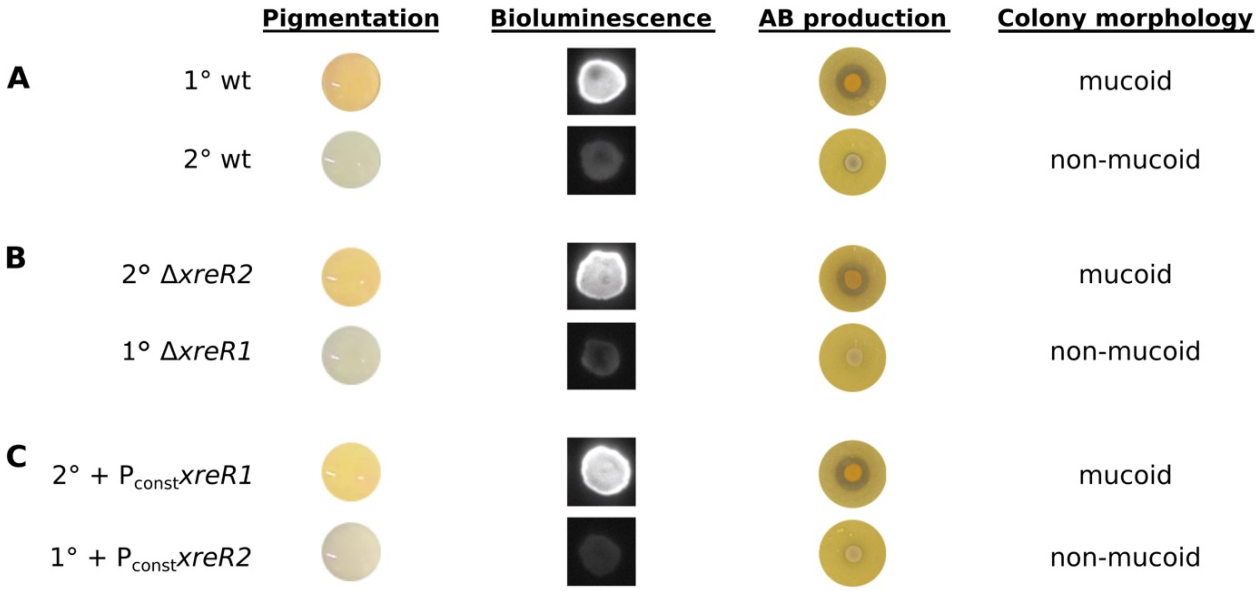


**Figure 4-2 Overview of phenotypic differences in 1° and 2° wt cells as well as XRE-mutation strains. A** While 1° cells are red pigmented, produce bioluminescence and antibiotics and form mucoid colonies, 2° cells lack all of these features. **B** These specific phenotypes could be reversed by deleting *xreR2* in 2° cells or *xreR1* in 1° cells, respectively. Here both created knock-out strains developed the phenotype of the respective other cell form. **C** The same effect of turning 2° into 1° and the other way around was gained by inserting extra copies of *xreR1* into 2° cells and extra copies of *xreR2* into 1° cells.

We went on investigating the effects of increased *xreR2* or *xreR1* levels. Therefore, we chromosomally integrated extra copies of *xreR1* into 2° cells (2° + $P_{const}$*xreR1*) and of *xreR2* into 1° cells (1° + $P_{const}$*xreR2*), respectively, each under the control of the constitutive promoter $P_{tac}$. As seen before in the knock-out strains, 2° cells overexpressing *xreR1* switched to the 1° phenotype while 1° cells containing increased *xreR2* levels exhibited 2°-specific characteristics (Tab. 4-1) both regarding pigmentation, bioluminescence, antibiotic synthesis and colony morphology (Fig. 4-2C).

The induced phenotypic switch in the deletion strains could successfully be reversed by chromosomally inserting extra copies of the respective gene (not shown) leading to three strains per phenotype, which were generated by solely altering *xreR2* or *xreR1* levels (Tab. 4-1).

**Table 4-1 List of resulting phenotypes caused by *xreR1* or *xreR2* expression levels.** The 1° wt strain as well as the strains 2° Δ*xreR2*, 2° + $P_{const}$*xreR1* and 1°Δ*xreR1* + $P_{const}$*xreR1* exhibit the 1° phenotype while 2° wt cells as well as the strains 1° Δ*xreR1*, 1° + $P_{const}$*xreR2* and 2° Δ*xreR2* + $P_{const}$*xreR2* show the 2° phenotype.

| **1° phenotype** | **2° phenotype** |
|---|---|
| 1° wt | 2° wt |
| 2° Δ*xreR2* | 1° Δ*xreR1* |
| 2° + $P_{const}$*xreR1* | 1° + $P_{const}$*xreR2* |
| 1° Δ*xreR1* + $P_{const}$*xreR1* | 2° Δ*xreR2* + $P_{const}$*xreR2* |

**Structural properties of XreR2 and XreR1** To get more insights about the function of XreR1 and XreR2 the amino acid composition of both were analyzed using Phyre2 (Kelley et *al.*, 2015). It turned out, that both *xreR1* and *xreR2* encode lambda (λ)

repressor-like proteins of the same superfamily, the XRE-transcriptional regulators. For XreR2 the highest homology was found to the DNA-binding protein Ner of the *Enterobacteria* phage Mu with the fold library ID d1nera_1. With coverage of 97%, 100% confidence and 59% sequence identity a structure XreR2 was predicted. According to this model it consists of five α-helices and no β-strands. Domain predictions revealed that the 69 amino acid long transcription factor solely consists of a lambda repressor like helix-turn-helix (HTH), also called Cro/C1 HTH DNA-binding domain. A signaling domain was not identified (Fig. 4-3A).

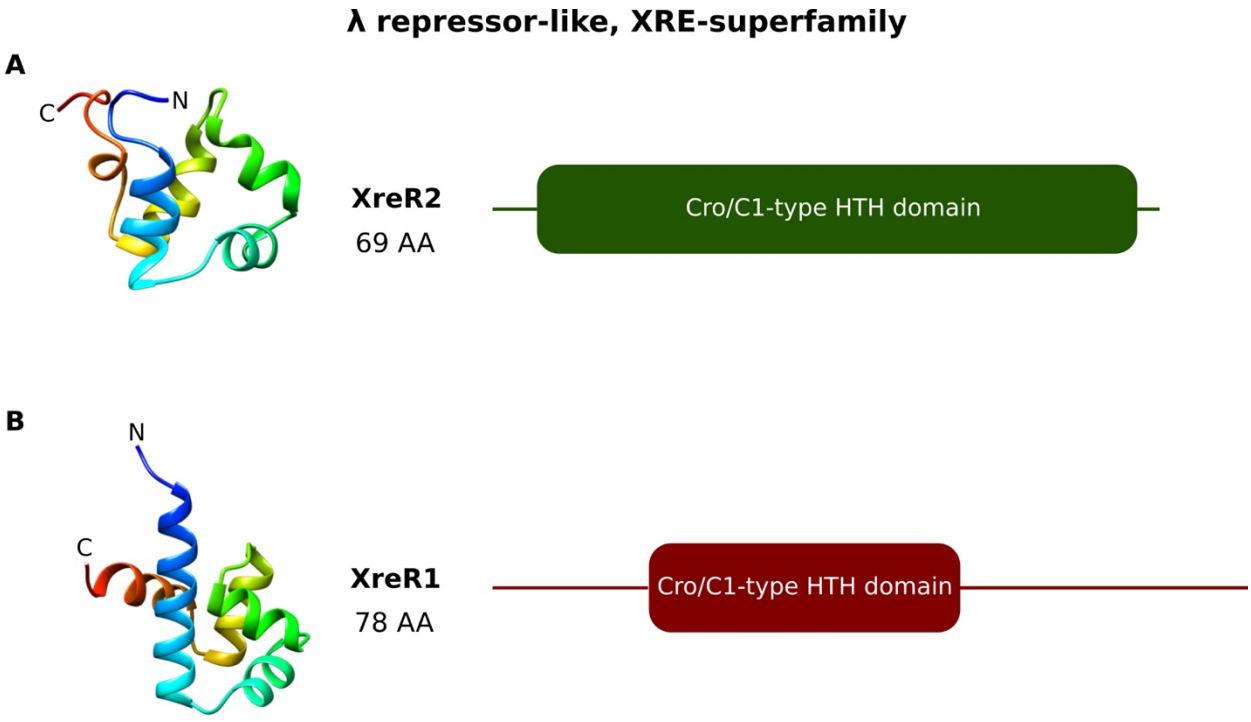


**Figure 4-3 Structure and domain prediction of XreR2 and XreR1. A** According to predictions the 69 amino acids (AA) long XreR2 comprises 5 helices and contains a lambda repressor-like (Cro/C1) HTH DNA-binding domain ranging from position 2 to 68. **B** XreR1 comprising 78 AA was predicted to belong to the same superfamily and also forms 5 helices with a Cro/C1 HTH domain reaching from AA 12 to 69.

Structure prediction for the slightly bigger 78 amino acid long protein XreR1 revealed a highly similar pattern. It is also built out of five α-helices and is predicted to only harbor a DNA-binding domain. With a coverage of 97%, 99.7% confidence and 42% sequence identity XreR1 was identified to belong to the SinR domain-like family (fold library ID:

d2b5aa1) which, according to prediction, also exclusively harbors a Cro/C1-type HTH-domain (Fig. 4-3B).

**Putative DNA targets of XreR1 and XreR2** Alteration of both, *xreR1* and *xreR2* levels were able to induce the respective other phenotype. However, the regulation mechanism was still unclear. As both transcriptional regulators exclusively harbor an HTH DNA-binding domain, we attempted to identify direct DNA targets. Here, we started with promoter regions of two of the most predominant 1°-specific traits: $P_{luxC}$ the promoter of the *lux* operon that is responsible for bioluminescence or to $P_{antA}$, the promoter of the *ant* operon, responsible for AQ production. Additionally, we analyzed binding of XreR1 and XreR2 to both $P_{xreR1}$ as well as $P_{xreR2}$ to investigate auto-regulatory functions as well as putative effects of one protein onto the expression of the respective other protein. Lastly, we examined interaction of XreR1 or XreR2 with the promoter region of the operon *PluDJC_21235/40* ($P_{pTAS}$). With 70% or 64% of identity those two genes respectively encode homologs of CcdA and CcdB a toxin/anti-toxin system (TAS) in *E. coli* and were thereby termed *ccdA*-like (*PluDJC_21235,* putative antitoxin) and *ccdB*-like (*PluDJC_21240,* putative toxin) for this study. However, CcdB-like seems to be truncated resulting in only 71% coverage of *E. coli* CcdB. In general, TAS are known to be involved in persister cell formation of different bacterial species another kind of phase variation (Wood et *al*., 2013). Since both genes of this putative TAS (pTAS) CcdAB-like are also known to be up-regulated in 2° compared to 1° cells (Eckstein et *al*. 2019) and because of its close proximity to *xreR2* as well as *xreR1* the pTAS might play a role in the phenotypic switching process of *P. luminescens*.

**Binding kinetics of XreR1 and XreR2 with different target regions** Initial MST analysis (data not shown) indicated binding of XreR2 to $P_{xreR2}$, $P_{pTAS}$ as well as $P_{xreR1}$. On the other XreR1 seemed to bind to its own promoter $P_{xreR1}$ as well as to $P_{xreR2}$. However,

none of both displayed binding to $P_{luxC}$ or $P_{antA}$ indicating no direct regulation of bioluminescence or AQ production by XreR1 and XreR2, respectively.

Next, we investigated the binding kinetics of XreR2 with the three identified DNA targets $P_{xreR2}$, $P_{pTAS}$ and $P_{xreR1}$ and of XreR1 with $P_{xreR1}$ and $P_{xreR2}$ using surface plasmon resonance (SPR) analysis. To do so, the respective promoter regions were immobilized onto streptavidin chips using biotin labeled DNA. Hereby, again binding of XreR2 to $P_{xreR1}$ (Fig. 4-4A), to its own promoter $P_{xreR2}$ (Fig. 4-4B) as well as to $P_{pTAS}$ (Fig. 4-4C) was indicated.

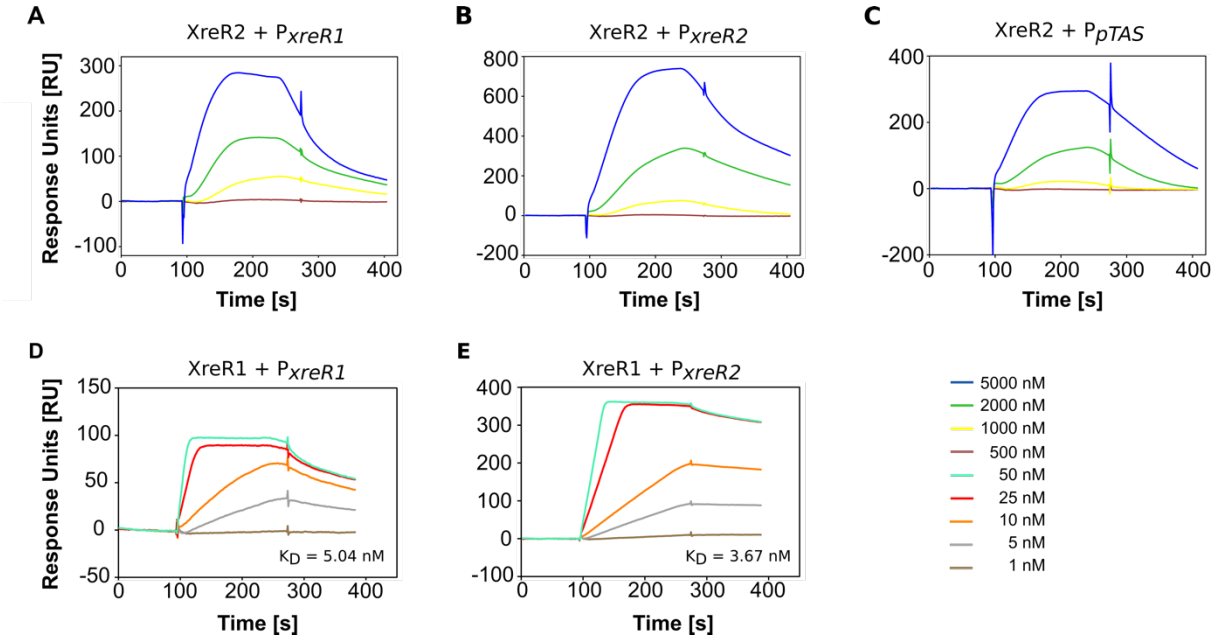


**Figure 4-4 Binding kinetics of XreR2 and XreR1 with different promoter regions via SPR.** The three promising promoters $P_{xreR2}$, $P_{xreR1}$ and $P_{pTAS}$ were respectively immobilized to a sensor chip and various concentrations of XreR2 and XreR1 were applied. Blue – 5000 nM; green - 2000 nM; yellow - 1000 nM; dark red - 500 nM; turquoise - 50 nM.; red - 25 nM; orange - 10 nM; grey - 5 nM; brown - 1 nM. All interactions show a strong on rate. Kinetics of XreR2 with $P_{xreR2}$ (**A**), and $P_{pTAS}$ (**B**) and $P_{xreR1}$ (**C**) indicate binding of the protein to all of the promoters while XreR1 strongly binds to its own promoter (**D**) as well as to $P_{xreR2}$ (**E**).

However, even upon applying 5000 nM of protein no saturation could be observed indicating a highly complex mode of action. The profile of the binding curves lets presume that the protein forms oligomers and is thereby able to bind to the DNA.

Consequently, no $K_D$ value could be calculated as the common algorithm does not fit this kind of binding profile.

XreR1 in turn bound with high affinity ($K_D$ = 5.04 nM; $k_a$= 1.54E+06 1/Ms; $k_d$= 6.16E-3 1/s) to its own promoter (Fig. 4D) and even stronger to $P_{xreR2}$ displaying a $K_D$ value of 3.67 nM ($k_a$= 6.35E+05 1/Ms; $k_d$= 1.47E-3 1/s; Fig. 4E). Both interactions seem to be very stable as the disassociation rate is very low.

**The XreR1/XreR2 network** SPR analysis revealed a high affinity of XreR1 to bind $P_{xreR2}$ as well as hints of XreR2 interacting with $P_{xreR1}$. Consequently, we attempted to investigate whether these bindings have activating or repressing effects using qRT-PCR.

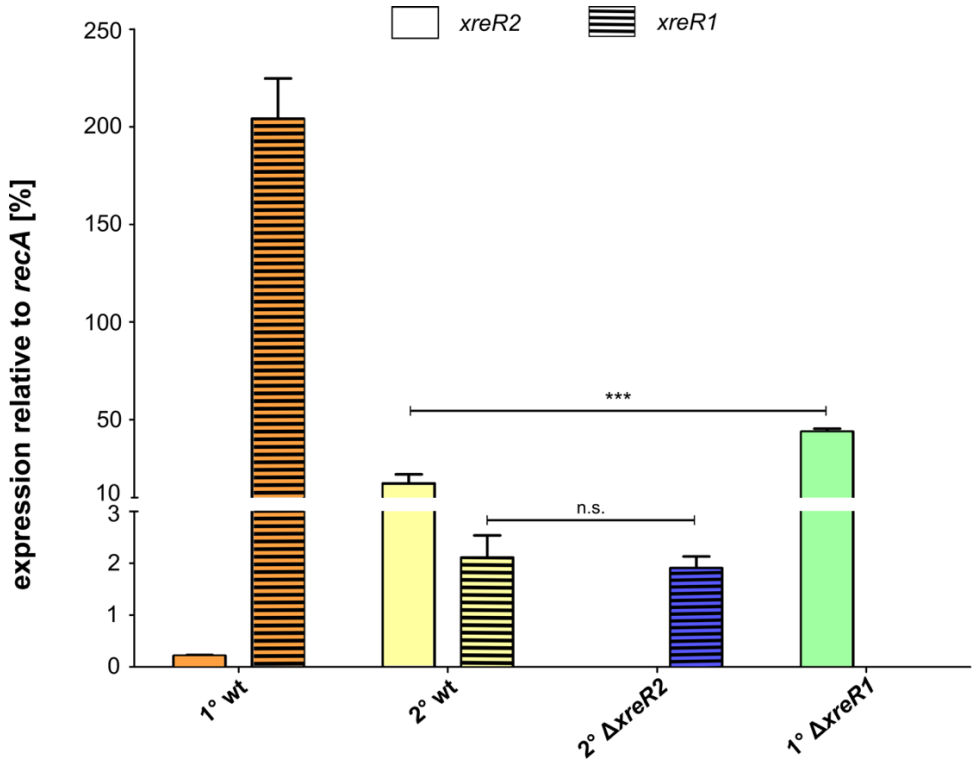


**Figure 4-5 Relative expression levels of xreR2 and xreR1 in wildtype and XRE-deletion strains.** To respectively compare gene expression of *xreR2* (blank bars) and *xreR1* (striped bars) in 1° (orange), 2° (yellow), 2°Δ*xreR2* (blue) and 1° Δ*xreR1* cells (green) mRNA was harvested during exponential growth phase and analyzed via qRT-PCR. Expression levels are depicted in percent, relative to expression of the housekeeping gene *recA*. The asterisks (***) indicate statistically significant differences with a p-value smaller than 0.001. Error bars represent the standard deviation of three independently performed experiments. N.s. not significant.

Here, *xreR2* seems to be negatively controlled by XreR1 as *xreR2* levels increased in the Δ*xreR1* strain (Fig. 4-5). On the other hand, no significant difference of *xreR1* expression between 2° wildtype and the 2° Δ*xreR2* strain could be observed (Fig. 4-5). Thus, although 2° Δ*xreR2* cells display the 1° phenotype, *xreR1* levels of 1° wildtype were not restored here. This leads to the assumption that *xreR1* is not under the control of XreR2 and that solely the absence of *xreR2* is sufficient to induce the 2° phenotype.

In addition to protein-DNA interaction we also analyzed a putative interaction between both proteins, XreR1 and XreR2. Therefore, we conducted bacterial two hybrid assays. And indeed, blue colored colonies of the *E. coli* BTH101 cells harboring both plasmids (pUT18-xreR2 and pKT25-xreR1) indicate interaction of XreR1 and XreR2, or vice versa (Fig. 4-6).

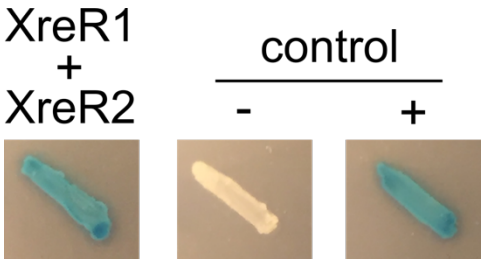


**Figure 4-6 Bacterial two hybrid assay of XreR1 and XreR2.** To analyze putative binding of both proteins E. coli BTH101 cells were co-transformed with pUT18-xreR2 and pKT25-xreR1 and plated on LB agar plates containing X-Gal and IPTG. The empty plasmids as well as pUT18-zip and pKT25-zip served as negative or positive controls, respectively. The blue color of the BTH101 cells pUT18-xreR2 and pKT25-xreR1 strongly indicates an interaction.

**Functionality of the putative toxin CcdB-like** The binding profiles of XreR2 with $P_{pTAS}$ obtained via SPR analysis strongly indicate interaction. However, the pTAS CcdAB-like is similar to the CcdAB TAS of *E. coli* where in absence of the anti-toxin (CcdA), the toxin (CcdB) targets the bacterial DNA-gyrase causing cell death by inducing DNA breaks (Bernard & Couturier, 1992). Therefore, we attempted to investigate if CcdB-like of *P. luminescens* still induces cell death or if it might has changed in

functionality and therefore could have assumed other regulatory tasks e.g. phenotypic switching of *P. luminescens*.

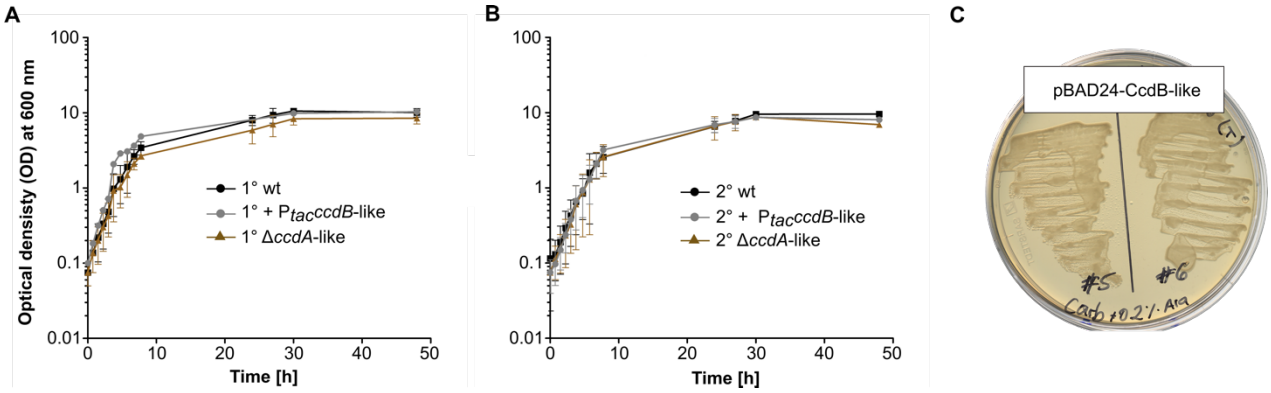


**Figure 4-7 Analysis of impaired growth caused by *ccdB*-like.** To analyze whether ccdB-like also acts as as a toxin we overexpression of *ccdB*-like in *P. luminescens* 1° as well as 2° cells. Furthermore, we deleted the cognate putative anit-toxin ccdA-like in both cell forms. and measured growth. Putative effects on the bacterias' fitness was analyzed by measuring growth over time comparing **A** 1° wt to the toxin overexpressing strain (1°+P$_{tac}$*ccdB*-like) and the strain lacking the anti-toxin 1° Δ*ccdA*-like as well as **B** 2° wt to the toxin overexpressing strain (2°+P$_{tac}$*ccdB*-like) and the strain lacking the anti-toxin 2° Δ*ccdA*-like. Additionally, we overexpressed *ccdB*-like in *E. coli* cells and monitored growth in agar plates (**C**).

To do so, we created knock-in strains overexpressing the *ccdB*-like gene in 1° and 2° cells. Additionally, we generated strains lacking the anti-toxin by deleting *ccdA*-like in 1° and 2°, respectively.

Neither toxin overexpressing 1° + P$_{tac}$*ccdB*-like cells nor the antitoxin knock-out strain 1 Δ*ccdA*-like exhibited a decrease in fitness as they grew perfectly fine when cultivated in liquid media (Fig. 4-7A). Furthermore, there were also no hints of increased cell death in the 2° + P$_{tac}$*ccdB*-like as well as the 2°Δ*ccdA*-like strain as they also showed growth behavior comparable to the wild type (Fig. 7B).

As the pTAS is similar to the CcdAB system of *E. coli* we then overproduced the toxin homolog CcdB-like in Dh5α-λ*pir* cells using the pBAD24 vector which harbors an arabinose inducible promoter. Upon arabinose addition we could not observe a

disadvantage in growth compared to the non-induced cells on agar-plates (Fig. 4-7C) as well as in liquid culture (not shown).

## Discussion

The appearance of two distinct phenotypically different cell forms makes *P. luminescens* a perfect model organism to study phenotypic heterogeneity. However, the regulation of phenotypic heterogeneity is still not completely understood. Two novel transcriptional regulators, XreR2 as well as XreR1, were identified to have major impact on phenotypic switching in *P. luminescens*. Both belong to the XRE (xenobiotic response element) - superfamily which is the second most frequently occurring regulator family in bacteria (Barragán et *al.* 2005). Proteins of this family are usually activated by interaction with environmental signals ranging from small effector molecules to large proteins (Bai et *al*., 1993; Fisher and Wray, 2002). Though, XreR2 and XreR1 were predicted to exclusively harbor a helix-turn-helix (HTH) DNA-binding domain similar to the Cro/C1 repressor protein of *λ* phage, comprising five $\alpha$ helices without any additional domain. In XRE-regulators this Cro/C1-HTH domain, always located N-terminally, (Roberts et *al*., 1977; Sauer et *al.*, 1982; Barragán et *al*., 2005) is highly conserved, while the C-terminal regulatory domain is variable (Kulinska et *al*., 2008). However, the XRE subfamily of ***λ***-like repressors is one of the best examples for simplest architectures as they almost entirely consist of a standalone HTH (Gehring et *al.* 1994). Several structures of Cro/C1-type transcriptional regulators have been resolved in the past. Here, just like for XreR2 and XreR1 the DNA-binding domain consists of five $\alpha$ helices which are highly conserved inside but much less at the extremities. Usually, the HTH which binds the DNA comprises the 2$^{nd}$ and 3$^{rd}$ helices. The remaining ones are involved in DNA-contacts and are referred to as recognition helices (Aggarwal et *al*., 1988).

We could successfully show that XreR2 binds to its own promoter. As expression of *xreR2* is essential to maintain the 2° phenotype of *P. luminescens*, it seems likely that

XreR2 positively auto-regulates itself - also taking into account that the regulator is about 500-fold up-regulated in 2° cells (Eckstein et *al*. 2019). Furthermore, we could show binding of XreR2 to $P_{pTAS}$, the promoter of the putative TAS system CcdAB-like (PluDJC_21245/50). Since both of these genes are also higher expressed in 2° than 1° cells (Eckstein et *al*. 2019) the system is also presumably activated by XreR2. However, this still has to be proven. Lastly, XreR1 also binds to its own promoter again suggesting a positive feedback loop.

Transcriptional regulators with phage-like HTH domains have usually repressing functions. However, in *Corynebacterium glutamicum* a member of the XRE family, ClgR, activates an operon encoding Clp proteases which then in turn recognize and degrade defective proteins (Gottesman et *al*., 1998). Furthermore, very recently an XRE transcriptional regulator of *Streptococcus suis,* SrtR, was found to be enhance the cells tolerance towards oxidative stress and high temperature (Hu et *al*., 2019).

Binding kinetics of XreR2 via SPR did not go into saturation for none of the tested promoters indicating no 1:1 binding of XreR2. Phage repressor-like proteins of the XRE superfamily are one example of proteins with the simplest HTH architecture. Almost every member of this family is built up by a standalone HTH. Among them some proteins harbor short extensions that are used to support protein folding and DNA contact (Aravind et *al*. 2005). Therefore, the initiate binding of XreR2 to its DNA targets could allow the protein to fold properly and so enables binding the specific site.

One of the best-studied XRE transcriptional regulators with a DNA-binding domain similar to that of the phage repressor proteins, C1 and Cro, is SinR of *Bacillus subtilis* (Lewis et *al.*, 1998) which represses biofilm formation by binding to the respective *eps* promoter. It has been shown that SinR represses the expression of *slrR* that encodes another XRE-family member, SlrR, which in turn represses SinR via direct binding. Thus, SinR and SlrR create a double negative feedback loop directly controlling genes involved

in cell separation and motility. Upon activation of that loop the cell gets time dependently locked in a high SlrR state (Chai et *al.*, 2010). The binding of XreR1 to $P_{xreR2}$ and the increase of *xreR2* levels in the 1°Δ*xreR1* strain also indicate a repression o *xreR2* by XreR1. Furthermore, both proteins seem to interact with each other. Therefore, XreR1 and XreR2 might also constitute an epigenetic switch comparable to the one of SinR and SlrR of *B. subtilis* (Fig. 4-8).

No binding of XreR2 or XreR1 to the $P_{luxC}$ or $P_{antA}$ promoter could be detected indicating no direct repression of bioluminescence or AQ-production. Here, it is worth mentioning that interaction assays were performed using either only XreR1 or XreR2. In the SinR/SlrR model of *B. subtilis* the respective genes are regulated by a complex of both proteins (Chai et *al.*, 2010). Therefore, a mixture of both, XreR1 and XreR2, might be needed to enable binding to promoter regions of phase specific phenotypes and thereby repressing or activating gene expression (Fig. 4-8).

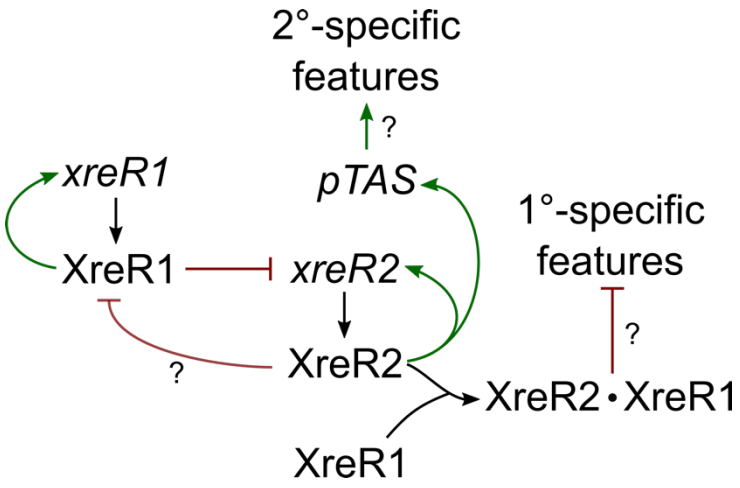


**Figure 4-8 Model of gene regulation via XreR1 and XreR2.** XreR1 binds to its own promoter - most probably leading to a positive feedback loop. Furthermore, it represses the expression of *xreR2* and thereby maintains the 1° phenotype. XreR2 in turn binds to XreR1 thereby putatively re-enforcing its own expression. The built XreR1-XreR2 complex might directly represses 1°-specific features inducing the 2° phenotype. Additionally, XreR2 most probably activates expression of the TAS-derived *ccdAB*-like system which could maintain the 2° phenotype by e.g. activating 2°-specific features. Green: activation; red: inhibition.

In *B. subtilis* the antagonist of SinR, SinI, gets activated during stationary phase and binds to SinR thereby releasing $P_{eps}$ and promoting biofilm formation (Gaur et *al.*, 1991; Bai et *al*., 1993). This suggests that the phenotypic switch is also reversible in *P. luminescens* DJC. However, the respective signal to trigger that conversion is still unknown.

Lastly, the role of the putative TAS CcdAB-like still remains elusive. There are several TAS which are described to be involved in persistence. Persister cell formation is one of the best-studied phenotypic heterogeneity forms using the bet-hedging strategy. Hereby, upon antibiotic treatment, single cells reversibly switch into a transient growth arrested state which allows them to survive the stress situation (Veening et *al*. 2008).

The CcdB protein of *E. coli* owes its toxicity to the last three C-terminal amino acid residues tryptophan (99), glycine (100) and isoleucine (Bahassi et *al*., 1995). Sequence analysis revealed that CcdB-like is also C-terminally truncated including the respective amino acids and thereby lost the amino acid residues responsible for CcdB toxicity in *E. coli*. This could explain the absence of an obvious phenotype in Δ*ccdA*-like or $P_{tac}$*ccdB*-like strains indicates that the CcdAB-like system arose from a TAS but owns a new function. The facts that the pTAS originates from another phenotypic heterogeneity inducing system, lies in close proximity to *xreR2* and *xreR1* and that XreR2 directly binds to its promoter strongly indicates an involvement in the process of phenotypic switching. As *ccdA*-like as well as *ccdB*-like were found to be up-regulated in 2° cells (Eckstein et *al.*, 2019) a positive regulation of it via XreR2 seems likely. The CcdAB-like system might help to maintain the 2° phenotype by e.g. activating 2°-specific features (Fig. 4-8).

Taken together, we identified two novel XRE-transcriptional regulators, XreR1 and XreR2, which play a major role in the process of phenotypic switching in *P. luminescens.* Both proteins interact with each other and are able to bind DNA and thereby display a complex regulatory network putatively including a double negative feedback loop. However, whether phase specific features are directly regulated via a XreR1-XreR2 complex or by some other proteins that are under the control of XreR1 or XreR2 has to be elucidated.

## Material and Methods

**Bacterial strains and growth conditions** *E. coli* strains MG1655 and DH5αλpir were used in this study. They were routinely grown at 37° C in LB medium [1% (w/v) NaCl; 1% (w/v) tryptone; 0.5% (w/v) yeast extract]. If necessary, 50 µg/ml antibiotic was added into the medium. All *P. luminescens* strains were cultivated aerobically in either LB medium or CASO medium [0.5% (w/v) NaCl, 0.5% (w/v) peptone from soy; 1.5% (w/v) tryptone] at 30°C. If necessary, the growth medium was supplemented with 50 µg/ml rifampicin (Sigma Aldrich). For preparation of agar plates, 1.5% (w/v) agar was added to the respective medium.

**RNA preparation** Total RNA from three independent cultures of DJC 1° or DJC 2° cells grown to optical densities at 600 nm (OD600) of 3 (mid-exponential growth phase) and 10 (early stationary growth phase) was extracted. Therefore, the pellets of harvested cells were resuspended in 500 µl ice-cold AE-buffer [20 mM NaAc pH 5.2, 1mM EDTA pH 8.0] then 500 µl Roti®-Aqua-P/C/I (Roth) and 25 µl 10% SDS was added. After vortexing the mixture was incubated for 30 min at 60° C with shaking. Subsequently the samples were placed into the fridge for one night. On the next day the samples were centrifuged with 16.100 rcf for 40 min at 0° C. Afterwards the supernatant was transferred into 5PRIME Phase Lock Gel Tubes (Quantabio), supplemented with 500 µl P/C/I and 50 µl 3M NaAc pH 5.2 and after mixing the tubes were centrifuged with 16.100 rcf for 10 min at 0°C. Then the supernatants were mixed with 1 ml 96% EtOH and put on -80° C for overnight precipitation. On day 3 samples were again centrifuged with 16.100 rcf for 30 min at 0°C but this time the supernatant was discarded. To wash the pellet 1 ml 80% EtOH was added and subsequently removed by centrifugation with 16.100 rcf for 10 min at 0°C. This washing step was repeated 2 times. Then the pellet was air dried for 60 min with

open lid and resolved in 100 µl DEPC-treated water. 5 µg of RNA were then treated with DNaseI to remove genomic DNA.

**qRT-PCR** To validate the whole-transcriptome data, quantitative reverse transcription-PCR (qRT-PCR) was carried out on three independent total RNA preparations, in each case in triplicates. cDNAs were synthesized during the run using Luna® Universal One-Step RT-qPCR Kit (NEB biolabs) therefore, the reactions were performed according to the protocol provided by the manufacturer. Reactions and melting curves were monitored in the LightCycler (BioRad). Differences in gene expression levels were calculated using the Pfaffl-Method (Pfaffl, 2001) with *recA* serving as housekeeping gene. All data are presented as a ratio of three independent biological replicates. Values are means ± the standard deviation.

**Generation of plasmids** To generate pNPTS-FAB-ΔxreR2 500 bp upstream (FA) and downstream (FB) of genomic *xreR2* were amplified by PCR using the primer pairs BamHI-xreR2-FA fwd + xreR2-FA-ovl-FB rev and xreR2-FB-ovl-FA fwd + xreR2-FB-EagI rev introducing a BamHI and a EagI restriction site to the 5′ end of the upstream fragment and the 3′ end of the downstream fragment, respectively. Overlap extension PCR was used to fuse the two PCR products which were then cloned into the pNPTs138-R6KT backbone using the BamHI and EagI restriction sites. Correctness of the plasmid was confirmed by PCR using primers check-pNPTS fwd and check-pNPTS rev.

pNPTS-FAB-ΔxreR1 was generated the same way, however with different restriction sites. Here EcoRI and EagI were used. Therefore, the respective primer pairs were EcoRI-xreR1-FA fwd + xreR1-FA-ovl-FB rev and xreR1-FB-ovl-FA fwd + xreR1-FB-EagI rev.

For pPINT-Ptac-xreR2 and pPINT-Ptac-xreR1 generation a *lacI*-$P_{tac}$ fragment (PstI-lacI_Ptac fwd: + Ptac-ovl-blank rev) was fused to either genomic *xreR2* (xreR2-ovl-Ptac fwd: + xreR2-EagI rev) or genomic *xreR1* (xreR1-ovl-Ptac fwd + xreR1-EagI rev) via overlap PCR, respectively, resulting in $P_{tac}$-*xreR2* and $P_{tac}$-*xreR1* each harboring a 3'-PstI and 5'-EagI restriction site. Afterwards the single fragments were cloned into the empty pPINT backbone. Correctness of the plasmids were checked by sequencing using the primers check-pPINT fwd and check-pPINT rev.

To generate pPNPTS-FAB-ΔccdA-like the up- and downstream flanking regions of genomic *ccdA-like* were amplified using the primer pairs BamHI-FA ccdA-like fwd + FA ovl FB ccdA-like rev and FB ovl FA ccdA-like fwd + FB ccdA-like-EagI rev. The resulting amplicons were then fused via overlap extension PCR and thereby FAB harboring a 5'-BamHI and 3'-EagI restriction site was generated. Using the respective restriction enzymes FAB was cloned into the empty pNPTs138-R6KT backbone.

pNPTS-FAB-ΔccdB-like was achieved by the same procedure. FA was amplified using the primer pair BamHI-FA ccdB-like fwd + FA ovl FB ccdB-like rev and FB was achieved by using primers FB ovl FA ccdB-like fwd and FB ccdB-like-EagI rev. Again, both flanking regions were fused via overlap extension OCR and the resulting FAB fragment was cloned into the pNPTs138-R6KT backbone using the restriction enzyme sites BamHI and EagI.

For pPINT-Ptac-ccdB-like generation again the *lacI*-$P_{tac}$ fragment was fused to genomic *ccdB*-like amplified with the primers ccdB-like ovl Ptac fwd and ccdB-like-EagI rev via overlap extension PCR. The resulting fragment was then cloned into the empty pPINT vector by utilizing the restriction enzymes PstI and EagI.

To gain the plasmid pBAD24-ccdB-like, genomic *ccdB*-like was amplified using the primers NheI-ccdB-like fwd and ccdB-like-XmaI rev. The thereby introduced restriction sites were used to clone the gene into the pBAD24 backbone downstream of the $P_{ara}$ promoter.

Correctness of all plasmids based on the pNPTs138-R6KT backbone were checked by sequencing using the primers: check-pNPTS fwd and check-pNPTS rev.

Integrational plasmids with pPINT backbone were sequenced with the primer pair check pPINT fwd + check-pPINT rev.

Rightness of the pBAD24-ccdB-like plasmid was confirmed by sequencing with the following primers: check-pBAD24 fwd + check-pBAD24 rev.

(For a list of all oligo sequences used in this study see Supplementary Table S1)

**Generation of knock out strains** For deletion of genomic *xreR2* in 2° cells or *xreR1* in 1° cells the plasmids pNPTS-FAB-ΔxreR2 and pNPTS-FAB-ΔxreR1 were used, respectively. The genes were depleted via double homologous recombination as described previously (Easom & Clarke, 2008). Therefore, the respective plasmid was conjugated from *E. coli* S17-1 λ*pir* into 1° or 2° cells and exconjugants were selected as $Rif^R Km^R$ colonies. The pNPTS138-R6KT plasmid contains the *sacB* gene and, after growth in LB broth (with no selection), putative mutants were identified by screening for $Rif^R$ $Suc^R$ $Km^S$ colonies. Successful deletion of *xreR2* or *xreR1* was confirmed by PCR using either the primer pair BamHI-xreR2-FA fwd/ xreR2-FB-EagI rev or EcoRI-xreR1-FA fwd/ xreR1-FB-EagI, respectively, followed by DNA sequencing.

**Insertion of extra gene copies into *P. luminescens* genome** To chromosomally insert constitutive expressed copies of either *xreR2*, *xreR1* or *ccdB*-like into 1° or 2°

cells, respectively, the non-coding intergenic region between the two genes *glmS* and *rpmE* was utilized. Therefore, the respective plasmids pPINT-Ptac-xreR2, pPINT-Ptac-xreR1 or pPINT-Ptac-ccdB-like were used. Insertion and backbone depletion were obtained via double homologous recombination as described above. Successful insertion of each gene was checked using again the primers check-pPINT fwd and check-pPINT rev followed by DNA sequencing.

**Bioluminescence bioassays** To analyze bioluminescence 1 ml LB were inoculated to an $OD_{600}$=1 with overnight cultures of the respective *P. luminescens* variant. Subsequently, 5 µl of were spotted onto LB plates and incubated at 30°C. After 48 hours bioluminescence was monitored using a Chemiluminescence Imager (Peqlab, Erlangen) using 5 min exposure time.

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

**Pigmentation** The development of red pigments was visually noted after 3 days of growth of *P. luminescens* DJC 1° and 2° cells on LB plates at 30°C

**Heterologous expression of *ccdB*-like in *E. coli*** E. coli Dh5α-λpir cells were transformed with the pBAD24-ccdB-like plasmid. To induce gene expression, $P_{ara}$ was activated by adding 0.2 % arabinose to the medium.

**Bioinformatical analysis** Structure prediction of XreR2 and XreR1 was performed by Phyre2 (Kelley et *al.*, 2015) and visualized using USCF Chimera 1.13.1 (Resource

for Biocomputing, Visualization, and Informatics). Additional domain prediction was performed by using InterPro (https://www.ebi.ac.uk/interpro/).

**Heterologous overproduction of recombinant XreR1 and XreR2**

*E. coli* BL21(DE3) pLysS harboring plasmid pET28-His-SUMO-XreR1 or pET28-His-SUMO-XreR1 was grown to exponential phase at 37°C. Expression of genes encoding N-terminally His-SUMO-tagged XreR1 ($His_6$-SUMO-XreR1) or XreR2 ($His_6$-SUMO-XreR2) was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the bacteria were incubated at 18°C over night. Subsequently, the cells were harvested and washed with at 6000 rpm for 30 minutes at 4°C. The cell pellet was frozen in liquid nitrogen and stored at -80°C until further use. Cells were resuspended in 0.2 ml/g lysis buffer [50 mM Tris/HCl pH 7.5, 5% glycerol (v/v), 10 mM MgCl2, 0.5 mM phenylmethane sulfonyl fluoride (PMSF), 1 mM dithiotreitol (DTT), 10 ng/ml DNAse] and lysed by passage through a high-pressure cell disrupter (Constant Systems). After centrifugation (1 hour at 45000 rpm and 4°C) of the disrupted cells, the supernatant containing the respective cytosolic $His_6$-SUMO-protein was incubated with $Ni^{2+}$-nitrilotriacetic acid (NTA) resin (Qiagen) preequilibrated with lysis buffer. After 1 h of incubation, the protein-resin complex was washed twice with washing buffer (50 mM Tris/HCl pH 7.5, 10% glycerol (v/v), 500 mM NaCl, 10 mM imidazole, 2 mM β-Mercaptoethanol (MeOH)). Finally, the His-SUMO-tagged protein was eluted in several fractions with buffer containing 250 mM imidazole, 50 mM Tris/HCl pH 7.5, 10% glycerol (v/v), 500 mM NaCl, 2 mM β-MeOH. Both proteins were dialyzed against XreR protein buffer (50 mM Tris/HCl pH 7.5, 10% glycerol (v/v), 500 mM NaCl, 2 mM β-MeOH) over night at 4°C. To cleave off the His-SUMO tag, 1 mg of the protease Senp2 per 500 mg protein was added to the respective dialysed His-SUMO-tagged protein and another 4 h step of dialysis against the XreR protein buffer was performed. Subsequently, the $Ni_{2+}$-NTA based affinity chromatography was repeated. As the tag was separated from the protein, the protein eluted in the flow

through while only the tag bound to the beads and were eventually eluted using elution buffer. Protein concentrations were determined using NanoDrop (ThermoFisher).

**Surface plasmon resonance (SPR) spectroscopy** SPR analysis was performed in a Biacore T200 (GE Healthcare, München) using carboxymethyl dextran sensor chips that were pre-coated with streptavidin (XanTec SAD500L, XanTec Bioanalytics GmbH, Düsseldorf). Promoter regions were 5'-biotinylated via PCR using the primers Btn-PxreR2 fwd and PxreR2 rev. for genomic Btn-$P_{xreR2}$ amplification. Genomic Btn-$P_{xreR1}$ was achieved using the primer pair Btn-PxreR1 fwd + PxreR1 rev. Lastly, Btn-$P_{pTAS}$ was amplified using Btn-Ptas fwd and Ptas rev.

Before immobilization of 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 biotinylated promoter DNA was injected using a contact time of 420 seconds and a flow rate of 10 µl/min. 1 M NaCl/50 mM NaOH/50 isopropanol was injected as a final wash step. Approximately 600 RU of $P_{xreR1}$ was captured onto flow cell 2, $P_{xreR2}$ onto flow cell 3 and $P_{pTAS}$ onto flow cell 4, respectively, of the chip. XreR2 or XreR1 were diluted in dialysis buffer and passed over flow cells 1 to 4 in different concentrations (0 nM, 0.1 nM, 1 nM, 5 nM, 10 nM, 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, 1000 nM, 2000 nM and 5000 nM) using a contact time of 180 sec followed by a 240 sec 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 µ/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.

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## Author contributions

S.E. and R.H. designed the experiments. S.E. created the deletion, insertion and complementation strains of *xreR2* and *xreR1*, performed the phenotypic comparison as well as the the qRT-PCR and overproduced and purified XreR2 and XreR1. M.S. constructed the *ccdB*-like and *ccdA*-like deletion strains as well as *ccdB*-like insertion strains and measured bacterial growth. S.E. performed the whole experiment with *E. coli* overexpressing *ccdB*-like. J.B. and S.E. conducted and evaluated SPR analyses. SE and R.H wrote the manuscript.

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

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Review

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# Regulation of Phenotypic Switching and Heterogeneity in *Photorhabdus luminescens* Cell Populations

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## Abstract

Phenotypic heterogeneity in bacterial cell populations allows genetically identical organisms to different behavior under similar environmental conditions. The Gram-negative bacterium *Photorhabdus luminescens* is an excellent organism to study phenotypic heterogeneity since their life cycle involves a symbiotic interaction with soil nematodes as well as a pathogenic association with insect larvae. Phenotypic heterogeneity is highly distinct in *P. luminescens.* The bacteria exist in two phenotypic forms that differ in various morphologic and phenotypic traits and are therefore distinguished as primary (1°) and secondary (2°) cells. The 1 cells are bioluminescent, pigmented, produce several secondary metabolites and exo-enzymes, and support nematode growth and development. The 2° cells lack all these 1°-specific phenotypes. The entomopathogenic nematodes carry 1° cells in their upper gut and release them into an insect's body after slipping inside. During insect infection, up to the half number of 1° cells undergo phenotypic switching and convert to 2° cells. Since the 2° cells are not able to live in nematode symbiosis any more, they cannot re-associate with their symbiosis partners after the infection and remain in the soil. Phenotypic switching in *P. luminescens* has to be tightly regulated since a high switching frequency would lead to a complete break-down of the nematode-bacteria life cycle. Here, we present the main regulatory mechanisms known to-date that are important for phenotypic switching in *P. luminescens* cell populations and discuss the biological reason as well as the fate of the 2° cells in the soil.



## Introduction

### The genus *Photorhabdus*

*Photorhabdus* spp. are Gram-negative enteric bacteria, which are close symbionts of heterorhabditid soil nematodes and are in turn highly pathogenic toward insects. Based on molecular biological analyses, the genus has been divided into three bacterial species: *Photorhabdus luminescens*, *Photorhabdus temperata* and *Photorhabdus asymbiotica* [1]. Recently, genome sequencing of already described as well as 11 new isolates identified 14 new *Photorhabdus* subspecies, which led to the proposal of re-organizing the existing taxonomy by raising several subspecies to species level [2]. However, all *Photorhabdus* species live in a close symbiotic interaction with nematodes of the family *Heterorhabditidae*. While *P. luminescens* and *P. temperata* are only highly pathogenic toward insects, *P. asymbiotica* is additionally able to infect humans [3]. *Photorhabdus* bacteria are the only terrestrial bacteria known to be bioluminescent due to bacterial luciferase production [4].

### The life cycle of *P. luminescens*

*P. luminescens* has a complex dualistic life cycle as on the one hand it maintains a mutualistic symbiosis with soil nematodes, and on the other hand, it is highly pathogenic toward a variety of insect species (Fig. 1). The bacteria colonize the upper gut of the infective juvenile stage (IJs) of the nematode *Heterorhabditis bacteriophora*. The IJs infect insect larvae by invading



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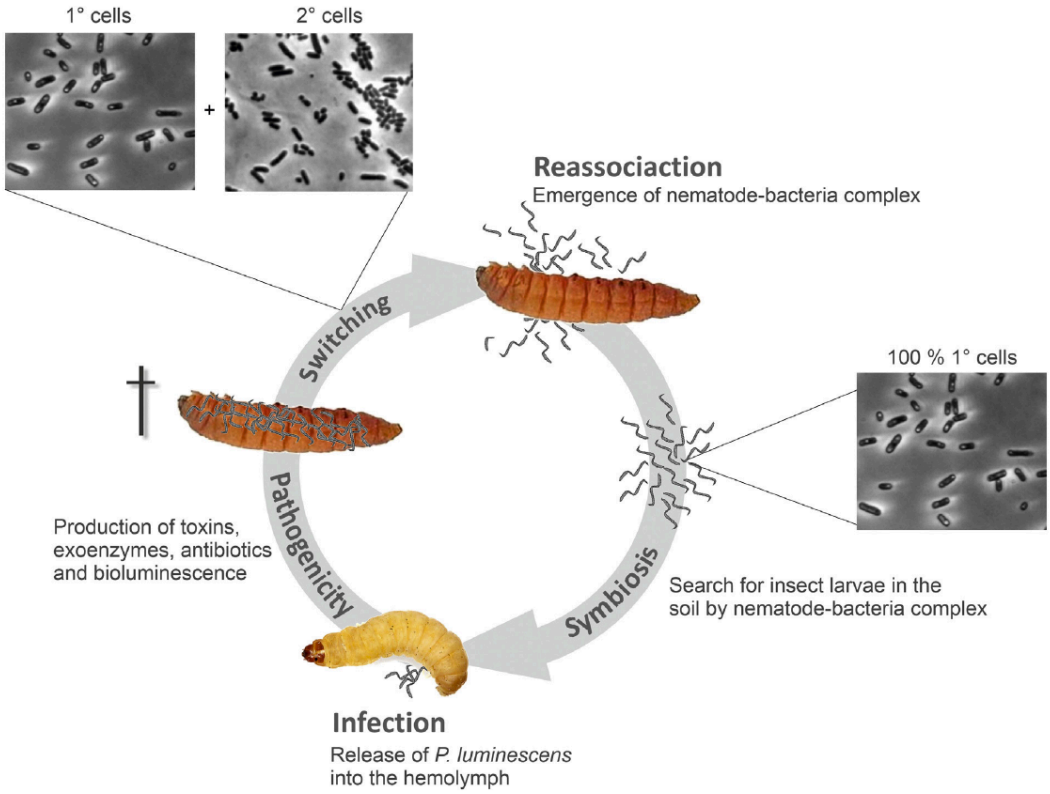


**Fig. 1.** Infection cycle and phenotypic heterogeneity of *P. luminescens*. After injected from the nematodes into the insect hemolymph, the bacteria replicate exponentially. After 48 h, the insect dies and the cells enter a prolonged stationary growth phase. In this growth phase, single cells undergo phenotypic switching. After 28 days, when the nutrients are depleted and novel generations of nematodes emerge from the cadaver, a significant subset of *P. luminescens* have converted to 2° cells. Among other phenotypic traits (e.g., pigmentation, bioluminescence, secondary metabolites, exoenzymes), 1° cells produce crystalline inclusion proteins that are visible in the light microscope, whereas 2° cells do not.

into the hemocoel of the insect. Once inside, the bacteria are directly released from the gut of the IJs into the insect's hemolymph by regurgitation. Then, the bacteria replicate rapidly, quickly establish a lethal septicemia in the host by production of a broad range of different toxins that effectively kill the insect victim within 48 h. Furthermore, *P. luminescens* produces several exoenzymes that bioconvert the insect's body into a rich food source, which is used for growth by the bacteria as well as by the nematodes. The bacteria support nematode development and reproduction, probably by providing essential nutrients that are required for efficient nematode proliferation [5,6]. In addition, the bacteria produce a huge range of secondary metabolites like several antibiotics to defend the insect cadaver from being affected by other bacteria and fungi [7]. Furthermore, the bacteria produce bacterial luciferase, which causes the cadaver to glow. When the nutrients are depleted, the IJs and bacteria re-associate and emerge from the carcass in search for a new insect host (Fig. 1; see Refs. [8,9] for reviews).

## Phenotypic Switching in *P. luminescens* Cell Populations

*P. luminescens* exists in two phenotypic different cell forms called primary (1°) and secondary (2°) cells. In the nematode gut, the population exclusively consists of 1° cells. During the infection, a large portion of the of the cells switch from 1° to 2° cells. Both cell forms are genetically identical but differ in distinct phenotypic traits [10]. 1° cells exhibit several phenotypical characteristics that are absent in all 2° cells [11,12]. Among these, most apparent is the production of extracellular enzymes like proteases, pigments, secondary metabolites like antibiotics, bioluminescence, the crystalline inclusion proteins CipA and CipB, and cell clumping factor [11–13].

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Moreover, both variants are morphologically distinct as 1° cells are long-shaped rods, whereas 2° cells are smaller short rods [14]. Interestingly, while both cell forms are equally virulent toward insects, only 1° cells are known to associate with the nematodes. Furthermore, 2° cells are unable to support nematode growth and development both in the insect cadaver and in culture (Fig. 2).

Moreover, heterogeneity of *P. luminescens* colonies has been described and the different colony forms were designated as P- and M-forms, which form larger and smaller colonies, respectively [15]. The pathogenic P-form switches to the mutualistic M-form to initiate mutualism in host nematode intestines. However, since a stochastic promoter inversion of the *mad* locus causes the switch between the two distinct forms, this phenomenon is not due to true phenotypic heterogeneity. The respective $P_{mad}$ promoter direction is assumed to be similar, so that both directions might be found in 1° and 2° cells.

Phenotypic switching of *P. luminescens* has previously also been referred to as phase variation [12]. However, this phenomenon is different from classical bacterial phase variations as both cell forms are genetically homogeneous [16]. Furthermore, classical phase variation involves reversible genetic events, occurs at significant frequency and is almost reversible. Both 1° and 2° cells are genetically identical. DNA rearrangements or modifications, genetic instability, or the loss of plasmids is not involved in *P. luminescens* 2° cell formation [10,16–18]. However, genome sequencing of several switched 2° colonies and comparison of their genome(s) to the respective 1° cells should be performed to finally prove that phenotypic switching of *P. luminescens* is due to true phenotypic heterogeneity of 1° and 2° cells.

The 1°-specific phenotypes are most distinct in the early post-exponential growth phase, which correlates with the establishment of *P. luminescens* as a saturating monoculture in the insect host and with the initiation of nematode feeding and development within the insect cadaver. After a couple of days in the insect host, 2° cells that are unable to support nematode development occur spontaneously. Therefore, it is argued that the 1°-specific characteristics are required for the symbiotic interaction with the nematode rather than for the pathogenic interaction with the insect. Since 2° cells develop not only inside the insect larvae but also after prolonged cultivation in the laboratory, it was suggested that phenotypic switching is a response to environmental stress [19,20]. It has been observed that low osmolarity of the culture medium seems to trigger phenotypic switching [16,21]. However, co-cultivation assays of labeled 1° and 2° cells revealed that 1° cells overgrew the 2° cells in the exponential phase, while 2° cells outcompeted the 1° cells in the stationary growth phase, regardless of the initial composition (A. Langer, R.H., LMU, unpublished). This is in accordance with the observation that after periods of starvation, 2° cells were able to restart growth 10 to 12 h earlier than 1° cells [22]. In *Escherichia coli*, the universal stress protein UspA is known to play an important role in the recovery upon periods of nutrient starvation [23]. Conformingly, in *P. luminescens* 2° cells, up-regulation of UspA was observed [24]. UspA also plays an important role in the protection of the cell against superoxide-generating agents. Comparison of the 1° and the 2° proteome

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

**Fig. 2.** Phenotypic differences of the *P. luminescens* 1° and 2° cells. In contrast to 1° cells, only reduced bioluminescence is visible in 2° cells. Cell clumping, protease activity, crystal protein production and symbiosis are absent from 2° cells. 1° and 2° cells are both pathogenic toward insects, whereas only insect cadavers that were infected with 1° cells are pigmented due to bacterial anthraqinone production. The figure was modified after Ref. [8]. "+++": high; "+": low; "-": no.

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identified up to 450 potential factors that are characteristic for either the one or the other cell form in the stationary growth phase [24]. In 2° cells, specific proteins involved in oxidative stress response, alternative energy metabolism and different translation factors are produced that are absent from 1° cells. Furthermore, the protein amounts of iron transporters and iron binding proteins as well as those responsible for consumption of several sugars and amino acids were affected in 2° cells. Moreover, compared to 1° cells, the number of molecular chaperones was strongly reduced in 2° cells [24].

## Regulation of Phenotypic Switching

Phenotypic switching of *P. luminescens* has to be tightly regulated. In principle, all cells are exposed to similar environmental conditions, stress or signal(s) in the insect cadaver. If 100% of the 1° cells would convert to 2° cells in the insect cadaver during nematode development, this would have fatal consequences on the viability of the bacteria-nematode symbiosis. Consequently, the complete life and infection cycle of the symbiosis partners would break down. Therefore, heterogenous regulation mechanisms have to ensure that phenotypic switching is only induced in individual cells of a population to induce phenotypic switching during the life cycle of *P. luminescens* ensuring that a sufficient portion of the bacteria stays 1°.

### Global regulation by the LysR-type receptor HexA

HexA has been identified as a major regulator that is supposed to act as master regulator of phenotypic heterogeneity in *P. luminescens* [19]. HexA belongs to the LysR-type transcriptional regulator family and is present in high amounts in 2° cells. Positive autoregulation of HexA is supposed to keep the high level and therefore maintaining the phenotype of the 2° cell form [19]. Accordingly, HexA is assumed to act as a repressor of 1°-specific genes. Deletion of the *hexA* gene in 2° cells resulted in the de-repression of the 1°-specific factors and restored the ability to support nematode growth and development [19]. Moreover, the overproduction of HexA in 1° cells was sufficient to induce the 2° phenotype, which supports the idea that high levels of HexA are mandatory for undergoing phenotypic switching [20]. Deletion of *hexA* in 2° cells caused attenuation in virulence against insect larvae, suggesting that HexA is also required for regulation of pathogenicity [19]. In contrast, in the plant pathogen *Erwinia carotovora* deletion of *hexA* increased virulence [25]. Moreover, the production of stilbene-derived small molecules that are important for symbiosis is up-regulated in the Δ*hexA* mutant, further indicating that HexA is involved in regulation of both symbiosis and pathogenicity in *P. luminescens* [26].

HexA is a homolog of the LysR-type regulator LrhA of *E. coli*, which is described to control stress response and motility via regulation of translation of the alternative sigma factor RpoS, small RNAs and the chaperone Hfq [27]. As HexA includes a predicted helix–turn–helix DNA-binding domain, interaction with the DNA was very likely. Similar to LrhA also for HexA, it is assumed that it has a highly complex regulation mechanism including small RNAs (Fig. 3) [19,27]. It has recently been described that HexA controls phenotypic heterogeneity in a versatile way, directly and indirectly [13]. It has been demonstrated that HexA does not directly affect bioluminescence. The respective *luxCDABE* operon is repressed at the post-transcriptional level, and transcriptional levels of the RNA chaperone gene *hfq* are also enhanced in 2° cells. This underlined the idea that small regulatory RNAs are presumably involved in regulation of phenotypic switching, which are under control of HexA [13]. That small regulatory RNAs are mainly involved in the regulation of the switching process is also supported by the fact that upon deletion of *hfq*, the bacteria are no longer able to maintain a healthy symbiosis with nematodes due to the abolishment of the production of all known secondary metabolites [28]. The *hexA* gene was highly up-regulated in the 1°Δ*hfq* strain, so that Hfq mediates regulation of secondary metabolism in *P. luminescens* via HexA. A further deletion of *hexA* besides *hfq* fully restored secondary metabolism [28].

Another phenotypic trait that is specific for 1° cells is cell clumping (see also below). 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. However, the binding kinetics of

**Fig. 3.** Model for the regulation of phenotypic switching in *P. luminescens*. Hfq mediates stability of *hexA* mRNA in 2° and not in 1° cells, which causes high levels of HexA in 2° cells repressing 1°-specific genes. Low HexA levels in 1° cells control cell clumping by directly controlling expression of the *pcfABCDEF* operon and indirect by controlling translation of the *luxCDABE* mRNA, which leads to light production. The heterogeneous strong activation of the *antABCDEFGHI* operon in 1° cells might be mediated via the ligand-bound AntJ. Enhanced HexA levels in 2° cells prevent the formation of cell clumping via repression of the $P_{pcfA}$ 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. The AstS/AstR two-component system controls timing of phenotypic switching via a probable stress or oxygen-derived signal (see text for details).

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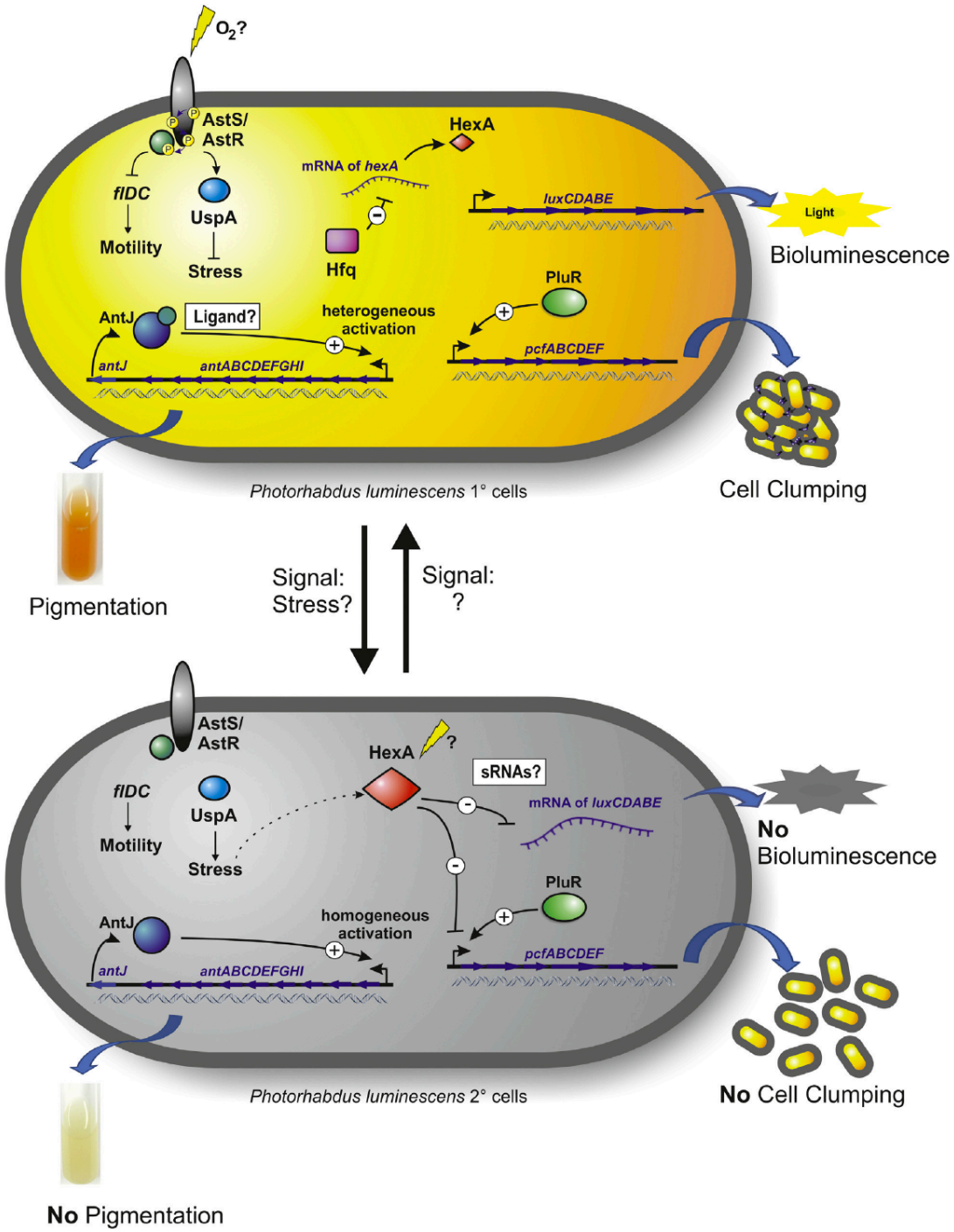


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HexA to the DNA revealed that a ligand is missing, which modulates DNA binding activity of HexA, because HexA alone was observed to have a high dissociation rate from the $P_{pcfA}$ promoter [13]. This is underlined by the fact that HexA has a C-terminal putative ligand binding site, which is believed to somehow affect HexA activity. Primary metabolites that indicate nutrient limitation have been speculated as HexA ligands, but overall, the chemical nature of this ligand is not yet known. In summary, HexA fulfills the task as repressor of 1° -specific features in 2° cells in a versatile way, directly and indirectly [13].

## Regulation of timing of phenotypic switching

It has been shown that *lrhA* expression, a homolog of *hexA* in *E. coli*, is under control of the Rcs phosphorelay system. Interestingly, AstS/AstR, a homologous system to Rcs, has been identified to control timing of phenotypic switching in *P. luminescens* [29]. *P. luminescens* cells lacking the response regulator AstR start to undergo phenotypic switching in culture 7 days earlier than the respective wild-type strain. However, in contrast to the situation in *E. coli*, expression of *hexA* is not under control of AstS/AstR. Proteome analysis of the Δ*astR* strain revealed that the AstS/AstR system positively regulates the expression of the gene encoding the universal stress protein UspA [29]. Such proteins occur in high concentrations during periods of stress, like oxidative and osmotic stress, as well as under stasis [30,31]. Therefore, the AstS/AstR pathway is believed to protect the cell from stress and thus prevent or delay phenotypic switching [20]. This supports the suggestion that global stress has a major impact on the signal inducing the switching process. Furthermore, the Δ*astR* mutant is hypermotile as the functional AstS/AstR system represses flagella formation [29]. However, motility is not under the control of HexA [19], revealing a different regulation mechanism for HexA than LrhA, which directly regulates motility in *E. coli* [32]. As this is only true under anaerobic conditions [33], it was suggested that there are at least two pathways controlling phenotypic switching, a HexA-dependent pathway and an $O_2$-dependent pathway via AstS/AstR. Although both pathways are somehow activated under global stress, no direct connection between the HexA and AstS/AstR regulation pathways is known to date [20] (Fig. 3).

## Regulation of pigmentation

Pigmentation caused by anthraquinones (AQ) is a distinct 1°-specific feature and absent from 2° cells. A novel type of transcriptional regulator named AntJ was found that activates expression of the *antABCDEFGHI* operon [34], which encodes the enzymes for AQ synthesis [34,35]. The development of a novel genetic tool for stable reporter gene integration into the *P. luminescens* genome made is possible to analyze reporter gene activity at the single cell level [36]. Using $P_{antA}$ reporter strains generated with this tool, bimodality of AQ production could be identified [34]. AntJ heterogeneously activates the AQ production in single *P. luminescens* 1° cells and blocks AQ production in 2° cells. AntJ is one of the rare examples of regulators that mediates heterogeneous gene expression by altering activity rather than copy number in single cells. AntJ contains a so called WYL-domain, which has yet only been found in bacteria [37]. The WYL-domain is predicted to be a putative ligand-binding domain, although any ligand(s) are unknown to date. The AntJ levels in 1° as well as in 2° cells are constant, which showed that transcriptional activation of the *antA-I* operon in single 1° cells is not mediated by a simple increase of AntJ. Therefore, a specific activation of AntJ was proposed to be mandatory for binding of AntJ to the $P_{antA}$ promoter and to promote heterogeneous AQ production. For that reason, AQ production in single 1° cells was suggested to be mediated by ligand-dependent activation of AntJ by a specific metabolite or protein, which is not present in 2° cells. A simple overexpression of *antJ* leads to a homogeneous activation of AQ production in 2° cells, which is though ligand-independently and only caused by the enhanced AntJ copy number in the cells. However, one additional chromosomal copy of *antJ* under control of its native promoter did not lead to AQ production in 2° cells but was sufficient to decrease heterogeneity of $P_{antA}$ activity in 1° cells. Only upon strong overexpression of *antJ*, AQ production was detectable in 2° cells, assuming that the influence of a putative inhibiting ligand might be out-competed by high AntJ copy numbers [34]. The presence or absence of a putative ligand must therefore drive activation of AntJ in single cells and mediate heterogeneity of $P_{antA}$ activation as a noise generator. Since no differences in heterogeneity of $P_{antA}$ activity in 1°Δ*hexA* cells were observed, heterogeneity of AQ production is presumably independent of the master regulator HexA [34] (Fig. 3).

## Quorum sensing and regulation of cell clumping

Cell-clumping caused by production of the *Photorhabdus* clumping factor (PCF) is another feature that is only detectable in 1° and not in 2° cells [13]. PCF is produced by enzymes that are encoded in the *pcfABCDEF* operon and is under control of bacterial quorum sensing (QS) [38]. QS via LuxI/LuxR-type systems with acyl-homoserine lactones as signals is well studied in many Gram-negative bacteria [39]. However, *P. luminescens* communicates via α-pyrones named photopyrones (PPYs) instead of acyl-homoserine lactones. The PPYs are produced by the pyrone synthase PpyS and sensed by the LuxR-type receptor PluR. At high cell density the PpyS/PluR system positively regulates the expression of the

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*pcfABCDEF* operon in 1° cells, which then leads to the production of PCF [38]. In *P. asymbiotica*, the *pcf* operon is under the control of the DarABC/PauR QS system that uses dialkylresorcinols instead of PPYs for communication [40]. As described above, it was shown that the expression of the *pcfABCDEF* operon was directly blocked upon HexA binding to the cognate $P_{pcfA}$ promoter in *P. luminescens*, so that HexA acts as a direct repressor for the *pcf* operon. The binding site of HexA is located upstream of the PluR binding site in the *pcfA* promoter region [13]. Furthermore, PPYs production is also reduced in 2° cells, so that cell–cell communication via the PpyS/PluR system is assumed to be predominantly present in 1° and not in 2° cells. Since *P. luminescens* harbors 40 LuxR solo receptors that have been supposed to be involved in cell–cell communication as well as inter-kingdom signaling [41], it is possible that 2° cells use another chemical language than 1° cells for QS.

## Biological Function of Phenotypic Switching

Phenotypic switching of *P. luminescens* might be a classical bet-hedging strategy that ensures survival of the community in any case. However, little is known about the role and the fate of the 2° cells. Since 2° cells are not able to re-associate with the nematodes, it has been suggested that they are better adapted to a life independent from their symbiosis partners. Therefore, phenotypic variation might be an adaptation for survival of the bacterial population that remains in soil after the nutrients of the insect are depleted and the nematodes together with the 1° cells have emerged from the cadaver [22]. However, 2° cells of *P. luminescens* have never been isolated from soil. For that reason, it is conceivable that 2° cells change into a kind of persister state to outlast periods of nutrient depletion until they somehow can re-enter the life cycle, but this has never been shown yet. However, it can be observed that nearly the complete *P. luminescens* population (1° and 2° cells) undergoes a switch into a non-culturable state upon pro-longed cultivation, meaning that the cells are viable but non-culturable (VBNC) any more (S.E. and R.H., unpublished). Those VBNC cells have also been described for other bacteria like *Salmonella* Typhimurium, *Vibrio cholerae*, *E. coli* or *Pseudomonas aeruginosa* [42–45], whereas only a small portion of the cells form VBNCs, which is in clear contrast to *P. luminescens*. The formation of VBNCs is often mediated by toxin/antitoxin systems in bacteria [46]. Since in *P. luminescens* more than 90 copies of those systems have been identified [47], it is likely that VBNC formation plays a major role to outlive long periods of starvation for *P. luminescens*. However, a correlation between VBNC formation and the high number of toxin/antitoxin systems in *P. luminescens* has not been found yet.

RNA-Seq analysis of 1° and 2° cells revealed a significantly decreased transcription of genes encoding 1°-specific features in 2° cells. Others that are homologous to factors involved in bacterial plant-interaction were induced in 2° cells (S.E. and R.H., unpublished). Furthermore, the up-regulation of several stress-related genes/proteins was identified in 2° cells [24]. This reveals a complete alternative life style for 2° cells in the soil, where the bacteria have to cope with starvation, temperature and osmotic stress, and to compete with other bacteria (Fig. 4). After remaining in the soil, the cells have to adapt and sustain in the rhizosphere, suggesting also an interaction with, for example, plant roots. Increased chemotactic motility of 2° cells toward plant root exudates would be the next step to further support a *Photorhabdus*–plant interaction in the future. Furthermore, VBNC formation has also been found to be important for the resistance of several phytopathogenic bacteria [48], so that a VBNC lifestyle of 2° cells on plants also seems possible.

It still remains unclear if *P. luminescens* 2° cells can reverse the switch and become 1° again. From the current state of knowledge, the phenotypic switching of *P. luminescens* is unidirectional, occurring only from 1° to 2° cells. However, a switch back from 2 ° to 1° cells has been observed for the closely related genus *Xenorhabdus nematophila* [49], implying that there must be a need or at least the possibility to reverse the switching process also for *P. luminescens* in nature. Therefore, it is likely that *P. luminescens* can also undergo a back-switch from 2° to 1° under specific environmental conditions or in presence of a specific, yet unknown, signal. However, such a signal might be found in the rhizosphere. It is possible that the signal(s) is derived from plants, nematodes or insects, which could force a decision of 2° cells to become 1° again, so that the cells can somehow re-enter the entomopathogenic life cycle.

## Conclusions and Future Work

The current knowledge of molecular mechanisms regulating phenotypic switching in *P. luminescens* cell populations has identified not only master regulators like HexA that are involved in the switching process but also those like AntJ that control further downstream regulation processes of phenotypic heterogeneity. Furthermore, regulation at the post-transcriptional level via small RNAs and the molecular chaperone Hfq seem to play a global role in the switching process. However, we are far away to fully understand this complex regulation network. It will be important in the near future to identify specific signals and ligands that control the activity of the specific regulators. This will help to understand how fine stochastic differences, concentrations or ligand affinities to specific regulators could make up the decision to induce the switching cascade in one cell, and to

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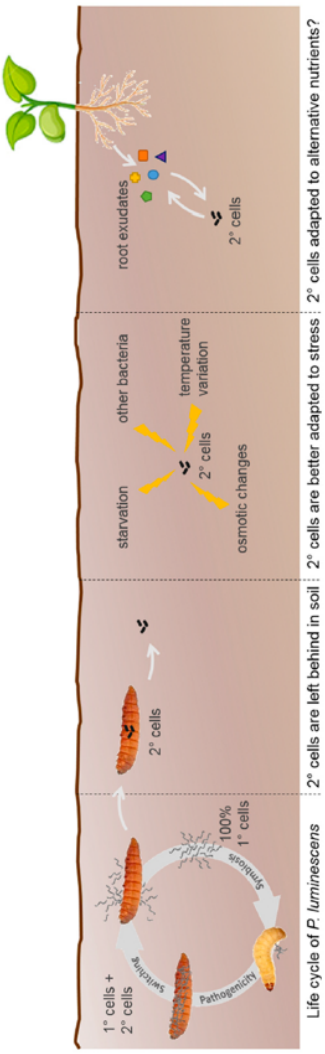


**Fig. 4.** Model for phenotypic switching of *P. luminescens* in nature and putative fate of the 2° cells. During insect infection, a specific subset of the 1° cells undergo phenotypic switching and convert to 2° cells. Since 2° cells are not able to support symbiosis and cannot re-associate with the nematodes, they remain in the soil when the infection cycle is finished. In the new environment, they have to adapt to several stress conditions like osmotic stress, variation in temperature and starvation and to compete with other bacteria. To survive in the rhizosphere, it is possible that the 2° cells are adapted to plant derived nutrients.

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block it in another cell in a homogenous environment. Furthermore, it will be important to understand the fate of 2° cells and therefore the biological reason for phenotypic heterogeneity in *P. luminescens*. A major focus will be laid on the molecular mechanism of VBNC formation, the interaction of *P. luminescens* with plants as well as the signal(s) and the back-switch of 2° to 1° cells. Since nematodes colonized by *P. luminescens* are commercially used as bio-insecticides in agriculture, the control of phenotypic switching and the signal(s) for the switching process are also major issues for the biotechnological use of *P. luminescens* as bio-insecticide.

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***Keywords:***
entomopathogenic bacteria;
phenotypic heterogeneity;
HexA;
AntJ;
PluR

***Abbreviations used:***
IJs, infective juveniles; AQ, anthraquinones; PCF, *Photorhabdus* clumping factor; QS, quorum sensing; PPYs, photopyrones; VBNC, viable but non-culturable.

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# 6 Concluding Discussion

Phenotypic heterogeneity is widespread within bacteria. *As Photorhabdus luminescens* exists in two distinct phenotypic forms, the primary (1°) and secondary (2°) cells it is an ideal organism to study the molecular mechanisms of this phenomenon. So far, only little is known about the regulation mechanism of phenotypic switching as well as of the advantages for the cell population to exist in two different forms. In this work, the regulation of phenotypic switching in *P. luminescens* and the general function of 2° cells was investigated. The laboratory strain *P. luminescens* subsp. *laumondii* TT01$^{Rif}$ is commonly used for scientific research. So far, it only has been described as spontaneous rifampicin resistant mutant of TT01. However, its genome has never been sequenced before. As a first step of this work, TT01 as well as TT01$^{Rif}$ were evaluated by performing comparative whole genome sequencing and analysis of phenotypic traits. Due to big differences in sequence and phenotypic characteristics the TT01$^{Rif}$ strain could be raised to the status of an independent isolate and has been renamed into *P. luminescens* subsp. *laumondii* DJC (Chapter 2). In the course of this work comparative transcriptome analysis of DJC 1° and 2° cells could proof the mediation of phenotypic differences at transcriptional level (Chapter 3). Furthermore, the obtained data suggest a better adaption of 2° cells to different stress conditions and alternative nutrients. Thus, they might be better adapted to a free living in soil (Chapter 3, Chapter 5). Via RNA-Seq analysis also two transcriptional regulators, XreR2 and XreR1, were identified to play a major role in phenotypic heterogeneity of *P. luminescens*. Altering the gene levels of *xreR2* or *xreR1* could induce a 100% switch to the respective other cell form (Chapter 4).

## 6.1 The complex regulation of phenotypic heterogeneity in *P. luminescens*

One of the most important characteristics that is absent in 2° cells of *P. luminescens* is the ability to support nematode growth and development (Boemare & Akhurst, 1988, Fig. 3-1A). Thereby, 2° cells cannot re-associate with the nematodes after the nutrients of the insect larvae are depleted. As a 100 % switching frequency would lead to a break-down of the bacteria's life cycle the switching process has to be tightly regulated. The LysR-type transcriptional regulator HexA was found to act as repressor of 1°-specific features (Joyce & Clarke 2003). Recently, it could be shown that *hexA* directly represses cell clumping and presumably indirectly inhibits bioluminescence at post-transcriptional level putatively involving small RNAs (Langer et *al*., 2017). By comparing the genomes of *P. luminescens* TT01 and DJC, among others, six new mobile genetic elements comprising inverted repeats (MITEs) were identified. Those repeats are related to transposases and can thereby be mobilized *in trans* by the cognate transposase (Filée et *al*., 2007). As they are considered to be non-coding these MITEs and their respective RNA might also play a role in the phenotypic switching of *P. luminescens*. However, this could not be proven so far.

Thus, the complete regulation mechanism of phenotypic heterogeneity is still puzzling. In the course of this work, two genes encoding two transcriptional regulators were found to be involved in the phenotypic switching process of *P. luminescens.* Both proteins belong to the superfamily of XRE-like transcriptional regulators which is the second most frequently occurring regulator family in bacteria (Barragán et *al.* 2005) and are therefore termed XreR1 (XRE-like regulator higher expressed in 1° cells) and XreR2 (XRE-like regulator higher expressed in 2° cells).

### 6.1.1 Regulation of phenotypic switching by XreR2 and XreR1

Comparative transcriptome analysis of 1° and 2° cells revealed up-regulation of *xreR2* in 2° cells while *xreR1* was higher expressed in 1° cells (Fig. 4-1). Domain prediction revealed that both proteins exclusively contain a λ phage repressor-like (Cro/C1) HTH DNA-binding domain and thus no SBD (Fig. 4-3). However, as phage repressor-like proteins harbor one of the simplest HTH DNA-binding architecture, in almost every protein of this family a standalone HTH is found (Aravind et *al*. 2005).

Proteins harboring such phage like repressor domain usually directly repress certain traits. However, some exceptions have already been described in literature. In *Corynebacterium glutamicum* e.g. the XRE-transcriptional regulator ClgR, activates an operon encoding Clp proteases which then in turn recognize and degrade defective proteins (Gottesman et *al*., 1998). Furthermore, very recently SrtR of *Streptococcus suis* was described to increase oxidative stress and high temperature tolerance (Hu et *al*., 2019).

Deletion of *xreR1* in 1° cells induced the switch to the 2° phenotype. Subsequently, overexpression of the same gene in 2° cells restored the 1° phenotype. Both with respect to the most predominant phenotypic characteristics like bioluminescence, pigmentation, antibiotic production and colony morphology (Fig. 4-2B and 2C). Therefore, an activation of such 1°-specific features via XreR1 seems likely (Fig. 6-1).

2° cells occur after prolonged cultivation whereby a signal derived by metabolic or environmental stress is thought to play a role (Joyce et *al*., 2006). Upon recognition of the yet unknown signal the respective receptor could then directly activate or repress the expression of *xreR2* and/or *xreR1*. Thus, conversion to the 2° phenotype is initiated. Here, high levels of *xreR2* are present while *xreR1* expression is low (Fig. 6-1). Since the 2° Δ*xreR2* strain exhibits 1°-specific features and 1° cells containing extra copies of *xreR2* become secondaries (Fig. 4-2B and 2C), XreR2 seems to repress 1°-specific

traits. Taken into account that *xreR2* is about 500-fold higher expressed in 2° cells than in 1° cells and its function to maintain the 2° phenotype, positive auto-regulation seems most likely (Fig. 6-1).

In *Bacillus subtilis* two XRE-transcriptional regulators constitute a double negative feedback loop directly controlling genes for e.g. motility and thereby creating an epigenetic switch. Here, SinR, a repressor of $P_{eps}$ and therefore biofilm formation during exponential growth inhibits expression of another XRE-transcriptional regulator SlrR. SlrR in turn binds to SinR thereby indirectly de-represses its own gene *slrR* as well as other SinR targets. Once that loop gets activated the cells are time dependently locked in a high SlrR state (Chai et *al*., 2010). XreR1 seems to inhibit *xreR2* expression by directly binding to $P_{xreR2}$ (Fig. 4-4E and Fig. 4-5) thereby presumably maintaining the 1° phenotype. Furthermore, both proteins interact with each other (Fig. 4-7). Whether this interaction has inhibitory or activating effects has to be elucidated, however these findings lead to the assumption that that XreR1 and XreR2 constitute a comparable epigenetic switch as SinR and SlrR (Fig. 6-1). Though, no binding of XreR2 or XreR1 to the $P_{luxC}$ or $P_{antA}$ promoter could be detected revealing no direct repression of bioluminescence and AQ-production, respectively. In *B. subtilis* the genes like such for autolysin or motility are regulated by a SinR/SlrR complex but neither by SinR nor by SlrR alone (Chai et *al*., 2010). Therefore, a mixture of XreR1 and XreR2 might be needed to observe binding activity. Furthermore, in *B. subtilis* binding of SinR to $P_{eps}$ is reversed during stationary phase as its antagonists SinI and SlrA get activated and thereby biofilm formation is promoted (Gaur et *al.*, 1991; Bai et *al*., 1993). Assuming a similar mode of action of XreR1 and XreR1 gives hints that the switch is also reversible in *P. luminescens*. However, the respective signal to trigger that conversion is still unknown.

Certainly, there are other putative molecular mechanisms for phenotypic switch-regulation via XreR1 and XreR2. As no direct targets with respect to 1°-specific

characteristics could be identified so far, it could be possible that they either activate other regulatory proteins or that they regulate expression at post-transcriptional level.

Post-transcriptional regulation in prokaryotes is commonly achieved via small RNAs (sRNAs) or RNA-binding proteins (RBPs). Usually the activity both types is regulated by again sRNAs. However, there are some RBPs which are activated by other regulatory proteins. The TRAP protein of *B. subtilis* e.g. is regulated by an anti-TRAP protein which binds near to the RNA-binding domain of the TRAP and thereby prevents its binding to mRNA targets (Snyder et *al*., 2004). Another example is the FliW protein from *B. subtilis* which is regulated the CsrA RBP by binding near to is active site (Assche et *al*., 2015).

As XreR1 and XreR2 are of small size (8,800 or 7,760 kDa, respectively) and only harbor an HTH-DNA binding domain, they could act similar to histone-like proteins whereby they would bind to specific DNA regions to enable or inhibit gene expression. As the name implies, histone-like proteins play the key role in re-arranging the chromosome involving the maintenance of the topological homeostasis of the cell as well as the organization of certain supercoiled loops (Dame et *al*., 2002). However, it has been shown that some of these proteins also fulfill regulatory functions. HU of *E. coli* e.g. binds to GalR, enabling a looped tetramer formation at the target *galP2* promoter and thereby inhibiting downstream expression (Aki & Adhya, 1997). Bacterial histone-like proteins can be divided into four major groups: HU (histone-like proteins *E. coli*, U93), H-NS (histone-like nucleoid structuring proteins), IHF (integration host factors) and FIS (factors for inversion stimulation) (Anuchin et *al*., 2010). However, neither XreR1 nor XreR2 show homology to any of the four groups. But as there are also histone-like proteins that cannot be put in any of those groups (Anuchin et *al*., 2010) a comparable function of XreR1 and XreR2 is still possible.

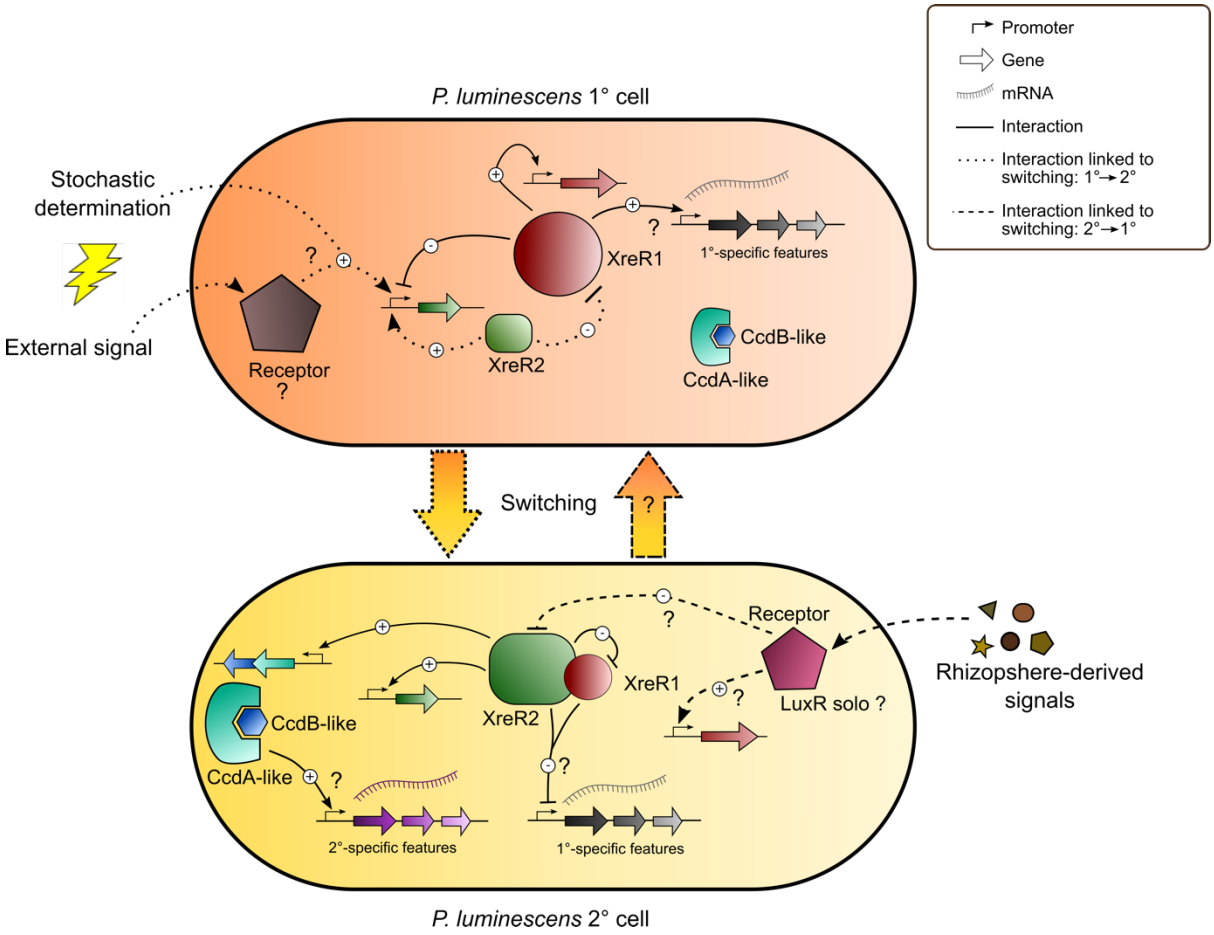


**Figure 6-1: Model of regulation of phenotypic switching in *P. luminescens* via XreR1 and XreR2.** In *P. luminescens* 1° cells high amounts of XreR1 inhibit expression of *xreR2* and therefore, XreR2 levels are low. Furthermore, 1°-specific features might get activated either directly or indirectly by XreR1. Upon recognition of a yet unknown signal or stochastic determination *xreR2* gets up-regulated. Binding of XreR2 to XreR1 putatively reinforces *xreR2* expression and thereby induces the 2° phenotype. Now, 1°-specific traits are presumably repressed by the XreR1-XreR2 complex. Furthermore, expression of the *ccdAB*-like is initiated by XreR2 thereby putatively maintaining the 2° phenotype. During free life in soil, rhizosphere-derived signals e.g. plant root exudates could be recognized by a specific LuxR-solo which in turn could reverse the switch back to the 1° phenotype.

Anyway, it still remains unclear how the expression of *xreR1* and *xreR2* is regulated. The LysR-type transcriptional regulator HexA has already been identified to act as a repressor of 1°-specific traits (Clarke & Joyce, 2003). Here, deletion of the *hexA* gene in 2° cells restored the 1° phenotype while extra copies of *hexA* in 1° cells were sufficient to induce the 2° phenotype. This is similar to the effects of XreR2 and contrary to those of XreR1. Therefore, a similar mode of action might take place. However, unlike HexA,

XreR2 seems not to be repressed by the RNA-chaperone Hfq since mRNA levels of *xreR2* were not altered in the Δ*hfq* strain (Nick Tobias, Helge Bode, personal communication) while *hexA* expression is about 60-fold increased (Tobias et *al*., 2017). Furthermore, the up-regulation of *hexA* in the Δ*hfq* strain did neither lead to an activation of *xreR2* nor to a repression of *xreR1* expression. Additionally, expression levels of both *xreR2* and *xreR1* were not altered in the Δ*hexA* strain (Nick Tobias, Helge Bode, personal communication). However, an interaction cannot be completely ruled out because although *hexA* is higher expressed, it could be inactive by the lack of a signal since the cognate signal for its SBD has not been identified yet.

Assuming an epigenetic switch, it could also be possible that up-regulation of *xreR2* or down-regulation of *xreR1*, respectively, is determined stochastically upon environmental signals. A model describing the *xreR1*/*xreR2*-dependent regulation of phenotypic switching is shown in Fig. 6-1.

### 6.1.2 The role of the CcdAB-like system in phenotypic phase variation

RNA-Seq analysis of 1° and 2° cells revealed an up-regulation of *ccdA*-like as well as *ccdB*-like in 2° cells. As described above XreR2 bound to the *ccdAB*-like promoter (Fig. 4-4C). These findings and the chromosomally close proximity to both *xreR2* and *xreR1* suggest a putative role in the process of phenotypic switching. Some TASs are described to be involved in persister cell formation which is one of the best-studied forms of bacterial phenotypic heterogeneity using the bet-hedging strategy. Hereby, usually upon antibiotic treatment, single cells change into a transient growth arrested state which allows them to survive the stress situation (Veening et *al*., 2008).

The most prominent example of a TAS controlling persistence is the HipAB system of *E. coli*. Here, once a threshold concentration of the toxin HipA is reached cells turn into

persister cells. HipA inactivates GltX (a Glu-tRNA synthetase) which activates RelA mediated (p)pp(G)pp synthesis. Increased (p)pp(G)pp levels in turn indirectly result in multidrug tolerance (Germain et *al*., 2013, Schumacher et *al*., 2009).

However, the *ccdAB*-like operon is highly similar to the *ccdAB* encoded TAS of *E. coli*. This F-plasmid based system is one of the first identified, and therefore well-established, TAS in *E. coli*. CcdAB is involved in plasmid maintenance via post-segregational killing (Maki et *al.*, 1996). Recently, chromosomal homologs of *ccdAB* were found in different *E. coli* strains including pathogenic ones. Here, it could be shown that these homologs as well as the *ccd* operon from the F plasmid, when chromosomally inserted, lead to persister cell formation while the plasmid encoded CcdB caused cell death to due inhibition of the DNA gyrase (Gupta et *al*., 2017). *P. luminescens* harbors no plasmids. Upon overexpression of the putative toxin *ccdB*-like in *P. luminescens* as well as in *E. coli* no decrease in fitness could be observed. Furthermore, deletion of the putative antitoxin *ccdA*-like in *P. luminescens* 1° as well as 2° cells did not lead to an obvious phenotype (Fig. 4-7). As TAS in general are described to be functional redundant (Dörr et *al*., 2010) and *P. luminescens* encodes for about 90 TAS it could be possible that another system compensated the phenotype. Indeed, another *ccdAB* homologous operon is found in the genome of *P. luminescens*. However, there are some hints that the CcdAB-like system evolved from a TAS but then changed in functionality. In *E. coli* the essential region of CcdB to fulfill the toxic activity lies C-terminally. The protein consists of 102 amino acids and only changes within the three amino acids (Trp99, Gly100 and Ile101) led to a non-cytotoxic protein (Bahassi et *al.*, 1995). CcdB-like of *P. luminescens* only consists of 74 amino acids homologous to the N-terminal part of CcdB. From these data the current theory arose that the CcdAB-like system in *P. luminescens* originates from a TAS, which is another phenotypic heterogeneity inducing regulatory system, but then developed another mode of action to contribute to phenotypic switching from 1° to 2° cells in *P. luminescens*. One possibility would be that the system gets

activated by high levels of XreR2 and then either directly or indirectly activates 2°-specific features (Fig. 6-1) to maintain the 2° phenotype.

## 6.2 The fate of *P. luminescens* 2° cells

In contrast to 1° cells, 2° cells lack most of the *P. luminescens* characteristic features such as bioluminescence, pigmentation or antibiotic production. Most important for studying the life cycle of *P. luminescens* is the fact, that 2° cells lost the ability to support the nematode's growth and development and are therefore not able to live in symbiosis anymore (Boemare & Akhurst, 1988). As a consequence, 2° are not able to re-associate with the nematodes and are therefore left behind in the nutrient-depleted carcass. The function of 2° cells and their fate in soil would be essential to understand the sociobiological aspects of the two phenotypic different cell forms of *P. luminescens*. RNA-Seq analysis performed in this work could not only confirm that all phenotypic differences of the 1° and 2° cell form are truly mediated at transcriptional level (Tab. 3-1) but also identified in total 638 genes that were differentially expressed (DEGs) in 2° compared to 1° cells.

### 6.2.1 Adaption of *P. luminescens* 2° cells to an alternative environment

A high portion of the DEGs including e.g. genes for metabolic functions as well as motility and stress related genes were higher expressed in 2° than in 1° cells (Fig. 3-2) indicating that 2° cells exhibit some yet unknown functions and might be better adapted to an alternative environment.

It has been shown already, that after experiencing a period of starvation 2° cells are able to restart growth after 3-4 hours and thus recover about 10 hours faster in comparison to 1° cells (Smigielski et *al.*, 1994). Consequently, earlier proteome analysis revealed an

up-regulation of proteins that could provide advantages under nutrient limiting and stressful conditions such as e.g. iron-scavenging proteins, chaperones or the transcriptional regulator Lrp (Turlin et *al*., 2006). Gene expression analysis revealed a higher expression of starvation related genes in 2° compared to 1° cells (Supplementary Table 3-S1).

The ability of 2° cells to deal with periods of starvation could be essential when they are left behind in soil and the nutrients of the carcass are depleted. All genes of the AST-pathway *(astABDE* and *PluDJC_15875*) are up-regulated in 2° cells. This pathway is induced in the presence of aspartate and arginine and under nitrogen limitation (Easom & Clarke, 2012; Schneider et *al*., 1998) and could help 2° cells to overcome starvation as in the rhizosphere high amounts of amino acids are present e.g. secreted by plant roots (Badri & Vivanco, 2009).

However, at some point they have to find a new source to feed on. Certainly, the nutrient composition present in the soil or rhizosphere differs from the one inside the insect larva. Indeed, we found DEGs indicating adaption of 2° cells to alternative nutrients. E.g. the HPA metabolism seems to be increased in 2° cells as all respective genes were up-regulated. Several bacteria as like *E. coli* are able to degrade 4-HPA to eventually metabolize it to pyruvate and succinate. As it is a common fermentation product of aromatic amino acids 4-HPA is also often found in soil as a result of plant material degradation by animals (Díaz et *al*., 2001). Therefore, enhanced capability to degrade 4-HPA could help 2° cells to grow in soil as it can be used as carbon source.

On the other hand, the respective gene cluster to degrade phenylpropanoids (*hcaCFE*, *hcaB*, *hcaD*) (Díaz et *al*., 1998) is down-regulated in 2° cells. Phenylpropanoid compounds most commonly derive from proteins (Díaz et *al*., 1998) which are in turn the main nutrient source inside the larvae. This indicates less affinity of 2° cells to this food source and thereby supports the theory of an alternative life style.

In soil new food resources might not occur right next to the insect carcass. Therefore, the capability of being motile would be of great benefit, especially for 2° cells. In the closely related strains *P. temperata* and *Xenorhabdus nematophila* motility was found to be a 1°-specific feature (Hodgson et *al*., 2003). However, *P. luminescens* DJC 2°cells appeared to have an increased expression of the complete flagella formation apparatus (Tab. 3-2) resulting in an increased swimming motility on soft-agar plates compared to 1° cells (Fig. 3-4). Thus, contrary to the situation in its close relatives, motility in *P. luminescens* DJC seems to be a 2°-specific feature. Furthermore, only 2° but not 1° cells chemotactically responded to serine as well as maltose (Fig. 3-5) presumably caused by the up-regulation of two genes encoding chemoreceptors (*PluDJC_09715 and PluDJC_09720*).

PluDJC_09715 *and* PluDJC_09720 are homologous to the methyl-accepting proteins (MCPs) Tsr and Tar of *E. coli*, which sense serine and Ala/Gly or maltose and Asp/Gly, respectively (Springer et *al*., 1977). This indicates that 2° cells are not only able to utilize these nutrients but can also actively move towards them.

In contrast to 1° cells, 2° cells exhibited increased swimming behavior upon addition of plant root exudates. As the majority of compounds in the rhizosphere such as amino acids or sugars as organic acids peptides, proteins or lipids directly derive from plant roots (Bais et *al.*, 2006; Walker, 2003; Lesuffleur et *al*., 2007) re-orientation of 2° cells towards this nutrient source makes totally sense. The wide variety of organic acids secreted by plants is known to serve bacteria as a nutrient source and initiate chemotactic movement (Haichar et *al.*, 2014). Thus, some additional MCPs involved in the response of 2° cells to other plant derived nutrients might be present in *P. luminescens* DJC.

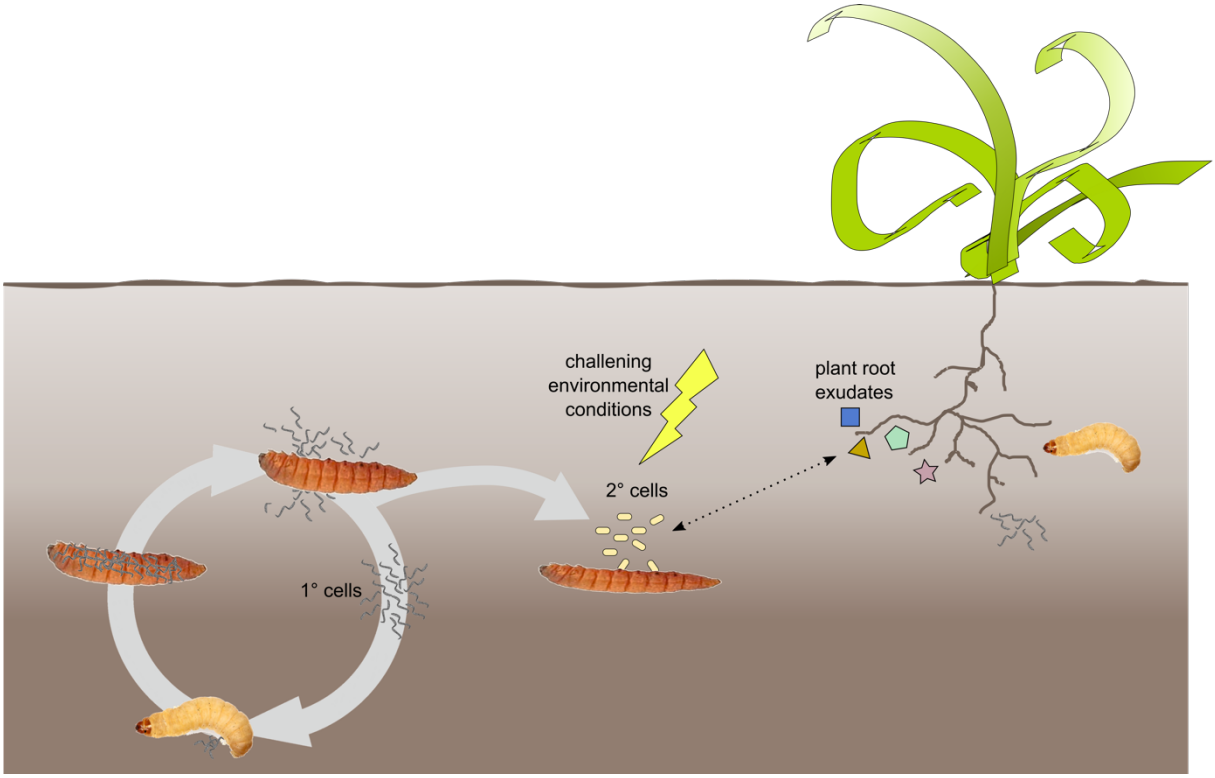


**Figure 6-2 Model for extended life cycle of *P. luminescens* and putative fate of the 2° cells in nature.** Since 2° cells are not able to support symbiosis and therefore cannot re-associate with the nematodes, they remain in the soil when the infection cycle is finished, and all nutrients of the larvae are depleted. In the new environment, they have to adapt to several stress conditions like variation in temperature and starvation and to compete with other bacteria. To survive in the rhizosphere, it is possible that the 2° cells are adapted to plant root exudates as alternative nutrient source.

Besides motility flagella are also described play a role in adhesion, invasion, or in the first steps of biofilm formation (Givaudan & Lanois, 2000; Josenhans & Suerbaum, 2002). For bacteria of the genus *Pseudomonas* involvement of *fliC* in plant root colonization has been shown already (Berg & Smalla, 2009; Lugtenberg et *al*., 2001). Furthermore, ectopic expression of *flhDC* led to hypermotility of *P. fluorescens* which directly correlated with the ability to colonize roots (Barahona et *al*., 2016; Redono-Nieto et *al*., 2013). As *fliC* as well as *flhDC* was also found to be up-regulated in 2° cells they might also be able to colonize plant roots. In the plant pathogen *Pseudomonas fluorescens* three MCPs CtaA, B and C, sensing amino acids, have been described to be involved in plant root colonization. However, additional chemotattractants seem to be involved since the *P. fluorescens* Δ*ctaABC* strain was still more competitive for root colonization than

non-motile *P. fluorescens* cells (Oku et *al*., 2012). Blast analysis revealed that PluDJC_09720 of *P. luminescens* is highly similar to an MCP of *Pseudomonas simiae*, which is described to be involved in plant root colonization (Cole et *al*., 2017). However, if PluDJC_09720 is involved in sensing plant-specific metabolites has not been proven yet.

Beside the importance of finding new nutrient sources in soil 2° cells would also have to deal with challenging environmental conditions different to those present inside the host.

Certainly, the microbiome in the rhizosphere differs from that present in the insect. Gram-negative bacteria harbor a unique class of glycoconjugates, the so-called Liposaccharides (LPS) present on top of the outer membrane. LPS consist of three different components the lipid A, the core oligosaccharide region and O-polysaccharide (O-antigen). The bacterial specificity is achieved by changes in the O-antigen region (Lerouge & Vanderleyden, 2002). LPS not only differs between bacterial species but also depends on environmental conditions. It has been shown previously that host-associated bacteria change their lipopolysaccharide (LPS) composition depending on the host niche. The gastritic disease causing bacterium *Helicobacter pylori* e.g. changes O-antigens synthesis depending on the pH (McGowan et *al*., 1998) and is thereby able to colonize different parts of the stomach. Furthermore, in some bacteria the O-antigen profile changes depending if they are inside or outside their host like e.g. the legume symbiont *Rhizobium etli* CE3 antigenically alters its LPS during growth outside of the plant (Duelli et *al*., 2001).

In 2° cells six *wbl* genes that play a role in the O-antigen biosynthesis of LPS in the cells (Derzelle et *al*., 2004) were either up- or down-regulated. This strongly indicates a specificity for environmental conditions other than those to which 1° cells are adapted.

Furthermore, the ability of 2° cells to deal with low (4°C) as well as high (37°C) temperatures is also of great benefit as the climate changes have a greater effect on the cells when living outside of the host.

Taken together, 2° cells seem to be well prepared for a life outside the larvae including challenging conditions such as nutrient limitation and temperature switches (Fig. 6-2). This sociobiology of two different cell forms could be a classical way of bed-hedging to ensure the bacteria's survival inside the host as well as when no host is available.

### 6.2.2 Rhizosphere-derived signals as trigger to become 1° again?

From the current state of knowledge phenotypic switching of *P. luminescens* only occurs unidirectional from the 1° to the 2° phenotype. However, as for the closely related genus *X. nematophila* a switch back from 2 ° to 1° cells was observed (Owuama, 2018) it seems likely that there is also a signal for *Photorhabdus* to reverse the switching process. This particular signal might be missing when working under laboratory conditions. 2° cells seem to be well prepared for an alternative environment such as e.g. soil. Therefore, the stimulus for reversing the switching might be found in the rhizosphere (Fig. 6-1) and sensed by a receptor specifically expressed by 2° cells. Transcriptome analysis revealed 14 LuxR solos being up-regulated in 2° cells. This type of transcriptional regulators consists of a LuxR-type receptor which lacks the cognate LuxI synthase (Subramoni & Venturi, 2009). *P. luminescens* harbors 40 LuxR solos which had been classified into four subgroups corresponding to their C-terminal signal binding domain. Comprising 34 clusters the group of LuxR solos with a PAS4 domain presents the biggest one (Brameyer et *al*., 2014). However, until today no signal specifically sensed by a PAS4-LuxR-solo of *P. luminescens* has been identified yet. 12 of the LuxR solos that are higher expressed in 2° cells harbor a PAS4 domain while the SBDs of the other two are still undefined (Brameyer et *al*., 2014).

It has been shown that LuxR-solos of plant-associated bacteria can respond to plant signaling molecules (Covaceuszach et *al*., 2013; Venturi & Fuqua, 2013), which might also be true for one or more LuxR solos up-regulated in 2° cells. Putatively, plant-derived signals might be the missing piece and trigger 2° cells to become 1° cells again. As 2° cells chemotactically respond to nutrients most possibly provided by plant root exudates (Fig. 3-5), they would be in proximity to the roots and thereby also be able to sense e.g. plant hormones. This could then cause either activation of other regulatory genes or direct repression of *xreR2* or promotion of *xreR1* expression could be initiated, respectively (Fig. 6-1). As heterorhabditid nematodes infect insect larvae, whose natural habitat is the rhizosphere as they devour plant roots, the newly re-switched 1° cells would be in the right spot to associate with the nematodes again and thereby re-enter the life cycle.

Another possibility is that the missing signal derives from insects instead of plants. In the fruit fly *Drosophila melangonaster* a PAS4-homologous domain has been shown to bind insect juvenile hormones (JHs) (Dubrovsky, 2005). Thus, PAS4 domains of *P. luminescens* are thought to also act as hormone or hormone-like receptors and therefore play an important role in inter-kingdom signaling (Heermann & Fuchs 2008). Upon stress situations insects release a several hormones and neurotransmitters like e.g. glucocorticoids and norepinephrine to mediate the stress response (Sternberg, 2006). The best-studied neurotransmitter in insects is the neurohormone octopamine (OA). The octopaminergic system of invertebrates is described as homolog of the noradrenergic systems of vertebrates (Roeder 1999). It was shown that upon infection with *X. nematophila* high concentrations of OA were released into the hemolymph to increase the activity of haemocytes (Dunphy & Downer 1994). After the larva's death the OA also diffuses into the soil. The ability to sense and maybe then move towards the source of the OA could lead to an activation of one of the PAS4-LuxR solos and induce the switch from the 2° to 1° phenotype. The possibility to re-enter the life cycle would be increased

as the reversed switching would occur near to dead larvae ideally containing nematodes. However, direct injection of only 2° cells into living *G. mellonella* larvae did not lead to a conversion into 1° cells (S.E., R.H. unpublished). Therefore, e.g. signals from 1° cells or the nematodes might be necessary.

## 6.3 Outlook

Phenotypic heterogeneity in *P. luminescens* DJC cell populations still poses a challenging field of research which has to be unraveled. It is of major interest to identify the signal which triggers 1° cells to convert into 2° cells and how the switching process is regulated.

XreR1 and XreR2 were found to play an important role in regulation of the switching. However, their mode of action remains elusive and needs to be investigated more deeply. As none of the proteins directly regulate any phenotype the downstream targets of the DNA binding proteins should be identified. This could be achieved by performing e.g. Chip-Seq analysis or DNase I footprinting assays. Furthermore, it should not be excluded that small RNAs are involved in phenotypic switching which could be adressed by conducting a high-throughput sequencing of small RNAs (Liu & Camilli, 2011). Additionally, XreR1 and XreR2 might control genes by forming a complex. Therefore, MST and SPR analysis with promoter regions of specific phenotypes should be repeated using a mixture of both proteins. On the other hand, XreR1 and XreR2 may regulate specific features indirectly rather than by direct binding. Therefore, transcriptome and/or proteome analysis of the deletion mutants compared to the wildtype could help to identify genes and proteins affected by one of the transcriptional regulators.

Do 2° cells actively interact with plants? Since the bacteria are used as bio-insecticide in agriculture, a putative interaction of 2° cells with plants and their fate in the soil is of great importance for biotechnology. Therefore, the impact of 2° cells on the growth of plants

and vice versa should be investigated. This can be achieved by either setting up native-like conditions with soil and plants or by directly inoculating the bacteria onto plant roots growing on plant-agar. Here, not only growth but also colonization of the roots by the bacteria can be monitored using microscopy, ideally using fluorescently labeled 2° cells. Native-like conditions could then also be expanded by adding insect larvae and/or nematodes as well as 1° cells. In the latter scenario both cell forms should be labeled with a resistance cassette to track them back to their origin as they might switch inside the soil.

A change of environment might also require a change of the cell-cell communication system. Both parts of the QS system found in *P. luminescens*, *ppyS* and the LuxR solo encoding *pluR*, were down-regulated in 2° cells. However, 16 other LuxR solos with yet unsolved function showed higher expression. It would be of great interest to analyze if one of these systems serves 2° cells as an alternative cell-cell communication system than PpyS/PluR.

Furthermore, it should be investigated if one or more of these LuxR solos respond to plant-derived compounds such as hormones, and if so, which genes are involved. Potential candidates might then be tested via SPR or a thermal stability assay. The latter enables high throughput screening 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).

Concludingly, as nematodes containing *P. luminescens* are already commercially used as bio-insecticide preventing crop failure, investigation on the fate of 2° cells in the soil including a putative interaction of 2° cells with plants is of great interest for agriculture. Further, the understanding of the complex regulation of phenotypic heterogeneity in *P. luminescens* might help to prevent enormous losses in industrial mass production of nematodes caused by phenotypic switching of 1° cells that should support the

nematode's development. Additionally, global principles of heterogeneous behavior could be transferred to other clinically and biotechnologically relevant microorganisms.

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# Curriculum vitae

## Personal Data

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## Education

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| 06/2019 – 12/2019 | PhD student in the group “Bacterial Infection Biology” of Prof. Dr. Ralf Heermann<br>*Johannes-von-Gutenberg-Universität, Mainz* |
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| 10/2012 – 03/2015 | Biology, Master of Science<br>*Technische Universität München, Munich*<br>Primary focus: Medical Biology<br>Secondary focuses: Genetics, Zoology<br>Project: Catalytic efficacy of organophosphorus hydrolases - kinetic quantification and structure-activity relationships (Grade 1.3)<br>Institute of Pharmacology and Toxicology*, Bundeswehr München, Munich* |
| 10/2009 – 10/2012 | Biology, Bachelor of Science<br>*Technische Universität München, Munich*<br>Project: Functional dissection of the paired domain of Pax6: roles in neural tube patterning and peripheral nervous system development (Grade 1.3)<br>Institute of Developmental Genetics*, Helmholtz-Zentrum München, Munich* |

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## Work experience

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| 04/2015 | Research Associate<br>Department for Cytogenetics and Reproductive Genetics<br>*Zentrum für Humangenetik und Laboratoriumsdiagnostik (MVZ) München, Munich* |
| 11/ 2014 – 03/2015 | Student Associate<br>Department for Cytogenetics and Reproductive Genetics<br>*Zentrum für Humangenetik und Laboratoriumsdiagnostik (MVZ) München, Munich* |
| 10/2014 – 03/2015 | Research Assistant<br>Department: Genes-Circuits-Behavior, Group: Baier<br>*Max Planck Institute of Neurobiology, Munich* |
| 11/2012 – 03/2014 | Research Associate<br>Institute of medical Informatics, Biometry and Epidemiology<br>*Ludwig-Maximilians-Universität München, Munich* |
| 11/2009 – 04/2012 | Student Associate<br>*Medical practice "Neurochirurgie Innenstadt", Munich* |

## Conferences

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| 27/03 – 29/03/19 | International conference: SPP1617 – Phenotypic heterogeneity and sociobiology of bacterial populations<br>*Hohenkammer, Germany*<br>**Poster:** Phenotypic heterogeneity of *Photorhabdus luminescens* – The influence of two novel XRE-transcriptional regulators on the switching process |
| 17/03 – 20/03/19 | Annual conference of the Association of general and applied Microbiology (VAAM)<br>*Mainz, Germany* |

| | |
|---|---|
| | **Poster:** Two novel XRE-transcriptional regulators play a major role in regulation of phenotypic heterogeneity in the insect pathogen *P. luminescens* |
| 23/09 – 25/09/218 | VAAM Symposium „How microorganism view their world”<br>*Marburg, Germany* |
| | **Talk + Poster:** From the host to soil – Regulation of phenotypic switching in insect pathogenic *P. luminescens* via two novel XRE-transcriptional regulators |
| 17/09 – 19/09/2018 | VAAM Summer School „Mechanisms of Gene Regulation”<br>*Tutzing, Germany* |
| | **Talk:** Identification of two novel XRE-transcriptional regulators that influence phenotypic switching of the insect pathogen *Photorhabdus luminescens* |
| 03/05 – 03/07/2018 | SPP1617 Progress Meeting – Phenotypic heterogeneity and sociobiology of bacterial populations<br>*Goettingen, Germany* |
| | **Talk:** Phenotypic heterogeneity of *Photorhabdus luminescens* – a way from insects to plants? |
| 07/03 – 07/07/2017 | Joint ICGEB-ICTP-APCTP Workshop on Systems Biology and Molecular Economy of Microbial Communities<br>*Trieste, Italy* |
| | **Poster:** The power of being different – phenotypic heterogeneity of the insect pathogen *Photorhabdus luminescens* |
| 06/26 – 06/30/2017 | EMBO EMBL Symposium 2017 „New Approaches and Concepts in Microbiology”<br>*Heidelberg, Germany* |
| | **Poster:** Bet hedging of insect pathogenic *Photorhabdus luminescens* – the power of individuality |
| 04/27 - 04/29/2017 | SPP1617 Progress Meeting – Phenotypic heterogeneity and sociobiology of bacterial populations<br>*Hohenkammer, Germany* |
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| | **Poster:** Phenotypic heterogeneity in *Photorhabdus luminescens* cell populations: a bet-hedging strategy? |
| 04/19 – 04/21/2016 | SPP1617 Progress Meeting – Phenotypic heterogeneity and sociobiology of bacterial populations<br>*Duesseldorf, Germany* |

## Publications

Eckstein S, Dominelli N, Brachmann A, Heermann R (2019) Phenotypic heterogeneity of insect pathogenic *Photorhabdus luminescens* - insights into the fate of secondary cells. *Appl Env Microbiol*, 85:e01910-19.

Eckstein S and Heermann R (2019) Regulation of phenotypic switching and heterogeneity in *Photorhabdus luminescens* cell populations. *J Mol Biol.* pii: S0022-2836(19)30211-6.

Zamora-Lagos M* and Eckstein S*, Langer A, Gazanis A, Pfeiffer F, Heermann R$^{+}$ and Habermann B$^{+}$ (2018) Phenotypic and genomic comparison of *Photorhabdus luminescens* subsp. l*aumondii* TT01 and a widely used rifampicin-resistant *Photorhabdus luminescens* laboratory strain. *BMC Genomics*. 19(1):854.

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Huettl RE, Eckstein S, Stahl T, Petricca S, Ninkovic J, Götz M, Huber AB (2015) Functional dissection of the Pax6 paired domain: Roles in neural tube patterning and peripheral nervous system development. *Dev Biol*. pii: S0012-1606(15)30051-8.