Heterogeneity and timing in the quorum sensing system of *Vibrio harveyi*

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“What is impossible for the individual, can be archived by many.”

F. W. Raiffeisen (1818-1888), german agrar politician and social reformer

Dedicated to my family, especially to my mother

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 an oral exam. The present dissertation (neither the entire dissertation nor parts) has not been presented to another examination board.

Munich, 2012/10/25 _____

Claudia Anetzberger

Eidesstattliche Versicherung

Hiermit versichere ich eidesstattlich, dass die vorliegende 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 vorliegende Dissertation liegt weder ganz, noch in wesentlichen Teilen einer anderen Prüfungskommision vor.

München, 25.10.2012 _____

Claudia Anetzberger

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Nomenclature

Gene products are numbered in a way that the first methionine of the wild type protein is designated “1” in the amino acid sequence. N-terminally affinity tags are left unconsidered.

Amino acid substitutions 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 is terminally added in one-letter code.

The full lengths proteins LuxS, Pfs, LuxU, LuxN, LuxQ produced in this study exhibit a hexahistidine (His_6) tag attached either directly or via a two amino acid (leucine and glutamine) linker (LuxN) to the N- (LuxS, Pfs) or the C-terminus (LuxU, LuxN, LuxQ) of the protein. The protein LuxP was produced removing its signal sequences (amino acids 1-21) and exhibit a gluthathion-sepharose tag (GST) attached to the C–terminus of the protein. The complete notation His_6-[protein], [protein]-His_6, or [protein]-GST was reduced to the designation [protein] by means of shortness.

Gen deletions are designated with Δ, gene replacements as follows: replaced gene::new gene.

List of Abbreviations

AA	amino acid
AAA	ATPases associated with a variety of cellular activities
AB medium	autoinducer bioassay medium
AHL	acyl-homoserine lactone
AI	autoinducer
AI-2	autoinducer-2
amp^R	ampicillin resistance
ARNT	Ah receptor nuclear translocator protein
a.u.	arbitrary unit
bp	base pair
CAI-1	*Vibrio cholerae* autoinducer-1
cam^R	chloramphenicol resistance
CF	culture fluid
CLSM	confocal laser scanning microscopy
CM	cytoplasmic membrane
CP	cytoplasm
DNA	desoxyribonucleic acid
DPD	dihydroxy pentandione
DSF	diffusible signaling factor
$FMNH_2$	reduced riboflavin-phosphate
HAI-1	*Vibrio harveyi* autoinducer-1
HAMP domain	domain present in histidine kinases, adenyl cyclases, methyl-accepting proteins and phosphatases
Hpt domain	histidine phosphotransfer domain
HTH motif	helix-trun-helix motif
kan^R	kanamycin resistance
LB	lysogeny broth
LM	lysogeny marine broth
mRNA	messenger RNA
P	phosphoryl group
PAB medium	pacific aquarium-based medium
PAS domain	PER, ARNT, SIM domain
PCR	polymerase chain reaction
PER	period circadian protein
PP	periplasm
PQS	*Pseudomonas* QS
QS	quorum sensing
REC domain	receiver domain
RNA	ribonucleic acid
SAB medium	shrimp aquarium-based medium
SIM	single-minded protein
sRNA	small regulatory RNA
SRH	second region of homology
sRNA	small RNA
tet^R	tetracycline resistance
TCS	two component system
UV	ultra violet

Publications and Manuscripts originating from this thesis

Original research papers

Chapter 2 / Publication 2
Anetzberger,C., Pirch,T., and Jung,K. Heterogeneity in quorum sensing-regulated bioluminescence of *Vibrio harveyi* (2009). *Molecular Microbiology* **73**(2), 267-277.

Chapter 3 / Publication 3
Anetzberger,C., Schell,U., and Jung,K. Single cell analysis of *Vibrio harveyi* uncovers functional heterogeneity in responses to quorum sensing signals (2012). *BMC Microbiology* **12**, 209.

Chapter 4 / Publication 4
Anetzberger,C., Reiger,M., Fekete,A., Schell,U., Stambrau,N., Plener,L., Kopka,J., Schmitt-Kopplin,Ph., Hilbi,H., and Jung,K. Autoinducers act as biological timers in *Vibrio harveyi* (2012). *PLoS One* **7**(10): e48310.

Book chapter

Book chapter included in chapter 1 of this thesis / Publication 1
Anetzberger,C., and Jung,K. Intercellular Communication – Introduction (2010). In Bacterial Signaling. Krämer R., and Jung K. (eds). Weinheim: WILEY-VCH Verlag GmbH & CoKGaA, 3-5.

Contributions to Publications and Manuscripts

Book chapter included in chapter 1 of this thesis
Claudia Anetzberger and Kirsten Jung wrote the book chapter.

Chapter 2
Claudia Anetzberger, Torsten Pirch and Kirsten Jung designed the study and wrote the paper. Claudia Anetzberger carried out all experiments, analyzed the data, and created all figures.

Chapter 3
Claudia Anetzberger and Kirsten Jung developed the concept of the study and wrote the paper. Claudia Anetzberger and Ursula Schell constructed all plasmids and reporter strains and carried out fluorescence microscopy. Claudia Anetzberger performed simultaneous fluorescence and luminescence microscopy. Claudia Anetzberger analyzed all data and created all figures.

Chapter 4
Claudia Anetzberger and Kirsten Jung designed the study. Claudia Anetzberger performed following experiments: synthesis of AI-2, determination of the AI-2 concentration in cell-free culture fluids, all bioluminescence and exoproteolysis measurements, and preparation of cell-free culture fluids for the determination of the concentration of HAI-1 and CAI-1. Claudia Anetzberger and Nina Stambrau performed *in vitro* phophorylation assays. Nina Stambrau constructed plasmid pNKQ. Agnes Fekete and Joachim Kopka determined the HAI-1 and CAI-1 concentrations in cell-free culture fluids using UPLC and GC-TOF-MS analysis, respectively. In parallel, Ursula Schell and Hubert Hilbi determined CAI-1 activity using *Vibrio cholerae* as reporter. In addition, Laure Plener used a *Vibrio harveyi* reporter to measure CAI-1 activity. Matthias Reiger carried out qPCR analysis for *luxR* and other autoinducer-regulated genes. Matthias Reiger, Nina Stambrau, Agnes Fekete, Hubert Hilbi and Joachim Kopka wrote the respective experimental procedures sections. Claudia Anetzberger and Kirsten Jung wrote the rest of the paper. Joachim Kopka created Fig. 3B and C. Matthias Reiger and Claudia Anetzberger created Fig. 5. Claudia Anetzberger created all other figures.

I herby confirm the above statements:

Claudia Anetzberger Prof. Dr. Kirsten Jung

Summary

Bacteria produce and excrete signaling molecules, so called autoinducers, which allow them to monitor their population density and/or their environment in a process best known as quorum sensing. The Gram-negative marine bacterium *Vibrio harveyi* regulates certain virulence factors like type III secretion, siderophore production, and exoproteolytic activity as well as biofilm formation and bioluminescence using quorum sensing. The bacterium produces three different autoinducers: HAI-1, a N-(3-hydroxybutyryl)-D-homoserine lactone, AI-2, a furanosylborate diester, and CAI-1, a (*Z*)-3-aminoundec-2-en-4-one. The autoinducers are recognized by the hybrid sensor kinases LuxN, LuxQ and CqsS. All information is transferred to the phosphotransfer protein LuxU and the response regulator LuxO via phosphorelay and further transduced into the copy number of the master regulator LuxR. LuxR induces/represses a multitude of genes/operons (>100) including the *lux*-operon responsible for the production of bioluminescence.

In order to understand how single cells behave within an autoinducer-activated community, autoinducer-induced processes were investigated in a homogeneous environment over time (chapter 2 and 3). Analysis of wild type single cells with respect to bioluminescence revealed that even at high cell densities only 70% of the cells of a population were bright. Moreover, fractionation of the population was found for autoinducer-controlled promoters (of genes coding for bioluminescence, exoproteolytic activity, and type III secretion) using reporter strains containing promoter::*gfp* fusions. These results indicated phenotypic heterogeneity of a genetic homogeneous population and were independent of the used cultivation medium, temperature or strain. An artificial increase of the autoinducer concentrations resulted in an all-bright cell population similar as observed for a *luxO* deletion mutant. Both, wild type and deletion mutant switched to biofilm formation at high cell density. However, the capability of the mutant to produce biofilm was significantly reduced. These data suggest that a population of the non-differentiating bacterium *Vibrio harveyi* takes advantages of division of labor.

In addition, a temporal variation of the autoinducer concentrations over time was found (chapter 4). The extracellular concentrations of the three autoinducers and quorum sensing-regulated functions of *Vibrio harveyi* were monitored in a growing culture. In

the early and mid-exponential growth phase only AI-2 was detectable and bioluminescence was induced. In the late exponential growth phase both, HAI-1 and AI-2 reached their maximum values, bioluminescence stayed high and exoproteolytic activity was induced. The stationary phase was characterized by equal concentrations of HAI-1 and AI-2, exoproteolytic activity reached its maximum, and CAI-1 activity was detectable in the culture fluids. Furthermore, only a stable and mature biofilm was formed, when HAI-1 and AI-2 were present in the above described ratios over time (chapter 5). CAI-1 had no influence on the biofilm formation in *Vibrio harveyi*. These results demonstrate that not the cell density *per se* is important, but that autoinducers rather control the development of a *Vibrio harveyi* population.

Zusammenfassung

Bakterien produzieren und sondern Signalmoleküle, sogenannte Autoinduktoren, in die Umgebung ab. Dieser Prozess, bestens bekannt als Quorum Sensing, ermöglicht ihnen ihre Zelldichte und/oder ihre Umgebung zu überwachen. Bei dem Gram-negativen marinen Bakterium *Vibrio harveyi* werden Virulenzfaktoren, wie das Typ III Sekretionssystem, die Produktion von Siderophoren oder exoproteolytische Aktivität, genauso wie die Biofilmbildung und Biolumineszenz über Quorum Sensing gesteuert. Das Bakterium produziert hierbei drei verschiedene Autoinduktoren: HAI-1, ein N-(3-Hydroxybutyryl)-D-homoserin Lakton, AI-2, ein Furanosylboratdiester und CAI-1, ein (*Z*)-3-Aminoundec-2-en-4-on. Die Autoinduktoren werden über die Hybridsensor-kinasen LuxN, LuxQ und CqsS wahrgenommen. Die gesamte Information wird über Phosphorylierungskaskaden auf das Phosphotransferprotein LuxU und den Antwortregulator LuxO übertragen und in die Kopienzahl des Masterregulators LuxR umgewandelt. LuxR induziert/reprimiert eine Vielzahl von Genen/Operonen (>100) einschließlich des *lux*-Operons, verantwortlich für die Produktion von Biolumineszenz.
Um zu verstehen wie sich einzelne Zellen in einer von Autoinduktoren aktivierenden Umgebung verhalten, wurden induzierende Prozesse über die Zeit untersucht (Kapitel 2 und 3). Die Analyse der Biolumineszenz einzelner Wildtyp Zellen zeigte, dass sogar bei hohen Zelldichten lediglich 70% der Zellen einer Population leuchten. Darüber hinaus wurden Reporterstämme, die eine Promoter::*gfp* Fusion (für Autoinduktor kontrollierte Gene verantwortlich für Biolumineszenz, exoproteolytische Aktivität oder Typ III Sekretion) tragen, untersucht und eine Aufteilung der Population in aktive und nicht aktive Zellen gefunden. Diese Ergebnisse weisen auf eine phänotypische Heterogenität einer genetisch homogenen Population hin und zeigten sich unabhängig von dem zur Kultivierung verwendetem Medium, der Temperatur oder dem Stamm. Eine künstliche Erhöhung der Autoinduktorkonzentration führte zu einer Zellpopulation mit lediglich leuchtenden Zellen genauso wie bei einer *luxO* Deletionsmutante. Beide, der Wildtyp und die Deletionsmutante, wechselten bei hoher Zelldichte zu Biofilmbildung. Jedoch war die Fähigkeit zur Biofilmbildung bei der Mutante signifikant reduziert. Anhand dieser Daten wird vorgeschlagen, dass eine Population des nicht differenzierenden Bakteriums *Vibrio harveyi* Arbeitsteilung nutzt.

Desweiteren wurde eine zeitliche Änderung der Autoinduktorkonzentrationen gefunden (Kapitel 4). Die extrazelluläre Konzentrationen der drei Autoinduktoren und Quorum Sensing regulierte Funktionen von *Vibrio harveyi* wurden in einer wachsenden Kultur untersucht. In der frühen und mittleren exponentiellen Wachstumsphase war nur AI-2 messbar und Biolumineszenz wurde induziert. In der späten exponentiellen Wachstumsphase erreichten beide, HAI-1 und AI-2, Maximalwerte, Biolumineszenz blieb hoch und exoproteolytische Aktivität wurde induziert. Die stationäre Phase wurde bestimmt durch gleiche Konzentrationen an HAI-1 und AI-2, exoproteolytische Aktivität erreichte ein Maximum und CAI-1 Aktivität war in den Kulturüberständen messbar. Außerdem wurde ein stabiler und reifer Biofilm nur dann gebildet, wenn HAI-1 und AI-2 in den vorher beschriebenen Verhältnissen über die Zeit vorhanden waren (Kapitel 5). CAI-1 hatte keinen Einfluss auf die Biofilmbildung in *Vibrio harveyi*. Diese Ergebnisse veranschaulichen, dass nicht die Zelldichte an sich wichtig ist, sondern, dass Autoinduktoren die Entwicklung einer *Vibrio harveyi* Population steuern.

1 Introduction

1.1 The language of bacteria

1.1.1 The simplest form of quorum sensing

"Quorum" is an old Latin term from the period of the Roman Empire and describes the number of senators that had to be present to make a legal decision. The simplest form of a quorum sensing (QS) system, first described in the bioluminescent, symbiotic living bacterium *Aliivibrio fischeri* (Engebrecht and Silverman, 1984; Milton, 2006; Shadel *et al*., 1990), is the production of one autoinducer (AI) by emitter cells. In *A. fischeri* this AI is an acyl-homoserine lactone (AHL), synthesized by LuxI. AI molecules can diffuse through the cytoplasmic membrane in both directions and accumulate in the surrounding media over time. With increasing cell density the extra- and therefore also the intracellular AI concentration rises. After reaching a certain threshold concentration the AI can interact with its cognate receptor - termed LuxR in *A. fischeri* - leading to changes in gene expression of QS-regulated processes in the responding cell. In intraspecies communication the cells are first emitters of and then responders to the signal. A scheme for this simple form of QS is shown in Fig. 1.1. Up to now over 70 of this LuxI-LuxR systems have been experimentally identified (Eberl and Riedel, 2011).

1.1.2 Discoveries in cell-cell communication and social bacterial behavior

"For a long time bacteria were regarded as dumb single-cell living organisms that ensure their survival by adapting to rapidly changing environmental conditions without communication. The concept of intercellular communication within bacterial populations originates from different discoveries in the 1960s and 1970s proposing the involvement of external factors excreted by bacteria themselves. In *Streptomyces griseus* aerial hyphae formation was found to be inducible by an old culture (Dondero and Scotti, 1957); 10 years later this compound was identified as γ-butyrollactone (A-factor) (Khokhlov *et al*., 1967). In 1965, Tomasz assumed that an external factor, "a hormone-like cell product", was important for genetic competence in *Streptococcus pneumoniae* (formerly known as *Pneumococcus*) (Tomasz, 1965), which was later shown to be a modified peptide. Hasting *et al*. found that *V. fischeri* [now called *Aliivibrio fischeri*,

Milton, 2006], a bioluminescent marine bacterium, produced light at high cell density, but not in dilute suspension (Nealson *et al.*, 1970). Light production could be stimulated by the addition of cell-free culture fluid."[1]

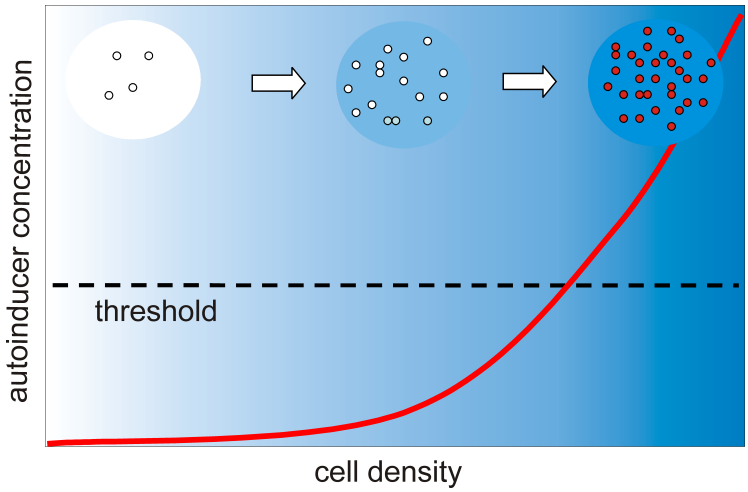

Figure 1.1 Generic scheme for a simple quorum sensing system. Bacterial cells (white circles) produce and emit small signaling molecules. These so-called autoinducers accumulate in the surrounding environment. With increasing cell density the autoinducer concentration rises (indicated by the red line and the gradient from white to blue) and reaches a certain threshold. The signal is recognized by receptors leading to changes in gene expression of quorum sensing-regulated genes. The cells respond to the emitted autoinducers (red circles). Emitter and responder are the same cells in intraspecies communication. Adapted after Keller and Surette, 2006.

"They called the responsible component "autoinducer", which was later identified as an AHL (Eberhard *et al.*, 1981). At this time autoinduction was defined as an environmental sensing system that allows bacteria to observe their own population density by monitoring the concentration of these AIs. At low cell density AIs are available in a low concentration, while at high cell density these compounds accumulate to the critical concentration required for activation of certain genes. The term "quorum sensing" was introduced by Greenberg in 1994 as cell-density linked, coordinated gene expression in populations that experience threshold signal concentrations to induce a synchronized population response (Fuqua *et al.*, 1994). "QS" spread like wildfire, making its way to virtually every following paper on autoinduction (Turovskiy *et al.*, 2007), and the scientific community accepted that bacterial social behaviors described, for example, for *Myxococcus* […], *Bacillus* or *Vibrio* are the norm and not the exception in the bacterial world (Shapiro, 1998). "[1]

[1] Anetzberger C. and Jung K., 2010 (Publication 1)

1.1.3 Important quorum sensing-regulated processes

In the last decades scientists start to change their view about bacteria from uniform, simple living cells to mutlicellular complex communities with individual behavior. Population heterogeneity was demonstrated for *Agrobacterium* (Goryachev *et al*., 2005), *Bacillus* (Chai *et al*., 2008; Kearns and Losick, 2005), *Escherichia* (Balaban *et al*., 2004; Keren *et al*., 2004) and *Pseudomonas* (Diggle *et al*., 2007). Cell-cell communication is important to coordinate the behavior of single cells within a population to share public goods. Non-differentiating bacteria especially use this kind of signal response.

"Processes controlled by QS are usually those that are unproductive when undertaken by an individual bacterium, but become effective when undertaken by a group. Thus, QS allows bacteria to behave like a multicellular organism. In addition to competence, bioluminescence and morphological differentiation, QS controls virulence factor secretion, biofilm formation and sporulation (Bassler and Losick, 2006)."[1]

Bioluminescence

Bioluminescence is a phylogenic widespread phenomenon and developed independently in bacteria, fungi, marine plankton and animals (such as fish, jellyfish, snakes, earthlings and bugs). Marine bioluminescence is mostly blue. On the one hand, blue light has the largest range under the sea. On the other hand, the majority of sea dwellers are only sensitive for blue light. Terrestrial bioluminescence is blue, green, yellow or orange. Bioluminescence results from an oxidation catalyzed by a luciferase. Besides the luciferase, molecular oxygen and a luciferin (aldehyde, benzothiazole, tetrapyrrole or flavins) have to be present. In bacteria the luciferin is a reduced riboflavin-phosphate, termed $FMNH_2$, associated with a long chain aldehyde (Cormier and Strehler, 1954). Bacterial bioluminescence is a characteristic reserved to marine bacteria of the genus *Vibrio* and *Photobacterium*. Up to now only one exception was described, the soil bacterium *Photorhabdus luminescence* (Tu and Mager, 1995). Bioluminescence can be important in enhancing the nutrient cycle (Hastings, 1983) or in the reduction of oxygen radicals (Seliger and McElroy, 1962; Thompson *et al*., 1986). Bioluminescence in *V. harveyi* can also play a role in the attraction of other cells of the

[1] Anetzberger C. and Jung K., 2010 (Publication 1)

same species to form aggregates or settle down on surfaces. *Vibrio* is able to recognize the generated blue light using specific receptors (Worthington *et al*., 2003). Bioluminescence is a QS-induced process in *V. harveyi* (Bassler *et al*., 1994).

Biofilm formation

For most microbes a life in surface-associated communities, termed biofilms, is expected. A biofilm is a self-produced matrix consisting of different types of polymeric substances and perused by water channels (Costerton *et al*., 1995). Principal components of the matrix are exopolysaccharides, proteins, enzymes and nucleic acids and can account for over 90% of the dry mass, leaving 10% for the cells themselves (Flemming and Wingender, 2010). Extracellular polymeric substances are mainly responsible to form the three-dimensional architecture of the biofilm and the preceding adhesion to surfaces. The formation of biofilms reflects the native growth condition of most bacterial species. The lifestyle within a biofilm differs totally from the planktonic one. Nutrient availability, metabolic cooperativeness, protection against the environment (like UV radiation and antibiotics), and the acquisition for new genetic traits (like horizontal gene transfer) are the ecological advantages (Davey and O'Toole, 2000). Bacterial biofilms posses optimal conditions for cell-cell communication (Dickschat, 2010) and are the most successful way of microbial life (Flemming and Wingender, 2010). Biofilm formation is a multifunctional and very complex process influenced in a positive way by QS in *V. harveyi*.

Type III secretion

V. harveyi is an opportunistic pathogen mainly for shrimp, but also for fish, squids or lobster (Austin *et al*., 2003; Austin and Zhang, 2006; Diggles *et al*., 2000). Virulence factors used in *V. harveyi* are a type III secretion system (Henke and Bassler, 2004a), cysteine proteases (Liu and Lee, 1999; Liu *et al*., 1997), metalloproteases (Teo *et al*., 2003), hemolysins (Zhang *et al*., 2001) and other exotoxins (Diggles *et al.,* 2000). Type III secretion systems are specialized secretion apparatuses to inject virulence factors like effector proteins directly in the cytoplasm of the host cell and used by a multitude of Gram-negative pathogenic bacteria like *Escherichia coli* (Jarvis *et al*., 1995), *Pseudomonas aeruginosa* (Hauser, 2009) *Yersinia spp.* (Cornelis, 2002) and *V.*

parahaemolyticus (Makino *et al*., 2003). In *V. harveyi*, *vscP*, the translocation protein of the type III secretion system, is repressed by QS (Henke and Bassler, 2004a).

1.1.4 The chemical vocabulary of bacteria

"The chemical vocabulary used by bacteria for communication is constantly increasing as new molecules are discovered. In general, Gram-negative QS bacteria communicate with AHLs (Visick and Fuqua, 2005) […], while Gram-positive bacteria predominantly communicate with short peptides that often contain chemical modifications (Waters and Bassler, 2005) […]."[1]

AHLs are typically synthesized by an enzyme of the LuxI family (Eberl, 1999; Fuqua *et al*., 2001; Greenberg, 2003). Therefore a reaction of S-adenosylmethionine with an acyl–acyl carrier protein occurs. A member of the LuxR family of transcriptional regulators typically detects the AHL signal. AHL signaling is found in *Aeromonas* (Vivas *et al*., 2004), *Agrobacterium* (Zhang *et al*., 1993), *Burkholderia* (Lewenza and Sokol, 2001), *Chromobacterium* (McClean *et al*., 1997) *Erwinia* (McGowan *et al*., 1995), *Pantoea* (Koutsoudis *et al*., 2006), *Pseudomonas* (Winson *et al*., 1995), *Ralstonia* (Flavier *et al*., 1997), *Rhodopseudomonas* (Schaefer *et al*., 2008), *Serratia* (Labbate *et al*., 2004), *Variovorax* (Leadbetter and Greenberg, 2000), *Vibrio* (Yang *et al*., 2011), and *Yersinia* (Atkinson *et al*., 2008). In most cases, this system has only a moderate specificity. Enzymes belonging to the LuxI family, such as LasI from *Pseudomonas aeruginosa*, often synthesize minor AHLs besides the predominant one (Winson *et al*., 1995). In Gram-positive bacteria a pre-protein is synthesized and processed into the active autoinducing peptide that is exported from the cell (Lyon and Novick, 2004). The structure is determined by the amino acid sequence. Therefore these signals are highly specific. Autoinducing peptides were used to group *Staphylococcus epidermidis* and *S. aureus* in specificity classes I to IV (Dufour *et al*., 2002). Autoinducing peptides are found in *Bacillus* (Grossman, 1995), *Clostridium* (Wuster and Babu, 2008), *Enterococcus* (Nakayama *et al*., 2006), *Lactobacillus* (Wuster and Babu, 2008), *Listeria* (Riedel *et al*., 2009), *Staphylococcus* (Mack *et al*., 1996; Novick and Geisinger, 2008), and *Streptococcus* (Fontaine *et al*., 2010).

[1] Anetzberger C. and Jung K., 2010 (Publication 1)

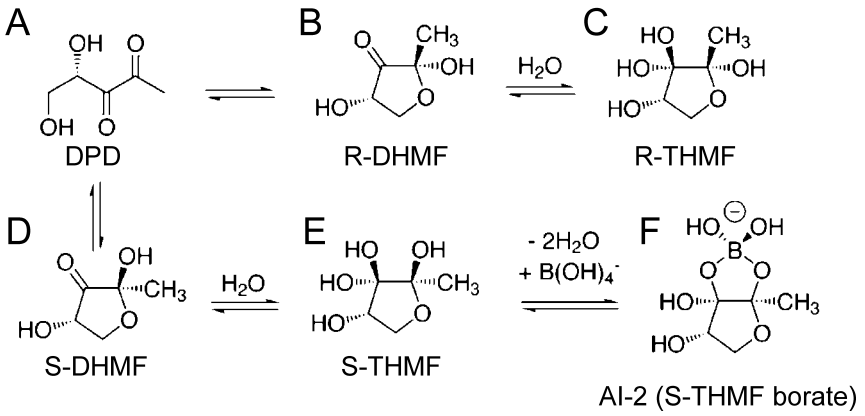

Figure 1.2 Biological forms of autoinducer-2 (AI-2). The precursor DPD (**A**, (4*S*)-4,5-dihydroxy-2,3-pentandione) is synthesized by different groups of bacteria. It exists in a fluent equilibrium with following forms: R-DHMF (**B**, (2R, 4*S*)-2,4-dihydroxy-2-methyldihydrofuran-3-one), R-THMF (**C**, (2R, 4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran), S-DHMF (**D**, (2*S*, 4*S*)-2,4-dihydroxy-2-methyldihydro-furan-3-one) and S-THMF (**E**, (2*S*, 4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran). In the presence of borate S-THMF is relocated to S-THMF borate (**F**, (2*S*, 4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate), the AI-2 of *Vibrio harveyi*.

"In addition, a family of compounds termed autoinducer-2, derived from the common precursor, 4,5-dihydroxy-2,3-pentanedione, has been found to be widespread in the bacterial world, and autoinducer-2 allows interspecies communication."[1]

It was therefore defined as a universal signal (Sun *et al*., 2004). But the common precursor 4,5-dihydroxy-2,3-petanedione exists in a fluent equilibrium with other furanones (Schauder *et al*., 2001) (Fig. 1.2) and the receptors of *Vibrio* and *Salmonella* recognize different AI-2 molecules (Chen *et al*., 2002; Miller *et al*., 2004) within the equilibrium (Fig. 1.2 F and C, respectively). So AI-2 systems have a species-specific part at the level of reception. 4,5-dihydroxy-2,3-petanedione is synthesized by degrading the key metabolic compound S-adenosyl-homocysteine, which also plays a role in thegeneration of AHLs. S-adenosyl-homocysteine is a cell toxin and the degradation is important for bacteria. It seems that the use of AHLs and AI-2 as signaling molecules is a secondary effect of metabolized products (Winzer *et al*., 2003). The structures of the main bacterial AIs described so far are drawn in Fig. 1.3.

[1] Anetzberger C. and Jung K., 2010 (Publication 1)

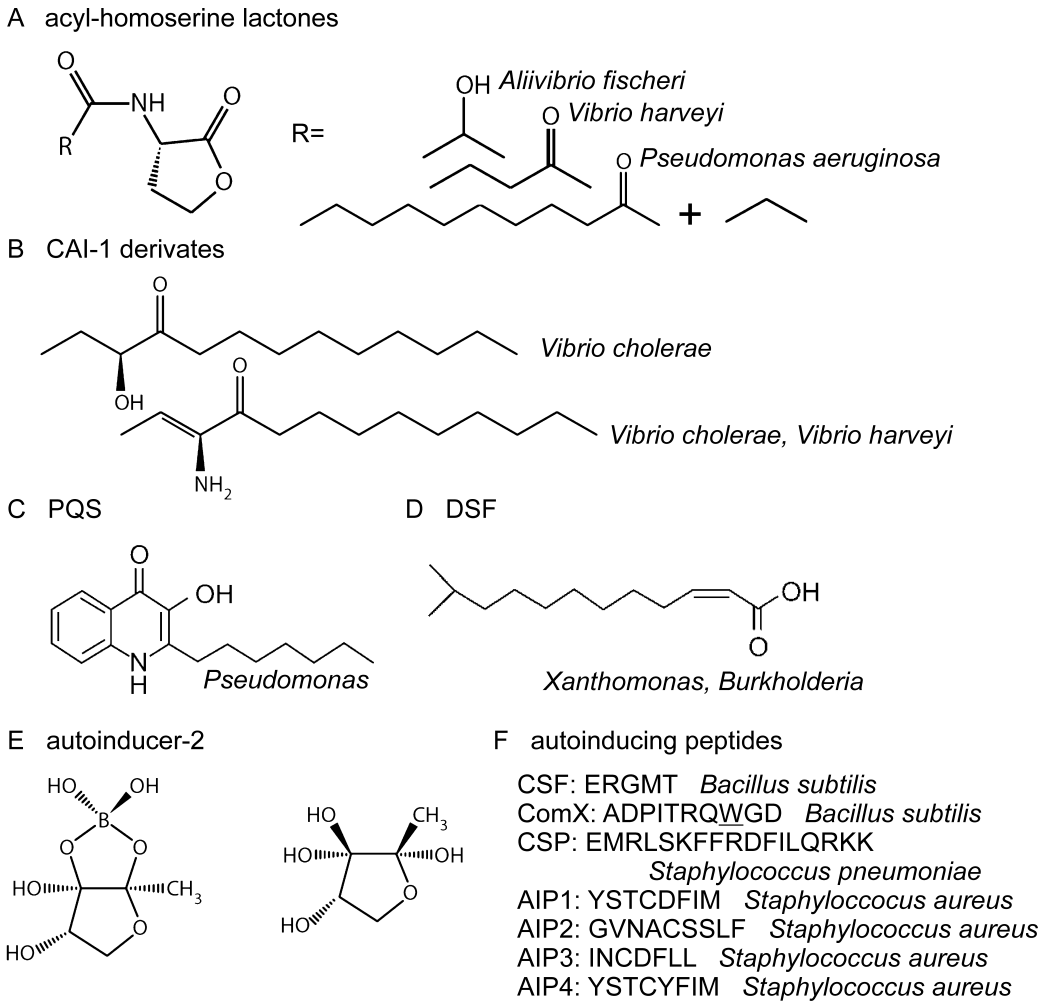

Figure 1.3 Structures of different quorum sensing molecules. Acyl-homoserine lactones (**A**) are produced by Gram-negative bacteria. Specifity is generated using different side chains. CAI-1 derivates (**B**) are the main autoinducers in *Vibrio cholera*, but also found in *Vibrio harveyi*. Other examples for signaling molecules produced by Gram-negative bacteria are PQS (**C**, *Pseudomonas* quorum sensing) and DSF (**D**, diffusible signaling factor) synthesized by *Pseudomonas* and *Xanthomonas* or *Burkholderia*, respecetively. Autoinducer-2 (**E**) is produced by Gram-positive and –negative bacteria. In addition Gram-positive bacteria like *Bacillus* and *Staphylococcus* communicate using autoinducing peptides (**F**). The one letter code is used to illustrate the peptides. The underlined amino acid is isoprenylated.

"The bacterial language is still very primitive and […] there are many more bacterial signal molecules that need to be discovered. A better understanding of interorganismic chemical communication will open new possibilities to manipulate bacterial behavior, including the blockage of pathogens from multiplying or the promotion of the growth of beneficial bacteria."[1]

[1] Anetzberger C. and Jung K., 2010 (Publication 1)

1.2 The bioluminescent bacterium *Vibrio harveyi*

1.2.1 Systematic characterization: *Vibrionaceae*

The model organism used in this thesis is the Gram-negative bioluminescent bacterium *V. harveyi*. According to Bergey's Manual of Systematic Bacteriology from 2005 (Farmer III and Janda, 2005), *Vibrionaceae* belong to the *γ-proteobacteria.*

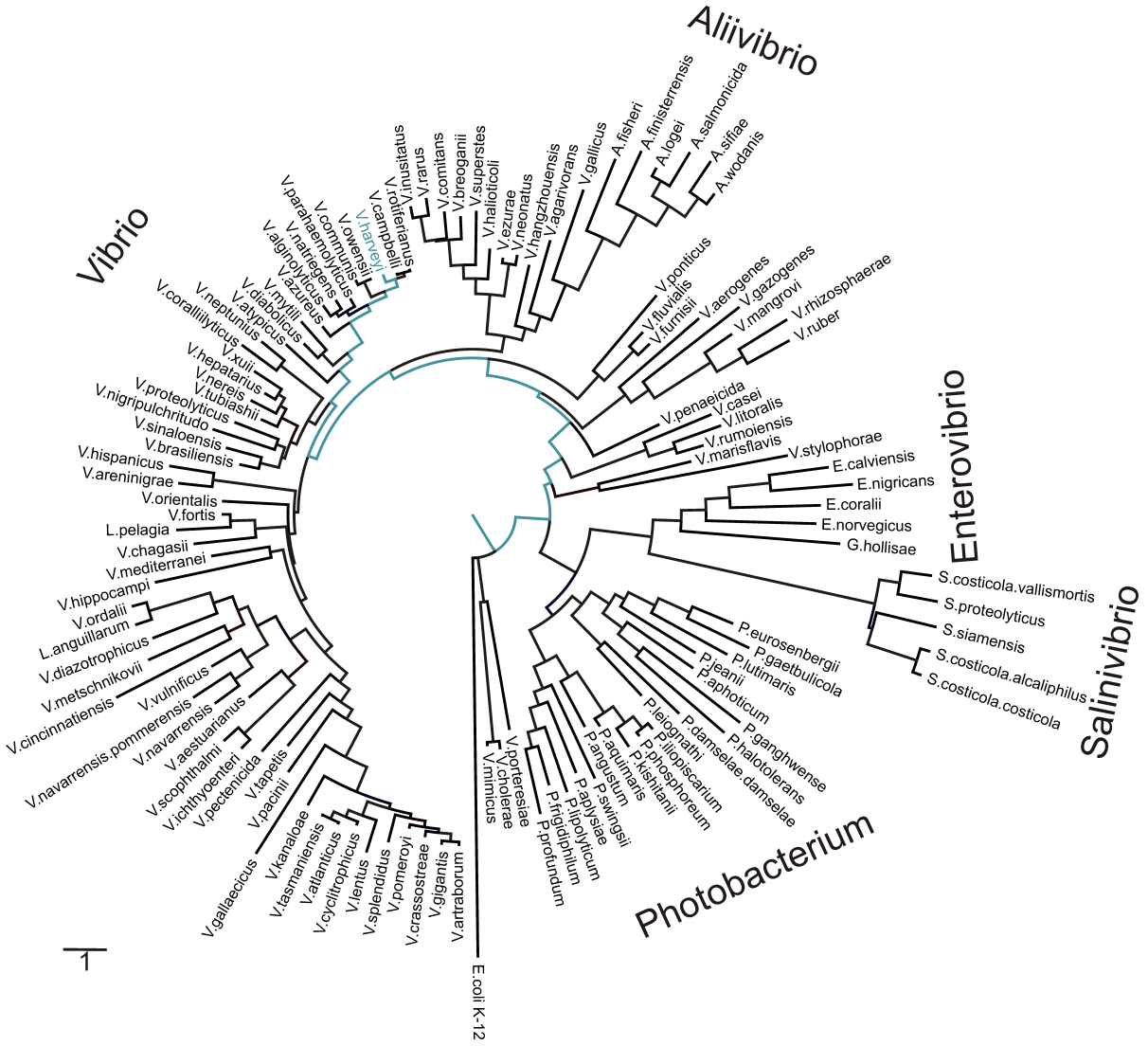

Figure 1.4 Systematic sorting of the members of the *Vibrionaceae*. The dendrogram is adapted after www.vibriobiology.net/species and based on 16S rRNA sequences. Branch lengths are in the same units as the evolutionary distances (number of base substitution per position). Used methods are the Neighbor-Joining method (Saitou and Nei, 1987) for the evolutionary history and the Jukes-Cantor method (Jukes and Cantor, 1969) for evolutionary distances. MEGA4 (Tamura *et al.*, 2007) and Figtree (http://tree.bio.ed.ac.uk/software/figtree) were utilized for phylogenetic analysis and drawing, respectively. *Vibrio harveyi* is highlighted in cyan.

They are mesophilic, chemoorganotrophic and facultative fermentative. They form polar flagella and small comma-shaped rods (0.5-0.8 x 1.4-2.6 µm) and are found in aquatic habitats, free-living or in symbiosis with eukaryotes. Most species are oxidase positive, require sodium, reduce nitrate to nitrite, and are able to ferment glycerol, D-fructose, and maltose. Up to now (last update august, 20th 2012 on www.vibriobiology.net) the International Committee on Systematics of Prokaryotes accepted 135 species within the *Vibrionaceae* grouped in 7 genus: *Aliivibrio* (6 species), *Catenococcus* (1), *Enterovibrio* (4), *Grimonita* (1), *Photobacterium* (22), *Salinivibrio* (6), and *Vibrio* (95). A dendrogram of sequenced *Vibrionaceae* according to the 16S rRNA is shown in Fig. 1.4. The pangenome of the sequenced *Vibrionaceae* contains 26,504 genes, whereas 488 genes belong to the coregenome (Thompson *et al*., 2009). The increase in the pangenom is caused by new strain specific genes and point out a reservoir of genetic diversity that allows survival in and occupation of different environmental niches. Unique genes allow the possessing strains to capture specific niches, e.g. the sensor kinase *rscS* in *A. fischeri* controls light-organ colonization (Mandel *et al*., 2009).

1.2.2 Ecological characterization: *Vibrio* as pathogen

V. cholerae was the first discovered *Vibrio* species. Filippo Pacini study cholera outbreaks in Florence and discovered the bacterium to be the causative agent in 1854 (Bentivoglio and Pacini, 1995), but without acceptance of the medical community. Robert Koch discovered the cholera bacterium again in 1883, when it was already accepted that microorganisms are able to indeed cause illness (Howard-Jonas, 1984). Cholera is a severe infectious disease of the small intestine with massive water and electrolyte secretion caused by the cholera toxin. These voluminous stools, termed rice water, lead to dehydration (Atia and Buchman, 2010). According to the World Health Organization a total of 221,226 cholera cases, including 4,946 deaths, were reported in 2009 (WHO, 2010). The case-fatality rate (ratio deaths to patients) was 2.24%. Other important human pathogens are *V. parahaemolyticus* and *V. vulnificus* causing gastroenteritis (Honda and Iida, 1993) and wound infection (Finkelstein *et al*., 2002), respectively. *V. anguillarum*, *V. salmonicida* and *V. vulnificus* are the main pathogens in fish species (Austin and Austin, 1999), whereas *V. harveyi* mainly causes damage in shrimp aquacultures (Lavilla-Pitogo *et al.*, 1998), sometimes leading to the death of the whole shrimp population. The caused damage by infectious diseases in aquacultures

account $ 9 billion per year worldwide, which accounts for 15% of the value (Tan *et al.*, 2007). Due to the misuse of antibiotics in aquacultures, many vibrios are resistant to several antibiotics (Cabello, 2006). Therefore the search for new therapeutics, like anti-QS molecules, becomes more and more important.

1.2.3 Genotypic and phenotypic characterization

The *V. harveyi* strain ATCC BAA-1116 [recently reclassified as *V. campellii* (Cano-Gomez *et al.*, 2011; Lin *et al.*, 2010)] was completely sequenced in 2007 (Bassler *et al.*, 2007). *V. harveyi* has two chromosomes and one plasmid. Chromosome I is 3,765,351 nucleotides long, whereas chromosome II consists of 2,204,018 base pairs. Chromosome I encodes more essential genes, chromosome II carries more species-specific genes. Both chromosomes have different origin of replications, but replicate synchronously. Plasmid pVIBHAR has 89,008 nucleotides and encodes 120 proteins. Chromosome I (II) has 3,721 (2,412) genes, encoding 3,561 (2,374) proteins and 143 (24) structural RNAs. The total GC amount accounts 45.4%. Horizontal gene transfer is important in vibrios. Natural competence (Meibom *et al.*, 2005) and bacteriophages (Vidgen *et al.*, 2006) may influence genomic features and cause phenotypic variation. *V. harveyi* is a marine bacterium, free-living or within and at the surface of marine animals, including squids (Fig. 1.5 A), corals, seahorses, oysters, prawns, lobsters, shrimps, turbot and milkfish (Owens and Busico-Salcedo, 2006).

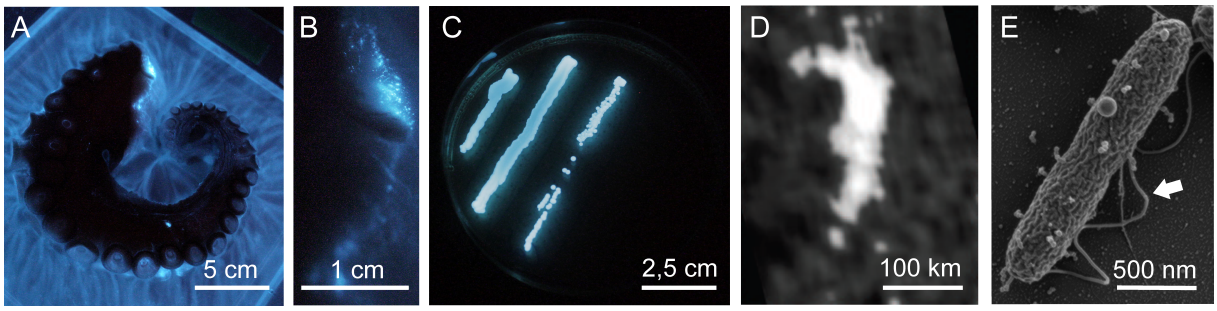

Figure 1.5 The bioluminescent mobile bacterium *Vibrio harveyi*. *Vibrio harveyi* lives free in the sea or in and on marine animals like squids (**A**) and shrimps. *Vibrio harveyi* is able to produce bioluminescence in the blue range of light in fluid culture (**A**), on natural surfaces (**A-B**) or on agar plates (**C**). It is thought, that *Vibrio harveyi* is responsible for the phenomenon of milky seas. A large area of surface water glows in the open sea. The satellite image, adapted from Miller *et al.*, 2005, shows such a milky sea (**D**). *Vibrio harveyi* is polar flagellated. The image shows a cell with a bundle of flagella (white arrow) and was taken via raster electron microscopy from Dr. Axel Müller and Prof. Dr. Gerhard Wanner, LMU Munich (**E**).

One outstanding characteristic of *V. harveyi* is the production of blue light in fluid culture or in colonies on agar plates or natural surfaces (Fig. 1.5 A-C). *V. harveyi* is rod-shaped, grows between 5° and 35°C, and is polar flagellated (Fig. 1.5 E). There are hints, that *V. harveyi* causes the milky sea effect in combination with an algae bloom in surface waters (Miller *et al.*, 2005). Milky seas are large areas with a uniform blue glow, which can cover 15,000 km² (Fig. 1.5 D). Up to now about 250 sights were documented. This phenomenon found its way in several tales, including "Twenty Thousand Leagues under the Sea" from Jules Verne, published in 1870.

1.3 The quorum sensing circuit of *Vibrio harveyi*

The QS system of *V. harveyi* consists of a complex phosphorelay system, combining the recognition of three different AIs: the AHL HAI-1 (Fig. 1.3 A), the tridecanone and main AI in *Vibrio cholerae* CAI-1 (Fig. 1.3 B), and the furanosyl-borate-diester AI-2 (Fig. 1.3 E, left). This cascade regulates cell density-dependent processes like bioluminescence, biofilm formation, exoproteolysis, type III secretion, and siderophore production.

1.3.1 Two component and phosphorelay systems

Information of intra- and extracellular signals is often transferred via phosphorylation cascades to effector proteins. The simplest from is the two component system (TCS), consisting of a histidine kinase and a response regulator.

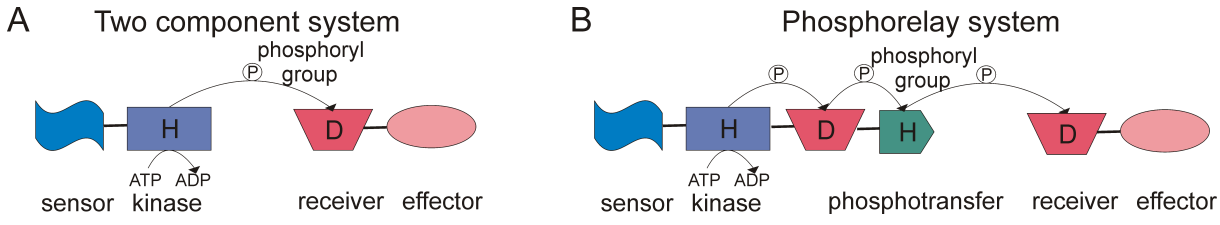

Figure 1.6 Two component and phosphorelay systems. The domain structures of a typical two component system (**A**) - characterized by one phosphotransfer - and a simple phosphorelay system (**B**) - containing multiple phosphotransfer reactions - are displayed. Arrows indicate the intra- and intermolecular transfer of the phophoryl group. H denotes the histidine, A the aspartate residue of the proteins.

The reversible reaction enfolds following steps: autophosphorylation of the histidine of the kinase, transfer of the phosphoryl group to the aspartate of the response regulator and concluding dephosphorylation of the response regulator (Stock *et al.*, 2000). The composition of classical TCSs (Fig. 1.6 A) is highly conserved. In contrast, phosphorelay systems (Fig. 1.6 B) show a high variability (Stock *et al.*, 2000) coming along with a multitude of histidine and aspartate residues and supplemental receptor and HPt (histidine phosphotransfer) domains. With increasing number of components and reactions the complexity rises. The different domains lie on isolated proteins or are located in combination with other domains on hybrid proteins (Perraud *et al.*, 1999; Stock *et al.*, 2000). The LuxN/LuxQ/CqsS-LuxU-LuxO system of *V. harveyi* shows additional complexity (Fig. 1.7).

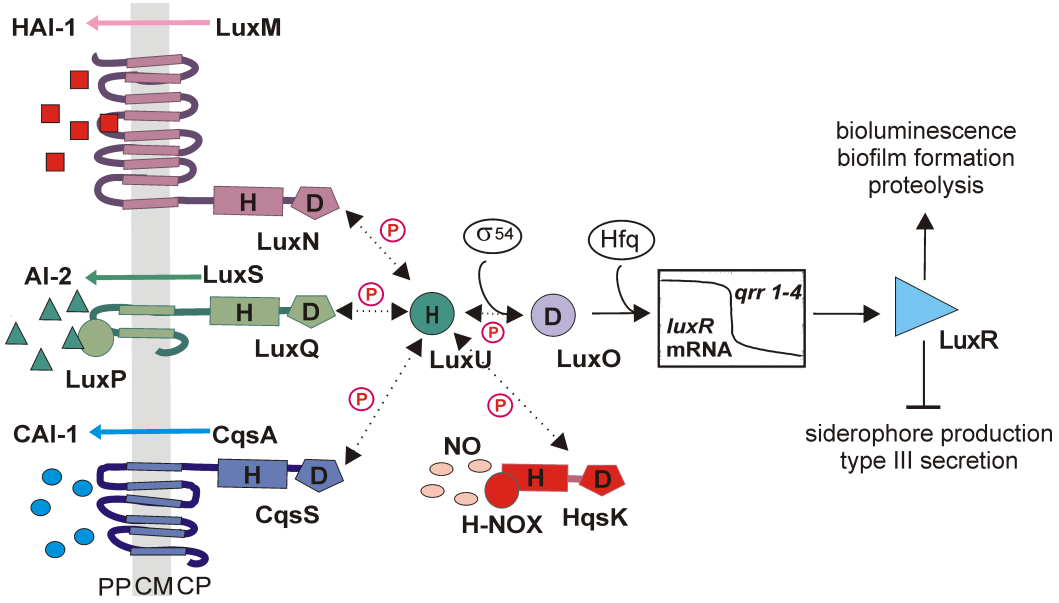

Figure 1.7 The complex quorum sensing circuit of *Vibrio harveyi*. *Vibrio harveyi* synthesizes the autoinducers HAI-1, AI-2 and CAI-1 via LuxM, LuxS, and CqsA, respectively. The hybrid sensor kinases LuxN, LuxQ (with the binding protein LuxP), and CqsS recognize the corresponding autoinducers. The higher their concentration, the lower is the autophosphorylation activity of the kinases. At low cell density, the phosphoryl group is transferred via phosphorelay including the histidine phosphotransfer protein LuxU to the σ^{54}-dependent transcriptional activator LuxO. Phosphorylated LuxO activates the transcription of five regulatory sRNAs (Qrr1-5), four of them destabilize together with the chaperon Hfq the mRNA of the master regulator LuxR. At high cell density, LuxO is dephosphorylated, and LuxR is produced. LuxR activates genes responsible for bioluminescence, biofilm formation and exoproteolytic activity. LuxR represses genes involved in type III secretion and siderophore production. A fourth circuit consisting of the soluble histidine kinase HqsK and the NO-sensing H-NOX introduces its information at the level of LuxU. Dashed lines marked with a 'P' indicate phosphotransfer reactions. H (histidine) and D (aspartate) denote the phosphorylation sites. CM, cytoplasmic membrane; CP, cytoplasm; PP, periplasm.

The three hybrid senor kinases, composed of a histidine and an aspartate domain, are membrane integrated and recognize their AI by interaction with the specific binding site: HAI-1 with LuxN, CAI-1 with CqsS, and AI-2 interacts with LuxQ via the binding protein LuxP (Chen *et al*., 2002; Freeman *et al*., 2000; Henke and Bassler, 2004b; Neiditch *et al*., 2005; Ng *et al*., 2010). The phosphoryl group is transferred to the histidine phosphotransfer protein LuxU (Bassler *et al*., 1993, Freemann and Bassler, 1999b; Henke and Bassler, 2004b), and further to the response regulator LuxO, an isolated protein (Freemann and Bassler, 1999a). At a low AI concentration, phosphorylated LuxO activates the transcription of five small regulatory RNAs (Qrr 1-5), four of them (Qrr1-4) destabilize together with the chaperone Hfq the transcript of the master regulator LuxR (Tu and Bassler, 2007). At a high AI concentration the transcript of LuxR is stable. LuxR regulates as activator or repressor a large number of genes (Lenz *et al*., 2004; Mok *et al*., 2003; Pompeani *et al*., 2008). The AIs are synthesized by the cells themselves using LuxM, LuxS and CqsA. Recently, a new circuit consisting of the soluble histidine kinase HqsK and the NO-sensing H-NOX was reported, which introduces its information at the level of LuxU (Henares *et al*., 2012). This extends the complexity of the QS system of *V. harveyi* even further.

1.3.2 The hybrid sensor kinases LuxN, LuxQ and CqsS

All three sensor kinases are membrane integrated and possess both a histidine and an aspartate domain. After autophosphorylation, an intramolecular phosphotransfer from the kinase domain to the receiver domain occurs. The absence (presence) of AIs leads to phosphorylation (dephosphorylation). Using three different AI signal transduction systems, *V. harveyi* is able to response differentiated at various situations.

LuxN has 849 amino acids (AAs), a relative molecular mass of 96 kDa and nine transmembrane domains with the N-terminus located in the periplasma (Jung *et al*., 2007). The conserved histidine at position 471 is autophosphorylated (Timmen *et al*., 2006). The phosphoryl group is transferred to the conserved aspartate at position 771, and further to LuxU. The kinase activity of LuxN is inhibited, if HAI-1 is present. In contrast, the phosphatase activity is not influenced by HAI-1 (Timmen *et al*., 2006). AAs implicated in the binding of HAI-1 are located in the transmembrane domains 4 (N133, T139), 5 (F162, F163, L166), 6 (F202, S205, T206, T214), and 7 (W224, P226, P227,

S232, E233) and the periplasmic loops 2 (V143, F151, I153, F155) and 3 (D219, F220, S221) (Swem *et al*., 2008).

LuxQ is 859 AAs long, has a relative molecular mass of 97 kDa and two transmembrane domains, connected via a periplasmic loop, consisting of two PAS domains (named after three PAS proteins: PER period circadian protein, ARNT Ah receptor nuclear translocator protein, SIM single-minded protein) (Gu *et al*., 2000). LuxP is bound to these PAS domains in presence and absence of AI-2 (Neiditch *et al*., 2005). Between the second transmembrane domain and the kinase domain lays a HAMP domain (present in histidine kinases, adenyl cyclases, methyl-accepting proteins and phosphatases), an additional PAS domain and a coiled-coil structure. The conserved residues responsible for the transfer of the phosphoryl group seem to be the histidine at position 492 and the aspartate at 785 (Stambrau, 2008).

CqsS is the third sensor kinase, consisting of 661 AAs and 76 kDa. Sequence comparison revealed the histidine at position 190 and the aspartate at position 613 as transfer residues. This third QS system plays only a minor role in *V. harveyi*, but is the main system in *V. cholerae* (Henke and Bassler, 2004b). CqsS has six transmembrane domains (Ng *et al*., 2011).

1.3.3 The phosphotransfer protein LuxU and the response regulator LuxO

The small protein LuxU has only 13 kDa and 114 AAs. LuxU is localized in the cytoplasm and has a 20 AAs long region, including the conserved histidine at position 58, that is conserved in the phophorelay protein family, such as in ArcB of *E. coli* (Kato *et al*., 1998), Spo0B of *B. subtilis* (Perraud *et al*., 1999) and Ypd1p of *Sacchromyces cerevisae* (Janiak-Spens and West, 2000). In absence of LuxU or the conserved histidine no induction of bioluminescence was seen. Thereby it was proofed that LuxU is essential for signal transduction in *V. harveyi* (Freeman and Bassler, 1999b).

The genes *luxU* and *luxO* are organized in one operon with an overlap of four AAs, denoting a collective translation of the two proteins. LuxO is a 50 kDa large cytoplasmic protein and belongs to the family of σ^{54}-dependent transcriptional activators (Lilley and Bassler, 2000). Those proteins are characterized by a N-terminal receiver domain (REC) with a conserved aspartate (D47 in LuxO) and a C-terminal helix-turn-helix motif (HTH) (from AAs 404 to 440 in LuxO). It is thought, that LuxU transfers the phosphoryl group

to the conserved aspartate at position 47. After changing the aspartate against alanine (D47A) or asparagine (D47N) no phosphorylation occours and the cells show a constitutive bright phenotype (Freeman and Bassler, 1999a). A phosphorylated status of LuxO, independent of the AI concentration, was mimicked by changing the aspartate against glutamate (D47E), causing a constitutive dark phenotype (Freeman and Bassler, 1999a). The HTH motif is responsible for DNA binding and the grouping to the NarL-family of response regulators (Galperin, 2006). In addition LuxO has an AAA^+ATPase domain ("ATPases associated with a variety of cellular activities") and belongs such as the protein NtrC to the AAA-superfamily (Cullen *et al*., 1996; Neuwald *et al*., 1999). Over 200 substitutes of this family are described in bacteria, eukaryotes and archeae (Confalonieri and Duguet, 1995; Patel and Latterich, 1998). Cellular activities of the AAA-proteins are DNA replication, proteolysis, generation of organelles and transmembrane transport (Vale, 2000). AAA^+ATPases are a subfamily of the Walker-ATPases, possessing two consensus motives, termed Walker A and B (Walker *et al*., 1982). The AAA^+ATPases show an additional conserved region, called SRH, for second region of homology (Swafield *et al*., 1992). The phosphotransfer from LuxU to LuxO was recently proofed (Wei *et al*., 2012).

1.3.4 The master regulator LuxR

The master regulator LuxR of *V. harveyi* is a TetR-type regulator and acts as an activator and a repressor of QS-regulated processes (Pompeani *et al*., 2008). LuxR consists of 211 AAs and is a 23 kDA large cytoplasmic protein. The consensus binding sequence was determined as TATTGATAAATTTATCAATAA with a dyad symmetry (Pompeani *et al*., 2008). LuxR binds upstream of QS-regulated genes such as *luxR*, *aphA*, *luxC*, *qrgB*, and *qrr4* (Pompeani *et al*., 2008) and expresses/inhibits directly or indirectly >100 genes of the QS regulon (Lenz *et al*., 2004; Mok *et al*., 2003; Waters and Bassler, 2006).

1.4 Scope of this thesis

The main objective of this thesis is to learn more about cell-cell communication in bacteria. Understanding how single cells communicate with each other and coordinate their behavior as a group will strengthen our knowledge on bacterial life. *V. harveyi* was

used as model organism possessing a complex QS circuit integrating the information of a multitude of systems.

Heterogeneity of AI-regulated bioluminescence was elucidated in the first study using luminescence microscopy with a high sensitivity camera. In order to find the advantage of a heterogeneous population, a *luxO* deletion mutant (mimicking a high AI concentration) with a homogeneous population was used. This mutant showed a significant reduction in biofilm formation. Same results were archived by adding exogenous AIs to the wild type. This result suggests that *V. harveyi* produces and/or keeps the AIs at non-saturating concentrations resulting in the generation of a bright and a dark subpopulation.

Heterogeneous behavior was also proved in the second analysis using reporter strains containing promoter::*gfp* fusions and fluorescence microscopy. Heterogeneity was found in QS-induced (bioluminescence, exoproteolytic activity) and -repressed (type III secretion) processes. Furthermore, a simultaneous analysis of two QS-induced processes indicated a division of labor.

Since the first two studies focusing on the single cell level indicated a regulation of the AI concentrations, the third study attempted to analyze the concentrations of the three produced AIs in parallel with QS-regulated processes within a growing culture. Different AI ratios were found in certain growth phases, regulating the onset time of the controlled functions bioluminescence and exoproteolytic activity. Further, the expression of AI-regulated genes, including *luxR*, was examined in dependence of the AI concentrations at distinct time periods. In addition, biochemical analysis of the phosphorelay system revealed interplay of the sensor kinases LuxN and LuxQ. This study shows that *V. harveyi* produces different ratios of AIs during certain growth phases to time QS-regulated processes.

To examine the influence of AIs as timer on a complex QS-regulated process, biofilm formation was analyzed using different QS mutants. Only the wild type was able to build a mature and stable matrix structure.

In conclusion, the data presented in this thesis will be included in a more detailed model of the QS system of *V. harveyi* and will enhance the knowledge of bacterial cell-cell communication.

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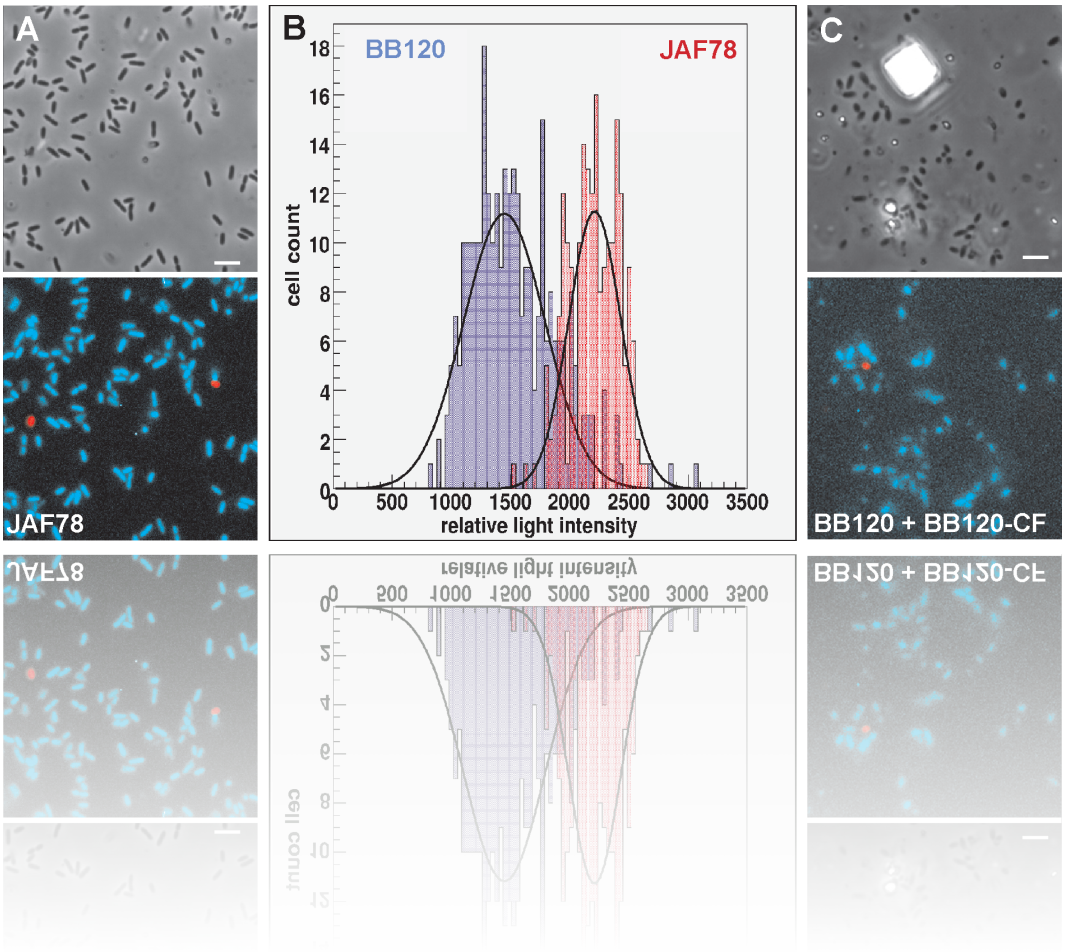
2 Heterogeneity in quorum sensing-regulated bioluminescence of *Vibrio harveyi*



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Heterogeneity in quorum sensing-regulated bioluminescence of *Vibrio harveyi*

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Summary

Quorum sensing (QS) refers to the ability of bacterial populations to read out the local environment for cell density and to collectively activate gene expression. *Vibrio harveyi*, one of the best characterized model organisms in QS, was used to address the question how single cells behave within a QS-activated community in a homogeneous environment. Analysis of the QS-regulated bioluminescence of a wild type strain revealed that even at high cell densities only 69% of the cells of the population produced bioluminescence, 25% remained dark and 6% were dead. Moreover, light intensities greatly varied from cell to cell at high population density. Addition of autoinducer to a bright liquid culture of *V. harveyi* increased the percentage of luminescent cells up to 98%, suggesting that *V. harveyi* produces and/or keeps the autoinducers at non-saturating concentrations. In contrast, all living cells of a constitutive QS-active mutant (Δ*luxO*) produced light. We also found that QS affects biofilm formation in *V. harveyi*. Our data provide first evidence that a heterogeneous population produces more biofilm than a homogeneous one. It is suggested that even a QS-committed population of *V. harveyi* takes advantage of heterogeneity, which extends the current view of QS-regulated uniformity.

Introduction

Bacterial populations are able to communicate via small diffusible molecules. Moreover, bacteria read out their local environment by producing and recognizing autoinducers for cell density, a phenomenon called quorum sensing (QS). Processes controlled by QS are useful for the population, like bioluminescence, virulence factor secretion, biofilm formation, sporulation and competence (Bassler and Losick, 2006). QS was first described in *Vibrio fischeri*, a symbiotic marine bacterium (Eberhard, 1972; Nealson, 1977; Greenberg *et al*., 1979; Kaplan and Greenberg, 1985). *Vibrio harveyi*, a Gram-negative marine bacterium, regulates cell density-dependent expression of multiple genes including those for bioluminescence (Bassler *et al*., 1994), siderophore production (Lilley and Bassler, 2000), an extracellular protease (Mok *et al*., 2003) and type III secretion (Henke and Bassler, 2004a). *V. harveyi* produces and responds to three different autoinducers (Waters and Bassler, 2005), which are recognized by membrane-integrated hybrid sensor kinases: HAI-1 by LuxN, AI-2 by LuxP/LuxQ and CAI-1 by CqsS (Freeman *et al*., 2000; Chen *et al*., 2002; Henke and Bassler, 2004b; Neiditch *et al*., 2005) (Fig. 1). HAI-1, an N-(3-hydroxybutyryl)-L-homoserine lactone and CAI-1, a 3-hydroxytridecan-4-one are *Vibrio* specific autoinducers (Cao and Meighen, 1989; Higgins *et al*., 2007). HAI-1 is the main autoinducer in *V. harveyi*, whereas CAI-1 is more important in *Vibrio cholerae*. In contrast, AI-2, a furanosylborate diester, is synthesized in Gram-negative and Gram-positive bacteria (Chen *et al*., 2002; Sun *et al*., 2004). Information of all three sensor kinases is transduced via phosphorelay to the response regulator LuxO (Freeman and Bassler, 1999). Phosphorylated LuxO activates the transcription of four small regulatory RNAs (sRNAs) that destabilize together with the chaperone Hfq the transcript for *luxR* (Tu and Bassler, 2007). The master regulator LuxR, a TetR-type protein, is both an activator and a repressor of a large number of QS-regulated genes (Mok *et al*., 2003; Lenz *et al*., 2004; Pompeani *et al*., 2008). Genetic studies revealed that LuxO is phosphorylated at low cell density resulting in a dark phenotype (no bioluminescence). When the threshold concentration of the corresponding autoinducers is reached, usually at high cell density, LuxO is dephosphorylated resulting in a bright, bioluminescent phenotype (Fig. 1). Besides studying the integration capacity of the rather complex signalling cascade by biochemical means (Timmen *et al*., 2006; Jung *et al*., 2007), we are interested in the regulation of gene expression at the single cell level (Megerle *et al*., 2008).

Up to now intercellular communication among non-differentiating bacteria via QS molecules is thought to synchronize the behaviour of all members of the group

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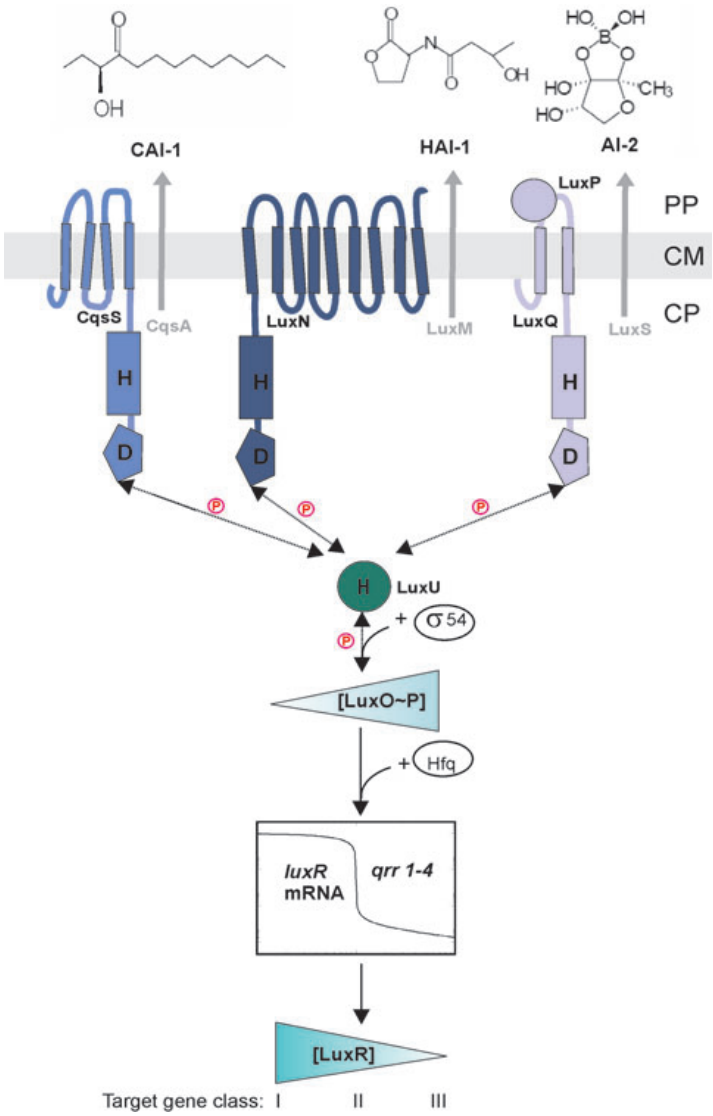

Fig. 1. The QS circuit of *V. harveyi*. The hybrid sensor kinases CqsS, LuxQ/LuxP and LuxN detect the concentration of the corresponding autoinducers. The higher the autoinducer concentration, the lower is their (auto)phosphorylation activity. The phosphoryl group is transferred via phosphorelay including the histidine phosphotransfer protein LuxU to the σ^{54}-dependent transcriptional activator LuxO. Phosphorylated LuxO (LuxO~P) activates transcription of four regulatory sRNAs (qrr1–4). Together with the chaperon Hfq these sRNAs destabilize the transcript of the master regulator LuxR. It is proposed that there is an ultrasensitive switch between degradation and accumulation of the *luxR* mRNA. This in turn produces a gradient of LuxR. According to their affinity to LuxR, QS target genes are grouped into three classes. The *lux* operon encoding luciferase belongs to the class I genes that have the lowest affinity and require a high concentration of LuxR. Lines marked with a 'P' indicate phosphotransfer reactions. The autoinducers CAI-1, HAI-1 and AI-2 are synthesized by CqsA, LuxM and LuxS respectively. *H* (histidine) and *D* (aspartate) denote the phosphorylation sites. *CM*, cytoplasmic membrane; *CP*, cytoplasm; *PP*, periplasm.

(Bassler and Losick, 2006; Keller and Surette, 2006). Here, we determined QS-induced bioluminescence in wild type *V. harveyi* at the single cell level. We demonstrate that even apparently non-differentiating bacteria are heterogeneous within a population to take advantage of division of work at high cell density.

Results

Heterogeneity of QS-regulated bioluminescence in V. harveyi

We examined induction of bioluminescence of wild type *V. harveyi* as the natural canonical QS-readout at the single cell level using luminescence microscopy in a dark chamber. After diluting a dense population of stationary phase cells (13 h of growth) 1000-fold into fresh mineral medium (AB medium), the percentage of bright cells slowly decreased to about 20% in the lag phase (Fig. 2A). Luciferase is a rather stable enzyme (Suadee *et al.*, 2007), which explains the relative high percentage of bright cells within the lag phase. It should be noted that the absolute cell number was rather low during lag phase, and bioluminescence of the averaged population was hardly detectable (Fig. 2A, purple dots). Within the exponential growth phase the percentage of bright cells increased and reached the maximum value (Fig. 2A, cyan part of bar chart). Unexpectedly, at no time all cells were bioluminescent. Even at high cell density on average 69%

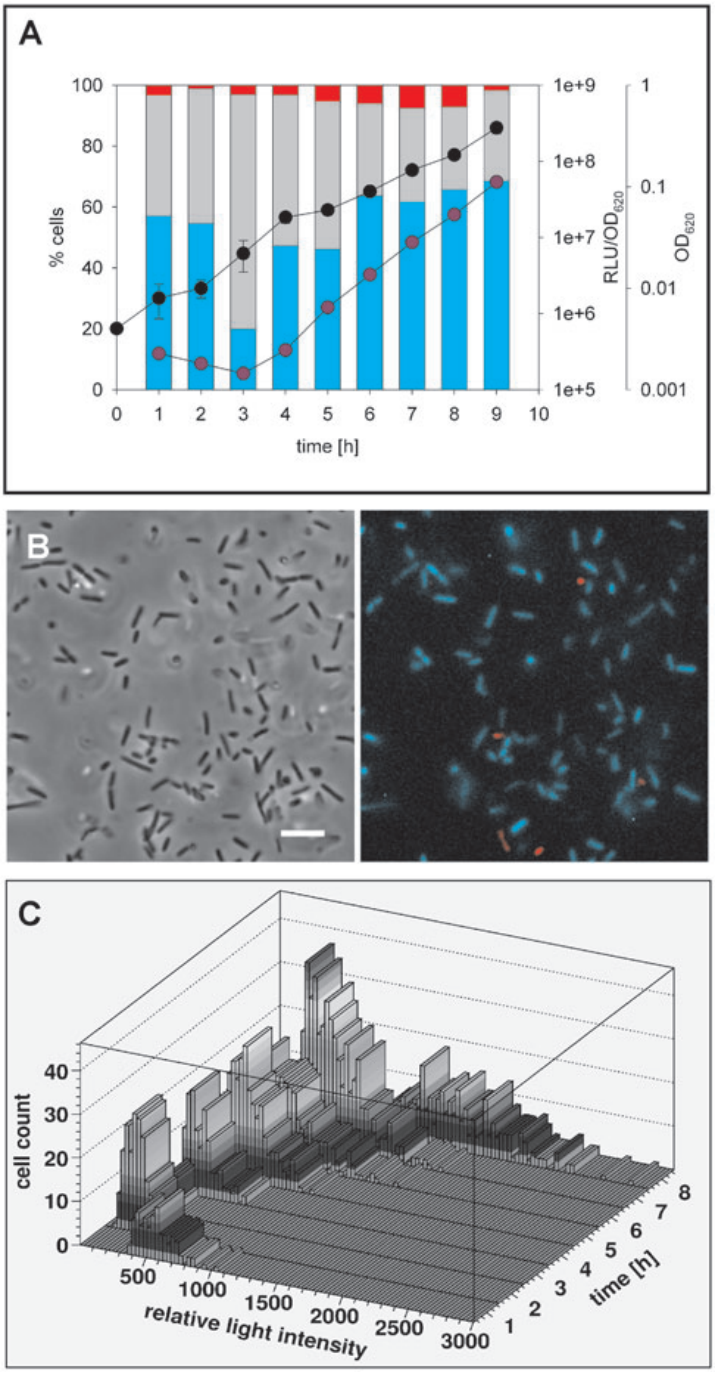

Fig. 2. Heterogeneity in QS-regulated bioluminescence of *V. harveyi*.
A. Time-dependent, quantitative analysis of bioluminescence production within a QS-activated population of *V. harveyi*. At time zero, a dense population of stationary phase cells (13 h of growth) was diluted 1000-fold into fresh AB medium, and growth was continued. At the times indicated samples were taken and analysed. Per time point three data sets are presented: (i) the total cell count is represented by a bar. The fractions of bright (cyan part), dark (grey part) and dead (red part) cells of the population are indicated with the colours. (ii) Growth was followed by measuring the optical density at 620 nm (black dots). (iii) The average luminescence (purple dots) was measured in a luminometer and is given in relative light units (counts s^{-1}) related to the optical density. The fractions of bright, dark and dead cells of *V. harveyi* wild type were determined after dividing the number of bright cells (luminescence images) or dead cells (cells stained with propidium iodide, fluorescence images) by the total cell number (phase contrast images). The remaining fraction represented the dark cells. For each time point between 300 and 700 cells were quantified. The cut-off value for a bright cell was > 50 units of relative light intensity above background.
B. Luminescence and phase contrast images of *V. harveyi* BB120 (wild type) after 8 h of growth. Phase contrast (left picture) and luminescence (right picture) images show the same field of view. Bright cells were recorded with a high-sensitivity camera and coloured cyan. Dead cells were stained with propidium iodide and recorded by fluorescence microscopy (red cells). Bar 2.5 μm.
C. Variation of light intensity of single bright cells of *V. harveyi* wild type. Luminescence images of all time points were analysed with respect to the variation of the relative light intensity of single cells. Between 150 and 350 bright cells were quantified for each time point. Relative light intensities of bright cells were plotted against the corresponding cell count in histograms. Cells with a relative light intensity below 50 were not considered in this diagram.

of the cells of a wild type population of *V. harveyi* produced bioluminescence (Fig. 2A, cyan part of bar chart), 25% of the cells remained dark (Fig. 2A, grey part of bar chart), and 6% of the cells were dead (Fig. 2A, red part of bar chart) (Table 1). Representative images obtained by luminescence and phase contrast microscopy are presented in Fig. 2B. The images show the same field of view of a sample taken from a culture at the late exponential growth phase (8 h). Thus, even at high cell density, the population was divided into two major subpopulations, one produced bioluminescence and the other not. In the stationary phase no further increase of the percentage of

Table 1. Heterogeneity of the wild type *V. harveyi* population after cultivation at different conditions.

Cultivation condition	Doubling time t_d (h)	Microscopy after (h)	Bright cells (%)	Dark cells (%)	Dead cells (%)
AB medium, 30°C	1.5	8	65.4 ±3.7	27.5 ± 3.3	7.1 ± 3.0
AB medium, 25°C	2.5	8	73.8 ± 2.7	22.9 ± 2.4	3.3 ± 0.6
AB medium, 15°C	7.0	8	59.1 ± 4.4	36.4 ± 3.2	4.5 ± 2.6
LM medium, 30°C	1.0	8	57.0 ± 1.2	41.8 ± 1.2	1.2 ± 1.3
PAB medium, 30°C	8.0	10	61.2 ± 0.6	38.5 ± 0.7	0.3 ± 0.1
SAB medium, 30°C	27.0	30	51.5 ± 3.4	44.9 ± 8.6	3.5 ± 5.0

V. harveyi BB120 was cultivated at different temperatures in AB medium (30°C, 25°C and 15°C) and in different media (AB, LM medium, in various salt water media) at 30°C. At the times indicated, cells were analysed by microscopy. About 700 cells per time point were examined, and the average distribution into bright, dark and dead cells of three independent experiments is given (see Fig. 2A for details). Standard deviations are indicated.
PAB, pacific aquarium-based; SAB, shrimp aquarium-based.

bright cells was observed (data not shown). It should be mentioned that cells started to aggregate at early stationary growth phase, a phenomenon that prevented accurate analysis of the whole population. Importantly, the fraction of free-living cells at this growth stage still exhibited heterogeneous luminescence behaviour (data not shown). We also tested the luminescence of aggregated cells, which were dissolved by detergent treatment before luminescence microscopy. None of these cells were found to be luminescent (data not shown). Routinely *V. harveyi* was cultivated in the mineral salt-based AB medium in which the cultures do not reach high cell densities (OD_{620} ~2.0). To analyse bioluminescence behaviour of a culture at higher cell density, *V. harveyi* was cultivated in rich LM medium, and the percentage of luminescent cells was determined over time. Although under this cultivation condition the cell density was significantly higher (OD_{620} ~5.0), a heterogenous population was formed (Table 1), indicating that the cell density in AB medium was not below the threshold for induction.

In parallel, the relative light units of the averaged population were determined using a luminometer. The population-wide average increase in bioluminescence corresponded with the temporal increase of the fraction of bright cells in the population (Fig. 2A). However, after 6 h of growth (exponential growth phase) total bioluminescence of the whole population further raised, while the percentage of bioluminescent cells remained constant. These results proposed that the expression of luciferase in cells of the bright subpopulation further increased. To test this hypothesis, individual luminescence intensities of about 150 (early time points of cultivation) to 350 cells were determined for each time point (Fig. 2C). Indeed, cells at high cell density (8 h of cultivation) were characterized by much higher light intensities than those at low cell density. It should be noted that the histograms of Fig. 2C represent the variation of light intensity of bright cells. Cells with a relative light intensity below 50 were designated as dark cells and were not considered in this diagram. Furthermore, it became evident that light intensity varied from cell to cell. The degree of variance increased considerably with higher cell densities (Fig. 2C, Fig. S1).

To exclude that the observed heterogeneity of the population was due to mutation, cells of a heterogeneous culture were plated. Subsequently, cultures of these colonies were analysed using luminescence microscopy. For all tested colonies a distribution into bright and dark subpopulations was found again, confirming that this distribution is an inherent property of a clonal population (data not shown).

In addition, we tested whether the heterogeneity found for the wild type population of *V. harveyi* BB120 was affected by the cultivation conditions. For this purpose, strain BB120 was cultivated at different temperatures (15°C, 25°C and 30°C), in different laboratory culture media (AB medium, LM medium) and in media closely resembling the natural free habitat [salt water from two aquaria (an aquarium with Pacific fishes and a shrimp aquarium) supplemented with arginine]. Changes in temperature and media affected the doubling times and hence maximal cell density, but heterogeneity within the population of *V. harveyi* BB120 was found in all media and at all tested temperatures (Table 1). Moreover, new bioluminescent strains were isolated from the surface of the European Seabass *Dicentrarchus labrax*. Two natural isolates that differed in their maximal light production were identified as *V. harveyi* with a sequence identity of 99.8% compared with *V. harveyi* ATCC BAA-1116 (BB120). Bioluminescence detection of single cells of the newly isolated strains revealed heterogeneous behaviour as well (data not shown). Therefore, the observed phenomenon was neither an exception for one strain nor the result of a specific cultivation condition.

Single cell bioluminescence of a constitutive QS-active V. harveyi *mutant*

Deletion of *luxO* introduces autoinducer-independent expression of QS genes (Freeman and Bassler, 1999).

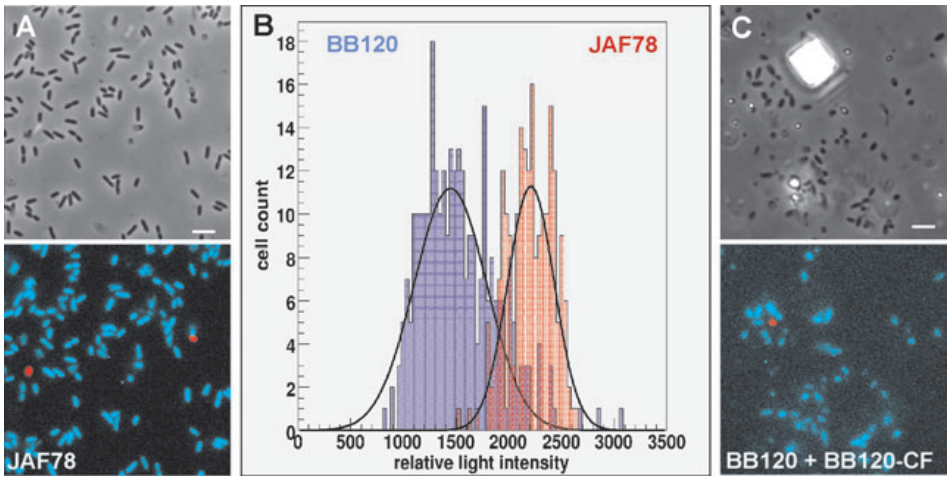

Fig. 3. Homogenous behaviour of a *V. harveyi* constitutive QS-active mutant (ΔluxO) or the wild type in the presence of additional autoinducers.
A. Luminescence of single cells of the *V. harveyi* mutant JAF78 (Δ*luxO*-Cam^R) after 8 h of growth. All living cells produced bioluminescence. Phase contrast (upper picture) and luminescence (lower picture) images show the same field of view. Bar 2.5 μm.
B. Comparison of the light intensities of single cells of *V. harveyi* mutant JAF78 (Δ*luxO*-Cam^R) and the wild type BB120 after 8 h of growth. Light intensity of about 200 (JAF78) and 300 (BB120) bright cells was quantified. The histogram for the light intensities of *V. harveyi* JAF78 is shown in red and the histogram for the wild type BB120 is shown in blue. For each histogram a Gaussian fit was performed (black curves).
C. Luminescence of single cells of the *V. harveyi* wild type BB120 after 8 h of growth in AB medium and exposure to fivefold concentrated cell-free fluid (CF) of a separate wild type culture. All living cells produced bioluminescence. Phase contrast (upper picture) and luminescence (lower picture) images show the same field of view. Bar 2.5 μm.

In contrast to the wild type, all living cells of the *V. harveyi luxO* null mutant (JAF78) produced bioluminescence (Fig. 3A), which was independent from cell density or growth phase (data not shown). Moreover, all living cells of the mutant produced significantly more light. The mean value of relative light intensities of mutant cells after 8 h of cultivation was 2199 compared with 1529 of the wild type (Fig. 3B). Although hardly seen by eye, image analysis revealed that light intensities of the mutant cells varied, too. However, the degree of variance (given as σ^2, whereby σ is the standard deviation) was 2.9-fold lower in case of the mutant compared with the wild type. The observed QS-independent variance of the mutant cells might be attributed to at least two factors: the metabolic state of each cell and a physical component. Different metabolic states of each cell result in variations in the cellular content of e.g. $FMNH_2$ or the long-chain aldehyde, affecting the luciferase reaction. Despite a biological explanation for the observed variance, the physical variance has to be taken into account. All pixels in the CCD camera are expected to have a nearly Gaussian electronic noise. In addition, the signal in a CCD pixel is expected to have a statistical error resulting from a Poissonian distributed photon counting rate.

The effect of autoinducers on the percentage of bright cells

To examine whether the cells of the dark subpopulation of *V. harveyi* were able to switch to a bright phenotype, we added autoinducer to a bright liquid culture after 8 h of cultivation, and samples were analysed after 30 min. The percentage of luminescent cells increased after addition of synthetic AI-2 (20 μM), or incubation in fivefold concentrated cell-free culture fluid to 83 ± 3% (± indicates the standard deviation, data not shown) and 98 ± 1% (Fig. 3C) respectively. This result suggests that *V. harveyi* produces and/or keeps the autoinducers at non-saturating concentrations resulting in the generation of bright and dark subpopulations.

Significance of the phenotypic heterogeneity within the V. harveyi *population*

In search for the biological significance of the described phenotypic heterogeneity, we compared growth behaviour of the wild type and the constitutive QS-active mutant JAF78 (Δ*luxO*). First, we observed that wild type cells of the stationary phase aggregated in liquid culture, while mutant cells remained apart (Fig. 4A). Second, mutant cells adhered hardly to coverslips. Adherence of these

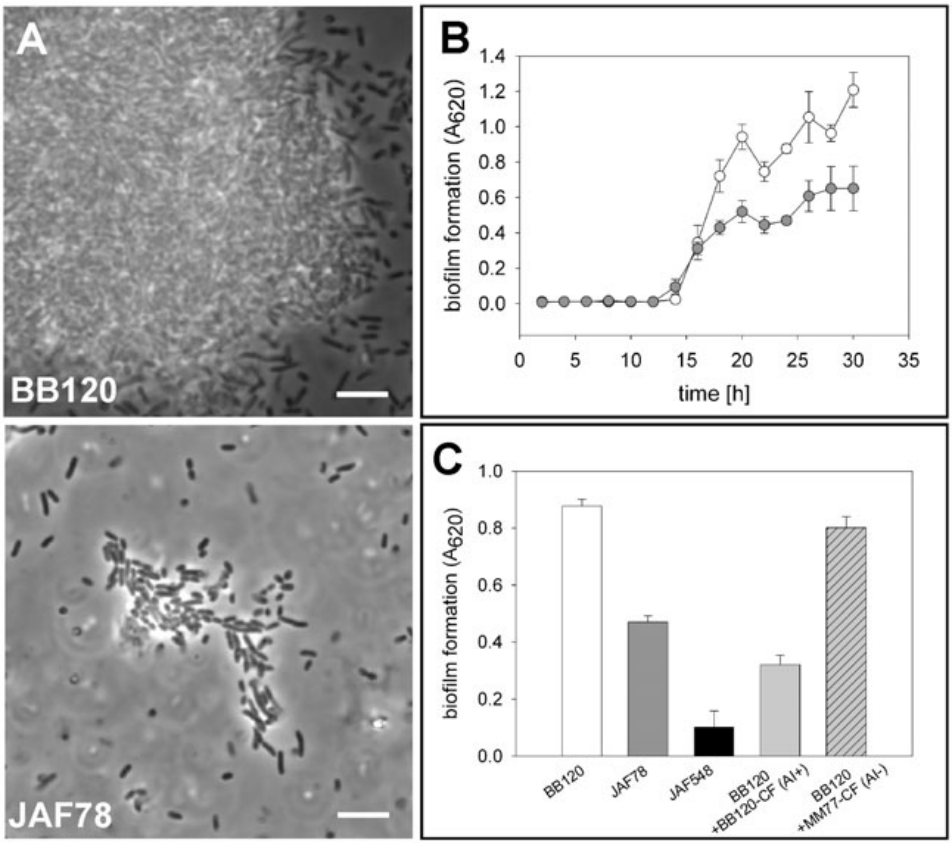

Fig. 4. Aggregation and biofilm formation of *V. harveyi*.
A. Phase contrast images of *V. harveyi* wild type BB120 (upper picture) and mutant JAF78 (Δ*luxO*-Cam^R) (lower picture). Cells were taken from stationary phase cultures. Bar 2.5 μm.
B. Biofilm formation of *V. harveyi* wild type BB120 (white) and mutant JAF78 (Δ*luxO*-Cam^R) (grey) was monitored over time. Cells were grown in AB medium in polystyrene microtiter plates. Biofilm formation was quantified after crystal violet staining. The experiment was performed in triplicates, and error bars indicate standard deviation of the mean.
C. Biofilm formation of *V. harveyi* wild type BB120 (white), the constitutive QS-active mutant JAF78 (Δ*luxO*-Cam^R, dark grey), the QS-negative mutant JAF548 (*luxO*D47E, black) and wild type BB120 either in the presence of fivefold concentrated cell-free fluid of a wild type BB120 culture containing all three autoinducers [+BB120-CF (AI+)] (light grey), or in the presence of fivefold concentrated cell-free fluid of the mutant MM77 (*luxLM*::Tn*5*, *luxS*::Cam^R) culture that lacks HAI-1 and AI-2 [+MM77-CF (AI–)] (striped light grey) was examined after 25 h of growth in polystyrene microtiter plates. Cells were grown and stained as described in (B). The experiment was performed in triplicates, and error bars indicate standard deviation of the mean.

cells was only possible when the coverslip was coated with poly L-lysine. *V. harveyi* is a serious pathogen for a wide range of marine animals, and biofilm formation is one of the virulence determinants (Austin and Zhang, 2006). Therefore, biofilm formation of the two strains was tested in polystyrene flat-bottom well plates. At the times indicated, wells were washed, and cells in the biofilm were visualized by staining with crystal violet. Both, wild type and mutant, switched to biofilm formation after 14 h of growth (Fig. 4B). Remarkably, the capability of the Δ*luxO* mutant to produce biofilm was significantly reduced (Fig. 4B and C). Consistently, the capability of wild type BB120 to form biofilm was also reduced when the culture was grown in the presence of fivefold concentrated cell-free fluid of a separate wild type culture that contained all three autoinducers (Fig. 4C). To exclude non-specific effects of cell-free culture fluids on growth, biofilm formation, etc., the experiment was repeated for wild type BB120 in the presence of fivefold concentrated cell-free fluid of a separate culture of *V. harveyi* mutant MM77 (Fig. 4C). *V. harveyi* MM77 lacks the genes encoding the autoinducer-synthases *luxS* and *luxM* (Mok *et al.*, 2003). Thus, this mutant is unable to produce AI-2 and HAI-1. Under this condition, the amount of produced biofilm by wild type BB120 was hardly affected (Fig. 4C). These results imply that heterogeneity of the population appears to be beneficial for biofilm formation. Moreover, *V. harveyi* seems to control the available autoinducer concentration

very tightly. An excess of autoinducers significantly lowered the amount of biofilm produced.

The correlation between biofilm formation and QS in *V. harveyi* is not well characterized yet. For that reason we tested the capability to form biofilm of the QS-negative *V. harveyi* mutant JAF548. This mutant is characterized by a dark phenotype independent of the autoinducer concentration (Freeman and Bassler, 1999). This phenotype is caused by a single point mutation in *luxO* resulting in the LuxO-D47E derivative which mimics the phosphorylated form of LuxO. Mutant JAF548 produced almost no biofilm (Fig. 4C). Cultures of mutants JAF78 and JAF548 grown in the absence or presence of fivefold concentrated cell-free fluid of a separate wild type BB120 culture showed indistinguishable biofilm formation (data not shown). These data provide a first indication that biofilm formation is positively regulated by QS. The suggestion is supported by the fact that an 'artificial heterogeneous' population consisting of 60% strain JAF78 and 40% strain JAF548 was unable to produce as much biofilm as the wild type (data not shown).

Discussion

Over the last two decades it became evident that bacteria communicate with each other using chemical signalling molecules. This chemical communication involves production, excretion, sensing and response to these signalling molecules. Especially non-differentiating bacteria use this kind of signal-response systems to synchronize their behaviour on a population-wide scale. Most QS-regulated processes are only beneficial when they are performed simultaneously by a large number of cells (Waters and Bassler, 2005).

Here, we determined the behaviour of single cells within a QS-activated community. Importantly, these studies were performed with a *V. harveyi* wild type strain using bioluminescence as natural QS readout. Our results indicate that a clonal high dense population forms two major subpopulations, a bright and a dark one, in a homogeneous environment. Moreover, light intensity varied considerably from cell to cell. Heterogeneous behaviour was found under all tested cultivation conditions. Therefore, it appears unlikely that the observed heterogeneity is attributed to specific laboratory conditions. Additionally, formation of subpopulations was also found for two newly isolated strains of *V. harveyi.* In contrast to the wild type strain, all cells of a Δ*luxO* mutant were luminescent. Light intensities of single cells within the mutant population were significantly higher, and the degree of variance was lower.

Population heterogeneity is a phenomenon that is known for *Bacillus* (Chai *et al*., 2008), *Agrobacterium* (Goryachev *et al*., 2005) and *Pseudomonas* (Diggle *et al*., 2007). Heterogeneity provides the prerequisite for multicellular behaviour of bacteria to share 'community goods' (Dunny *et al*., 2008), such as degradative enzymes, or material for an extracellular matrix. In most of these processes, positive feedback plays a fundamental role, as it can generate bistable and multistable outputs. In this way, two or more phenotypically different subpopulations stably develop out of a clonal population in one culture. These subpopulations are characterized by distinct gene expression states (Smits *et al*., 2006). The lactose and arabinose utilization systems display bistable (all-or-nothing) responses at non-saturating concentrations of lactose or arabinose respectively (Ozbudak *et al*., 2004; Megerle *et al*., 2008). In these systems bistability is caused by the autocatalytic positive feedback of the corresponding sugars on their uptake proteins. Positive feedback loops in QS-regulated processes are less well characterized. In *V. fischeri* the autoregulatory feedback seems to be dependent on LuxR and LuxI (Sayut *et al*., 2007). In *V. harveyi,* QS-controlled gene expression is rather complex, and the three autoinducers, HAI-1, AI-2 and CAI-1, display synergistic activity. Thus, all three autoinducers are required to induce maximal bioluminescence (Henke and Bassler, 2004b). We found that the percentage of luminescent cells of a bright liquid culture of wild type *V. harveyi* significantly increased after addition of synthetic AI-2 or incubation of cells in fivefold concentrated cell-free culture fluid. This result suggests that *V. harveyi* produces/self-limits the autoinducers at subsaturating concentrations. The QS signalling cascade comprises an ultrasensitive switch that is regulated by the level of phosphorylated LuxO and hence the expression of four sRNAs. (Lenz *et al*., 2004) (Fig. 1). By this means a gradient of the master regulator LuxR is generated, which in turn induces expression of different classes of QS-target genes (Tu and Bassler, 2007). The first gene of the *lux*-operon encoding luciferase *luxC* belongs to the class I genes (Waters and Bassler, 2006). Induction of these genes requires a high intracellular concentration of LuxR. Recently, for both, *luxR* and *luxC,* the LuxR-binding sites upstream of the corresponding genes were identified. Both bioinformatic prediction and electrophoretic mobility shift assays uncovered multiple binding sites of LuxR to the *luxC* promoter (Pompeani *et al*., 2008). Altogether, the autoinducer-dependent phosphorylation state of LuxO in combination with the sRNA triggered level of *luxR* mRNA and a putative positive feedback of LuxR might account for the observed heterogeneity. Some cells reach the threshold concentration of LuxR required for induction of the *lux* operon and some cells not, which results in the generation of a bright and a dark subpopulation. In addition, the bright subpopulation displayed a high cell to cell variance, which is in agreement with the proposed LuxR gradient and the sub-

saturating autoinducer concentrations. The mechanism, how *V. harveyi* controls self-limitation of autoinducers, needs further investigation. Temporal synthesis, degradation or uptake of autoinducers are conceivable possibilities (Xavier and Bassler, 2005).

In a recent study HAI-1 and/or AI-2 responsive *qrr4-gfp* expression was studied at single cell resolution. In contrast to our results homogenous behaviour of the reporter strain population was found (Long *et al*., 2009). The difference in the behaviour of the wild type and the reporter strain populations can be explained as follows. Expression of the *gfp* fusion in the reporter strains was independent of LuxR (compare Fig. 1), and all strains lacked the corresponding autoinducer synthases. According to our theory, a variable cellular LuxR concentration contributes to heterogeneous behaviour of the *V. harveyi* wild type strain. In addition, our results imply that *V. harveyi* self-limits the autoinducer concentration. Putative feedback loops associated with the synthesis and hence final autoinducer concentration were eliminated in the reporter strains used by Long *et al*. (2009).

We found that cells of the wild type population had a much higher tendency to aggregate. Hence we tested the capability of *V. harveyi* to form biofilm. According to our results a heterogeneous population, such as the wild type, produced more biofilm than a homogenous one, such as the constitutive QS-active mutant JAF78 or the wild type in the presence of additional autoinducers. In contrast, the QS-negative mutant JAF548 (LuxO-D47E) was hardly able to produce biofilm, proposing that genes required for biofilm formation are under positive control of QS. This is the first indication for a correlation between QS and induction of biofilm formation in *V. harveyi*. In *V. cholerae*, the LuxR homologue HapR is a negative regulator of biofilm formation, while in most of the other examined *Vibrio* species biofilm formation is positively controlled by QS (Yildiz and Visick, 2009). Thus, cells of the dark subpopulation might not be regarded as QS-inactive. The LuxR level in these cells might be below the threshold concentration to induce class I genes, such as *luxC*. Consequently, these cells save the energy for biosynthesis of luciferase, which can accumulate up to 5% of total soluble protein in the stationary phase (Reeve and Baldwin, 1982), and $NADPH + H^+$ and ATP required for luminescence. However, the level of LuxR in these cells appears to be sufficient to induce QS-regulated class II or III genes encoding gene products required for biofilm formation. Thus, the dark cells might be responsible for providing material for an extracellular matrix. In this way, the population would take advantage of division of work at high cell density. In line with this hypothesis, we found that an 'artificial heterogenous' population consisting of strains JAF78 and JAF548 was unable to restore the biofilm capability of wild type. The results described here are reminiscent of the interconnection between population heterogeneity and biofilm formation found in *Bacillus* (Chai *et al*., 2008) and *V. cholerae* (Matz *et al*., 2005). Heterogeneous behaviour is also advantageous from an evolutionary point of view. According to the game theory, heterogeneity in populations benefits the individual by maximizing its own fitness resulting in an evolutionary advantage of the whole population (Wolf *et al*., 2005; Davidson and Surette, 2008).

To sum up, our results reveal that even apparently non-differentiating bacteria form a heterogeneous population. The results extend the current view on QS-dependent synchronized behaviour, and will initiate future studies on the molecular basis of the implemented bi-/multistability as well as the significance of heterogeneous populations from the medical, ecological and evolutionary point of view.

Experimental procedures

Bacterial strains and growth conditions

The *V. harveyi* strains BB120 (wild type) (Bassler *et al*., 1997), MM77 (*luxLM*::Tn*5*, *luxS*::Cam^R) (Mok *et al*., 2003), JAF78 (Δ*luxO*-Cam^R) and JAF548 (*luxO*D47E) (Freeman and Bassler, 1999) were used throughout this study. *V. harveyi* cells were cultivated in AB medium (Greenberg *et al*., 1979) or LM medium (20 g l^{-1} NaCl, 10 g l^{-1} tryptone, 5.0 g l^{-1} yeast extract), and incubated aerobically in a rotary shaker at 30°C. When appropriate, the medium was supplemented with chloramphenicol (33 μg ml^{-1}). Salt water from a pacific and a shrimp aquarium was sterile-filtered (0.20 μm), and supplemented with 10 mM L-arginine. To obtain culture fluids, the cultures were centrifuged at 5000 *g* for 15 min. Culture fluids were then filtrated (0.20 μm), lyophilized when required and stored in concentrated form at –20°C or used immediately.

New *V. harveyi* strains were isolated from the surface of the European Seabass *D. labrax*. The whole dead fish was incubated in artificial sea water (46.4 g l^{-1} NaCl, 49.2 g l^{-1} $MgSO_4 \times 7\ H_2O$, 3.0 g l^{-1} KCl, 5.8 g l^{-1} $CaCl_2 \times 2\ H_2O$) at room temperature for 30 h. Bright colonies were transferred to sea water agar plates [20.0 g l^{-1} agar, 750 ml artificial sea water, 250 ml basal-medium (20.0 g l^{-1} peptone, 12.0 g l^{-1} yeast extract, 4.0 g l^{-1} $CaCO_3$, 12 ml l^{-1} glycerol)] and incubated at room temperature for 24 h. Strains were purified and cultivated in AB medium. The isolated strains were identified as *V. harveyi* with a sequence identity of 99.8% compared with *V. harveyi* ATCC BAA-1116 (http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genome&Cmd=Search&TermToSearch=txid338187[orgn]) after sequencing a 566 bp fragment within the V3 region of the 16S rRNA gene using primers 341f (5′-CCTACGGGAAGCAG-3′) and 907r (5′-CCGTCAATTCCTTTGAGTTT-3′). Cycle sequencing was performed according to standard protocols.

Single cell analysis

Cells of an overnight culture were diluted (1000-fold) into fresh medium, and growth was continued under aerobic

conditions. At the times indicated, cells were collected by centrifugation at 5000 *g* for 10 min. The culture fluid was saved for the subsequent steps. Cell pellets were resuspended in small volumes of the corresponding culture fluid. To separate cells within a biofilm, the culture was treated with 0.05% (w/v) Tween20 for 10 min. 20 μl of the cell suspension was stained with 5 μM propidium iodide (staining of dead cells), and incubated in the middle of a coverslip in the dark for 20 min. All coverslips were pretreated with poly L-lysine (0.05 g l^{-1}) to fix the cells on the surface. Subsequently, cells were washed twice with the corresponding air-saturated culture fluid directly on the slip to remove non-fixed cells. Cells were examined using a customized inverted Leica microscope DMI 6000 B (all light-emitting diodes were inactivated) and the high-sensitivity camera iXON (Andor, 12 bit) in a flow cell (Pecon). This flow cell has an upper and a lower coverslip separated by a perfusion ring. Via this ring the chamber was filled with medium. Cells were fixed on the lower coverslip. All cells were monitored using phase-contrast microscopy. Dead cells were identified using fluorescence imaging with the bandpass-filter BP546/12 and the emission-filter 605/75. Bright cells were identified using luminescence imaging without any filter in a dark room. All lights had to be switched off, and the chip of the CCD-camera was exposed to the luminescent cells for 240 s. Images were manually analysed by counting about 700 cells per image. Light intensity of single cells was determined by using ImageJ 1.37c (National Institute of Health, http://rsb.info.nih.gov/ij), and population analysis was performed using Root 5.20/00 (CERN). The cut-off value for bright cells was > 50 units of relative light intensity above background. Aggregated cells of *V. harveyi* BB120 and JAF78 were monitored using phase contrast microscopy.

Quantitative biofilm assay

To assess biofilm formation, cells of an overnight culture of *V. harveyi* were adjusted to an absorbance of $A_{620} = 0.02$ by dilution with AB medium, and 100 μl was inoculated into a polystyrene 96 flat-bottom well plate (Sarstedt). The culture was grown at 30°C with shaking. At the times indicated, wells were washed with distilled water or saline twice, and cells in a biofilm were visualized by staining with 0.5% (w/v) crystal violet for 20 min according to Wakimoto (Wakimoto *et al*., 2004). Absorption of crystal violet was determined in a microplate reader (Tecan Sunrise) at 620 nm.

Synthesis of AI-2

AI-2 was synthesized *in vitro* using the enzymes LuxS and Pfs, and purified over boric acid resin according to Schauder and Lenz (Schauder *et al*., 2001; Lenz *et al*., 2004). For this purpose, LuxS and Pfs were produced and purified as described before (Schauder *et al*., 2001; Sperandio *et al*., 2003; Xavier and Bassler, 2005). Purity and yield of AI-2 were indirectly assayed according to Lozada-Ramirez (Lozada-Ramirez *et al*., 2006) by the method of Ellman (Ellman *et al*., 1961). The biological activity of AI-2 was determined using *V. harveyi* MM77 in a bioluminescence based reporter assay (Bassler *et al*., 1993).

Bioluminescence assay

Luminescence produced by *V. harveyi* strains was determined in a Centro LB960 (Berthold Technology) for 0.1 s, and data are reported as relative light units [counts s^{-1}] related to the optical density.

Acknowledgements

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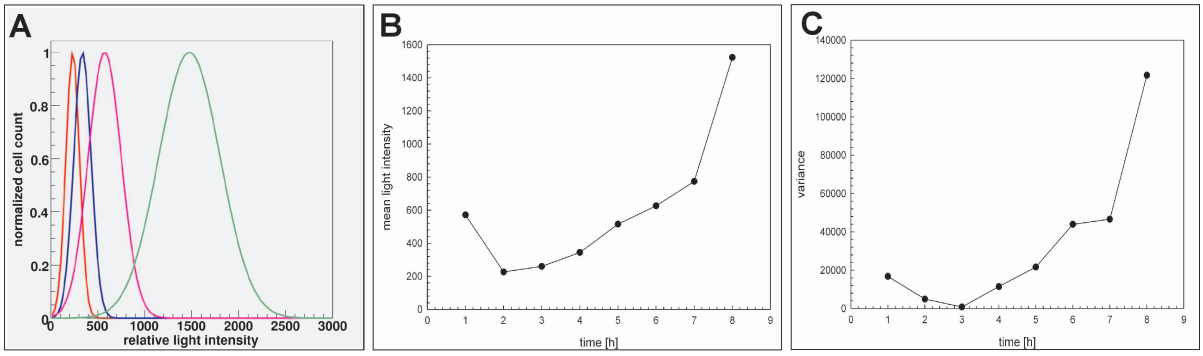
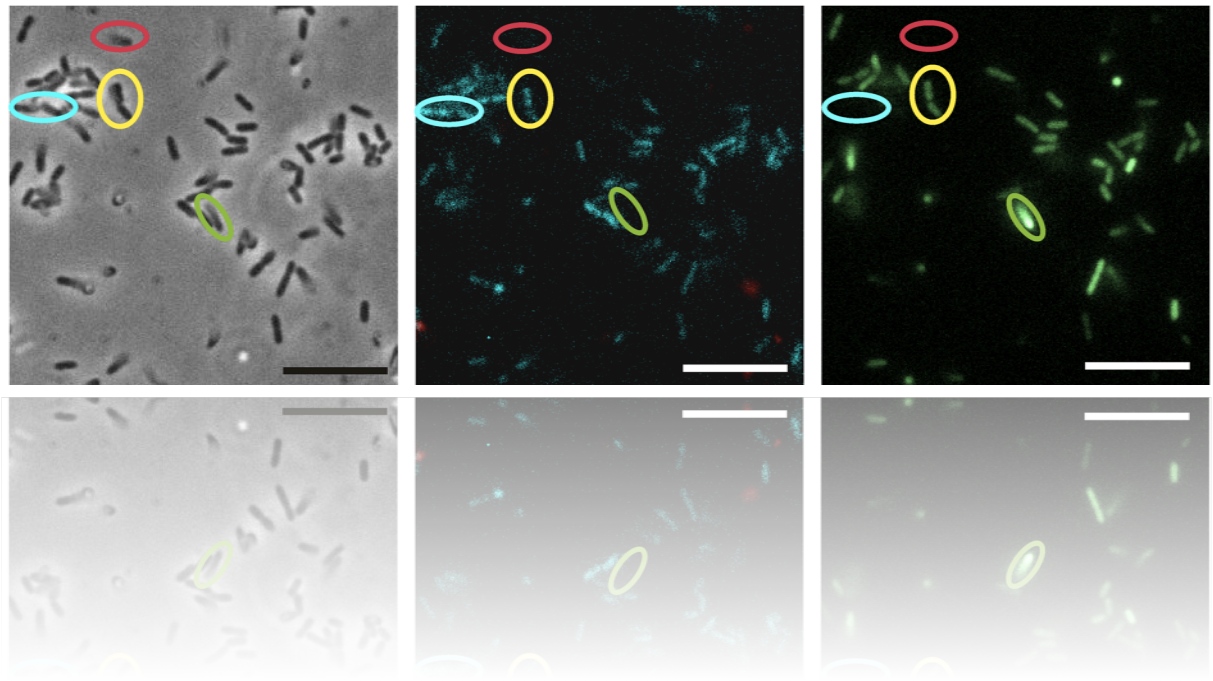


SFig. 1: Alterations of light intensity of *Vibrio harveyi* wild-type BB120 cells. The relative light intensities of single bright cells were quantified (Fig. 2C). The cell count of the mean relative light intensity was normalized to 1 for each time point. The Gaussian-fitted curves of the normalized cell count plotted against the relative light intensity after 2 h (red), 4 h (blue), 6 h (purple) and 8 h (green) are shown in **(A)**. For each time point, these curves were fitted to determine the mean light intensity **(B)** and the variance **(C)**.

3 Single cell analysis of *Vibrio harveyi* uncovers functional heterogeneity in responses to quorum sensing signals



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Single cell analysis of *Vibrio harveyi* uncovers functional heterogeneity in response to quorum sensing signals

Claudia Anetzberger[1], Ursula Schell[1,2] and Kirsten Jung[1*]

Abstract

Background: *Vibrio harveyi* and closely related species are important pathogens in aquaculture. A complex quorum sensing cascade involving three autoinducers controls bioluminescence and several genes encoding virulence factors. Single cell analysis of a *V. harveyi* population has already indicated intercellular heterogeneity in the production of bioluminescence. This study was undertaken to analyze the expression of various autoinducer-dependent genes in individual cells.

Results: Here we used reporter strains bearing promoter::*gfp* fusions to monitor the induction/repression of three autoinducer-regulated genes in wild type conjugates at the single cell level. Two genes involved in pathogenesis - *vhp* and *vscP*, which code for an exoprotease and a component of the type III secretion system, respectively, and *luxC* (the first gene in the *lux* operon) were chosen for analysis. The *lux* operon and the exoprotease gene are induced, while *vscP* is repressed at high cell density. As controls *luxS* and *recA,* whose expression is not dependent on autoinducers, were examined. The responses of the promoter::*gfp* fusions in individual cells from the same culture ranged from no to high induction. Importantly, simultaneous analysis of two autoinducer induced phenotypes, bioluminescence (light detection) and exoproteolytic activity (fluorescence of a promoter::*gfp* fusion), in single cells provided evidence for functional heterogeneity within a *V. harveyi* population.

Conclusions: Autoinducers are not only an indicator for cell density, but play a pivotal role in the coordination of physiological activities within the population.

Keywords: Bioluminescence, Exoprotease, Type III secretion, Autoinducer, Division of labor, Subpopulation

Background

Populations of genetically identical bacteria are conventionally regarded as being phenotypically homogeneous. Over the past decade however, it has become apparent that bacterial cell clones are not necessarily functionally homogeneous. For example, heterogeneity within clonal *Bacillus sp.* populations has been extensively investigated [1,2]. We previously observed heterogeneous behavior of quorum sensing (QS) regulated bioluminescence in a *V. harveyi* population [3]. Even at high cell densities, the population was found to comprise two subpopulations: two-thirds of all cells exhibited luminescence, while the rest remained dark.

QS is a form of cell to cell communication, which involves production, excretion and sensing of signaling molecules, the autoinducers (AIs) (see [4] for review). The Gram-negative marine bacterium *V. harveyi* (recently reclassified as *Vibrio campbellii* [5]) produces three different AIs. HAI-1 belongs to the group of acylhomoserine lactones used by many Gram-negative species [6]. CAI-1, a long-chain ketone, is the main AI in *V. cholerae*, whereas it seems to be less important in *V. harveyi* [7]. AI-2, a furanosyl borate diester derived from 4,5-dihydroxy-2,3-pentandione, is widespread in the bacterial world [8,9]. The three AIs are recognized by three hybrid sensor kinases located in the cytoplasmic membrane (Figure 1): HAI-1 by LuxN, AI-2 by LuxQ (in concert with its binding protein LuxP) and CAI-1 by CqsS [7,8,10-12]. Information is

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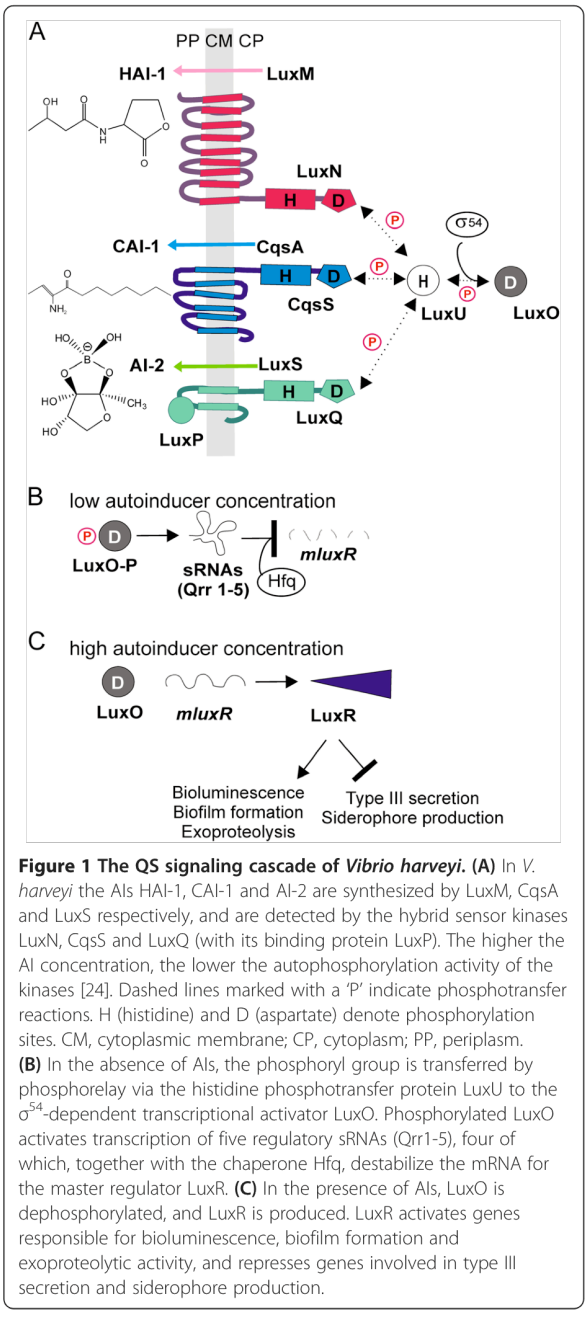

Figure 1 The QS signaling cascade of *Vibrio harveyi*. **(A)** In *V. harveyi* the AIs HAI-1, CAI-1 and AI-2 are synthesized by LuxM, CqsA and LuxS respectively, and are detected by the hybrid sensor kinases LuxN, CqsS and LuxQ (with its binding protein LuxP). The higher the AI concentration, the lower the autophosphorylation activity of the kinases [24]. Dashed lines marked with a 'P' indicate phosphotransfer reactions. H (histidine) and D (aspartate) denote phosphorylation sites. CM, cytoplasmic membrane; CP, cytoplasm; PP, periplasm. **(B)** In the absence of AIs, the phosphoryl group is transferred by phosphorelay via the histidine phosphotransfer protein LuxU to the σ^{54}-dependent transcriptional activator LuxO. Phosphorylated LuxO activates transcription of five regulatory sRNAs (Qrr1-5), four of which, together with the chaperone Hfq, destabilize the mRNA for the master regulator LuxR. **(C)** In the presence of AIs, LuxO is dephosphorylated, and LuxR is produced. LuxR activates genes responsible for bioluminescence, biofilm formation and exoproteolytic activity, and represses genes involved in type III secretion and siderophore production.

transduced via phosphorelay to LuxU and further to the response regulator LuxO [13]. A recently described new circuit consisting of the NO-sensing H-NOX and the soluble histidine kinase HqsK also feeds its information to the QS network at the level of LuxU [14]. Phosphorylated LuxO activates the transcription of five small regulatory RNAs (Qrr 1-5). Four of these, acting together with the chaperone Hfq, destabilize the transcript that encodes the master regulator LuxR [15,16]. LuxR is both an activator and a repressor of a large number (> 100) of genes [17,18]. Several feedback loops regulate the level of LuxR in the cell. These involve the autorepression of *luxR* [19], the induction of *qrr2-4* transcription by LuxR [20], the autorepression of *luxO* [21], the down-regulation of the translation of *luxO* and *luxMN* by *qrr* sRNAs [21,22], and the direct repression by AphA, an antagonist of LuxR [23].

V. harveyi is an opportunistic pathogen mainly for shrimps, but also for fish, squids and lobsters [25-27] and causes major losses in shrimp aquaculture [28]. The response to QS signals is of interest in this context, because genes regulated by QS encode proteins required for biofilm formation [3] and virulence factors, such as siderophores [29], type III secretion (e.g. *vscP*) [30] and exoproteolytic activity (e.g. *vhp*) [17,31], in addition to bioluminescence (using the *lux* system) [32].

Here we focused on the single cell analysis of fluorescent reporter strains bearing plasmids containing promoter::*gfp* fusions, which allowed us to simultaneously monitor the expression of two AI-regulated genes in single cells.

Results

AI-regulated bioluminescence correlates well with the activity of the corresponding promoter::*gfp* fusion

To expand our previous findings on heterogeneous behavior of a *V. harveyi* population found for bioluminescence [3] to other AI-regulated genes, we decided to construct promoter::*gfp* fusions. It was important to use a wild type genetic background to monitor bioluminescence as a marker for an intact QS cascade in each strain. Therefore, all promoter::*gfp* fusions are plasmid based. To set up the reporter system we tested first a plasmid containing a promoter::*gfp* fusion of the constitutively expressed housekeeping gene *recA* to estimate the degree of heterogeneity in the expression of this gene [33]. Wild type cells conjugated with this plasmid were grown to the exponential growth phase, stained with propidium iodide to identify dead cells (about 5%), and single cells in the same field of view were analyzed in phase contrast and fluorescence modes. Images were analyzed using ImageJ. Luminescence and fluorescence intensities of each living cell are expressed as intensity values per cell after normalization to the same cell size. All living cells were fluorescent, indicating expression of *recA* in all cells. Fluorescence intensities were determined in about 1,400 cells. The average fluorescence intensity was calculated to be 1,017 a.u./cell [(a.u.) arbitrary units] with a standard deviation of 9.9% (data not shown). For comparison all living cells of strain BB120*gfp* containing a chromosomal encoded *gfp* were fluorescent and showed an average fluorescence intensity of 1,085 a.u./cell with a standard deviation of 10.5% (data not shown). Testing for statistical significance (with $\Delta\mu > 1\sigma$ proving a significant difference) revealed

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that these two fusions were not significantly different, with $\Delta\mu = 0.45\ \sigma$. These results indicated that plasmid and chromosomal encoded genes exhibit a comparable expression pattern at the single cell level. Furthermore, promoter::*gfp* fusions of constitutively expressed genes result in fluorescence of all living cells.

After that, a plasmid containing a promoter::*gfp* fusion for the *lux* operon in addition to the intact *luxCDABE* operon was constructed to test whether bioluminescence in single cells correlated with the fluorescence intensity of the corresponding P_{luxC}::*gfp* fusion. The wild type strain conjugated with a plasmid encoding a P_{luxC}::*gfp* fusion was grown to the mid-exponential growth phase, and single cells in the same field of view were analyzed in phase contrast (Figure 2A left), bioluminescence (Figure 2A middle) and fluorescence (Figure 2A right) modes. Intensity data for 450 living bacteria were acquired and depicted in a correlation plot, with each dot representing a single cell (Figure 2B). There was a strong correlation between bioluminescence and fluorescence ($r = 0.84$, $p < 0.001$) (Figure 2B), indicating that the P_{luxC}::*gfp* fusion reliably mirrors natural bioluminescence induction.

We analyzed the third construct, which contains a P_{vhp}::*gfp* fusion. *vhp* encodes an exoprotease. Bacteria were cultivated as described above, and 300 living cells were quantitatively analyzed with respect to bioluminescence and fluorescence intensities (Figure 2C, D). Here, single cell analysis revealed no correlation between bioluminescence and fluorescence ($r = 0.06$, $p = 0.28$) (Figure 2D). This is reflected in the fact that luminescent cells were not necessarily fluorescent and vice versa (Figure 2D). In addition, the culture contained a larger fraction of luminescent than fluorescent cells. This finding is in agreement with our observation that exoproteolytic activity does not coincide with bioluminescence during growth of *V. harveyi* (unpublished observation). Overall, these data indicate that promoter::*gfp* fusions provide a reliable mean to monitor AI-regulated gene expression at the single cell level in *V. harveyi*.

Expression of various AI-regulated genes is heterogeneous

Next we analyzed the time-dependent expression of three AI-regulated genes and two AI-independent genes at the single cell level. In addition to the P_{luxC}::*gfp*, the

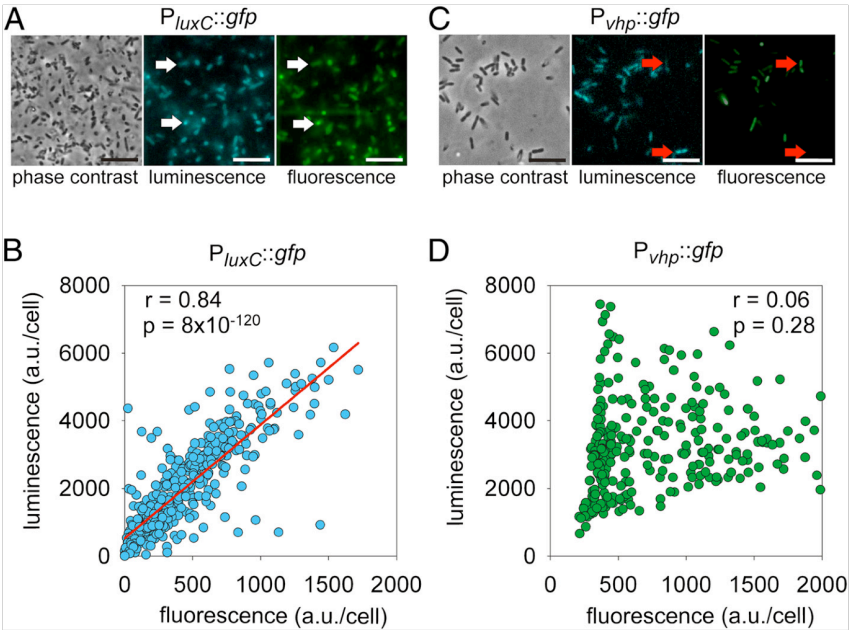

Figure 2 Characterization of AI-regulated gene activity in *V. harveyi* strains containing promoter::*gfp* reporter fusions. *V. harveyi* strains containing P_{luxC}::*gfp* **(A, B)** and P_{vhp}::*gfp* **(C, D)** reporter fusions were grown to the mid-exponential growth phase ($OD_{600} = 0.2$), and single cell analysis was performed. 450 (P_{luxC}::*gfp*) and 300 (P_{vhp}::*gfp*) cells were individually analyzed using ImageJ. In panels **B** and **D**, fluorescence and bioluminescence levels (normalized for cell size and expressed in arbitrary units) are plotted for individual cells bearing the reporter fusions indicated. The correlation coefficient r and the p-value are indicated. A regression line could be drawn only for strain P_{luxC}::*gfp* (red). Panels **A** and **C** show phase-contrast (left), bioluminescence (middle) and fluorescence (right) views of cells expressing promoter::*gfp* fusions for *luxC* and *vhp*, respectively. The images in each row show the same field of view. Note the tight correlation between luminescence and *luxC* reporter expression in panel **A**. White arrows indicate two cells displaying signals of equal intensity in the bioluminescence and fluorescence channels. In panel **C** red arrows point to cells that exhibit high bioluminescence and low fluorescence or vice versa. Scale bar = 2.5 µm.

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P_{vhp}::*gfp* and the P_{recA}::*gfp* strains described above, strains with P_{vscP}::*gfp* and P_{luxS}::*gfp* fusions were generated. The *vscP* gene encodes a translocation protein of the type III secretion system and the product of *luxS* is involved in the synthesis of AI-2. Our preliminary experiments and a microarray study indicated that *luxS* expression is not dependent on AIs (unpublished observation; [34]). For all experiments, wild type cells (conjugated with one of the plasmids containing promoter::*gfp* fusions for *luxC*, *vhp*, *vscP*, *luxS*, or *recA*) from an overnight culture were diluted about 10,000-fold into fresh medium, effectively returning the cells to an environment without extracellular AIs (time 0). Cultures were then grown until the end of the exponential or into the early stationary growth phase (12 or 15 hours). When a suitable cell number was reached (usually after 8 hours of growth = early exponential growth phase), cells were collected and analyzed by microscopy as described above. First, the average fluorescence per cell was determined for each of the five fusions (Figure 3A) as well as for the BB120 strain without any fusion to determine the autofluorescence of *V. harveyi* (about 100 a.u./cell background fluorescence) (data not shown). As expected the mean values of cells containing P_{luxS}::*gfp* or P_{recA}::*gfp* did not change significantly over time (Figure 3A). In contrast, the measurements revealed induction of *luxC* and *vhp*, and repression of *vscP* over time (Figure 3A). The *luxC* promoter was induced up to 100-fold (10.000 a.u./cell compared to 100 a.u./cell) during the exponential growth phase. The *vhp* promoter was maximally induced (40-fold) in the early stationary phase. Conversely, the *vscP* promoter was repressed 8-fold over the course of the exponential growth phase.

At the single cell level we found that *luxC* was induced in a subpopulation during the early exponential growth phase (Figure 3B). Over time more and more cells induced *luxC*, but a substantial fraction of the population (about 20%) did not activate the *luxC* promoter at all (Figure 3B).

Promoter activity of P_{vhp}::*gfp* was detected only in a minority of the population (20%) at early times (8 hours) (Figure 3C). The percentage of fluorescent cells increased slowly over the exponential growth phase. Therefore, we decided to analyze this promoter also during early stationary growth. By the time the population had entered the stationary growth phase (15 hours) 80% of the cells had initiated transcription of *vhp*. In the remaining 20% the promoter was silent.

Single cell analysis of the population containing P_{vscP}::*gfp* in the early exponential phase (8-9 hours) revealed two distinct subpopulations exhibiting high (about 50% of the population) and low fluorescence (Figure 3D). As the cell density further increased, the signal level in the former decreased, so that the two subpopulations eventually fused into one, which was characterized by low fluorescence. In parallel, we investigated the promoter activity of the two QS-independent genes *luxS* and *recA* at the single cell level. Although fluorescence was detectable in all cells of the strain containing the P_{luxS}::*gfp* fusion, we observed that a small fraction (< 10%) of the population expressed *luxS* at a constant low level (Figure 3E). The reason for this phenomenon is unknown. Moreover, all living cells of the strain containing the P_{recA}::*gfp* fusion showed comparable fluorescence intensity, which resulted in one peak independent of the growth phase of the population (Figure 3F).

Overall, these data show that all the AI-regulated promoters tested are expressed heterogeneously within expanding populations of *V. harveyi* (Figure 3). Strikingly, this heterogeneity of expression was observed for both AI-induced genes and an AI-repressed gene.

The deletion of *luxO* causes an AI-independent expression of all QS-regulated genes [13]. Thus, *V. harveyi* JAF78 (Δ*luxO*) is characterized by an all-bright phenotype [3]. We conjugated this strain with plasmids containing promoter::*gfp* fusions for *luxC*, *vhp*, or *vscP* and analyzed single cell expression at the mid-exponential growth phase. All living cells of JAF78 conjugated with either of the plasmids containing a P_{luxC}::*gfp* or a P_{vhp}::*gfp* fusion showed fluorescence, whereas no fluorescence was detectable in JAF78 conjugated with the plasmid encoding P_{vscP}::*gfp* (data not shown). Moreover, average intensities of the P_{luxC}::*gfp* and the P_{vhp}::*gfp* fusions were significantly higher and the standard deviation was lower in the JAF78 strain compared to the BB120 strain (Table 1). These data are consistent with the luminescence behavior of JAF78 versus BB120 cells at the single cell level [3]. These results indicate that heterogeneous promoter activity is dependent on AIs.

Simultaneous analysis of two AI-induced genes reveals division of labor

Next we analyzed the induction of two AI-induced genes in cells of the same reporter strain. For this study we used cells containing the P_{vhp}::*gfp* fusion and monitored the induction of both fluorescence and bioluminescence in 1,150 cells simultaneously. Cells were grown to the transition from exponential into early stationary growth to ensure that both genes are readily expressed (see Figure 3). Different types of response were found among cells in the same field of view. Some cells exhibited high levels of bioluminescence and medium or no fluorescence (Figure 4A-C, cyan circle). Cells expressing the converse pattern were also observed (Figure 4A-C, green circle), as were others that showed medium-intensity signals in both channels (Figure 4A-C, yellow circle). While the majority of bacteria simultaneously expressed both phenotypes at different levels, some of the population produced neither fluorescence nor bioluminescence (Figure 4A-C, red circle). Very

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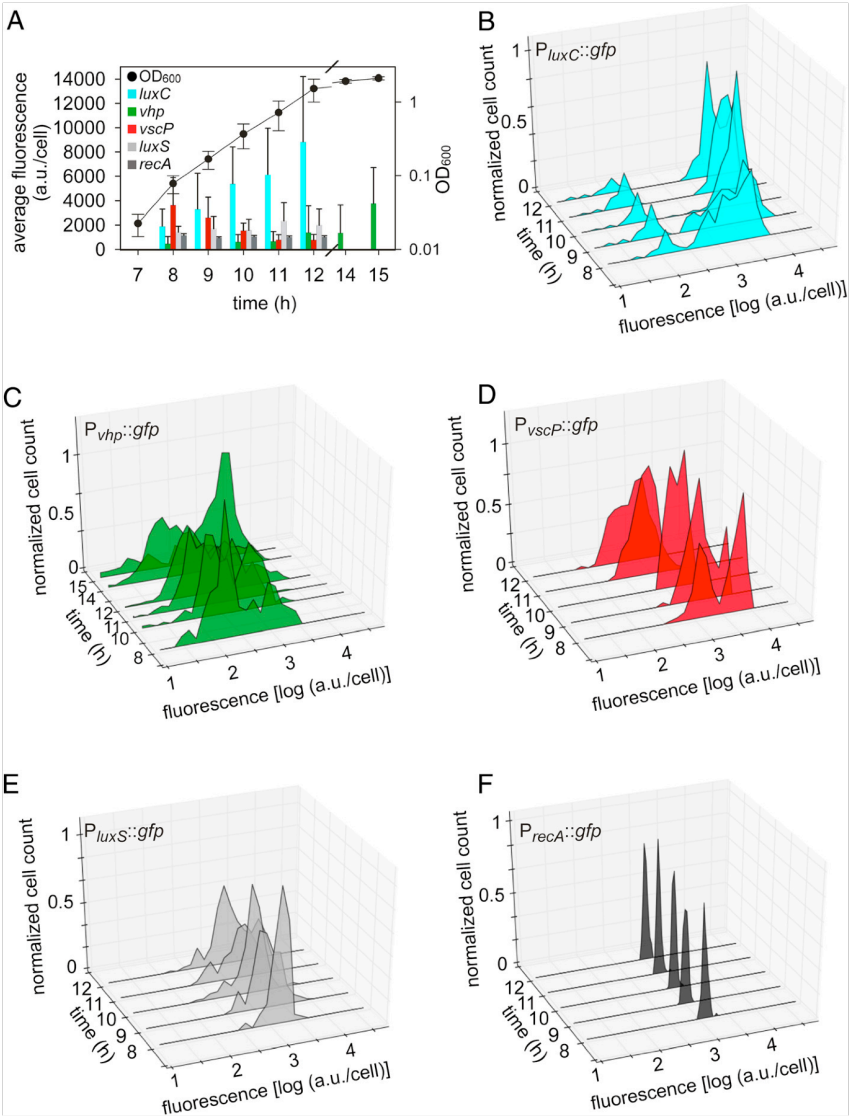

Figure 3 Growth-dependent analysis of the expression of AI-regulated genes at the single cell level. *V. harveyi* conjugants that carried one of the plasmids pCA2, pCA3, pCA4, pCA5, and pCA1 containing a promoter::*gfp* fusion driven by the *luxC* (blue), *vhp* (green), *vscP* (red), *luxS* (grey), or *recA* (dark grey) promoter, respectively, were cultivated, and at the indicated times the optical density (OD_{600}) was determined **(A)** and single cell analysis was performed **(B-F)**. At each time point the average fluorescence of the population was determined **(A)**. The activity of *luxC* **(B)**, *vhp* **(C)**, *vscP* **(D)**, *luxS* (**E**), and *recA* **(F)** promoters was followed in a growing population over time. Fluorescence levels were normalized for cell size and expressed in arbitrary units.

few cells were found to exhibit high-intensity signals in both channels.

To compare induction of bioluminescence and fluorescence (P_{vhp}::*gfp*), the intensities of each were calculated for every single living cell and evaluated in two histograms. Subsequently, cells were grouped in "no", "medium", or "high signal intensity". The borderline between the two peaks in each histogram (fluorescent or luminescent; similarly to Figure 3) was used to classify between "no intensity" and "bright intensity". Moreover, the bright cells were classified into "medium" and "high intensity". Therefore, the 0.9 quantile was chosen to distinguish between cells with truly high intensity (10%) and cells with medium intensity (90%). Based on these groups for bioluminescence and fluorescence, six types of intensity classes were defined (Figure 4D). Some of the cells (12.7%) showed no fluorescence and luminescence. Both medium fluorescence and luminescence were found in 32.4% of the cells. The

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Table 1 Characterization of the constitutive QS-active *V. harveyi* mutant JAF78 containing promoter::*gfp* reporter fusions

Promoter fusion	Average fluorescence [a.u./cell]		Standard deviation σ [a.u./cell] (%)	
	JAF78	BB120	JAF78	BB120
P_{luxC}::*gfp*	4490	3370	1347 (30)	3033 (90)
P_{vhp}::*gfp*	730	620	226 (31)	614 (99)

V. harveyi JAF78 (Δ*luxO*) cells were grown to the mid-exponential growth phase, analyzed at the single cell level as described in Figure 3, and compared with the wild type BB120.

majority of Vibrios (54.4%) showed an unequal behavior, such as high fluorescence and no luminescence and vice versa (3.0%), medium fluorescence and no luminescence and vice versa (42.5%), and high fluorescence and medium luminescence and vice versa (8.9%). Only 0.5% of the population exhibited both high fluorescence and high luminescence intensities. These data indicate that individual cells are essentially unable to induce the *lux* operon and the gene encoding the protease simultaneously at high levels. The heterogeneous response of AI-dependent genes gives rise to a division of labor in a genetically homogenous population of *V. harveyi*.

Discussion

Here we show that several AI-regulated genes are heterogeneously expressed in populations of *V. harveyi* wild type cells. We found that the promoters of *luxC*, *vscP* and *vhp* – genes that are important for bioluminescence, type III secretion and exoproteolysis, all show wide intercellular variation in their responses to AIs. In contrast, *luxS*, an AI-independent gene, is expressed in an essentially homogeneous manner. Homogenous promoter activities

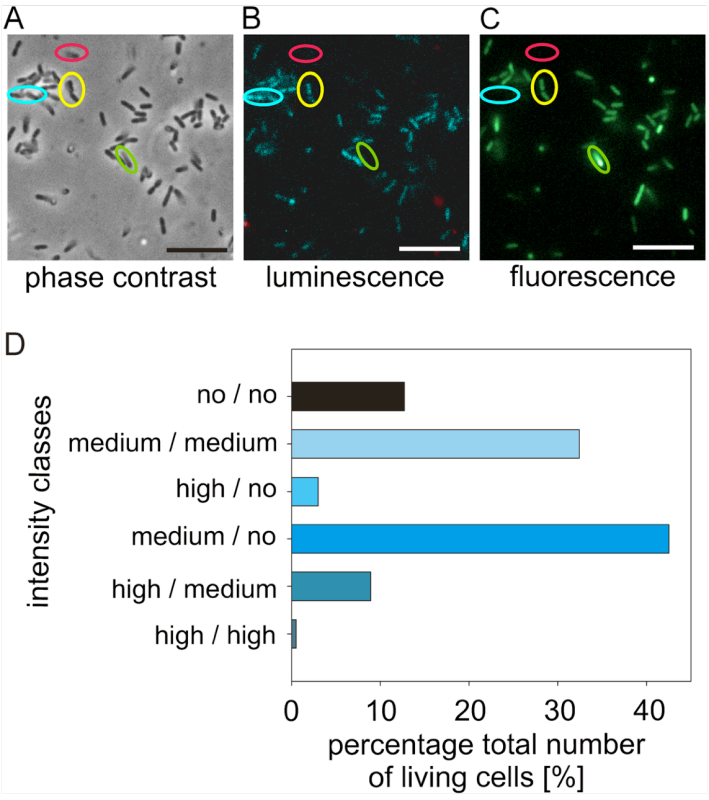

Figure 4 Simultaneous monitoring of AI-regulated bioluminescence and induction of P_{vhp}::*gfp*. The P_{vhp}::*gfp* reporter strain enables simultaneous measurement of two AI-dependent phenotypes, bioluminescence and exoproteolysis. Cells were cultivated, and single cell analysis was performed at the transition to the stationary phase. Panels **A-C** show a representative set of images of the same field viewed by phase contrast **(A)**, luminescence **(B)**, and fluorescence **(C)** microscopy. The yellow circle marks a cell with medium luminescence and fluorescence intensity. The blue circle indicates a cell with high luminescence intensity and no fluorescence. The green circle surrounds a cell with high fluorescence intensity and no luminescence. The red circle marks a dark cell (no fluorescence, no luminescence). The bar is 2.5 μm. Luminescence and fluorescence intensities (in a.u./cell) were quantitatively analyzed for 1,150 cells. For each channel the cells were grouped according to their signal intensity in no, medium, or high. (The separation in these groups is described in detail in the results part). Panel **D** shows the distribution of the various intensity classes plotted as percentage of the total number of cells analyzed.

for *luxC*, *vscP* and *vhp* were found after conjugation of *V. harveyi* mutant JAF78, which expresses QS-regulated genes in an AI-independent manner, with the corresponding plasmids. These findings extend our original observations on the heterogeneous induction of bioluminescence, the canonical readout of QS in *V. harveyi* [3].

Based on these results, we hypothesize that AIs act to drive phenotypic diversification in a clonal population. A heterogeneous response to AIs has also been described for the bioluminescent phenotype of individual *Aliivibrio fischeri* cells [35,36]. In addition, single cell analysis of *Listeria monocytogenes* has indicated that the Agr QS system induces heterogeneity within the population and does not primarily sense cell density [37]. In *Salmonella enterica* promoters that show a high level of phenotypic noise have been identified [38]. The genes concerned are involved in flagella biosynthesis or associated with virulence and host-pathogen interactions. Single cell analysis revealed heterogeneous expression of the cardinal virulence factor of *S. enterica*, the type III secretion system, which is crucial for host manipulation and elicitation of the disease [39]. The fraction of type III secretion-positive cells increased from < 10% to 60% during the late exponential growth phase. In *V. harveyi* we found a decrease from 60% to < 20% of cells that express *vscP*. Even though the regulation clearly differs, a fractionation of the population into producing and non-producing cells was found in both organisms. Proteases also play important roles in pathogenesis, e.g. in *Pseudomonas aeruginosa* [40], *Legionella pneumophila* [41], and *V. harveyi* [42]. Our results indicate a fractionation of the population into cells with and without exoproteolytic activity, suggesting an advantage for the whole population to produce 'public goods' only in a subpopulation.

Moreover, we simultaneously examined the expression of two AI-dependent phenotypes in one reporter strain. Based on the very good correlation between luminescence and fluorescence (P_{luxC}::*gfp* fusion) for the *lux* promoter (see Figure 2) we used bioluminescence (*lux* operon) and fluorescence (P_{vhp}::*gfp)* as read-outs. Nevertheless, it is worth mentioning that bioluminescence is the result of an enzymatic reaction, which might be affected by other factors. The strain was cultivated until the early stationary phase when both genes were readily expressed (Figure 3A). Only 32.4% of these cells were characterized by equal fluorescence and luminescence intensity, whereas 12.7% did neither induce fluorescence nor luminescence. These apparently non-responding cells might express other AI-regulated phenotypes. Surprisingly, very few cells (0.5% of the 1,150 cells examined) activated both *luxC* and *vhp* at high levels. In the majority of cells (54.4%), transcriptional levels of the two genes clearly differed. High-level induction of both of these AI-induced genes at the same time seems to be excluded in the wild type. Previous results with *V. harveyi* mutant JAF78 (AI-independent gene expression), indicated that all living cells were bright, but biofilm formation was significantly (2-fold) reduced compared to the wild type (70% bioluminescent cells). Moreover, the artificial increase of the AIs concentration within the wild type population resulted in the same phenotype (98% bioluminescent cells, 2-fold reduction in biofilm formation) [3]. Overall, these data suggest division of labor in AI-regulated processes in the non-differentiating bacterium *V. harveyi.* This conclusion is in line with earlier suggestions according to which AI-dependent gene regulation seems to support the evolution of cooperation among bacteria [43,44]. AI-regulated cooperation could be viewed as a superimposition of and interaction between two cooperative behaviors, namely a cooperative communication system that coordinates cooperative behavior to produce 'public goods', such as exoenzymes, exopolysaccharides, and siderophores.

Conclusions

Our results reveal heterogeneous expression of three AI-regulated genes in *V. harveyi.* Furthermore, simultaneous analysis of bioluminescence and exoproteolysis in single cells by transcriptional analysis of a corresponding promoter::*gfp* fusion provided evidence for a division of labor. Based on these results, it is suggested that AIs not only serve as indicators for cell density but also play a pivotal role in the diversification of the population, and the coordination of QS-regulated processes.

Methods

Bacterial strains and culture conditions

Strains and their genotypes are listed in Table 2. *V. harveyi* strains BB120 and JAF78 after conjugation with plasmids were used throughout this study. *Escherichia coli* BW29427 was used for conjugation and was cultivated in lysogenic broth (LB) [45] supplemented with diaminopimelic acid (1 mM) at 37°C with aeration. For conjugation, *V. harveyi* was grown in autoinducer bioassay (AB) medium [46] with aeration at 30°C. Biparental mating of *V. harveyi*, either BB120 or JAF78, and *E. coli* BW29427 was performed on agar plates (1.5% w/v) containing Luria marine (LM) medium (1% w/v tryptone, 2% w/v NaCl, 0.5% w/v yeast extract) supplemented with diaminiopimelic acid (1 mM) at 30°C. Fluorescent reporter strains were cultivated in LM medium supplemented with tetracycline (12 $\mu g^{*}mL^{-1}$) at 30°C with aeration.

Plasmid construction

DNA manipulations were performed using standard procedures [53,54].

Deoxyribonucleoside triphosphates, restriction endonucleases, alkaline phosphatase and T4 DNA ligase were obtained from New England BioLabs. Phusion DNA

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Table 2 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference
Escherichia coli BW29427	*thrB1004 pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD*)567 Δ*dapA1341::* [*erm pir (wt)*]	[47]
Vibrio harveyi BB120	wild type, ATCC BAA-1116 [reclassified as *Vibrio campbellii*]	[5,48]
Vibrio harveyi JAF78	Δ*luxO*-Cam^R	[13]
pLAFRII	cosmid vector, Tet^R	[49]
pBK-miniTn*7-gfp3*	mini-Tn*7* transposon delivery plasmid	[50]
pBAD24	pBR322 ori, Amp^R	[51]
pBAD24*gfp*	pBAD24 carrying *gfpmut3*	[52]
pBAD24*gfptet*^{R}	pBAD24 carrying *gfpmut3*, Tet^R	This work
pCA1	pBAD24 carrying P$_{recA}$::*gfpmut3*, Tet^R	This work
pCA2	pBAD24 carrying P$_{luxC}$::*gfpmut3*, Tet^R	This work
pCA3	pBAD24 carrying P$_{vhp}$::*gfpmut3*, Tet^R	This work
pCA4	pBAD24 carrying P$_{vscP}$::*gfpmut3*, Tet^R	This work
pCA5	pBAD24 carrying P$_{luxS}$::*gfpmut3*, Tet^R	This work

polymerase (Finnzymes) and Taq polymerase (Roche) were used for PCR cloning reactions and control PCRs, respectively. DNA extraction and purification kits were provided by Südlabor (for plasmids) and by MO BIO Laboratories (for genomic DNA). Primer sequences are available upon request. Plasmids pCA2, pCA3, and pCA5 were constructed using two-step PCRs [55] to link 500 bp of the upstream flanking regions of the corresponding genes (including the native promoter) with *gfptet*^{R}. Plasmids pCA1 and pCA4 were constructed by amplification of *gfptet*^{R} and 500 bp of the upstream regions of *vscP* and *recA* (including the native promoter), and generating a PstI recognition site between the two amplificates. EcoRI (or XbaI) and HindIII (or SphI) recognition sites were introduced upstream and downstream of the constructs, respectively. Upstream flanking regions were amplified from the genomic DNA of *V. harveyi* BB120. *gfptet*^{R} was amplified from pBAD24*gfptet*^{R} (constructed for this work by fusing the promoter-less *gfpmut3* [56] from pBAD24*gfp* [52] to *tet*^{R} with a constitutive promoter amplified from pLAFRII [57], in pBAD24). In all plasmids the start codon of *gfp* replaced the start codon of the original gene. All PCR fragments were restricted with suitable restriction enzymes and ligated into the similarly treated vector pBAD24. Plasmid structures were verified by sequencing prior to transformation of *E. coli* BW29427. The transformants were then used for mating.

Construction of fluorescent *Vibrio harveyi* strains

To introduce the plasmids containing promoter::*gfp* fusions driven by the *recA*, *luxC*, *vscP*, *luxS* and *vhp* promoters into *V. harveyi*, a modified protocol for conjugation of *V. harveyi* [7] based on biparental filter mating was used. Mating was achieved by mixing stationary phase cultures (diluted to OD_{600} = 0.6) of *E. coli* BW29427, carrying the *tra* genes (for conjugation) on the genome and one of the donor plasmids pCA1, pCA2, pCA3, pCA4, and pCA5 with the recipient *V. harveyi* BB120 (or JAF78) at a ratio of 1:4 (donor to recipient). The mixtures (500 μl volume) were incubated on micropore (45 μm) filters (Millipore) on LM agar plates supplemented with diaminopimelic acid (1 mM) at 30°C for three days. The mixed cultures were then resuspended in 1 ml of LM medium supplemented with tetracycline (12 μg*mL^{-1}) and incubated at 30°C with aeration for 1 h. Selection of transconjugant *V. harveyi* cells was carried out on LM plates containing tetracycline (12 μg*mL^{-1}) and polymyxin B (10 μg*mL^{-1}) at 30°C overnight. Polymyxin B was added to prevent growth of *E. coli* cells.

A chromosomal inserted *gfp* fusion was generated in strain BB120 using the mini-Tn*7* transposon system (using plasmid pBK-miniTn*7-gfp3*), which leads to an insertion downstream of *glmS* (encoding a glucosamine-6-phosphate activated ribozyme) via homologous recombination [50]. The insertion was verified by control PCR and subsequent sequencing.

Single cell fluorescence and bioluminescence microscopy

To measure promoter activity of P$_{luxC}$::*gfp*, P$_{luxS}$::*gfp*, P$_{vscP}$::*gfp*, P$_{vhp}$::*gfp*, and P$_{recA}$::*gfp* in individual cells, *V. harveyi* BB120 (or JAF78) cells conjugated with one of the donor plasmids were cultivated in LM medium supplemented with tetracycline (12 μg*mL^{-1}) in Erlenmeyer flasks on a rotary shaker at 30°C overnight. Cultures were then diluted 10,000-fold in LM supplemented with tetracycline and incubated on a rotary shaker (to ensure sufficient aeration as well as homogenous AI distribution) at 30°C. At the indicated times about 10^5 cells were collected by centrifugation (5,000 × g for 10 min). At least 1 mL of the cell-free culture fluid was saved, air-saturated and stored on ice until use. The cell pellet was resuspended in a small volume of the corresponding culture fluid. Propidium iodide (5 mM, dissolved in phosphate-buffered saline) was added to 20 μL of this cell suspension to stain dead cells (red fluorescence), and the suspension was immediately transferred onto a coverslip and incubated in the dark for 20 min to allow cells to adhere. All coverslips were pretreated with poly L-lysine (0.05 g*L^{-1}) to fix the cells on the surface. Subsequently, cells were washed twice with the corresponding air-saturated culture fluid directly on the coverslip to remove non-adherent cells. Phase contrast and fluorescence images were taken at room temperature using a customized inverted Leica DMI 6000 B microscope, an oil-immersion objective and a high-sensitivity iXON CCD camera (Andor). Fluorescence microscopy was performed using the bandpass filters BP546/12 (red) and

BP470/40 (green) and the emission filters 605/75 (red) and 525/50 (green). Luminescent cells were identified by bioluminescence microscopy without any filter in a Pecon flow chamber to ensure sufficient oxygen supply [3]. The exposure time for imaging of luminescent cells with the cooled (-80°C) CCD camera was set to 240 s. Phase-contrast, bioluminescence and/or fluorescence images were obtained from the same fields of view.

Single cell analysis

Images were analyzed using ImageJ 1.37c (National Institute of Health http://rsb.info.nih.gov/ij). A screen depicting the contours of the cells was created from the phase contrast image using the self-programmed PlugIn CellEvaluator (Prof. Dr. J. Rädler, LMU Munich). This screen was superimposed on the background-corrected fluorescence and bioluminescence images. Intensities were determined for each cell and normalized by cell size. The correlation coefficient r is defined as the covariance of two variables (here fluorescence and luminescence) divided by the product of their standard deviations. A value of $|r| = 1$ indicates 100% correlation. The p-value is a measure of the probability that the correlation is due to chance. Time-lapse histograms were generated using Matplotlib (http://matplotlib.sourceforge.net).

Abbreviations

AI: Autoinducer; QS: Quorum sensing; a.u: Arbitrary units.

Competing interests

The authors declare no competing interests.

Authors' contributions

CA and KJ developed the concept of the study and wrote the paper. CA and US constructed all plasmids used in this study, conjugated all strains, and carried out fluorescence microscopy. CA performed simultaneous fluorescence and luminescence microscopy. CA and KJ analyzed all data and created all figures. All authors read and approved the final manuscript.

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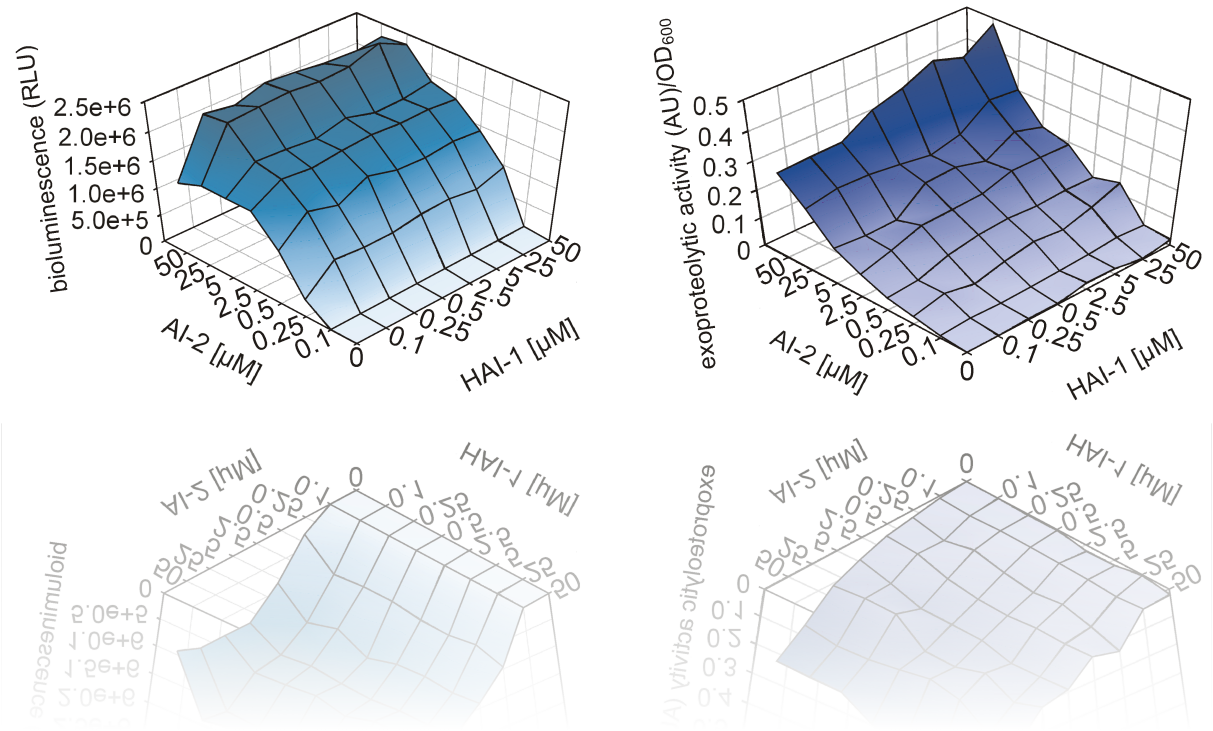
4 Autoinducers act as biological timers in *Vibrio harveyi*



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Autoinducers Act as Biological Timers in *Vibrio harveyi*

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Abstract

Quorum sensing regulates cell density-dependent phenotypes and involves the synthesis, excretion and detection of so-called autoinducers. *Vibrio harveyi* strain ATCC BAA-1116 (recently reclassified as *Vibrio campbellii*), one of the best-characterized model organisms for the study of quorum sensing, produces and responds to three autoinducers. HAI-1, AI-2 and CAI-1 are recognized by different receptors, but all information is channeled into the same signaling cascade, which controls a specific set of genes. Here we examine temporal variations of availability and concentration of the three autoinducers in *V. harveyi*, and monitor the phenotypes they regulate, from the early exponential to the stationary growth phase in liquid culture. Specifically, the exponential growth phase is characterized by an increase in AI-2 and the induction of bioluminescence, while HAI-1 and CAI-1 are undetectable prior to the late exponential growth phase. CAI-1 activity reaches its maximum upon entry into stationary phase, while molar concentrations of AI-2 and HAI-1 become approximately equal. Similarly, autoinducer-dependent exoproteolytic activity increases at the transition into stationary phase. These findings are reflected in temporal alterations in expression of the *luxR* gene that encodes the master regulator LuxR, and of four autoinducer-regulated genes during growth. Moreover, *in vitro* phosphorylation assays reveal a tight correlation between the HAI-1/AI-2 ratio as input and levels of receptor-mediated phosphorylation of LuxU as output. Our study supports a model in which the combinations of autoinducers available, rather than cell density *per se*, determine the timing of various processes in *V. harveyi* populations.

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Introduction

The term "quorum sensing", introduced by Peter Greenberg in 1994 [1], refers to a concept according to which bacteria constantly produce and excrete low-molecular-weight signaling molecules, called autoinducers (AIs), into the medium. As cell numbers increase, so does the concentration of AIs. At a defined threshold AI concentration, the population expresses a synchronized, AI-specific response – usually a phenotype, such as virulence, light production or biofilm formation, which is more effective when deployed by a group of cells rather than a single bacterium.

Vibrio harveyi strain ATCC BAA-1116 (recently reclassified as *Vibrio campbelli* [2,3]), one of the best studied model organisms for quorum sensing, produces and responds to three different classes of AIs: the species-specific HAI-1 [N-(3-hydroxybutyryl)-homoserine lactone], AI-2 (furanosyl borate diester), which is synthesized by many bacterial species, and the genus-specific CAI-1 [(*Z*)-3-aminoundec-2-en-4-one (Ea-C8-CAI-1)] [4–7]. These three AIs are recognized by the three membrane-bound hybrid sensor kinases LuxN, LuxQ (in cooperation with the periplasmic AI-2-binding protein LuxP) and CqsS respectively (Fig. 1) [5,6,8–10]. Information on AI concentrations is transduced by the sensor kinases via phosphorelay to the histidine phosphotransfer protein LuxU and further to the response regulator LuxO [11]. Recently, a new circuit consisting of the soluble histidine kinase HqsK and the NO-sensing H-NOX was reported, which feeds its information into the network at the level of LuxU [12]. At low cell densities (low AI concentration) phosphorylated LuxO activates the transcription of five small regulatory RNAs; four of these (Qrr1-4), together with the RNA chaperone Hfq, act to destabilize the *luxR* transcript [13]. At high cell densities (high AI concentration) LuxO is dephosphorylated and LuxR is produced [11]. A direct inhibitory effect of HAI-1 on the kinase activity of LuxN has already been demonstrated [14]. LuxR in turn activates and represses large numbers of genes [15]. At high AI concentrations, genes involved in bioluminescence [16], biofilm formation [17] and extracellular proteolysis [18] are induced, while genes for type III secretion [19] and siderophore production [20] are repressed.

Several feedback loops are known to regulate the content of LuxR in the cells. These involve autorepression of *luxR* [21], activation of *qrr2-4* transcription by LuxR [22], autorepression of *luxO* and repression of *luxO* translation by Qrr sRNAs [23], repression by AphA, a recently described antagonist of LuxR [24], and down-regulation of *luxMN* translation by Qrr sRNAs [25].

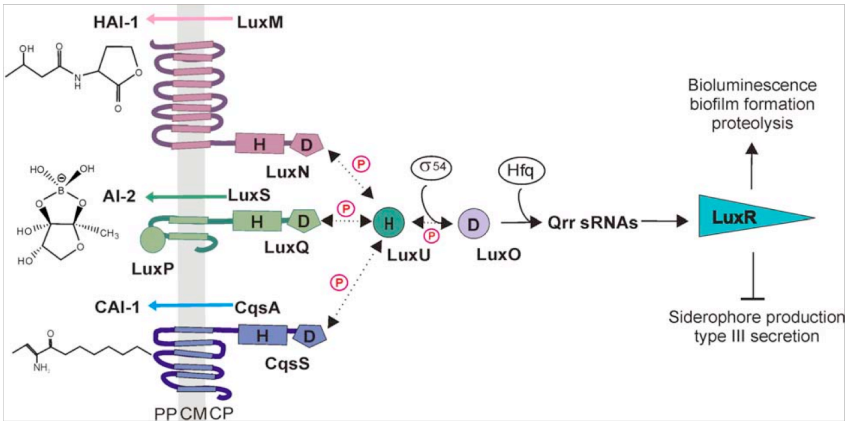

Figure 1. The quorum sensing circuit in *Vibrio harveyi*. In *V. harveyi* the three autoinducers HAI-1, AI-2 and CAI-1 are synthesized by the synthases LuxM, LuxS and CqsA. The cognate hybrid sensor kinases LuxN, LuxQ together with LuxP, and CqsS detect each autoinducer and effectively measure their concentrations: the higher the autoinducer concentration, the lower is the autophosphorylation activity of the hybrid kinases. The phosphoryl groups are transferred via phosphorelay including the histidine phosphotransfer protein LuxU to the σ^{54}-dependent transcriptional activator LuxO. Phosphorylated LuxO in turn activates transcription of five regulatory sRNAs, four of which (Qrr1-4) are active. Together with the RNA chaperone Hfq, these sRNAs destabilize the transcript that codes for the master regulator LuxR. The LuxR content is further regulated by additional feedback regulation (see text for details). Autoinducers activate genes required for bioluminescence, biofilm formation and proteolysis and repress genes involved in type III secretion and siderophore production. Dashed lines indicate phosphotransfer reactions. *H* (histidine) and *D* (aspartate) denote the phosphorylation sites. *CM*, cytoplasmic membrane; *CP*, cytoplasm; *PP*, periplasm.
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In spite of detailed knowledge of the complex signaling cascade, it is still unclear why *V. harveyi* produces three AIs but channels all information into a single signaling cascade. Moreover, we have previously shown that extracellular concentrations of AIs correlate with the degree of cell-to-cell variance in the expression of bioluminescence [17]. We have therefore examined the pattern of accumulation of the three AIs in a growing culture of the wild type strain, from the early exponential (10^6 cells*mL^{-1}, $OD_{600} = 0.001$) to the stationary growth phase ($2*10^9$ cells*mL^{-1}, $OD_{600} = 2$). It should be noted here that, in previous studies, the expression of AI-regulated genes has been analyzed predominantly by studying their responses to exogenously provided AIs [18,26]. We have also monitored the time course of *luxR* transcript levels and diverse AI-regulated processes. Our data suggest a model in which the precise composition of the AIs present in certain growth phases, rather than the cell density *per se*, is the more important influence on AI-regulated gene expression. This model is supported by *in vitro* phosphorylation studies.

Materials and Methods

Strains and growth conditions

The *V. harveyi* strains listed in Table 1 were cultivated in autoinducer bioassay (AB) medium [27], and incubated aerobically on a rotary shaker at 30°C. When necessary, the medium was supplemented with chloramphenicol (33 μg*mL^{-1}). Overnight cultures were diluted 5,000-fold into fresh AB medium and grown for a further 20 h. Samples were taken every hour, and cells were removed by centrifugation at 5,000× g for 15 min. The culture fluids were then filtered (0.20 μm) and stored at −20°C or used immediately. To measure the cell density of a *V. harveyi* culture the optical density at 600 nm was determined for values larger than 0.01. For cultures with an $OD_{600}<0.01$ the number of colony-forming units was determined directly, and the optical density was calculated ($OD_{600} = 1$ corresponds to 10^9 cells*mL^{-1}).

Escherichia coli strains listed in Table 1 were grown in lysogenic broth (LB) [28] or KML medium [1% (w/v) tryptone, 1% (w/v) KCl, 0.5% (w/v) yeast extract] and incubated aerobically in Erlenmeyer flasks on a rotary shaker at 37°C. When necessary, the medium was supplemented with ampicillin (100 μg*mL^{-1}) or chloramphenicol (33 μg*mL^{-1}).

Cloning of luxN and luxQ

For overexpression of *luxN* and *luxQ* in *E. coli* TKR2000 each gene was inserted into plasmid pKK223-3, in which expression is under control of the *tac* promoter. To use the natural Shine Dalgarno box of *kdpD*, plasmid pPV5-1 (*kdpD* in pKK223-3 [29]) was used, and *kdpD* was replaced by *luxN* or *luxQ*. For ease of cloning, a KpnI site was first inserted downstream of the start codon of *kdpD* by two-step PCR [30] resulting in plasmid pPV5-10. *luxN* and *luxQ* were amplified from genomic DNA by PCR using the primer pairs LuxN/KpnIsense and LuxN/HindIIIantisense, and LuxQ/KpnIsense and LuxQ/HindIIIantisense. The PCR fragments were restricted with KpnI and HindIII and cloned into plasmid pPV5-10 to obtain plasmids pNKN and pNKQ. Sequences of the primers used are available on request.

Preparation of inverted membrane vesicles

E. coli TKR2000 was transformed with plasmids pNKN and pNKQ, encoding wild type LuxN and LuxQ. Each protein carried a His-tag at the C-terminus, attached either directly (LuxQ) or via a two-amino acid linker (LeuGln, LuxN). Inside-out membrane vesicles were prepared as described [14].

Heterologous production of LuxP and LuxU

LuxP was produced in and purified from *E. coli* MDAI-2 transformed with the plasmid pGEX_LuxP as described before [10]. LuxU was produced and purified exactly as described before, using *E. coli* JM109 transformed with plasmid pQE30LuxU-6His [14]. All proteins were stored at −80°C prior to use.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Genotype or description	Reference
V. harveyi BB120	wild type ATCC BAA-1116	[64]
V. harveyi MM77	*luxM*::Tn*5*, *luxS*::Cm^r	[18]
V. harveyi JAF78	Δ*luxO*::Cm^r	[11]
V. harveyi JAF548	*luxO*-D47E	[11]
V. harveyi JMH634	Δ*luxM*, Δ*luxS*, *cqsA*::*Cm*^{r}	[8]
V. harveyi JMH626	Δ*luxN*, *luxQ*::Tn5, *cqsA*::*Cm*^{r}	[8]
V. cholerae MM920	Δ*cqsA*, Δ*luxQ*, pBB1	[33]
E. coli TKR2000	Δ*kdpFABCDE thi rha lacZ nagA trkA405 trkD1 atp706*	[65]
E. coli MDAI-2	*luxS*::Tet^r-derivative of *E. coli* W3110	[66]
E. coli JM109	*recA1 endA1 gyrA96 traD36 thi hsdR17 supE44* λ^- *relA1* Δ*(lac-proAB)/F′ proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15	[67]
pPV5-1	*kdpD* in pKK223-3	[29]
pPV5-10	pPV5-1 with KpnI site after the start codon of *kdp*	This work
pNKN	*luxN* in pPV5-10	This work
pNKQ	*luxQ* in pPV5-10	This work
pGEX_LuxP	*luxP* in pGEX-4T1	[10]
pQE30LuxU-6His	*luxU* in pQE30	[14]
pQE30LuxS-6His	*luxS* in pQE30	[41]
pQE30Pfs-6His	*pfs* in pQE30	[41]
pTS-6	*cqsA* in pGEM-T-Easy	[34]

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Analytical procedures

Protein concentrations were determined by the method of Peterson [31] using bovine serum albumin as standard. Proteins were fractionated by SDS-PAGE [32]. His-tagged Lux proteins on immunoblots were labeled with mouse monoclonal antibodies directed against the His-tag (Qiagen) and detected by incubation with alkaline phosphatase-conjugated anti-mouse IgG (GE Healthcare) according to the manufacturer's instructions. Quantitative Western blots were scanned with 300 dpi resolution in 256 gray scales and imported as TIFF files into ImageQuant 5.0 (GE Healthcare). The amount of Lux proteins associated with membrane vesicles was quantified by comparison with the total amount of purified His-tagged LuxN.

Determination of autoinducer concentrations in cell-free culture medium

HAI-1 was quantified by UPLC using an Acquity UPLC System with a 2996 PDA detector controlled by Empower software (Waters). The system was equipped with an Acquity 2.1×100 mm BEH C18 column packed with 1.7-µm particles (Waters), which was maintained at a constant temperature of 60°C. The Sample Manager was kept at 27°C. Aliquots (5 µl) of sample were injected via a partial loop with needle overfill, and all samples were analyzed three times. Water (Biosolve) containing 3% acetonitrile (Biosolve) served as the mobile phase, and isocratic elution was applied at a flow rate of 0.9 mL*min^{-1} causing a back-pressure of 770 bar. Detection was performed at 195 nm with a scan rate of 20 Hz. The analysis time for each injection was set to 3 min, and all sample constituents were eluted from the column. The retention time for HAI-1 (0.579 min) and the UV-Vis spectra of the peak provided the criteria for identification of the compound and assessment of its purity. A standard solution of HAI-1 was used for calibration and quantification of the analyte.

To determine the concentration of AI-2 in culture fluids, LuxP-GST (2.5 mg*mL^{-1}) was added, and the mixture was incubated at 30°C for 30 min. The LuxP-AI-2 complex was then separated from the culture fluid by centrifugation of the sample through a NMWL filter (Millipore) with a 30,000-dalton cut-off, so that the protein-AI-2 complex was retained on the filter. To dissociate the complex, the membrane was washed in water, and the extract was kept at 50°C for 10 min and filtered again. The filtrate containing the AI-2 molecules was subsequently used in a bioluminescence assay with *V. harveyi* MM77 as reporter strain. To obtain a calibration curve, standard solutions of synthetic AI-2 (0 to 50 µM) were tested in the reporter assay with *V. harveyi* MM77 (see below). The fitted lines for the HAI-1 and AI-2 concentrations presented in Figure 2 were generated using the following equations:

HAI-1:

$$f = 10.8/\left(1 + e^{-\frac{x-10.9}{1.2}}\right).$$

AI-2: $f = 13.5/\left(1 + e^{-\frac{x-6.5}{0.6}}\right)$ for 1 to 9 h and $f = 9.8 + 858.8e^{-0.6x}$ for 9 to 20 h.

CAI-1 levels in cell-free cultures fluids of wild type *V. harveyi* were determined using the *V. cholerae* reporter strain MM920 [33,34] or the *V. harveyi* reporter strain JMH626 in a bioluminescence assay, incubating a fresh diluted culture of the reporter strain with cell-free culture fluids [50% (v/v)]. In parallel, CAI-1 was analyzed by GC-TOF-MS. Metabolites in culture fluids prepared as described above were chemically modified by sequential methoxyamination and trimethylsilylation, as described earlier [35,36]. Gas chromatography coupled to electron impact ionization-time of flight-mass spectrometry was performed using an Agilent 6890N24 gas chromatograph coupled to a Pegasus III time-of-flight mass spectrometer (LECO, St. Joseph, USA).

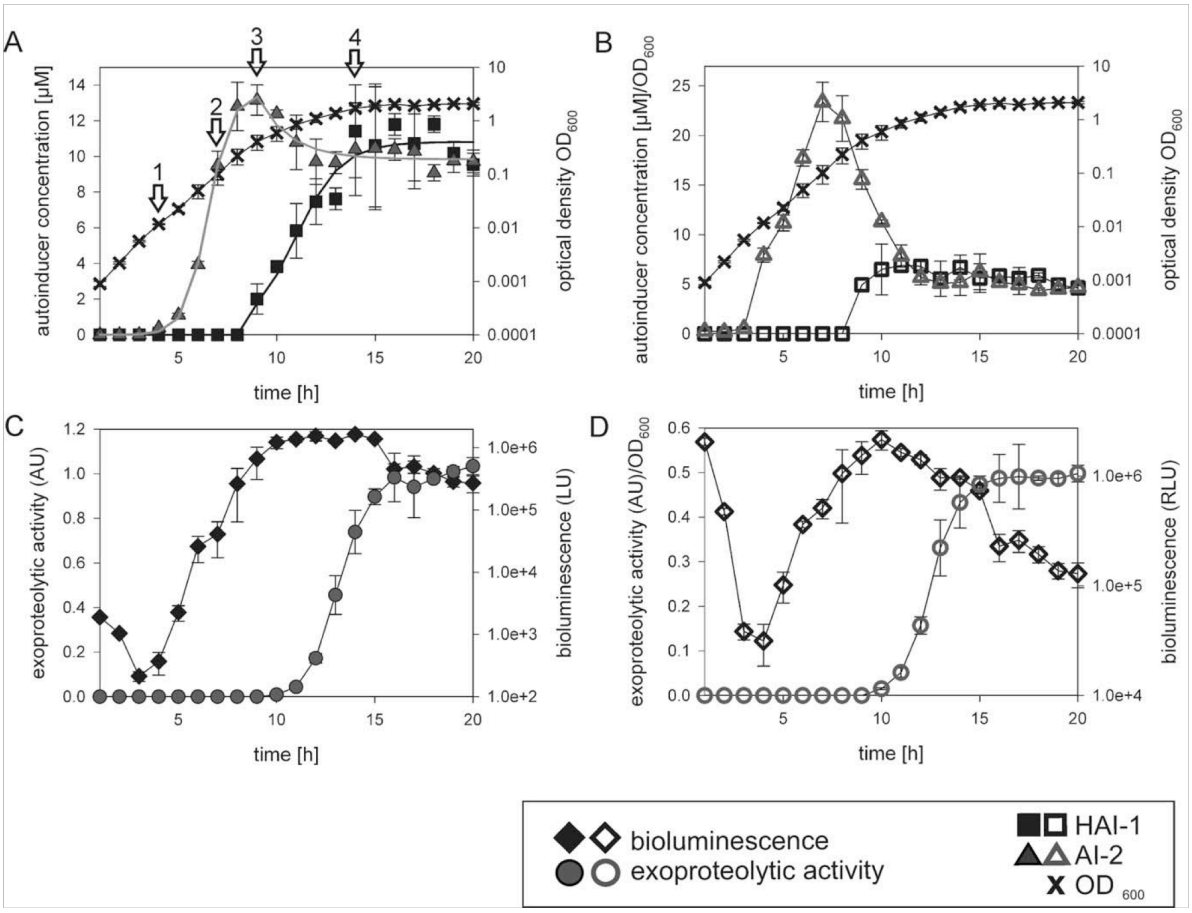

Figure 2. Time course of HAI-1 and AI-2 production (A, C), bioluminescence and exoproteolytic activity (B, D) during growth of *V. harveyi*. Cells of an overnight culture of *V. harveyi* BB120 were diluted 5,000-fold in fresh AB medium and cultivated aerobically at 30°C. Samples were taken at the times indicated and autoinducer concentrations in the medium, bioluminescence levels and exoproteolytic activity were determined. (A, B) Extracellular HAI-1 concentrations were determined by UPLC (black squares). AI-2 was captured with the binding protein LuxP, and quantified by bioassay (gray triangles). In parallel, the CFU and the optical density (OD_{600}, black crosses) were determined. Closed symbols (A) indicate the extracellular concentrations of the autoinducers. Open symbols (B) indicate autoinducer concentrations normalized relative to the OD_{600} value. The arrows (A) mark the time points chosen for transcriptional analysis (see Fig. 6). (C, D) The same samples were analyzed for bioluminescence level (light units, LU) and exoproteolytic activity (AU). Closed symbols (C) indicate bioluminescence levels (black diamonds) and exoproteolytic activity (gray circles) as absolute values; open symbols (D) are normalized to the corresponding optical density. All experiments were performed in triplicate and error bars indicate standard deviations of the mean.
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Chromatograms were acquired with CHROMATOF software 1.00, Pegasus driver 1.61 (Leco; http://www.leco.de). Selective ion traces and peak heights were extracted from the NetCDF CHROMATOF export, and processed using the TagFinder software [37]. Compounds that accumulated (relative to their levels in the sample taken after 7 h of cultivation) were filtered according to significance (p) using Students t-test and the Kruskal-Wallis test. The mass spectrum of modified CAI-1 was generated under manual supervision by automated deconvolution (CHROMATOF software 1.00). Replicate mass spectra and retention indices [38] were uploaded to the Golm Metabolome Database, http://gmd.mpimp-golm.mpg.de [39,40]. Available compound information may be retrieved from http://gmd.mpimp-golm.mpg.de/search.aspx using the "A" identifier code (see legend to Fig. 3). The fitted line for the CAI-1 concentration presented in Figure 3 was generated using the following equation: $f = 118.7 + 537.4/\left(1 + e^{-\frac{x-12.6}{0.7}}\right)$.

Synthetic autoinducers

HAI-1 (D- and L-isomers) was purchased from the University of Nottingham and dissolved in a minimal volume of acetonitrile [10% (v/v)], diluted with water to a concentration of 100 mM and stored at −20°C. DPD, the precursor of AI-2, was synthesized *in vitro* using S-adenosyl-homocysteine (Sigma-Aldrich) and the enzymes LuxS and Pfs, followed by purification over boric acid resin [41]. LuxS and Pfs were produced heterologously in *E. coli* JM109 transformed with plasmid pQE30LuxS-6His or pQE30Pfs-6His, respectively, and purified as described before [41,42]. Purity and yield of AI-2 were indirectly determined as described [43] using the method of Ellman [44]. The biological activities of both

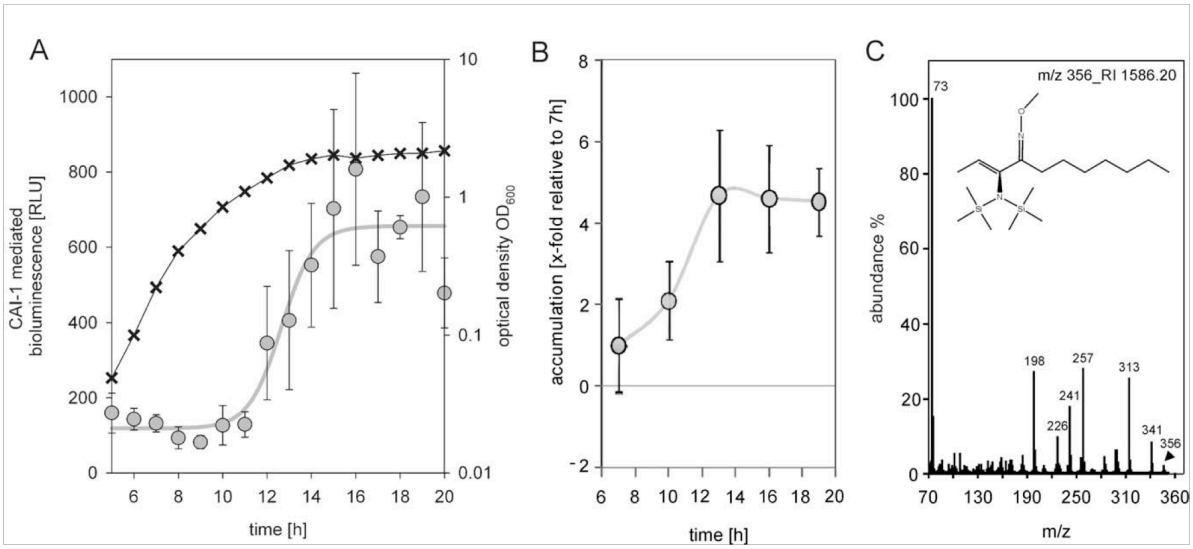

Figure 3. Alterations in CAI-1 activity during growth of *V. harveyi*. (A) CAI-1 activity was determined in cell-free culture fluids (the same samples as described in Fig. 2) using a bioassay with *V. cholerae* MM920 as reporter strain. Levels of CAI-1 mediated bioluminescence are indicated by light gray dots. A curve is presented to guide the eye. The optical density (OD_{600}) is plotted as crosses. All experiments were performed at least in triplicate, and error bars indicate standard deviations of the mean. (B, C) Unbiased GC-TOF-MS profiling was used to identify signaling molecules that accumulated in the medium after 7 h of growth. (B) Single-ion responses with defined retention indices (RI) close to that expected for Ea-C8-CAI-1 were tested for significant increases between 7 h and 19 h of cultivation, $p<1.0\ 10^{-4}$. The data were presented as x-fold accumulation in comparison to the 7 h time point. The replicate mass spectrum and respective retention index may be retrieved from the Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de/) using the identifier code A158016 (m/z 356_RI 1586.20). (C) Representative mass spectrum of candidate signaling molecules possibly representing Ea-C8-CAI-1. The mass of compound A158016 corresponds to Ea-C8-CAI-1 (arrow), which was modified with trimethylsilylated methoxyamine. Its chemical structure is shown. All experiments were performed at least in triplicate. Error bars indicate standard deviations of the mean. Time courses were interpolated by smoothed lines using MS-EXCEL software.
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AIs were determined using the bioluminescence based reporter assay [45] and *in vitro* phosphorylation experiments with LuxN/LuxQ and LuxU.

Bioluminescence assay

Luminescence produced by *V. harveyi* strains was determined in microtiter plates in a Centro LB960 (Berthold Technology) for 0.1 s, and data are reported as light units (LU) or relative light units (RLU) [counts*s^{-1}] per OD_{600} unit. All measured data were below the saturation range of the instrument ($2.2*10^6$ LU). To determine the dose-dependent effect of HAI-1 or AI-2, strain MM77 (*luxM*::Tn*5 luxS*::Cm^r) was used as reporter. Overnight cultures of strain MM77 were diluted 1:100 in AB medium containing culture fluids [50% (v/v)] or various concentrations of synthetic HAI-1 and AI-2. Cells were grown until the mid-exponential growth phase and analyzed as described above.

Protease assay

Exoproteolytic activity of *V. harveyi* strains was measured by incubating hide powder azure (Sigma-Aldrich) in phosphate-buffered saline (PBS, pH 7.2) with cell-free culture fluids at 37°C. The reaction was stopped with trichloracetic acid [6.7% (v/v)] after 2 h, and the absorbance at 600 nm was measured [46]. The activity is expressed as the difference between initial and final absorption after 2 h (AU). The assay was adapted to microtiter plates using 0.5 mg hide powder azure, 100 µl PBS and 100 µl culture fluid per well. For standardization, protease K (Sigma-Aldrich) was used. When indicated the metalloprotease inhibitor EDTA (5 mM) and the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (1 mM) were added prior to incubation [47].

Kinetic analysis of the transcriptional response of AI-induced/repressed genes by qRT-PCR

V. harveyi strains BB120 and JMH634 were cultivated as described above. Samples were withdrawn, and RNA was isolated as described before [48]. 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 *luxA*, *luxR*, *vhpA*, *vopN*, *vscP* and *recA*. Duplicate samples from three independent biological experiments were used, and the C_T 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 C_T method [49].

Phosphorylation and dephosphorylation assays

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 25°C. The sensor kinases LuxQ and LuxN were tested as full-length membrane integrated proteins in inverted membrane vesicles. To incorporate LuxP into LuxQ-bearing membrane vesicles, vesicles were subjected to three cycles of freezing and thawing.

A typical reaction mixture for a phosphorylation assay (total volume 150 µl) contained 7.5 mg*mL^{-1} (LuxQ) or 5 mg*mL^{-1} (LuxN) membrane proteins, and 0.36 mg*mL^{-1} purified LuxU. LuxP was added at a concentration of 0.96 mg*mL^{-1}. For experiments involving both kinases, the concentration of each kinase was halved. The reaction was started by addition of radiolabeled Mg^{2+}-ATP, typically 100 µM [γ-^{32}P] ATP (specific radioactivity of 0.94 Ci*mmol^{-1}; Perkin Elmer) and 110 µM $MgCl_2$. At various times thereafter, the reaction was terminated by the addition of SDS loading buffer [32], followed by separation of the proteins on SDS-polyacrylamide gels. Gels were dried at 80°C on filter paper, exposed to a phosphoscreen for at least 24 h, and subsequently scanned using a PhosphorImagerSI (GE Healthcare). Different dilutions of [γ-^{32}P] ATP were used to generate a calibration curve for quantification of the signal intensities of phosphorylated proteins using ImageQuant software (Molecular Dynamics V5.0; GE Healthcare). All enzymatic activities were calculated as mean values of at least three independent experiments. The gels shown are representative of each set of experiments.

For dephosphorylation assays LuxU was first phosphorylated using LuxN. In this case, the reaction mixture contained twice the usual amounts of LuxN and LuxU. After 10 min of incubation, membrane vesicles were removed by centrifugation (100,000 x *g*, 15 min, 4°C), and ATP was removed by gel filtration (Sephadex G25 columns, GE Healthcare). Dephosphorylation of phosphorylated LuxU (0.18 mg*mL^{-1}) was then initiated by the addition of 110 µM $MgCl_2$ and membrane vesicles containing LuxQ (3.75 mg*mL^{-1}). Phosphorylated LuxU was quantified as described above.

Results

Patterns of accumulation of the three autoinducers change during growth of V. harveyi

The extracellular concentrations of the three AIs were determined in a wild type population of *V. harveyi* (strain BB120, now *V. campbellii* ATCC BAA-1116) grown in liquid AB medium at regular intervals (Fig. 2). To start the experiment, a dense inoculum from an overnight culture was diluted 1:5,000 into fresh medium at time 0. The concentration of the furanosylborate diester AI-2 increased rapidly, whereas the acyl-homoserine lactone HAI-1 remained undetectable for the first 8 h (Fig. 2A; the detection limit for HAI-1 using UPLC was 0.5 µM). The concentration of AI-2 reached a maximum of 13.2±0.8 µM near the end of the exponential growth phase, decreased thereafter to about 10 µM (9.6±0.6 µM) and remained constant at this concentration during the stationary phase. The concentration of HAI-1 increased continuously after 9 h of cultivation (late exponential growth phase) and reached a maximal concentration of about 10 µM (10.6±2.7 µM) in the stationary phase. Thus, during the early and mid-exponential growth phases only AI-2 is present in detectable amounts, in the late exponential growth phase AI-2 predominates over HAI-1, and the stationary phase is characterized by essentially equal molar concentrations of HAI-1 and AI-2. Note that most reports on quorum sensing in *V. harveyi* have relied on cell density measurements, most commonly in the range between 10^6 and 10^8 cells*mL^{-1} [8], values that correspond to the early and mid-exponential growth phases (OD_{600} ranging from 0.001 to 0.1).

To estimate the productivity of the population, the measured concentrations of HAI-1 and AI-2 were normalized to the corresponding cell density (Fig. 2B). Remarkably, this revealed that the normalized AI-2 concentration actually decreases significantly when the population enters the stationary phase. In contrast, the normalized HAI-1 concentration remained constant once the maximal level was reached (Fig. 2B). These data suggest that, in the case of AI-2, *V. harveyi* either ceases to produce this AI at a certain point and/or the bacterium has other ways of reducing the number of AI-2 molecules present in the medium. The time course for HAI-1 productivity per cell corresponds to the typical threshold-mediated regulation in quorum sensing.

We were unable to determine exact concentrations for CAI-1 in cell-free culture fluids. Instead, variations in CAI-1 levels were measured using the *V. cholerae* MM920 reporter strain [33,50]. High CAI-1 activity was detectable in the stationary phase, while only a low basal level of active CAI-1 was present during exponential growth (Fig. 3A). In parallel, *V. harveyi* JHM626 was used as reporter strain, which revealed comparable results with a basal CAI-1 activity during exponential growth and a high activity within the stationary phase (data not shown). These results were supported by GC-TOF-MS profiling (Fig. 3B and 3C). A compound which mass corresponds to that of the *V. harveyi*-specific Ea-C8-CAI was identified in the culture fluids of cells grown to the stationary phase, but not in cell-free culture fluids isolated from cultures in exponential growth phase (Fig. 3C). It is worth mentioning here that, in addition to this compound, six other metabolites (Fig. 3C) accumulated in late-stage cultures (relative to their levels in the exponential growth phase). Their chemical structures are still unknown.

In summary, the three AIs produced by wild type *V. harveyi* exhibit distinct patterns of accumulation in growing cultures. Consequently, the various growth phases are associated with different levels and blends of extracellular AI-2, HAI-1 and CAI-1.

Induction of luminescence and exoproteolytic activity is postponed during growth of V. harveyi

The *luxCDABE* operon encoding the luciferase in *V. harveyi*, as well as a distantly located gene encoding an extracellular metalloprotease, is induced in an AI-dependent manner [18]. Bioluminescence and exoproteolytic activity were determined in samples taken from the same liquid culture of *V. harveyi* BB120 as described above. Residual bioluminescence decreases upon dilution of the cells but, after a short lag phase, bioluminescence begins to rise rapidly (Fig. 2C). This renewed onset of bioluminescence occurred at a cell density of 2.5*10^6 cells*mL^{-1} (OD_{600} = 0.0025), which is in agreement with earlier reports [8]. It is important to note that, at this stage of growth, AI-2 is essentially the only AI present (Fig. 2A). Bioluminescence reached its maximal value in the late exponential growth phase (Fig. 2C), shortly after AI-2 peaked and HAI-1 had attained its half-maximal concentration (Fig. 2A). Thereafter, bioluminescence intensity decreased. As illustrated in Figure 2D, when normalized with respect to cell number, bioluminescence intensity displays a typical sharp decrease in the absence of AIs, an increase during the exponential growth phase, and a slow decrease thereafter.

In the next experiment we determined the time course of the AI-dependent induction of exoproteolytic activity in cell-free culture fluids from a growing *V. harveyi* culture. Since AI-dependent induction of a gene encoding a putative exoprotease has only been described at the transcriptional level [18], it was first necessary to test whether the detectable exoproteolytic activity was indeed regulated under the control of AIs. For this purpose we analyzed the exoproteolytic activity in culture fluids of various mutants that had been grown to the stationary phase (Fig. 4A). The exoproteolytic activity measured for the wild type strain was comparable to the activity seen in the AI-independent, constitutively active mutant JAF78 (Δ*luxO*-Cm^r). The quorum sensing

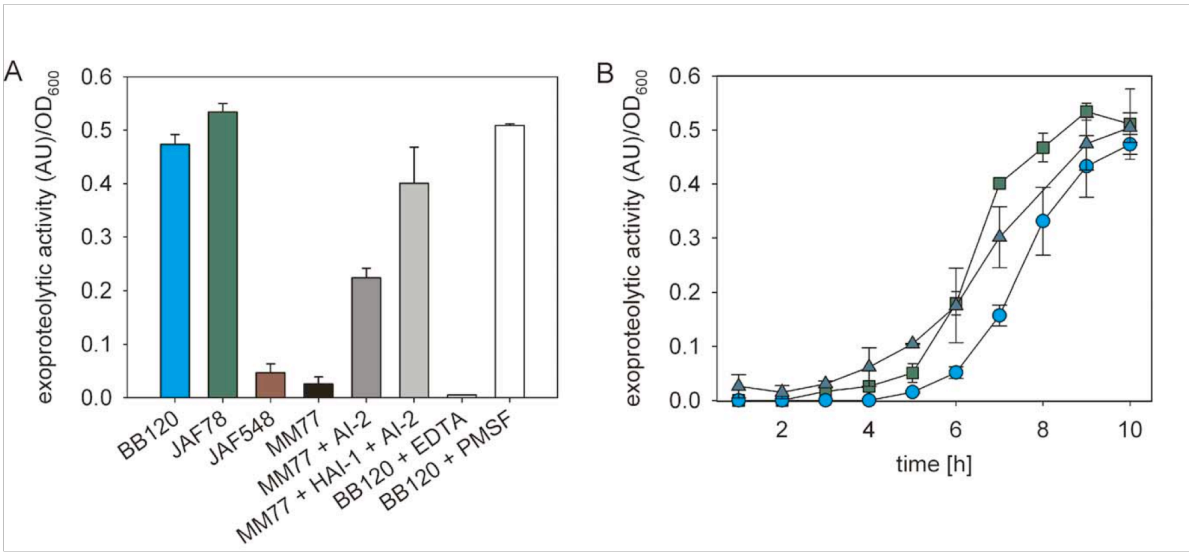

Figure 4. Exoproteolytic activity of *V. harveyi* mutants. (A) Exoproteolytic activity was analyzed in cell-free culture fluids of the wild type BB120 (blue) in comparison to the autoinducer-independent, constitutively active mutant JAF78 (Δ*luxO*) (green), and the quorum sensing negative mutant JAF548 (*luxO*-D47E) (red). Furthermore, the exoproteolytic activity produced by the autoinducer synthase mutant MM77 (*luxM*::Tn*5 luxS*::Cm^r) in the absence (black) or in the presence of AI-2 (gray) or HAI-1 and AI-2 (each 10 μM) (light gray) was determined. Culture fluids were obtained from cells grown to the stationary phase. All experiments were performed in triplicate, and error bars indicate standard deviations of the mean. To classify the type of exoprotease detected, the metalloprotease inhibitor ethylenediaminetetraacetic acid (EDTA, 5 mM) (white, striped to the right) or the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF, 1 mM) (white) was added to the activity assay. (B) Time courses of the exoproteolytic activity of growing cells of strains BB120 (wild type, blue circles), JAF78 (autoinducer-independent, constitutively active mutant, green squares), and BB120 in the presence of synthetic HAI-1 (10 μM), which was added at time point 0 (dark gray triangles). All experiments were performed in triplicate, and error bars indicate standard deviations of the mean.
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negative mutant JAF548 (*luxO*-D47E), as well as the mutant MM77 (*luxM*::Tn5, *luxS*::Cm^r) which is unable to produce HAI-1 or AI-2, exhibited very low activities. Proteolytic activity could be restored in mutant MM77 by adding both HAI-1 and AI-2 at physiological concentrations (Fig. 4A). These data confirmed that the exoproteolytic activity determined in the culture fluids of *V. harveyi* is regulated by AIs. Furthermore, this protease belongs to the metalloproteases, since it was inhibited by ethylenediaminetetraacetic acid (EDTA), but was insensitive to phenylmethylsulfonyl fluoride (PMSF) (Fig. 4A).

Analysis of a wild type *V. harveyi* population indicated that exoproteolytic activity was absent during the first 10 h of cultivation (Fig. 2C). Subsequently, activity coincided with the increase in the HAI-1 concentration, reaching a maximum in the stationary phase (after 15 h of cultivation; Fig. 2C). Normalization of the proteolytic activity to the corresponding optical density did not significantly alter the shape of the hyperbolic curve (Fig. 2D).

To test whether the appearance of HAI-1 in the medium times the induction of exoproteolytic activity, we added an excess of HAI-1 to a culture at time 0. In this case, exoproteolytic activity was first observed in the mid-exponential growth phase (at 8 h), significantly earlier than in the untreated wild type population (at 10 h) (Fig. 4B). Although HAI-1 clearly influences the onset of the induction of the exoproteolytic activity, this phenotype did not immediately develop after addition of synthetic HAI-1. Similarly, mutant JAF78 did not show constitutive exoproteolytic activity (Fig. 4B). These results unambiguously indicate the involvement of further, as yet unknown, regulatory mechanisms. These control mechanisms might be effective at the level of transcription or enzymatic activity or protein export.

In summary, induction of the exoprotease is temporally decoupled from the onset of bioluminescence, despite the fact that the corresponding genes are primarily under the control of the same signaling cascade. This notion supports the idea that different blends of AIs drive different outputs.

Bioluminescence and exoprotease activity are the result of different AI combinations

To experimentally test this idea we monitored the induction of bioluminescence and exoproteolytic activity in the *V. harveyi* mutant MM77 (*luxM*::Tn5, *luxS*::Cm^r) after adding different concentrations and mixtures of AI-2 and HAI-1. Induction of bioluminescence showed a linear dependence on AI-2 concentration over the range from 0.1 to 5 μM (Fig. 5A). At very high concentrations (25 μM and 50 μM) no further increase was found.

When we tested D-HAI-1, we also found that bioluminescence was induced, albeit with much reduced efficacy. The level of bioluminescence induced by D-HAI-1 was only 0.06% of that observed following the addition of the same concentration (5 μM) of AI-2 (Fig. 5A). The functionality of the D-HAI-1 used was confirmed by *in vitro* phosphorylation experiments with LuxN and LuxU (data not shown). The L-HAI-1 isomer caused no significant induction (data not shown), which is in agreement with the known stereospecificity of *V. harveyi* HAI-1 [51]. The dark phenotype of a *luxS/cqsA* mutant, which produces only HAI-1 [8], is compatible with the low intensity of bioluminescence induced by HAI-1 observed here. By contrast, in the *luxM/luxS* mutants KM413 [26] and KM135 [18] (which are comparable to the MM77 strain used in this study), bioluminescence could be induced by HAI-1 (either by synthetic HAI-1 [26] or HAI-1 containing culture fluids [18]).

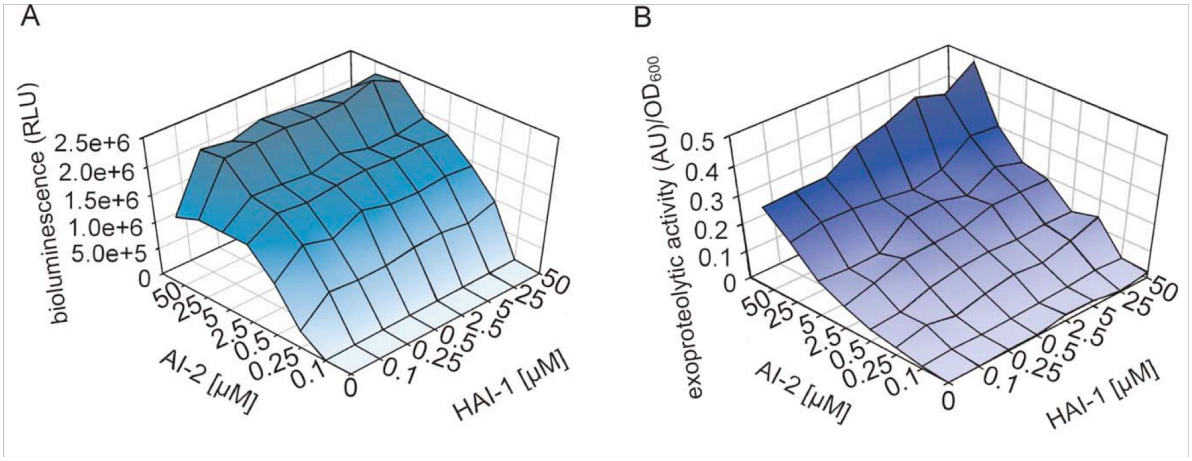

Figure 5. Dose-dependent effects of HAI-1 and AI-2 on bioluminescence and exoproteolytic activity of *V. harveyi*. The autoinducer synthase negative mutant *V. harveyi* MM77 (*luxM*::Tn*5 luxS*::Cm^r) was used to analyze the dose-dependent effects of HAI-1 and AI-2. Strain MM77 was cultivated in the presence of varying concentrations (0, 0.1, 0.3, 0.5, 2.5, 5, 25 and 50 μM) of HAI-1 and/or AI-2, and levels of bioluminescence (A) and exoproteolytic activity (B) in the culture fluids were determined. Light levels and exoproteolytic activities were expressed relative to the optical density of the culture, and values are displayed in a 3D mesh. All experiments were performed in triplicate, and mean values are shown. The standard deviations were below 5%.
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It is important to note that all these mutants are able to produce CAI-1. In our experiments bioluminescence was measured of mid-exponentially grown cells, when CAI-1 was not detectable (see Fig. 3A). In former studies CAI-1 might be responsible for bioluminescence induction, because cells were analyzed after 14–16 h of growth [18,26]. Note that, as described above, HAI-1 is at no time the sole AI to be found in a wild type culture, and our results indicate that induction of bioluminescence by HAI-1 is dependent on the presence of other AIs.

We therefore tested the effects of HAI-1 and AI-2, applied in different molar ratios, on the induction of bioluminescence (Fig. 5A). Importantly, bioluminescence increased when both HAI-1 and AI-2 were present (Fig. 5A). This effect (about 2-fold) was particularly pronounced at the lowest AI-2 concentrations tested (0.1 μM and 0.25 μM) and a low concentration of HAI-1 (0.1 μM); no further increase was observed upon exposure to higher concentrations of HAI-1. Thus, while AI-2 is able to induce bioluminescence in *V. harveyi* ($1.3*10^6$ RLU, for AI-2 at 0.25 μM) on its own, the simultaneous presence of HAI-1, which has only a minor effect by itself ($1.2*10^3$ RLU, for HAI-1 at 0.25 μM), significantly increases the level of bioluminescence observed ($2.5*10^6$ RLU for HAI-1 and AI-2, each at 0.25 μM).

Then we tested the dose-dependent effect of AIs on the induction of the exoprotease. An increase in the AI-2 concentration led to a concomitant increase in the exoproteolytic activity. HAI-1 induced this activity too, but to a much lesser degree (between 5% and 15%; see Fig. 5B). Finally, a mixture of HAI-1 and AI-2 resulted in maximal exoproteolytic activity (Fig. 5B). These results correlate with the onset of exoproteolytic activity in a growing wild type population at a time when both HAI-1 and AI-2 are present in the medium (Fig. 2C).

luxR transcription levels follow the pattern of AIs accumulation in a growing V. harveyi population

Next we analyzed the level of the transcript encoding the master regulator LuxR at different time points during growth (see arrows in Fig. 2A), which are characterized by different concentrations/blends of the AIs (1– early exponential growth phase = low concentration of AI-2; 2– mid-exponential growth phase = high concentration of AI-2; 3– late exponential growth phase = blend of AI-2 and HAI-1; 4– stationary phase = blend of AI-2, HAI-1 and CAI-1). As a control, the synthase negative mutant JMH634, which is unable to produce AI-2, HAI-1, and CAI-1, was analyzed at essentially the same stages of growth. Cells were cultivated, RNA was isolated, cDNA was synthesized, and levels of the *luxR* transcript were determined by qRT-PCR (Fig. 6A). Changes in *luxR* mRNA levels relative to the *recA* transcript were calculated using the C_T method [49]. The level of *luxR* mRNA in the wild type increased with the buildup in AI-2 concentration (time points 1 and 2), and rose further when HAI-1 appeared in the medium (time point 3). The maximal transcript level was measured at the time when all three AIs were present (time point 4; 54-fold induction compared to the mutant). The number of transcripts per cell (calculated according to [52]) revealed an increase from 0.9, 2.2, 4.2 to 11.0 transcripts per cell from the early exponential to the stationary growth phase. In the mutant JMH634 0.2 *luxR* transcripts per cell were detectable, indicating that *luxR* is not completely repressed in the absence of AIs. However, the effects of extremely low concentrations of LuxR on cell physiology are still unknown. The number of LuxR proteins per cell is difficult to deduce from these data, due to the numerous feedback mechanisms. Nevertheless, it is expected that the number of transcripts is reflected in the number of LuxR molecules produced (see [52] for quantitative data), which in turn is the primary parameter that determines the responses of different gene classes (Fig. 1).

AI-regulated genes harbor different transcription profiles

Transcript levels were also determined for four AI-regulated genes [26]. The experiments were essentially the same as described above for *luxR*. The profiles for *luxA* (which codes for a subunit of luciferase), *vhpA* (an exoprotease), *vopN* (an outer membrane protein) and vscP (a putative translocation protein in type III secretion) transcripts all differed in detail (Fig. 6B–E). *luxA* was induced by up to 1,500-fold at stages when AI-2 was the major AI

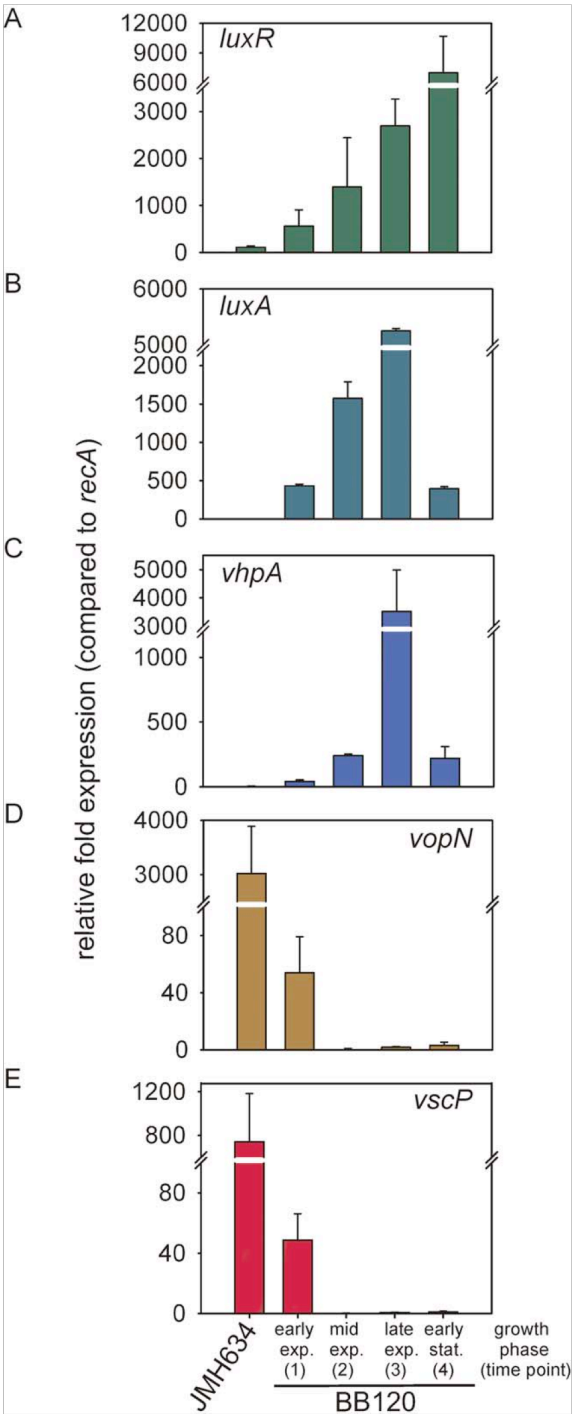

Figure 6. Transcriptional analysis of AI-regulated genes. Cells of the wild type (BB120) and the autoinducer-negative mutant JMH634 were grown as described in Fig. 2. Total RNA was isolated at four different time points (marked by the arrows in Fig. 2A), which are characterized by different concentrations/blends of the AIs: 1– early exponential growth phase = low concentration of AI-2; 2– mid-exponential growth phase = high concentration of AI-2; 3– late exponential growth phase = blend of AI-2 and HAI-1; 4– stationary phase = blend of AI-2, HAI-1 and CAI-1. Levels of *luxR* (A), *luxA* (B), *vhpA* (C), *vopN* (D), *vscP* (D) and *recA* (as reference) transcripts were determined by qRT-PCR for each time point. Changes in transcript levels (expressed relative to *recA*) were calculated using the C_T method [49]. Since transcript levels of the corresponding genes in mutant JMH634 did not change significantly over time, only one time point (3) is shown. All experiments were performed in triplicate, and error bars indicate standard deviations of the mean.
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in the medium (time points 1 and 2; Fig. 6B). When HAI-1 became available the *luxA* transcript level increased further (time point 3; 3-fold additional increase). At time point 4 (LuxR level highest), the transcript level of *luxA* was low. Luciferase is a stable protein, which might explain the transcriptional down-regulation. Nevertheless, the drop in *luxA* transcript level coincides with the decline in bioluminescence described above (Fig. 2).

In contrast, levels of the *vhpA* transcript increased very slightly between time points 1 and 2, while the maximum value was found at time point 3, when both HAI-1 and AI-2 were present (750-fold induction at time point 3 compared to mutant JMH634; Fig. 6C). Thereafter the transcript level decreased. Increasing AI-2 concentrations are associated with increased repression of *vopN* and *vscP* (time points 1 and 2; Fig. 6D, E). HAI-1 and CAI-1 have no additional effect (time points 3 and 4, Fig. 6D, E).

In conclusion, different combinations of AIs present at certain growth stages drive different AI-regulated processes, and thus determine their timing and succession.

HAI-1 and AI-2 act synergistically on the phosphorylation cascade of V. harveyi

We performed *in vitro* phosphorylation assays to test the effects of different inputs, specifically, different ratios of HAI-1 and AI-2, on the LuxN and LuxQ (LuxP)-mediated phosphorylation of LuxU as output. The full-length hybrid kinases LuxN and LuxQ (tagged with 6 histidine residues) were expressed in the *E. coli* strain TKR2000. This strain lacks the F_1/F_o-ATPase, and inverted membrane vesicles can be used directly for phosphorylation experiments. Analogously to a biochemical characterization of the HAI-1-recognizing kinase LuxN described earlier [14], an initial characterization of the AI-2-sensing LuxQ in interplay with LuxP (LuxPQ) was performed (Fig. 7). Western blot analysis using purified protein revealed that LuxN and LuxQ were incorporated into the lipid bilayers of membrane vesicles, and accounted for about 2.7% and 1.8% of all membrane proteins (data not shown). Since the LuxQ-LuxP interaction does not change in the presence of AI-2 [10], all studies were performed with LuxQ and purified LuxP in a molar ratio of 1:1. LuxPQ was able to phosphorylate LuxU in a time-dependent manner (Fig. 7A). The LuxPQ kinase activity was determined to be in the same range as the LuxN kinase activity (initial rates 300 and 200 pmol*min^{-1}*mg^{-1} kinase protein, respectively) (Fig. 7B). Addition of AI-2 inhibited the LuxPQ kinase activity (Fig. 7) in a concentration-dependent manner (data not shown), with half-maximal inhibition occurring at 5 μM AI-2. Importantly, even at the highest AI-2 concentration tested (30 μM), LuxU phosphorylation was still detectable (data not shown). These findings are reminiscent of the incomplete inhibitory effect of HAI-1 on the LuxN kinase activity [14]. When each AI was added to its cognate kinase at a concentration of 10 μM, LuxU phosphorylation by LuxPQ and LuxN was inhibited to comparable extents (Fig. 7B, 61% and 57%, respectively). Moreover, HAI-1 had no effect on LuxPQ-mediated phosphorylation of LuxU, and AI-2 had no effect on LuxN-mediated phosphorylation of LuxU (data not shown). LuxPQ also

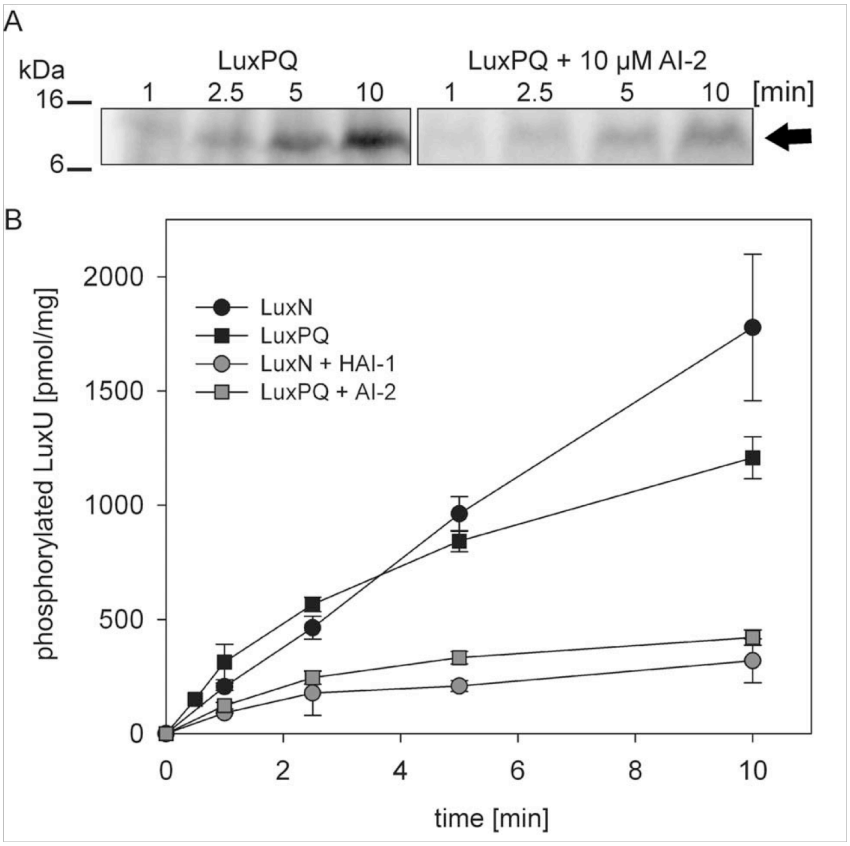

Figure 7. Phosphorylation activity of LuxQ. Inverted membrane vesicles prepared from *E. coli* TKR2000 containing full-length LuxQ were incubated with purified LuxP, purified LuxU and (where indicated) with 10 μM AI-2 (A). The phosphorylation reaction was started by adding 100 μM [γ-^{32}P] ATP at time 0. At the indicated times, the reaction was terminated, and radiolabeled proteins were separated by SDS-PAGE, and visualized by autoradiography. The arrow indicates phosphorylated LuxU. Phosphorylated LuxU was quantified with ImageQuant using [γ-^{32}P] ATP as standard (B). Phosphorylation experiments were also performed in the presence or absence of 10 μM HAI-1 using membrane vesicles containing full-length LuxN and phosphorylated LuxU was quantified accordingly (B).
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catalyzed the dephosphorylation of phospho-LuxU, and this reaction was unaffected by the presence of AI-2 (data not shown).

In order to simulate the situation *in vivo*, we designed an experiment in which the total rate of LuxN and LuxPQ (molar ratio LuxN:LuxQ = 1:1) mediated LuxU phosphorylation was assayed in the presence of various combinations of AIs. In so doing, we utilized the physiological concentrations of HAI-1 and AI-2 we had found to be present in a growing wild type culture *in vivo* (Fig. 2). In the absence of AIs, LuxU was readily phosphorylated (initial rate of 250 pmol*min^{-1}*mg^{-1} kinase protein, 100%). Addition of increasing amounts of AI-2 led to concomitant inhibition of LuxU phosphorylation (Fig. 8, simulated time points 1–8 h). Upon supplementation with HAI-1, a significant increase in inhibition (from 35% to 50%) was observed (Fig. 8, simulated time points 8 and 9 h). Moreover, the use of HAI-1 and AI-2 in ratios characteristic of longer cultivation times resulted in a linear increase in inhibition although the slope was lower than for AI-2 alone (Fig. 8, simulated time points 9 to 14 h). However, the highest combined concentration of the two AIs (HAI-1:AI-2 = 1:1) tested was insufficient to completely inhibit LuxN/LuxPQ-mediated phosphorylation of LuxU (Fig. 8). These findings thus leave room for the input of the third (the CAI-1 responsive CqsS) and fourth (the NO-sensing H-NOX/HqsK) systems. Unfortunately, synthetic CAI-1 is not commercially available and therefore could not be included in the phosphorylation experiments thus far.

In conclusion, the sensory part of the complex signaling cascade responds sensitively to various concentrations and blends of AIs by generating distinct outputs at the level of phosphorylated LuxU. Subsequently, these signals are transduced by the same signaling cascade via LuxO and Qrr to *luxR*, which permits fine-tuning of the level of the *luxR* transcript (Fig. 6A) and thus enables tight control of LuxR-regulated genes.

Discussion

Like *V. harveyi*, other bacterial species also use more than one AI for quorum sensing. For example, *Staphylococcus aureus* [53,54] and *Vibrio cholerae* [55] produce and respond to two, *Pseudomonas aeruginosa* [56] and *Aliivibrio fischeri* [55,57] to three different AIs. Here we report time and growth phase-dependent alterations in the onset and concentration of each of the three *V. harveyi* AIs in liquid culture. Importantly, during the shift from low to high cell density (10^6 to 10^8 cells/mL, OD_{600} = 0.001 to 0.1) that occurs in

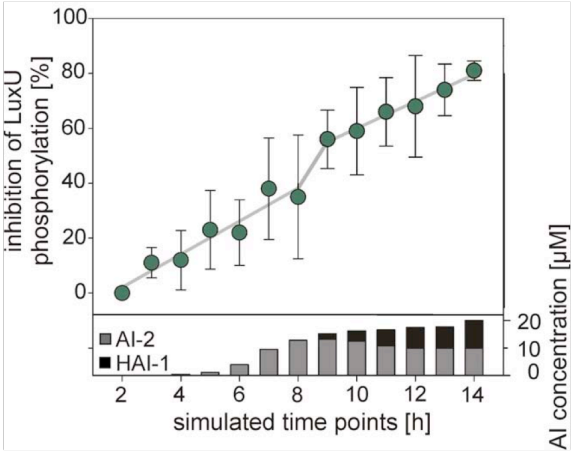

Figure 8. Effects of various concentrations of HAI-1 and AI-2 on LuxN- and LuxQ- mediated phosphorylation of LuxU. LuxN- and LuxQ-bearing membrane vesicles, together with purified LuxP and LuxU, were incubated with 100 μM [γ-^{32}P] ATP, and the effects of AI-2 and HAI-1 on the initial rate of LuxU phosphorylation were tested. AI-2 and HAI-1 were added at physiological concentrations (see Fig. 2), indicated in the lower part of the graph (HAI-1 in black, AI-2 in gray). Phosphorylated LuxU was quantitatively analyzed as described in Fig. 7. The degree of inhibition is expressed as the percentage reduction in the initial rate of LuxU phosphorylation measured in the presence of the indicated concentrations/blends of autoinducers relative to that seen in the absence of autoinducers. All experiments were performed in triplicate, and error bars indicate standard deviations of the mean.
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the exponential growth phase, AI-2 is the major AI in the medium. HAI-1 first becomes detectable and increases in concentration during the late exponential growth phase. Under our growth conditions, CAI-1 was detected at readily measurable levels only in the stationary phase. Since we were unable to determine the molar concentration of CAI-1, the sensitivity of our method is unknown. Therefore, we cannot exclude that physiological relevant low concentrations of CAI-1 are present at earlier growth phases. The delay in secretion of HAI-1 relative to AI-2 is in good agreement with data in a recent report [57], although the two sets of results are not strictly comparable, because different growth media were used (the more complex LM medium in [57], AB medium here). Moreover, a previous study has shown that CAI-1 activity peaks in the stationary phase when cells are grown in LM medium [8], in agreement with our observations. These data show that different stages in the expansion of a *V. harveyi* culture are characterized by distinct AI profiles: the early exponential growth phase by low AI-2, the mid-exponential growth phase by high AI-2, the late exponential growth phase and the early stationary phase by a blend of AI-2 and HAI-1, and the later stationary phase by a combination of AI-2, HAI-1 and CAI-1. This classification corresponds well with the staggered expression of bioluminescence and exoproteolytic activity during growth of wild type *V. harveyi*. Although both phenotypes are dependent on AI-controlled genes and hence on the same signaling cascade, they are not induced simultaneously. The onset of bioluminescence occurs, and light levels reach their maximum, in the exponential growth phase, whereas exoproteolytic activity only sets in after the transition into the stationary phase. These findings are supported by our reporter strain analysis, which indicated that AI-2 is sufficient for induction of bioluminescence and that HAI-1 (even at a low concentration) acts synergistically to enhance light production. In contrast, both HAI-1 and AI-2 were required to induce exoproteolytic activity. Other AI-regulated phenotypes seem also to be affected by different combinations of AIs. Based on our experiments, full repression of *vscP* and *vopN* requires only AI-2. Furthermore, the sRNA Qrr4 can be induced by HAI-1 or AI-2, but full induction is attained only when both are present together [39]. The effects of HAI-1 and AI-2 on the promoter activities of AI-regulated genes have been analyzed previously using promoter::*gfp* fusions [26], and these studies permitted differentiation between three groups of genes. The first group requires both AIs for activity; either HAI-1 or AI-2 can induce the second set, but both are necessary for full activity, and either HAI-1 or AI-2 is sufficient to induce full activity of the third. Remarkably, we observed a tight correlation between the various inputs and the level of the *luxR* transcript that encodes the master regulator of the signaling cascade. With each additional AI, levels of *luxR* mRNA increased. The highest level was measured when all three AIs were present simultaneously. Curiously, no gene is yet known to be regulated by LuxR at this late growth stage.

LuxR activates and represses more than 100 genes, and both the numbers and relative affinities of its binding sites vary for different genes [15]. The level of extracellular AIs as input is translated into a particular intracellular concentration of LuxR. A low LuxR concentration in the cell seems to be sufficient for the induction of *luxA* and hence for bioluminescence, and for the repression of *vopN* or *vscP*. At later growth stages, levels of the *luxR* transcript increase, and *vhpA*, which codes for a protease, is induced to a maximal level. In agreement with this, full induction of the exoproteolytic activity requires both HAI-1 and AI-2, and hence a higher copy number of LuxR than does the induction of bioluminescence.

The transcriptional analysis raises questions regarding the molecular mechanism of down-regulation of gene expression. For example, significantly decreased transcript levels were determined for *luxA* and *vhpA* during stationary phase. It is still unclear whether LuxR or AphA [24] – a transcriptional regulator that acts in the opposite manner to LuxR – or other components of the stationary phase control network are responsible for this phenomenon.

Our *in vitro* data on receptor-mediated phosphorylation of LuxU, the protein which gathers all information, reveal a very tight correlation between various inputs and outputs. The rate of LuxU phosphorylation decreases linearly with the physiological increase in the AI-2 concentration, and the decrease continues as HAI-1 is added to the mix. Remarkably, the activities of the two histidine kinases LuxN and LuxPQ exhibit some degree of cooperativity, because the effects of AI-2 and HAI-1 were non-additive (Fig. 8). Even at a low concentration, HAI-1 had a significant effect on the inhibition of LuxU phosphorylation (Fig. 8). Furthermore, the blend of AI-2 and HAI-1 available in the late stationary phase did not suffice to prevent LuxU phosphorylation, indicating that the system has capacity to spare for the integration of information, e.g. from the CAI-1/CqsS and NO H-NO/HqsK circuits. Cooperativity between the different histidine kinases is supported by earlier *in vivo* measurements with mutants lacking one or two histidine kinases [8]. Mutants lacking either LuxN or CqsS or the corresponding double mutant required a higher cell density (according to our data a higher AI-2 concentration) to induce bioluminescence. In contrast, in a mutant lacking LuxQ, a lower HAI-1 and/or CAI-1 concentration was sufficient for luminescence induction. Thus, deletion of kinases has a greater or lesser effect on the sensitivity of the quorum sensing system depending on the AIs to which each responds.

The *in vitro* data also complement a comprehensive study on input-output relationships in various feedback-loop mutants [25]. There, it was clearly demonstrated that feedbacks affecting the cellular concentrations of LuxR as well as LuxO ensure a broad and graded response to HAI-1 and AI-2, and prevent switch-like on-off behavior. Here we found that the receptor-mediated input ensures a graded output already at the level of phosphorylated LuxU. Thus far, our *in vitro* studies have used equal quantities of LuxN and LuxPQ. In future experiments we will integrate the other histidine kinases, and test different ratios of the histidine kinases to take into account the recently described positive *luxMN* feedback loop and the increased sensitivity to HAI-1 [25].

The stable succession of different AI-regulated processes might facilitate the proliferation of *V. harveyi* in the ocean. Bioluminescence might attract organisms of the same species to form aggregates or to settle down on surfaces. *V. cholerae* is known to possess blue-light photoreceptors [58]. Based on genome analyses, *V. harveyi* also possesses genes encoding proteins with a BLUF domain, a sensor for blue light. Bioluminescence improves the nutrient cycle [59] as well as the metabolization of oxygen, and thereby reduces the number of oxygen radicals [60,61]. In this way microcolonies could benefit from light production during the infection of shrimps. In addition, *V. harveyi* might use additional AI-2 that is produced by other species. Later, when its population has reached a certain cell density, *V. harveyi* produces and responds to the species-specific HAI-1. Subsequently, HAI-1 boosts bioluminescence induction. At this growth stage, which coincides with stationary growth and the beginning of biofilm formation [17], the population starts to produce an exoprotease. Exoenzymes might be useful for the recycling of dead cells during stationary growth or for the release of single cells from aggregates. Exoproteases are also important for the pathogenicity of some *Vibrio* species [62,63]. By utilizing the species-specific HAI-1 to induce the exoprotease, *V. harveyi* ensures that the products of exoproteolysis are made available to its own kind. Unfortunately, no gene is known which is under the control of CAI-1 in the stationary phase. Nonetheless, it is suggested that *V. harveyi* needs all three AIs to time the onset and duration of certain AI-regulated processes during different stages of growth.

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Author Contributions

Conceived and designed the experiments: CA NS KJ. Performed the experiments: CA MR AF US NS LP JK. Analyzed the data: CA MR AF US NS LP JK KJ HH PSK. Contributed reagents/materials/analysis tools: JK KJ HH PSK. Wrote the paper: CA KJ.

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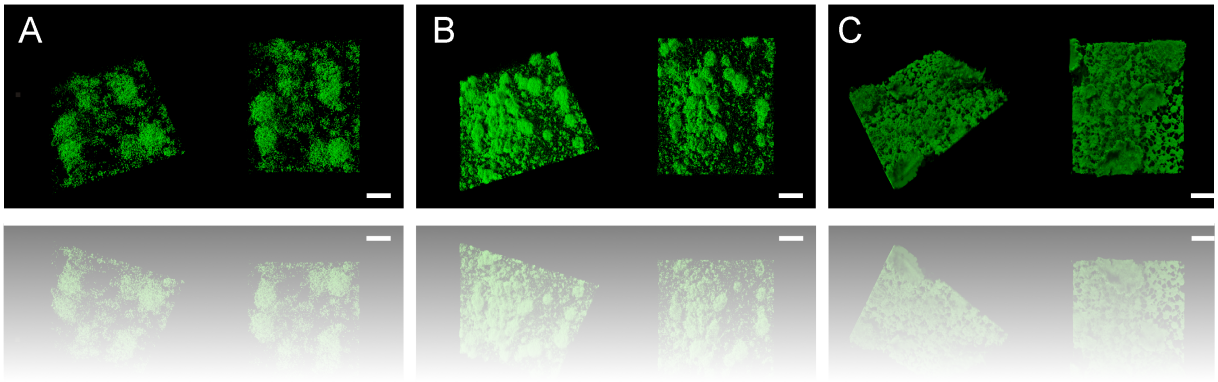
5 Unpublished data: Autoinducer-dependent biofilm formation in *Vibrio harveyi*



5.1 Introduction

Most microbes are expected to live in surface-associated communities, encased in a self-produced matrix surrounded by water channels, termed biofilms (Costerton *et al*., 1995). The formation of biofilms reflects the native growth condition of most bacterial species. In the majority of cases, the bacterial cells themselves form less than 10% of the dry mass of the biofilm. The matrix can compose over 90% (Flemming and Windenger, 2010) and consists of different types of polymeric substances. These extracellular polymeric substances are mainly responsible for adhesion to surfaces and other cells and form the frame for the three-dimensional architecture of the biofilm. Exopolysaccharides, proteins, enzymes, and nucleic acids are the principal components. The lifestyle within a biofilm is totally different than that of planktonic individual cells and has a multitude of advantages. It has been described that extracellular polymeric substances provide protection from environmental stresses like pH shifts, osmotic shocks, desiccation, and UV radiation (Flemming, 1993). The matrix can physically prevent antimicrobial agents, like antibiotics, to enter the biofilm (Gilbert *et al*., 1997) and sequester cations, metals and toxins (Decho, 1990; Flemming, 1993). The water channels within have been compared to a primitive circulatory system providing an effective region to exchange nutrients and metabolites with the aqueous phase, enhancing nutrient availability (Costerton *et al*., 1995; Davey and O'Toole, 2000). Also the acquisition of new genetic traits, like horizontal gene transfer or transduction, within the biofilm community, is discussed (Davey and O'Toole, 2000).

The bacterial biofilm is the most successful form of microbial life (Flemming and Windenger, 2010) and possesses optimal conditions for cell-cell communication and interactions (Dickschat, 2010). QS and biofilm formation are often interconnected processes in bacteria (Bassler and Losick, 2006; Miller and Bassler, 2001; Parsek and Greenberg, 2005; Waters and Bassler, 2005). QS enables the secretion of extracellular polymeric substances (Nadell *et al*., 2008). Biofilm formation is a very complex behavior and many processes have to be activated to get a mature and stable biofilm. Biofilms of different QS mutants were examined using phase contrast microscopy, a stain with crystal violet and confocal laser scanning microscopy (CLSM). Magnitude and structure of these biofilms were compared to the wild type. In addition, the quantity of secreted exopolysaccharides and the synthesis of flagella were analyzed. Our results reveal that

the presence of HAI-1 and AI-2 and their ratios at distinct growth phases are important for a stable and three-dimensional biofilm structure in *Vibrio harveyi*.

5.2 Material and Methods

5.2.1 Bacterial strains, culture conditions, cell-free culture fluids and synthetic autoinducers

V. harveyi strains BB120 (wild type), JAF78 (Δ*luxO-Cm*^{r}), MM77 (*luxLM*::Tn5, *luxS*:: *Cm*^{r}), JMH626 (Δ*luxN*, *luxQ*::Tn5, *cqsA*::*Cm*^{r}) (see Tab. 1 for references), and JAF548 (*luxO* D47E) (Freeman and Bassler, 1999) were used throughout this study *V. harveyi* cells were, when not mentioned differentially, cultivated in AB medium (Greenberg *et al*., 1979), and incubated aerobically in a rotary shaker at 30°C. When appropriate, the medium was supplemented with chloramphenicol (33 µg*mL^{-1}), kanamycin (100 µg*mL^{-1}), or gentamycin (100 µg*mL^{-1}, *gfp* tagged strains).

To obtain cell-free culture fluids, cultures were centrifuged at 5000 x g for 15 min. Culture fluids were then filtrated (0.20 µm), lyophilized until a 10-fold concentration was reached, and stored at -20°C or used immediately. Culture fluids were used in an end concentration of 1-fold. Synthetic HAI-1 was purchased from the University of Nottingham. AI-2 was synthesized *in vitro* like described before (Anetzberger *et al*., 2009).

5.2.2 Phase contrast and luminescence microscopy

Microscopy was performed using the customized inverted Leica microscope DMI 6000 B, an oil objective (100-fold magnification) and the high-sensitivity and cooled (-80°C) CCD camera iXON (Andor). Cells were incubated in Erlenmeyer flasks or in culture dishes (for monitoring biofilms). At the times indicated, an appropriate number of cells was collected. Culture fluid was gained and saved for the subsequent steps. Cells were resuspended in small volumes of the corresponding culture fluid. 20 µl of cell suspension were incubated in the middle of a cover slip in the dark for 20 min to allow adherence. All cover slips were pretreated with poly L-lysine (0.05 g l^{-1}) to fix the cells on the surface. Subsequently, cells were washed twice with the corresponding air-saturated culture fluid directly on the slip to remove non-fixed cells. Luminescent cells

were identified using bioluminescence imaging without any filter in a flow chamber to ensure sufficient oxygen concentration. A Pecon flow chamber consisting of an upper and a lower cover slip separated by a perfusion ring was used. Via this ring the chamber was filled with the air-saturated culture fluid. The chip of the CCD-camera was exposed to the luminescent cells for 240 s. Phase contrast microscopy was used to visualize all cells for comparison with luminescence images of planktonic cells, aggregates or biofilms and to monitor stained flagella. All images were edited using Image J 1.37c.

5.2.3 Confocal Laser Scanning Microscopy (CLSM)

For structure analysis of produced biofilms, used strains were tagged with the fluorescent protein *gfp* using the mini-Tn7 transposon system like described before (Lambertsen *et al*., 2004). In short *V. harveyi* strains BB120, JAF78, and MM77 were tagged by introducing the delivery plasmid pBK-miniTn7-*gfp*2 and the helper plasmids pUX-BF13 (Bao *et al*., 1991), carrying the transposase genes, and pRK600 (Kessler *et al*., 1992), carrying the RP4/RK2 conjugation system, via filter mating using *Escherichia coli* WM3064 (Gödeke *et al*., 2011) as donor. The tag inserts downstream of gene *glmS*. Insertion was proofed via control PCRs and fluorescent microscopy. Strain JMH626 was tagged with Syto9 Green fluorescent nucleic acid stain (Invitrogen) right before use.
Biofilms were cultivated at 30°C in AB medium in custom-made three-channel flow cells with individual channel dimensions of 1 by 4 by 40 mm. Microscope cover slips (Roth) were used as a colonization surface, glued onto the channels with silicone (Sista-Henkel), and left to dry for 24 h at room temperature prior to use. Assembly, sterilization, and inoculation of the flow system were performed essentially as previously described (Thormann *et al*., 2004). Microscopic visualization of biofilms and image acquisition were conducted using an inverted Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems) equipped with ×10/0.3 Plan-Neofluar and ×63/1.2 W C-Apochromate objectives. For displaying biofilm images, CLSM image stacks were processed using the IMARIS software package (Bitplane AG, Zürich, Switzerland) and Adobe Photoshop.

5.2.4 Swimming behavior on solidified medium

Swimming was analyzed using LM or AB swimming plates containing 0.25% agar. 1 µl of

a bacterial suspension with an optical density OD_{600}=1 was dropped in the middle of the plate. After an appropriate time of cultivation, the plates were photographed and the diameter of the bacterial lawn was measured.

5.2.5 Staining of flagella

The staining of flagella was performed according to Heimbrook (Heimbrook *et al*., 1989). One drop of cell suspension of exponential or stationary cultures was transmitted to a microscope slide. After covering the suspension with the cover slip and removal of excess suspension, staining suspension was added at the side of the cover slip. The stain attaches to the flagella and makes it visible using phase contrast microscopy. The stain consists of two suspensions (I and II), which were combined right before usage in the ratio 10:1 (I:II). Suspension I contains 2 g tannin in 10 ml 5% phenol and 10 ml saturated aluminiumpotassiumsulphate-12-hydrate. Suspension II is 12 g crystal violet in 100 ml 96% ethanol.

5.2.6 Northern blot analysis

To visualize the expression of genes *luxAB* (luciferase), *vscP* (type III secretion) and *flgA* (flagellin synthase) *V. harveyi* JAF78 and JAF548 were cultivated in AB medium until late exponential growth. Cells were harvested and RNA was extracted according to the protocol described earlier (Fritz *et al*., 2009). RNA (20 µg per lane) were separated by gel electrophoresis and blotted on membranes (Northern blot) (Fritz *et al*., 2009). Blotted membranes were hybridized with [α^{32}P]dCTP labelled DNA probes corresponding to DNA single strands for *luxAB* (370 bp), *vscP* (300 bp) and *flgA* (761 bp). A 518 bp large DNA single strand, which was complementary to the 16S rRNA, was also tested to ensure that equal quantities of RNA were loaded.

5.2.7 Quantitative biofilm assay

To assess biofilm formation, cells of *V. harveyi* strains were inoculated in AB medium into a polystyrene 96 flat-bottom well plate (Sarstedt). Cultivation occurred aerobically in a rotary shaker at 30°C. After 25 h, wells were washed twice with saline, and cells in a biofilm were visualized by staining with 0.5% (w/v) crystal violet for 20 min according to Wakimoto (Wakimoto *et al.*, 2004). Absorption of crystal violet was determined in a

microtiter plate reader (Tecan Sunrise) at 620 nm.

5.2.8 Exopolysaccharide assay

Exopolysaccharides were isolated and extracted like described before by Enos-Berlage (Enos-Berlage and McCarter, 2000). In short 100 µl of a culture with an optical density OD_{600}=1 were droped on an AB plate and incubated over night. Cells were scraped from the plate and resuspended in an appropriate volume of phosphate buffered saline. After repeating steps of shaking and centrifugation, RNaseA, DNaseI, $MgCl_2$ and protease were added. A two-fold extraction with phenol-chloroform was followed by a precipitation with 100% ethanol. Extracted polysaccharides were washed with 70% ethanol and resuspended in Bidest. The quantity of polysaccharides was determined using the colorimetric phenol–sulfuric acid test (Dubois *et a*l., 1956). To 1 ml of a sugar suspension (samples or suspensions with 10 to 100 µg glucose for calibration) 25 µl of 80% phenol and 2.5 ml concentrated sulphuric acid were added. After incubation for 20 min at 25-30˚C the generated color was stable. Absorbance was measured at 488 nm.

5.3 Results

5.3.1 Developmental stages of a *Vibrio harveyi* wild type biofilm

In general, bacterial biofilm formation can be distinguished in five stages (Fig. 5.1A). First, single cells swim free in the medium (Fig. 5.1A a) and start to interact by forming cell-cell or cell-surface contacts (Fig. 5.1A b). More and more cell aggregates attach to the surface (Fig. 5.1A c). These aggregates maturate to monolayers and three-dimensional microcolonies (Fig. 5.1A d). The last stage is the formation of a stable and mature three-dimensional structure (Fig. 5.1A e) consisting of extracellular matrix, in which the cells are embedded or moving within. Single cells can leave the biofilm and start again a planktonic way of life (Fig. 5.1A e). All described stages were found in a *V. harveyi* wild type culture (Fig. 5.1B). Bacteria were incubated in cell culture dishes. At different time points samples with planktonic cells and formed biofilms were investigated under the microscope using phase contrast. In addition bioluminescence was analyzed using a high sensitivity camera (Fig. 5.1B c, f, i, k, m, o, q, s) and flagella were stained for the first 10 h of cultivation (Fig. 5.1B a, d, g). In the first stage the cells

were free swimming (Fig. 5.1B a) and were only connected during cell division (Fig. 5.1B b). A part of the population started to produce luminescence (Fig. 5.1B b, c).

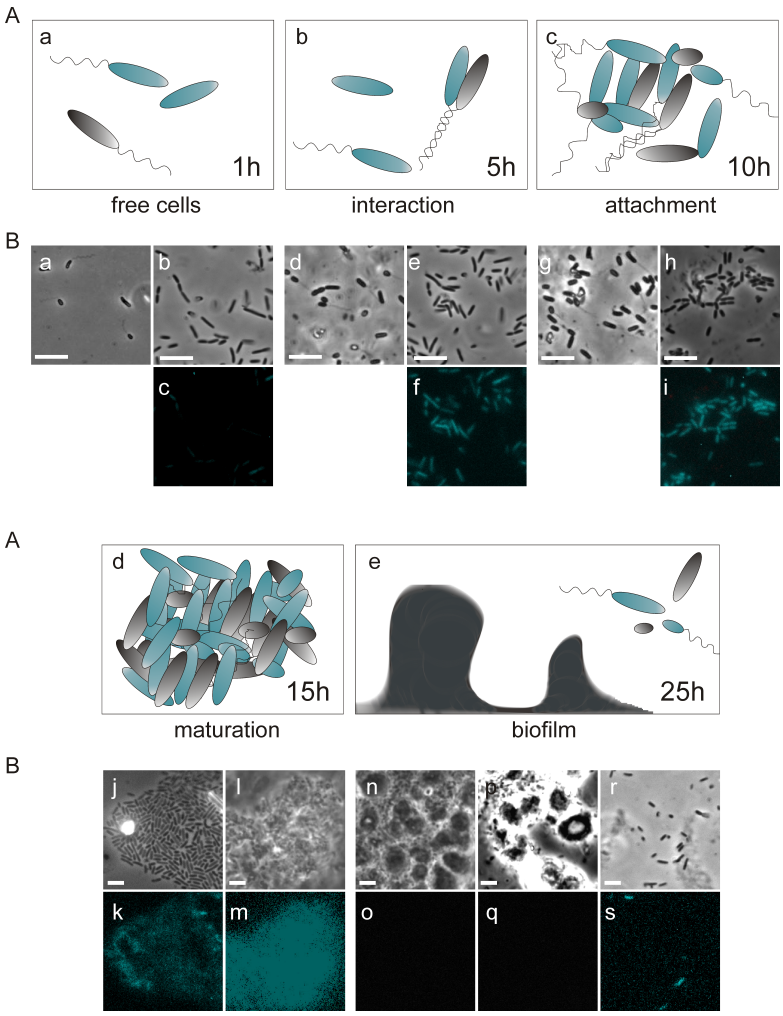


Figure 5.1 Development of a *Vibrio harveyi* biofilm. (A) A scheme for the five stages of biofilm formation is drawn: free cells (a), cell-cell and cell-surface interaction (b), attachment of cell aggregates and single cells (c), maturation (monolayers and three-dimensional structures) (d) and mature biofilm (e). In addition, heterogeneity in bioluminescence production is schematically indicated by gray (dark) and cyan (bright) cells. Time points of the different stages in liquid culture are included. (B) For all stages phase contrast and luminescence pictures of the same image section are shown (b/c, e/f, h/i, j/k, l/m, n/o, p/q and r/s). For the last stage pictures of the biofilm (n/o and p/q) and planktonic cells (r/s) were taken. Flagella staining was performed for the first 10 h of cultivation (a, d, and g). The bar is 5 µm in length.

The second stage was characterized by cell-cell contact mostly via flagella (Fig. 5.1B d). More cells produced luminescence with a higher light intensity (Fig. 5.1B e, f). Within the

exponential growth phase (third stage) more and huger cell aggregates appeared (Fig. 5.1B g, h) and the luminescence reached a maximum in light intensity produced by about 70% of the cells (Fig. 5.1B h, i). In the fourth stage (stationary phase) monolayers and three-dimensional structures were formed consisting of dark and bright cells (Fig. 5.1B j-m). However, the overall luminescence of the culture was lower than in the third stage (Anetzberger *et al.*, 2012a). In the last stage a mature biofilm with bubble and mushroom like structures was formed (Fig. 5.1B n, p). Within these structures the cells were moving, but did not produce light (Fig. 5.1B o, q). Only planktonic cells produced the low luminescence of the last stage (Fig. 5.1B r, s).

5.3.2 QS mutants of *Vibrio harveyi* produce different biofilm structures than the wild type

To get an insight in the influence of the synthesized AIs on the complex process of biofilm formation in *V. harveyi*, different QS mutants were used in this study and compared with the wild type (Tab. 1). Mutant JAF78, a *luxO* deletion mutant, shows a constitutive bright phenotype independent of the prevalent AI concentration. A high AI concentration is always mimicked (AIs-ON). Strain MM77 is only able to synthesize one AI, CAI-1 (HAI-1/AI-2-OFF). Cells of this strain are characterized by a dark phenotype. Since CAI-1 seems to have only a minor role in the production of bioluminescence, another strain was analyzed in comparison to test the influence of CAI-1 on biofilm formation. Mutant JMH626 is not able to recognize neither HAI-1 nor AI-2, and is not able to synthesize CAI-1 (AIs-OFF).

Table 5.1 Used strains/mutants of *Vibrio harveyi*

Strain	Relevant genotype	Phenotype luminescence	Reference
BB120	Wild type (ATCC-BAA1116) [reclassified: *V. campbellii*]	Bright at high cell density	Bassler *et al*., 1997, Lin *et al*., 2012
JAF78	Δ*luxO*-Cm$^{r:}$	Constitutive bright	Freeman and Bassler, 1999
MM77	*luxLM*::Tn5, l*uxS*::Cm^{r}	Dark	Mok *et al*., 2003
JMH626	Δ*luxN*, *luxQ*::Tn5, *cqsA*::Cm^{r}	Dark	Henke and Bassler, 2004

As a first step we compared the biofilm structures of the different QS mutant strains using CLSM in a hydrodynamic flow chamber (Fig. 5.2). The wild type produced a stable

biofilm with mushroom like structures (Fig. 5.2A), whereas mutant JAF78 showed less biofilm with similar structures (Fig. 5.2B). Mutant MM77 only produced a two-dimensional biofilm with rare mushroom like structures (Fig. 5.2C). The biofilm of strain JMH626 was identical to that of MM77 (Fig. 5.2D). The third QS system of *V. harveyi* CqsA/CqsS with CAI-1 as AI seems to plays no role in biofilm formation.

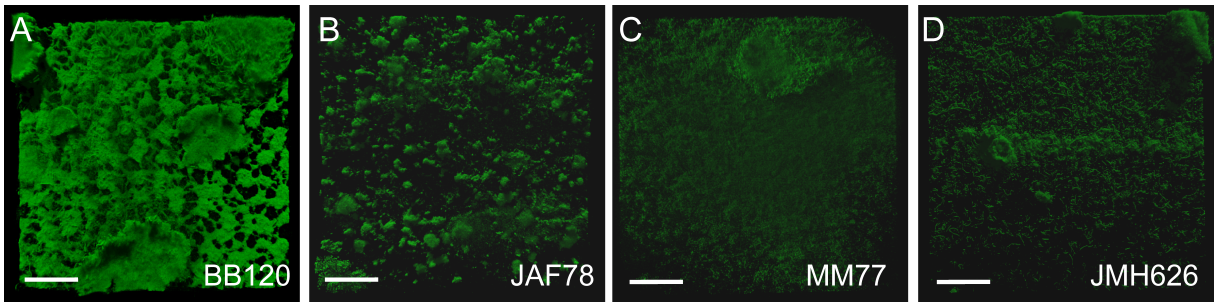


Figure 5.2 Biofilm structures of different *Vibrio harveyi* strains. Wild type BB120 (A), AIs-ON strain JAF78 (B), HAI-1/AI-2-OFF strain MM77 (C) and AIs-OFF strain JMH626 (D) were incubated in flow chambers and analyzed using CLSM. Top views of all strains after cultivation for 24 h are shown. The bar is 50 µm in length.

5.3.3 QS regulates the formation of a mature and stable biofilm in *V. harveyi*

The get a more detailed understanding of the influence of AIs on the different stages of biofilm formation four developmental stages (according to the wild type) were looked at: interaction, attachment, maturation and biofilm. The three *V. harveyi* strains BB120 (wild type), MM77 (HAI-1/AI-2-OFF) and JAF78 (AIs-ON) were used. Strain MM77 was favored over strain JMH626 (AIs-OFF), because synthetic HAI-1 and AI-2 can be added and induce AI-regulated functions in MM77.

All tested strains formed microcolonies in the interaction stage (data not shown) and cell aggregates in the attachment stage (Fig. 5.3A, E and I), but only the wild type began to form three-dimensional structures. The wild type biofilm maturated (Fig. 5.3B) and formed a stable structure with mushroom like outgrowths and a height of 45 µm (Fig. 5.3C and D). Strain MM77 only formed aggregates on the surface (Fig. 5.3J) and ended with a monolayer with rare and isolated outgrowths (Fig. 5.3K and L). JAF78, not able to produce a stable three-dimensional structure in the maturation stage (Fig. 5.3F), formed at last a biofilm that resembled the wild type biofilm, but had only a height of 25 µm (Fig. 5.3G and H). Conspicuous for JAF78 was, that the biofilm structure was more stabilized

via cell-cell than cell-surface interaction. Therefore, cell clouds above the biofilm were seen (Fig. 5.3H).

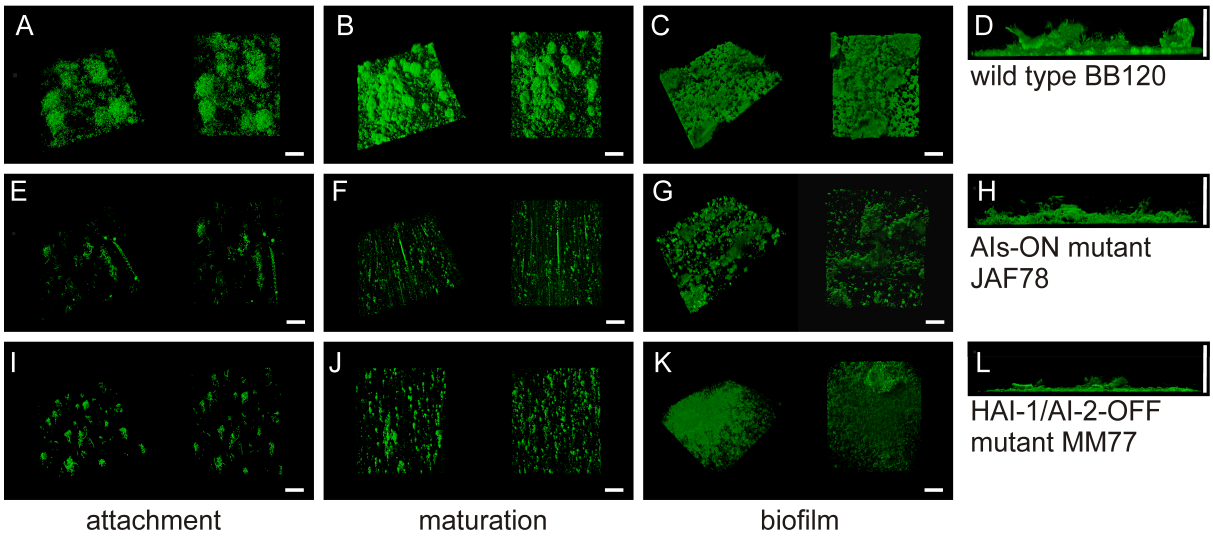


Figure 5.3 Development of a biofilm structure of wild type (BB120) *Vibrio harveyi* compared to quorum sensing mutants (JAF78 and MM77). Cells of the wild type BB120 (A-D), the AIs-ON strain JAF78 (E-H) and the HAI-1/AI-2-OFF strain MM77 (I-L) were incubated in flow chambers and following stages of biofilm formation were analyzed using CLSM: attachment, maturation and biofilm. For each stage one three-dimensional picture (left) and one top view (right) are shown. For the last stage also side views are displayed (D, H and L). The bar is 50 µm in length.

5.3.4 QS has an impact on flagella synthesis and hence on initial stages during biofilm formation in *V. harveyi*

In most bacteria flagella mediated motility is involved in initial stages (free swimming, cell-cell and cell-surface interaction) of biofilm formation by enhancing the movement towards and along the surface (O'Toole, 2000). In *Vibrio spp.* the impact seems to extend beyond attachment (Yildiz and Visick, 2009). In many *Vibrio spp.*, like *V. cholerae*, *V. parahaemolyticus* or *V. vulnificus*, flagella mediated motility is important in biofilm formation. Lack of the flagella lowers the ability to attach on surfaces (Watnick and Kolter, 1999; Enos-Berlage *et al.*, 2005; Lee *et al.*, 2004). We analyzed the influence of AIs on the swimming behavior and the synthesis of flagella in *V. harveyi* using solidified medium and phase contrast microscopy after staining of the flagella, respectively. The diameter of the bacterial lawn grown on solidified medium was measured. Nearly no differences between the wild type and the mutants JAF78 (AIs-ON) and MM77 (HAI-

1/AI-2-OFF) were found on solidified rich complex medium like described before (Lilley and Bassler, 2000). In contrast the same experiment on solidified minimal medium revealed significant differences between the wild type, the AIs-ON mutant JAF78 and the HAI-1/AI-2-OFF mutant MM77 (Fig. 5.4A). JAF78 showed no swimming on solidified minimal medium within the first 25 h and only 20% of the cells in a liquid culture produced a flagellum within the exponential growth phase (Fig. 5.4B).

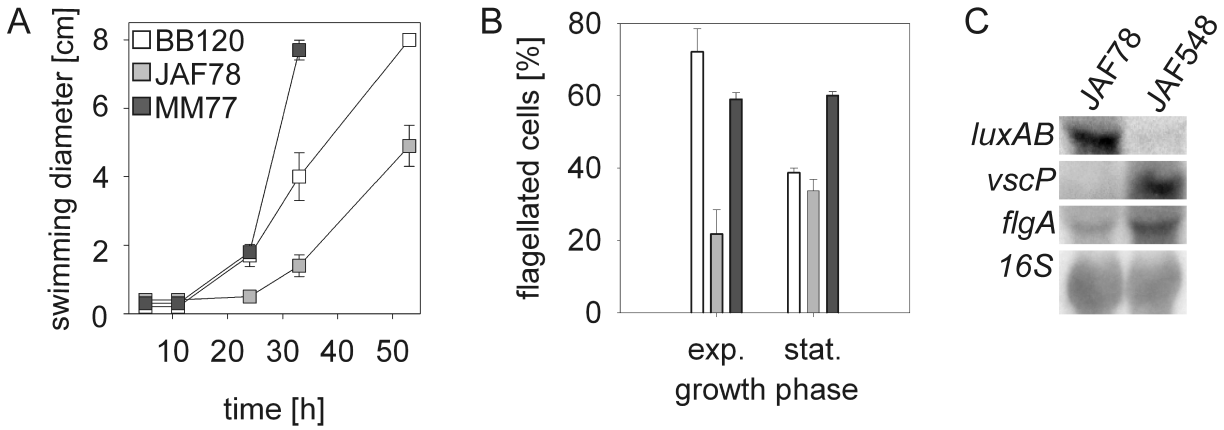


Figure 5.4 Swimming behavior and synthesis of flagella in *Vibrio harveyi*. (A) Strain BB120 (white), JAF78 (gray) and MM77 (dark gray) were cultivated on AB swimming plates. After 5 h, 11 h, 24 h, 33 h and 50 h the swimming diameter was measured. Strain MM77 reached the border of the plate after 50 h (data was not included). All experiments were performed in triplicate. (B) Flagella of exponential and stationary grown cells of strain BB120 (white), JAF78 (gray) and MM77 (dark gray) were stained and monitored using phase contrast microscopy. At least three pictures, each containing about 100 (exp. growth phase) or 200 to 400 cells (stat. phase), of every strain and growth phase were quantified. (C) Northern blot analysis of *flgA* (flagellin synthase) was performed for strains JAF78 (AIs-ON) and JAF548 (QS negative mutant). As controls the AI-induced gene *luxAB* (luciferase) and the AI-repressed gene *vscP* (type III secretion) were explored. To ensure identical RNA quantities the 16S gene was analyzed.

In contrast MM77 and the wild type demonstrated an identical swimming behavior in the first 25 h. 60% and 75% of the cells of MM77 and the wild type, respectively, were flagellated within the exponential growth phase (Fig. 5.4B). In the stationary phase about 40% of cells of JAF78 were flagellated, which corresponds to the wild type (Fig. 5.4B). These two strains also showed identical swimming behavior after 25 h, whereas MM77 formed larger swimming diameters (Fig. 5.4A). The percentage of flagellated cells of MM77 remained high (Fig, 5.4B). High AI concentrations (like mimicked in strain JAF78) repress the synthesis of flagella in *V. harveyi*. However, other regulatory impacts seem to be important since the AIs-ON mutant JAF78 shows swimming behavior and

also forms a flagellum (Fig. 5.4A and B). Transcriptional analysis via Northern blot (Fig. 5.4C) comparing strains JAF78 (AIs-ON) and JAF548, a QS constitutive negative mutant - caused by a single point mutation in *luxO* (D47E) mimicking the phosphorylated form of LuxO - confirmed these results. Transcripts of *flgA* (flagellin synthase) were traceable in strain JAF78, but highly reduced compared to mutant JAF548. The AI-repressed gene *vscP* (type III secretion system), used as an AI-negative regulated control, showed no band in the AIs-ON strain (Fig. 5.4C).

5.3.5 QS has an influence on biofilm formation and on the production of exopolysaccharides, one component of the matrix, in *Vibrio harveyi*

A measurement of the biofilm formation over time for the wild type and strain JAF78 (AIs-ON) was published before (Anetzberger *et al*., 2009). In both strains biofilm formation was detectable since the early stationary phase. JAF78 produced two times less biofilm than the wild type over the whole analyzed time period. The same result was archived after adding concentrated cell-free wild type culture fluid to a growing wild type (Anetzberger *et al*., 2009) or 5-fold concentrated synthetic AIs (data not shown). Accordingly, mutant MM77 (HAI-1/AI-2-OFF) was incubated in culture plates and analyzed for biofilm formation with subsequent quantification (Fig. 5.5).

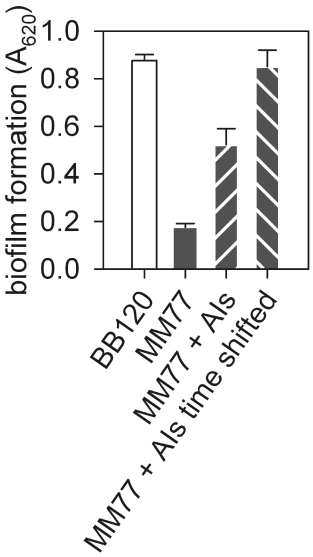


Figure 5.5 Induction of biofilm formation in strain MM77 of *Vibrio harveyi*. Cultures of MM77 (HAI-1/AI-2-OFF) incubated without or with synthetic exogenously added HAI-1 and AI-2 (10 µM each) were grown in culture plates for 25 h. For quantification the biofilms were stained with crystal violet and displayed as measured absorbance at 620 nm. MM77 without AIs is illustrated in dark gray. AIs were added at the beginning of the experiment (dark gray, stripped to the right) or time shifted (AI-2 after 2 h, HAI-1 after 6 h) (dark gray, stripped to the left). Wild type BB120 (white) was used for comparison. All experiments were performed at least in triplicate.

Only a marginal biofilm formation was determined for MM77. Biofilm formation could be induced by adding AIs exogenously. After adding both AIs, HAI-1 and AI-2, at physiological concentrations at the beginning of cultivation about 60% biofilm compared to the wild type was formed. A recently performed analysis of the AI concentrations of *V. harveyi* in a growing culture (Anetzberger *et al*., 2012a), revealed that HAI-1 and AI-2 are synthesized to different time points. After adding AI-2 and HAI-1 to these time points, i.e. AI-2 2 h and HAI-1 6 h after the beginning of the experiment (dilution 1:100), MM77 produced as much biofilm than the wild type.

Mature biofilms consists of an extracellular matrix that enables attachment to the surface and cohesion of the cells. Exopolysaccharides, a component of the extracellular matrix, are the most common part of a *Vibrio* biofilm and responsible genes/operons have been identified for numerous *Vibrio spp*. (Yildiz and Visick, 2009). The magnitude of exopolysaccharide production was determined in the different QS strains of *V. harveyi* (Fig. 5.6).

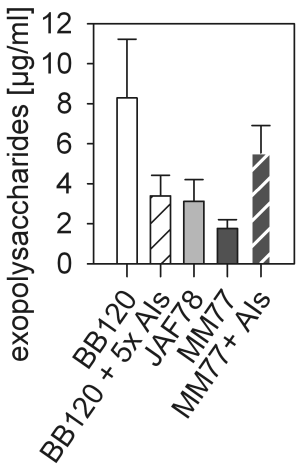


Figure 5.6 Synthesis of exopolysaccharides in different strains of *Vibrio harveyi*. Exopolysaccharides were determined by using the acid phenol method and are displayed as bars in µg/ml cell culture. Performed experiments are: wild type BB120 (white), BB120 + exogenous added AIs (5-fold concentrated) (stripped white), AIs-ON mutant JAF78 (gray), HAI-1/AI-2-OFF mutant MM77 (dark gray) and MM77 + exogenous added AIs (physiological concentrations) (stripped dark gray). All experiments were performed at least in triplicate.

The results were comparable to those for the quantitative biofilm analysis (Fig. 5.5; Anetzberger *et al*., 2009). Strain JAF78 (AIs-ON) and the wild type incubated with additional AIs (5-fold concentrated) produced 37% and 41% of exopolysaccharides compared to the wild type. This is in accordance with our finding, that the biofilm of JAF78 has a 2-fold reduced height (Fig. 5.3). Strain MM77 (HAI-1/AI-2-OFF) alone showed a percentage of 20%. This explains why MM77 only forms small aggregates on the surface (Fig. 5.3). The percentage increased after induction with AIs (physiological concentrations) to 66%. Exogenous added AIs could induce the production of

exopolysaccharides in MM77, but not in the same range as the wild type. Originating in the method, the experiment with adding AIs time shifted, like in the method before (staining with crystal violet), was not accomplishable, because the cells were not grown in fluid culture, but within a bacterial lawn on solidified medium. AIs could only be added at the beginning of the experiment. The formation of exopolysaccharides determines the formation of a biofilm matrix in *V. harveyi*.

5.4 Discussion

Bacteria are often referred to as single cell organisms. But studying microbial development in the last decades revealed that also bacteria are capable of collective behaviour and complex differentiation (O'Toole *et al*., 2000), like described for *Caulobacter* (Shapiro *et al*., 1971), *Bacillus* (Losick *et al*., 1986), or *Myxococcus* (Kaplan, 1991). Another form of multicellular behavior of bacteria was described in the last years. Population heterogeneity provides the possibility to share 'public goods' such as degradative enzymes or material for an extracellular matrix and is known for *Bacillus spp.* (Chai *et al*., 2008), *Agrobacterium spp.* (Goryachev *et al.*, 2005), and *Pseudomonas spp.* (Diggle *et al*., 2007). In addition, population heterogeneity was also found for the public goods bioluminescence, exoprotelytic activity, and type III secretion in *Vibrio spp.* (Anetzberger *et al*., 2009; Anetzberger *et al*., 2012b). Microbial development is also found for the formation of multicellular surface-associated assemblages termed biofilms with a spatiotemporal regulation of gene expression (O'Toole *et al*., 2000; Vlamakis *et al*., 2008). *V. harveyi* synthesize the AHL HAI-1 and the widespread AI-2. Both have a function in biofilm formation, whereas CAI-1, the third AI, seems not to be important. A mutant not able to synthesize neither HAI-1 nor AI-2 (MM77) showed the same lack in biofilm formation than a mutant with inactivated genes encoding the CAI-1 synthase and the HAI-1 and AI-2 kinases (JMH626). For these mutants the QS system is in a constitutive low AI status (for MM77 only for HAI-1 and AI-2) within all analyzed growth phases. The transcripts of the master regulator LuxR are destabilized, exopolysaccharide production is repressed, and flagella synthesis is induced. Biofilm formation is hardly induced. A monolayer with rare outgrowths is formed. Mutant MM77 is able to form stable cell-cell and cell-surface interactions using its flagella, but

does not produce exopolysaccharides, the main component of the biofilm matrix, at high concentrations. Therefore only a two-dimensional biofilm develops. The AIs-ON mutant JAF78 (or the wild type incubated with an excess of AIs) is in a constitutive high AI status. The low number of synthesized flagella causes reduced cell-cell and cell-surface interactions, but exopolysaccharides are produced. This results in a biofilm with reduced stability. In comparison to the wild type less exopolysaccharides and hence less biofilm matrix is synthesized. This may be explained by the additional consumption of energy for the increased QS-induced processes, at least bioluminescence and exoproteolytic activity. In particular bioluminescence is a high energy consuming process. Furthermore, all cells of strain JAF78 (or the wild type incubated with an excess of AIs) produce light at high intensity, whereas maximal 70% of a wild type population are bright (Anetzberger *et al.*, 2009). The dark part of the wild type population may have energy resources left and might be responsible for the production of other QS-regulated processes like biofilm formation. Only for the wild type a mature and stable three-dimensional biofilm was archived. The wild type shows regulated time courses of the AIs to distinguish between different growth phases (Anetzberger *et al.*, 2012a) in order to induce/repress genes when needed, e.g. genes responsible for flagella and exopolysaccharide synthesis important for biofilm formation.

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6 Concluding Discussion

The interest on cell density-dependent gene regulation, best known as quorum sensing (QS), continuously increased within the scientific world in the last decades. Even industrial companies have a great interest in the detailed analysis of QS mechanisms to enhance the production of antibiotics and proteases in bacteria and to prevent biofilm formation. Biofilms protect the cells within from antibiotics, and the appearance of resistances, mainly in aquacultures, became a major problem (Lavilla-Pitogo *et al*., 1998). Consequently, the point of view has changed to use anti-QS mechanisms instead of antibiotics to disturb the system in order to circumvent the formation of aggregates and biofilms in industry, but also in the medicine sector to establish new therapeutical treatments (Federle and Bassler, 2003).

In the last decades, the marine bacterium *Vibrio harveyi* turned out to be the perfect model organism to study QS. It has a very complex QS system, combining characteristics of both, Gram-negative and Gram-positive bacteria (Miller and Bassler, 2001; Mok *et al*., 2003). Signal recognition and transfer occur according to the principle of bacterial two component systems (Kofoid and Parkinson, 1988; Parkinson and Kofoid, 1992). Thereby, the information of a multitude of different systems is channeled into one signaling cascade. A model of the QS system of *V. harveyi* (shown in Fig. 1.7) was created based on sequence analysis and knockout mutants (Bassler *et al*., 1994a; Bassler *et al*., 1994b; Freeman and Bassler, 1999; Freeman *et al*., 2000; Henares *et al*., 2012; Henke and Bassler, 2004; Schauder *et al*., 2001). Biochemical analysis proofed the phosphotransfer from LuxN/LuxQ/CqsS to LuxU and further to LuxO and the dephosphorylation of LuxU caused by LuxN/LuxQ/CqsS *in vitro* (Antzberger *et al*., 2012; Stambrau, 2008; Timmen *et al*., 2006, Wei *et al*., 2012).

To enhance the knowledge on cell-cell communication of *V. harveyi*, this work focused on single cell analysis of AI-regulated processes and the measurement of these processes in parallel with the prevalent AI concentrations during growth of a wild type population. Since *V. harveyi* is one of the rare bacteria able to produce bioluminescence, this AI-induced phenotype could be examined in a microscopic analysis using wild type *V. harveyi* cells and a high-sensitivity camera. Transcriptional fluorescent reporter strains were constructed to analyze additional AI-regulated processes at the single cell

level. In addition different AI-induced processes were analyzed in a growing wild type culture during exponential and stationary growth. Furthermore, different QS mutants were used to examine the influence of AIs on various regulated phenotypes. The new results provide more detailed insights into the QS system of *V. harveyi* and enhance the knowledge on bacterial cell-cell communication.

6.1 Bacterial cell-cell communication – predominant perspectives

QS is a form of cell-cell communication mainly used by non-differentiating bacteria and was investigated extensively during the last decades. The prevalent hypothesis says, that QS-regulated genes are expressed/repressed in dependence of the actual cell density by measuring the excreted AIs to optimize processes that are beneficial for the whole population (Miller and Bassler, 2001). More ideas are present in the literature whereof diffusion sensing (Redfield, 2002), efficiency sensing (Hense *et al*., 2007), and confinement-induced QS (Carnes *et al*., 2009) are presented in more detail. For diffusion sensing AIs are excreted in order to determine the actual diffusion force. The production of degrading enzymes or transport systems is only beneficial at low diffusion. The evolution of diffusion sensing is easier to understand than the evolution of cell density sensing, describing a complex social behavior. It is an autonomic activity of a single cell leading to a direct fitness enhancement. The third hypothesis, efficiency sensing, is a mixture of the two previous ones combining cell density, limitation of the material exchange, and spatial distribution. The cells test the efficiency of producing metabolic costly extracellular effectors (e.g. proteases, siderophores) by releasing cheap diffusible AIs. Since the AI concentration is influenced by the same factors as the concentration of the effectors, both concentrations are highly correlated. The costs for a homoserine lactone (like HAI-1)/AI-2 amount to only ~10/0-1 ATP molecules compared to ~200 ATP molecules for the production of an oligopeptide used as extracellular effector (Keller and Surette, 2006). Besides, measuring with high sensitivity can reduce the concentration of AIs even further and only one molecule needs to be produced to measure the efficiency of a multitude of effectors. So efficiency sensing is a cost server. All perspectives described so far depend strictly on the prevalent local AI concentration and should operate at the single cell level as well. Carnes *et al.* developed a physical

system to isolate individual cells to examine confinement as a mechanism to induce QS (Carnes *et al*., 2009). The authors proved autoinduction for an individual, chemically and physically isolated *Staphylococcus aureus* cell. Isolated *S. aureus* cells are able to sense confinement through an increased extracellular AI concentration. Autoinduced cells activate genes needed for survival in such an environment together with virulence factors. This extends the current models for cell-cell communication by the additional benefit of a better survival of the individual cell. In confinement-induced QS selection occurs at the level of the individual and makes the inclusion of a complex social behavior for the evolution and maintenance of QS unnecessary (Carnes *et al*., 2009). The four perspectives are presented in Table 6.1.

Table 6.1 Quorum-, diffusion-, efficiency- and confinement-induced quorum sensing – a comparison

Perspective	Quorum sensing	Diffusion sensing	Efficiency sensing	Confinement-induced QS *
How and why cells sense	Emission and sensing of small diffusible molecules (AIs), regulation of gene expression			
What cells sense	Cell density	Mass-transfer properties of the environment	Combination of cell density, mass-transfer and spatial cell distribution	Confinement through increased extracellular AI concentration
Why cells sense	Detection of situations in which cell density is sufficient to make a coordinated response	Detection of situations in which mass-transfer is sufficiently limited for producing extracellular effectors	Estimation of the efficiency of producing extracellular effectors	Detection of a situation where confinement occurs to activate virulence factor pathways and to induce genes needed for survival in such an environment
Benefit, evolution	Group fitness	Individual fitness	Group and individual fitness	Individual fitness

Adapted after Hense *et al*., 2007; * Carnes *et al*., 2009

The major disadvantages of QS concern evolutionary stability (outcompeting through cheaters) and complexity (complex environment, spatial distribution). The theory of diffusion sensing avoids evolutionary problems by the assumption that each cell measures the diffusion force as an autonomous activity. However, the problem concerning a complex environment remains, since both QS and diffusion sensing rely on analysis in cell-mixed liquid cultures. The theory of efficiency sensing also stands in complex natural environments, where spatial distribution often has a greater influence

on the AI concentration than the cell density. Confinement-induced QS describes the advantage of a single cell to produce AIs in isolation and/or as a pathogen and is therefore independent of cell density and spatial distribution. It depends on the bacterium, the living condition, and the analyzed phenotype which perspective describes the situation of cell-cell communication best. The problems of QS are for example reduced in microcolonies being a clonal population (evolution) and minimizing diffusion (complexity) (Hense *et al*., 2007). There are examples for AI-regulated phenotypes where indeed the cell density and not the diffusion force (mass-transfer) are important. Type III secretion would not be effective when the neighboring cells are bacteria rather than host cells (in a biofilm). Therefore, the cells turn off type III secretion at high cell density (Hense *et al*., 2007). Also bioluminescence (produced enzymatically inside the cell) does not involve the release of diffusible effectors and is only reasonable at high cell density (Hense *et al*., 2007).

6.2 Quorum sensing and phenotypic heterogeneity

The prevalent assumption, coming along with all discussed perspectives before, is that all bacteria of a QS-committed population respond uniformly after the AIs reached a certain threshold concentration (Bassler and Losick, 2006; Keller and Surette, 2006). However, more and more results reveal significant cell-to-cell variations in gene expression of bacteria, which cause bimodal, multimodal or Gaussian distributions and therefore phenotypic differences within an isogenic population (Bongaerts *et al*., 2002; Elowitz *et al*., 2002). In order to test this prevalent assumption luminescence and fluorescence microscopy in combination with a high sensitivity camera were used to analyze the light intensity of single *V. harveyi* wild type (chapter 2) and fluorescent reporter cells (containing promoter fusions for QS-regulated genes) (chapter 3). Both, luminescence and fluorescence microscopy, revealed population heterogeneity. The heterogeneous behavior was consistent in different tested conditions (medium, temperature) and in different *V. harveyi* wild type strains (newly isolated strains). These findings on the single cell level disprove the view that communication via AIs leads to a synchronization of all cells of the population (Bassler and Losick, 2006; Keller and Surette, 2006), at least for *V. harveyi* (this thesis). Also for *Aliivibrio fischeri* (Perez and

Hagen, 2010) and *Listeria monocytogenes* (Garmyn *et al*., 2011) a heterogenous response for AI-regulated genes was found. In addition, phenotypic heterogeneity was described for *Agrobacterium* (Goryachev *et al*., 2005), *Bacillus* (Chai *et al*., 2008) and *Pseudomonas* (Diggle *et al.*, 2007) and is suggested to cause a selective advantage in fast fluctuating environments (Acar *et al*., 2008; Kussell and Leibler, 2005).

6.2.1 Origin of phenotypic heterogeneity

In the lactose and arabinose utilization systems of *E. coli* a bistable response (also called an all-or-nothing response) occurs at non-saturating concentrations of the sugar molecules (Megerle *et al*., 2008; Ozbudak *et al*., 2004). Here, bistability is caused by the autocatalytic positive feedback of the sugars to their uptake proteins. Positive feedback loops are less well characterized in QS-regulated processes. In *A. fischeri* it seems to depend solely on LuxI and LuxR (Sayut *et al*., 2007), whereas in *V. harveyi*, with its highly complex QS system, only one positive feedback loop was described so far, the so-called LuxN loop (Teng *et al*., 2011). This loop induces an increased transcription of *luxN*, encoding the sensor kinase recognizing HAI-1, when the HAI-1 concentration rises and causes therefore a higher sensitivity towards HAI-1 (Teng *et al*., 2011). We suggest that the source for heterogeneity in the QS system of *V. harveyi* relies on the heterogeneous distribution of the master regulator LuxR from cell to cell. Single cell analysis of a reporter strain with a LuxR-mcherry fusion protein revealed a graded LuxR concentration from 200 to 1200 copies per cell after induction with AIs (Teng *et al*., 2010). LuxR induces in turn the expression of different classes of QS-regulated genes (Tu and Bassler, 2007). In addition, we propose that *V. harveyi* tightly regulates the AI concentrations. Adding additional AIs could increase the percentage of bioluminescent cells from 70% to 98% (chapter 2). The QS signaling cascade contains an ultrasensitive switch regulated by the level of phosphorylated LuxO (dependent on the AI concentrations) and therefore the expression of sRNAs influencing the LuxR concentration (Lenz *et al*., 2004). Altogether, the phosphorylation state of LuxO in combination with the sRNA triggered level of *luxR* mRNA and positive feedback regulations might explain the observed heterogeneity of QS-regulated processes. Some cells reach the threshold concentration of LuxR required for the induction of a QS-regulated gene/operon and some do not. This results in a heterogeneous population.

Beyond that a high cell-to-cell variance in induction was found, which is in agreement with the proposed LuxR distribution from cell to cell and the regulated AI concentrations.

6.2.2 Phenotypic heterogeneity and pathogenesis

Bacteria cause major damage by infectious diseases (Tan *et al*., 2007) and are the main problems of aquacultures used in seafood industry. Vibriosis, an infection with high mortalities, is found in almost any type of cultured sea organisms from fish over crustaceans to molluscs (Defoirdt *et al*., 2007). *V. harveyi* causes vibriosis mainly in shrimps and is often responsible for the death of the whole shrimp population grown in aquaculture (Lavilla-Pitogo *et al*., 1998). It is becoming more and more obvious, that there is a link between QS and pathogenesis by the regulation of certain phenotypes and virulence factors responsible for the disease by AIs, like proteases, hemolysins, and siderophores (Natrah *et al*., 2011). In this work heterogeneous promoter activity was found for an exoprotease and the type III secretion system of *V. harveyi* using single cell analysis (chapter 3). Also in *S. enterica* a heterogeneous expression of the type III secretion system *ttss-1* was found (Sturm *et al*., 2011). Type III secretion enables the cell to manipulate the host and initiate the disease. The authors found out that the percentage of the TTSS-1$^+$ subpopulation increased from <10% to 60% during the late exponential growth phase, despite a major growth penalty compared to the TTSS-1$^-$ cells. AI-regulated processes could only evolve, if cells investing their individual resources for the benefit of the whole population reproduce better as cells living selfishly (Redfield, 2002). In order to explain the evolution and maintenance of TTSS-1$^+$ and TTSS-1$^-$ subpopulations the authors predict, according to the 'division of labor' model, that the TTSS-1$^+$ phenotype must confer an advantage from a 'public good', i.e. increased growth in the gut lumen and enhanced transmission.

6.2.3 Phenotypic heterogeneity, public goods and division of labor

Population heterogeneity enables non-differentiating bacteria to behave multicellularly to share 'public goods' (also called 'community goods' or 'commun goods') (Dunny *et al*., 2008). The investment of a public good is costly for the individual cell, but benefits the community. Degradative enzymes, material for a biofilm matrix, siderophores, and

putatively bioluminescence are public goods. Resource supply and the level of relatedness of nearby cells are important factors in the evolution of cooperation (Brockhurst *et al*., 2008; Buckling *et al*., 2007). In case when a public good is needed for growth, virulence declines with decreasing relatedness of the cells (Buckling and Brockhurst, 2008). The production of a public good must provide a direct benefit to the reproduction rate (direct or indirect via kin cells) of the acting cell that outweighs the investment (West *et al*., 2006). But as the production of public goods benefits all individuals, cells that do not bear the costs or cheaters may evolve. In *V. harveyi* also not all cells show expression of public goods. Promoter activity of a gene encoding an exoprotease was found in maximal 80% of the cells. Also bioluminescence was only activated in a fraction (maximal 75%) of the population. Interestingly, cells with a high promoter activity for the gene encoding the exoprotease did not show high luminescence values and vice versa (chapter 3). Here we propose that the cells of a *V. harveyi* wild type population divide labor in QS-regulated processes. In order to find the advantage of a heterogeneous population (= wild type) a constitutive QS-positive mutant was analyzed according to QS-regulated processes. In this *luxO* deletion mutant a high AI concentration at all times is mimicked independently of the prevalent AI concentration. As assumed all living cells of the deletion mutant showed luminescence with a significant lower variance than the wild type (chapter 2). Exoproteolytic activity was induced earlier in the deletion mutant than in the wild type and biofilm formation was significantly reduced compared to the wild type (chapter 4 and 5). We suggest that dark cells use their saved energy to produce extracellular substances to build a biofilm matrix. This is the first indication of division of labor in a QS-committed population. The mutant phenotype could be mimicked in the wild type by adding additional AIs to the culture, indicating that a tight regulation of the AI concentrations causes heterogeneity and consequently division of labor in *V. harveyi*.

6.3 Timing in the quorum sensing system of *Vibrio harveyi*

V. harveyi produces three different AIs, the homoserine lactone HAI-1, the furanosyl-borate diester AI-2, and the aminoundecenone CAI-1. Nevertheless, it is still unanswered why *V. harveyi* produces three different types of AIs. To address this question we

quantified the concentrations of the three AIs in a growing culture of *V. harveyi* and analyzed their influence on different QS-regulated processes.

6.3.1 Growth phase-dependent regulation of autoinducers

We found alterations in the onset of the production of the signaling molecules and their concentrations during the growth of a *V. harveyi* wild type population (chapter 4). According to these alterations of the three AIs produced, certain growth phases are characterized by distinct ratios of AIs.

In the literature examples for bacteria adjusting the concentration of AHLs growth phase-dependently via synthesis and degradation were found e.g. for *P. putida* (Fekete *et al*., 2010), *Bradyrhizobium japonicum* (Lindemann *et al*., 2011), and *Aeromonas hydrophila* (Swift *et al*., 1997). *Agrobacterium tumefaciens*, a plant pathogen, produces a 3-oxo-octanoyl-homoserine lactone and tightly regulates the concentration of this molecule over synthesis and degradation resulting in distinct concentrations within certain steps during host interaction (Haudecoeur and Faure, 2010). Input signals are described to be tightly regulated at the level of synthesis (Metha *et al*., 2009). In addition, AI systems can be deactivated if the conditions do not favor the AI-regulated phenotype (Schuster and Greenberg, 2006), often at the transition to the stationary phase, during starvation or after other changing environmental or metabolic conditions (Gonzalez and Keshavan, 2006; Newton and Fray, 2004; Yates *et al*., 2002; Zhang *et al*., 2004).

Lactonases and acylases are common in the bacterial world and are said to play an integral part in QS systems using AHLs. We found a HAI-1-degrading activity in the cytosol of *V. harveyi* and identified a cytosolic lactonase (VIBHAR_02708), able to degrade the HAI-1 *in vitro* by hydrolyzing the ester bond of the lactone ring (Schell, 2010). An influence of this lactonase on the QS system of *V. harveyi in vivo* still needs to be proved. Alterations of the extracellular AI-2 concentration did not correlate with the transcriptional regulation of *luxS*, one gene required for its synthesis in *V. harveyi* (unpublished observation), *E. coli* (Xavier and Bassler, 2005), *Salmonella* (Beeston and Surette, 2002), and *Shewanella* (Bodor *et al*., 2011). In *E. coli* and *S. typhimurium* active AI-2 transport via the Lsr transporter system, which is induced by AI-2, and subsequent metabolisation was described (Taga *et al*., 2003; Xavier and Bassler, 2005) and accounts

for the resulted decrease in concentration. Since no Lsr homologues are found in *Vibrio spp*., another ABC type transporter called Rbs was proposed to uptake AI-2 (Rezzonico and Duffy, 2008). The proposed binding protein RbsB (VIBHAR_06143) of *V. harveyi* was able to complex AI-2 *in vitro* (unpublished observation). However, knockout mutants for RbsB or RbsC (transport unit) did not show altered AI-2 concentration curves (Reiger, unpublished observation).

Mechanisms to degrade AIs are part of so-called quorum quenching systems. AI-quenching mechanisms endow the possessing bacteria to successfully combat other groups of microorganisms by disturbing their QS system. Besides bacteria also eukaryotes use quorum quenching to repel bacterial infections. QS often regulates genes responsible for host colonization. Plant roots activate defense mechanisms in the presence of AHLs of *P. putida* (Schuhegger *et al*., 2006). Mammalian cells respond to AHLs by inducing inflammation or respiratory reflexes to prevent bacterial invasions (Tizzano *et al*., 2010). Therefore a down-regulation of AHLs might be advantageous for bacteria to evade host responses. Also shrimps, as the main host for *V. harveyi*, might activate defense mechanisms in the presence of AHLs. In summary, quorum quenching is important to regulate the AI concentrations within the same species, to disturb QS in other species, and to evade host responses.

The three AIs of *V. harveyi* show alterations according to appearance and concentrations over time (chapter 4). Only for CAI-1 a clear distinction in low (exponential growth phase) and high (stationary phase) cell density is seen. HAI-1 is induced in the mid-exponential growth phase, reaches its maximum at the late exponential growth phase and remains constant thereafter. AI-2 is induced in the early exponential growth phase, reaches a maximum in the mid exponential growth phase, decrease afterwards and is adjusted to the same concentration as HAI-1 in the stationary phase. *V. harveyi* does not use AIs to monitor the cell density *per se*, but rather to distinguish between different growth phases by producing distinct ratios of AIs.

6.3.2 Timing of quorum sensing-regulated processes

Like described before, different time periods are distinguishable during the growth of a *V. harveyi* population based on variations in the AI concentrations. At time point 1 and 2 (lag and early exponential growth phase) only AI-2 is present, at time point 3 (mid and

late exponential growth phase) the concentrations of HAI-1 and AI-2 reach their maxima, and at time point 4 (stationary phase) all three AIs, HAI-1, AI-2 and CAI-1, are available. These time points correspond with the postponed time course for bioluminescence, exoproteolytic activity, and biofilm formation (chapter 4 and 5). These different time curves can be explained by the different blends of AIs available at certain growth phases. For the induction of bioluminescence AI-2 alone was sufficient, the addition of HAI-1 further increased luminescence. Production of exoprotease was induced when both, HAI-1 and AI-2 were available. Biofilm formation was induced in the stationary phase but HAI-1 and AI-2 alone are sufficient for full induction. CAI-1 plays no role in biofilm formation of *V. harveyi* (chapter 5). In addition, it was already reported that HAI-1 and AI-2 are needed for repression of a gene encoding the type III secretion system (Mok *et al*., 2003). The sRNA *qrr4* can be induced either by HAI-1 or by AI-2, but both have to be present for full induction (Long *et al*., 2009). By disturbing the AI ratio by adding HAI-1/AI-2 in excess within the lag phase, bioluminescence and exoproteolytic activity are slightly influenced (higher bioluminescence value, earlier onset time of exoprotease activity) (chapter 4), whereas biofilm formation is drastically disturbed (chapter 5). Similar results were achieved with a *luxO* deletion mutant, mimicking high AI concentrations to all times. In contrast to bioluminescence (*lux*-operon) and exoproteolytic activity (gene *vhp*), a multitude of genes and operons, coding e.g. flagella and exopolysaccharide synthesis, have to be induced/repressed to form a stable and mature matrix. A tight timing of processes involved in biofilm formation is important, conducted via AI ratios. Also for *V. cholerae* the timing of HapR (analog to LuxR of *V. harveyi*) expression is crucial for biofilm thickness, detachment rates and hence intestinal colonization efficiency (Liu *et al*., 2007). In addition, it seems to be important to produce different virulence factors at various infection stages. QS induces exoprotease, caseinase and gelatinase activities in *V. harveyi*, whereas phospholipases and a chintinase are repressed (Defoirdt *et al*., 2010, Natrah *et al*., 2011). The production and detection of AIs might offer a way to time virulence factor expression (Ohtani *et al*., 2002). The signal molecule PhrA of *B. subtilis* was described as a QS signal which simultaneously acts as a timing mechanism, which delays sporulation when DNA damages are present (Jabbari *et al*., 2011). All examples support the consumption that AIs are able to act as tight timers of QS-regulated processes.

6.4 Pheromone sensing - a new perspective

In this thesis the complex cell-cell communication system of *V. harveyi* was analyzed mainly on the output level. AI-regulated processes are coordinated by producing certain AI ratios to different growth phases (chapter 4 and 5). In *V. harveyi* AIs are responsible for a perfect timing of the induction/repression of genes of the cell-cell communication regulon and do not simply monitor cell density. Besides the timer function, AIs cause heterogeneity in QS-regulated processes (chapter 2 and 3). These results are now included in the complex cell-cell communication circuit of *V. harveyi* (Fig. 6.1).

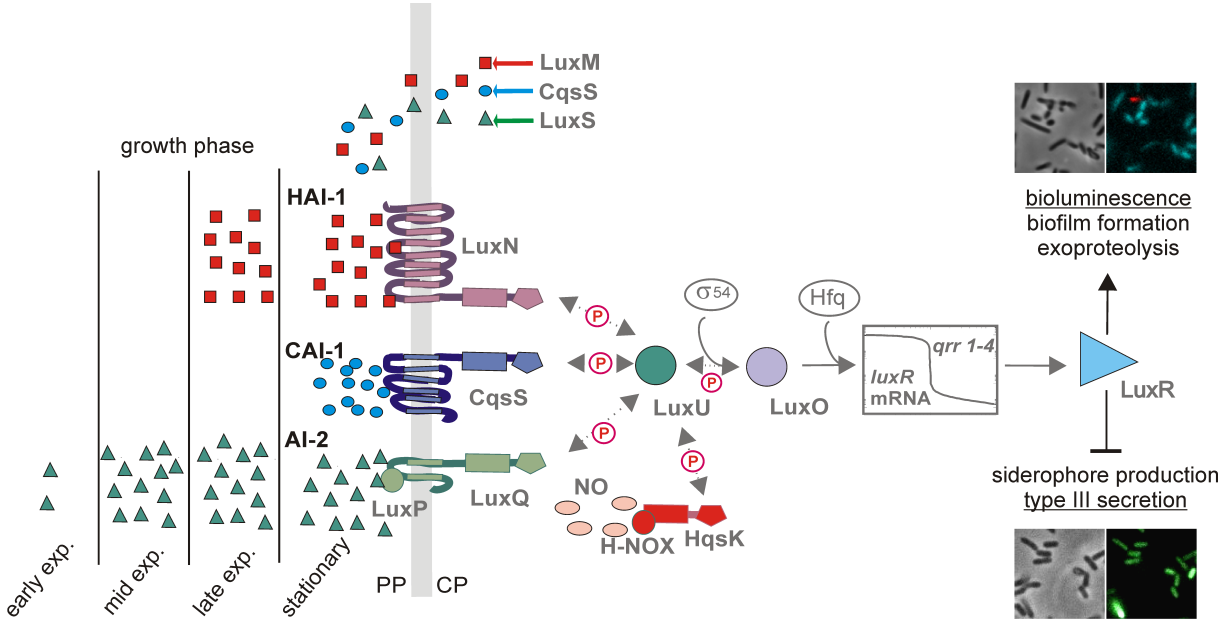

Figure 6.1 Timing and heterogeneity in the cell-cell communication circuit of *Vibrio harveyi*. The autoinducers HAI-1 (red squares), AI-2 (green triangles) and CAI-1 (blue circles) are synthesized during different growth phases. Steps for signal perception and integration are shown in gray and occur essentially like described in Fig. 1.7. Heterogeneous behavior was directly found according to bioluminescence (upper left picture: phase contrast, upper right picture: bioluminescence in cyan) and indirectly using transcriptional reporter strains for exoproteolysis (not shown) and type III secretion (lower left picture: phase contrast, lower right picture: fluorescence in green). CP, cytoplasm; PP, periplasm.

All perspectives described before on cell-cell communication (quorum, diffusion, efficiency and confinement-induced quorum sensing) assume that bacteria produce constantly AIs and that AI-dependent genes are induced in all cells of the population after reaching a certain threshold concentration (synchronized response).

According to our results, *V. harveyi* produces different AIs to distinguish between different phases during the growth of the population to ensure the induction of AI-

regulated processes when needed. For example bioluminescence is induced in the exponential growth phase, whereas exoproteolytic activity is maximal within the stationary phase. A tight timing is very important for complex behavior, like biofilm formation, where a multitude of genes has to be induced when needed. We suggest, that *V. harveyi* uses AIs for a species-specific chemical communication to organize certain behavior or developmental processes within its own population. This regulation pattern is reminiscent of "pheromone sensing" (Karslon und Luscher, 1959). Pheromones are molecules that are secreted by an individual, and received by a second individual of the same species in which they release a specific reaction, for example, a definite behavior or developmental process. Pheromones are known for mammalians to find and identify mates, to regulate the level of aggression and social dominance and the recognition of kin and non-kin (Zufall and Leinders-Zufall, 2007). In addition, no synchronized, but a heterogeneous response was found. It is assumed, that a division of labor occurs within an AI-committed *V. harveyi* population to ensure full availability of extracellular factors needed with simultaneous reduction of the costs for a single cell. In Figure 6.2 a generic scheme for QS and the new proposed pheromone sensing of *V. harveyi* are compared with each other. To compare the suggested perspective of pheromone sensing with the other perspectives described before, the main questions (see table 6.1) are discussed in more detail:

1. How and why do the cells sense? – Like for the other perspectives the cells produce and emit AIs, which were recognized to lead to the regulation of gene expression.
2. What do the cells sense? – The cells sense the prevalent growth phase by measuring the (growth phase specific) ratio of different produced AIs.
3. Why do the cells sense? – The cells detect the growth phase to induce certain processes needed in this growth phase or to time different processes for a complex behavior important for the whole population.
4. What are the benefits? – On the first look the benefits are mainly on the side of group fitness, but on the other side by including heterogeneous behavior also an individual fitness enhancement occurs by the division of labor.

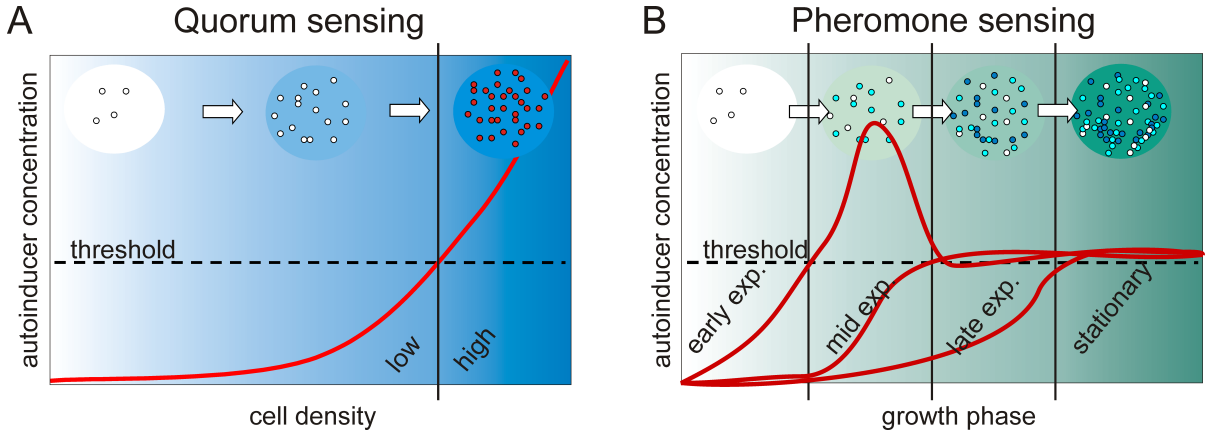

Figure 6.2 Generic scheme for a quorum sensing system (A) compared to the scheme for pheromone sensing of *Vibrio harveyi* (B). Bacterial cells (white circles) produce and emit autoinducers, which accumulate in the surrounding environment. With increasing cell density the autoinducer concentration rises (indicated by the red lines and the gradient from white to blue/green) and reaches a certain threshold concentration. The signal is recognized by receptors leading to changes in gene expression of quorum-/pheromone sensing regulated genes. (A) The classical scheme for quorum sensing describes that all cells respond to the emitted autoinducers after reaching a certain threshold concentration (red cells). (B) The suggested pheromone sensing of *V. harveyi* describes that not the cell density *per se* is important for the induction of certain genes, but that the cells can distinguish between different phases of growth (by producing different ratios of autoinducers) to induce phase specific autoinducer-regulated processes (cyan and blue cells). Also a complex behavior, like biofilm formation (indicated by cell clumping) can be regulated.

The main problem associated with pheromone sensing persists in the evolution of cheaters, which benefit from the production of public goods without investing in the production. Are the dark cells cheaters? A heterogeneous population (according to bioluminescence) can be shifted to an all-bright population by adding a high AI concentration to the population. In parallel, cells of a heterogeneous culture were plated and cultures of these colonies were analyzed using luminescence microscopy. For all tested colonies a distribution into bright and dark subpopulations was found again, confirming that this distribution is an inherent property of a clonal population (chapter 2). These results exclude that the dark cells are mutants. But are they cheaters? Cheaters should have a fitness enhancement and outcompete the whole population after certain generations. However, growth analysis revealed no differences in growth rate in a QS-negative and therefore dark strain compared to the wild type and an increase of dark cells was never found independent of the generation of cultivation (unpublished

observation). Besides, dark cells were not necessarily exoprotease-negative. So the percentage of inactive cells reduces from ~25%, if only one process is included to ~12%, if two processes are included (chapter 3). It is suggested that there are no inactive cells, if all QS-regulated processes were included. Besides, the formation of a dark and a bright subpopulation (according to bioluminescence) was found to be independent of the cultivation (medium, temperature) and the used *V. harveyi* wild type strain (chapter 2). Therefore, the observed phenomenon is neither an exception for one strain nor the result of a specific cultivation condition and seems to be a common and important feature for cell-cell communication in *V. harveyi*.

6.5 Outlook

Of the remaining questions regarding cell-cell communication and division of labor in *V. harveyi* the following are the most interesting ones: How does the same circuit time AI-regulated processes differently? Does one process inhibits the others? What is the role of the CqsA/S system in *V. harveyi*? What is the influence of different blends of the three AIs on the phosphorylation of LuxU via the kinases?

In order to answer these questions, the following experiments should be performed:

Simultaneous analysis of the three AI-induced functions bioluminescence, exoproteolytic activity, and biofilm formation should be performed on the single cell level. A reporter strain with promoter fusions using different fluorophores for the mentioned functions has to be constructed and analyzed under the microscope. Thus the percentage of active cells for each phenotype in dependence of different AI ratios/growth phases can be determined. In addition, inhibiting effects of one QS-regulated function on the others could be shown.

The influence of synthetic CAI-1 should be tested on AI-regulated phenotypes. Furthermore, transcriptional analysis comparing different conditions concerning growth phase, medium, etc. should be performed to find CAI-1-regulated processes.

Phosphorylation assays containing the three kinases LuxN, LuxQ (in combination with the AI-2 binding protein LuxP), and CqsS with all thinkable combinations of AIs are important to fully understand the QS circuit of *V. harveyi*. In addition the fourth discovered circuit H-NOX/HqsK should be included.

6.6 References to Concluding Discussion

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Poster award prize at the annual meeting of the VAAM 2011 in Karlsruhe.

Invited Talks:

Anetzberger C.
Heterogeneity and timing in the quorum sensing system of *Vibrio harveyi*
John Innes Centre Norwich, Department of Molecular Microbiology, Prof. Dr. Buttner
Young Microbiologists Mini-Symposium, 2012/04/23

Anetzberger C.
Heterogeneity in quorum sensing regulated bioluminescence of *Vibrio harveyi*
LMU Munich, Institute for Biophysics, Prof. Dr. Rädler
Lunch Seminar: Soft Matter and Biophysics, 2008/12/09

Poster presentations at conferences:

Lorenz N., **Anetzberger C**., Reiger M., and Jung K.
Signal Integration via the Lux-Phosphprelay in *Vibrio harveyi*
Mechanisms of Gene Regulation 2012
2012/10/03-05, Wartaweil (Ammersee)

Reiger M., **Anetzberger C**., and Jung K.
Playing around with signaling molecules
CAS conference – Synthetic Biology
2012/06/23-25, Munich

Anetzberger C., and Jung K.
Heterogeneity and timing in autoinducer regulated processes of *Vibrio harveyi*
VAAM conference 2012 (**VAAM travel grant**)
2012/03/18-21, Tübingen

Reiger M., **Anetzberger C.**, and Jung K.
Characterization of AHL-lactonases and their influence on the quorum sensing system of *Vibrio harveyi*
VAAM conference 2012 (**VAAM travel grant**)
2012/03/18-21, Tübingen

Anetzberger C., Thormann K., Stambrau N., and Jung K.
The ratio of autoinducers is important for induction of quorum sensing regulated processes in *Vibrio harveyi*
FEMS conference 2011 (**FEMS young scientist grant**)
2011/06/26-30, Geneva (Suisse)

Anetzberger C., Stambrau N., and Jung K.
The ratio of autoinducers determines quorum sensing regulated phenotypes in *Vibrio harveyi*
VAAM conference 2011 (**VAAM travel grant** and **Poster prize**)
2011/04/03-06, Karlsruhe

Anetzberger C., Fekete A., and Jung K.
Quorum sensing in *Vibrio harveyi*: Autoinducers act as a timer
Gordon Research Conference "Sensory Transduction in Microorganisms"
2010/01/24-29, Ventura (USA)

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Heterogeneity in quorum sensing regulated bioluminescence of *Vibrio harveyi*
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Gordon Research Conference "Microbial Stress Response"
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Anetzberger C., Pirch T., and Jung K.
Vibrio harveyi as a test system for quorum sensing effective molecules
Workshop Functional Genomics and Industrial Biotechnology
2008/06/23-25, Graz (Austria)

Anetzberger C., Pirch T., and Jung K.
Analysis of quorum sensing dependent functions of *Vibrio harveyi* at single cell level
VAAM conference 2008 (**VAAM travel grant**)
2008/03/09-11, Frankfurt

Pirch T., **Anetzberger C.**, Kraxenberger T., and Jung. K.
Serach for quorum sensing effective molecules from a metagenomic library
3rd European Conference on Prokaryotic Genomics (DECHEMA)
2007/10/07-10, Göttingen