Information processing and disturbance of the quorum sensing cascade of *Vibrio harveyi*

Dissertation

Zur Erlangung des Doktorgrades

der Naturwissenschaften

(Dr. rer. nat.)

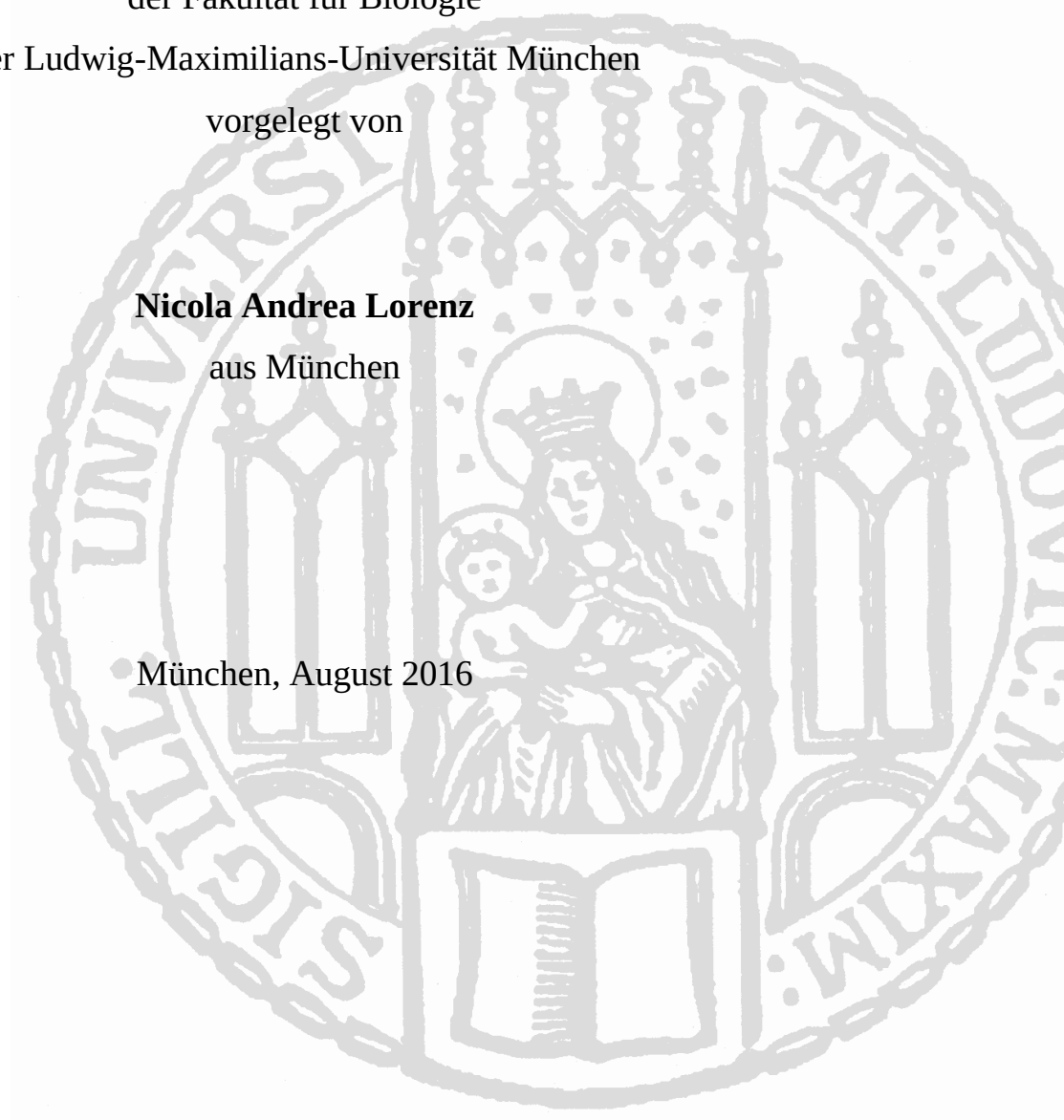
der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

vorgelegt von

Nicola Andrea Lorenz

aus München



München, August 2016

Gutachter:

1. Prof. Dr. Kirsten Jung
2. Prof. Dr. Marc Bramkamp

Datum der Abgabe: 22.08.2016
Datum der mündlichen Prüfung: 03.11.2016

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt wurde. Des Weiteren erkläre ich, dass ich nicht anderweitig ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Die folgende Dissertation liegt weder ganz, noch in wesentlichen Teilen einer anderen Prüfungskommission vor.

München, 22.08.2016

Statutory Declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources. As well I declare, that I have not submitted a dissertation without success and not passed the oral exam. The present dissertation (neither the entire dissertation nor parts) has not been presented to another examination board.

Munich, 22.08.2016

Contents

Nomenclature

Gene products are numbered in a way that the first methionine/valine of the wild type protein is designated “1” in the amino acid sequence (if present: independently of the N-terminal affinity tag). N-terminal and C-terminal affinity tags are marked in genes and proteins corresponding to their position (e.g. 6His-LuxN or LuxN-6His). Amino acid substitutions in proteins are termed as follows: The native amino acid is designated in one-letter code, followed by the respective amino acid position in the protein. The amino acid introduced by (site-directed) mutagenesis is terminally added in one-letter code (Example: CqsS F175C). Deletions of genes are marked by “Δ”.

The strain *Vibrio campbellii* ATCC BAA-1116 was formerly misidentified as *Vibrio harveyi*. In the introduction and the discussion part of this thesis as well as in the unpublished manuscript this strain will throughout be designated as *V. harveyi* adapted to the former publications. However, in two publications (Lorenz *et al.* 2016, Zhao *et al.* 2016) presented here, the strain is already referred to as *V. campbellii* but it has to be emphasized that this is the identical strain as used for the other studies.

Abbreviations

AA	amino acid
AB medium	autoinducer bioassay medium
AHL	acyl-homoserine lactone
AI	autoinducer
AU	arbitrary unit
bp	base pair
CAI-1	*Vibrio cholerae* autoinducer-1
CM	cytoplasmic membrane
CP	cytoplasm
DNA	desoxyribonucleic acid
DPD	dihydroxy pentandione
GFP	green fluorescent protein
HAI-1	*Vibrio harveyi* autoinducer-1
HCD	high cell density
HPt protein	histidine phosphotransfer protein
HTH	helix-turn-helix
LB	lysogeny broth
LCD	low cell density
LM	lysogeny marine broth
mRNA	messenger RNA
P	phosphoryl group
PAGE	polyacryamide gel electrophoresis
PCR	polymerase chain reaction
PP	periplasm
QQ	quorum quenching
QS	quorum sensing
REC domain	receiver domain
RNA	ribonucleic acid
sRNA	small regulatory RNA
TCS	two component system
TEM	transmission electron microscopy
TM	transmembrane

UV	ultra violet
VOC	volatile organic compounds
vs	versus

Publications and Manuscripts Originating from this Thesis

Chapter 2:

Plener, L.[*], Lorenz, N.[*], Reiger, M., Ramalho, T., Gerland, U., Jung, K. (2015). The phosphorylation flow of the *Vibrio harveyi* quorum-sensing cascade determines levels of phenotypic heterogeneity in the population. *J Bacteriol* 197:1747-1756.

[*] Authors contributed equally

Chapter 3:

Lorenz, N., Yen Shin J. and Jung K. (2016). Activity, abundance and localization of quorum sensing receptors in *Vibrio harveyi* (Manuscript).

Chapter 4:

Zhao, W., Lorenz, N., Jung, K., Sieber, S.A. (2016). Fimbrolide natural products disrupt bioluminescence of *Vibrio harveyi* by targeting autoinducer biosynthesis and luciferase activity. *Angew Chem Int Ed* 55 (3):1187-1191.

Chapter 5:

Lorenz, N., Reiger, M., Toro-Nahuelpan, M., Brachmann, A., Poettinger, L., Lassak, J., Jung, K. (2016). Identification and initial characterization of prophages in *Vibrio campbellii*. PloS One 11(5): e0156010. doi:10.1371/journal.pone.0156010

Contributions to Publications and Manuscripts presented in this Thesis

Chapter 2:

Nicola Lorenz, Laure Plener and Kirsten Jung designed the experiments. Laure Plener and Nicola Lorenz constructed all plasmids. Laure Plener performed bioluminescence assays and fluorescence microscopy of all kinase mutants. Nicola Lorenz constructed all kinase and kinase/synthase mutants, conducted qRT-PCR and carried out fluorescence microscopy of the synthase mutants. Matthias Reiger constructed the synthase mutants. Tiago Ramalho and Ulrich Gerland developed the single cell analysis software. Laure Plener, Nicola Lorenz and Kirsten Jung wrote the manuscript.

Chapter 3:

Nicola Lorenz, Jae Yen Shin and Kirsten Jung designed the experiments. Nicola Lorenz carried out all experiments. Jae Yen Shin helped with fluorescence microscopy and analyzed the resultant data. Nicola Lorenz and Kirsten Jung wrote the manuscript.

Chapter 4:

Weining Zhao, Nicola Lorenz, Kirsten Jung and Stephan A. Sieber designed the experiments. Nicola Lorenz generated all *Vibrio* mutants, performed bioluminescence and growth assays of *V. harveyi*. Weining Zhao performed all other experiments. Weining Zhao and Stephan A. Sieber wrote the manuscript.

Chapter 5:

Nicola Lorenz, Matthias Reiger, Jürgen Lassak, Laure Plener and Kirsten Jung designed the concept of the study. Jürgen Lassak constructed the plasmid pNPTS-138-R6KT-GFP. Lisa Poettinger constructed plasmids for the promoter fusions and prepared phage concentrate following mitomycin C treatment. Mauricio Toro-Nahuelpan carried out TEM analysis.

Andreas Brachmann performed sequencing of phage DNA. Matthias Reiger and Andreas Brachmann evaluated data from the sequencing. Nicola Lorenz performed growth assays, fluorescence microscopy, qRT-PCR and prepared phage concentrates of heat induced cells. Nicola Lorenz, Matthias Reiger, Jürgen Lassak and Kirsten Jung wrote the manuscript.

We hereby confirm the above statements:

Nicola Lorenz Kirsten Jung

Summary

Vibrio harveyi is a marine, bioluminescent bacterium. The ability to produce light is regulated in a cell density dependent manner called quorum sensing (QS). This communication relies on the production, excretion and perception of small molecules called autoinducers (AIs). In *V. harveyi* QS relies on three parallel systems involving three different AIs (AI-2, HAI-1, CAI-1) produced by three synthases (LuxS, LuxM, CqsA) and recognized by their cognate sensors, specifically hybrid histidine kinases (LuxQ, LuxN, CqsS). The information about the presence of the AIs is processed in one cascade comprising a phosphorelay involving the three sensors, the histidine phosphotransfer (HPt) protein LuxU, the response regulator LuxO, an Hfq/small RNA (sRNA) switch and the transcriptional regulator LuxR. LuxR is responsible for the induction and repression of numerous QS-regulated genes e.g. for bioluminescence. The inhibition of QS is called quorum quenching (QQ) and could be used as a strategy to prevent bacteria from developing group behaviors which can be detrimental to health or industry such as biofilm formation or virulence. This thesis focuses on understanding the information processing in the complex QS system of *V. harveyi* and on how QS could be disturbed in this bacterium.

In the first part, this thesis focuses on the elucidation of the impact of each AI and its associated sensor on QS activation at the population and the single-cell level (Chapter 2). QS activation was found to be homogeneous in the presence of all AIs. However, under conditions which result in mixed kinase and phosphatase activities of the sensors, QS activation was heterogeneous. We showed that the ratios of kinase to phosphatase activities and therefore the pools of phosphorylated LuxU/LuxO per cell are important for the signaling output as well as noise in the output. The ability to generate heterogeneous gene expression is an inherent feature due to the complex architecture of the cascade.

The second part of the study focuses on the characterization of the sensors combining *in vivo* and *in vitro* approaches (Chapter 3). Their localization was investigated via fluorescence microscopy. Cluster formation of the sensors could be shown as well as differences in their abundance on the transcript and the protein level. The respective activity of each sensor was assessed by an *in vitro* approach and uncovered differences in their kinase but not phosphatase activities. Finally, it was shown that the direct influence of AIs on the sensors was restricted to inhibition of the kinase activity whereas the phosphatase activity was not affected by the presence of AIs.

Fimbrolides are natural products synthesized by the alga *Delisea pulchra* and are known to be potent QS disruptors. A comprehensive proteomic study to unravel potential direct and irreversible targets of fimbrolides was the focus of the third part of this thesis (Chapter 4). LuxS, the AI-2 synthase as well as LuxE, a part of the luciferase complex, were found to be directly inhibited by fimbrolide compounds.

The induction of lysogenic prophages in *V. harveyi* was investigated in Chapter 5. Lysogenic prophages in the genome of *V. harveyi* were characterized and the lytic lifecycle of at least four prophages could be induced upon mitomycin C or heat treatment. Two different phages could be detected via transmission electron microscopy. The phage mediated lysis of *V. harveyi* could be responsible for bacterial population dynamics in the natural habitat.

Zusammenfassung

Vibrio harveyi ist ein marines, biolumineszentes Bakterium. Die Produktion der Biolumineszenz erfolgt in Abhängigkeit der Zelldichte, und der zugrunde liegende Prozess wird als Quorum Sensing (QS) bezeichnet. Diese Form der Kommunikation basiert auf der Synthese und Wahrnehmung kleiner Moleküle, sogenannter Autoinduktoren (AI). In *V. harveyi* werden drei Autoinduktoren (AI-2, HAI-1, CAI-1) von drei Synthasen (LuxS, LuxM, CqsA) produziert und von drei spezifischen Hybridhistidinkinasen (LuxQ, LuxN, CqsS) wahrgenommen. Die Information über die Anwesenheit der AI wird in eine Kaskade integriert und mit Hilfe eines Phosphorelays über das Histidinphosphotranferprotein LuxU auf den Antwortregulator LuxO übertragen. P-LuxO kontrolliert die Expression von regulatorischen sRNAs, die die Kopienzahl des Transkriptionsregulator LuxR beeinflussen. LuxR ist für die Induktion und Repression zahlreicher QS-regulierter Phänotypen z.B. für die Produktion von Biolumineszenz, verantwortlich. Die QS Inhibierung wird als Quorum Quenching (QQ) bezeichnet und könnte dazu beitragen zelldichteabhängige Prozesse (z.B. Biofilmbildung oder Virulenz) zu bekämpfen. Die vorliegende Arbeit konzentriert sich auf die Aufklärung der Signalintegration in die komplexe QS Kaskade von *V. harveyi* und darauf wie der Prozess des QS beeinflusst werden kann.

Im ersten Teil dieser Arbeit steht die Aufklärung der individuellen Einflüsse der AI und deren Sensoren bei der QS Aktivierung auf Populations- sowie auf Einzelzellebene im Mittelpunkt (Kapitel 2). Es konnte gezeigt werden, dass in Anwesenheit aller AI die QS Aktivierung auf Einzelzellebene homogen ist. Wenn die Kaskade allerdings um einen Input reduziert wurde, resultierte das in einem heterogenen Output. Das Verhältnis von Kinase- zu Phosphataseaktivität der Hybridhistidinkinasen und damit die Menge an phosphoryliertem LuxU/LuxO pro Zelle ist wichtig für den QS Output sowie für den Grad an Heterogenität innerhalb der Population. Die Fähigkeit zur heterogenen Genexpression ist eine Eigenschaft, die in der komplexen Architektur der Kaskade verankert ist.

Der zweite Teil der vorliegenden Studie fokussiert auf die Charakterisierung der Sensoren mittels kombinierter *in vivo* und *in vitro* Ansätze (Kapitel 3). Die Lokalisation der Sensoren wurde mithilfe von Fluoreszenzmikroskopie untersucht. Hierbei konnte eine Clusterbildung beobachtet werden. Zudem wurden Unterschiede im Mengenverhältnis der Sensoren zueinander auf Protein- sowie auf Transkriptebene festgestellt. Die entsprechenden enzymatischen Aktivitäten der Sensoren wurden *in vitro* analysiert und wiesen Unterschiede

in den Kinase- jedoch nicht in den Phosphataseaktivitäten auf. Letztendlich konnte gezeigt werden, dass die AI bei der Inhibierung der Kinase- nicht jedoch bei der Stimulierung der Phosphataseaktivität eine Rolle spielen.

Bei Fimbroliden handelt es sich um Naturstoffe die als QS-Quencher bekannt sind und von der Alge *Delisea pulchra* synthetisiert werden. Eine umfassende Proteomstudie zur Aufdeckung potenzieller direkter und irreversibler Angriffsziele fehlte bislang und war Gegenstand des dritten Teils dieser Arbeit (Kapitel 4). LuxS, die AI-2 Synthase, und LuxE, ein Protein des Luziferasekomplexes, wurden als spezifisch durch Fimbrolide inhibierte Proteine identifiziert.

Die Induktion von lysogenen Prophagen von *V. harveyi* wurde in Kapitel 5 untersucht. Lysogene Prophagen im Genom von *V. harveyi* wurden charakterisiert und der lytische Zyklus von vier Prophagen konnte durch Mitomycin C Zugabe oder Hitzeschock ausgelöst werden. Zwei unterschiedliche Phagen konnten mittels Transmissionselektronenmikroskopie (TEM) detektiert werden. Die durch Phagenfreisetzung induzierte Lyse von *V. harveyi* Zellen könnte für spezifische Populationsdynamiken im natürlichen Habitat verantwortlich sein.

1 Introduction

1.1 Quorum Sensing

For a long time, it was thought that single bacteria in populations act independently of each other. Over the years it was elucidated that bacteria are in fact able to communicate. In the 70s Hastings and colleagues showed that *Vibrio harveyi* and *Vibrio fischeri*, two marine bacteria, produce bioluminescence at high cell density (HCD) but not in a diluted culture (Nealson *et al.*, 1970). This cell-cell communication is now referred to as Quorum Sensing (QS). In the simplest form of QS, the bacteria measure their population density via the production, excretion and sensing of specific signaling molecules. This process allows the cells to synchronize their behavior in a cell-density dependent manner by altering the gene expression upon a certain threshold concentration of signaling molecules (Figure 1.1) (Fuqua *et al.*, 1994, Keller & Surette, 2006). Tasks that are usually controlled via this process are more useful if undertaken by a group than by individual cells. Thereby, the bacteria can act as a multicellular organism.

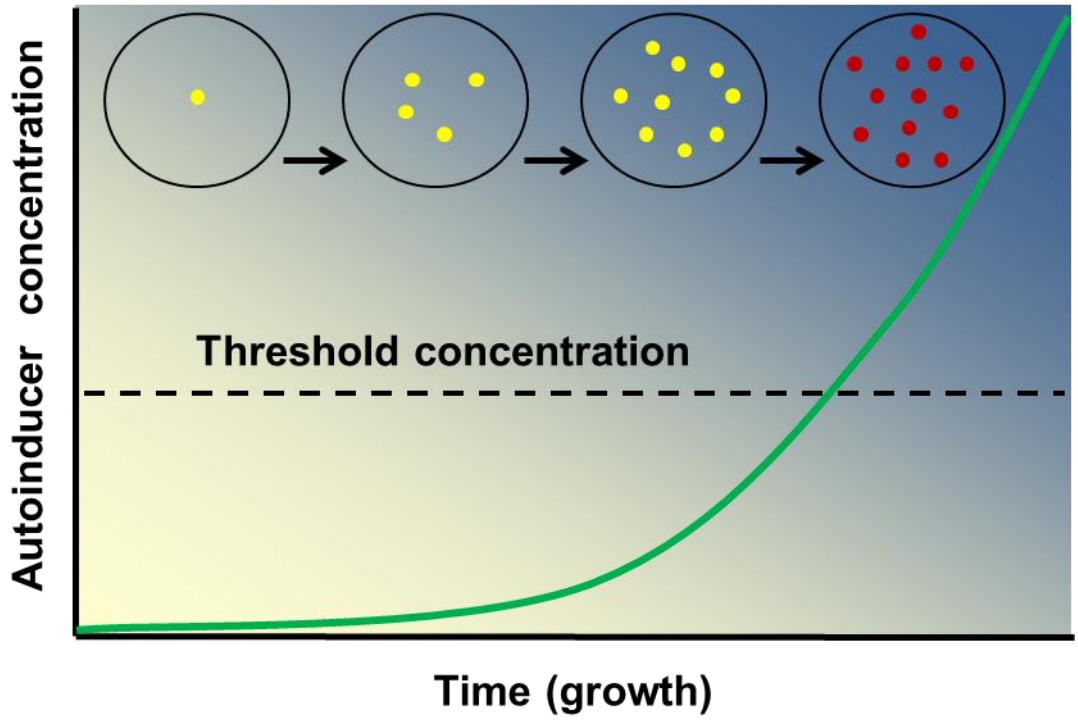


Figure 1.1 General scheme for QS. Bacterial cells (yellow dots) synthesize and excrete signaling molecules. These autoinducers (AIs) accumulate in the surrounding environment with rising cell densities (indicated by the green line and the gradient from yellow to blue). After reaching a threshold concentration, the perception of the signal leads to changes in the gene expression of QS-regulated genes. The cells respond to the emitted AI (red dots). In case of intra-species communication, the emitter and responder are the same cells. Figure adapted after Keller and Surette, 2006.

The QS-regulated processes include for example the regulation of competence, virulence, biofilm formation and sporulation. Formerly, it was assumed that the phenomenon QS is restricted to a few bacterial species, but over the years it became apparent, that intercellular communication is rather the rule than the exception (Bassler & Losick, 2006). The signaling molecules, also called autoinducers (AIs), can be of different chemical nature. Gram-negative bacteria mainly communicate using acyl-homoserine lactones (AHLs) (Visick & Fuqua, 2005). In *V. fischeri* these AHLs are synthesized by LuxI-type synthases and recognized by the cytoplasmic receptor LuxR. If the AHLs reach a threshold concentration, they bind to LuxR, which also functions as a DNA-binding transcriptional activator, and subsequently binds to the operon encoding the luciferase. It is interesting to note, that the AHL-LuxR complex also activates the *luxI* transcription resulting in a positive feedback loop which ensures that the whole population switches to the QS-ON mode and thereby produces light. A wide variety of Gram-negative bacteria use AHLs as signaling molecules for intra-species communication (Fuqua *et al.*, 2001, Eberl, 1999, Greenberg, 2003). The specificity of these AHLs is maintained by varying the length of the acyl moieties (between four and 18 C atoms). In the acyl chain the third C atom can either be a carbonyl group, a hydroxyl group or a methylene moiety (Whitehead *et al.*, 2001).

In contrast, Gram-positive bacteria, for example *Streptococcus* or *Bacillus* species, communicate mostly using short peptides comprising various chemical modifications and membrane-bound histidine kinases as receptors. The signal transduction is mediated by a phosphorylation cascade that eventually activates a DNA-binding response regulator (Hakenbeck & Stock, 1996). Peptide signals are not diffusible across the membrane, therefore specific exporters are needed (Kleerebezem *et al.*, 1997, Lyon & Novick, 2004). One example of a process regulated by QS in Gram-positive bacteria is the regulation of competence in *Bacillus subtilis* (Turgay *et al.*, 1998). The competent state is achieved at the transition between logarithmic and stationary phase, where due to cell lysis, increased levels of exogenous DNA are available (Miller & Bassler, 2001). Overall, both ways of QS-communication are designed to exhibit high specificity in the signals. However, one additional cell-cell communication system is the LuxS/AI-2 pathway. This pathway is found in Gram-negative as well as in Gram-positive bacteria. AI-2s all derive from a common precursor, 4,5-dihydroxy-2,3 pentanedione (DPD) (Schauder *et al.*, 2001). The receptors of AI-2 in *V. harveyi* and *Salmonella enterica* bind different stereoisomers. AI-2 represents a more universal language, which can lead to the communication between different species. Interestingly, bacterial QS molecules can also be recognized by eukaryotes which are

colonized by the bacteria (Hartmann & Schikora, 2012). In the initial steps of symbiosis between *Rhizobium meliloti* and its plant host, the early signals are produced by the plant. Flavonoid molecules are produced in the rhizosphere by the plant roots (Peters *et al.*, 1986).

1.2 The marine bacterium *Vibrio harveyi*

V. harveyi (recently reclassified as *V. campbellii* (Lin *et al.*, 2010)) is a versatile free living marine γ-proteobacterium. Due to the formation of either a polar flagellum or peritrichous flagella it is able to swim and swarm, respectively (Allen & Baumann, 1971, McCarter, 2001) (Figure 1.2 A). Besides its pelagic lifestyle it can be found on the surface of algae or as a pathogen in shrimp or fish (Jiravanichpaisal *et al.*, 1994, Catap *et al.*, 2003). An outstanding characteristic of this bacterium is its ability to produce bioluminescence (Figure 1.2 B). It is assumed that *V. harveyi* is primarily responsible for the so called "milky sea effect". Mariners of the 17th century already described this effect of a blue glowing sea that lasts from several hours to a few days and then disappears fast again. This milky sea can cover up to 16,000 km^2 and can even be detected from space (Figure 1.2 C) (Miller *et al.*, 2005). By the time *V. harveyi* colonizes the haptophyte *Phaeocystis*, the bacterium excretes AIs which lead to bioluminescence (Lapota *et al.*, 1988).

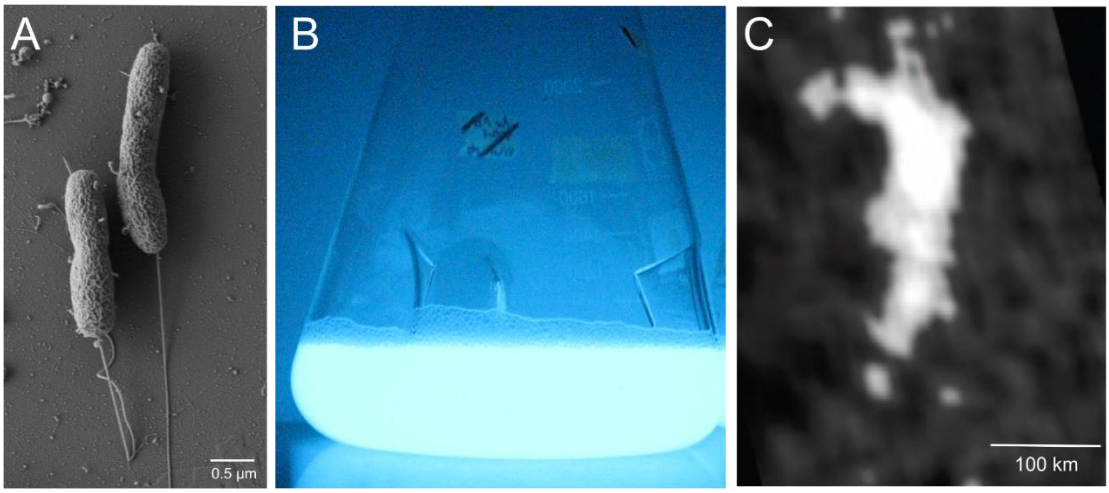


Figure 1.2 The marine bioluminescent bacterium *V. harveyi*. A) The γ-proteobacterium *V. harveyi* rod shaped and polar flagellated (picture was taken by Dr. Axel Müller and Prof. Dr. Gerhard Wanner) **B)** Bioluminescence produced by wild type *V. harveyi* cells in a flask (picture taken by Dr. Axel Müller) **C)** Satellite image taken at the coast of Somalia showing the milky sea effect, a large area of glowing water. The satellite image was adapted from Miller *et al.*, 2005.

1.2.1 The quorum sensing system of *Vibrio harveyi*

V. harveyi, in contrast to *V. fischeri*, responds to three different classes of AIs that are channeled into one complex QS cascade. HAI-1, an acyl-homoserine lactone [*N*-(3-hydroxybutyryl)-homoserine lactone] is produced by the AI synthase LuxM (Cao & Meighen, 1989). As this AI is only produced by *V. harveyi* and close relatives it is supposed to be an

intra-species signal. The second AI CAI-1, a long-chain amino ketone [*(Z)*-3-aminoundec-2-en-4one] (Ea-C8-CAI-1) is produced by the synthase CqsA and synthesized by several *Vibrio* species, mainly by *Vibrio cholerae* (Ng *et al.*, 2011). The third synthase LuxS produces AI-2, a furanosyl borate diester (Chen *et al.*, 2002) (Figure 1.3 A).

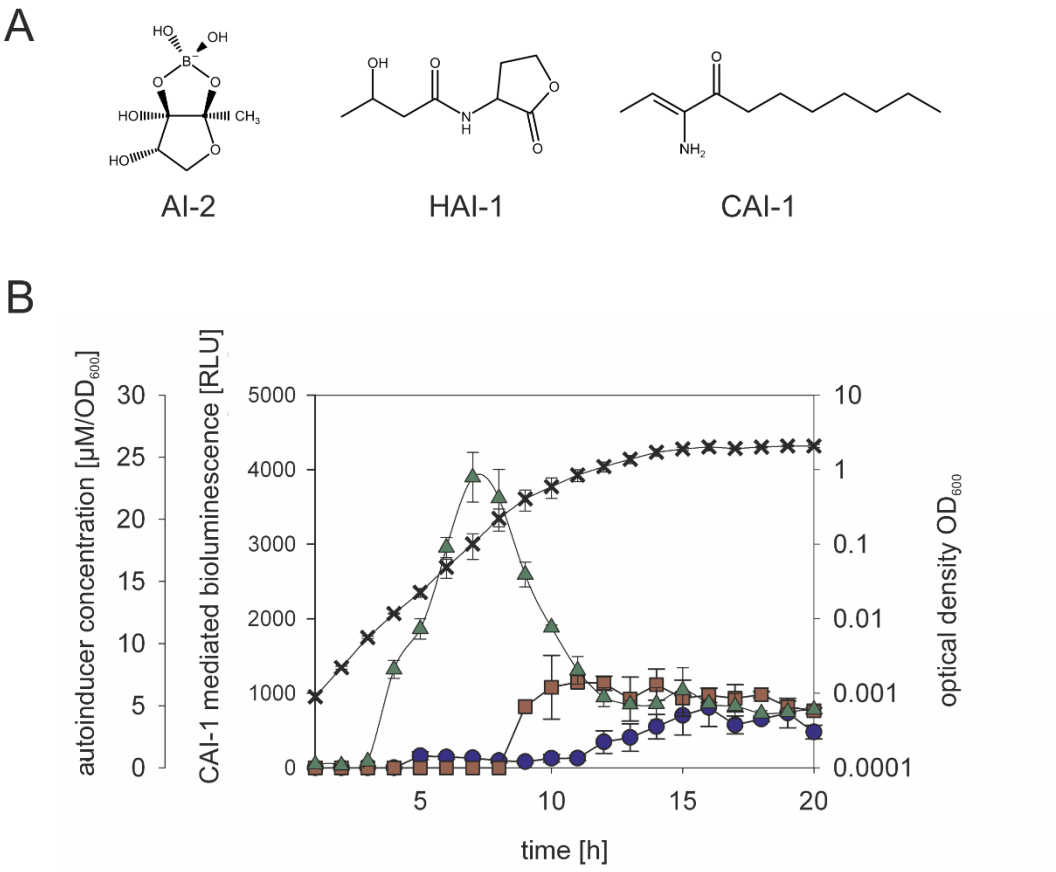


Figure 1.3 Chemical nature and timing of the AIs produced by *V. harveyi.* A) Structure of the three AIs produced by *V. harveyi*. AI-2, a furanosyl borate diester is produced by LuxS. HAI-1 is an acyl-homoserine lactone produced by LuxM for intra-species communication. CAI-1, a hydrophobic long-chain amino ketone produced by the synthase CqsA. **B)** Time course of AI-2, HAI-1 and CAI-1 production during growth of *V. harveyi*. Crosses indicate the optical density of the bacteria (OD_{600}). AI-2 (green triangles) and HAI-1 (red brown squares) concentrations are normalized by the OD_{600}. Blue circles indicate bioluminescence mediated by CAI-1 enriched in dichloromethane. Figure B) was modified from Anetzberger *et al.* 2012.

AI-2 is a widespread molecule that is produced by several Gram-positive and Gram-negative bacteria. It was elucidated recently in our group, that these AIs exhibit temporal variations regarding their availability and concentration (Figure 1.3 B). While the AI-2 concentration increases in the exponential growth phase, HAI-1 and CAI-1 were detected starting in the late exponential phase (Anetzberger *et al.*, 2012). The three AIs (HAI-1, CAI-1 and AI-2) are perceived by three specific hybrid histidine kinases (LuxN, CqsS and LuxQ in combination with the periplasmic protein LuxP, respectively). Information on the availability and concentration of the AIs is subsequently transduced via phosphorelay to the histidine

phosphotransfer (HPt) protein LuxU and ultimately to the response regulator LuxO (Figure 1.4). At low cell density (LCD), hence low AI concentrations, the hybrid histidine kinases autophosphorylate and subsequently transfer the phosphoryl group to the HPt protein LuxU. LuxU in turn phosphorylates the response regulator LuxO, and P-LuxO, together with the sigma factor 54, induces the expression of five regulatory small RNAs (Qrr 1-5) (Figure 1.4) (Freeman & Bassler, 1999a, Lenz *et al.*, 2004, Lilley & Bassler, 2000). These Qrrs, together with the chaperon Hfq, destabilize and degrade transcripts of the master regulator *luxR*. Therefore, the QS phenotypes are in an OFF state (Tu & Bassler, 2007).

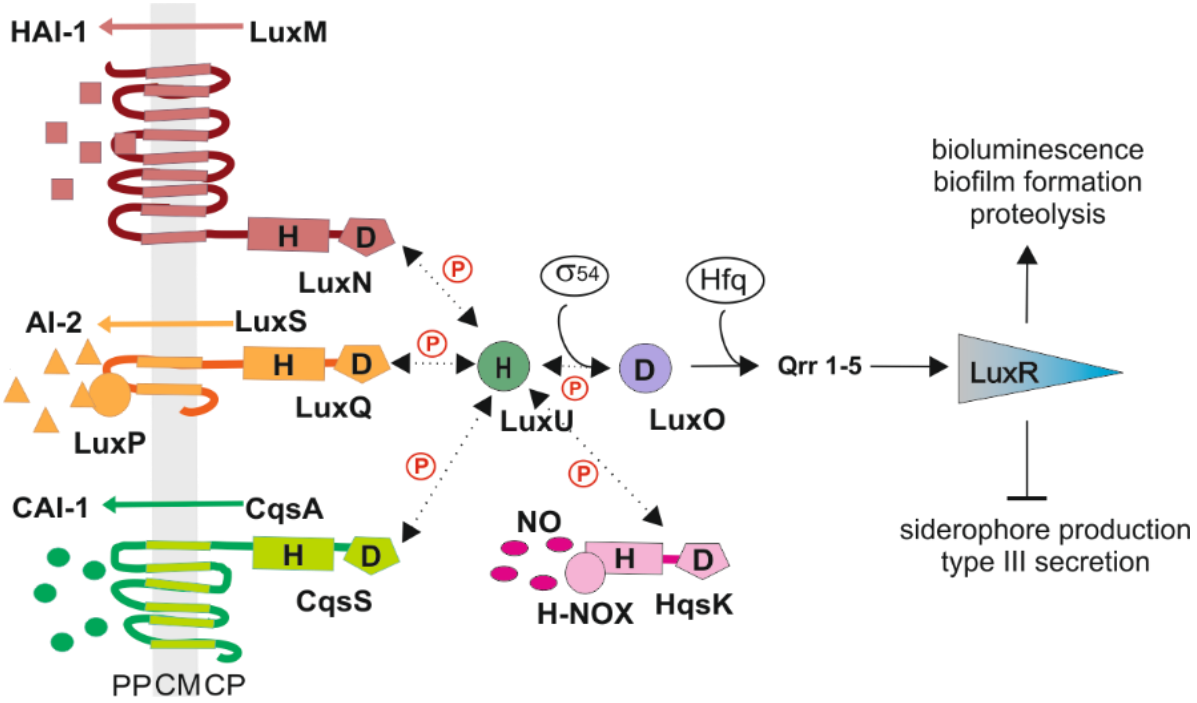


Figure 1.4 The QS cascade of *V. harveyi.* Three synthases (LuxM, LuxS and CqsA) synthesize three AIs (HAI-1, AI-2 and CAI-1). At LCD, equivalent to low AI concentrations, the sensors autophosphorylate and transfer the phosphoryl group via phosphorelay to the histidine phosphotransfer protein LuxU and then to the response regulator LuxO. P-LuxO, together with σ^{54}, induces the expression of five regulatory small RNAs (Qrr 1-5). These sRNAs along with the chaperon Hfq, destabilize the transcript of the QS master regulator LuxR. At HCD, equivalent to high AI concentrations, the AIs are perceived by the corresponding hybrid histidine kinases LuxN, LuxQ (together with the periplasmic binding protein LuxP) and CqsS and the kinase activities are inhibited. Thereby the phosphate is drained from the cascade and LuxR is produced. LuxR induces the gene expression required for bioluminescence, biofilm formation and proteolysis and represses the expression of genes for siderophore production and type III secretion systems. An additional system comprising the soluble histidine kinase HqsK and the NO sensor H-NOX integrates the information of NO levels by entering the QS cascade through phosphotransfer to LuxU. H indicates histidine and D aspartate (phosphorylation sites). PP, periplasm; CM, cytoplasmic membrane; CP, cytoplasm. Dotted lines stand for phosphotransfer and continuous arrows show transcriptional or posttranslational regulatory interactions. Figure adapted from (Plener *et al.*, 2015).

At high cell densities, the AIs accumulate and upon perception of their cognate AI the three receptors inhibit their kinase activity leading to dephosphorylation of P-LuxU and thereby the phosphoryl groups are drained from the cascade (Timmen *et al.*, 2006). Unphosphorylated LuxO cannot induce the expression of the sRNAs which leads to the production of the master regulator LuxR. Eventually, LuxR induces the expression of several QS-controlled

phenotypes as bioluminescence (Bassler *et al.*, 1994), proteolysis (Mok *et al.*, 2003) and biofilm formation (Anetzberger *et al.*, 2009). However, LuxR can also act as a repressor for genes encoding a type III secretion system and siderophores (Henke & Bassler, 2004, Lilley & Bassler, 2000) (Figure 1.4).

Moreover, in 2012 it was shown that *V. harveyi* integrates information about nitric oxide concentrations and thereby information about the environment in the QS pathway. H-NOX is functioning as a NO sensor and NO/H-NOX (heme-nitric oxide/oxygen) regulates the activity of the kinase HqsK (H-NOX-associated quorum sensing kinase) which is also transduced by phosphotransfer to LuxU (Figure 1.4) (Henares *et al.*, 2012).

1.2.2 The hybrid histidine kinases LuxN, LuxQ and CqsS

Usually sensor kinases consist of N-terminal transmembrane sensing domains, dimerization histidine phosphotransfer (DHp) domains and C-terminal catalytic ATP-binding (CA) domains (Dutta *et al.*, 1999). The three AI receptors LuxN, LuxQ and CqsS contain an additional C-terminal receiver domain harboring a conserved aspartate residue. The autophosphorylation at the conserved histidine residue in the kinase domain is followed by an intramolecular phosphotransfer to the conserved aspartate residue in the response regulator domain. The phosphorylation cascade takes place if the AIs are not available and in turn the kinases are dephosphorylated in the presence of the AIs.

LuxN consists of 849 amino acids (AA), nine transmembrane domains with the N-terminus located in the periplasm and comprises a molecular mass of 96 kDa (Jung *et al.*, 2007). The conserved, catalytic histidine residue is located at position 471 (Timmen *et al.*, 2006). The phosphorylated aspartate residue is at position 771 in the receiver domain. It was shown that the kinase activity is inhibited upon perception of HAI-1 whereas the phosphatase activity remains unaffected (Timmen *et al.*, 2006). The HAI-1 binding domain is composed of TM helices 4, 5, 6 and 7 as well as the periplasmic loops 2 and 3 (Swem *et al.*, 2008). It was elucidated recently that the leucine at position 166 specifies the AHL tail length which can be recognized. Decreasing the size of the residue at position 166 correlated with increasing length of the optimal AHL tail detected (Ke *et al.*, 2015).

LuxQ harbors 859 AA, two transmembrane domains consisting of two PAS domains and has a relative molecular mass of 97 kDa. LuxQ binds the periplasmic protein LuxP at the PAS domains in the presence and absence of AI-2, monitoring its AI-2 occupancy (Neiditch *et al.*, 2005). The binding of AI-2 causes a conformational change within LuxP and this in turn

stabilizes the arrangement in which two LuxPQ monomers are asymmetrically associated. This asymmetric structure is supposed to be responsible for the inhibition of the kinase activity of both LuxQ (Neiditch *et al.*, 2006). The conserved residues are histidine at position 492 and aspartate at position 785 (Stambrau, 2008).

The hybrid histidine kinase CqsS harbors six membrane domains and consists of 681 AA (Ng *et al.*, 2011). According to sequence comparisons the conserved catalytic residues are presumably the histidine at position 190 and aspartate at position 613. It was found that the residue F175 is important for CAI-1 chain length preference. The presence of a bulky AA like phenylalanine in *V. harveyi* increases selectivity, while the presence of a small residue like cysteine in *V. cholerae* relaxes chain length specificity (Ng *et al.*, 2011).

1.2.3 The histidine phosphotransfer protein LuxU and the response regulator LuxO

The signals of the hybrid histidine kinases are transduced via the histidine phosphotransfer (HPt) protein LuxU to the response regulator LuxO. It was shown that both proteins are important for QS signal transduction as in the absence of LuxU or LuxO the density dependent expression of the *lux* operon is abolished (Freeman & Bassler, 1999a, Freeman & Bassler, 1999b). It is likely that both genes are translated together as they are organized in one operon (Freeman & Bassler, 1999b). LuxU is localized in the cytoplasm and comprises only 13 kDa. The conserved histidine residue is located at position 58 in a region consisting of 20 AA, which is highly conserved in the phosphorelay protein family. Other members of this family are proteins like ArcB in *Escherichia coli* (Kato *et al.*, 1998), Spo0B of *B. subtilis* (Perraud *et al.*, 1999) and Ypd1p of *Saccharomyces cervevisae* (Janiak-Spens & West, 2000). But unlike other phosphotransferases e.g. Spo0B LuxU is monomeric (Ulrich *et al.*, 2005).

LuxO is a σ^{54}-dependent transcriptional regulator composed of 467 AA and a molecular weight of 52 kDa (Lilley & Bassler, 2000). The conserved aspartate residue lies in the N-terminal receiver domain at position 47 (Freeman & Bassler, 1999a). The C-terminal helix-turn-helix (HTH) domain is responsible for DNA-binding (Galperin, 2006). Between these two domains the σ^{54}-binding site is located which is crucial for the interaction between the sigma factor and the DNA (Rombel *et al.*, 1998). Furthermore, LuxO harbors an AAA^{+} ATPase-domain (ATPases associated with diverse cellular activity). Members of these families are subfamilies of Walker-ATPases harboring two consensus motives (Walker A and Walker B) (Walker *et al.*, 1982). Additionally, they harbor another conserved region within the ATPase domain, the SRH (Second Region of Homology). The phosphorylation of the aspartate in the receiver domain activates the C-terminal DNA-binding domain for ATP

hydrolysis and leads to opening and activation of σ^{54}-dependent promoters (Freeman & Bassler, 1999a, Lilley & Bassler, 2000). A *V. harveyi* LuxO mutant lacking the receiver domain is constitutively active *in vivo* demonstrating that LuxO is negatively regulated by its receiver domain and phosphorylation within the receiver domain releases the negative regulation (Freeman & Bassler, 1999a).

1.2.4 The master regulator LuxR

LuxR resembles the master regulator of the QS cascade in *V. harveyi* as it acts as a repressor as well as an activator for QS-regulated processes at high and low cell densities (van Kessel *et al.*, 2013a). It belongs to the TetR-type regulators and consists of 211 AAs (Pompeani *et al.*, 2008). LuxR regulates (positively and negatively) the expression of 625 genes, like *aphA*, *luxC* and *luxR*, directly or indirectly (Mok *et al.*, 2003, Lenz *et al.*, 2004, Waters & Bassler, 2006, van Kessel *et al.*, 2013a). It was shown that the consensus binding site of LuxR at repressed promoters is a symmetric palindrome (van Kessel *et al.*, 2013b). However, in promoters activated by LuxR the binding site is asymmetric and contains only half of the palindrome (van Kessel *et al.*, 2013b). Another transcription factor, AphA, is absent at HCD but acts to fine-tune QS gene expression at LCD (Rutherford *et al.*, 2011, van Kessel *et al.*, 2013a). AphA regulates the expression of 167 genes and LuxR/AphA coregulate 77 genes (van Kessel *et al.*, 2013a).

1.3 Integration of quorum sensing signals in *Vibrio harveyi*

Complex signal transduction pathways, like the QS cascade of *V. harveyi*, are often needed to correctly integrate signals. In situations where cells concurrently detect multiple chemical communication cues, these signals have to be integrated and the underlying information finally guides the behavior of the cells (Pawson & Scott, 2010). For instance, the chemotaxis circuit in *E. coli* has to integrate multiple positive and negative signals and generates an integrated response (Khan *et al.*, 1995). As the QS cascade in *V. harveyi* is highly complicated with three different inputs that channel all in the same cascade, it is crucial for the cells to integrate the signals and maintain the transmission fidelity. The QS cascade comprises five feedback loops (Figure 1.5 A and B). Protein levels of the response regulator LuxO are tightly controlled via two feedback loops. LuxO negatively autoregulates its own transcription, independent of its phosphorylation state, by directly binding to its own promoter region (Figure 1.5 A and B) (Tu *et al.*, 2010). Additionally the *luxO* mRNA is controlled by the small Qrr RNAs via sequestration (Figure 1.5 A) (Feng *et al.*, 2015). Thereby, the LuxO protein levels are precisely regulated and the Qrr production is tightly controlled. This

supports the fine-tuning in the cascade and ensures an accurate response to changes in AI levels.

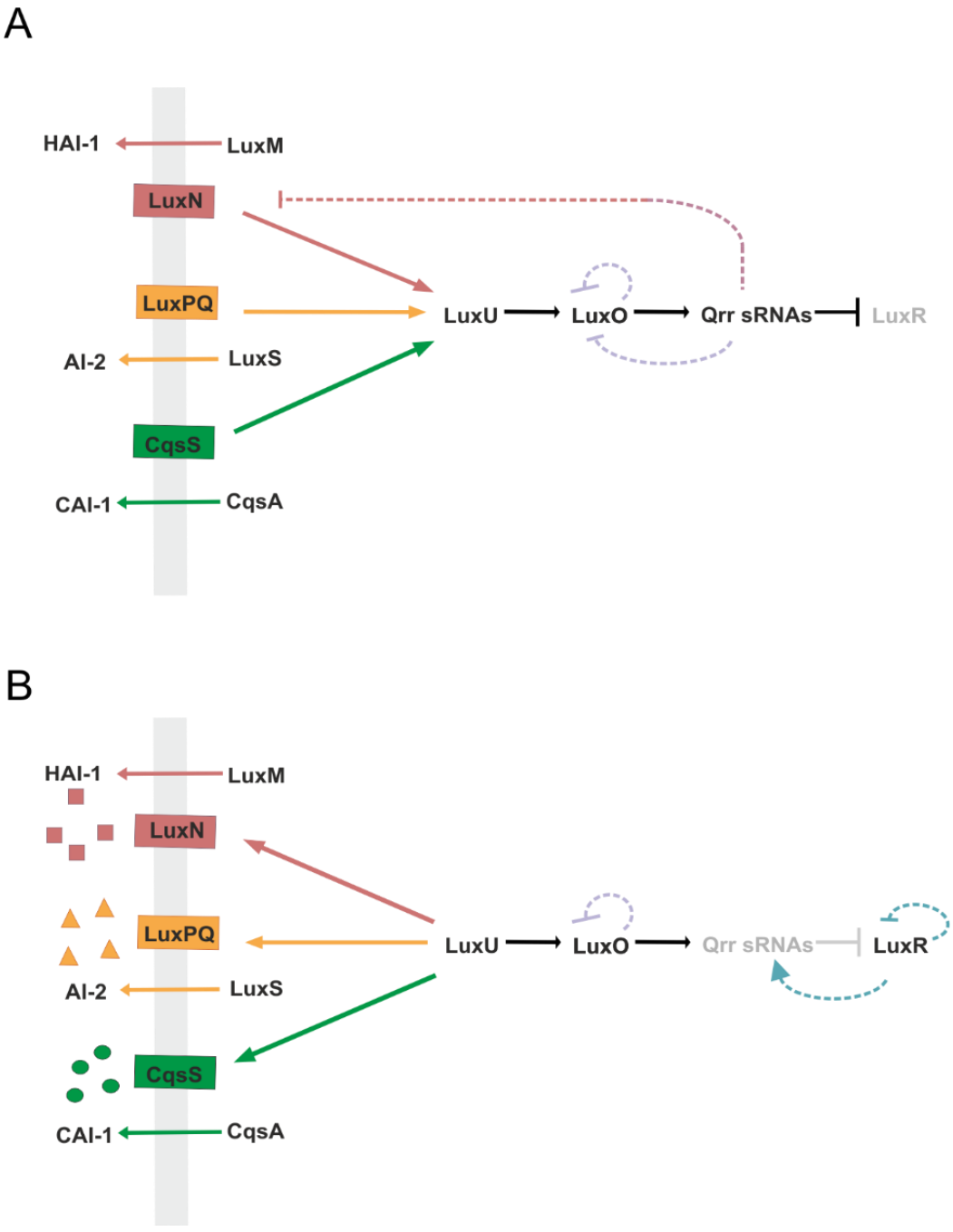


Figure 1.5 Schematic QS cascade of *V. harveyi* with feedback loops. The QS cascade harbors five feedback loops that fine-tune the integration of the AI signals to eventually control the abundance of the master regulator LuxR. **A) Predominant feedback loops at LCD.** Under LCD conditions (low AI concentrations) three feedback loops (dotted lines) are predominant. **B) Predominant feedback loops at HCD.** Under HCD conditions three feedback loops (dotted lines) are predominant. A detailed description of the feedback loops can be found in the main text. Figure adapted from Teng *et al.* 2011.

The master regulator LuxR directly autoregulates its own transcription by functioning as an autorepressor and directly activates the transcription of the Qrr sRNAs by binding to the promoter regions (Figure 1.5 B) (Chatterjee *et al.*, 1996, Tu *et al.*, 2008). Furthermore, it was elucidated recently that the Qrr sRNAs directly downregulate the translation of *luxMN* by

direct base pairing (Figure 1.5 A). Therefore, it is possible for *V. harveyi* to adjust the relative sensitivity to AI signals. With increasing HAI-1 concentrations the transcript levels of *luxMN* increase and this guarantees a higher response to the intra-species signal (Teng *et al.*, 2011). On the opposite, at low cell densities, meaning low LuxN copy numbers, the cells are more responsive to AI-2.

1.4 Quorum Quenching

The term Quorum Quenching (QQ) refers to all processes involved in the disturbance of QS (Dong *et al.*, 2001). QQ molecules differ extremely in their nature (chemical compounds or enzymes), their mode of action (QS signal degradation, competitive inhibition) as well as in their specific targets (Grandclement *et al.*, 2016). The ability for the disturbance of QS by a bacterial population could give an advantage in specific environments, where different populations compete for limiting resources. Several pathogenic bacteria use QS to coordinate their virulence. Therefore, the hosts ability to disrupt cell-to-cell communication could be essential to prevent colonization (Waters & Bassler, 2005). Up to now numerous AHL-degrading or modifying enzymes have been reported and can be grouped in four distinct classes. Lactonases, which are able to open the homoserine lactone ring (Uroz *et al.*, 2008), and acylases, which cleave AHLs at the amide bond and thereby release fatty acid and homoserine lactone (Lin *et al.*, 2003). Furthermore, reductases which convert 3-oxo-substituted AHL to their cognate 3-hydroxyl-substituted AHL (Bijtenhoorn *et al.*, 2011) and cytochrome oxidases which catalyze oxidation of the acyl chain (Chowdhary *et al.*, 2007). Some bacteria even degrade their own AIs and thereby terminate the QS activities. *Agrobacterium tumefaciens* produces the AttM AHL lactonase to degrade the AI in late growth stage, where group behaviors are hypothesized to be disadvantageous for the population (Zhang *et al.*, 2002). In *E. coli* AI-2 concentration is adjusted through active import via the Lsr system and phosphorylation by the kinase LsrK to P-AI-2 which leads to an elevated degradation of the AI (Xavier & Bassler, 2005). Some rhizospheric bacterial strains produce volatile organic compounds (VOC) such as dimethyl disulfide and thereby inhibit AHL-based QS regulation (Chernin *et al.*, 2011). Various eukaryotic mechanisms that counteract bacterial QS have been described. The Australian red microalga *Delisea pulchra* produces a mixture of halogenated furanones, which harbor structural similarities to AHLs, on its surface as a natural defense mechanism to prevent microbial biofouling (Givskov *et al.*, 1996). It was shown that these natural furanones disrupt AHL- as well as AI-2 mediated signaling in *V. harveyi* (Ren *et al.*, 2001). The natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone inhibits the QS-regulated gene expression in *V.*

harveyi by decreasing the DNA-binding activity of the master regulator LuxR (Defoirdt *et al.*, 2007). The fact that this natural furanone blocks all three channels by acting at the end of the QS cascade makes it a good choice for the protection of different hosts from pathogenic Vibrios (Tinh *et al.*, 2007, Defoirdt *et al.*, 2006). Furthermore, the chances to develop resistances are quite low, as the furanone shows no effect on the growth of Vibrios and hence no selective pressure on them (Defoirdt *et al.*, 2006, Manefield *et al.*, 2000).

1.5 Bacteriophages

Viruses are the most abundant entities on earth (Weitz & Wilhelm, 2012). Viruses targeting bacterial cells are so called bacteriophages and most if not all bacterial species are targeted by bacteriophages (Canchaya *et al.*, 2003). Furthermore, seawater contains up to $2.5x10^8$ virus particles per milliliter and is therefore one of the richest natural sources for free viruses (Bergh *et al.*, 1989). Bacteriophages and genetic elements, like pathogenicity islands and prophage-like elements make up an extensive amount of bacterial genomes (Nanda *et al.*, 2015). Phages penetrate the bacterial plasma membrane in order to release genetic material into the cytoplasm (Ackermann & DuBow, 1987). Depending on their life cycle, phages can be divided into two groups, lytic or lysogenic phages. During the lytic cycle (Figure 1.6 A), transcription and replication of the phage DNA takes place by hijacking the bacterial cell machineries. Subsequently, the viral components are encapsulated to form new complete phages. Each bacterial cell is filled with typically 100-200 viruses, which are released for new infections upon lysis of the bacterial cell (Ackermann & DuBow, 1987). Well known representatives for this group are the *E. coli* infecting phages T4 and T7 (Simon, 1972, Saigo, 1975).

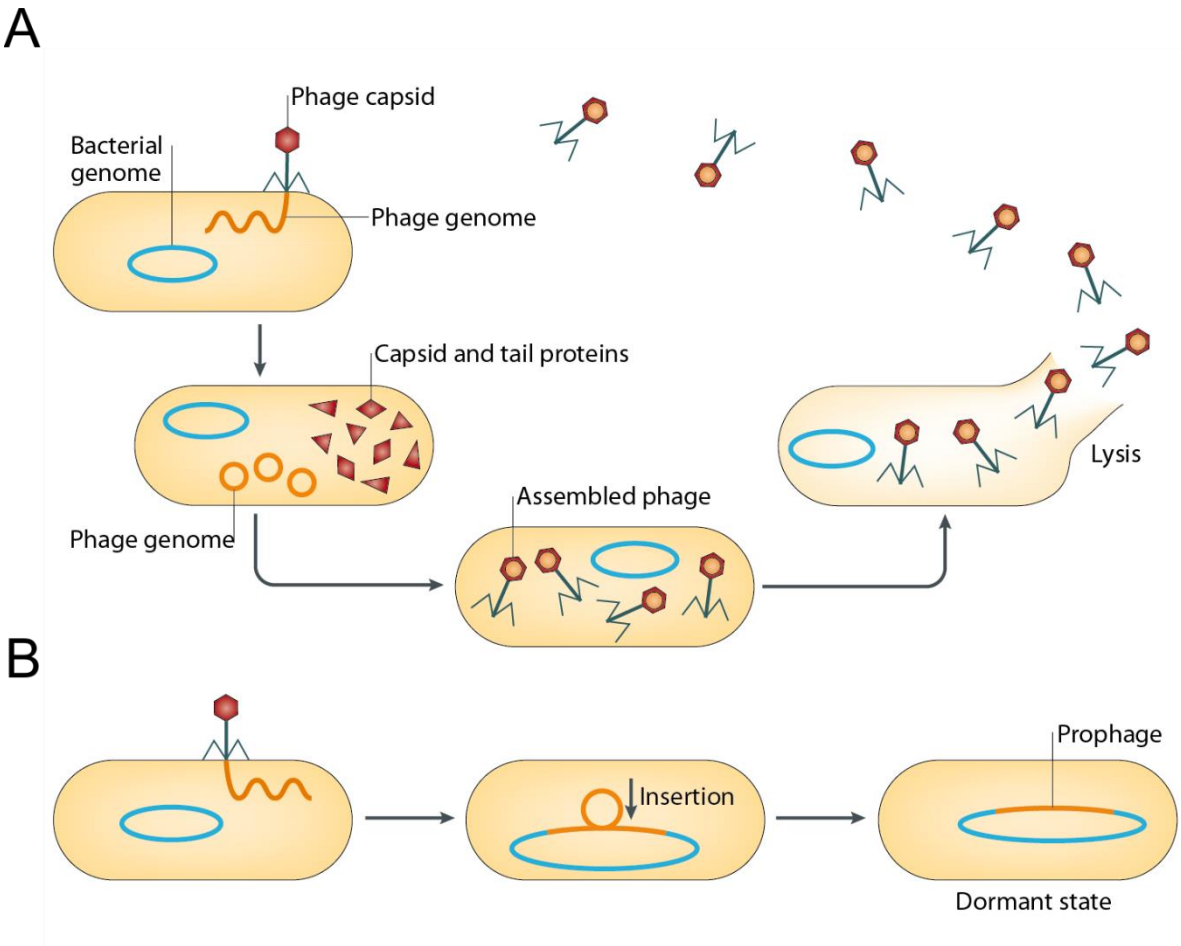


Figure 1.6 Scheme of lytic (A) and lysogenic (B) phage life style. A) Subsequently after the injection of the viral DNA, the bacterial machineries are used for genome replication, phage capsid and tail protein synthesis. Phage particles are assembled and released after bacterial lysis. **B)** Temperate phages exhibit a lysogenic lifestyle. Therefore, the virus genome is integrated in the bacterial chromosome. This dormant state does not lead to immediate bacterial cell death or production of new phage particles. The prophages replicate together with the bacterial host. Figure modified from Feiner *et al*., 2015 (Feiner *et al.*, 2015).

On the contrary, the lysogenic life cycle does not result in an immediate replication and release of new phages (Figure 1.6 B). Instead, it leads to an integration of the injected phage DNA into the hosts genome and replicates as part of the bacterial chromosome. The expression of the viral genes, which would be necessary for phage replication are repressed, but other phage genes can be expressed in a state called lysogenic conversion. The expression of these genes could even benefit the fitness of the bacterium (Little, 2005). This so called prophage normally remains dormant until the lytic cycle is induced.

1.5.1 Mechanisms responsible for the conversion from lysogenic to lytic life style

Upon a change to stressful conditions leading to DNA damage, temperate phages switch from lysogenic to lytic lifestyle (Little, 1996). Subsequently, the phage genome is excised and lytic genes, which support DNA replication, assembly of viral particles, DNA packaging and lysis of the bacterial host are expressed. The factors causing DNA damage can be either of intrinsic

or extrinsic nature. It was shown that spontaneously induced SOS response can lead to the induction of prophages (Nanda *et al.*, 2014). Ongoing replication, during growth, can result in sporadic DNA damage which leads to a derepression of genes required for the SOS response (Cox *et al.*, 2000). Furthermore, it was elucidated for a *recA* deficient mutant that no recognizable spontaneous prophage induction took place. In addition to the intrinsic factors which affect RecA or the DNA and thereby induce the SOS response, several extrinsic factors, like UV radiation or reactive oxygen species, as well as the antibiotic mitomycin C, heat stress or pH are known to result in DNA damage (Lee *et al.*, 2012, Tomasz, 1995, Choi *et al.*, 2010). Moreover, for phages that are in the lysogenic state it was shown that even in the absence of stress conditions a subpopulation of the cells exhibits spontaneous prophage induction (Nanda *et al.*, 2014, Lwoff, 1953). For phage genome integration and excision, specific phage DNA encoded recombinases (integrases and excisionases) are necessary. Crucial for the specificity of the recombination are attachment sites in the bacterial genome (*attB*) and in the phage genome (*attP*) (Nash, 1981). However, a random integration in the bacterial genome was shown for some phages, like phage Mu (Harshey, 2012).

1.5.2 Impact of prophages on the fitness of bacteria

As mentioned in chapter 1.5 during the lysogenic cycle the phage genome is integrated in the bacterial genome. The phage is described as a prophage and the bacterial cell as lysogen. Strikingly, around 25% of phage genomes occur as prophages (Casjens, 2005). This is not surprising as phage genomes harbor various genes which provide a fitness advantage for the host bacteria if they are expressed (Bondy-Denomy & Davidson, 2014). Changes of the hosts phenotype mediated by phages are referred to as “lysogenic conversion”.

One advantageous effect of prophages is their inhibition of infection through other phages, including themselves, the so called “superinfection exclusion” (Suttle, 2005). In several cases, bacteria are converted from harmless bacteria to significant pathogens through the integration of a phage. For example, *V. cholerae* was turned from a harmless marine bacterium to the causative agent of the disease cholera by the acquisition of prophage VPIΦ encoding the toxin co-regulated pilus. In humans, this resembles a colonizing factor and furthermore a receptor for infection by phage CTXΦ, which is responsible for production of cholera toxin (Waldor & Mekalanos, 1996, Karaolis *et al.*, 1999). In *Shewanella oneidensis* MR-1 it was shown that spontaneous phage induction in the early growth phase is escorted by the release of DNA and necessary for proper biofilm formation (Gödeke *et al.*, 2011). As elucidated recently in *Enterococus faecalis*, AI-2 induces phages and thereby supports the distribution of virulence

genes via lateral gene transfer (Rossmann *et al.*, 2015). As described in Chapter 1.2.1 AI-2 is also a part of the QS cascade in *V. harveyi* and it is possible that lysis mediated by potential phages in the bacterium could cause a population breakdown.

1.6 Scope of this thesis

QS is a bacterial communication process and involves the production and perception of chemical signals called AIs. *V. harveyi* harbors an exceptionally complex QS cascade, characterized by the production and perception of three different AI signals and by processing the underlying information in one common phosphorelay system. Despite the fact that the cascade is already well studies, there are still unsolved questions, as most of the studies deprived the system by one input. Within this thesis the information processing capacity shall be investigated regarding all inputs and by investigating the resultant specific outputs. This study aims to understand why such a complex QS architecture has evolved. Do the signals employ a form of hierarchy with regard to QS activation? Therefore, the individual impact of each subsystem to the QS output shall be clarified. In order to investigate potential differences between individual cells, the output should be assessed at the population as well as at the single-cell level. The AI sensors are bifunctional enzymes and can employ kinase as well as phosphatase activity depending on the absence or presence of the AIs. Therefore, their individual enzymatic activities shall be investigated *in vitro*. Furthermore, the amounts and localization of the QS components could influence the information processing capacity and QS activation and shall therefore be determined.

Inter- and intra-species communication often plays a role in virulence regulation of pathogens, therefore strategies that interfere with QS could serve as new treatments against bacterial diseases. Hence, cellular targets of natural QS disruptors should be elucidated and their mode of action should be characterized in *V. harveyi*. Furthermore, due to the newly identified link between QS and prophage induction in *E. faecalis* potential intact prophages in the genome of *V. harveyi* shall be discovered and an initial characterization should be performed.

1.7 References for Introduction

Ackermann, H.W. & M.S. DuBow, (1987) *Viruses of Prokaryotes: General properties of bacteriophages*. CRC Press, Boca Raton, FL.

Allen, R.D. & P. Baumann, (1971) Structure and arrangement of flagella in species of the genus *Beneckea* and *Photobacterium fischeri*. *J Bacteriol* **107**: 295-302.

Anetzberger, C., T. Pirch & K. Jung, (2009) Heterogeneity in quorum sensing-regulated bioluminescence of *Vibrio harveyi*. *Mol Microbiol* **73**: 267-277.

Anetzberger, C., M. Reiger, A. Fekete, U. Schell, N. Stambrau, L. Plener, J. Kopka, P. Schmitt-Kopplin, H. Hilbi & K. Jung, (2012) Autoinducers act as biological timers in *Vibrio harveyi*. *PLoS One* **7**: e48310.

Bassler, B.L. & R. Losick, (2006) Bacterially speaking. *Cell* **125**: 237-246.

Bassler, B.L., M. Wright & M.R. Silverman, (1994) Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. *Mol Microbiol* **13**: 273-286.

Bergh, O., K.Y. Borsheim, G. Bratbak & M. Heldal, (1989) High abundance of viruses found in aquatic environments. *Nature* **340**: 467-468.

Bijtenhoorn, P., H. Mayerhofer, J. Müller-Dieckmann, C. Utpatel, C. Schipper, C. Hornung, M. Szesny, S. Grond, A. Thürmer, E. Brzuszkiewicz, R. Daniel, K. Dierking, H. Schulenburg & W.R. Streit, (2011) A novel metagenomic short-chain dehydrogenase/reductase attenuates *Pseudomonas aeruginosa* biofilm formation and virulence on *Caenorhabditis elegans*. *PLoS One* **6**: e26278.

Bondy-Denomy, J. & A.R. Davidson, (2014) When a virus is not a parasite: the beneficial effects of prophages on bacterial fitness. *J Microbiol* **52**: 235-242.

Canchaya, C., C. Proux, G. Fournous, A. Bruttin & H. Brussow, (2003) Prophage genomics. *Microbiol Mol Biol Rev* **67**: 238-276.

Cao, J.G. & E.A. Meighen, (1989) Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. *J Biol Chem* **264**: 21670-21676.

Casjens, S.R., (2005) Comparative genomics and evolution of the tailed-bacteriophages. *Curr Opin Microbiol* **8**: 451-458.

Catap, E.S., C.R. Lavilla-Pitogo, Y. Maeno & R.D. Travina, (2003) Occurrence, histopathology and experimental transmission of hepatopancreatic parvovirus infection in *Penaeus monodon* postlarvae. *Dis Aquat Organ* **57**: 11-17.

Chatterjee, J., C.M. Miyamoto & E.A. Meighen, (1996) Autoregulation of *luxR*: the *Vibrio harveyi lux*-operon activator functions as a repressor. *Mol Microbiol* **20**: 415-425.

Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B.L. Bassler & F.M. Hughson, (2002) Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**: 545-549.

Chernin, L., N. Toklikishvili, M. Ovadis, S. Kim, J. Ben-Ari, I. Khmel & A. Vainstein, (2011) Quorum-sensing quenching by rhizobacterial volatiles. *Environ Microbiol Rep* **3**: 698-704.

Choi, J., S.M. Kotay & R. Goel, (2010) Various physico-chemical stress factors cause prophage induction in *Nitrosospira multiformis* 25196--an ammonia oxidizing bacteria. *Water Res* **44**: 4550-4558.

Chowdhary, P.K., N. Keshavan, H.Q. Nguyen, J.A. Peterson, J.E. Gonzalez & D.C. Haines, (2007) *Bacillus megaterium* CYP102A1 oxidation of acyl homoserine lactones and acyl homoserines. *Biochemistry* **46**: 14429-14437.

Cox, M.M., M.F. Goodman, K.N. Kreuzer, D.J. Sherratt, S.J. Sandler & K.J. Marians, (2000) The importance of repairing stalled replication forks. *Nature* **404**: 37-41.

Defoirdt, T., R. Crab, T.K. Wood, P. Sorgeloos, W. Verstraete & P. Bossier, (2006) Quorum sensing-disrupting brominated furanones protect the gnotobiotic brine shrimp *Artemia*

franciscana from pathogenic *Vibrio harveyi*, *Vibrio campbellii*, and *Vibrio parahaemolyticus* isolates. *Appl Environ Microbiol* **72**: 6419-6423.

Defoirdt, T., C.M. Miyamoto, T.K. Wood, E.A. Meighen, P. Sorgeloos, W. Verstraete & P. Bossier, (2007) The natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone disrupts quorum sensing-regulated gene expression in *Vibrio harveyi* by decreasing the DNA-binding activity of the transcriptional regulator protein LuxR. *Environ Microbiol* **9**: 2486-2495.

Dong, Y.-H., L.-H. Wang, J.-L. Xu, H.-B. Zhang, X.-F. Zhang & L.-H. Zhang, (2001) Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature* **411**: 813-817.

Dutta, R., L. Qin & M. Inouye, (1999) Histidine kinases: diversity of domain organization. *Mol Microbiol* **34**: 633-640.

Eberl, L., (1999) N-acyl homoserinelactone-mediated gene regulation in gram-negative bacteria. *Syst Appl Microbiol* **22**: 493-506.

Feiner, R., T. Argov, L. Rabinovich, N. Sigal, I. Borovok & A.A. Herskovits, (2015) A new perspective on lysogeny: prophages as active regulatory switches of bacteria. *Nat Rev Micro* **13**: 641-650.

Feng, L., Steven T. Rutherford, K. Papenfort, John D. Bagert, Julia C. van Kessel, David A. Tirrell, Ned S. Wingreen & Bonnie L. Bassler, (2015) A Qrr noncoding RNA deploys four different regulatory mechanisms to optimize quorum-sensing dynamics. *Cell* **160**: 228-240.

Freeman, J.A. & B.L. Bassler, (1999a) A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi*. *Mol Microbiol* **31**: 665-677.

Freeman, J.A. & B.L. Bassler, (1999b) Sequence and function of LuxU: a two-component phosphorelay protein that regulates quorum sensing in *Vibrio harveyi*. *J Bacteriol* **181**: 899-906.

Fuqua, C., M.R. Parsek & E.P. Greenberg, (2001) Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet* **35**: 439-468.

Fuqua, W.C., S.C. Winans & E.P. Greenberg, (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* **176**: 269-275.

Galperin, M.Y., (2006) Structural classification of bacterial response regulators: diversity of output domains and domain combinations. *J Bacteriol* **188**: 4169-4182.

Givskov, M., R. de Nys, M. Manefield, L. Gram, R. Maximilien, L. Eberl, S. Molin, P.D. Steinberg & S. Kjelleberg, (1996) Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. *J Bacteriol* **178**: 6618-6622.

Gödeke, J., K. Paul, J. Lassak & K.M. Thormann, (2011) Phage-induced lysis enhances biofilm formation in *Shewanella oneidensis* MR-1. *ISME J* **5**: 613-626.

Grandclement, C., M. Tannieres, S. Morera, Y. Dessaux & D. Faure, (2016) Quorum quenching: role in nature and applied developments. *FEMS Microbiol Rev* **40**: 86-116.

Greenberg, E.P., (2003) Bacterial communication and group behavior. *J Clin Invest* **112**: 1288-1290.

Hakenbeck, R. & J.B. Stock, (1996) Analysis of two-component signal transduction systems involved in transcriptional regulation. *Methods Enzymol* **273**: 281-300.

Harshey, R.M., (2012) The Mu story: how a maverick phage moved the field forward. *Mob DNA* **3**: 21.

Hartmann, A. & A. Schikora, (2012) Quorum sensing of bacteria and trans-kingdom interactions of N-acyl homoserine lactones with eukaryotes. *J Chem Ecol* **38**: 704-713.

Henares, B.M., K.E. Higgins & E.M. Boon, (2012) Discovery of a nitric oxide responsive quorum sensing circuit in *Vibrio harveyi*. *ACS Chem Biol* **7**: 1331-1336.

Henke, J.M. & B.L. Bassler, (2004) Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi*. *J Bacteriol* **186**: 6902-6914.

Janiak-Spens, F. & A.H. West, (2000) Functional roles of conserved amino acid residues surrounding the phosphorylatable histidine of the yeast phosphorelay protein YPD1. *Mol Microbiol* **37**: 136-144.

Jiravanichpaisal, P., T. Miyazaki & C. Limsuwan, (1994) Histopathology, biochemistry, and pathogenicity of *Vibrio harveyi* infecting black tiger prawn *Penaeus monodon*. *J Aquat Anim Health* **6**: 27-35.

Jung, K., T. Odenbach & M. Timmen, (2007) The quorum-sensing hybrid histidine kinase LuxN of *Vibrio harveyi* contains a periplasmically located N terminus. *J Bacteriol* **189**: 2945-2948.

Karaolis, D.K., S. Somara, D.R. Maneval, Jr., J.A. Johnson & J.B. Kaper, (1999) A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. *Nature* **399**: 375-379.

Kato, M., T. Mizuno & T. Hakoshima, (1998) Crystallization of a complex between a novel C-terminal transmitter, HPt domain, of the anaerobic sensor kinase ArcB and the chemotaxis response regulator CheY. *Acta Crystallogr D Biol Crystallogr* **54**: 140-142.

Ke, X., L.C. Miller & B.L. Bassler, (2015) Determinants governing ligand specificity of the *Vibrio harveyi* LuxN quorum-sensing receptor. *Mol Microbiol* **95**: 127-142.

Keller, L. & M.G. Surette, (2006) Communication in bacteria: an ecological and evolutionary perspective. *Nat Rev Microbiol* **4**: 249-258.

Khan, S., J.L. Spudich, J.A. McCray & D.R. Trentham, (1995) Chemotactic signal integration in bacteria. *Proc Natl Acad Sci U S A* **92**: 9757-9761.

Kleerebezem, M., L.E. Quadri, O.P. Kuipers & W.M. de Vos, (1997) Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Mol Microbiol* **24**: 895-904.

Lapota, D., C. Galt, J.R. Losee, H.D. Huddell, J.K. Orzech & K.H. Nealson, (1988) Observations and measurements of planktonic bioluminescence in and around a milky sea. *J Exp Mar Biol Ecol* **119**: 55-81.

Lee, K.M., Y. Park, W. Bari, M.Y. Yoon, J. Go, S.C. Kim, H.I. Lee & S.S. Yoon, (2012) Activation of cholera toxin production by anaerobic respiration of trimethylamine N-oxide in *Vibrio cholerae*. *J Biol Chem* **287**: 39742-39752.

Lenz, D.H., K.C. Mok, B.N. Lilley, R.V. Kulkarni, N.S. Wingreen & B.L. Bassler, (2004) The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* **118**: 69-82.

Lilley, B.N. & B.L. Bassler, (2000) Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. *Mol Microbiol* **36**: 940-954.

Lin, B., Z. Wang, A.P. Malanoski, E.A. O'Grady, C.F. Wimpee, V. Vuddhakul, N. Alves Jr, F.L. Thompson, B. Gomez-Gil & G.J. Vora, (2010) Comparative genomic analyses identify the *Vibrio harveyi* genome sequenced strains BAA-1116 and HY01 as *Vibrio campbellii*. *Environ Microbiol Rep* **2**: 81-89.

Lin, Y.H., J.L. Xu, J. Hu, L.H. Wang, S.L. Ong, J.R. Leadbetter & L.H. Zhang, (2003) Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. *Mol Microbiol* **47**: 849-860.

Little, J.W., (1996) The SOS Regulatory System. In: Regulation of Gene Expression in *Escherichia coli*. Boston, MA: Springer US, pp. 453-479.

Little, J.W., (2005) Lysogeny, prophage induction, and lysogenic conversion. In: Phages. American Society of Microbiology, pp.

Lwoff, A., (1953) Lysogeny. *Bacteriol Rev* **17**: 269-337.

Lyon, G.J. & R.P. Novick, (2004) Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria. *Peptides* **25**: 1389-1403.

Manefield, M., L. Harris, S.A. Rice, R. de Nys & S. Kjelleberg, (2000) Inhibition of luminescence and virulence in the black tiger prawn (*Penaeus monodon*) pathogen *Vibrio harveyi* by intercellular signal antagonists. *Appl Environ Microbiol* **66**: 2079-2084.

McCarter, L.L., (2001) Polar flagellar motility of the *Vibrionaceae*. *Microbiol Mol Biol Rev* **65**: 445-462.

Miller, M.B. & B.L. Bassler, (2001) Quorum sensing in bacteria. *Annu Rev Microbiol* **55**: 165-199.

Miller, S.D., S.H. Haddock, C.D. Elvidge & T.F. Lee, (2005) Detection of a bioluminescent milky sea from space. *Proc Natl Acad Sci U S A* **102**: 14181-14184.

Mok, K.C., N.S. Wingreen & B.L. Bassler, (2003) *Vibrio harveyi* quorum sensing: a coincidence detector for two autoinducers controls gene expression. *EMBO J* **22**: 870-881.

Nanda, A.M., A. Heyer, C. Kramer, A. Grunberger, D. Kohlheyer & J. Frunzke, (2014) Analysis of SOS-induced spontaneous prophage induction in *Corynebacterium glutamicum* at the single-cell level. *J Bacteriol* **196**: 180-188.

Nanda, A.M., K. Thormann & J. Frunzke, (2015) Impact of spontaneous prophage induction on the fitness of bacterial populations and host-microbe interactions. *J Bacteriol* **197**: 410-419.

Nash, H.A., (1981) Integration and excision of bacteriophage lambda: the mechanism of conservation site specific recombination. *Annu Rev Genet* **15**: 143-167.

Nealson, K.H., T. Platt & J.W. Hastings, (1970) Cellular control of the synthesis and activity of the bacterial luminescent system. *J Bacteriol* **104**: 313-322.

Neiditch, M.B., M.J. Federle, S.T. Miller, B.L. Bassler & F.M. Hughson, (2005) Regulation of LuxPQ receptor activity by the quorum-sensing signal autoinducer-2. *Mol. Cell* **18**: 507-518.

Neiditch, M.B., M.J. Federle, A.J. Pompeani, R.C. Kelly, D.L. Swem, P.D. Jeffrey, B.L. Bassler & F.M. Hughson, (2006) Ligand-induced asymmetry in histidine sensor kinase complex regulates quorum sensing. *Cell* **126**: 1095-1108.

Ng, W.L., L.J. Perez, Y. Wei, C. Kraml, M.F. Semmelhack & B.L. Bassler, (2011) Signal production and detection specificity in *Vibrio* CqsA/CqsS quorum-sensing systems. *Mol Microbiol* **79**: 1407-1417.

Pawson, C.T. & J.D. Scott, (2010) Signal integration through blending, bolstering and bifurcating of intracellular information. *Nat Struct Mol Biol* **17**: 653-658.

Perraud, A.L., V. Weiss & R. Gross, (1999) Signalling pathways in two-component phosphorelay systems. *Trends Microbiol* **7**: 115-120.

Peters, N.K., J.W. Frost & S.R. Long, (1986) A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. *Science* **233**: 977-980.

Plener, L., N. Lorenz, M. Reiger, T. Ramalho, U. Gerland & K. Jung, (2015) The phosphorylation flow of the *Vibrio harveyi* quorum sensing cascade determines levels of phenotypic heterogeneity in the population. *J Bacteriol* **197**: 1747-1756.

Pompeani, A.J., J.J. Irgon, M.F. Berger, M.L. Bulyk, N.S. Wingreen & B.L. Bassler, (2008) The *Vibrio harveyi* master quorum-sensing regulator, LuxR, a TetR-type protein is both an activator and a repressor: DNA recognition and binding specificity at target promoters. *Mol Microbiol* **70**: 76-88.

Ren, D., J.J. Sims & T.K. Wood, (2001) Inhibition of biofilm formation and swarming of *Escherichia coli* by (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone. *Environ Microbiol* **3**: 731-736.

Rombel, I., A. North, I. Hwang, C. Wyman & S. Kustu, (1998) The bacterial enhancer-binding protein NtrC as a molecular machine. *Cold Spring Harb Symp Quant Biol* **63**: 157-166.

Rossmann, F.S., T. Racek, D. Wobser, J. Puchalka, E.M. Rabener, M. Reiger, A.P. Hendrickx, A.K. Diederich, K. Jung, C. Klein & J. Huebner, (2015) Phage-mediated dispersal of biofilm and distribution of bacterial virulence genes is induced by quorum sensing. *PLoS Pathog* **11**: e1004653.

Rutherford, S.T., J.C. van Kessel, Y. Shao & B.L. Bassler, (2011) AphA and LuxR/HapR reciprocally control quorum sensing in vibrios. *Genes Dev* **25**: 397-408.

Saigo, K., (1975) Polar DNA ejection in bacteriophage T7. *Virology* **65**: 120-127.

Schauder, S., K. Shokat, M.G. Surette & B.L. Bassler, (2001) The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Mol Microbiol* **41**: 463-476.

Simon, L.D., (1972) Infection of *Escherichia coli* by T2 and T4 bacteriophages as seen in the electron microscope: T4 head morphogenesis. *Proc Natl Acad Sci U S A* **69**: 907-911.

Stambrau, N., (2008) Der LuxP/AI-2- und LuxQ-abhängige Signaltransduktionsweg des Quorum sensing-Systems von *V. harveyi*. In: Dissertation, LMU München. LMU München, pp.

Suttle, C.A., (2005) Viruses in the sea. *Nature* **437**: 356-361.

Swem, L.R., D.L. Swem, N.S. Wingreen & B.L. Bassler, (2008) Deducing receptor signaling parameters from *in vivo* analysis: LuxN/AI-1 quorum sensing in *Vibrio harveyi*. *Cell* **134**: 461-473.

Teng, S.W., J.N. Schaffer, K.C. Tu, P. Mehta, W. Lu, N.P. Ong, B.L. Bassler & N.S. Wingreen, (2011) Active regulation of receptor ratios controls integration of quorum-sensing signals in *Vibrio harveyi*. *Mol Syst Biol* **7**: 491.

Timmen, M., B.L. Bassler & K. Jung, (2006) AI-1 influences the kinase activity but not the phosphatase activity of LuxN of *Vibrio harveyi*. *J Biol Chem* **281**: 24398-24404.

Tinh, N.T., N.D. Linh, T.K. Wood, K. Dierckens, P. Sorgeloos & P. Bossier, (2007) Interference with the quorum sensing systems in a *Vibrio harveyi* strain alters the growth rate of gnotobiotically cultured rotifer *Brachionus plicatilis*. *J Appl Microbiol* **103**: 194-203.

Tomasz, M., (1995) Mitomycin C: small, fast and deadly (but very selective). *Chem Biol* **2**: 575-579.

Tu, K.C. & B.L. Bassler, (2007) Multiple small RNAs act additively to integrate sensory information and control quorum sensing in *Vibrio harveyi*. *Genes Dev* **21**: 221-233.

Tu, K.C., T. Long, S.L. Svenningsen, N.S. Wingreen & B.L. Bassler, (2010) Negative feedback loops involving small regulatory RNAs precisely control the *Vibrio harveyi* quorum-sensing response. *Mol Cell* **37**: 567-579.

Tu, K.C., C.M. Waters, S.L. Svenningsen & B.L. Bassler, (2008) A small-RNA-mediated negative feedback loop controls quorum-sensing dynamics in *Vibrio harveyi*. *Mol Microbiol* **70**: 896-907.

Turgay, K., J. Hahn, J. Burghoorn & D. Dubnau, (1998) Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. *EMBO J* **17**: 6730-6738.

Ulrich, D.L., D. Kojetin, B.L. Bassler, J. Cavanagh & J.P. Loria, (2005) Solution structure and dynamics of LuxU from *Vibrio harveyi*, a phosphotransferase protein involved in bacterial quorum sensing. *J Mol Biol* **347**: 297-307.

Uroz, S., P.M. Oger, E. Chapelle, M.T. Adeline, D. Faure & Y. Dessaux, (2008) A *Rhodococcus qsdA*-encoded enzyme defines a novel class of large-spectrum quorum-quenching lactonases. *Appl Environ Microbiol* **74**: 1357-1366.

van Kessel, J.C., S.T. Rutherford, Y. Shao, A.F. Utria & B.L. Bassler, (2013a) Individual and combined roles of the master regulators AphA and LuxR in control of the *Vibrio harveyi* quorum-sensing regulon. *J Bacteriol* **195**: 436-443.

van Kessel, J.C., L.E. Ulrich, I.B. Zhulin & B.L. Bassler, (2013b) Analysis of activator and repressor functions reveals the requirements for transcriptional control by LuxR, the master regulator of quorum sensing in *Vibrio harveyi*. *MBio* **4**.

Visick, K.L. & C. Fuqua, (2005) Decoding microbial chatter: cell-cell communication in bacteria. *J Bacteriol* **187**: 5507-5519.

Waldor, M.K. & J.J. Mekalanos, (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**: 1910-1914.

Walker, J.E., M. Saraste & N.J. Gay, (1982) *E. coli* F1-ATPase interacts with a membrane protein component of a proton channel. *Nature* **298**: 867-869.

Waters, C.M. & B.L. Bassler, (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* **21**: 319-346.

Waters, C.M. & B.L. Bassler, (2006) The *Vibrio harveyi* quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. *Genes Dev* **20**: 2754-2767.

Weitz, J.S. & S.W. Wilhelm, (2012) Ocean viruses and their effects on microbial communities and biogeochemical cycles. *F1000 Biol Rep* **4**: 17.

Whitehead, N.A., A.M. Barnard, H. Slater, N.J. Simpson & G.P. Salmond, (2001) Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol Rev* **25**: 365-404.

Xavier, K.B. & B.L. Bassler, (2005) Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in *Escherichia coli*. *J Bacteriol* **187**: 238-248.

Zhang, H.B., L.H. Wang & L.H. Zhang, (2002) Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. *Proc Natl Acad Sci U S A* **99**: 4638-4643.

2 The phosphorylation flow of the *Vibrio harveyi* Quorum-Sensing cascade determines levels of phenotypic heterogeneity in the population

Plener, L.[*], Lorenz, N.[*], Reiger, M., Ramalho, T., Gerland, U., Jung, K. (2015). The phosphorylation flow of the *Vibrio harveyi* quorum-sensing cascade determines levels of phenotypic heterogeneity in the population. *J Bacteriol* 197:1747-1756. doi: 10.1128/JB.02544-14

[*] Authors contributed equally

Abstract

Quorum sensing (QS) is a communication process that enables a bacterial population to coordinate and synchronize specific behaviors. The bioluminescent marine bacterium *Vibrio harveyi* integrates three autoinducer (AI) signals into one quorum sensing cascade comprising a phosphorelay involving three hybrid sensor kinases: LuxU; LuxO, an Hfq/small RNA (sRNA) switch; and the transcriptional regulator LuxR. Using a new set of *V. harveyi* mutants lacking genes for the AI synthases and/or sensors, we assayed the activity of the quorum-sensing cascade at the population and single-cell levels, with a specific focus on signal integration and noise levels. We found that the ratios of kinase activities to phosphatase activities of the three sensors and, hence, the extent of phosphorylation of LuxU/LuxO are important not only for the signaling output but also for the degree of noise in the system. The pools of phosphorylated LuxU/LuxO per cell directly determine the amounts of sRNAs produced and, consequently, the copy number of LuxR, generating heterogeneous quorum-sensing activation at the single-cell level. We conclude that the ability to drive the heterogeneous expression of QS-regulated genes in *V. harveyi* is an inherent feature of the architecture of the QS cascade.

Full-text article:

http://jb.asm.org/content/197/10/1747.long

Supplemental Material:

http://jb.asm.org/content/197/10/1747/suppl/DCSupplemental

3 Activity, abundance and localization of Quorum Sensing Receptors in *Vibrio harveyi*

Manuscript

Nicola Lorenz[1], Jae-Yen Shin[1] and Kirsten Jung[1].

[1]Munich Center for Integrated Protein Science (CiPSM) at the Department of Biology I, Microbiology, Ludwig-Maximilians-Universität München, Martinsried, Germany

Running Title: hybrid histidine kinases in *Vibrio*

To whom correspondence should be addressed: Prof. Dr. K. Jung, Ludwig-Maximilians-Universität München, Department Biologie I, Bereich Mikrobiologie, Großhaderner Str. 2-4, D-82152 Martinsried, Germany. Tel:+49-89-2180-74500; Fax:+49-89-2180-74520; E-mail: jung@lmu.de

Abstract

Quorum sensing (QS) is a process enabling a bacterial population to communicate via small molecules called autoinducers (AIs). This intercellular communication process allows single cells to synchronize their behavior within a population. The marine bacterium *Vibrio harveyi* ATCC BAA-1116 processes the information of three AI signals into one QS cascade. The AIs are perceived by three hybrid histidine kinases (LuxN, Lux(P)Q and CqsS) and the information is transduced via phosphorelay to the histidine phosphotransfer (HPt) protein LuxU and subsequently to the response regulator LuxO. Hence, the level of phosphorylated LuxO depends on the AI concentrations. P-LuxO controls the expression of small regulatory RNAs in a σ^{54}-dependent manner. These sRNAs, together with the chaperon Hfq, destabilize the transcript of the master regulator *luxR*. LuxR is responsible for the induction and repression of several genes (e.g. for bioluminescence, exoprotease and siderophore production). It was shown that the ratio between P-LuxU/LuxU and therefore P-LuxO/LuxO is crucial for the output and potential noise in the system. However, these studies were performed *in vivo* by analyzing corresponding deletion mutants. The specific impact of the inherent enzymatic activities of each sensor as well as their abundance was not known so far. Upon combined *in vivo* and *in vitro* approaches we report here, that the hybrid histidine kinases not only differ regarding their abundance, with LuxN being more abundant than LuxQ and CqsS, but also with respect to their enzymatic activities *in vitro*, in particular their kinase activities. LuxN showed the highest capacity to phosphorylate LuxU while the phosphatase activity was comparable to LuxQ and CqsS. Furthermore, our results indicate, that at least LuxN and LuxQ form cluster in the membrane.

Introduction

Vibrio harveyi ATCC-BAA 1116 (reclassified as *V. campbellii*) (Lin *et al.*, 2010) is a marine, free-living bacterium, which can be found on the surface of algae or as pathogen in shrimp or fish. One outstanding characteristic of this Gram-negative microorganism is its ability to produce bioluminescence in a cell density dependent manner called quorum sensing (QS). This communication process allows the cells to coordinate their behavior within a population by altering gene expression, upon a threshold concentration of signaling molecules (Keller & Surette, 2006). *V. harveyi* responds to three different classes of autoinducers (AIs) and the information is channeled into one phosphorelay cascade. The first AI is HAI-1 an acyl-

homoserine lactone [*N*-(3-hydroxybutyryl)-homoserine lactone], which is produced by the synthase LuxM and is a signaling molecule specific for *V. harveyi* (Cao & Meighen, 1989). The second one is AI-2, a furanosyl borate diester, synthesized by LuxS and can be regarded as a global signal as it is produced by various bacterial species (Chen *et al.*, 2002). CAI-1 is a long-chain amino ketone [*(Z)* 3-aminoundec-2-en-4one] (Ea-C8-CAI-1), produced by CqsA and is specific to members of the *Vibrio* genus (Ng *et al.*, 2011). It was shown recently that these AIs follow a distinct synthesis pattern, meaning they are not produced constitutively but differ in concentration and even growth phase. While the concentration of AI-2 increases during the exponential growth phase, HAI-1 and CAI-1 cannot be detected until late exponential phase (Anetzberger *et al.*, 2012). The AIs HAI-1, AI-2 and CAI-1 are perceived by three different receptors, the hybrid histidine kinases, LuxN, LuxQ (together with the periplasmic binding protein LuxP) and CqsS, respectively (Figure 3.1). These membrane-bound receptors comprise a transmitter domain, containing a dimerization and histidine phosphotransfer domain (DHp) and a catalytic and ATP-binding (CA) domain including the conserved histidine residue. Hybrid histidine kinases also contain a C-terminal receiver domain harboring a conserved aspartate residue. At low cell density (LCD) and therefore low AI concentrations the receptors act as kinases, thereby they get autophosphorylated at the conserved histidine residue and subsequently transfer the phosphoryl group to their conserved aspartate. Then, the phosphoryl group is transferred to the histidine phosphotransfer (HPt) protein LuxU, which in turn phosphorylates the response regulator LuxO at its conserved aspartate residue (Freeman & Bassler, 1999). P-LuxO is activated and induces, together with the sigma factor σ^{54}, the transcription of five small regulatory RNAs (Qrr1-5) (Lenz *et al.*, 2004). These small RNAs, together with the RNA chaperone Hfq, destabilize and degrade the mRNA of the master regulator *luxR*. Therefore, the positively regulated QS phenotypes remain in an OFF state (Tu & Bassler, 2007).

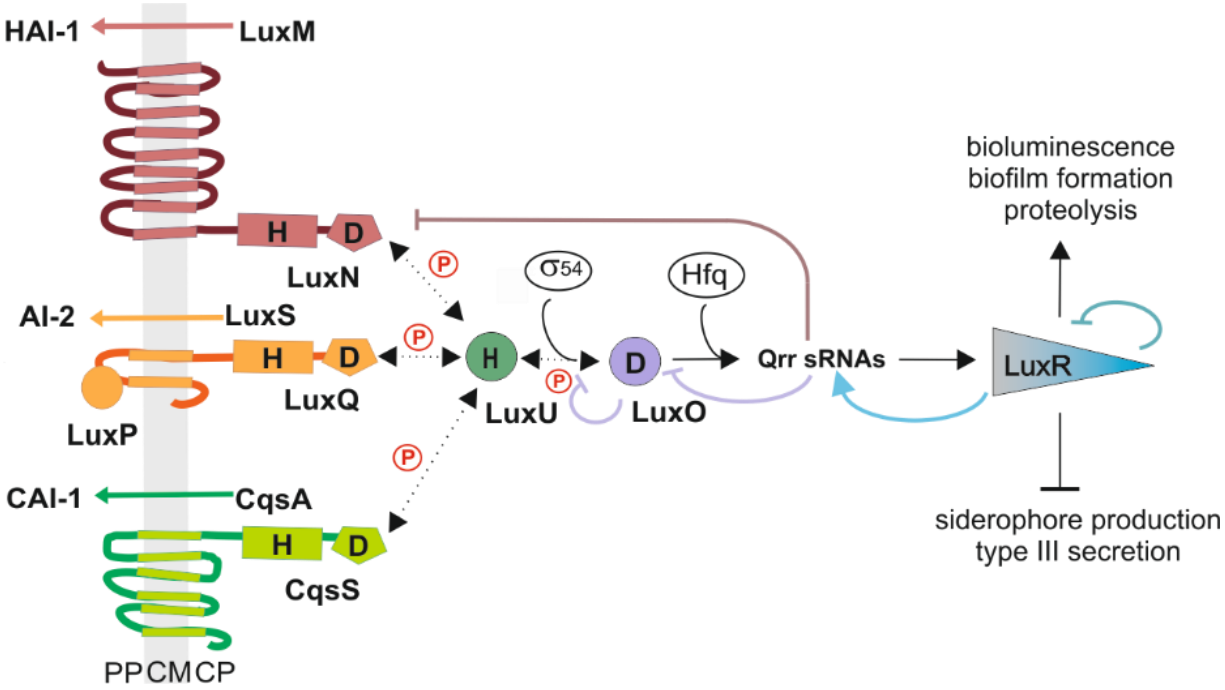


Figure 3.1 The QS cascade of *V. harveyi*. LuxM, LuxS and CqsA synthesize three different AIs (HAI-1, AI-2 and CAI-1). At low cell density and therefore low AI concentrations, the receptors autophosphorylate and transfer the phosphoryl group via phosphorelay, via the HPt protein LuxU, to the response regulator LuxO. P-LuxO, together with σ^{54}, induces the expression of five regulatory small RNAs (Qrr 1-5). These sRNAs along with the chaperon Hfq, destabilize the transcript of the QS master regulator LuxR. At HCD, meaning high AI concentrations, the AIs are sensed by the corresponding hybrid histidine kinases LuxN, LuxQ (together with the periplasmic binding protein LuxP) and CqsS, which leads to an inhibition of the kinase activity. Thereby the phosphate is drained from the cascade and LuxR is produced. LuxR induces the gene expression required for bioluminescence, biofilm formation and proteolysis and represses the expression of genes for siderophore production and type III secretion systems. H indicates histidine and D aspartate (phosphorylation sites). PP, periplasm; CM, cytoplasmic membrane; CP, cytoplasm. Dotted arrows indicate phosphotransfer and continuous arrows show transcriptional or posttranslational regulatory interactions. Figure adapted from Plener *et al.* (Plener *et al.*, 2015).

After accumulation and binding of the AIs to the receptors at high cell density (HCD), their kinase activities are inhibited, leading to dephosphorylation of LuxU and thereby the phosphoryl groups are drained from the cascade (Timmen *et al.*, 2006). LuxO is inactive and the sRNAs are not expressed anymore. Eventually, LuxR is produced and induces the expression of genes responsible for bioluminescence (Bassler *et al.*, 1994), biofilm formation (Anetzberger *et al.*, 2009) and represses genes encoding a type III secretion system (Henke & Bassler, 2004) as well as siderophores (Lilley & Bassler, 2000). Moreover, the complex QS cascade in *V. harveyi* comprises five feedback loops: LuxO and LuxR negatively regulate their own transcription by binding to the corresponding promoter regions (Tu *et al.*, 2010, Chatterjee *et al.*, 1996). LuxR directly activates the transcription of the sRNAs (Tu *et al.*, 2008). The sRNAs in turn control *luxO* mRNA via sequestration (Feng *et al.*, 2015). Furthermore, the translation of *luxMN* is negatively controlled by the sRNAs (Qrr 1-5) (Teng *et al.*, 2011).

It was shown recently that the kinase to phosphatase activity ratios of the hybrid histidine kinases and therefore the amount of phosphorylated LuxU/LuxO are important for the

signaling output and hence for the degree of noise in the system (Plener *et al.*, 2015). The copy number of the master regulator LuxR is directly determined by the pools of P-LuxU and P-LuxO and accordingly the amount of sRNAs per cell (Plener *et al.*, 2015). Using various *V. harveyi* mutants the impact of each subsystem was studied for QS activation at the population and single-cell level. It was found that in the presence of all AIs the output was homogeneous while in the absence of one or two AIs the QS activation varied from cell to cell (Plener *et al.*, 2015). However, this study focused on the cascade utilizing *in vivo* approaches with different receptor and/or synthase deletion mutants. In our opinion a comprehensive characterization of the sensors is important to elucidate the contribution of underlying molecular mechanisms to the generation of the differential impact of the AIs/sensors. In this study we identified differences in the kinase but not in the phosphatase activities between the hybrid histidine kinases *in vitro*. Furthermore, we demonstrate, that the copy number of the kinases differs not only on the transcript but also on the protein level. Eventually, here we show that LuxN and LuxQ form clusters *in vivo*, which might contribute to regulation processes within the complex QS signaling cascade.

Materials and Methods

Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Table 3.1. The *Escherichia coli* strains were aerobically grown in lysogeny broth (LB) (10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract) at 37°C in a rotary shaker. The *V. harveyi* strains were cultivated in autoinducer bioassay (AB) medium (Greenberg *et al.*, 1979) or Luria marine (LM) medium (20 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract) and were grown aerobically in a rotary shaker at 30°C. When required, media were solidified by using 1.5% (w/v) agar. If necessary, media were supplemented with 50 µg/ml kanamycin sulfate and/or 100 µg/ml ampicillin sodium salt. The conjugation strain *E. coli* WM3064 was grown in the presence of 300 µM meso-diaminopimelic acid (DAP).

For microscopy and in gel fluorescence *V. harveyi* cells were grown overnight in LM medium and afterwards inoculated 1:1,000 in fresh AB medium. For the in gel fluorescence approach cells were harvested in the early or late exponential phase (OD_{600} ~0.08 and ~0.7 respectively).

Table 3.1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference or source
Bacterial strains		
E. coli DH5α-λpir	F$^-$φ80d*lacZ* ΔM15 Δ (*lacZYA-argF*)U169 *recA1 hsdR17 deoR thi*-1 *supE44 gyrA96 relA1/λpir*	(Miller & Mekalanos, 1988)
E. coli WM3064	*thrB*1004 *pro thi rpsL hsdS lacZ* ΔM15 RP4-1360 Δ(*araBAD*)567 Δ*dapA*1341::[*erm pir*(wt)]	W. Metcalf, University of Illinois, Urbana-Champaign
E. coli JM109	*recA1 endA1 gyrA96 traD36 thi hsdR17 supE44 λ$^-$ relA1 Δ(lac-proAB)*/F' *proA$^+$B$^+$ lacI^q lacZD*M15	(Yanisch-Perron *et al.*, 1985)
Rosetta (DE3) pLysS	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysSRARE2 (Cam^R)	Novagen
E. coli MDAI-2	*luxS::*Tet^r-derivative of *E. coli* W3110	(DeLisa *et al.*, 2001)
V. harveyi ATCC BAA-1116	wild type	(Bassler *et al.*, 1997)
V. harveyi ATCC BAA-1116 *ΔluxNΔluxQΔcqsS*	wild type *ΔluxNΔluxQΔcqsS*	(Plener *et al.*, 2015)
V. harveyi ATCC BAA-1116 P$_{luxN}$-*luxN-mNeonGreen*	Integration of P$_{luxN}$-*luxN-mNeonGreen* at the native locus in *V. harveyi* ATCC BAA-1116	This study
V. harveyi ATCC BAA-1116 P$_{luxQ}$-*luxQ-mNeonGreen*	Integration of P$_{luxQ}$-*luxQ-mNeonGreen* at the native locus in *V. harveyi* ATCC BAA-1116	This study
V. harveyi ATCC BAA-1116 P$_{cqsS}$-*cqsS-mNeonGreen*	Integration of P$_{cqsS}$-*cqsS-mNeonGreen* at the native locus in *V. harveyi* ATCC BAA-1116	This study
Plasmids		
pGEX_LuxP	*luxP* in pGEX-4T1	(Neiditch *et al.*, 2005)
pQE30LuxU-6His	*luxU* in pQE30	(Timmen *et al.*, 2006)
pNKN	*luxN* in pPV5-10	(Anetzberger *et al.*, 2012)
pNKQ	*luxQ* in pPV5-10	(Anetzberger *et al.*, 2012)

pKK223-3 cqsS F175C	*cqsS F175C* in pKK223-2	This study
pNPTS138-R6KT-GFP	*mobRP4*$^{+}$ *ori*-R6K *sacB gfp; Km*^{r}	(Lorenz *et al.*, 2016)
pNPTS138-R6KT-mNeonGreen	*mobRP4*$^{+}$ *ori*-R6K *sacB mNeonGreen; Km*^{r}	This study
pNPTS138-R6KT P_{luxN}-*luxN-mNeonGreen*	*mobRP4+ ori*-R6K P_{luxN}-*luxN-mNeonGreen*; Km^{r}	This study
pNPTS138-R6KT P_{luxQ}-*luxQ-mNeonGreen*	*mobRP4+ ori*-R6K P_{luxQ}-*luxQ-mNeonGreen*; Km^{r}	This study
pNPTS138-R6KT P_{cqsS}-*cqsS-mNeonGreen*	*mobRP4+ ori*-R6K P_{cqsS}-*cqsS-mNeonGreen*; Km^{r}	This study

Generation of full-length protein fluorophore hybrids

Molecular methods were carried out according to standard protocols (Sambrook, 1989) or according to manufacturer´s instructions. Kits for the isolation of plasmids and purification of PCR products were purchased from Südlabor (Gauting, Germany). Enzymes were purchased from New England Biolabs (Frankfurt, Germany) and Fermentas (St. Leon-Rot, Germany). Chemically competent cells of *E. coli* were transformed with the corresponding plasmids (Inoue *et al.*, 1990).

mNeonGreen (Shaner *et al.*, 2013) (Allele Biotechnology, San Diego) was used to replace GFP in the vector pNPTS138-R6KT-GFP after restriction with SpeI and PspOMI. For construction of the full-length receptor mNeonGreen fusions (P_{luxN}-*luxN-mNeonGreen*, P_{luxQ}-*luxQ-mNeonGreen* and P_{cqsS}-*cqsS-mNeonGreen*) the coding sequence as well as promoter sequence of *luxN*, *luxQ* and *cqsS* were ligated into pNPTS138-R6KT-mNeonGreen after restriction with EcoRI/BamHI (in case of *luxN*) or BamHI/PspOMI (for *luxQ* and *cqsS*) (primer sequences can be found in Table 3.2). Chromosomal insertions of the fluorophore fusions into *V. harveyi* were achieved by integrating the resultant suicide vectors via RecA dependent single homologous recombination as described previously (Fried *et al.*, 2012). The conjugative plasmid transfer from donor strain *E. coli* WM3064 containing the required plasmid into *V. harveyi* was performed as described before. Therefore, the donor and the recipient strain were cultivated in LB medium up to an OD_{600} of 0.8 - 1.0, supplemented with 300 µM *meso*-diaminopimelic acid (DAP) for the growth of *E. coli* WM3064. Single colonies were checked for chromosomal integration via performance of a PCR with the genomic DNA.

Table 3.2 Primers used in this study

Name	**Sequence**
Fluorophor fusions	
mNeonGreen PspOMI s	CCGGGCCCATGGTGAGCAAGGGCGAGGAGGAT
mNeonGreen SpeI as	CCACTAGTTTACTTGTACAGCTCGTCCATGCC
LuxN sense 500 bp up	CGCGATACTTGGCTCAGCTACGCGCCA
LuxN BamHI ohne Stop as	CCGGATCCTTCTCTCTCAGCTTCACAAGC
LuxQ +300 bp up BamHI s	CCGGATCCCGACGTAGCATTAGGTGCA
LuxQ ohne stop PspOMI as	CCGGGCCCGGTTCTTTCTACCAAGAA
Up CqsS +500 bp BamHI s	CCGGATCCGCTCGATTAGATGCATGGTTT
cqsS PspOMI as	TAGGGCCCAATCCAGTTCGCAATCTTGTC
CqsS overproduction	
cqsS EcoRI s	GATAAAGAATTCATGGACGCGATTCGCAAAGTAT ATCAG
CA cqsS HindIII as	CAAGAGAAGCTTCTAATGATGATGATGATGATGA ATCCAGTTCGCAATCTTGTCG
CqsSF175C_s	TTGGTAACTTGTGCTACTTCCGA
CqsSF175C_as	TTTCGGAAGTAGCACAAGTTACC
qRT PCR	
luxN 142 s	GCAGCTTATATTGCGTACTCGGTGTG
luxN 293 as	GCAAAAGCAAATGCGAAGAAGGAAGC
luxQ 197 s	GCCACTTTGCGGCGATCCAGATACA
luxQ 353 as	GTTAGAAAACGGAATTCTGGTGTGTGC
cqsS 217 s	GCCTATTATCAAGTGGTCACAACGCT
cqsS 363 as	CATCACCGATGTGATATGCACGAGC
recA 240 s	GCTAACTCTTGAGCTTATTGCTGCTG
recA 395 as	AGCGCTTGCTCACCTGTGTCTGGC
Sequencing	
M13_uni -21	GTAAAACGACGGCCAGT
M13_reverse -29	AACAGCTATGACCATG
CqsS_V456_s	TTCTCGATGTTCCTGCGGTTC
CqsS_L223_s	TTAAGTGATGAAGATGTGAC
pKK176	AAATCACTGCATAATT

Analysis of transcription levels via qRT-PCR

V. harveyi ATCC BAA-1116 was cultivated as described above, and samples were harvested by centrifugation every hour. RNA was isolated as described before (Fritz *et al.*, 2009). The RNA was then used as template for random-primed first-strand cDNA synthesis according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) (iQ5 real-time PCR detection system, Biorad) was performed using the synthesized cDNA, a SYBR-green

detection system (Biorad) and specific internal primers for *recA*, *luxN*, *luxQ*, *cqsS, luxO* and *luxU* (Table 3.2). The CT value (cycle threshold) was determined after 40 cycles using the iQ software (Biorad). Values were normalized with reference to *recA* and relative changes in transcript levels were calculated using the comparative CT method (Schmittgen & Livak, 2008).

Preparation of inverted membrane vesicles

E. coli Rosetta (DE3) pLysS was transformed with plasmid pNKN, pNKQ and pKK223-3-cqsS-F175C encoding wild type LuxN, LuxQ or CqsS-F175C respectively. Membrane vesicles were prepared as described before (Timmen *et al.*, 2006).

Heterologous production of LuxP and LuxU

E. coli MDAI-2 was transformed with the plasmid pGEX_LuxP and purified as described elsewhere (Neiditch *et al.*, 2005). LuxU was overproduced using *E. coli* JM109 transformed with pQE30LuxU-6His, and purified as described in (Timmen *et al.*, 2006). All proteins were stored at -80 °C.

Phosphorylation assay

Phosphorylation reactions were performed in phosphorylation buffer (50 mM Tris/HCl pH 8.0, 10 % (v/v) glycerol, 500 mM KCl, 2 mM DTT) at room temperature. The hybrid histidine kinases LuxQ, CqsS F175C and LuxN were used as full-length membrane integrated proteins in membrane vesicles. LuxQ containing membrane vesicles were added at final concentrations of 1 mg/ml. Via comparative Western Blot the amounts of LuxN and CqsS-F175C membrane vesicles needed were calculated as ratio based on LuxQ. The reaction mixture contained 0.36 mg/ml LuxU and 0.1 mg/ml LuxP unless otherwise indicated. To incorporate LuxP into LuxQ containing membrane vesicles, three cycles of freezing and thawing were performed. Unless otherwise indicated, AI-2, HAI-1 and CAI-1 (*V. cholerae)* were added in a final concentration of 10 µM. The phosphorylation reaction was started by adding radiolabeled Mg^{2+}-ATP, typically 100 µM [γ-^{32}P] ATP (0.94 Ci/mmol; Perkin-Elmer, Rodgau-Jügesheim, Germany) and 110 µM $MgCl_2$, and stopped at various time points by the addition of SDS loading buffer (Jung *et al.*, 1997), followed by separation of the proteins on a SDS-PAGE (Laemmli, 1970). Gels were dried at 80 °C on filter paper, exposed to a phosphoscreen for at least 24 h and scanned using a Typhoon Trio variable mode imager (GE Healthcare, München, Germany). Quantification of the bands was performed using ImageQuant (GE Healthcare, München, Germany).

For dephosphorylation assays, LuxU was phosphorylated using Lux(P)Q. In this case the phosphorylation buffer contained 10 mM $CaCl_2$ instead of $MgCl_2$ and twice the amount of Lux(P) and LuxU. After 10 min incubation at room temperature, membrane vesicles were removed by centrifugation (45,000 rpm, 15 min, 4°C), and ATP and $CaCl_2$ were removed by gel filtration (Sephadex G25 columns, GE Healthcare). Dephosphorylation of P-LuxU was started by the addition of 110 µM $MgCl_2$, 100 µM ATP-γ-S, and membrane vesicles containing the respective receptor. As described above, the reaction was stopped at the indicated time points, samples were subjected to SDS-PAGE and exposed to a phosphoscreen.

In gel fluorescence

Cells were cultivated as described above. In the early and late exponential growth (OD_{600} ~0.08 and ~0.7 respectively) phase, cells were harvested by centrifugation (5,000 rpm, 15 min, 4°C). The pellet was resuspended in TG buffer (50 mM Tris/HCl pH 8.0, 10% (v/v) glycerol) supplemented with lysozyme, DNase and PMSF (0.5 mM) and thereby concentrated 200 fold. After lysis at 37°C for 15 min an ultracentrifugation (Sorvall Discovery M120) was conducted (45,000 rpm, 15 min, 4°C). The pellet was resuspended in TG buffer (and diluted when indicated). Samples were mixed accordingly with solubilization buffer (200 mM Tris/HCl (pH 8.8), 20% (v/v) glycerol, 5 mM EDTA (pH 8.0), 0.02% (w/v) bromphenol blue (aliquots of 700 µl)). Before use 200 µl 20% (w/v) SDS and 100 µl 0.5 M DTT (Drew *et al.*, 2006)) were added. SDS-PAGE was performed according to Laemmli (Laemmli, 1970) and run at 150 V for 2 h in the dark to avoid bleaching of the fluorophore. In gel-fluorescence was analyzed using a Typhoon Trio scanner (Amersham Biosciences) with a 488 nm laser and a 526 nm emission filter. Quantification of fluorescence was performed using ImageQuant (GE Healthcare, München, Germany).

Fluorescence microscopy

Cells were grown in AB medium until the late exponential growth phase and fluorescence microscopy was conducted. For imaging, 3 µl of cells were dropped on a 1% (w/v) AB-agarose pad. A DeltaVision Elite microscope (GE Healthcare, Applied Precision) equipped with a CoolSnap HQ2 CCD camera was used. Images were taken with a 100x oil PSF U-Plan S-Apo 1.4 NA with an exposure time of 2 s using a 475/28 nm excitation and 525/48 nm emission wavelengths. Analysis was performed using ImageJ.

Results

The quorum sensing receptors show differences in kinase but not phosphatase activities *in vitro*

In vitro phosphorylation assays were performed to determine the enzymatic activity of each hybrid histidine kinase. For this purpose, genes of the full-length hybrid histidine kinases were overexpressed, membrane vesicles were prepared and directly used for phosphorylation experiments. Initial characterizations of kinase and phosphatase activities for LuxN and Lux(P)Q, but not for CqsS, had already been performed in the past, and the results suggested that their kinase activities were in the same range and that the presence of AIs only influences the kinase but not the phosphatase activity (Anetzberger *et al.*, 2012, Timmen *et al.*, 2006). However, the concentration dependent inhibition of the kinase activities by the AIs was incomplete, meaning that even at high HAI-1 and AI-2 concentrations phosphorylated LuxU was still detectable (Timmen *et al.*, 2006, Anetzberger *et al.*, 2012). Nonetheless, to quantitatively compare enzymatic activities and to avoid day to day variation, we believe it is crucial to perform the assays for all hybrid histidine kinases at the same time and under the exactly same conditions. Since *V. harveyi* CAI-1 can only be enriched in dichloromethane, which is interfering with our phosphorylation assay and CAI-1 is not commercially available, we introduced an amino acid substitution at position 175 (Phe to Cys). This variant CqsS-F175C was shown to recognize and respond to soluble CAI-1 from *Vibrio cholerae* (Ng *et al.*, 2011). To ensure that equal protein amounts were used for the phosphorylation assays, we quantified the amount of the receptors in the membrane vesicles via Western blot (data not shown).

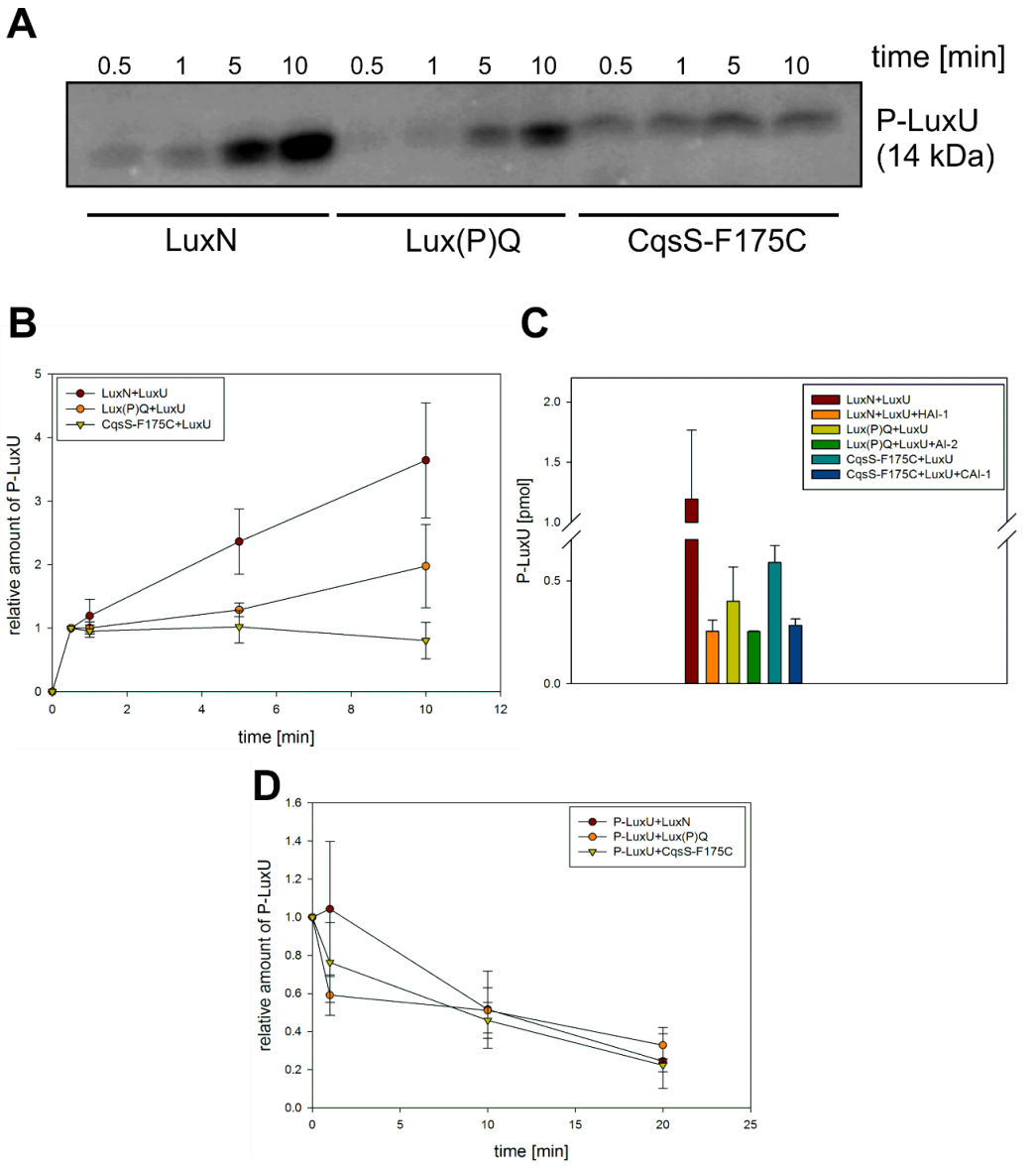


Figure 3.2 *In vitro* enzymatic activities of the QS receptors LuxN, Lux(P)Q and CqsS-F175C. A) Time-dependent phosphotransfer from LuxN, Lux(P)Q and CqsS-F175C to the Hpt protein LuxU. Membrane vesicles containing the hybrid histidine kinases LuxN, LuxQ, and CqsS-F175C, respectively were mixed with LuxU, and the reaction was started with [γ^{32}-P] Mg^{2+} ATP. For LuxQ mediated phosphorylation, LuxP had been incorporated into the vesicles. At the indicated time points the phosphorylation reaction was stopped, proteins were separated by SDS-PAGE followed by exposure of the gels to a phosphoscreen. The autoradiograph corresponding to LuxU protein size is representative for three independent experiments. **B) Time dependent LuxU phosphorylation.** LuxU was phosphorylated by LuxN, Lux(P)Q or CqsS-F175C respectively and the reaction was stopped at indicated time points. P-LuxU was quantified and the relative amounts were calculated as factor of P-LuxU after 0.5 min of phosphorylation. **C) Influence of AI on the kinase activity of the QS receptors.** When indicated HAI-1, AI-2 or CAI-1 was added to the assay mixture in a concentration of 10 µM prior incubation. Ten minutes after incubation the reaction was stopped and P-LuxU was quantified. **D) Dephosphorylation of P-LuxU.** A mixture of P-LuxU and LuxN, Lux(P)Q or CqsS-F175C, respectively was incubated. At the indicated time points the reactions were terminated, proteins separated via SDS-PAGE and the amount of P-LuxU was quantified. Values are calculated as relative factor of the control assay without addition of hybrid histidine kinases. All quantifications were performed with ImageQuant (GE Healthcare, München, Germany) using [γ^{32}-P] Mg^{2+} ATP as standard. Shown are the mean values and standard deviations of three independent experiments.

All three hybrid histidine kinases phosphorylated LuxU in a time dependent manner (Figure 3.2 A and B). The relative amount of P-LuxU was calculated as a factor based on the concentration of P-LuxU after 0.5 min to avoid day to day variations based on different amounts of P-LuxU (Figure 3.2 B). The fold-increase in P-LuxU differed depending on the

receptors present in the reaction (Figure 3.2 B). After ten minutes, the highest amount of P-LuxU was obtained after phosphorylation with LuxN. Membrane vesicles containing CqsS-F175C showed a comparable initial rate of LuxU phosphorylation, however the saturation level of P-LuxU was already reached after one minute (Figure 3.2 B). Altogether the capacity of the receptors to phosphorylate LuxU showed the following order LuxN>Lux(P)Q>CqsS-F175C. Furthermore, the addition of each AI inhibited the respective kinase activity. It is interesting to note that despite the fact that LuxN shows the strongest kinase activity, all kinase activities were inhibited to the same extent (Figure 3.2 C). However, the inhibitory effects of all three AIs were incomplete and residual kinase was still detectable, which is in agreement with previous studies (Anetzberger *et al.*, 2012). In order to investigate the corresponding phosphatase activities, LuxU was phosphorylated with γ^{32}-P ATP via Lux(P)Q, purified and used for subsequent analysis. All three receptors were able to specifically dephosphorylate P-LuxU in a time-dependent manner (Figure 3.2 D). P-LuxU alone was stable for the duration of the assay, excluding the possibility that the decrease of P-LuxU is due to instability of the protein and/or the phosphorylation (Figure S 1). The dephosphorylation of P-LuxU was calculated as fold-reduction based on P-LuxU incubated without the receptors. All three receptors dephosphorylated P-LuxU to a comparable extent in a similar timeframe (Figure 3.2 D). However, none of the hybrid histidine kinases was able to entirely dephosphorylate P-LuxU.

Receptor transcript levels vary during growth phases

To investigate potential differences in quantity of the QS components, we performed qRT-PCR to directly compare transcript levels. From a growing wild type culture, samples were taken every hour and RNA was extracted (Figure 3.3 C). Overall, *luxN* was the most transcribed kinase with its highest transcript levels in the late exponential growth phase (Figure 3.3 A). The transcription of *luxQ* and *cqsS* did not differ significantly and, except for a slight peak for *luxQ* after three hours, remained constant (Figure 3.3 A).

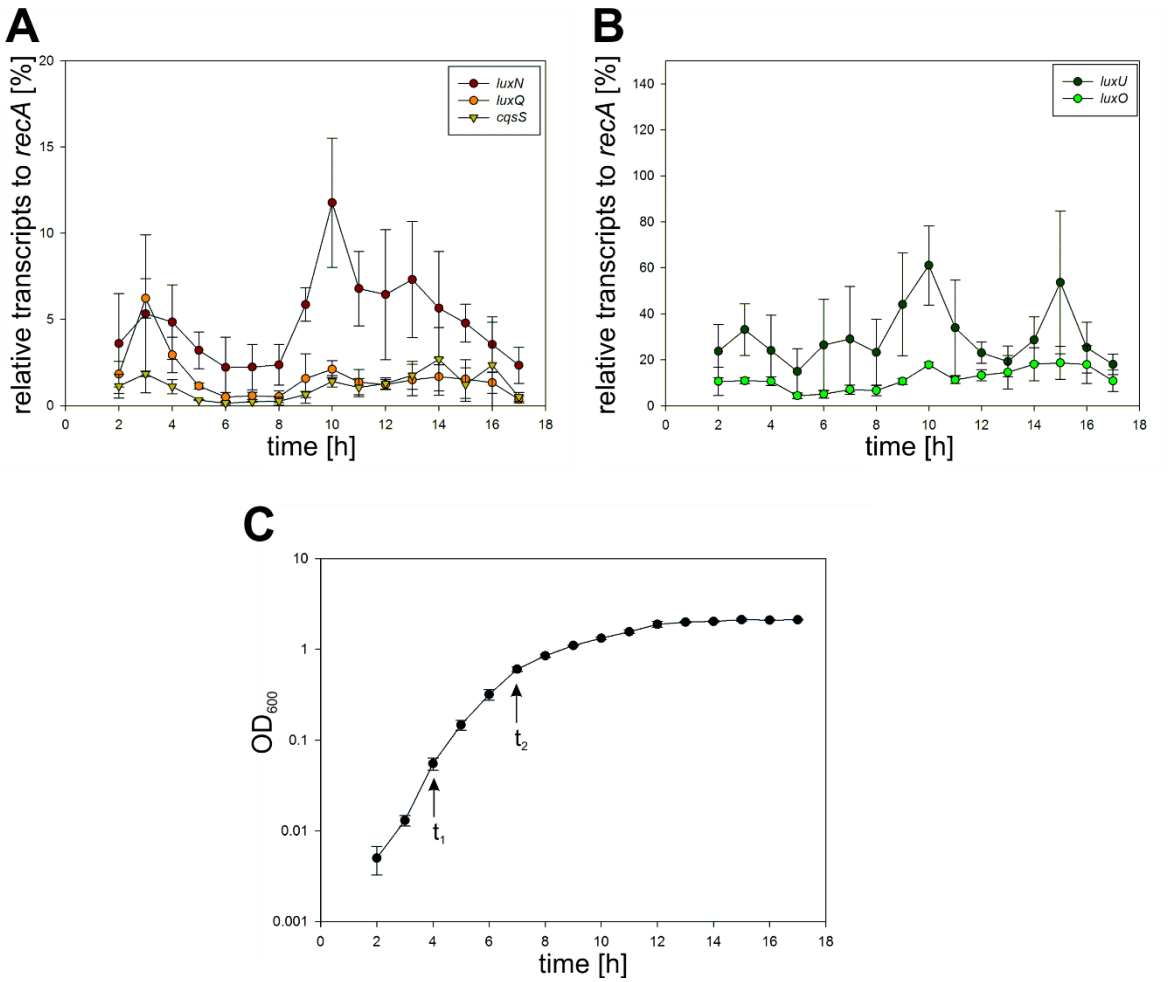


Figure 3.3 A) and B) Transcriptional analysis of the components of the QS cascade in *V. harveyi.* Cells of an overnight culture of *V. harveyii* were diluted 5,000 fold in fresh AB medium and cultivated aerobically at 30°C. Samples were taken every hour and total RNA was isolated. Transcript levels of *luxN*, *luxQ*, *cqsS*, *luxU* and *luxO* were determined via qRT-PCR. Transcript levels relative to *recA* were calculated using the C_t method (Schmittgen & Livak, 2008). Experiments were performed in triplicates and error bars represent the standard deviation of the mean. **C) Optical densities (OD_{600}) determined for *V. harveyi* corresponding to time points for RNA extraction.** t_1 and t_2 indicate time points used for further experiments.

Moreover, we could show that the transcript levels of the kinases were overall lower compared to *luxU*, coding for the HPt protein, or *luxO*, coding for the response regulator at any time (Figure 3.3 A and B). The transcript levels of *luxU* were 2-fold higher than the mRNA levels of *luxO* (Figure 3.3 B).

The quorum sensing receptors are clustered in *V. harveyi* and differ in abundance

In order to elucidate potential cluster formation of the receptors we compared the wild type with a triple kinase mutant (*V. harveyi ΔluxNΔluxQΔcqsS)* by performing electron cryotomography. This method was successfully used to identify cluster formation in bacterial chemoreceptors (22). However, we could not find any differences between the wild type and the deletion mutant (Ariane Briegel, data not shown). This could either be due to the low receptor expression levels or to the lack of cluster formation. Subsequently, we constructed

reporter strains harboring full-length C-terminal fluorophore fusions of the hybrid histidine kinases to investigate their subcellular localization as well as the corresponding protein levels. As our first attempts to visualize the receptors using GFP or mCherry failed, presumably due to the low expression levels, we decided to use mNeonGreen (NG), since this fluorophore is known to exhibit a higher brightness compared to e.g. GFP (Shaner *et al.*, 2013). All protein fluorophore fusions were integrated at the native locus in the chromosome in order to investigate their localization at native protein levels. Cells were grown until late exponential growth phase (see Figure 3.3 C t_2) and subsequently used for imaging on agar pads. The non-tagged *V. harveyi* wild type was used as control for background fluorescence to ensure the observed signals are no artefacts. The signal in the control was significantly lower compared to the reporter strains harboring the receptor fluorophore fusions. LuxN-NG clustered in the membrane and exhibited the strongest fluorescence signal (Figure 3.4 upper panel). LuxQ-NG was found to cluster as well however appeared to be localized mainly at the cell poles (Figure 3.4 second panel from top). In cells harboring CqsS-NG the fluorescence was rather homogeneously distributed in the membrane (Figure 3.4 third panel from top). The negative control with the non-tagged wild type using the same imaging settings confirmed that the clusters seen in the reporter strains were no artefacts (Figure 3.4 bottom panel).

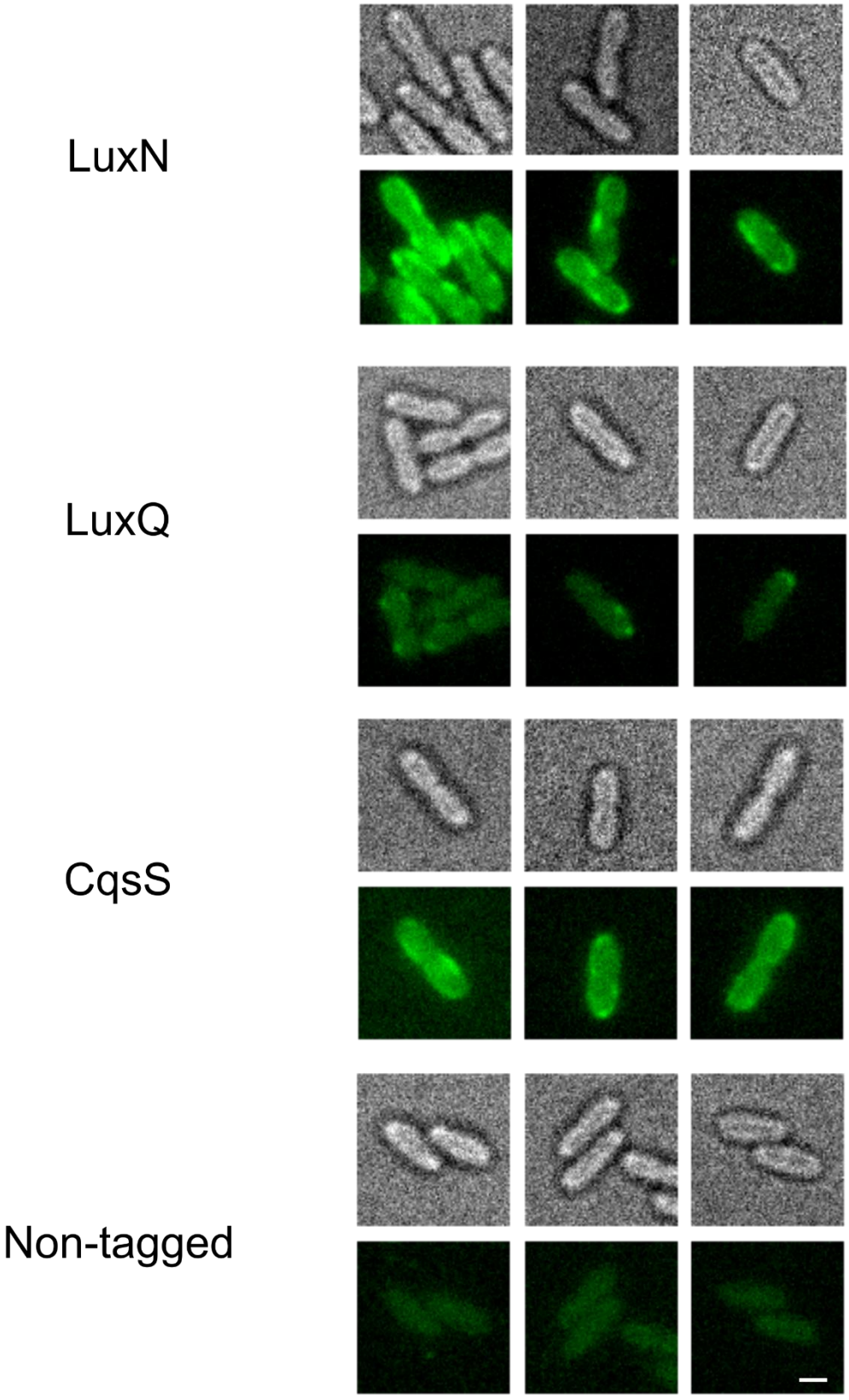


Figure 3.4 Localization of the hybrid histidine kinases. Brightfield (always upper row) and Fluorescence microscopy (lower row) images of LuxN-NG, LuxQ-NG, CqsS-NG and non-tagged wild type (from top to bottom). Fluorescence microscopy (exposure time 2 s) was performed using cells in the late exponential growth phase and analyzed on 1% (w/v) AB agarose pads. Protein-fluorophore hybrids are indicated by increased fluorescence compared to background. Scale bar 1 µm.

To gain quantitative results regarding the abundance of the single receptors we performed in gel fluorescence assays. For this assay we used the same reporter strains as for the microscopy approach. The cells were harvested in the early and late exponential growth phase respectively (for time points see Figure 3.3 C t_1 and t_2), lysed, the membrane fractions were prepared and subsequently analyzed via SDS-PAGE. For each reporter strain (LuxN-NG, LuxQ-NG and CqsS-NG) a specific band (corresponding to the size of the protein and fluorophore) could be observed, which was not present in the non-tagged wild type control (Figure 3.5 A). In the early exponential growth phase LuxN was the most abundant kinase, followed by LuxQ and CqsS (Figure 3.5 A). Quantification of the band intensities using ImageQuant and normalization to LuxQ showed that the amount of LuxN was three times higher than LuxQ, whereas CqsS showed the lowest copy number and was two-fold lower compared to LuxQ (Figure 3.5 B). In the late exponential growth phase specific bands for each of the fusions could be observed as well. However, the distinct expression pattern differed from the one in the early exponential phase. LuxN was still the predominantly expressed kinase, but its ratio relative to the other kinases was further increased: LuxN was more abundant than LuxQ by a factor of 8 ± 3. LuxQ and CqsS showed comparable protein amounts with CqsS being slightly more abundant than LuxQ (factor 1.24 vs 1) (Figure 3.5 C). It is interesting to note, that the differences observed on the transcript levels could be found as well on the protein level (compare Figure 3.3 and Figure 3.5).

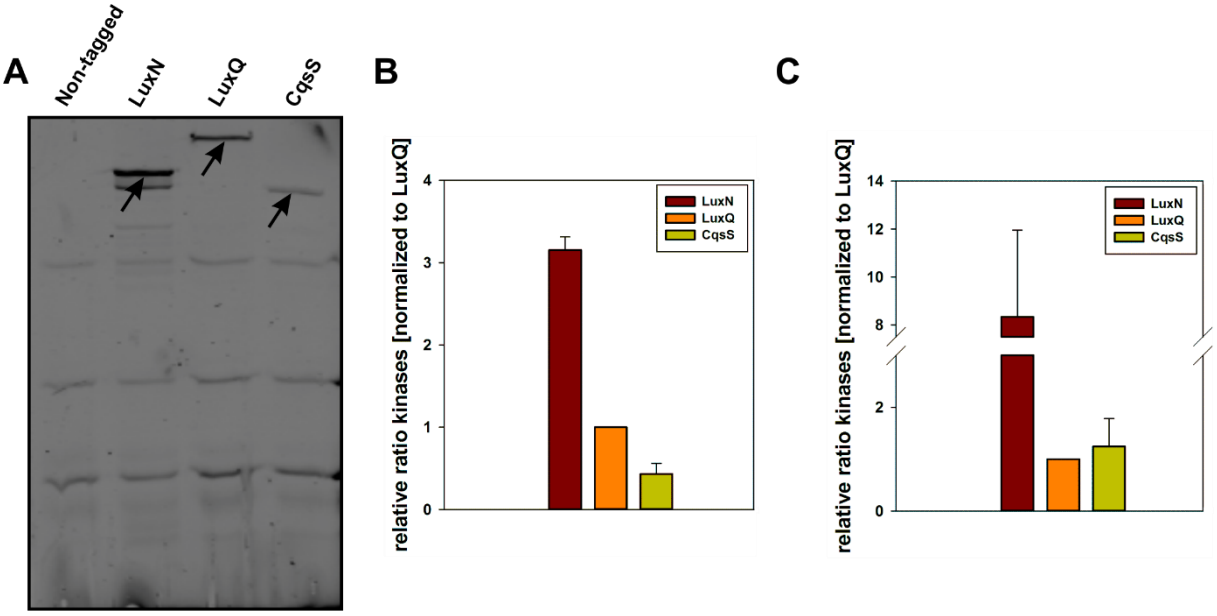


Figure 3.5 In Gel Fluorescence. A) Membrane fractions of the non-tagged wild type or cells harboring LuxN-NG, LuxQ-NG or CqsS-NG harvested in the early exponential growth phase (time point t_1 Fig. 3 C). Cell densities were adjusted (OD_{600} 0.08) and concentrated 200-fold. SDS-PAGE was run for 1.5 h at 150 V in the dark and scanned using Typhoon Trio scanner (Amersham Biosciences) with a 488 nm laser and a 526 nm emission filter. Arrows indicate the bands corresponding to LuxN-NG, LuxQ-NG and CqsS-NG. Gel pictures are representative of three independent experiments. **B) Quantification of specific LuxN-NG, LuxQ-NG, CqsS-NG in the early exponential growth phase (t_1 Fig 3 C).**

Quantification was performed based on band intensities on the scanned SDS gels. All values were normalized to LuxQ and therefore represent protein-fluorophore abundance as a factor of LuxQ abundance. **C) Quantification of specific LuxN-NG, LuxQ-NG, CqsS-NG in the late exponential growth phase (t_2 Fig. 3 C).** Quantification was performed based on band intensities on the scanned SDS gels. All values were normalized to LuxQ and therefore represent protein-fluorophore abundance as a factor of LuxQ abundance. Bars in **B)** and **C)** represent mean values from three independent experiments and standard deviations of the mean are indicated.

Discussion

Among prokaryotes sensing and integration of multiple environmental signals is important for the adaption of specific behaviors. For example, *E. coli* integrates positive and negative signals to generate an integrated chemotactic response (Khan *et al.*, 1995). In the case of cell-cell communication, several bacterial species are known to synthesize and respond to multiple AIs/environmental signals. *V. cholerae* integrates four inputs in one cascade (Jung *et al.*, 2015). *V. harveyi* perceives three different AIs (AI-2, HAI-1 and CAI-1) with three different sensors, specifically hybrid histidine kinases (Lux(P)Q, LuxN and CqsS) (Waters & Bassler, 2006), and integrates the information in one phosphorelay cascade. AI-2 is made by various bacterial species, while HAI-1 is solely made and sensed by *V. harveyi.* CAI-1 with an 8-carbon tail is perceived by *V. harveyi* CqsS, whereas *V. cholerae* CqsS can perceive CAI-1 with an 8- or 10-carbon tail. Accordingly, these systems are supposed to be specific for inter-species, intra-species and intra-genus communication, respectively (Federle & Bassler, 2003, Henke & Bassler, 2004, Ng *et al.*, 2011). To monitor the availability of the AIs it is crucial that the hybrid histidine kinases exhibit two enzymatic activities, they act as kinases as well as phosphatases. It is assumed that this bifunctionality ensures robustness in the output response (Siryaporn *et al.*, 2010). It was shown recently that the ratio of kinase and phosphatase activities of the different receptors and hence the extent of phosphorylated LuxU and LuxO is crucial for the signaling output and also for potential noise in the system (Plener *et al.*, 2015). This study focused on *in vivo* approaches based on deletion mutant studies and showed that the output response varies dependent on the presence or absence of different receptors and/or the AIs. In order to fully understand the impact of each AI/receptor pair it is also necessary to investigate the specific enzymatic properties of each receptor.

By performing *in vitro* phosphorylation assays with adjusted amounts of the kinases and the HPt protein LuxU, the inherent enzymatic activities were characterized and directly compared. The initial rate of phosphorylation was comparable for all receptors, but after prolonged incubation LuxN had the highest capacity to phosphorylate LuxU followed by LuxQ and CqsS-F175C (Figure 3.2 A and B). Upon AI addition the kinase activities were inhibited, which is in accordance with previous studies (Timmen *et al.*, 2006, Anetzberger *et al.*, 2012). It is interesting to note, that despite the fact that LuxN was able to phosphorylate

the highest amount of LuxU, upon supplementation with equimolar concentrations for all three AIs, all kinase activities were inhibited to the same extent. However, none of the three AIs was able to inhibit LuxU phosphorylation entirely (Figure 3.2 C) which is supported by the finding that only in the presence of all AIs the induction of bioluminescence was homogeneous in the population (Plener *et al.*, 2015). The receptors also exhibit phosphatase activity, which is crucial upon accumulation of the AIs to switch the QS cascade to an ON state by draining the phosphoryl groups out of the cascade. In contrast to the observed differences in kinase activities, the found phosphatase activities were comparable between the three receptors (Figure 3.2 D) and not influenced upon addition of the AIs (data not shown). Consequently, the phosphatase activity is not regulated by the stimulus and ensures constant dephosphorylation of P-LuxU. However, the dephosphorylation of P-LuxU is accelerated by the AI mediated inhibition of the kinase activity.

In addition to the inherent enzymatic activities which could be responsible for the observed distinct outputs based on differential inputs, the abundance of the various receptors should also be taken in account. For example it was shown that LuxN/HAI-1 had the lowest impact on activation of the *lux* operon *in vivo* (Plener *et al.*, 2015). This could not only be due to stronger or weaker kinase/phosphatase activity, but rather rely on differences in protein copy numbers. For this purpose, we initially investigated transcript levels of all components of the QS cascade and could show that compared to *luxU* (encoding the HPt protein) or *luxO* (encoding the response regulator), the hybrid histidine kinases are transcribed to a lower extent (Figure 3.3). It is likely that the ratio of phosphorylated LuxU or LuxO is crucial for the output. LuxN is the most transcribed receptor with a peak after ten hours of cultivation (Figure 3.3 A). This correlates well with the fact that at lower cell density, meaning lower HAI-1 concentrations, the sRNAs lead to degradation of *luxMN* transcripts (Teng *et al.*, 2011). This downregulation is alleviated at higher cell densities with increasing AI concentrations (Teng *et al.*, 2011). It was shown that the AIs follow a distinct pattern, with AI-2 being the first produced in the early exponential growth phase followed by HAI-1 and CAI-1 in the late exponential phase (Anetzberger *et al.*, 2012). Hence, upon accumulation of AIs, meaning less sRNAs are expressed, the *luxMN* transcripts are not degraded anymore and thus explains the rapid increase of *luxN* transcripts after ten hours. For the transcripts of *luxQ* we observed a peak as well, however no feedback loop is known by now which could lead to this increase in mRNA levels (Figure 3.3 A).

We constructed various reporter strains harboring mNeonGreen fusions for all receptors (LuxN-NG, LuxQ-NG, CqsS-NG). After imaging the cells in the late exponential growth phase we could observe differences in the relative abundance between the different receptors. LuxN seemed to be the most abundant receptor compared to CqsS and LuxQ (Figure 3.4). Furthermore, we observed differences in their subcellular localization. We found LuxN to form larger clusters compared to LuxQ, but these clusters were not solely located at the cell poles. LuxQ in turn formed small spots which appeared close to the poles. CqsS seemed to be not specifically located, but spread across the membrane (Figure 3.4). In chemotaxis, clustering of chemoreceptors ensures an increase in specificity, amplification of the signal and integration of multiple signals with a wide dynamic range (Maddock & Shapiro, 1993, Sourjik, 2004). Thereby the cells respond to even small changes in the concentration of an attractant or repellant (Sourjik, 2004). With the conduction of in gel fluorescence of the membrane fractions prepared from the reporter strains, we obtained concise information about the abundance of the receptors. Already in the early growth phase LuxN was more abundant (factor 3 compared to LuxQ) than LuxQ or CqsS (factor ~0.5 compared to LuxQ) (Figure 3.5). However, in the late exponential growth phase LuxN copy number increased even more extensively (LuxN factor ~8 compared to LuxQ), while CqsS protein levels were slightly elevated compared to LuxQ (Figure 3.5). The negative feedback loop on *luxMN* relies on the presence of the sRNAs. Consequently, increasing concentrations of AIs at higher cell densities lead to less sRNAs and therefore elevated amounts of *luxN* transcripts and finally higher levels of LuxN (Teng *et al.*, 2011).

The presence of a feedback loop on *luxN* but not *luxQ* shows that at later stages the cells increase the possibility to sense HAI-1 by increasing LuxN copy numbers and therefore concentrate on the intra-species signal. At lower cell densities the perception of CAI-1 or AI-2 results in a stronger impact on the output. In bacterial chemotaxis the extracellular stimuli are sensed either via direct binding or via indirect binding through periplasmic binding proteins (Neumann *et al.*, 2010). Direct binding of the ligands leads to a higher dynamic range, while binding via periplasmic binding proteins results in high sensitivity and a narrower dynamic range (Neumann *et al.*, 2010). HAI-1 and CAI-1 are directly sensed by LuxN and CqsS respectively, while the perception of AI-2 is dependent on the periplasmic binding protein LuxP and therefore prone to disturbances. The fact that LuxN directly senses HAI-1, is high abundant and clustered in the membrane contributes to the hypothesis of a high dynamic range. LuxN is assumed to play a prominent role during the transition of high to low cell density gene expression. Due to the high LuxN levels and strong kinase activity the transition

is accelerated. Eventually, LuxO will be phosphorylated rapidly, sRNAs are produced and the gene expression switches to LCD specific gene expression. This could be due to e.g. the removal of HAI-1 or because the cells are shed from a biofilm.

Our results show in more detail, that the architecture of the QS cascade, the presence of the AIs and also the abundance and the enzymatic state of the sensors are crucial for information processing and impact on the output. Consequently, *V. harveyi* integrates the specific information contained in each signal to subtle allow behavioral changes that are crucial for the adaption to certain niches in the environment. Feedback loops on sensor numbers enable the bacteria to focus on one signal or to monitor the developmental stage (Mehta *et al.*, 2009). Furthermore, under natural conditions it might be advantageous for the cells to correctly respond to different mixtures of AIs for example during the development of a biofilm. Altogether our data contribute to the understanding of QS signal integration in *V. harveyi* by bringing both a qualitative (relative activity of the receptors) and a quantitative (abundance of the receptors) approach together.

References for Manuscript

Anetzberger, C., T. Pirch & K. Jung, (2009) Heterogeneity in quorum sensing-regulated bioluminescence of *Vibrio harveyi*. *Mol Microbiol* **73**: 267-277.

Anetzberger, C., M. Reiger, A. Fekete, U. Schell, N. Stambrau, L. Plener, J. Kopka, P. Schmitt-Kopplin, H. Hilbi & K. Jung, (2012) Autoinducers act as biological timers in *Vibrio harveyi*. *PLoS One* **7**: e48310.

Bassler, B.L., E.P. Greenberg & A.M. Stevens, (1997) Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J Bacteriol* **179**: 4043-4045.

Bassler, B.L., M. Wright & M.R. Silverman, (1994) Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. *Mol Microbiol* **13**: 273-286.

Cao, J.G. & E.A. Meighen, (1989) Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. *J Biol Chem* **264**: 21670-21676.

Chatterjee, J., C.M. Miyamoto & E.A. Meighen, (1996) Autoregulation of *luxR*: the *Vibrio harveyi lux*-operon activator functions as a repressor. *Mol Microbiol* **20**: 415-425.

Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B.L. Bassler & F.M. Hughson, (2002) Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**: 545-549.

DeLisa, M.P., J.J. Valdes & W.E. Bentley, (2001) Quorum signaling via AI-2 communicates the "Metabolic Burden" associated with heterologous protein production in *Escherichia coli*. *Biotechnol Bioeng* **75**: 439-450.

Drew, D., M. Lerch, E. Kunji, D.J. Slotboom & J.W. de Gier, (2006) Optimization of membrane protein overexpression and purification using GFP fusions. *Nat Methods* **3**: 303-313.

Federle, M.J. & B.L. Bassler, (2003) Interspecies communication in bacteria. *J Clin Invest* **112**: 1291-1299.

Feng, L., Steven T. Rutherford, K. Papenfort, John D. Bagert, Julia C. van Kessel, David A. Tirrell, Ned S. Wingreen & Bonnie L. Bassler, (2015) A Qrr noncoding RNA deploys four different regulatory mechanisms to optimize quorum-sensing dynamics. *Cell* **160**: 228-240.

Freeman, J.A. & B.L. Bassler, (1999) A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi*. *Mol Microbiol* **31**: 665-677.

Fried, L., J. Lassak & K. Jung, (2012) A comprehensive toolbox for the rapid construction of *lacZ* fusion reporters. *J Microbiol Methods* **91**: 537-543.

Fritz, G., C. Koller, K. Burdack, L. Tetsch, I. Haneburger, K. Jung & U. Gerland, (2009) Induction kinetics of a conditional pH stress response system in *Escherichia coli*. *J Mol Biol* **393**: 272-286.

Greenberg, E.P., J.W. Hastings & S. Ulitzur, (1979) Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. *Arch Microbiol* **120**: 87-91.

Henke, J.M. & B.L. Bassler, (2004) Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi*. *J Bacteriol* **186**: 6902-6914.

Inoue, H., H. Nojima & H. Okayama, (1990) High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**: 23-28.

Jung, K., B. Tjaden & K. Altendorf, (1997) Purification, reconstitution, and characterization of KdpD, the turgor sensor of *Escherichia coli*. *J Biol Chem* **272**: 10847-10852.

Jung, S.A., C.A. Chapman & W.L. Ng, (2015) Quadruple quorum-sensing inputs control *Vibrio cholerae* virulence and maintain system robustness. *PLoS Pathog* **11**: e1004837.

Keller, L. & M.G. Surette, (2006) Communication in bacteria: an ecological and evolutionary perspective. *Nat Rev Microbiol* **4**: 249-258.

Khan, S., J.L. Spudich, J.A. McCray & D.R. Trentham, (1995) Chemotactic signal integration in bacteria. *Proc Natl Acad Sci U S A* **92**: 9757-9761.

Laemmli, U.K., (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lenz, D.H., K.C. Mok, B.N. Lilley, R.V. Kulkarni, N.S. Wingreen & B.L. Bassler, (2004) The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* **118**: 69-82.

Lilley, B.N. & B.L. Bassler, (2000) Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. *Mol Microbiol* **36**: 940-954.

Lin, B., Z. Wang, A.P. Malanoski, E.A. O'Grady, C.F. Wimpee, V. Vuddhakul, N. Alves Jr, F.L. Thompson, B. Gomez-Gil & G.J. Vora, (2010) Comparative genomic analyses identify the *Vibrio harveyi* genome sequenced strains BAA-1116 and HY01 as *Vibrio campbellii*. *Environ Microbiol Rep* **2**: 81-89.

Lorenz, N., M. Reiger, M. Toro-Nahuelpan, A. Brachmann, L. Poettinger, L. Plener, J. Lassak & K. Jung, (2016) Identification and Initial Characterization of Prophages in *Vibrio campbellii*. *PLoS One* **11**: 10.1371/journal.pone.0156010.

Maddock, J.R. & L. Shapiro, (1993) Polar location of the chemoreceptor complex in the *Escherichia coli* cell. *Science* **259**: 1717-1723.

Mehta, P., S. Goyal, T. Long, B.L. Bassler & N.S. Wingreen, (2009) Information processing and signal integration in bacterial quorum sensing. *Mol Syst Biol* **5**: 325.

Miller, V.L. & J.J. Mekalanos, (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* **170**: 2575-2583.

Neiditch, M.B., M.J. Federle, S.T. Miller, B.L. Bassler & F.M. Hughson, (2005) Regulation of LuxPQ receptor activity by the quorum-sensing signal autoinducer-2. *Mol Cell* **18**: 507-518.

Neumann, S., C.H. Hansen, N.S. Wingreen & V. Sourjik, (2010) Differences in signalling by directly and indirectly binding ligands in bacterial chemotaxis. *EMBO J* **29**: 3484-3495.

Ng, W.L., L.J. Perez, Y. Wei, C. Kraml, M.F. Semmelhack & B.L. Bassler, (2011) Signal production and detection specificity in *Vibrio* CqsA/CqsS quorum-sensing systems. *Mol Microbiol* **79**: 1407-1417.

Plener, L., N. Lorenz, M. Reiger, T. Ramalho, U. Gerland & K. Jung, (2015) The phosphorylation flow of the *Vibrio harveyi* quorum sensing cascade determines levels of phenotypic heterogeneity in the population. *J Bacteriol* **197**: 1747-1756.

Sambrook, J., (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schmittgen, T.D. & K.J. Livak, (2008) Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* **3**: 1101-1108.

Shaner, N.C., G.G. Lambert, A. Chammas, Y. Ni, P.J. Cranfill, M.A. Baird, B.R. Sell, J.R. Allen, R.N. Day, M. Israelsson, M.W. Davidson & J. Wang, (2013) A bright monomeric green fluorescent protein derived from *Branchiostoma lanceolatum*. *Nat Methods* **10**: 407-409.

Siryaporn, A., B.S. Perchuk, M.T. Laub & M. Goulian, (2010) Evolving a robust signal transduction pathway from weak cross-talk. *Mol Syst Biol* **6**: 452-452.

Sourjik, V., (2004) Receptor clustering and signal processing in *E. coli* chemotaxis. *Trends Microbiol* **12**: 569-576.

Teng, S.W., J.N. Schaffer, K.C. Tu, P. Mehta, W. Lu, N.P. Ong, B.L. Bassler & N.S. Wingreen, (2011) Active regulation of receptor ratios controls integration of quorum-sensing signals in *Vibrio harveyi*. *Mol Syst Biol* **7**: 491.

Timmen, M., B.L. Bassler & K. Jung, (2006) AI-1 influences the kinase activity but not the phosphatase activity of LuxN of *Vibrio harveyi*. *J Biol Chem* **281**: 24398-24404.

Tu, K.C. & B.L. Bassler, (2007) Multiple small RNAs act additively to integrate sensory information and control quorum sensing in *Vibrio harveyi*. *Genes Dev* **21**: 221-233.

Tu, K.C., T. Long, S.L. Svenningsen, N.S. Wingreen & B.L. Bassler, (2010) Negative feedback loops involving small regulatory RNAs precisely control the *Vibrio harveyi* quorum-sensing response. *Mol Cell* **37**: 567-579.

Tu, K.C., C.M. Waters, S.L. Svenningsen & B.L. Bassler, (2008) A small-RNA-mediated negative feedback loop controls quorum-sensing dynamics in *Vibrio harveyi*. *Mol Microbiol* **70**: 896-907.

Waters, C.M. & B.L. Bassler, (2006) The *Vibrio harveyi* quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. *Genes Dev* **20**: 2754-2767.

Yanisch-Perron, C., J. Vieira & J. Messing, (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**: 103-119.

4 Fimbrolide natural products disrupt bioluminescence of *Vibrio harveyi* by targeting autoinducer biosynthesis and luciferase activity.

Zhao, W., Lorenz, N., Jung, K., Sieber, S.A. (2016). Fimbrolide natural products disrupt bioluminescence of *Vibrio harveyi* by targeting autoinducer biosynthesis and luciferase activity. *Angew Chem Int Ed* 55 (3):1187-1191. doi: 10.1002/anie.201508052

Abstract

Vibrio is a model organism for the study of quorum sensing (QS) signaling and is used to identify QS-interfering drugs. Naturally occurring fimbrolides are important tool compounds known to affect QS in various organisms; however, their cellular targets have so far remained elusive. Here we identify the irreversible fimbrolide targets in the proteome of living *V. harveyi* and *V. campbellii* via quantitative mass spectrometry utilizing customized probes. Among the major hits are two protein targets with essential roles in *Vibrio* QS and bioluminescence. LuxS, responsible for autoinducer 2 biosynthesis, and LuxE, a subunit of the luciferase complex, were both covalently modified at their active-site cysteines leading to inhibition of activity. The identification of LuxE unifies previous reports suggesting inhibition of bioluminescence downstream of the signaling cascade and thus contributes to a better mechanistic understanding of these QS tool compounds.

Full-text article:

http://onlinelibrary.wiley.com/doi/10.1002/anie.201508052/pdf

Supplemental Material:

http://onlinelibrary.wiley.com/store/10.1002/anie.201508052/asset/supinfo/anie201508052-sup-0001-misc_information.pdf?v=1&s=dc444ad65b2965d381280c749a918774060a8e36

5 Identification and initial characterization of prophages in *Vibrio campbellii*

Lorenz, N., Reiger, M., Toro-Nahuelpan, M., Brachmann, A., Poettinger, L., Lassak, J., Jung, K. (2016). Identification and initial characterization of prophages in *Vibrio campbellii*. PloS One 11(5): e0156010. doi:10.1371/journal.pone.0156010

Abstract

Phages are bacteria targeting viruses and represent the most abundant biological entities on earth. Marine environments are exceptionally rich in bacteriophages, harboring a total of 4x1030 viruses. Nevertheless, marine phages remain poorly characterized. Here we describe the identification of intact prophage sequences in the genome of the marine γ-proteobacterium *Vibrio campbellii* ATCC BAA-1116 (formerly known as *V. harveyi* ATCC BAA-1116), which presumably belong to the family of *Myoviridae*. One prophage was found on chromosome I and shows significant similarities to the previously identified phage ΦHAP-1. The second prophage region is located on chromosome II and is related to *Vibrio* phage kappa. Exposure of *V. campbellii* to mitomycin C induced the lytic cycle of two morphologically distinct phages and, as expected, extracellular DNA from induced cultures was found to be specifically enriched for the sequences previously identified as prophage regions. Heat stress (50°C, 30 min) was also found to induce phage release in *V. campbellii*. Notably, promoter activity of two representative phage genes indicated heterogeneous phage induction within the population.

Full-text article:

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0156010

Supplemental Material:

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0156010#sec016

7 Concluding discussion

Bacteria are able to communicate by a mechanism called quorum sensing (QS). They are secreting small molecules, called AIs, and by the simultaneous perception of the latter the cells are able to measure their density and species complexity in the population. Numerous AIs have been identified as QS molecules, however the chemical nature of those molecules can be of diverging nature. While Gram-positive bacteria mainly use peptides to communicate, Gram-negative bacteria produce and sense different small molecules e.g. acyl homoserine lactones, AI-2 a furanosyl borate diester, fatty acids or quinolones (Chen *et al.*, 2002, Churchill & Chen, 2011, Lee & Zhang, 2015, Winans, 2011). Many QS systems are composed of multiple overlapping signaling pathways. In theory, a simple one-to-one pathway should be sufficient to measure cell density. What are the benefits of evolving a complex QS cascade? There are several bacterial species known to produce and sense more than one AI. The plant pathogen *Ralstonia solanacearum* responds to two different AIs and in this case one system is able to induce the second one according to a many-to-one hierarchical system (Genin & Denny, 2012). *Vibrio harveyi* is an ideal model organism to study a complex QS system as it processes the information of three different AIs in one signaling cascade according to a parallel many-to-one circuit. Signal recognition is mediated by transmembrane hybrid histidine kinases according to the bacterial two component system principle. In the past, most of the studies consisted solely of *in vivo* approaches and were performed after reducing the QS cascade to two inputs by deleting the CqsA/CqsS synthase/sensor pair. However, to address the question why such a complex cascade evolved, it is crucial to investigate the QS cascade with all its inputs. To elucidate the role of each subsystem with respect to information processing and individual input-output relations the impact of each AI/sensor pair was investigated using *in vivo* (at the population and at the single-cell level) and *in vitro* approaches (Chapter 2 and 3). Furthermore, the discovery of potential strategies to disturb cell-cell communication or to initiate a population breakdown could play an important role in combatting pathogenic bacteria and are therefore also focus of this work (Chapter 4 and 5).

7.1 Architecture of the quorum sensing cascade

The complex QS cascade of *V. harveyi* combines characteristics of Gram-positive and Gram-negative bacteria in a parallel many-to-one system (Miller & Bassler, 2001, Mok *et al.*, 2003). Despite the fact that the QS cascade in *V. harveyi* is already well understood at the mechanistic level there are still open questions inherent to its complexity. The cascade is

composed of three transmembrane hybrid histidine kinases (LuxN, Lux(P)Q, CqsS) which channel their AI information via LuxU to LuxO (Bassler *et al.*, 1993, Freeman & Bassler, 1999, Henke & Bassler, 2004, Neiditch *et al.*, 2005, Swem *et al.*, 2008). Each of the AIs is assumed to have its own meaning as AI-2, HAI-1 and CAI-1 resemble inter-species, intra-species and intra-genus signals (Federle & Bassler, 2003, Henke & Bassler, 2004, Ng *et al.*, 2011). HAI-1 is only produced by *V. harveyi* and close relatives (Cao & Meighen, 1989), while AI-2 is produced by Gram-positive as well as Gram-negative species (Chen *et al.*, 2002). CAI-1 and analogs are synthesized by different *Vibrio* species. CAI-1 produced by *V. harveyi* carries an 8-carbon tail, while CAI-1 from *V. cholerae* comprises a 10-carbon tail. CqsS from *V. harveyi* displays high specificity for the endogenous produced CAI-1 compared to CqsS from *V. cholerae* which does not discriminate accurately between the ligands (Ng *et al.*, 2011). It is known that not all AIs are present at the same time but follow a specific pattern, with AI-2 being the first AI to be produced followed by HAI-1 in the exponential growth phase and CAI-1 in the late exponential growth phase (Anetzberger *et al.*, 2012). Furthermore, AI synthesis depends via S-adenosylmethionine directly on the metabolic state of the cell. Moreover, *V. harveyi* monitors the availability of nitric oxide (NO) by a fourth system (H-NOX/HqsK) which is enhancing biofilm formation through to QS activation (Henares *et al.*, 2012, Henares *et al.*, 2013). It is likely that each AI signal could be translated into a particular output and therefore the inherent specific information could allow for distinct behavioral changes.

7.1.1 Signal hierarchy and information processing in the quorum sensing cascade

To investigate differences regarding the impact of the individual AI/receptor pairs on the QS output (e.g. induction of the luciferase operon), AIs were added artificially to a synthase deletion mutant (produces no AIs) (Chapter 2). LuxN/HAI-1 was found to have the least, while CqsS/CAI-1 had a medium impact on QS activation *in vivo*. LuxQ/AI-2 was found to have the greatest impact (Chapter 2). The possibility that the uncovered differences in the output strength were due to unnatural concentration and/or timing of the added AIs, was excluded by the results obtained from different synthase mutants which implicated the same hierarchy (Chapter 2). In order to monitor the availability of the AIs it is essential that the hybrid histidine kinases exhibit two enzymatic activities, they act as kinases as well as phosphatases. This bifunctionality is supposed to lead to robustness in the output (Siryaporn *et al.*, 2010). This is a common principle in bacteria, but interestingly likewise in eukaryotic organisms like *Arabidopsis thaliana*, which harbors ten comparable sensors (Mizuno, 2005). The phosphorelay systems in *A. thaliana* are also known to respond to hormone-like

molecules (Inoue *et al.*, 2001, Sheen, 2002), which makes it likely that these sensory systems exhibit an advantage in signal integration.

The information of the three hybrid histidine kinases in *V. harveyi* is merged in the phosphorylation state of the HPt protein LuxU and subsequently transferred to the response regulator LuxO. In order to conceive different levels of P-LuxO, regulation of the kinase activity is necessary. The kinase activities in *V. harveyi* differed *in vitro*, with LuxN being capable to phosphorylate a greater amount of LuxU than the other sensors (Chapter 3). LuxQ and CqsS were able to phosphorylate equivalent amounts of LuxU. However, upon addition of the AIs the kinase activity of all three sensors was inhibited to the same extent and the sensors exhibited predominantly phosphatase activity. The phosphatase activities between the sensors were comparable and not influenced by the AIs. Likewise, in *V. cholerae* the kinase activity of CqsS is inhibited by CAI-1 but the phosphatase activity is constant to ensure rapid dephosphorylation of P-LuxO (Wei *et al.*, 2012).

However, using *in vitro* approaches only the inherent enzymatic activity is examined, but for the *in vivo* output the abundance of the QS components is fundamental as well and contributes to the total kinase/phosphatase activity. Transcriptional analysis indicated that the kinases are overall less transcribed, but show a differential transcript level pattern. *luxN* was the most transcribed kinase with a peak after ten hours, followed by *luxQ* and *cqsS*. The peak of *luxN* transcript levels correlates well with the fact that the sRNAs lead to degradation of the *luxN* transcripts at LCD (low HAI-1 concentrations) (Teng *et al.*, 2011). With increasing AI concentrations this downregulation is eased. Overall *luxU* and *luxO* were transcribed to higher levels compared to the sensors, with *luxU* transcripts being double as abundant as *luxO* (Chapter 3). This makes it likely that *luxO* serves as a bottleneck in the cascade. A comparable abundance pattern was found on the protein level. Already in the early exponential growth phase LuxN was more present than LuxQ and CqsS. This difference became even more prominent during growth (Chapter 3). In the late exponential growth phase LuxN was eight times more abundant than CqsS. Consistently, this increase in LuxN protein level can be explained by the lack of the feedback loop (degradation of *luxMN* transcripts) at higher cell density and therefore higher AI concentrations (Teng *et al.*, 2011, Anetzberger *et al.*, 2012). In addition, we observed a differential subcellular localization. LuxQ and LuxN seem to form cluster, while CqsS appears to be equally distributed over membrane (Chapter 3). A well-known example for receptor clustering are the chemoreceptor arrays (Maddock & Shapiro, 1993, Briegel *et al.*, 2012). In *E. coli* the chemotaxis proteins localize to the cluster

of receptors and form large sensory cluster (Sourjik & Berg, 2000). The chemotactic response is extremely sensitive (Mao *et al.*, 2003) and the signal is amplified during the transmission of the signal via phosphorylation to the flagellar motors (Segall *et al.*, 1986).

7.1.2 Role of the architecture of the quorum sensing cascade

One advantage for bacteria to integrate multiple inputs in one cascade, is the possibility to decipher information incorporated in the signals. The AIs produced by *V. harveyi* are thought to mediate inter- and intra-species as well as intra-genus communication. By processing all signals in one shared cascade *V. harveyi* could assess its own population density and also the abundance of other species for example in mixed species communities. Furthermore, it allows to monitor the developmental stage of the population. The specific information in each signal could lead to appropriate changes depending on the environmental niche. The specificity of the signals is maintained at different stages of the cascade. The presence of a feedback loop to adjust transcript levels of *luxN* but not *luxQ* or *cqsS* ensures that at LCD the responsivity of HAI-1 is lower due to less LuxN receptors compared to the situation at HCD. Hence, a feedback loop on one sensor allows the cells to concentrate on distinct signals (Mehta *et al.*, 2009).

Under the tested conditions HAI-1 plays a more prominent role at later time points once the cascade is already triggered by AI-2. It seems likely that at this stage it is beneficial for the population to concentrate on their species specific signal. Moreover, it was shown for the chemotaxis in *Escherichia coli* that direct binding of ligands leads to a higher dynamic range (concentration range in which differences are perceived) (Neumann *et al.*, 2010). LuxN and CqsS sense their cognate AIs (HAI-1 and CAI-1, respectively) directly, while LuxQ perceives the presence of AI-2 via the periplasmic binding protein LuxP. Consequently, AI-2 sensing primary relies on the availability of LuxP and is therefore less sensitive to concentration fluctuations. Furthermore, while *V. cholerae* CqsS is able perceive different CAI-1s, CqsS from *V. harveyi* is highly specific for the endogenous CAI-1 (Ng *et al.*, 2011). Additionally, it was shown that LuxN is highly specific for the cognate acyl homoserine lactone (Ke *et al.*, 2015), which leads to efficient signaling. This leads to the hypothesis that *V. harveyi* prefers to perceive its endogenous signals and keeps its “conversation” private.

It is important to embed these findings in a global context as *V. harveyi* has different environmental niches. *V. harveyi* is a pathogen of crustaceans or fish, can be free living in the sea or on the surface of algae. It is advantageous for cells to correctly respond to mixtures of AIs in order to develop a biofilm or to monitor their developmental stage (Mehta *et al.*, 2009).

This hypothesis is supported by the described timing of the AIs during growth of *V. harveyi* (Anetzberger *et al.*, 2012). Under natural conditions, for example while colonizing shrimps it was shown that the various AIs are of varying importance towards the QS-regulated virulence depending on the crustacean host. AI-2 and CAI-1 are important for virulence towards the brine shrimp larvae *Artemia franciscana*, in contrast HAI-1 and AI-2 are needed for the infection of the giant freshwater prawn *Macrobrachium rosenbergii* (Defoirdt & Sorgeloos, 2012, Pande *et al.*, 2013). These studies point to the hypothesis that diverging signaling molecules are needed to specifically infect different hosts.

A sort of AI hierarchy can also be found in the QS system of *Vibrio fischeri*. *V. fischeri* harbors two QS systems, namely *ain* and *lux*, which produce two different acyl homoserine lactones as signaling molecules. Both were shown to bind to LuxR, however with different specificity (Schaefer *et al.*, 1996). The *ain* system activates luminescence gene expression at lower cell density than the *lux* system (Lupp *et al.*, 2003). Furthermore, both systems are important for colonization of the squid *Euprymna scolopes*. While the *ain* system is important in the early colonization, the *lux* system regulates factors needed at later stages of colonization (Lupp & Ruby, 2005). *V. cholerae* QS follows the parallel many-to-one principle like *V. harveyi*. In contrast, the cells are able to regulate gene expression QS-dependent as long as one of the four signaling pathways is functional suggesting that the sensors in this case are redundant. A mutant lacking all four systems is avirulent while a deletion of three pathways only had a minor effect on virulence (Jung *et al.*, 2015). Thereby it is suggested that signal integrity is maintained and commitment to QS due to small signal changes is prevented (Jung *et al.*, 2016).

In conclusion, our data support the hypothesis that the QS cascade of *V. harveyi* is a "coincidence detector" where each signal is able to activate QS, however to a different extent (Henke & Bassler, 2004). The observed differences in the strength of each AI/sensor pair on the output are suggested to be generated by the specific enzymatic activities, the different mechanisms in AI perception (direct vs indirect) as well as varying protein amounts (e.g. the sensors). The different impact of the AIs eventually results in distinct LuxR concentrations. Interestingly, the promoters of QS controlled target genes harbor divergent affinities for LuxR and are therefore activated at particular LuxR concentrations (Waters & Bassler, 2006).

7.2 Cell-to-cell variations in quorum sensing activation

Bacterial populations can be composed of phenotypically different cells, despite the fact that they are genetically homogeneous. In order to elucidate the information processing capacity

and specific input-output relations of the *V. harveyi* QS cascade with respect to potential cell-to-cell variations, the impact of every AI/receptor pair was determined at the single-cell level, using a chromosomal integrated promotor-fluorophore fusion (P_{luxC}-*mCherry*) in the background of different deletion mutants. Under laboratory conditions, meaning all AIs are produced and sensed by the cells, QS activation was homogeneous at the single cell level (Chapter 2). The same was true in the absence of the AIs, the QS cascade was OFF throughout the population and accordingly fluorescence production was prevented. On the contrary, in the absence of one or two AIs, the QS activation showed high variability between the single cells with regard to fluorescence production. In this situation not all sensors are in the same enzymatic mode and the resulting ratio of kinase to phosphatase activity generates a heterogeneous output (Figure 7.1). Moreover, within a population of cells deleted for all synthases and additionally deleted for two sensors (LuxQ/CqsS) even in the absence of any AI, a part of the population switched the cascade in an ON state (Chapter 2).

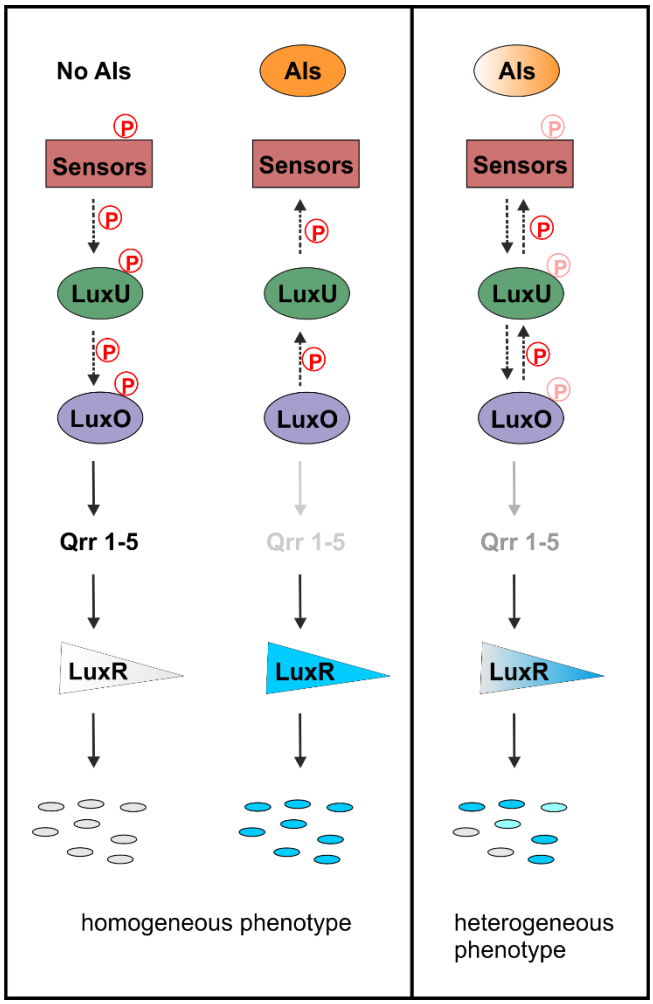


Figure 7.1 Scheme for the generation of cell-to-cell variations on level of *lux* operon induction in *V. harveyi* (from left to right). Under conditions when all sensors are in the kinase mode (e.g. absence of AIs) they autophosphorylate and transfer the phosphoryl group via LuxU to LuxO. This leads to expression of the Qrr sRNAs and degradation of *luxR* mRNA. The population is homogeneously QS-OFF. Under conditions when all sensors are in phosphatase mode (e.g. saturating AI

concentrations) P-LuxU gets dephosphorylated and the phosphoryl groups are drained from the cascade. LuxR is produced and the population is homogeneously QS-ON. Under conditions (e.g. low AI concentration) when some sensors are in the kinase and some are in the phosphatase mode full activation of the QS cascade is prevented and results in high cell-to-cell variations.

7.2.1 Mechanisms generating phenotypic heterogeneity

But where are the switches which can lead to a heterogeneous output under certain conditions? The already mentioned heterogeneity observed for the AI-negative mutant with deletions for LuxQ and CqsS (thereby loss of their enzymatic activities) already a hint as it was independent from any AI perception. The data from the *in vitro* enzymatic activities point against the hypothesis that the LuxN kinase activity is not strong enough to keep the cascade in an OFF state (Chapter 3). The possibility that LuxN is less abundant than the other sensors can also be excluded because in-gel fluorescence and microscopy revealed that LuxN is the most expressed kinase (Chapter 3). If we take in account the negative feedback loop on *luxMN* transcripts (Teng *et al.*, 2011) we could imagine, that due to the high levels of sRNAs, which are a direct result of the high kinase activity and abundance of LuxN molecules (in this mutant the only sensor) over time a high amount of the *luxMN* transcripts is degraded, leading to less production of LuxN and consequently reduced phosphotransfer which ends in production of LuxR and thus the onset of bioluminescence. Accordingly, not only the presence of one AI is important but also the inherent state of the cell.

Another likely source of noise are fluctuations in the amount of the cascade components (Long *et al.*, 2009). This is supported by studies performed with single synthase deletion mutants (two AIs are still produced) which presumably harbor low levels of sRNAs as two sensors are in the phosphatase mode and thereby the levels of P-LuxO are low. In these mutants the addition of a second *luxR* copy reduced noise in the bioluminescence expression, because the initially low amount of sRNAs are certainly outcompeted by the second copy of *luxR* and ultimately higher LuxR protein levels (Chapter 2). Hence, the opposite takes place in the double synthase mutants. The presence of two sensors being in the kinase mode lead to high sRNA levels and consequently the ratio between sRNAs and *luxR* transcripts cannot be reversed. Thus, the level of noise increases in those mutants. The ability to exhibit heterogeneous behavior seems to be anchored in the architecture and the regulation of the components of the complex QS cascade. Based on our transcriptional analysis via qRT-PCR it seems likely that *luxO* serves as a bottleneck, because it is less transcribed than *luxU* (Chapter 3). This is supported by the fact that several of the known feedback loops ensure low LuxO levels (degradation of *luxO* mRNA by the Qrr sRNAs, autorepression of *luxO*). Additionally,

the phosphorylation state of LuxO seems to be crucial for the output and the decision if one cell will be in a QS ON or OFF state. Mutants which are deprived for AI-2 or CAI-1 production, still showed high cell to cell variations even after 24 h of incubation (supplement Chapter 2). One explanation could be that AI-2 is taken up as it was shown for *E. coli* (Xavier & Bassler, 2005) or that the AI e.g. CAI-1 is not stable and therefore degraded. Consequently, a cell that possesses high levels of sRNAs and hence P-LuxO is prevented from switching to the ON state.

It is interesting to note that we could never observe bistability as it was reported for the competence development in *Bacillus subtilis* (Cahn & Fox, 1968) or the regulation of chemotaxis in *E. coli* (Korobkova et al., 2004). In these two examples it was shown that the bistability is generated by a positive feedback loop in the system. Whereas, the fluorescence distribution in our experiments was always Gaussian with a single peak and differed only in the width of the distribution (Chapter 2). It is interesting to note that in contrast to the QS-regulated bioluminescence in *V. fischeri*, in which a positive feedback loop acts to reactivate the system (the AI activates LuxR and LuxR in turn induces the expression of the AI synthase) (Williams *et al.*, 2008), none of the numerous feedback loops in the cascade of *V. harveyi* is known to reactivate the system so far. The *in vivo* data showed, in agreement with previous studies (Long *et al.*, 2009), that the integration of the AIs allows gradually increasing output. The more AIs are present, the more the QS cascade is in an ON state. In addition, our study demonstrated that at low concentrations, the low QS output is due to some cells with almost full activation of their QS system rather than a basal expression of QS traits in all cells. With increasing AI concentrations, the QS activation tends to be homogeneous at the single cell level and therefore resembles a robust output.

7.2.2 Potential role of phenotypic heterogeneity

In a laboratory culture when all AIs are present QS activation was homogeneous. However, in their natural habitat *V. harveyi* faces completely different conditions. Inside a biofilm where AIs are concentrated, cells would exhibit QS ON phenotypes (e.g. bioluminescence, proteolysis). However, on top of a biofilm, where nutrients and AIs are washed away the cells would express completely different phenotypes (e.g. siderophore production). This phenotypic heterogeneity could be a form of division of labor. This phenomenon is known for several bacterial species. A clonal population of S*almonella enterica* subsp. e*nterica* serovar Typhimurium differentiates in two subpopulations while infecting the gut. One part of the population expresses *ttss-1* (type three secretion system 1) specific virulence genes in order to

invade the gut epithelium and the other part is *ttss-1* OFF and remains in the gut lumen. By this the two subpopulations fulfill complementary roles in the infection process and thus they exhibit a form of division of labor (Ackermann *et al.*, 2008, Hautefort *et al.*, 2003). For *B. subtilis* it was shown that motile cells, biofilm-producing and sporulating cells have different niches within a biofilm and this localization is highly dynamic (Vlamakis *et al.*, 2008). Phenotypic heterogeneity is also favorable when cells are growing in a rapidly changing environment as it was shown that a heterogeneous population is fitter than a homogeneous one (Thattai & van Oudenaarden, 2004). Certainly, at least one part of the population would always exhibit the appropriate phenotypes and survive.

The architecture of the QS cascade of *V. harveyi* with multiple parallel QS systems integrated in one common phosphorelay leads to homogeneous QS activation at the single-cell level under saturating conditions (e.g. under laboratory conditions). However, the ability to generate heterogeneity in the activation of QS-regulated genes seems to be an inherent feature of the architecture of the cascade and is hypothesized to be beneficial for the population under specific conditions.

7.3 Quorum Quenching - the disturbance of quorum sensing

Processes which interfere with QS are called quorum quenching (QQ) (Dong *et al.*, 2001). Prokaryotes as well as eukaryotes evolved strategies to disrupt cell-cell communication. Several bacterial species rely on QS for pathogenesis through the cell-density dependent regulation of proteases, hemolysins and siderophores (Natrah *et al.*, 2011). Therefore, these anti-QS mechanisms could play a prominent role in combatting pathogenic bacteria by preventing e.g. their adherence and could serve as an alternative for antibiotic treatment (Federle & Bassler, 2003). *Staphylococcus delphini* secretes two compounds, namely yayurea A and B, which were found to quench QS in *V. harveyi*. Upon addition of the compounds, the LuxN-mediated phosphorylation of LuxU is stimulated and thereby the QS signaling is suppressed, leading to e.g. low bioluminescence level. Furthermore, the growth of several Gram-negative beta- and gamma-proteobacteria was inhibited by the addition of these compounds (Chu *et al.*, 2013). As *S. delphini* was initially isolated from dolphins and is a marine bacterium as well, it is conceivable that they share a habitat with *V. harveyi*. The production of yayurea A and B could function in self-protection and competitiveness within a shared environment for *S. delphini* (Chu *et al.*, 2013). Higher marine organisms are able to synthesize natural compounds which interfere with QS as well (Blunt *et al.*, 2012). Fimbrolides, in fact halogenated furanones, are produced by the marine algae *Delisea pulchra*

and were shown to be potent QS disruptors (de Nys *et al.*, 1993). Not only are those compounds potent against the QS system of *V. harveyi* but they also interfere with QS systems of other clinically relevant pathogens (Kutty *et al.*, 2013, Hentzer *et al.*, 2002). Although brominated furanones and fimbrolides are commonly investigated compounds, hitherto potential QS related targets were tested using directed approaches (Defoirdt *et al.*, 2007). In Chapter 4 irreversible fimbrolide binding partners in *V. harveyi* were elucidated by performing a global proteomic approach. The complex QS cascade of *V. harveyi* comprises a multitude of potential targets to disrupt QS (Chapter 2 and 3). Therefore, several natural brominated furanones were synthesized to dissolve their mode of action on the QS cascade in *V. harveyi* (Chapter 4). Compounds which led to reduced growth of the bacteria were excluded. The brominated furanone F1 and the corresponding probe FP3 were selected for further studies as their addition led to the strongest bioluminescence reduction. Using gel-free mass spectrometry, specific and irreversible fimbrolide binding partners were identified. These targets included LuxS, PhaB and LuxE among others. This is interesting as all three proteins play or are assumed to play a role in QS. LuxS is the synthase responsible for AI-2 production (Schauder *et al.*, 2001). LuxE, a part of the luciferase complex, is an acyl-protein synthetase and responsible for the ATP-dependent activation of fatty acids (Boylan *et al.*, 1989). PhaB is an acetoacetyl-CoA reductase and participates in polyhydroxybutyrate synthesis which is assumed to serve as an energy repository for bioluminescence (Miyamoto *et al.*, 1998). However, investigation of a *phaB* deletion mutant showed, that this target is not responsible for the observed reduction in bioluminescence as the mutant showed the same luminescence pattern as the wild type. The identification of LuxS as target of the fimbrolides is in agreement with previous reports (Figure 7.2) (Zang *et al.*, 2009). Though, the experiments revealed a different residue as ligand attachment site (the catalytic Cys83 instead of Cys128). But LuxS cannot solely be responsible for the observed phenotype, as a strain deleted for *luxS* was also inhibited in bioluminescence production upon fimbrolide treatment. Rather a target downstream of LuxO in the cascade accounts for this inhibition since a *luxO* deletion mutant was still inhibited in bioluminescence production upon fimbrolide addition.

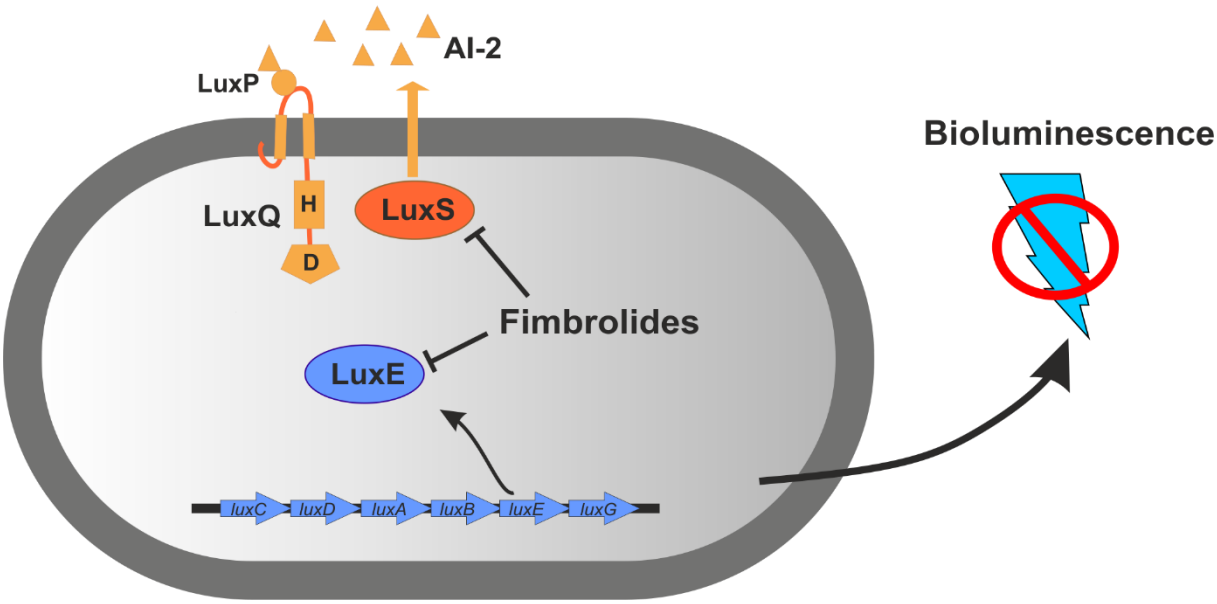


Figure 7.2 Scheme of cellular fimbrolide targets of the QS pathway in *V. harveyi*. Fimbrolides irreversibly bind to LuxS (orange), the AI-2 synthase, and LuxE (blue), a subunit of the luciferase complex. Thereby the activity of LuxS and LuxE are inhibited and this leads to bioluminescence inhibition

LuxE was found to be an irreversible target of fimbrolide binding as well (Figure 7.2). Therefore, it is likely that the largest impact, at least on the bioluminescence inhibition, is achieved by fimbrolide binding to the catalytic residue Cys362 of LuxE and thereby inhibiting the corresponding enzymatic activity (Figure 7.2). It was shown that furanones block QS in *V. harveyi* by binding to the master regulator LuxR and thereby prevent binding of LuxR to the target promoters (Defoirdt *et al.*, 2007). LuxR was not identified as a target in this global proteomic approach. Nevertheless, LuxR cannot be excluded as a direct fimbrolide target, but the results indicate that LuxR is at least not an irreversible target. Manefield *et al.* showed that upon binding of furanones, LuxR is destabilized by a factor of 100 (Manefield *et al.*, 2002). It could be that LuxR simply is degraded and therefore could not be identified in the proteome assay.

Given the fact that these natural furanones interfere with QS in a multitude of ways and moreover at different stages in the cascade makes them an interesting tool for the development of new treatments against pathogenic bacteria. A multitude of pathogens use different QS signals to regulate virulence. It was shown that AI-2 is important for the expression and distribution of virulence factors of *Enterococcus faecalis* (Rossmann *et al.*, 2015) and its concentration is critical for biofilm formation of e.g. *Actinomyces naeslundii* and *Streptococcus oralis* (Rickard *et al.*, 2006). Contact lenses coated with fimbrolides exhibited reduced adhesion of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Serratia marcescens* and *Acanthamoeba* sp. between 67 and 92% (Zhu *et al.*, 2008). As LuxS, the

synthase of AI-2 in *V. harveyi*, was found to be inhibited by fimbrolides in the presented proteomic approach one could imagine a potential role of these compounds in combatting bacterial infections by attenuating virulence and biofilm formation. Especially with the knowledge, that AI-2 is important for infection of the brine shrimp *A. franciscana* and the giant freshwater prawn *M. rosenbergii* (Pande *et al.*, 2013, Defoirdt & Sorgeloos, 2012). The disruption of QS by a natural and a synthetic furanone was already shown to protect *A. franciscana* from the pathogenic *V. harveyi* (Defoirdt *et al.*, 2006). Based on the fact that *V. harveyi* pathogenicity is a severe threat to shrimp farms, fimbrolides could contribute to a new strategy besides antibiotic treatments and thereby reducing the risk of increased antibiotic resistances.

7.4 The role of prophages

Bacteria and viruses live in close contact in their specific environment. Numerous bacteria, for example in the human gut, can live as harmless commensals, but switch to pathogenicity and cause fatal infections. Recently, a novel mechanism was described on how phages contribute to virulence. It was shown that *E. faecalis* uses the universal AI, AI-2, for the induction of virulence genes. At high AI-2 concentrations and therefore HCD, phage release is triggered and hence the neighboring cells are infected (Rossmann *et al.*, 2015). This novel link between QS, specifically AI-2 concentration, and phage release was interesting given the fact that *V. harveyi* comprises a QS cascade combining the input of three different AIs. Moreover, putative prophage sequences were identified in more than 70% of the sequenced genomes (Canchaya *et al.*, 2003) and marine environments are exceptionally rich in free virus particles (Bergh *et al.*, 1989). An initial bioinformatical analysis suggested the presence of five putative prophages on each of the two chromosomes (Chapter 5). However, manual curation of the data regarding the presence of specific phage related sequences, e.g. *att* sites for recombination of the DNA, reduced the amount of putative intact prophage regions to two on each chromosome. One of the putative prophage regions on chromosome I showed similarities to *Halomonas* phage ΦHAP-1, belonging to the family of *Myoviridae* (Mobberley *et al.*, 2008). The second presumably intact prophage on chromosome I shares orthologous proteins with the filamentous *Vibrio* phage VfO4K68 (Chang *et al.*, 2002). On chromosome II two presumably intact phage regions were identified as well. A *Vibrio* kappa-like phage and *Vibrio* phage VfO3K6-like phage (Nasu *et al.*, 2000). *Vibrio* phage kappa belongs to the family of *Myoviridae* likewise as phage ΦHAP-1 (Guidolin & Manning, 1987) and the *Vibrio* phages VfO4K68 and VfO3K6 belong to the genus *Inovirus.* In contrast to the filamentous *Inoviruses, Myovidridae* are characterized by a head-to-tail morphology. The cytotoxic

antibiotic mitomycin C is a DNA-damaging agent and a potent SOS response inducer (Wei *et al.*, 2001). The addition of mitomycin C to a growing culture is a common approach in studying prophage induction. Sequencing of concentrated extracellular DNA from a mitomycin C induced culture supernatant revealed significantly increased amounts of DNA, mapping the four bioinformatically identified prophage regions (factor 1.2-2.2). Transmission electron microscopy (TEM) of phage concentrates revealed the presence of two morphologically different phages, however none showed a filamentous phenotype but rather comprised an icosahedral head structure and a contractile tail (Chapter 5). Upon mitomycin C treatment at least two prophages were shifted from the lysogenic to the lytic cycle (Figure 7.3). The identified phages differed significantly in head diameter size (45 nm compared to 70 nm). It was shown recently that the genome size of phages can directly correlate with their physical size (Cui *et al.*, 2014). Therefore, the bigger phage in the TEM is assumed to be the *Vibrio* phage kappa-like, as this one comprises the larger genomic region. The other identified head structure is supposed to belong to the ΦHAP-1-like phage (Chapter 5). The lack of filamentous phages could be due to the possibility that they are less abundant than the head-to-tail bacteriophages or get destroyed during the concentration process. Either less filamentous phages are built upon mitomycin C treatment or it was not possible to precipitate those phages performing ultracentrifugation with polyethylene glycol. For other filamentous phages precipitation was performed with 40% saturated ammonium sulfate (Nasu *et al.*, 2000). This could serve as an alternative strategy for future experiments.

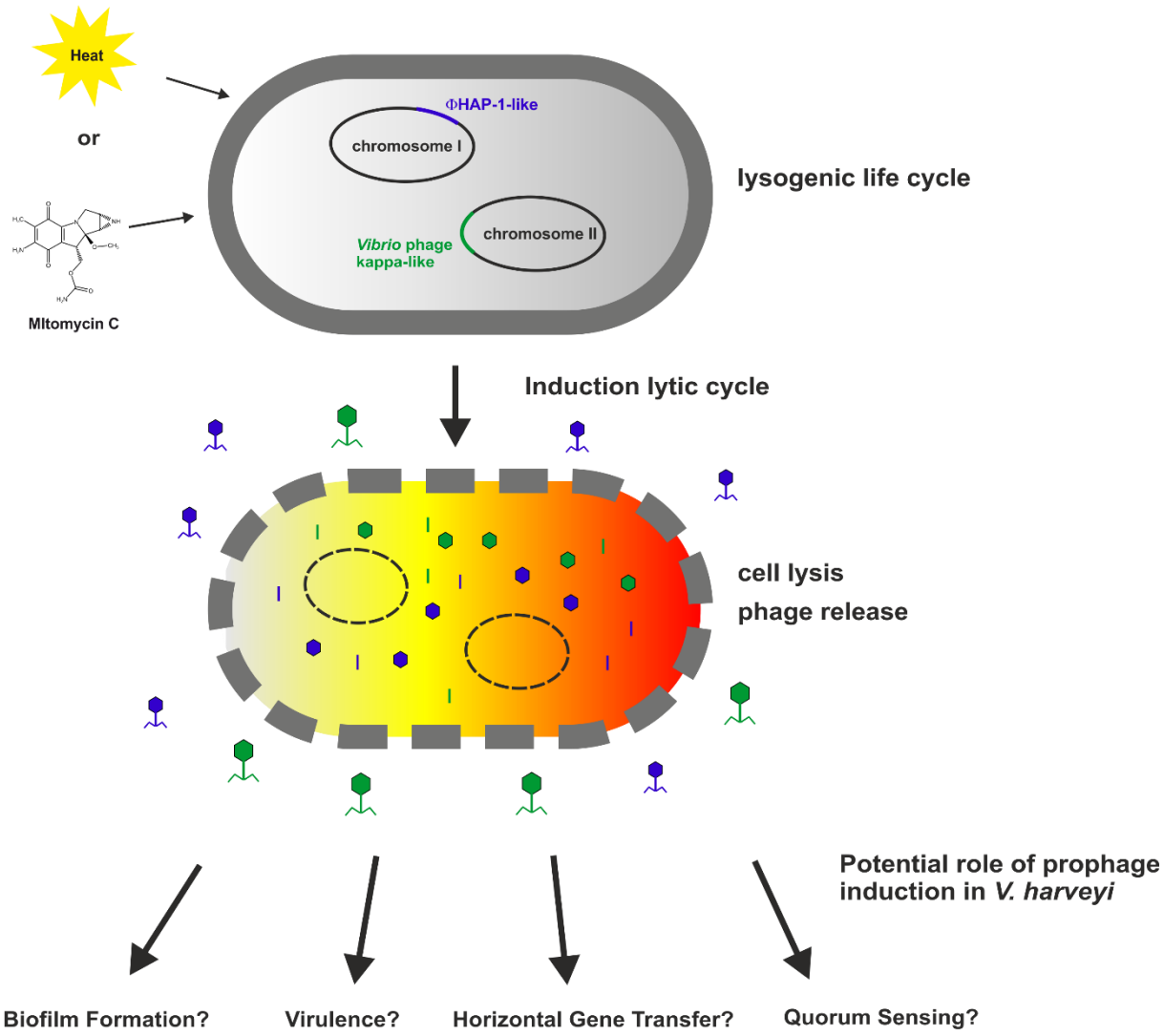


Figure 7.3 Scheme of prophage induction in *V. harveyi* upon mitomycin C or heat treatment. Mitomycin C and heat treatment induces the lytic cycle of at least two different prophages: a ΦHAP-1-like phage (blue) and a *Vibrio* phage kappa-like phage. The host cell machinery is used for the production of new phages. Eventually, the bacterial cell lyses and the phages are released. The impact of prophage induction on host physiology remains to be investigated.

The sequence homologies of the phages released from *V. harveyi* to already known phages suggests that they differ in their host range. The *Vibrio* kappa-like phage seems to infect mainly *Vibrio* species but homologous proteins were also identified in certain Alteromonads (Gerdes & Romig, 1975). The identified ΦHAP-1-like phage originates from *Halomonas aquamarina* (Mobberley *et al.*, 2008), and its presence in *V. harveyi* suggests a broader infection spectrum. Accordingly, we found genes homologous to phage ΦHAP-1 in the genomes of other alpha-, beta- or gamma-proteobacteria like *Wolbachia*, *Burkholderia* or *Shewanella* species. The failed efforts to reinfect *Vibrio* cells using phage concentrates could be explained by a resistance mechanism called superinfection exclusion, which is a common principle of bacterial cells to be protected against further phages including themselves (Bondy-Denomy & Davidson, 2014). The superinfection exclusion can be achieved through

several mechanisms e.g. by preventing entrance of phage DNA in a bacterial cell via alterations in cell surface or other components of the cell envelope (Labrie *et al.*, 2010). Due to the natural habitat of *V. harveyi* for example on the surface of algae, cells were exposed to heat stress which should resemble a rather natural stress situation and resulted likewise to mitomycin C in the induction of two morphologically different phages (Figure 7.3) (Chapter 5). It is likely that *V. harveyi* encounters temperature shifts during the algae colonization close to the water surface. Recently, it was shown for *Corynebacterium glutamicum* that upon spontaneously induced SOS response only a fraction of cells undergoes lysis as a result of prophage induction (Nanda *et al.*, 2014). Likewise, the activity dynamics of two phage related promoters (one belonging to the ΦHAP-1-like and one to the *Vibrio* phage kappa-like) upon heat stress (45-55°C) showed cell-to-cell variations. After incubation at 45°C the fluorescence levels in single cells differed from no fluorescence via medium to high fluorescence (Chapter 5). These high cell-to-cell variations could be a form of bet-hedging (Veening *et al.*, 2008). For *Shewanella oneidensis* and *Streptococcus pneumoniae* it was described that it is beneficial for the population if only a part induces prophages (Gödeke *et al.*, 2011, Carrolo *et al.*, 2010). Thus, the release of extracellular DNA promotes proper biofilm formation. Furthermore, due to the high cell densities in biofilms, phage-induced lysis provides a vast pool of extracellular DNA for horizontal gene transfer (Madsen *et al.*, 2012). Moreover, it was shown to be advantageous for Shiga toxin-producing *E. coli* if only a part of the population induces the prophages harboring genes coding for the toxin like a sacrifice as a form of "bacterial altruism" (Łoś *et al.*, 2012). In this context it is interesting to note that also the prophages in the genome of *V. harveyi* harbor potential virulence related genes (e.g. genes encoding hemolysins and chitinases). However, with increasing temperatures we observed less cell-to-cell variations as most of the cells showed strong promoter activities. Furthermore, the percentage of dead cells increased and hence could be the result of a population collapse due to lysis of the cells. Bacterial cells and phages interact in various ways. As *V. harveyi* is a model organism for the investigation of QS and the newly identified link between AI-2 and phage induction in *E. faecalis* made it interesting to identify potential inducible prophages in the genome of *V. harveyi*. Our initial experiments towards the elucidation of a potential QS influence in the induction of prophages failed, but this could be due to the possibility that we investigated conditions in which a link between QS and prophage induction would not be favorable. We merely compared the promoter activities of selected phage-related genes in AI producing cells (wild type), in a mutant deleted for the AI synthases (QS-OFF) and in a mutant deprived for the response regulator LuxO (QS-ON) regarding induction at the single

cell level. In this initial approach we could not detect significant differences (data now shown).

V. harveyi belongs to the severest pathogens in shrimp farms (Austin & Zhang, 2006, Defoirdt & Sorgeloos, 2012). The observed bacterial lysis upon prophage induction could serve as basis for a novel strategy of phage therapy to combat the problem of luminous vibriosis in shrimp farms, especially with the emerging problem of antibiotic resistances (Stalin & Srinivasan, 2016). *V. harveyi* is thought to contribute among others to the so called milky sea effect (Lapota *et al.*, 1988). The so far unknown reason for the sudden breakdown of luminescence could likely be due to a population collapse caused by phage induction. Several other natural stresses besides heat can cause SOS response induction in bacteria and therefore lead to induction of prophages (Nanda *et al.*, 2015). One might be the exposure to UV light which could also play a role in the milky sea effect as the algae are close to the surface and thereby the bacteria, which are colonizing the algae, as well. UV light induction of SOS response and a corresponding prophage induction was also shown for *E. coli* (Lamont *et al.*, 1989). In this context it is worth mentioning that a bioinformatical analysis of the upstream region of the investigated prophage related promoter revealed a putative binding site for RecA and LexA (for the ΦHAP-1-like phage promoter) which both play a role in the SOS-response (Žgur-Bertok, 2013). Furthermore, heterogeneous expression for LexA-regulated genes was shown in *E. coli* and relies on stochastic factors as well as the binding affinity of LexA to the SOS boxes (Kamenšek *et al.*, 2010).

7.5 Outlook

To further complete the knowledge on QS in *V. harveyi* some questions remain open. At the level of information processing, it would be interesting to investigate if a QS repressed gene shows the same output dynamics like the positively regulated genes encoding the luciferase at the single-cell level. Furthermore, most studies to understand the processing of information are performed under laboratory conditions, it would be intriguing to analyze *V. harveyi* cells in their natural habitat as free living in sea water, where AIs are washed away or while colonizing a shrimp at the single-cell level with regard to potential bet-hedging and/or division labor strategies. Within this thesis it was found that at least LuxN and LuxQ cluster in the membrane. In a next step it should be analyzed if e.g. LuxN and LuxQ form joint cluster using a strain harboring both receptor-fluorophore fusions. High resolution microscopy approaches could be utilized to further dissolve the discovered clusters. Possibly also the third

sensor CqsS forms cluster but these could not be discovered using epifluorescence microscopy.

After the successful characterization of two inducible prophages in the genome of *V. harveyi*, a more detailed investigation of a potential link between QS and prophage induction would be of interest. Alternative strategies to switch from lysogenic to lytic lifestyle should be applied and may also result in an induction of the other potential prophages which were identified within the bioinformatic approaches. To unravel a potential link between QS and prophage induction another possibility could be to add an excess of AIs to a growing culture and analyze for lysis of the cells. Moreover, a potential influence of prophage induction on *V. harveyi* biofilm formation, as it was shown for *S. oneidensis*, should be assessed in another study.

7.6 References for Concluding Discussion

Ackermann, M., B. Stecher, N.E. Freed, P. Songhet, W.-D. Hardt & M. Doebeli, (2008) Self-destructive cooperation mediated by phenotypic noise. *Nature* **454**: 987-990.

Anetzberger, C., M. Reiger, A. Fekete, U. Schell, N. Stambrau, L. Plener, J. Kopka, P. Schmitt-Kopplin, H. Hilbi & K. Jung, (2012) Autoinducers act as biological timers in *Vibrio harveyi*. *PLoS One* **7**: e48310.

Austin, B. & X.H. Zhang, (2006) *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. *Lett Appl Microbiol* **43**: 119-124.

Bassler, B.L., M. Wright, R.E. Showalter & M.R. Silverman, (1993) Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Mol Microbiol* **9**: 773-786.

Bergh, O., K.Y. Borsheim, G. Bratbak & M. Heldal, (1989) High abundance of viruses found in aquatic environments. *Nature* **340**: 467-468.

Blunt, J.W., B.R. Copp, R.A. Keyzers, M.H. Munro & M.R. Prinsep, (2012) Marine natural products. *Nat Prod Rep* **29**: 144-222.

Bondy-Denomy, J. & A.R. Davidson, (2014) When a virus is not a parasite: the beneficial effects of prophages on bacterial fitness. *J Microbiol* **52**: 235-242.

Boylan, M., C. Miyamoto, L. Wall, A. Graham & E. Meighen, (1989) Lux C, D and E genes of the *Vibrio fischeri* luminescence operon code for the reductase, transferase, and synthetase enzymes involved in aldehyde biosynthesis. *Photochem Photobiol* **49**: 681-688.

Briegel, A., X. Li, A.M. Bilwes, K.T. Hughes, G.J. Jensen & B.R. Crane, (2012) Bacterial chemoreceptor arrays are hexagonally packed trimers of receptor dimers networked by rings of kinase and coupling proteins. *Proc Natl Acad Sci U S A* **109**: 3766-3771.

Cahn, F.H. & M.S. Fox, (1968) Fractionation of transformable bacteria from ocompetent cultures of *Bacillus subtilis* on renografin gradients. *J Bacteriol* **95**: 867-875.

Canchaya, C., C. Proux, G. Fournous, A. Bruttin & H. Brussow, (2003) Prophage genomics. *Microbiol Mol Biol Rev* **67**: 238-276.

Cao, J.G. & E.A. Meighen, (1989) Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. *J Biol Chem* **264**: 21670-21676.

Carrolo, M., M.J. Frias, F.R. Pinto, J. Melo-Cristino & M. Ramirez, (2010) Prophage spontaneous activation promotes DNA release enhancing biofilm formation in *Streptococcus pneumoniae*. *PLoS One* **5**: e15678.

Chang, B., H. Miyamoto, H. Taniguchi & S. Yoshida, (2002) Isolation and genetic characterization of a novel filamentous bacteriophage, a deleted form of phage f237, from a pandemic *Vibrio parahaemolyticus* O4:K68 strain. *Microbiol Immunol* **46**: 565-569.

Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B.L. Bassler & F.M. Hughson, (2002) Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**: 545-549.

Chu, Y.Y., M. Nega, M. Wolfle, L. Plener, S. Grond, K. Jung & F. Gotz, (2013) A new class of quorum quenching molecules from *Staphylococcus* species affects communication and growth of gram-negative bacteria. *PLoS Pathog* **9**: e1003654.

Churchill, M.E.A. & L. Chen, (2011) Structural basis of acyl-homoserine lactone-dependent signaling. *Chem Rev* **111**: 68-85.

Cui, J., T.E. Schlub & E.C. Holmes, (2014) An allometric relationship between the genome length and virion volume of viruses. *J Virol* **88**: 6403-6410.

de Nys, R., A.D. Wright, G.M. König & O. Sticher, (1993) New halogenated furanones from the marine alga *Delisea pulchra* (cf. *fimbriata*). *Tetrahedron* **49**: 11213-11220.

Defoirdt, T., R. Crab, T.K. Wood, P. Sorgeloos, W. Verstraete & P. Bossier, (2006) Quorum sensing-disrupting brominated furanones protect the gnotobiotic brine shrimp *Artemia franciscana* from pathogenic *Vibrio harveyi*, *Vibrio campbellii*, and *Vibrio parahaemolyticus* isolates. *Appl Environ Microbiol* **72**: 6419-6423.

Defoirdt, T., C.M. Miyamoto, T.K. Wood, E.A. Meighen, P. Sorgeloos, W. Verstraete & P. Bossier, (2007) The natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone disrupts quorum sensing-regulated gene expression in *Vibrio harveyi* by decreasing the DNA-binding activity of the transcriptional regulator protein LuxR. *Environ Microbiol* **9**: 2486-2495.

Defoirdt, T. & P. Sorgeloos, (2012) Monitoring of *Vibrio harveyi* quorum sensing activity in real time during infection of brine shrimp larvae. *ISME J* **6**: 2314-2319.

Dong, Y.-H., L.-H. Wang, J.-L. Xu, H.-B. Zhang, X.-F. Zhang & L.-H. Zhang, (2001) Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature* **411**: 813-817.

Federle, M.J. & B.L. Bassler, (2003) Interspecies communication in bacteria. *J Clin Invest* **112**: 1291-1299.

Freeman, J.A. & B.L. Bassler, (1999) Sequence and function of LuxU: a two-component phosphorelay protein that regulates quorum sensing in *Vibrio harveyi*. *J Bacteriol* **181**: 899-906.

Genin, S. & T.P. Denny, (2012) Pathogenomics of the *Ralstonia solanacearum* species complex. *Annu Rev Phytopathol* **50**: 67-89.

Gerdes, J.C. & W.R. Romig, (1975) Complete and defective bacteriophages of classical *Vibrio cholerae*: Relationship to the kappa type bacteriophage. *J Virol* **15**: 1231-1238.

Gödeke, J., K. Paul, J. Lassak & K.M. Thormann, (2011) Phage-induced lysis enhances biofilm formation in *Shewanella oneidensis* MR-1. *ISME J* **5**: 613-626.

Guidolin, A. & P.A. Manning, (1987) Genetics of *Vibrio cholerae* and its bacteriophages. *Microbiol Rev* **51**: 285-298.

Hautefort, I., M.J. Proenca & J.C. Hinton, (2003) Single-copy green fluorescent protein gene fusions allow accurate measurement of *Salmonella* gene expression *in vitro* and during infection of mammalian cells. *Appl Environ Microbiol* **69**: 7480-7491.

Henares, B.M., K.E. Higgins & E.M. Boon, (2012) Discovery of a nitric oxide responsive quorum sensing circuit in *Vibrio harveyi*. *ACS Chem Biol* **7**: 1331-1336.

Henares, B.M., Y. Xu & E.M. Boon, (2013) A nitric oxide-responsive quorum sensing circuit in *Vibrio harveyi* regulates flagella production and biofilm formation. *Int J Mol Sci* **14**: 16473-16484.

Henke, J.M. & B.L. Bassler, (2004) Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi*. *J Bacteriol* **186**: 6902-6914.

Hentzer, M., K. Riedel, T.B. Rasmussen, A. Heydorn, J.B. Andersen, M.R. Parsek, S.A. Rice, L. Eberl, S. Molin, N. Hoiby, S. Kjelleberg & M. Givskov, (2002) Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology* **148**: 87-102.

Inoue, T., M. Higuchi, Y. Hashimoto, M. Seki, M. Kobayashi, T. Kato, S. Tabata, K. Shinozaki & T. Kakimoto, (2001) Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. *Nature* **409**: 1060-1063.

Jung, S.A., C.A. Chapman & W.L. Ng, (2015) Quadruple quorum-sensing inputs control *Vibrio cholerae* virulence and maintain system robustness. *PLoS Pathog* **11**: e1004837.

Jung, S.A., L.A. Hawver & W.L. Ng, (2016) Parallel quorum sensing signaling pathways in *Vibrio cholerae*. *Curr Genet* **62**: 255-260.

Kamenšek, S., Z. Podlesek, O. Gillor & D. Žgur-Bertok, (2010) Genes regulated by the *Escherichia coli* SOS repressor LexA exhibit heterogeneous expression. *BMC Microbiol* **10**: 283.

Ke, X., L.C. Miller & B.L. Bassler, (2015) Determinants governing ligand specificity of the *Vibrio harveyi* LuxN quorum-sensing receptor. *Mol Microbiol* **95**: 127-142.

Korobkova, E., T. Emonet, J.M. Vilar, T.S. Shimizu & P. Cluzel, (2004) From molecular noise to behavioural variability in a single bacterium. *Nature* **428**: 574-578.

Kutty, S.K., N. Barraud, A. Pham, G. Iskander, S.A. Rice, D.S. Black & N. Kumar, (2013) Design, synthesis, and evaluation of fimbrolide-nitric oxide donor hybrids as antimicrobial agents. *J Med Chem* **56**: 9517-9529.

Labrie, S.J., J.E. Samson & S. Moineau, (2010) Bacteriophage resistance mechanisms. *Nat Rev Micro* **8**: 317-327.

Lamont, I., A.M. Brumby & J.B. Egan, (1989) UV induction of coliphage 186: prophage induction as an SOS function. *Proc Natl Acad Sci U S A* **86**: 5492-5496.

Lapota, D., C. Galt, J.R. Losee, H.D. Huddell, J.K. Orzech & K.H. Nealson, (1988) Observations and measurements of planktonic bioluminescence in and around a milky sea. *J Exp Mar Biol Ecol* **119**: 55-81.

Lee, J. & L. Zhang, (2015) The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein Cell* **6**: 26-41.

Long, T., K.C. Tu, Y. Wang, P. Mehta, N.P. Ong, B.L. Bassler & N.S. Wingreen, (2009) Quantifying the integration of quorum-sensing signals with single-cell resolution. *PLoS Biol* **7**: e68.

Łoś, J.M., M. Łoś, A. Węgrzyn & G. Węgrzyn, (2012) Altruism of Shiga toxin-producing *Escherichia coli*: recent hypothesis versus experimental results. *Front Cell Infect Microbiol* **2**: 166.

Lupp, C. & E.G. Ruby, (2005) *Vibrio fischeri* uses two quorum-sensing systems for the regulation of early and late colonization factors. *J Bacteriol* **187**: 3620-3629.

Lupp, C., M. Urbanowski, E.P. Greenberg & E.G. Ruby, (2003) The *Vibrio fischeri* quorum-sensing systems *ain* and *lux* sequentially induce luminescence gene expression and are important for persistence in the squid host. *Mol Microbiol* **50**: 319-331.

Maddock, J.R. & L. Shapiro, (1993) Polar location of the chemoreceptor complex in the *Escherichia coli* cell. *Science* **259**: 1717-1723.

Madsen, J.S., M. Burmolle, L.H. Hansen & S.J. Sorensen, (2012) The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunol Med Microbiol* **65**: 183-195.

Manefield, M., T.B. Rasmussen, M. Henzter, J.B. Andersen, P. Steinberg, S. Kjelleberg & M. Givskov, (2002) Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. *Microbiology* **148**: 1119-1127.

Mao, H., P.S. Cremer & M.D. Manson, (2003) A sensitive, versatile microfluidic assay for bacterial chemotaxis. *Proc Natl Acad Sci U S A* **100**: 5449-5454.

Mehta, P., S. Goyal, T. Long, B.L. Bassler & N.S. Wingreen, (2009) Information processing and signal integration in bacterial quorum sensing. *Mol Syst Biol* **5**: 325.

Miller, M.B. & B.L. Bassler, (2001) Quorum sensing in bacteria. *Annu Rev Microbiol* **55**: 165-199.

Miyamoto, C.M., W. Sun & E.A. Meighen, (1998) The LuxR regulator protein controls synthesis of polyhydroxybutyrate in *Vibrio harveyi*. *Biochim Biophys Acta* **1384**: 356-364.

Mizuno, T., (2005) Two-component phosphorelay signal transduction systems in plants: from hormone responses to circadian rhythms. *Biosci Biotechnol Biochem* **69**: 2263-2276.

Mobberley, J.M., R.N. Authement, A.M. Segall & J.H. Paul, (2008) The temperate marine phage ΦHAP-1 of *Halomonas aquamarina* possesses a linear plasmid-like prophage genome. *J Virol* **82**: 6618-6630.

Mok, K.C., N.S. Wingreen & B.L. Bassler, (2003) *Vibrio harveyi* quorum sensing: a coincidence detector for two autoinducers controls gene expression. *EMBO J* **22**: 870-881.

Nanda, A.M., A. Heyer, C. Kramer, A. Grunberger, D. Kohlheyer & J. Frunzke, (2014) Analysis of SOS-induced spontaneous prophage induction in *Corynebacterium glutamicum* at the single-cell level. *J Bacteriol* **196**: 180-188.

Nanda, A.M., K. Thormann & J. Frunzke, (2015) Impact of spontaneous prophage induction on the fitness of bacterial populations and host-microbe interactions. *J Bacteriol* **197**: 410-419.

Nasu, H., T. Iida, T. Sugahara, Y. Yamaichi, K.S. Park, K. Yokoyama, K. Makino, H. Shinagawa & T. Honda, (2000) A filamentous phage associated with recent pandemic *Vibrio parahaemolyticus* O3:K6 strains. *J Clin Microbiol* **38**: 2156-2161.

Natrah, F.M., H.A. Ruwandeepika, S. Pawar, I. Karunasagar, P. Sorgeloos, P. Bossier & T. Defoirdt, (2011) Regulation of virulence factors by quorum sensing in *Vibrio harveyi*. *Vet Microbiol* **154**: 124-129.

Neiditch, M.B., M.J. Federle, S.T. Miller, B.L. Bassler & F.M. Hughson, (2005) Regulation of LuxPQ receptor activity by the quorum-sensing signal autoinducer-2. *Mol Cell* **18**: 507-518.

Neumann, S., C.H. Hansen, N.S. Wingreen & V. Sourjik, (2010) Differences in signalling by directly and indirectly binding ligands in bacterial chemotaxis. *EMBO J* **29**: 3484-3495.

Ng, W.L., L.J. Perez, Y. Wei, C. Kraml, M.F. Semmelhack & B.L. Bassler, (2011) Signal production and detection specificity in *Vibrio* CqsA/CqsS quorum-sensing systems. *Mol Microbiol* **79**: 1407-1417.

Pande, G.S., F.M. Natrah, P. Sorgeloos, P. Bossier & T. Defoirdt, (2013) The *Vibrio campbellii* quorum sensing signals have a different impact on virulence of the bacterium towards different crustacean hosts. *Vet Microbiol* **167**: 540-545.

Rickard, A.H., R.J. Palmer, Jr., D.S. Blehert, S.R. Campagna, M.F. Semmelhack, P.G. Egland, B.L. Bassler & P.E. Kolenbrander, (2006) Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth. *Mol Microbiol* **60**: 1446-1456.

Rossmann, F.S., T. Racek, D. Wobser, J. Puchalka, E.M. Rabener, M. Reiger, A.P. Hendrickx, A.K. Diederich, K. Jung, C. Klein & J. Huebner, (2015) Phage-mediated dispersal of biofilm and distribution of bacterial virulence genes is induced by quorum sensing. *PLoS Pathog* **11**: e1004653.

Schaefer, A.L., B.L. Hanzelka, A. Eberhard & E.P. Greenberg, (1996) Quorum sensing in *Vibrio fischeri*: probing autoinducer-LuxR interactions with autoinducer analogs. *J Bacteriol* **178**: 2897-2901.

Schauder, S., K. Shokat, M.G. Surette & B.L. Bassler, (2001) The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Mol Microbiol* **41**: 463-476.

Segall, J.E., S.M. Block & H.C. Berg, (1986) Temporal comparisons in bacterial chemotaxis. *Proc Natl Acad Sci U S A* **83**: 8987-8991.

Sheen, J., (2002) Phosphorelay and transcription control in cytokinin signal transduction. *Science* **296**: 1650-1652.

Siryaporn, A., B.S. Perchuk, M.T. Laub & M. Goulian, (2010) Evolving a robust signal transduction pathway from weak cross-talk. *Mol Syst Biol* **6**: 452-452.

Sourjik, V. & H.C. Berg, (2000) Localization of components of the chemotaxis machinery of *Escherichia coli* using fluorescent protein fusions. *Mol Microbiol* **37**: 740-751.

Stalin, N. & P. Srinivasan, (2016) Molecular characterization of antibiotic resistant *Vibrio harveyi* isolated from shrimp aquaculture environment in the south east coast of India. *Microb Pathog* **97**: 110-118.

Swem, L.R., D.L. Swem, N.S. Wingreen & B.L. Bassler, (2008) Deducing receptor signaling parameters from *in vivo* analysis: LuxN/AI-1 quorum sensing in *Vibrio harveyi*. *Cell* **134**: 461-473.

Teng, S.W., J.N. Schaffer, K.C. Tu, P. Mehta, W. Lu, N.P. Ong, B.L. Bassler & N.S. Wingreen, (2011) Active regulation of receptor ratios controls integration of quorum-sensing signals in *Vibrio harveyi*. *Mol Syst Biol* **7**: 491.

Thattai, M. & A. van Oudenaarden, (2004) Stochastic gene expression in fluctuating environments. *Genetics* **167**: 523-530.

Veening, J.W., W.K. Smits & O.P. Kuipers, (2008) Bistability, epigenetics, and bet-hedging in bacteria. *Annu Rev Microbiol* **62**: 193-210.

Vlamakis, H., C. Aguilar, R. Losick & R. Kolter, (2008) Control of cell fate by the formation of an architecturally complex bacterial community. *Genes Dev* **22**: 945-953.

Waters, C.M. & B.L. Bassler, (2006) The *Vibrio harveyi* quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. *Genes Dev* **20**: 2754-2767.

Wei, Y., W.L. Ng, J. Cong & B.L. Bassler, (2012) Ligand and antagonist driven regulation of the *Vibrio cholerae* quorum-sensing receptor CqsS. *Mol Microbiol* **83**: 1095-1108.

Wei, Y., A.C. Vollmer & R.A. LaRossa, (2001) In vivo titration of mitomycin C action by four *Escherichia coli g*enomic regions on multicopy plasmids. *J Bacteriol* **183**: 2259-2264.

Williams, J.W., X. Cui, A. Levchenko & A.M. Stevens, (2008) Robust and sensitive control of a quorum-sensing circuit by two interlocked feedback loops. *Mol Syst Biol* **4**: 234-234.

Winans, S.C., (2011) A new family of quorum sensing pheromones synthesized using S-adenosylmethionine and Acyl-CoAs. *Mol Microbiol* **79**: 1403-1406.

Xavier, K.B. & B.L. Bassler, (2005) Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in *Escherichia coli*. *J Bacteriol* **187**: 238-248.

Zang, T., B.W. Lee, L.M. Cannon, K.A. Ritter, S. Dai, D. Ren, T.K. Wood & Z.S. Zhou, (2009) A naturally occurring brominated furanone covalently modifies and inactivates LuxS. *Bioorg Med Chem Lett* **19**: 6200-6204.

Žgur-Bertok, D., (2013) DNA Damage Repair and Bacterial Pathogens. *PLoS Pathog* **9**: e1003711.

Zhu, H., A. Kumar, J. Ozkan, R. Bandara, A. Ding, I. Perera, P. Steinberg, N. Kumar, W. Lao, S.S. Griesser, L. Britcher, H.J. Griesser & M.D. Willcox, (2008) Fimbrolide-coated antimicrobial lenses: their in vitro and in vivo effects. *Optom Vis Sci* **85**: 292-300.

Supplemental Material Chapter 3

Activity, abundance and localization of Quorum Sensing Receptors in *Vibrio harveyi*

Nicola Lorenz[1], Jae-Yen Shin[1] and Kirsten Jung[1].

[1]Munich Center for Integrated Protein Science (CiPSM) at the Department of Biology I, Microbiology, Ludwig-Maximilians-Universität München, Martinsried, Germany

Running Title: hybrid histidine kinases in *Vibrio*

To whom correspondence should be addressed: Prof. Dr. K. Jung, Ludwig-Maximilians-Universität München, Department Biologie I, Bereich Mikrobiologie, Großhaderner Str. 2-4, D-82152 Martinsried, Germany. Tel:+49-89-2180-74500; Fax:+49-89-2180-74520; E-mail: jung@lmu.de

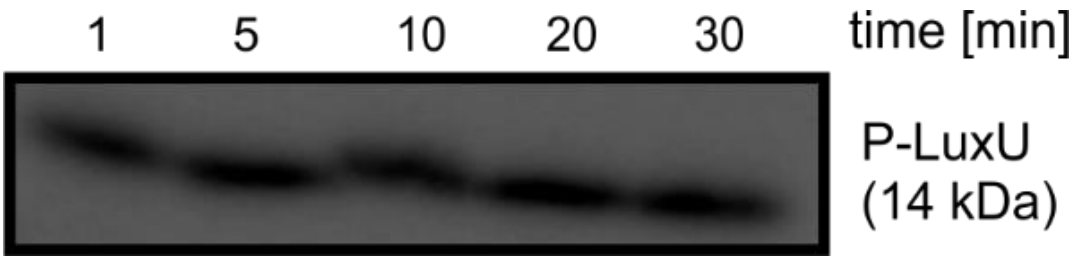


Figure S 1 Stability of P-LuxU *in vitro.* LuxU was initially phosphorylated via Lux(P)Q with [γ^{32}-P] Mg^{2+} ATP. After purification P-LuxU was incubated at room temperatures for distinct times. At the indicated times the phosphorylation reaction was stopped, proteins were separated by SDS-PAGE followed by exposure of the gels to a phosphoscreen. The autoradiograph corresponding to LuxU protein size is representative of three independent experiments.

Danksagung

Abschließend möchte ich mich bei allen Leuten bedanken, die zum Entstehen dieser Arbeit beigetragen haben.

Zuerst möchte ich mich bei Frau Prof. Dr. Kirsten Jung für die herzliche Aufnahme in ihre Arbeitsgruppe sowie die Betreuung und Unterstützung während der letzen Jahre bedanken. Ich habe mich stets wohl gefühlt und die Arbeit hat mir immer viel Freude bereitet.

Des Weiteren möchte mich bei meinem gesamten Prüfungskomitee insbesondere bei Prof. Dr. Marc Bramkamp bedanken, der sich als Zweitgutachter zur Verfügung gestellt hat.

Beim DFG Schwerpunkt für "Phenotypic heterogeneity and sociobiology of bacterial populations" möchte ich mich für die Möglichkeit bedanken, an diversen wissenschaftlichen Konferenzen teilzunehmen. CiPSM woman danke ich für die Finanzierung mehrere projektspezifischer Sachmittel.

Ein großer Dank geht auch an unsere Kollaboratoren Prof. Dr. Stephan Sieber, Weining Zhao, Prof. Dr. Ulrich Gerland, Dr. Tiago Ramalho, Dr. Andreas Brachmann und Mauricio Toro-Nahuelpan für die erfolgreiche Zusammenarbeit.

Weiterhin möchte ich mich bei der AG Bramkamp, im Besonderen bei Juri Bach, Giacomo Giacomelli und Catriona Donovan, für die Hilfe mit dem Deltavision und Elyra Mikroskop bedanken.

Für die tolle Atmosphäre und Hilfsbereitschaft in der Arbeit möchte ich mich bei allen gegenwärtigen sowie ehemaligen Mitgliedern der AG K. Jung, AG H. Jung und AK R. Heermann bedanken. Ein besonderer Dank geht dabei an Dr. Jürgen Lassak, Dr. Jae-Yen Shin, Dr. Laure Plener, Dr. Matthias Reiger, Dr. Luitpold Fried, Dr. Hannah Schramke, Dr. Susanne Bracher, Angela Glaeser, Jannis Brehm, Ralph Krafczyk, Christopher Maier, Laura Lindenthal und Wolfram Volkwein. Ebenfalls danken möchte ich "meinen" ehemaligen Studenten Franziska Häfele, Johannes Redwitz, Lisa Poettinger und Georg Stabl.

Ganz speziell möchte ich noch Laure (Lord P aka Schlaure), Hannah (Schrumpi aka Frau mit dem roten Hut aka Hannak aka Frau Schrammle) und Angi (GG aka Geli Glaeser aka Frangi) danken für die tolle Zeit die wir auch außerhalb der Arbeit verbracht haben. An dieser Stelle sei auch dem Jannis (Jannibär aka Jannis-Pannis) gedankt, der sich (erschreckend) nahtlos ins Mädels-/Satelliten-/Deobüro eingefügt hat. Bruno Pinheiro danke ich unter anderem für die interessanten Einblicke in die brasilianische Kultur (Wonderland). Wolfram Volkwein möchte ich noch herzlich dafür danken, dass er ein Korinthenkacker ist ;-).

Meiner lieben Anna möchte ich von ganzem Herzen für alles danken. Für unsere langjährige Freundschaft sowie die Einblicke in ihre Forschung zur Aneignung des Mobiltelefons durch Frauen im Kontext der Geschlechterbeziehungen in Gilgit-Baltistan ;-).

Ebenfalls danken möchte ich meiner gesamten Familie insbesondere meiner Tante Bettina sowie meiner Oma. Der größte Dank gebührt meinen Eltern Monika und Werner Lorenz sowie meinem Bruder Marco. Danke, Danke, Danke, dass ihr immer für mich da wart und stets an mich geglaubt habt.