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Acyl homoserine lactone signaling in members of the *Vibrionaceae* family

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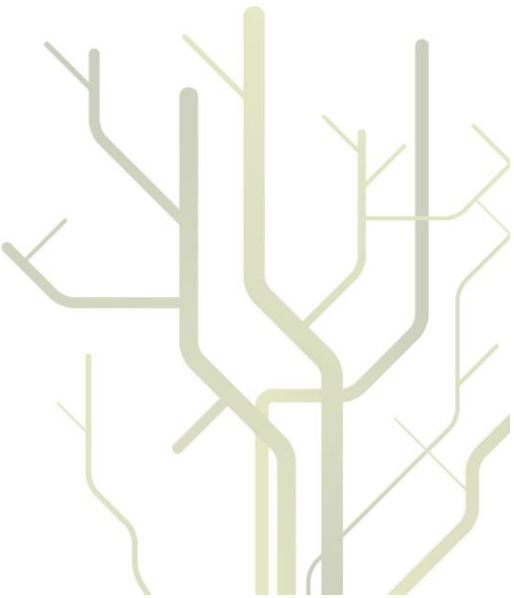

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“In the fields of observation chance favors only the prepared mind.”

- Louis Pasteur, French chemist and microbiologist

"What the mind of man can conceive and believe, it can achieve"

- Napoleon Hill, author of many books on formulas to achieve success for average person

Content

Acknowledgement

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Abbreviations

ACP – Acyl Carrier Protein
AHL – Acyl homoserine lactone
ATP – Adenosine triphosphate
CCSM – Cell communication signaling molecule
CoA – Coenzyme A
CZE – Capillary zone electrophoresis
DNA – Deoxyribonucleic acid
GC – gas chromatography
Gfp – Green flurescent protein
HCD – Higher cell density
HSL – Homoserine lactone
kDa – kilo Dalton
LCD – Lower cell density
MS – Mass Spectrometery
MTA – S-Methyl-5′-Thioadenosine
m / z – mass to charge ratio
NADH – Nicotinamide adenine dinucleotide
QS – Quorum sensing
OD – Optical density
rRNA– ribosomal Ribonucleic acid
SAH – S-adenosylhomocysteine
SAM – S-adenosyl methionine
SPE – solid phase extraction
SRM – Single reaction monitoring
TLC – Thin layer chromatography
UV – ultra violet

List of Papers

Paper 1

Amit Anand Purohit, Jostein A Johansen, Hilde Hansen, Hanna Kirsti Leiros, Alexander Kashulin, Christian Karlsen, Arne Smalås, Peik Haugen, and Nils Peder Willassen. **Presence of acyl-homoserine lactones in 57 members of the *Vibrionaceae* family.** Submitted to Journal of Applied Microbiology.

Paper 2

Amit Anand Purohit, Hilde Hansen, Ane Mohn Bjelland, Stefanie Kellermann, Hanna Kirsti Schroder Leiros, Jostein A Johansen, and Nils Peder Willassen**. Temperature dependent regulation of quorum sensing in *Aliivibrio salmonicida* LFI1238.** To be submitted.

Paper 3

Amit Anand Purohit, Hanna-Kirsti S. Leiros, Hilde Hansen, Jostein A Johansen, Peik Haugen, Annette Bayer, Magnus Engqvist, Arne Smalås and Nils Peder Willassen. **Preliminary analysis of the substrate specificity of acyl homoserine lactone (AHL) synthase VanM from *Vibrio anguillarum*.** To be submitted.

Summary

Quorum sensing (QS) is the regulation of gene expression in response to cell density. Bacteria produce, release and receive small diffusible molecules called auto-inducers that increase in concentration as a function of cell density and in response to fluctuation in concentration change their gene expression. One of such cell density dependent or cell-to-cell communication signaling molecules is acyl-homoserine lactone (acyl-HSL or AHL) which are mainly used by Gram negative bacteria. **Paper 1** presents a method for detection of 15 different AHLs using ultra high performance liquid chromatography and high resolution mass spectrometry. The limit of detection and limit of quantification was determined for all 15 AHLs. This method not only uses low amount of sample but also reduces the sample processing and data collection time. The method was successfully applied on 57 different *Aliivibrio*, *Photobacterium* and *Vibrio* strains. Detected AHLs were mapped on a phylogenetic tree generated by 16S rRNA gene sequence data and it was found that bacteria producing similar AHLs have an evolutionary relationship.

In **paper 2,** the method was taken forward to analyze the QS systems in *Aliivibrio salmonicida* LFI1238. Many mutants were created targeting genes involved in QS and were analyzed with reference to the effect on AHL production. AHLs synthesized by AHL synthases AinS and LuxI, were identified. LuxI synthase was producing seven AHLs; N-3-oxo-butyl HSL (3-oxo-C4-HSL), N-butyl HSL (C4-HSL), N-hexanoyl HSL (C6-HSL), N-3-oxo-hexanoyl HSL (3-oxo-C6-HSL), N-octanoyl HSL (C8-HSL), N-3-oxo-octanoyl HSL (3-oxo-C8-HSL), and N-3-oxo-decanoyl HSL (3-oxo-C10-HSL). On the other hand AinS synthase was responsible for producing N-3-hydroxy-decanoyl HSL (3-OH-C10-HSL). LitR mutant was able to reduce the AinS produced AHL and the effect was more prominent at a lower temperature. The *lux* operon of the *A. salmonicida* LFI1238 has two *luxR* genes and we speculate that they work as hetero dimers. Other mutants involved in the QS system such as *luxS*, *luxP*, *luxO*, and *qrr* were also tested and it was found that they did not affected the production of AHLs.

In **paper 3** an AHL synthase, VanM (LuxM family member) from *Vibrio anguillarum* NB10 was expressed, purified and stabilized. The activity was verified by the method developed in paper 1 and the reaction rate kinetics of the enzyme against variety of substrates was conducted. The enzyme was found to have a preference for C6 carbon chain length and a hydroxy group at the third position. A phylogenetic tree was also constructed based on the similar amino acid sequences to VanM enzyme and it revealed the presence of at least three types of the enzyme.

It can be concluded, from the results of these studies that the method for detection of AHLs has been improved and has increased our knowledge about the QS systems present in *Vibrionaceae* family members and *A. salmonicida*. During this study expression, purification and preliminary

substrate specificity analysis of VanM from LuxM family has also been performed. VanM does not have any known 3 dimensional homologue structure in the protein data bank. We hope that the techniques and knowledge, developed and learnt will be very useful for further research in future.

Sammendrag

Bakterier produsere små organiske molekyler som benyttes til kommunikasjon mellom celler i en prosess kalt Quorum sensing (QS). Bakterier syntetiserer, frigir og mottar disse molekylene, også kalt auto-indusere, og responsen er ofte relatert til endret genuttrykk. En type slike signalmolekyler er acyl-homoserin-laktoner (acyl HSL eller AHL), som i hovedsak brukes til kommunikasjon hos Gram negative bakterier. Paper 1 presenterer en forbedret fremgangsmåte for deteksjon av 15 forskjellige AHLer ved hjelp av ultra HPLC og høy-oppløst massespektrometri. Deteksjonsgrensen og kvantifiseringsgrensen ble bestemt for alle 15 AHLene. Denne metoden bruker ikke bare et lite prøvevolum, men reduserer også tiden som brukes til behandling av prøven og innsamlings av data. Denne metoden ble benyttet til å detektere AHLer i 57 forskjellige *Aliivibrio, Photobacterium* og *Vibrio* stammer. De detekterte AHLene ble plottet på et fylogenetisk tre basert på 16S rRNA gensekvenser og et evolusjonært slektskap ble funnet mellom bakterier som produserte like AHL mønster.

I paper 2, ble metoden benyttet til å analysere mekanismene i QS systemene til bakerien *Aliivibrio salmonicida* LFI1238. Flere mutanter av *A. salmonicida* ble gererert ved å inaktivere gener involvert i QS og studere deres AHL og biofim produksjon Først fant vi at AHLer blir syntetisert av AHL syntasene AinS og LuxI. LuxI syntase produserte sju AHLer; N-buytanoyl HSL (C4-HSL), N-3-okso-buytanoyl HSL (3-oxo-C4-HSL) ,N-heksanoyl HSL (C6-HSL), N-3-okso-heksanoyl HSL (3-oxo-C6-HSL), N-oktanoyl HSL (C8-HSL), N-3-okso-oktanoyl HSL (3-oxo-C8-HSL), og N-3-okso-dekanoyl HSL (3-oxo-C10-HSL). AinS syntase derimot produserte kun N-3-hydroksy-dekanoyl HSL (3-OH-C10-HSL). Inaktivering av master regulatoren LitR i QS systemet redusere 3-OH-C10-HSL produsert av AinS og effekten var mest fremtredende ved lav temperatur. Inaktivering av to luxR regulator gener i Lux operonet, indikerer regulering av operonet vha en luxR1/luxR2 heterodimer.

I paper 3 ble AHL syntasen VanM fra *V. anguillarum NB10* som er i LuxM familien, uttrykt, rensing og stabilisert. Aktiviteten ble målt vha metoden utviklet i paper 1 og kinetiske analyser ble utført med ulike substrater. Enzymet ble funnet å ha en preferanse for substraer med en kjedelengde på seks karboner, og en hydroksylgruppe i den tredje posisjonen på substratet. Et fylogenetisk tre ble også konstruert basert på homologe aminosyresekvenser av VanM og minst tre typer enzymer ble identifiset i denne LuxM familien.

Fra studien kan vi konkludere med at metoden for påvisning av AHLer er forbedret, og ved å benytte denne metoden har økt vår kunnskap om funksjonen av QS systemene i Vibrionaceae og

spesielt i bakterien *A. salmonicida*. I løpet av dette studiet har uttrykk, rensing og stabilisering VanM også blitt gjennomført. VanM har ingen kjent tre dimensjonal struktur i protein databaken (PDB). Vi håper at våre teknikker, erfaringer og resultater vil være nyttfor fremtidig forskning.

I. Introduction

Part 1. *Vibrionaceae* family and Quorum Sensing

1.1. *Vibrionaceae* family

Currently the bacterial kingdom is divided into 30 phylas (Euzeby, 1997), (http://www.bacterio.cict.fr/) where one of the phylum is the *Proteobacteria* phylum, described for the first time in 1980s as "purple bacteria and their relatives" (Woese, 1987, Stackebrandt et al., 1988). As shown in Figure 1, this phylum is again divided into six sections or classes. One of the class is *gammaproteobacteri*, which again is divided into 15 different orders and the *Vibrionaceae* family which belong to the *Vibrionales* order (Garrity, 2005).

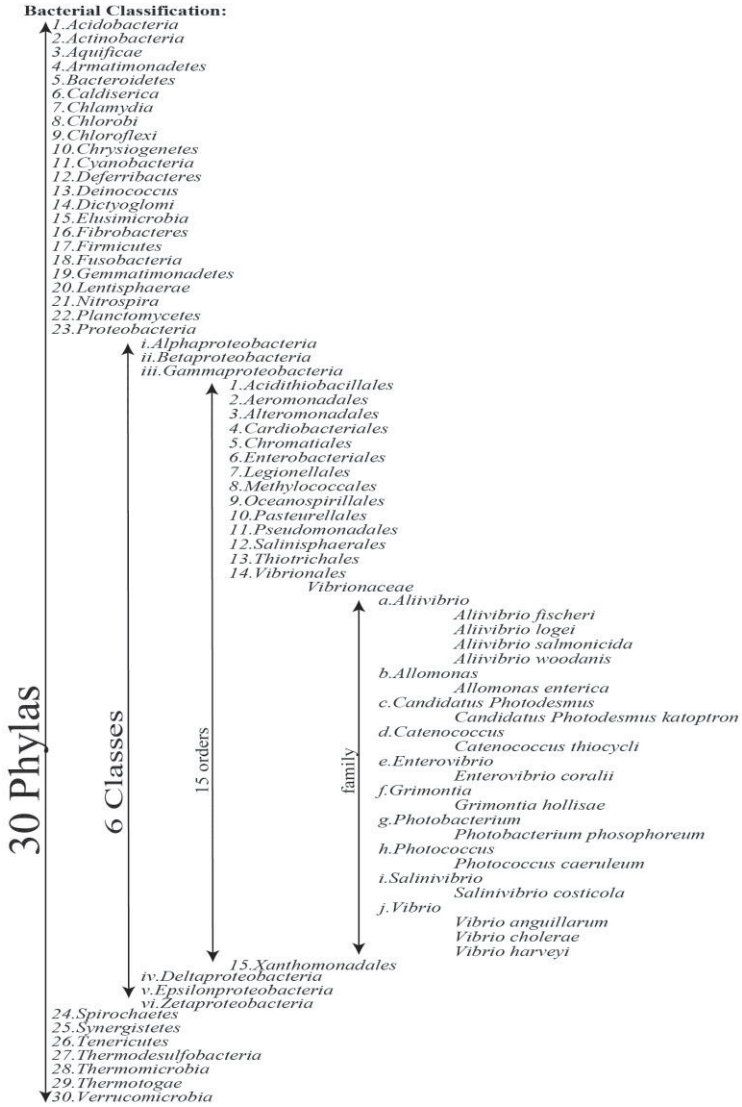


Figure 1: List of 30 phylas subdivided into classes, orders and family including the *Vibrionaceae* family. Figure is based on information from (http://www.bacterio.cict.fr/) (Euzeby, 1997).

There have been changes with respect to classification of *Vibronaceae* family members in the previous years, some of the examples are discussed in this section. *Allomonas enterica* has been evaluated based on DNA-DNA hybridization and phenotypically analyzed to be very close to *Vibrio fluvialis*. Hence, *A. enterica* has become synonymous to *Vibrio fluvialis* (Farmer, 1989). Bacteria in the *Beneckea* genus has also been renamed as *Vibrios*, some examples are *alginolytica, campbellii, gazogenes, harveyi, natriegens, nereis (nereida), nigripulchritudo, parahaemolytica, pelagia, splendida* and *vulnifica* (Baumann et al., 1980). Similarly, *Listonella* (Thompson et al., 2011) and *Lucibacterium* (Baumann et al., 1980) have also been renamed as *Vibrios*. On the other hand genus like *Enhydrobacter*, (Kawamura et al., 2012) and *Aeromonas* (Colwell et al., 1986) has been removed from *Vibrionaceae* and put in the new orders .

1.1.1. General description of *Vibrionaceae* family

Vibrio Pacini (also called *Vibrio cholerae Pacini*) is the type strain both for family *Vibrionaceae* as well as for the genus *Vibrios*. It was first described by Filippo Pacini more than 150 years ago as *Vibrions* from the fact that they moved rapidly back and forth (Nardi, 1954). *Vibrio* are Gram negative bacteria and their shape could be small, straight, slightly curved, curved, or comma-shaped rods (Baumann et al., 1984). Some marine *Vibrios* are reported to have shape resembling L forms (Onarheim et al., 1994). In liquid media they have polar flagella enclosed in a sheath for motility, whereas they have numerous lateral flagella on solid media (Garrity, 2005). Most *Vibrios* can grow in medium containing D-glucose and ammonium as the sole carbon and nitrogen sources. All *Vibrios* have the capability of fermenting D-glucose producing acid and have also a normal respiratory metabolism, which classifies them as facultative anaerobes. Other sugars that can also be used are D-fructose, maltose and in addition to glycerol (Baumann et al., 1984). Sodium ions are essential for growth of all species and the salt concentration for optimal growth ranges from 5 to 700 mM (0.029–4.1%). Moreover, most species grow well in media containing a seawater base (Baumann et al., 1984). Members of this genus are oxidase positive and reduce nitrate to nitrite. All the *Vibrios* species can be easily grown at 20-30ºC, however some also prefer temperature as low as 4ºC or as high as 35-37ºC. Some strains are also bioluminescent. Many are human pathogens causing diarrhea or extra-intestinal infections. Many species also cause infections in other vertebrates and several invertebrates. The GC content of the DNA in *Vibrios* ranges from 38 to 51% (Garrity, 2005).

1.1.2. Members of *Vibrionaceae* family

Currently there are more than 9000 members that have been reported to belong to the family *Vibrionaceae* as per the NCBI's taxonomy browser

(http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=641). However, only 62 bacteria (see Table 1) have been sequenced or their sequencing project is on-going currently.

Table 1: List of *Vibrionaceae* family members whose genomes have been sequenced fully or partially.

No	Genera; specie	Brief description	Reference or assembly no. or bioproject no.
1.	*Aliivibrio fischeri*	Found in seawater and symbiotic in marine animals.	(Ruby et al., 2005)
2.	*Aliivibrio logei*	Isolated from size fractionation of seawater	Vib-5S-186
3.	*Aliivibrio salmonicida*	Fish pathogen	(Hjerde et al., 2008)
4.	*Enterovibrio calviensis*	Isolated from plum island by size fractionation of seawater	Vib-1F-211
5.	*Enterovibrio norvegicus*	Isolated from plum island (MA,USA) by size fractionation of seawater	Vib-FF-33
6.	*Grimontia hollisae*	Could be a causative organism for human diarrhea	ASM17651v1
7.	*Photobacterium angustum*	Found in marine organisms	ASM15326v1
8.	*Photobacterium damselae*	Isolated from marine fishes and human infections	(Kim et al., 2008)
9.	*Photobacterium leiognathi*	Found in light organs of marine fishes	ASM21149v1
10.	*Photobacterium profundum*	Isolated from sea sediments	(Vezzi et al., 2005)
11.	*Photobacterium sp.* SKA34	Isolated from the North Atlantic at a depth of 2-5 meters	ASM15332v1
12.	*Vibrio alginolyticus*	Dissolves alginic acid from seaweed. Also found in human soft tissue infections	ASM17605v1
13.	*Vibrio anguillarum*	Fish pathogen	(Naka et al., 2011)
14.	*Vibrio aerogenes*	Gas producing	PRJNA171492
15.	*Vibrio aestuarianus*	Pertaining to an estuary	PRJDB354
16.	*Vibrio azureus*	Environmental bacteria	PRJDB356
17.	*Vibrio brasiliensis*	Found to be associated with bivalve larvae cultures	ASM18925v1
18.	*Vibrio breoganii*	Isolate from plum island, live preferentially on macroalgae.	ASM28088v1
19.	*Vibrio campbellii*	Ocean water	(Amaral et al., 2012)
20.	*Vibrio caribbenthicus*	Isolated from a marine sponge	Vcar_1.0
21.	*Vibrio cholera*	Causes severe diarrheal disease in humans	(Heidelberg et al., 2000)
22.	*Vibrio comitans*	Environmental bacteria	PRJDB357
23.	*Vibrio coralliilyticus*	Coral pathogen	(Santos Ede et al., 2011)
24.	*Vibrio crassostreae*	Isolated from the haemolymph of oysters (Crassostrea gigas)	VibCrassostreae-9ZC13
25.	*Vibrio cyclitrophicus*	Isolated from creosote contaminated marine sediment	Vibcyclo-ZF-14_1.0
26.	*Vibrio ezurae*	Environmental bacteria	PRJDB359
27.	*Vibrio fluvialis*	Causes diarrhoea in humans	PRJDB353
28.	*Vibrio furnissii*	Found in fresh water areas	(Lux et al., 2011)
29.	*Vibrio gazogenes*	Environmental bacteria	PRJDB352
30.	*Vibrio genomosp*	Environmental bacteria	Vib-ZF-129
31.	*Vibrio halioticoli*	Found in the gut of *Haliotis discus hannai*	PRJDB351
32.	*Vibrio harveyi*	Shrimp Pathogen	(Espinoza-Valles et al., 2012)
33.	*Vibrio ichthyoenteri*	Pathogenic to flounder larva	ASM22260v1
34.	*Vibrio inusitatus*	Environmental bacteria	PRJDB349
35.	*Vibrio kanaloae*	Environmental bacteria	Vib-5S-149
36.	*Vibrio marisflavi*	Environmental bacteria	PRJNA171491
37.	*Vibrio mediterranei*	Found in see water, plankton and sediments	PRJDB348
38.	*Vibrio metschnikovii*	Widely distributed in the environment	ASM17615v1
39.	*Vibrio mimicus*	Occurs in aquatic environment and cause diarrheal disease in humans	(Hasan et al., 2010)
40.	*Vibrio natriegens*	Found in salt marsh mud and coastal seawater	PRJDB346
41.	*Vibrio navarrensis*	Found in river water, irrigation water and sewage	PRJNA43075
42.	*Vibrio nereis*	Found in seawater	PRJDB347
43.	*Vibrio nigripulchritudo*	Found in seawater	ASM22268v1
44.	*Vibrio ordalii*	Fish pathogen	(Naka et al., 2011)
45.	*Vibrio orientalis*	Found in seawater and shrimps	ASM17623v1
46.	*Vibrio owensii*	Marine bacteria	PRJNA174229
47.	*Vibrio parahaemolyticus*	Cause diarrheal disease in humans and also found in marine environment	(Makino et al., 2003)
48.	*Vibrio proteolyticus*	Found in marine animals	PRJDB344
49.	*Vibrio rarus*	Environmental bacteria	PRJDB342
50.	*Vibrio rotiferianus*	Marine pathogen	(Chowdhury et al., 2011)
51.	*Vibrio rumoiensis*	First identified at a fish product processing plant in the drainage.	Vib-1S-45
52.	*Vibrio sagamiensis*	Found in seawater	PRJDB343
53.	*Vibrio scophthalmi*	Found in marine organisms	ASM22258v1
54.	*Vibrio sinaloensis*	Environmental bacteria	ASM18927v1
55.	*Vibrio shilonii*	Isolated from the coral Oculina patagonica in the Mediterranean Sea	ASM18153v1
56.	*Vibrio sp.* EJY3	Marine agarolytic bacterium	(Roh et al., 2012)
57.	*Vibrio sp.* Ex25	Isolated from a deep-sea hydrothermal vent community	ASM2482v1
58.	*Vibrio splendidus*	Found in marine environment	ASM9146v1
59.	*Vibrio superstes*	Environmental bacteria	PRJDB341

60.	*Vibrio tasmaniensis*	Isolated from Atlantic salmon	Vib-1F-267
61.	*Vibrio tubiashii*	Found in larvae and juveniles of bivalve molusks	(Temperton et al., 2011)
62.	*Vibrio vulnificus*	Human diseases and marine environment	(Chen et al., 2003)

1.1.3. Bacteria involved in this study from *Vibrionaceae* family

For the purpose of the study, two different bacteria belonging to *Vibrionaceae* family have been used and they are described in brief.

Vibrio anguillarum (V. anguillarum)

This bacteria was previously named as *Listonella anguillarum*, and has been recently re-classified into *V. anguillarum* (Thompson et al., 2011). With the help of DNA–DNA hybridization experiments along with confirmation by the structural similarity of superoxide dismutase and 16S rRNA gene sequence comparison it has been shown that the most related species is *V. ordalii* (Garrity, 2005). However, they are very different when comparing phenotypic and virulence properties because *V. ordalii* has about 600 kilo bases less and lack many important genes for virulence (Naka et al., 2011). *V. anguillarum* is the main causative agent of vibriosis (defined as haemorrhagic septicaemia) in fish (Woo, 2006).

Aliivibrio salmonicida (A. salmonicida)

This bacterium was formally called *Vibrio salmonicida* and has in a recent report been reclassified as *Aliivibrios* (Urbanczyk et al., 2007). It is a causative agent of cold water vibriosis in salmonids and cod, a disease now kept under control due to successful vaccination (Bornø et al., 2010). The disease is called cold-water vibriosis because it appears at low water temperature (>10°C) and mainly in late autumn, winter or early spring (Enger et al., 1991) and it is named salmonicida because of its ability to kill salmon. The whole genome comparison has shown that the *A. salmonicida* is highly related to *Aliivibrio fisheri* (*A. fischeri*) (Hjerde et al., 2008).

1.2. Quorum sensing

The literal meaning of the word “quorum” is defined as the number of people (officers or members) required to pass a vote in favor of a resolution or to carry out any business transaction. In biological context living cells do reach a quorum or a certain cell density and the ability to sense this population density is defined as quorum sensing (QS). In simpler words QS is a system which depends on cell density and involves receiving a stimulus and producing a response to it. QS are reported in bacteria where diffusible small molecules called auto-inducers are produced in a cell-density dependent manner. For more thorough review on QS see Miller et

al (Miller and Bassler, 2001). These small molecules are received as stimuli and a coordinated response is observed with increase or decrease of certain gene expression. QS are also reported in some social insects where QS is used to determine where to nest. For review on QS in insects see Visscher et al (Visscher, 2007).

1.2.1. QS in bacteria

QS was first identified and explored more than 30 years ago in luminous marine bacteria *A. fischeri* (Ruby and Nealson, 1976). However some researchers have proven that functions other than density sensing are involved with QS for instance conjugal transfer of plasmid (Fuqua and Winans, 1994), swarming (Eberl et al., 1996), diffusion sensing (Redfield, 2002), iron chelation (Kaufmann et al., 2005) and antibiotic biosynthesis (Wood and Pierson, 1996, Lowery et al., 2009, Ueda et al., 2010).

Eukaryotic cells and organisms are usually believed to be associated with exchange of chemical signals between cells (Miller and Bassler, 2001, Mitchell et al., 2011). Moreover, bacteria were earlier not considered to have any cell-cell communication systems. However, recent development in this field has shown that many bacteria communicate with their surrounding by secreting chemical substances to coordinate a particular response (Shank and Kolter, 2009). The signals are not only used directly or indirectly to regulate gene expression but they are also used to differentiate between species (Miller and Bassler, 2001, Ng and Bassler, 2009). Surprisingly, recent reports also show inter-kingdom communications (Lowery et al., 2008, Mellies and Lawrence-Pine, 2010, Curtis and Sperandio, 2011, Di Cagno et al., 2011). From these reports there is good evidence that the ability to communicate is very important for both bacterial survival and interaction with the natural environment.

QS system comprise of small organic molecules or oligopeptides as auto-inducer or signalling molecules (Miller and Bassler, 2001). Signals can be produced either by enzymes or expressed as peptides and can diffuse freely in and out of the cells or excreted by cell membrane transporters (Ng and Bassler, 2009). Then these signals bind to receiver proteins which are either membrane bound or present in the cytoplasm. The receiver protein then causes changes in gene expression either directly or indirectly by starting a signalling cascade, which eventually leads to changes in gene expression. Both Gram positive and negative bacteria have the QS system (Miller and Bassler, 2001). Till date oligopeptide signalling molecules have only been found in Gram positive bacteria. LuxS is an AI synthase found in both Gram positive and negative bacteria but its receivers are described only in Gram negative bacteria (Reading and

Sperandio, 2006). Many QS systems other than oligopeptides based are well studied in Gram negative bacteria and they are described later in the section.

1.2.2. Definition of a QS signal

With increasing number of signals being detected (both in number and type) over the last couple of decades, there has arisen a need for defining what is a signal and what is not a signal. Keeping this in mind some researchers have set up certain criteria for characterizing a molecule as a cell-to-cell signal molecule (CCSM), for review on this topic see Winzer et al and Monds and O'Toole et al (Winzer et al., 2002, Monds and O'toole, 2008).

These criteria are as follows:

- CCSM should be produced at a certain phase of growth (example lag, log or stationary), and it should be produced at certain conditions or as a response to a change in condition of the environment.
- CCSM should be present in good amounts outside the cell and it should also bind to a receptor.
- There should be a response once a critical threshold concentration of CCSM has been reached.
- There should also be a cellular response to neutralize or destroy the CCSM.
- The concentration of CCSM should not be at a toxic level.
- The last and the most difficult to prove is that the CCSM is adaptive to the level of the community. This is hard to show as it involves evolutionary experiments, for example assessing whether a functional signaling network provides a fitness advantage in a challenging environment among competitors.

1.2.3. Different QS systems in *Vibrionaceae* family

In the *Vibrionaceae* family, three major QS systems have been studied. Table 2 shows the three major systems and their main signaling molecules. The systems are described in detail in the following sections.

Table 2: Three main QS systems and their respective signals.

System	Signal
LuxS/LuxPQ	AI-2
AHL producing	AHLs
CqsA/CqsS	CAI-1

1.2.4. LuxS/LuxPQ system in prokaryotes

LuxS/LuxPQ is one of the three major QS systems where LuxS is responsible for the production of 4,5-dihydroxy-2,3-pentanedione (DPD), as shown in Figure 2, which undergoes a rearrangement in presence of boron to form the auto-inducer molecule AI-2 (Chen et al., 2002). It is widely distributed in both Gram negative (Surette et al., 1999) and positive bacteria (Miller and Bassler, 2001). It attracted wide spread attention as it is believed to be responsible for not only inter-species but also for inter-kingdom communication (Walters and Sperandio, 2006). Many different types of methyl transferase reactions in prokaryotic and eukaryotic systems are carried out by methyl transferase enzymes, using SAM as the methyl donor (Fontecave et al., 2004). The methylation reaction converts SAM into SAH and accumulation of SAH is toxic to the living cell.

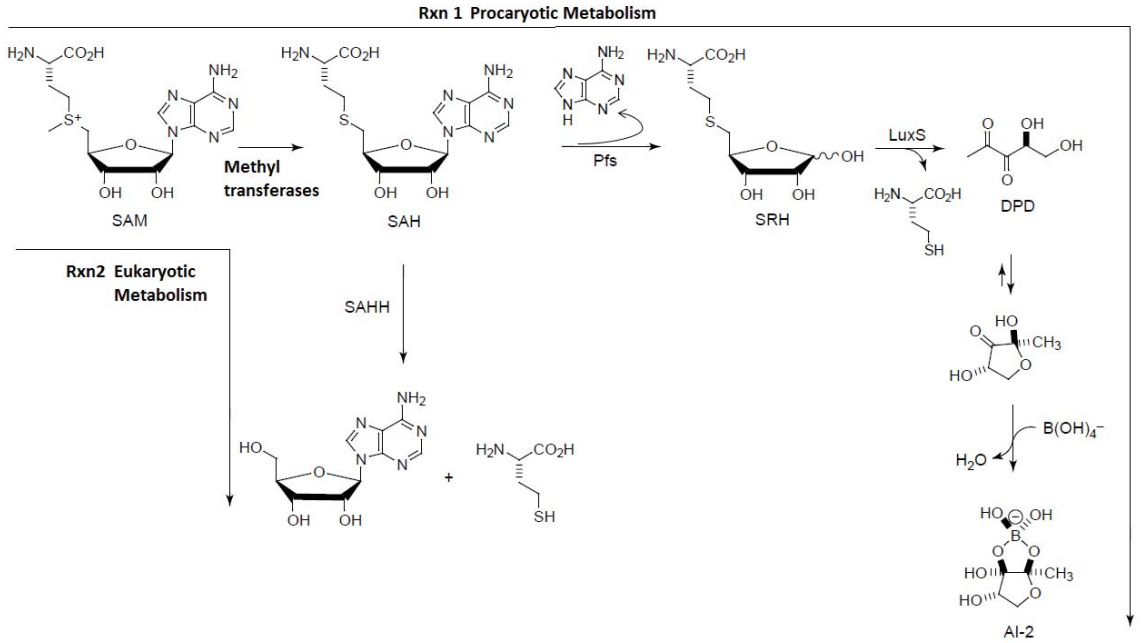


Figure 2: SAM metabolism in prokaryotics and eukaryotic systems.

Reaction1 (Rxn 1) is taking place in bacteria and used for the production of auto-inducer AI-2 by bacteria, whereas reaction Rxn 2 occurs in eukaryotes. SAM = S-adenosylmethionine, SAH = S-adenosylhomocysteine, SRH = S-

ribosylhomocysteine, SAHH = enzyme SAH hydrolase, see text for further details. Figure adapted from Pei and Zhu et al (Pei and Zhu, 2004).

The detoxification reactions in eukaryotes involve using an enzyme SAH hydrolase (SAHH), which hydrolyzes SAH into adenosine and homocysteine (Figure 2 (Rxn 2)). Although some prokaryotes use the SAHH enzyme, most prokaryotes utilize a two-step mechanism to convert SAH first into S-ribosylhomocysteine (SRH) by removing adenine base using the nucleosidase (Pfs). The second step involve removal of a thioether bond from SRH to give homocysteine and DPD using the enzyme LuxS, Figure 2 (Rxn 1), (Pei and Zhu, 2004). In a recent study the *Escherichia coli* (*E.coli*) LuxS mutant were able to synthesize low amounts of AI-2 through ribulose-5-phosphate, which resulted from glucose degradation via the oxidative pentose phosphate pathway (Tavender et al., 2008). Two thermo-stable microbes *Thermotoga maritime* and *Pyrococcus furiosus* were also reported to produce AI-2 in absence of LuxS under hydrothermal conditions (Nichols et al., 2009). Table 3 shows some homologues proteins in different bacteria from this system. The main receiver protein for the AI-2 signal is LuxP. The crystal structure of LuxP from *V. harveyi* reviled an AI-2 molecule with boron atom (Chen et al., 2002) while in the crystal structure of LsrB (also an AI-2 binding protein) from *Salmonella typhimurium*, it was found that (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (R-THMF), a non borate form of AI-2, was bound to the receiver protein without the boron atom (Miller et al., 2004). This shows that both R-THMF and AI-2 can function as signals.

Table 3: LuxS/LuxPQ system homologous proteins in different bacteria.

QS system Component	Description	Identity (%)	Accession number
Synthase (LuxS family of enzymes)	LuxS *V. harveyi* 1DA3	100	ZP_06175640.1
	LuxS *A. fischeri* ES114	87	AAW85040.1
	LuxS *A. salmonicida* LFI1238	87	YP_002262164.1
	LuxS *V. anguillarum* M93Sm	85	ABE98251.1
	LuxS *Streptococcus pneumonia* 05-447 (Gram positive)	33	AFC91867.1
Receptors (Two component histidine kinase)	LuxP/LuxQ *V. harveyi* 1DA3	100/100	ZP_06177838.1/ ZP_06177837.1
	LuxP/LuxQ *A. fischeri* ES114	55/38	YP_204090.1/ YP_204091.1
	LuxP/LuxQ *A. salmonicida* LFI1238	Frame shifted/38	Frame shifted/ YP_002262426.1
	VanP/VanQ *V. anguillarum* NB10	63/60	AAS98247.1/ AAS98248.1

1.2.5. AHL and different AHL producing systems

AHL was the very first QS signal to be detected (Eberhard et al., 1981). It consists of a lactonised homoserine moiety attached by an amide bond to an acyl side chain.

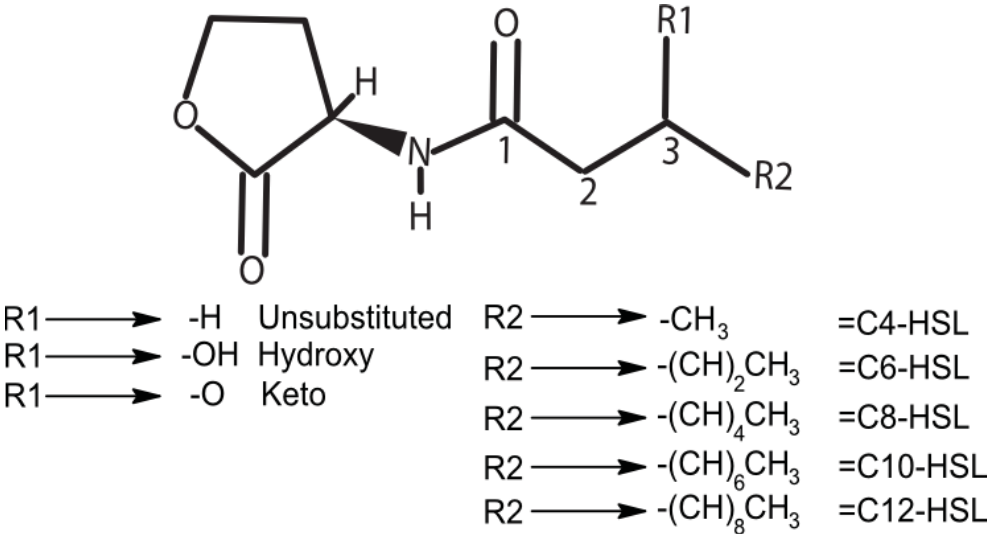


Figure 3: Structure of Acyl homoserine lactone.
R1 group is defined as substitutions on the third carbon and R2 group is defined as acyl chain length. Figure adapted from Annous et al (Annous et al., 2009).

The acyl chains can vary with even carbon numbers starting from C4, C6, C8 to up to 12 carbons or even more (Krick et al., 2007). Odd number of carbons in the side chains has also been reported (Lithgow et al., 2000, Morohoshi et al., 2004, Pomini and Marsaioli, 2008). Usual substitutions are on the third position (R1 in Figure 3) with hydrogen (un-substituted), a keto (3-oxo), or a hydroxy (3-OH). However, there are exceptions to the above described set of rules. In a recent study methyl branched AHLs has been reported in the bacterium *Aeromonas culicicola* (Thiel et al., 2009). In another report, an isovaleryl analogue of AHL was detected in the soybean symbiont *Bradyrhizobium japonicum* (Lindemann et al., 2011). Moreover, bacteria can accept certain substrates from the environment and design new classes of autoinducer, for example plant symbiont *Rhodopseudomonas palustris* was reported to produce an aroyl-homoserine lactone called *p*-coumaroyl-HSL. Here the plant produced *p*-coumarate which was incorporated into the signaling molecule instead of a fatty acid (Schaefer et al., 2008). For a review on AHL structures see Churchill and Chen et al (Churchill and Chen, 2011).

There are three different types of AHL producing systems known so far, namely the LuxI/LuxR, the LuxM/LuxN system and the newly discovered HdtS system. These systems are described in the following section.

(a). LuxI/LuxR system

LuxI/LuxR is an integrated part of the *lux* operon, which is involved in bioluminescence. It is believed that the *lux* genes (*luxCDABEG*) is vertically inherited in the *Vibrionaceae* family (Urbanczyk et al., 2008). In *A. fischeri* the LuxI synthase is known to produce C6-HSL and 3-oxo-C6-HSL while VanI from *V. anguillarum* has been shown to produce 3-oxo-C10-HSL (Kuo et al., 1994, Milton et al., 1997). Receivers for these systems are called LuxR, a two domains response regulator were the 1st domain binds AHL and the 2nd regulates target gene expression, which regulates the transcription of *lux* operon. There are many homologues of this system found in other bacteria (see Table 4) and they may play an important role in bacterial communication. For review on different LuxI/LuxR systems and their role in cross system communication see Smith et al Stevens et al and Strassmann et al (Smith et al., 2011, Stevens et al., 2011, Strassmann et al., 2011).

Table 4: LuxI/LuxR homologues in different bacteria.

QS system	Description	Identity (%)	Accession number
Synthases (LuxI family of enzymes)	LuxI *A. fischeri* ES114	100	AAQ90197.1
	LuxI *A. salmonicida* LFI1238	80	YP_002265246.1
	VanI *V. anguillarum* NB10	49	P74945.1
Receptors	LuxR *A. fischeri* ES114	100	AAQ90196.1
	LuxR1 *A. salmonicida* LFI1238	57	AAM46717.1
	LuxR2 *A. salmonicida* LFI1238	55	AAM46724.1
	VanR *V. anguillarum* NB10	32	AAC45213.1

(b). LuxM/LuxN system

LuxM is an AHL synthase and it's product was identified as 3-OH-C4-HSL in *V. harveyi* (Cao and Meighen, 1989). The gene was initially wrongly annotated as two proteins, LuxM and LuxL, which was belived to be necessary for the production of AHL (Bassler et al., 1993). Later Milton et al (Milton et al., 2001) while working on VanM from *V. anguillarum* found that a sequencing error in the *LuxM* gene from *V. harveyi* gave rise to the two proteins. The gene has now been corrected and renamed as LuxM (as a single protein). AinS is a homologues AHL synthase found in *A. fischeri* and is responsible for C8-HSL production (Hanzelka et al., 1999). The receivers for the LuxM, VanM and AinS products have been recognized as LuxN, VanN and AinR in *V. harveyi*, *V. anguillarum* and *A. fischeri*, respectively (see Table 5).

Table 5: LuxM/LuxN homologues in different bacteria.

QS system Component	Description	Identity (%)	Accession number
Synthase (LuxM family of enzymes)	LuxM *V. harveyi* 1DA3	100	A7MRY3.1
	AinS *A. fischeri* ES114	24	YP_204420.1
	AinS *A. salmonicida* LFI1238	23	YP_002262635.1
	VanM *V. anguillarum* M93Sm	63	AAG01003.1
Receptors (Histidine Kinase)	LuxN *V. harveyi* 1DA3	100	AAC36808.1
	AinR *A. fischeri* ES114	41	YP_204419.1
	AinR *A. salmonicida* LFI1238	40	YP_002262634.1
	VanN *V. anguillarum* NB10	76	AAG01004.1

(c). HdtS system

HdtS has been identified as a third AHL producing enzyme. It was first shown in *Pseudomonas fluorescens* F113 (*P. fluorescens* F113) as a synthase responsible for the production of 3-OH-C14:1-HSL (1 is the number of unsaturated carbon), C10-HSL, and C6-HSL (Laue et al., 2000). In *Nitrosomonas europaea* strain Schmidt, HdtS was believed to be responsible for the production of AHLs (C6-HSL, C8-HSL and C10-HSL) as no other known AHL synthases like AinS or LuxI homologues were detected (Burton et al., 2005). In *V. vulnificus* also AHL production is reported but again no AinS or LuxI homologues could be identified (Valiente et al., 2009). Possible receiver protein for the HdtS system has yet not been reported.

1.2.6. CqsA/S system

A QS system which was identified for the first time in *V. cholera* was named cholera quorum sensing system (CqsA/S) (Miller et al., 2002). CqsA an enzyme (aminotransferase) which utilizes pyridoxal phosphate (PLP) to synthesize CAI-1, identified as (S)-3-hydroxytridecan-4-one (Higgins et al., 2007). A crystal structure of CqsA has been reported (Kelly et al., 2009). The 3D structure was solved by molecular replacement using the well studied homologous protein, 3-amino-7-oxononanoate synthase (AONS), which is involved in biotin biosynthesis. As CqsA and AONS structures were largely similar, it was initially believed that the substrates

and activity would also be similar. Hence (S)-2-Amino-butyrate and decanoyl Coenzyme A (CoA) were used as substrates. However, it turned out that the product of CqsA was not CAI-1 but Amino-CAI-1 (Wei et al., 2011). The mechanism for the conversion to CAI-1 could not be elucidated. It was realized that the reaction rate was very slow and SAM was found to be a better substrate as compared to (S)-2-amino butryate (Wei et al., 2011). It was shown that decanoyl CoA and SAM were converted into (S)-3-aminotridecan-4-one (Ea-CAI-1), followed by a spontaneous conversion into tridecane-3, 4-dione (DK-CAI-1) and finally to CAI-1 (Figure 4). In the final step, a *V. cholera* oxido-reductase protein VC1059 and NADH were crucial. CAI-1 is believed to bind to membrane bound receptors CqsS, which also starts a phosphorylation cascade similar to LuxPQ, see signal transduction section (1.2.7 (ii)) for more details.

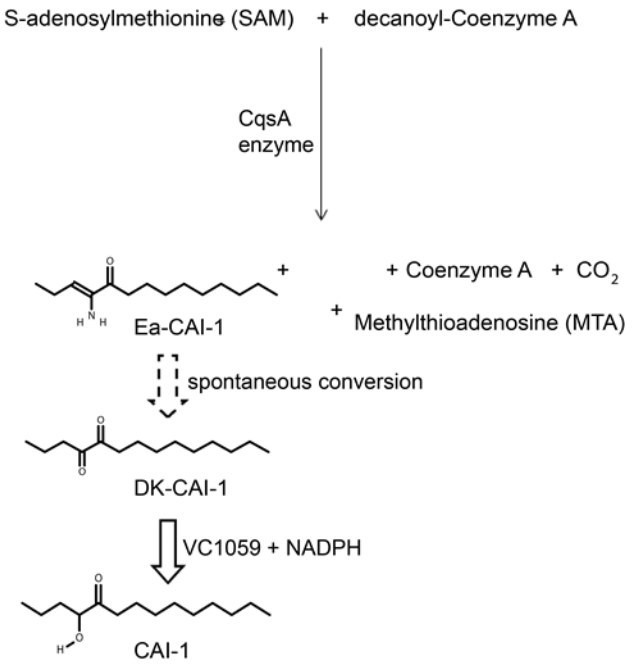


Figure 4: Reaction of CqsA converting the substrates decanoyl CoA and SAM into (S)-3-aminotridecan-4-one (Ea-CAI-1), followed by spontaneous conversion to tridecane-3, 4-dione (DK-CAI-1), and finally to (S)-3-hydroxytridecan-4-one (CAI-1) molecules. The oxido-reductase (VC1059) is the main enzyme identified which is essential for the last step. Figure redrawn from (Wei et al., 2011).

1.2.7. QS in *Vibrionaceae* family members

QS systems have been studied in a number of bacteria. Some of the model bacteria belonging to the *Vibrionaceae* family will be discussed in the following sections.

(i). QS in *A. fischeri*

There are three QS systems in *A. fischeri*, the LuxS/LuxPQ, the AinS/AinR and the LuxI/LuxR systems. The QS in *A. fischeri* is well studied with respect to bioluminescence (Ruby and Nealson, 1976, Meighen, 1991, Meighen, 1994). In *A. fischeri* bioluminescence is mainly controlled by the LuxI/LuxR system and through the master regulator LitR. The AinS/AinR and LuxS/LuxPQ systems also influence the light production (Lupp et al., 2003). Transcription of *lux* operon is activated by a LuxR dimer upon binding of the auto-inducer 3-oxo-C6-HSL produced by LuxI (Schaefer et al., 1996) or C8-HSL produced by AinS. C8-HSL binds to AinR and LuxR (Lupp et al., 2003).

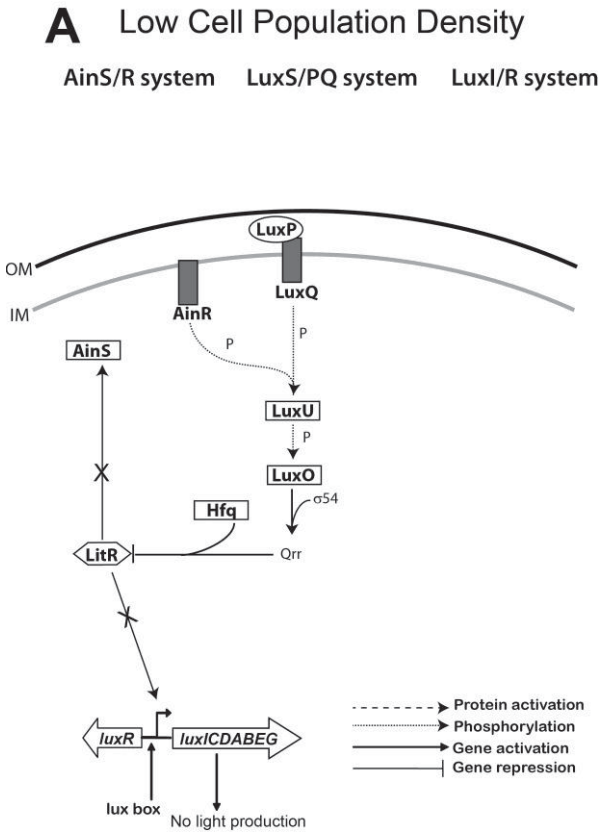


Figure 5(A)**:** QS systems in *A. fischeri* at low cell population density.
OM and IM are outer and inner membranes. Figure adapted from Lupp et al (Lupp et al., 2003) and Milton et al (Milton, 2006).

AHLs are not produced in *A. fischeri* at low cell population density and C8-HSL is the first AHL to be produced at intermediate cell density, which binds to LuxR and starts the transcription of the *lux* operon (Lupp et al., 2003). The *lux* operon consists of all genes for bioluminescence production (*luxCDABEG*) along with auto-inducer synthase gene *luxI*. Thereafter the *luxI* gene

is expressed and LuxI enzyme starts producing the AHLs at higher cell density (Figure 5 (A), (B) and (C)).

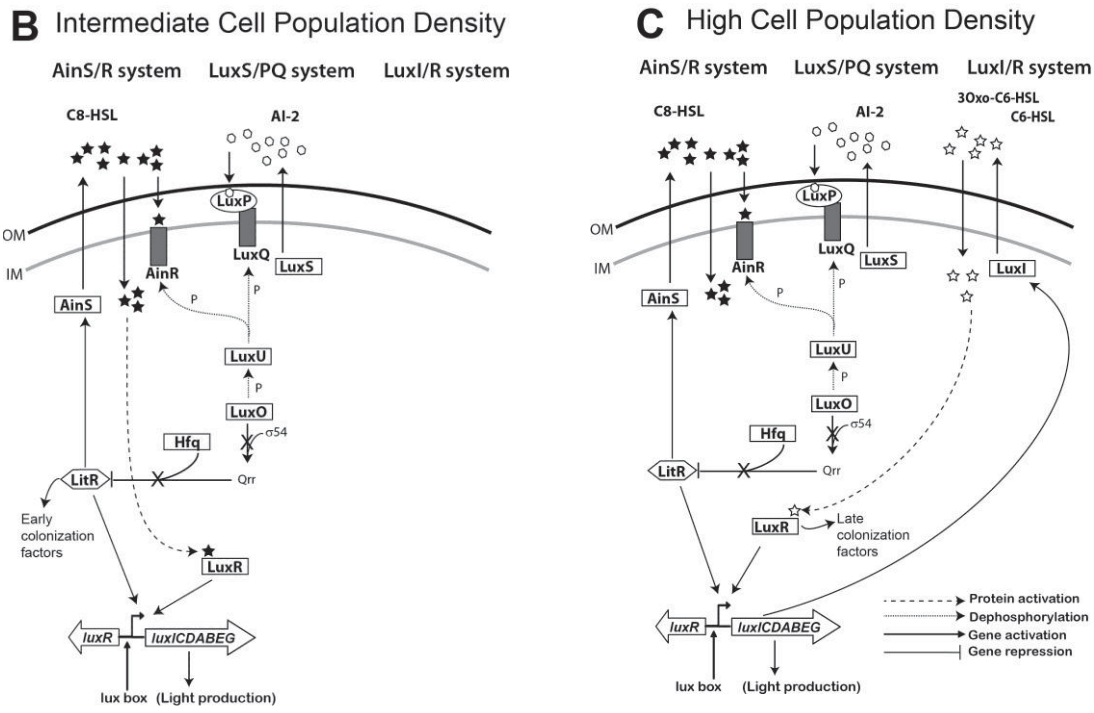


Figure 5(B) & (C)**:** QS systems in *A. fischeri* at (B) intermediate and (C) high cell population density. Figure adapted from Lupp et al (Lupp et al., 2003) and Milton et al (Milton, 2006).

In *A. ficheri* LitR is responsible for both expression of AinS directly and expression of LuxI indirectly via controlling the expression of LuxR (Lupp et al., 2003). For review on QS systems in vibrios see Milton et al (Milton, 2006). The phosphorylation cascade is similar to that of *V. harveyi*, as discussed in the next section.

(ii). QS in V. harveyi

V. harveyi also have three QS systems, out of which LuxS/LuxPQ and LuxM/LuxN systems are homologues to the *A. fischeri* systems LuxS/LuxPQ and AinS/AinR respectively. The third system CqsA/CqsS system is homologues to Cqs system in *V. cholerae*. The *lux* operon in *V. harveyi* is slightly different from the operon in *A. fischeri* as it lacks the AHL binding LuxR response regulator. On the other hand *V. harveyi* has a LitR homologue (tetR fold protein) defined as LuxR, which controls the expression of the *lux* operon. Moreover, the *luxI* gene is not present as in the original LuxI/LuxR system found in *A. fischeri* (Bassler et al., 1993, Bassler et al., 1994a). QS receptors LuxN, LuxPQ and CqsS are dual functional, two component enzymes that has both kinase and phosphatase activities. At low cell population density (Figure 6 A) the

auto-inducer levels are low and the receptors function as kinases, resulting in phosphorylation of conserved histidine residues by ATP.

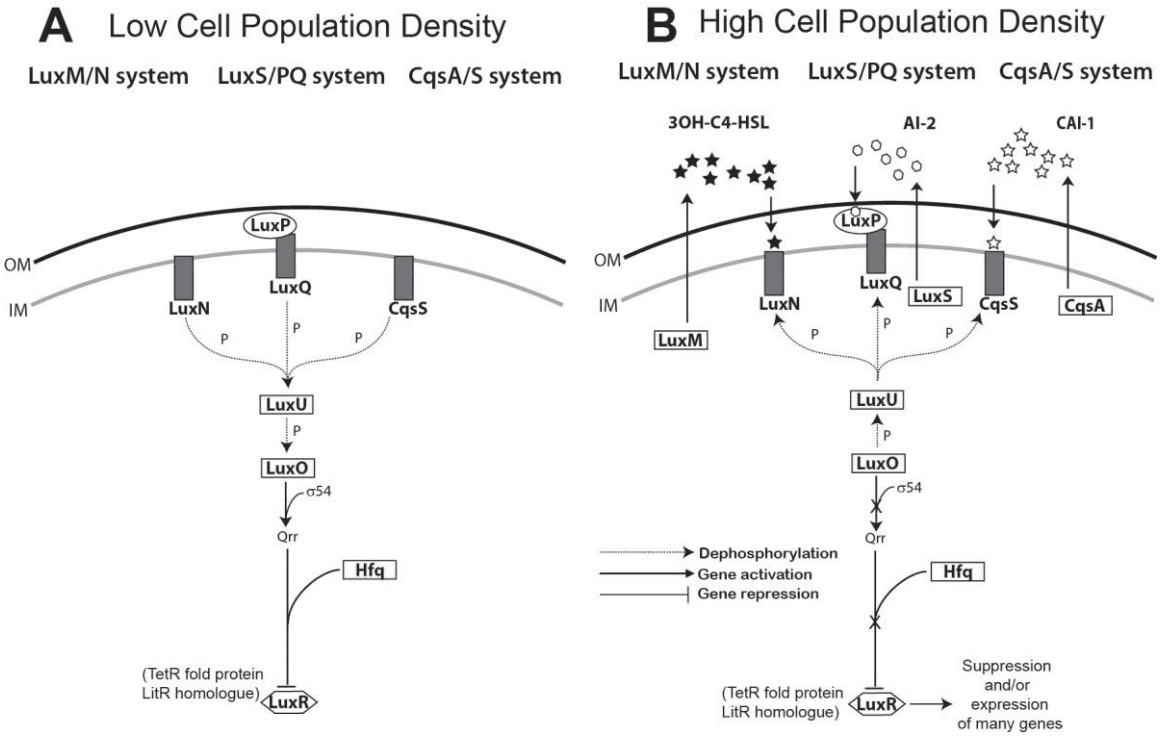


Figure 6: QS systems in *V. harveyi* at low (A) and high (B) cell population density.
Figure adapted from Milton et al (Milton, 2006).

The phosphate group is transferred to a conserved aspartate residue in the C-terminus of the receptor and then subsequently to a single protein, LuxU, which in-turn transfers the phosphate to a response regulator LuxO (Bassler et al., 1994b, Lilley and Bassler, 2000). Phosphorylated LuxO transcribes *qrr*1-5 sRNA (*A. fischeri* has only one *qrr*) and destabilize the QS master regulator *luxR* mRNA and hence LuxR is not produced (Tu and Bassler, 2007). At higher cell densities, (Figure 6 B), the concentration of auto-inducers increases above a certain threshold and the receptors (LuxN, LuxPQ and CqsS) shifts from kinase to phosphatase in activity. This result in de-phosphorylation of LuxO through LuxU, which stops the transcription of *qrr*1-5 sRNA and the QS master regulator, transcription factor LuxR is expressed. See Table 6 for some homologous QS regulators in different bacteria. For a review on QS phosphorylation in *V. harveyi* see Ng et al (Ng and Bassler, 2009).

Table 6: Homologous QS regulators from different bacteria.

QS system	Description	Identity	Accession

		(%)	number
Regulators (with conserved histidine residue)	LuxU *V. harveyi* ATCC BAA-1116	100	A7MVC1.1
	LuxU *A. fischeri* ES114	38	YP_204321.1
	LuxU *A. salmonicida* LFI1238	38	YP_002263271.1
	VanU *V. anguillarum* M93Sm	46	AAS98244.1
Regulator (with conserved aspartate residue)	LuxO *V. harveyi* 1DA3	100	ZP_06176439.1
	LuxO *A. fischeri* ES114	69	YP_204320.1
	LuxO *A. salmonicida* LFI1238	50	YP_002264649.1
	VanO *V. anguillarum* NB10	78	YP_004566681.1
Regulator with tetR fold	LuxR *V. harveyi* 1DA3	100	ZP_06176280.1
	LitR *A. fischeri* ES114	59	YP_205560.1
	LitR *A. salmonicida* LFI1238	61	YP_002263961.1
	VanT *V. anguillarum* NB10	80	AAL59612.1

(iii). QS in *V. anguillarum*

In *V. anguillarum*, four systems are described. The first system VanI/VanR is homologous to *A. fischeri* LuxI/LuxR system (Milton et al., 1997) but it lacks the *lux* operon completely. The second system VanM/VanN is the other AHL system homologous to *A. fischeri* AinS/AinR. However, it seems that VanM somehow controls the production of AHLs of VanI (Milton et al., 2001), see Figure 7. The third system is also an *A. fischeri* LuxS/LuxPQ homologue. LitR homologue VanT, LuxU homologue VanU and LuxO homologue VanO are present. However it is found that VanU, activates while VanO represses expression of *vanT* mRNA and the expression of VanT is negatively self regulated by VanT controlling its own and *vanOU* promoter. (Croxatto et al., 2004). The fourth system is predicted to be homologous to the CqsA/CqsS system, for a review on QS in vibrios see Milton et al (Milton, 2006).

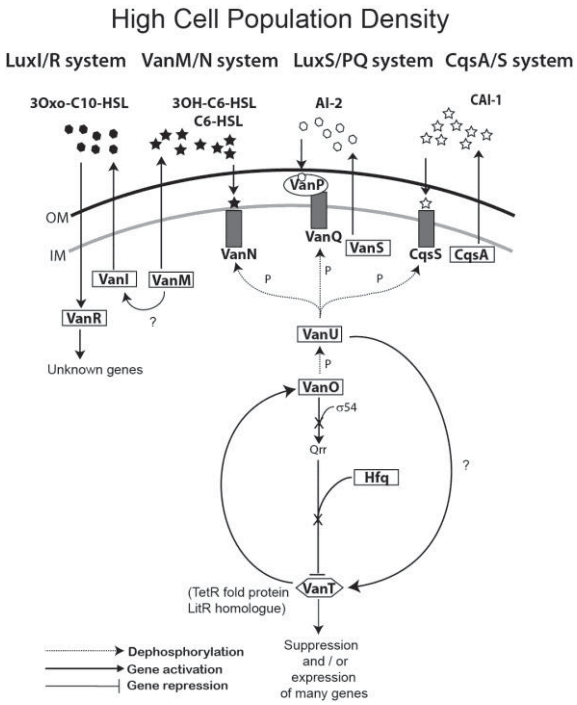


Figure 7: QS systems in *V. anguillarum* at high cell population density.
Figure adapted from Milton et al (Milton, 2006).

(iv). QS in *A. salmonicida*

In the *A. salmoncida* strain LFI1238 genome four QS systems have been identified (Hjerde et al., 2008). The four QS systems were identified as AinS/AinR, LuxI/LuxR, LuxM/LuxN and LuxS/LuxPQ, respectively. Among them only two are believed to be functional as they have intact genes (they are AinS/AinR and LuxI/LuxR), see Figure 8. The AHL synthase *luxM* gene is missing from LuxM/LuxN system, along with a frame-shift mutation in the gene *luxN*. At the same time, the *luxP* gene in the LuxS/LuxPQ system contains a frame-shift mutation. It is believed that if it is subjected to "programmed frameshifting", their proteins can be expressed (Hjerde et al., 2008).

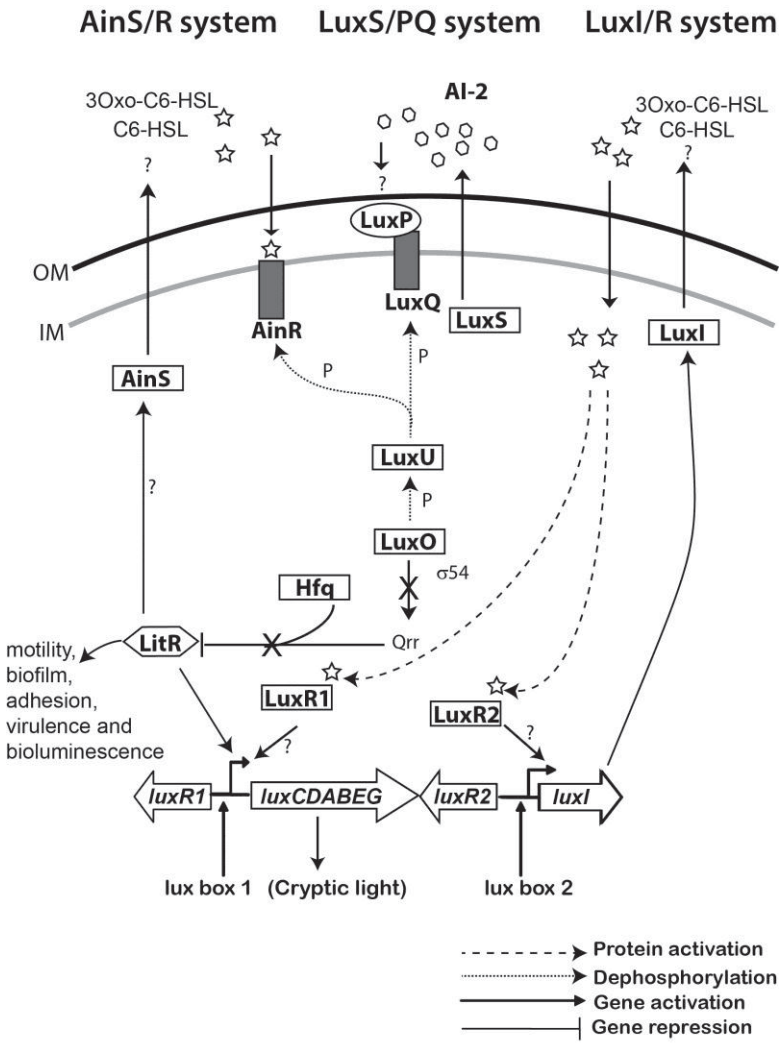


Figure 8: Proposed QS systems in *A. salmonicida.*

In a previous report (Nelson et al., 2007) the *lux* operon of *A. salmonicida* strain NCMB 2262 has been reported as novel with respect to arrangement of genes, as compared to that of *A. fischeri* and *V. harveyi lux* operons. The main difference is that the *luxR* gene is found in two copies, referred to as luxR1 and luxR2 (65% identity), where the luxR2 is placed at the other end of the operon followed by the *luxI* gene. However, the main machinery for the light production *luxAB* (luciferase) and *luxCDE* (aliphatic-aldehyde synthesis) have the same organization. *A. salmonicida* has been shown to have cryptic bioluminescence, meaning that the bacteria needs addition of exogenous aldehyde to produce light and it is due to a mutation in *luxD* gene (Fidopiastis et al., 1999, Manukhov et al., 2011). The *lux* operon of the strain LFI1238 also shows a similar arrangement and property of cryptic bioluminescence (Hjerde et al., 2008, Bjelland et al., 2012).

Some strains of *A. salmonicida* (NCIMB 2262, VS81, VS201, VS224, VS288, VS289, VS297, and VS399) have been investigated for AHL production and only two types (3-oxo-C6-

HSL and C6-HSL) of AHLs have been reported (Bruhn et al., 2005). In a recent study LitR was reported to suppress motility, adhesion, cell-to-cell aggregation, biofilm formation in *A. salmonicida* and also shown to be sensitive to the concentration of salt in the medium (Bjelland et al., 2012). LitR was also shown to influence the cryptic bioluminescence but AHL production was not affected. The LitR mutant was also shown to have reduced mortality effect on Atlantic salmon as compared to the wild type. This indicates that LitR is a master regulator in the QS regulation.

Part 2. AHL biochemistry

2.1. AHL synthases

As discussed earlier there are two well-defined AHL systems – the LuxI/LuxR and LuxM/LuxN. Their respective synthases are called LuxI and LuxM. The recently identified HdtS has not been reported to produce AHLs in any *Vibironaceae* family member and hence is therefore not included in this section.

2.1.1. LuxI family of synthases

LuxI from *A. salmonicida* LFI1238 and VanI from *V. anguillarum* NB10 belong to LuxI family of AHL synthases (Table 4). Among the three systems described above, the LuxI family of AHL synthases is the most widely distributed in the *Vibrionaceae* as well as in other families, see review Churchill et al (Churchill and Chen, 2011). There are three homologues structures reported till date. The first was EsaI (PDB 1K4J) from *Pantoea stewartii* which is known to produce 3-oxo-C6-HSL (Watson et al., 2002). The second to be solved was LasI (PDB 1RO5) from *Pseudomonas aeruginosa* (*P. aeruginosa*), which produces 3-oxo-C12-HSL (Gould et al., 2004) and the third was TofI from *Burkholderia glumae* (PDB 3P2H) (Chung et al., 2011), which is responsible for the C8-HSL production.

2.1.2. LuxM family of synthases

The LuxM type of AHL synthases is found only in the *Vibrionaceae* family. LuxM from *V. harveyi* is reported to produce 3-OH-C4-HSL (Cao and Meighen, 1989), AinS from *A. fischeri* has been reported to produce C8-HSL (Kuo et al., 1994, Hanzelka et al., 1999) and VanM from *V. anguillarum* is been reported to produce 3-OH-C6- and C6-HSL (Milton et al., 2001). Not much is known about the structural and functional properties of the enzymes, but knockout studies have revealed some interesting features. An AinS knockout mutant of has been shown to

turn the acetate switch off in *A. fischeri* (Studer et al., 2008). While the wild type releases weak acids (mainly acetate) into the media and removes the excreated acids, the AinS mutant is not able to remove the excreated acids and eventually dies because of the acidic medium. Similarly a VanM knockout strain of *V. anguillarum* has a complete shutdown of AHL production indicating that the AHL synthase VanM also controls the AHL production via VanI (the LuxI family member) (Milton et al., 2001). The exact mechanism is not known, it could be at the substrate level or protein expression as DNA binding capability of LuxM family of enzymes has yet not been explored.

2.1.3. Comparisions of LuxI and LuxM families

The LuxI and LuxM enzyme families are very well characterized (Churchill and Chen, 2011). The enzymes use SAM as substrate for the homoserine lactone part of the product and acyl-ACP as the acyl chain provider. However other substrates have also been reported, for example homoserine lactone has been used as substrate in place of SAM as shown with the enzyme RhlI from *P. aeruginosa* (Jiang et al., 1998). This indicates that some bacteria may utilize degradation products of signal molecules and convert them into a signal for their communication. Similarly p-Coumarate (from the environment) has been shown to be taken up by the bacteria *Rhodopseudomonas palustris* and converted into aryl homoserine lactone with the help of AHL synthase RpaI (Schaefer et al., 2008). On the other hand most bacterial AHLs synthtases cannot use the acyl chain without the help of a Coenzyme A (CoA) or Acyl carrier protein (ACP). LuxI (Schaefer et al., 1996) from *A. fischeri* has been shown to use only Acyl-ACP as substrate and not acyl-CoA, whereas RhlI (Jiang et al., 1998), RpaI (Schaefer et al., 2008) and the LuxM homologue AinS (Hanzelka et al., 1999) from *A. fischeri* were shown to use both Acyl-CoA and Acyl-ACP as acyl donors. Acyl-ACP was most often shown to be a better substrate than Acyl-CoA. The LuxM homologues for example AinS in *A. fischeri* (Hanzelka et al., 1999) and VanM in *V. anguillarum* (Milton et al., 2001) are about 40-45 (KDa) whereas LuxI (Schaefer et al., 1996) and homologues for example VanI (Milton et al., 1997) are smaller enzymes (20-25 KDa).

2.2. Substrates

There are two substrates for the enzymatic formation of AHLs. The first is SAM, which donates the lactonized homoserine part, and an acyl chain doner, usually an acyl carrier protein (ACP) or acyl coenzyme A (acyl CoA).

2.2.1. SAM (S-adenosylmethionine)

SAM is a biological sulfonium compound and the second most widely used enzyme substrate after adenosine triphosphate (ATP), (Cantoni, 1975). Most reactions involving SAM are mainly driven due to the electrophilic character of the carbon centers that are adjacent to the positively charged sulfur atom. SAM is biosynthesized when a methionine molecule reacts with ATP by using the enzyme SAM synthase (Markham et al., 1980) as shown in Figure 9.

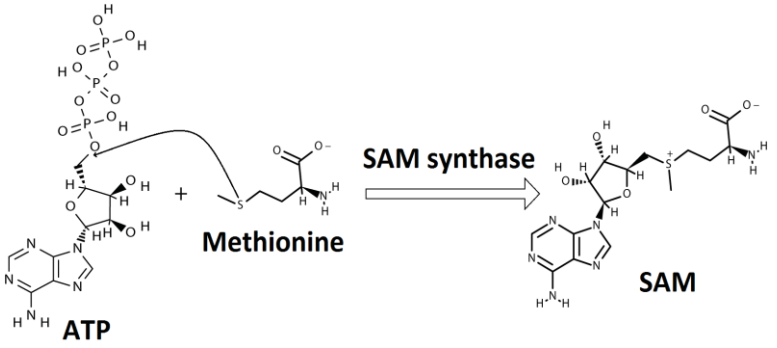


Figure 9: Biosynthesis of SAM starting from ATP and methionine.
Figure adapted from Fontecave et al (Fontecave et al., 2004) (review).

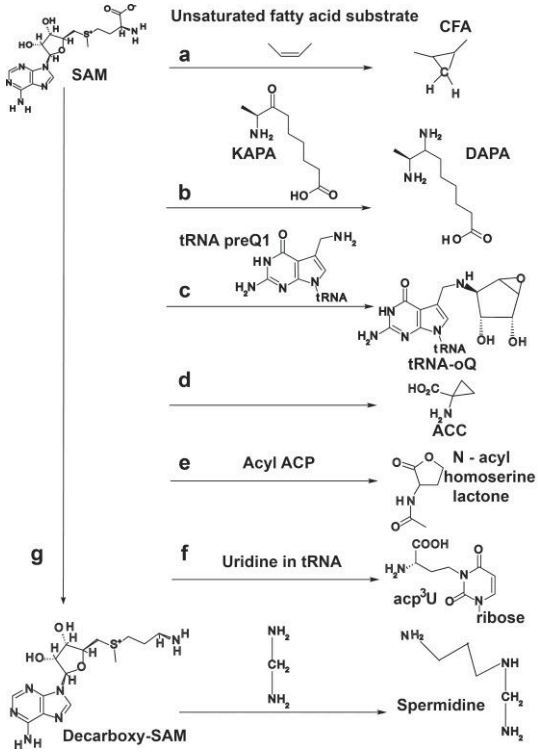


Figure 10: Important biological reactions involving the cofactor SAM.

(a) Methylation reaction (e.g. Cyclopropane fatty acids (CFAs) synthesis), (b) donating amino group (7,8-diaminopelargonic acid (DAPA) synthesis), (c) transferring ribosyl group, (d) forming a source of aminoalkyl group (e.g. 1-aminocyclopropane-1-carboxylic acid (ACC) synthesis), (e) donating homoserine for AHL formation (f) aminocarboxyproply group transfer and (g) forming decarboxy-SAM to form spermidine.
Figure adapted from Fontecave et al (Fontecave et al., 2004).

Within the cell environment SAM plays many roles. Some of the major reactions carried out by SAM in a living cell are described in Figure 10 (a-g), for review on important reactions of SAM see Fontecave et al (Fontecave et al., 2004).

2.2.2. Acyl carriers

In previous reports (Cao and Meighen, 1993, Val and Cronan, 1998) it has been proven that by blocking the fatty acid biosynthesis pathway, the AHL production is also blocked. Both acyl-ACP and acyl-CoA are intermediates in the fatty acid synthesis. However, which carrier becomes the donor of the acyl chain is enzyme dependent. Fatty acid biosynthesis is a very vast field of study as there are several pathways that lead to initiation, elongation and finally transferring the acyl moiety. Hence for the sake of clarity only some major pathways are shown in the Figure 11, which could be related to AHL synthesis (Cronan and Rock, 1996, Hoang et al., 2002).

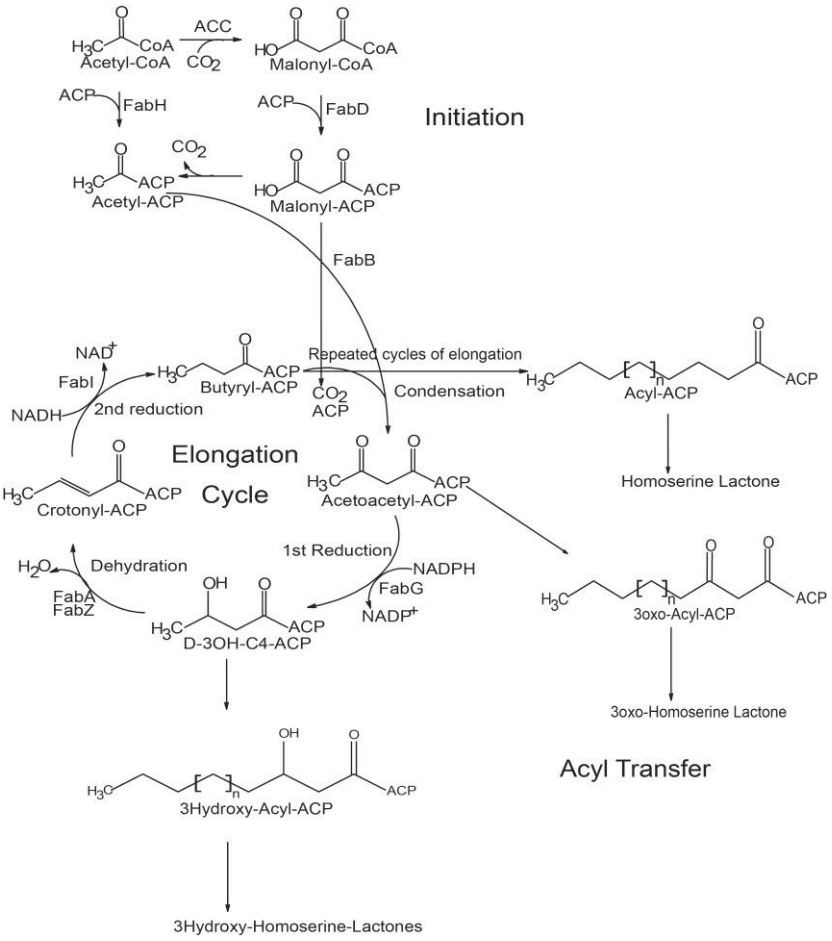


Figure 11: Scheme of some major stages involved in fatty acid biosynthesis.

Enzymes involved are called Fab from fatty acid biosynthesis. First stage is initiation which uses enzymes like Acetyl-CoA carboxylase (ACC), FabD, B, and H. Second stage is elongation cycle involving FabG, A, Z and I.

Finally acyl transfer stage where acyl-ACPs could either go to respective AHL synthases or they could be taken up by other pathways like Lipid A, Hemolysin, Phospholipids or other biosynthetic pathway. Figure redrawn from Hoang et al (Hoang et al., 2002).

2.3. Mechanism of AHL synthesis

As mentioned before the AHL synthesis involves two substrates, SAM and acyl-ACP/acyl-CoA. This mechanism (as shown in Figure 12(a)) was described for the first time in the study of TraI (LuxI type) from *Agrobacterium tumefaciens* (*A. tumefaciens*), where it is believed to be a ping pong reaction mechanism (More et al., 1996). This indicated that the substrates acyl-ACP and SAM would interact simultaneously leading to the formation of the product through lactonization of SAM and acyl-transferase reaction. However, in a later report a sequential, ordered reaction mechanism was proposed for RhlI (LuxI type) AHL synthase from *P. aeruginosa* (Parsek et al., 1999). In this mechanism SAM binds first to the enzyme forming a SAM-enzyme complex, followed by the acyl-ACP joining the enzyme-SAM-complex to make a second complex. Then the apo ACP leaves the complex followed by release of AHL and finally the MTA molecule, leaving the enzyme ready to bind the next SAM molecule for another reaction (Figure 12(b)). There are two types of reactions taking place within the enzyme, first is acylation and the second is lactonization of the homoserine moiety. It has been revealed that acylation precedes lactonization by providing N-butyryl-S-adenosylmethionine to the enzyme which resulted in C4-HSL formation (Parsek et al., 1999). The next part of the enzyme reaction involving the lactonization mechanism still remains unclear (Raychaudhuri et al., 2005).

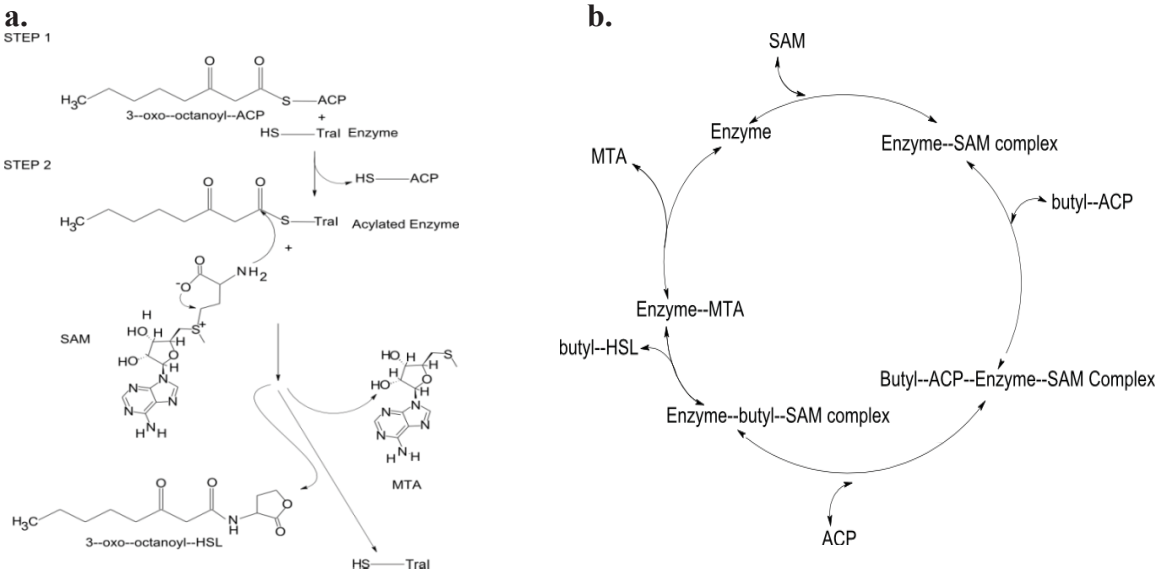


Figure 12 (a): Proposed ping-pong reaction mechanism for AHL production by TraI from *A. tumefaciens*. (b): Proposed sequential reaction mechanism of the enzyme RhlI from *P. aeruginosa*. ACP (acyl carrier protein), MTA (methylthioadenosine) and SAM (S-adenosylmethionine). Figure adapted from More et al (More et al., 1996) and Parsek et al (Parsek et al., 1999).

Although it remains an enigma as to why certain enzymes prefer to produce one or two AHLs as major products, there are some reports shedding light on it. In the study by Raychaudhuri et al. (Raychaudhuri et al., 2005) on RhlI from *P. aeruginosa* three different ACPs were identified in the genome. Two of the ACPs were found to be good substrates while the third ACP was poor. It was also confirmed by microarray data that the gene expression of one of the ACP's gene was controlled by QS. This indicates that there might be several ACPs present in the system but only one or two are capable to take the acyl chain to a particular enzyme. RhlI make C4-HSL as the major product and it has been shown that RhlI also makes C6-HSL, though not as efficiently as C4-HSL (Jiang et al., 1998, Parsek et al., 1999). LasI (LuxI type) from *P. aeruginosa* is responsible for making 3-oxo-C12-HSL. However, in an *in-vitro* experiment it was shown that by not providing the right cofactor (NADH in place of NADPH) to a fatty acid synthesis enzyme, FabG (beta-ketoacyl-ACP reductase) resulted in failure of the elongation cycle. This led to increased amounts of shorter chained 3-oxo-HSL product synthesized by LasI (Hoang et al., 2002). When AHL synthase TraI from *A. tumefaciens* and HtdS from *P. fluorescens* F113 were expressed in *E.coli* it produces the same AHLs as under the native expression conditions (Val and Cronan, 1998, Laue et al., 2000). On the other side LasI from *P. aeruginosa* expressed in *E.coli* produced a high proportion of unusual AHLs with acyl chains consisting of an odd number of carbons (Gould et al., 2006). This suggests that more work needs to be done in order to understand the molecular mechanisms behind the AHL production.

2.4. Enzyme kinetics and cold adapted regulation of QS

Simplest form of one substrate enzyme kinetics can be explained as follows:

$$[E] + [S] \underset{K_{-1}}{\overset{K_1}{\leftrightarrow}} [ES] \overset{K_2}{\rightarrow} [E] + [P]$$

Where $[E]$ is the enzyme concentration, $[S]$ is the substrate concentration, and $[P]$ is the product formed. At the equilibrium phase the rate of formation (K_1[E][S]) and rate of disappearance (K_{-1}+K_2[ES]) of enzyme substrate complex $[ES]$ is equal (Michaelis and Menten, 1913, Michaelis et al., 2011). Hence we can write $[ES] = K_1[E][S]/K_{-1} + K_2$ and if we define K_m (Michaelis constant) as $K_{-1}+K_2/ K_1$ then we can write $[ES] = [E][S]/K_m$. In first order rate reaction

constant K_2 can be defined as K_{cat} and the velocity of the product formation can be defined by Michaelis-Menten equation $v = ([E]_0[S]K_{cat}/K_m + [S])$ where $[E]_0$ is the total enzyme concentration. The maximum velocity (V_{max}) of the product formation at saturated substrate concentration can be explained as $V_{max}=[E]_0K_{cat}$ and hence Michealis-Menten equation can also be written as $v = V_{max}([S]/[S] + K_m)$. The temperature dependence of these rate equations is described by Arrhenius equation as follows:

$K = Ae^{-E_a/RT}$.

Where K is the rate constant, A is the pre-exponential factor related to molecular collision frequency and steric factors, E_a is the activation energy, R is the gas constant and T is the absolute temperature in Kelvins. This equation explains that any increase in temperature will result in exponential reduction in reaction rate. Cold adapted enzymes can handle this situation by decreasing the activation energy of the reaction (Hoyoux et al., 2004, Fedøy et al., 2007).

Part 3. Detection and verification of AHLs

3.1. Introduction

The first identification of AHLs was performed by extraction, separation and detection, using mass spectrometry (MS) and nuclear magnetic resonance (NMR) (Eberhard et al., 1981). Later bioassays became more common as they were less time consuming, economical and did not required a broad knowledge of the molecules or biochemical pathways (Bassler et al., 1997). The first bacteria to be investigated were *A. fischeri* (Eberhard et al., 1981), *V. harveyi* (Cao and Meighen, 1989), *Erwinia carotovora* (Bainton et al., 1992), *A. tumefaciens* (Zhang et al., 1993), *P. aeruginosa* (Pearson et al., 1994), *Yersinia enterocolitica* (Throup et al., 1995) and *V. anguillarum* (Milton et al., 1997). The phenotypic traits monitored were bioluminescence (Meighen, 1994), plasmid transfer (Zhang et al., 1993), antibiotic production (Chhabra et al., 1993), swarming motility (Givskov et al., 1998) and virulence (Jones et al., 1993, Pirhonen et al., 1993, Pearson et al., 1994). Then came the era of detection of multiple systems (Bassler et al., 1994a) and multiple AHLs in the same bacteria of *A. fischeri* (Kuo et al., 1994), *P. aeruginosa* (Pearson et al., 1995) and *V. anguillarum* (Milton et al., 2001). This was followed by different AHL synthases and receivers being identified (Winson et al., 1995). New developments in QS studies are still occurring like detection of new QS systems, new correlation with old systems, for example AHL related to quinolone (Heeb et al., 2011, Huse

and Whiteley, 2011), phenazine (Dietrich et al., 2006), indole (Lee et al., 2007) and modification of AHLs to form tetramic acids (Kaufmann et al., 2005, Lowery et al., 2009).

3.2. Extraction

In order to get sufficient amounts of signal molecules and decrease the background of contaminants, a common procedure is to extract the molecule of interest in an organic solvent. The organic solvent is supposed to be volatile, solubilize the signal molecule of interest and it should evaporate quickly on rotary vacuum pumps. This method is called liquid-liquid extraction method, it is widely used but it has some disadvantages like volatile liquid could be toxic, it could spill, due to evaporation the extraction is not always uniform and not all type of signalling molecules are extracted with one given solvent. In the past, different solvents have been used like chloroform, di-chloromethane, hexane and ethyl acetate (Brelles-Marino and Bedmar, 2001). In a previous report AHLs were screened for extraction solvent and it was found that dichloromethane and chloroform were better solvents and it was also reported that extraction yield was not media dependent but it increased with decreased in polarity of the AHL (Morin et al., 2003).

The other method is solid phase extraction (SPE). The biggest disadvantage of SPE cartilages is that they are expensive and cannot be reused, however it is less time consuming and usually the extraction is reproducible. Li et al reported as much as 100 ml of spiked media was extracted with no loss of sample (Li et al., 2006). For detailed procedures using SPE see (Frommberger et al., 2005, Schupp et al., 2005).

3.3. Methods for identification

Once extraction of the signalling molecule is done, it has to be analysed. Usually it is done either by biological methods or by non-biological methods. Some of the methods are described in the following sections.

3.3.1. Biological methods

A. Bioassay (Biosensing on solid medium)

Bioassay is performed on agar plates with bacteria containing different reporter genes. In some cases bacteria were mutated so they do not produce any AHL but AHL present in the sample triggers the reporter gene to produce an effect, which can be monitored qualitatively (Mcclean et al., 1997). In some reports the diameter of the zone produced by the biosensor bacteria is also used as a quantitative measure (Buch et al., 2003, Schwenteit et al., 2011). One disadvantage of

this method is that it is not possible to determine the precise structure (Ravn et al., 2001). Most common bacterium for this type of assay is *A. tumefaciens*. This bacterium is sensitive to 3-oxo-AHLs and upon receiving the molecules it expresses β-galactocidase, which in turn breaks down Xgal present on the agar plate to give blue color. In this strain the *traI* (AHL synthase) gene has been deleted and when AHL binds TraR it starts expressing *traG::lacZ* fusion reporter gene present on a plasmid (Cha et al., 1998, Zhu et al., 2003). The other bacteria is *Chromobacterium violaceum* (*C. violaceum*) CV026 which is more sensitive to C4 to C8 un-substituted homoserine lactones (Mcclean et al., 1997). The AHLs induce the production of a purple compound violacein which is visualized on agar plate. Some important landmark reports using these techniques are described in Table 7.

B. Bioluminescence (Biosensing in liquid medium)

This method was developed after multiple QS systems were identified and recently 25 members of *Vibrionaceae* family were screened by this method (Yang et al., 2011). Most bioluminescence assays have been performed using mutated *V. harveyi* strains (described in Table 8). It has been reported that bioluminescence assay can detect extremely low amount of AHLs especially in patients suffering from cystic fibrosis (Chambers et al., 2005). Another popular method is the green fluorescence protein (Gfp) based monitor bacteria strains, for example *Pseudomonas putida* (*P.putida*) (Steidle et al., 2001). There are many more bacteria that have been used in the past as AHL sensors like *E.coli*, *Aeromonas hydrophila*, *B. cepacia*, *V. fischeri*, *P. fluorescens and P. aeruginosa* with different reporter genes, for a review on this topic see (Fuqua and Winans, 1996, Dickschat, 2010, Wang et al., 2011). Table 7 describes and compares both the Biosensor and Bioluminescence methods.

3.3.2. Non-biological methods

Non-biological methods comprises usually of a separation technique of the extracted AHLs followed by a detection method. In some cases separation techniques are combined with biological methods and/or analytical instruments. Advantage, disadvantages and applications of some of the techniques are described below.

A. Separation techniques

(i). Thin layer chromatography (TLC)

TLC is a powerful technique both for analysis and separation of different AHLs, that can be analysed either by biological methods or analytical techniques (Shaw et al., 1997). Extracts are spotted on TLC plates and allowed to run in a mixture of organic solvents. Plates are usually

C18 silica matrix plates. Separated AHLs are either visualized with UV or by chromic agents like potassium dichromate in sulphuric acids. Alternatively, spots could be scraped off and the material obtained extracted with either dichloromethane or ethyl acetate for further analysis (Brelles-Marino and Bedmar, 2001). One disadvantage is that no structural information can be obtained and interference of metabolites is hard to avoid, for example two unknown spots found in a report which could not be verified (Buchholtz et al., 2006).

(ii). Liquid chromatography (LC)

This is one of the most commonly used separation technique, unlike TLC, which is performed on plates, LC are performed in columns, usually C8 or C18 with reverse phase chromatography. It has been reported to work in conjunction with biosensors as well as with mass spectrometry (MS) (Chambers et al., 2005, Cataldi et al., 2008). Ultra performance liquid chromatography has been reported with different detection methods like spectrophotometer (Li et al., 2006) and MS (Fekete et al., 2007) detectors. Nano-LC has been reported with small sample injection volumes of 1-5 µl but with a limitation that gradient cannot be made (Frommberger et al., 2004). Although it is one of the most common technique used in AHL analysis, it faces serious disadvantages such as it is time consuming, needs method optimization and long time for processing large number of samples (Wang et al., 2011).

Table 7: Comparisons of different methods
a: Comparison of biosensor methods of AHL detection. b: Comparison of different chromatographic techniques used to purify AHLs after extraction.

a.

Method	Biosensors on agar media	Biosensors in liquid
Principle	Zone formation of colour developed B-galactosidase Violacein production	Bioluminescence Green fluroscence
Qualitative	Yes	Yes
Quantitative	Yes	Most sensitive (in pM)
Advantage	Low sample volume Less expensive Less specialization Used in conjunction of TLC and HPLC	Low sample volume Less expensive Less specialization Used in conjunction of TLC and HPLC
Disadvantage	No structural information Time consuming	No structural information Time consuming

b.

Method	Thin layer chromatography	Gas chromatography	Liquid chromatography	Capillary zone electorphoresis
Principle	Extracts are spotted on plates and run in a mixture of organic solvents	GC followed by Mass spectrometry (EI)	LC is for separation used with Biosensors or MS	Separation on the basis of charge
Qualitative	Yes	Yes	Yes	Yes
Quantitative	Yes	Yes (poor) µM	Yes	Yes
Advantage	Low sample volume Less expansive Less specialization Used in conjunction with other detection techniques	Direct injection after extraction	Many variation UPLC, Nano, preparative and analytical Flexible with detection method	Distinguish between homoserine as degradation product and homoserine from intact AHLs
Disadvantage	Metabolites can interfere No structural information	Low volatility and instability of AHL at high temperature Used only with MS	Longer time for optimization and processing	AHLs cannot be reused

B. Analytical methods

Analytical methods are usually the final part of any non-biological analysis which mainly comprises identification of AHL.

Mass spectrometry

One of the most common analytical instruments used in the detection of AHLs, is MS. It not only provides the qualitative identification by measuring AHL's molecular mass but with advancement in this technique it is also possible to measure AHL quantitatively (Cataldi et al., 2008, Cataldi et al., 2009). Detection of AHLs by MS is usually being performed in a positive ion mode where AHL acquires a positive charge by adduct formation with hydrogen or sodium ion. The main advantage of MS technique is the accuracy and ability to tell the structure of the AHL detected. However the biggest disadvantage is large amount of sample required, cost of the instrument and need of a trained user.

3.4. Methods to study QS

The most important part of QS studies involves identifying the signalling molecule(s) and the synthases(s) involved in production of these molecules, receptors responsible for receiving the signals, signal transduction proteins, and transcription regulators involved in final expression of a gene or set of genes responsible for a particular phenotype.

Following the very first auto-inducer for bioluminescence identified as 3-oxo-C6-HSL in *A. fischeri*, researchers turned to the genes responsible for the light production (Eberhard et al., 1981). The *lux* operon was identified, cloned and expressed in *E. coli* and with the help of transposon mutagenesis each gene in the operon was investigated (Engebrecht and Silverman, 1984). LacZ was used as a reporter gene to verify that LuxR was responsible for expression of the operon and the expression was increased 10,000 fold by binding of the AI to LuxR. It was not until a *luxI* mutant in *A. fischeri* was created by gene deletion, that C8-HSL was identified to be produced by AinS and verified by recombinant protein expression in *E.coli* (Kuo et al., 1994). In the same report a LuxI dependent product, C6-HSL, was also reported. 3-OH-C4-HSL was identified in *V. harveyi* (Cao and Meighen, 1989), this was followed by mutagenesis done with nitrosoguanidine (Cao et al., 1989) to produce a dark mutant (non-bioluminescent), which upon addition of AHL was producing light. Similarly analysis of many QS gene mutants showed the presence of multiple systems involved in bioluminescence in *V. harveyi* (Bassler et al., 1994a).

In a recent study, three LuxR-type receptors in *P. aeruginosa*, (LasR, RhlR and QscR) were mutated and it was revealed that at least 37 genes were regulated in response to AHLs using microarray (Chugani and Greenberg, 2010). In a novel approach for identifying AHL receptors, affinity chromatography has developed with modified structures of AHLs. Upon binding of the modified AHL with the target protein, the AHL will be available for binding to a tag which allows easy isolation and detection of the targeted receptor protein (Dubinsky et al., 2009).

Table 8: QS signals, systems and methods of detection.

Bacteria species and Strains	System	Molecule/Method	Reference
Aliivibrio salmonicida NCIMB2262,VS81,VS201,VS224, VS288, VS289, VS297, VS399	ND/ND ND/ND	C6, 3-oxo-C6/ Bioassay (a b) HPLC-HR-MS AHL/Bioassay (a, d, f)	(Bruhn et al., 2005) (Garcia-Aljaro et al., 2012)
Aliivibrio fischeri MJ11, ES114	LuxI/LuxR AinS/AinR	3-oxo-C6-HSL /HPLC-MS C6-HSL/ HPLC-MS C8-HSL/ HPLC-MS	(Eberhard et al., 1981, Kuo et al., 1994)
Aliivibrio logei VIB414	ND/ND ND/ND	AHL/Bioassay (b, c) and Biolum (i) AHL/Bioassay (a)	(Yang et al., 2011) (Garcia-Aljaro et al., 2012)
Grimontia hollisae VIB314	ND/ND	AHL/Bioassay (b, c) and Biolum (i)	(Yang et al., 2011)
Listonella pelagia VIB305	ND/ND	AHL/Bioassay (b, c) and Biolum (i)	(Yang et al., 2011)
Photobacterium damselae VIB289	ND/ND	AHL/Bioassay (b, c) and Biolum (i)	(Yang et al., 2011)
Photobacterium leiognathi	ND/ND	AHL/Bioassay (a)	(Garcia-Aljaro et al., 2012)
Photobacterium ganghwense	ND/ND	AHL/Bioassay (a, d, f)	(Garcia-Aljaro et al., 2012)
Photobacterium phosphoreum P100, FR7	ND/ND	3-OHC8-HSL,Bioassay (a), TLC,HPLC-MS	(Flodgaard et al., 2005)
Vibrio aestuarianus VIB281	ND/ND ND/ND	AHL/Bioassay (b, c) and Biolum (i) AHL/Bioassay (a)	(Yang et al., 2011) (Garcia-Aljaro et al., 2012)
Vibrio anguillarum NB10, 19264	 VanI/VanR VanM/N	ND / Bioassay (a b) HPLC-MS, TLC 3-oxoC10-HSL 3-OHC6-HSL, C6-HSL AHL/Bioassay (a, b, d, f)	(Bruhn et al., 2005) (Milton et al., 1997) (Milton et al., 2001) (Garcia-Aljaro et al., 2012)
Vibrio campbellii LMG21361, LMG21362, LMG21363, LMG22888, LMG22889, LMG22890, LMG22895,VIB285	ND/ND ND/ND ND/ND ND/ND	AHL/Biolum (i) AHL/Biolum (i) and (ii) AHL/Bioassay (b, c) and Biolum (i) Bioassay (d, f) GCMS, HPLC-MS	(Defoirdt et al., 2006) (Defoirdt et al., 2008) (Yang et al., 2011) (Cuadrado-Silva et al., 2013)
Vibrio chagasii	ND/ND ND/ND	AHL/Bioassay (b, c) and Biolum (i) AHL/Bioassay (a)	(Yang et al., 2011) (Garcia-Aljaro et al., 2012)
Vibrio cholera (isolated from *Acanthurus coeruleus*)	ND/ND	AHL/Bioassay (a)	(Garcia-Aljaro et al., 2012)
Vibrio cincinnatiensisi VIB287	ND/ND	AHL/Bioassay (b, c) and Biolum (i)	(Yang et al., 2011)
Vibrio diazotrophicus VIB290	ND/ND ND/ND	AHL/Bioassay (b, c) and Biolum (i) AHL/Bioassay (a)	(Yang et al., 2011) (Garcia-Aljaro et al., 2012)
Vibrio fluvialis VIB292	ND/ND ND/ND	AHL/Bioassay (b, c) and Biolum (i) AHL/Bioassay (a, d, f)	(Yang et al., 2011) (Garcia-Aljaro et al., 2012)
Vibrio furnissii VIB293	ND/ND	AHL/Bioassay (b, c) and Biolum (i)	(Yang et al., 2011)
Vibrio gazogenes VIB294	ND/ND	AHL/Bioassay (b, c) and Biolum (i)	(Yang et al., 2011)

Vibrio harveyi LMG22891, LMG22893, LMG22894, BB120, VIB 571, VIB 645	LuxM/LuxN ND/ND ND/ND	3-OHC4-HSL/ HPLC-MS ND/Biolum (i) and (ii) AHL/Bioassay (b, c) and Biolum (i) AHL/Bioassay (a) Bioassay (d, f) GCMS, HPLC-MS	(Cao and Meighen, 1989) (Defoirdt et al., 2008) (Yang et al., 2011) (Garcia-Aljaro et al., 2012) (Cuadrado-Silva et al., 2013)
Vibrio ichthyoenteri DA3; (isolated from water)	AHL detected ND/ND	AHL/Bioassay (b, c) and Biolum (i) AHL/Bioassay (a)	(Li et al., 2010) (Garcia-Aljaro et al., 2012)
Vibrio lentus	ND/ND	AHL/Bioassay (a)	(Garcia-Aljaro et al., 2012)
Vibrio mediterranei VIB296	ND/ND ND/ND	AHL/Bioassay (b, c) and Biolum (i) AHL/Bioassay a, d, f	(Yang et al., 2011) (Garcia-Aljaro et al., 2012)
Vibrio metschnikovii	ND/ND ND/ND	AHL/Bioassay (b, c) and Biolum (i) AHL/Bioassay (a, b, d, f)	(Yang et al., 2011) (Garcia-Aljaro et al., 2012)
Vibrio mimicus VIB298	ND/ND ND/ND	AHL/Bioassay (b, c) and Biolum (i) AHL/Bioassay (a)	(Yang et al., 2011) (Garcia-Aljaro et al., 2012)
*Vibrio parahaemolyticus*BB22	LuxM	AHL / Biolum (i)	(Bassler et al., 1997)
Vibrio pomeroyi	ND/ND	AHL/Bioassay (b, c) and Biolum (i)	(Yang et al., 2011)
Vibrio proteolyticus VIB306	ND/ND ND/ND	AHL/Bioassay (b, c) and Biolum (i) AHL/Bioassay (a, d)	(Yang et al., 2011) (Garcia-Aljaro et al., 2012)
Vibrio rotiferianus	ND/ND	AHL/Bioassay (a, b, d, f)	(Garcia-Aljaro et al., 2012)
Vibrio sp. UST950701, UST010723 *Vibrio sp.* (23-6PIN), (11-6DEP)	ND/ND ND/ND	Bioassay (b, e) C6-HSL GCMS, TLC Bioassay (d, f) GCMS, HPLC-MS	(Huang et al., 2007) (Cuadrado-Silva et al., 2013)
Vibrio splendidus DMC-1 DTC-5, VIB308	ND/ND ND/ND ND/ND	AHL/Bioassay (a, b, c) HPLC-MS AHL/Bioassay (b, c) and Biolum (i) AHL/Bioassay (a, d)	(Bruhn et al., 2005) (Yang et al., 2011) (Garcia-Aljaro et al., 2012)
Vibrio tubiashii VIB309	ND/ND	AHL/Bioassay (b, c) and Biolum (i)	(Yang et al., 2011)
Vibrio vulnificus ATCC27562, NCIMB2136, 94-8-108, 98-2-32, YJ016, CECT 5164, CG100, ATCC 33816, CECT 4606, 94-9-119, CECT 4608, CECT 4602, CECT 4604, CECT 4867, CECT 4917, CECT 4999, CECT 5763, CCUG 38521, C1, 94-9-123, CECT 5198, CECT 5769, CECT 7030, CECT 7029 95-8-161, 95-8-162, 95-8-6, 162, 11028	ND/ND LuxM/ LuxI absent ND/ND ND/ND ND/ND	AHL / Bioassay (a, b) C4-HSL/ Bioassay (a, b) HPLC-MS C4-, 3-oxo-C10- and 3-oxo-C12-HSLs C6-, 3-oxo-C8- and 3-oxo-C14-HSLs HPLC-MS/MS AHL/Bioassay (a)	(Bruhn et al., 2005) (Valiente et al., 2009) (Morin et al., 2003) (Garcia-Aljaro et al., 2012)

LuxM/ LuxI systems produce AHLs. ND/ND is for systems not determined.
1. Bioassay on Agar plates (Ravn et al., 2001),

(a) *A. tumefaciens* –NT1 (pZLR4)(Cha et al., 1998). Beta-galactocidase which breakdown X-gal results in blue colour. Sensitive to 3-oxo-AHL

(b) *C. violaceum*– CV026(Mcclean et al., 1997). Sensitive to unsubstituted C4 and C8 HSL and these AHLs induce production of the purple compound violacein.

(c) *A. tumefaciens* reporter strain KYC55 (pJZ372)(pJZ384)(pJZ410)(Zhu et al., 2003).

(d) *P. putida* F117 (pAS-C8) and (pKR-C12) AHLs sensor strains (Steidle et al., 2001) green fluorescence protein used for detection.

(e) *A. tumefaciens* A136 (traI-lacZ fusion) (pCF218)(pCF372) AHL biosensor.

A. tumefaciens KYC6 (3-oxo-C8-HSL overproducer as positive control) for AHL assay (Fuqua and Winans, 1996, Mclean et al., 2004).

(f) *E. coli* MT102 (pSB403) or (pSB401) (Winson et al., 1998) Lux operon based bioluminescence on the agar medium.

2. TLC reverse phase C18 (Shaw et al., 1997)
3. HPLC-MS - High pressure liquid chromatography mass spectrometry
4. GCMS - Gas chromatography mass spectrometry
5. Bioluminesence (Biolum)

(i) *V. harveyi* strains

BB170 (AI-2 sensor, LuxM sensor mutant) and BB886 (AI-2 sensor mutant, LuxM sensor) BB120 wild type (Greenberg et al., 1979, Bassler et al., 1997)

JAF375 harveyi (LuxN and LuxQ mutants) (Freeman and Bassler, 1999)

(autoinducer 1) (sensor AHL-), (sensor AI-2-), (sensor CAI-1+)

JMH597 harveyi (LuxN and CqsS mutants) (Henke and Bassler, 2004)

(autoinducer 2) (sensor AHL-), (sensor AI-2+), (sensor CAI-1-)

JMH612 harveyi (LuxPQ and CqsS mutants) (Henke and Bassler, 2004)

(autoinducer 1) (sensor AHL+), (sensor AI-2-), (sensor CAI-1-)

(ii) plasmid pBB1 carrying *V. harveyi* luxCDABE inserted in *Vibrio cholera* by conjugation (Lenz et al., 2004)

II. Aims of the study

The main objective of this study was to improve our understanding of the bacterial communication in the *Vibrionaceae* family, with the main emphasis on AHL detection and quantification.

Secondary objectives:

(1) To develop an efficient method for extraction and accurate determination of AHLs and use this method to screen available strains of *Vibrionaceae* species for AHLs (Paper 1).

These objectives were achieved by the following studies:

a. Establishment of effective extraction strategy.
b. Development of detection method with low limits of detection and quantification.
c. Development of efficient pipeline for determination by improvement of sample processing and analysis time

(2) Exploring the QS systems in *A. salmoncida* using AHL detection/quantification in knockouts mutants of targeting genes involved in the QS systems (Paper 2).

The goal was achieved by:

a. Creation of QS mutants by plasmid insertion or by complete gene deletion.
b. Using mutants to identify the AHL synthases products and regulation of operons.

(3) To characterize the VanM synthase from *V. anguillarum* (Paper 3).

The goal was achieved by:

a. Expression and purification of VanM
b. Determination of substrate specificity

III. Summary of the main results

Paper1: Presence of acyl-homoserine lactones in 57 members of the *Vibrionaceae* family

- A HPLC-MS based method for detection of AHLs was established using SRM (single reaction monitoring) mode method. AHLs were extracted with ethyl acetate from spent culture medium supernatant, dried and re-dissolved in 20% acetonitrile and before injection on HPLC-MS.

- In total 15 AHLs were screened and their LOD, LOQ and Linearity range were determined. Accuracy and precision of the method for detecting the AHLs was also evaluated.

- To compare the method with previously published data we analyzed *Aliivibrio fischeri* ES114 and found six AHLs - three more than previously reported.

- Fiftyseven bacteria (7 *Vibrio*, 5 *Photobacterium* and 45 *Aliivibrio* isolates) were phylogenetically classified into 6 clades. Clade I members were mainly aliivibrios and produced maximum number of AHLs (8-11 in number) with 3-oxo-C6-HSL in highest quantities. From clade II only *A. fischeri* ES114 was tested which produced C8 as the dominating AHL. Clade III members were *A. woodanis* producing mainly 3-OH-C10-HSL. Clade IV contains aliivibrios which do not produced AHLs. Clade V was vibrios clade with different AHL profiles. Finally the Clade VI members were photobacteriums, which did not produce any AHLs.

Paper 2: Temperature dependent regulation of quorum sensing in *Aliivibrio salmonicida* LFI1238

- In this study several mutants of QS system were constructed in psychrophilic fish pathogenic bacteria *Aliivibrio salmonicida* LFI1238.

- Using high-performance liquid chromatography with mass spectrometry (HPLC-MS/MS) it was found that LuxI was responsible for producing 7 AHLs and AinS produced only 1 AHL.

- AHL production was found to be temperature dependent and the production was higher at 6 and 12°C, compared to at 16°C.

- The master regulator of QS, LitR was found to regulate both the synthases. However, AinS was more tightly regulated as compared to LuxI synthase. Both AinS and LuxI AHL systems were also involved in biofilm formation.

- Both *luxR* genes on the *lux* operon were found to be necessary for the production of LuxI AHLs. This indicates heterodimer formation between them (LuxR1 and LuxR2) for the expression of *luxI* gene.

- Other genes studied (*luxS*, *luxP*, *luxO*, and *qrr*) did not influence the AHL production.

Paper 3: Preliminary analysis of the substrate specificity of acyl homoserine lactone (AHL) synthase VanM from *Vibrio anguillarum*

- The VanM synthase from *V. anguillarum* was produced in *E. coli* BL21 (DE3) RIL cell lines, as a fusion protein with intein and chitin binding protein tag to homogeneity.

- VanM uses SAM as the lactone doner and acyl-Acyl Carrier Protein (acyl-ACP) or acyl-coenzyme A (acyl-CoA) as the acyl chain provider. VanM produced the product C6-HSL with both the substrates and it was indicated that C6-ACP was a better substrate than C6-CoA.

- Substitution in the 3rd position and chain length specificity were tested with different CoAs. Upon screening of 3-OH-C4-CoA, 3-oxo-C4-CoA and C4-CoA it was found that 3-OH-C4-CoA had the highest turnover. Using different chain length ((C4-C10)-CoA) it was found that C6 chain length had the most optimum activity.

IV. Discussion

4.1. General Discussion

In the first paper we have established method for AHL detection and it was successfully applied to analyze 57 bacterial strains of *Aliivibrios*, *Photobacterium* and *Vibrios*. In the work presented in the second paper we mutated several genes belonging to the QS system of *A. salmonicida* LFI1238. We were able to detect eight different types of AHLs that were produced by the two AHL synthases LuxI and AinS. We also studied the effect of QS gene deletions on the synthases and this revealed a complex QS system, which was temperature dependent. Finally in the third paper, we expressed the main synthase present in *Vibrionaceae* family (LuxM type) and measured its kinetics. During the investigation we found that 3-OH position of the AHL was most preferred by the enzyme along with the C6 chain length.

4.2. Detection of AHLs

AHL quantification has been improving since the first AHL was detected (Eberhard et al., 1981). Apart from MS, most common procedures for quantification were based on bioassays (Mcclean et al., 1997) and TLC (Shaw et al., 1997) using known standards at different concentrations. While bioassays have limitation since only some types of AHLs can be detected and problems with interference of bacterial metabolites, MS have proven to be the best choice. The main drawback of using MS for high throughput analysis has been larger amount of sample required and time consuming extraction and analysis procedures (see Table 9). One report have even mentioned using solid phase extraction (SPE) for improving efficiency, however this actually adds to the cost of analysis to the already expensive HPLC-MS method (Englmann et al., 2007). We have optimized the liquid-liquid extraction (LLE) procedure which uses small amount of sample and eventually reduces the time of sample preparation and analysis (~200 samples per day).

Table 9 : Mass spectrometry development and AHL detections through the years ESI Positive ion electrospray; FAB Fast atom bombardment; CID Collision induced dissociation; FD Field desorption; CI Chemical ionization; EI Electron impact ionization; MS is mass spectrometry (it is used where type of mass analyzer is not mentioned); FTICR Fourier-Transform-Ion-Cyclotron-Resonance; TOF Time of Flight mass spectrometer; SPE solid phase extraction.

Ionization technique in -MS	Bacteria	Culture volume	Extraction solvent	Study	References
FD-MS	*Photobacterium fischeri*	6 liters	Chloroform	Structural Identification of AHL	(Eberhard et al., 1981)
CI-MS	*Vibrio harveyi*	6 liters	Chloroform	Structural Identification of AHL	(Cao and Meighen, 1989)
CI-MS	*Pseudomonas aeruginosa*	6 liters	Ethyl acetate	Structural Identification of AHL	(Pearson et al., 1994)
CI and EI with magnetic sectors	*Vibrio fischeri*	4 liters	No extraction	Multiple AHLs of luminescence	(Kuo et al., 1994)
FAB-MS	*Vibrio anguillarum*	4 liters	DCM	The LuxM homologue VanM and vanI	(Milton et al., 1997, Milton et al., 2001)
FAB-MS	*Chromobacteriurn Violaceum* ATCC 31532	4 liters	DCM	detection of AHLs by Chromobacteriurn violaceum	(Mcclean et al., 1997)
ESI and CID-Triple quadrupole	*Pseudomonas fluorescens* 2-79	500 ml	Ethyl acetate	Detecting and characterizing AHLs by TLC	(Shaw et al., 1997)
ESI-MS	*Pseudomonas fluorescens* F113	20 ml	DCM	HdtS third protein family capable of AHL biosynthesis	(Laue et al., 2000)
ESI-MS	*Yersinia pseudotuberculosis YPIII* *Pseudomonas aeruginosa PAO1*	20 ml	DCM	AHLs undergo lactonolysis in a pH, Temperature, and Acyl Chain Length-Dependent Manner	(Yates et al., 2002)
ESI-MS	*Vibrio vulnificus*	50 ml	DCM	Detection and quantification of AHLs	(Morin et al., 2003)
ESI-LCQ ion trap	*Burkholderia cepacia*	500 µl	Chloroform	Method development	(Frommberger et al., 2004)
ESI-LCT orthogonal TOF	*Vibrio anguillarum*	50 ml	Ethyl acetate	Influence of growth conditions and serotype	(Buchholtz et al., 2006)
ESI-Triple quadrupole	Plasmid expressed in *Escherichia coli* *Vibrio fischeri PAO1*	10 ml	Ethyl acetate	Specificity of AHL Synthases examined by Mass Spectrometry	(Gould et al., 2006)
ESI-MS	*Pseudomonas aeruginosa* PAO1, *Vibrio fischeri MJ-1* *Vibrio harveyi*	3-5 ml	Ethyl acetate	Method development	(Makemson et al., 2006)
FTICR	*Burkholderia cepacia*	40 ml	SPE	The hydrolysis of unsubstituted AHLs	(Englmann et al., 2007)
	Pseudomonas putida IsoF	200 ml		AHL production and degradation	(Fekete et al., 2010)
ESI-LTQ-FTICR	*Serratia liquefaciens*	10 ml	Chloroform	Profiling AHLs	(Cataldi et al., 2008)
ESI triple quadrupole	*Tenacibaculum maritimum*	100 ml	DCM	AHL production and degradation	(Romero et al., 2010)
ESI-Ion trap	Coral associated bacteria	15 ml	Ethyl acetate	QS characterization	(Golberg et al., 2011)
ESI- LTQ orbitrap	*Burkholderia pseudomallei*	10 ml	Ethyl acetate	AHLs produced by BpsI/BpsR in biofilm	(Gamage et al., 2011)
ESI-triple-quadrupole	*Pseudomonas aeruginosa*	1ml	Ethyl acetate	Method development	(Ortori et al., 2011)

4.3. AHLs extraction

Several solvents have been used in the past to extract AHLs such as chloroform (Eberhard et al., 1981, Cao and Meighen, 1989), ethyl acetate (Kuo et al., 1994), and dichloromethane (Milton et al., 1997). However, with increase in the number and types of AHLs extracted, it is becoming important to screen for a solvent which can be good enough for all the AHLs. Upon screening 15 different AHLs (ranging from C4 to C12 with or without substitutions) with different solvents we found ethyl acetate to be comparatively better than others. However analyzing the C4s chained AHLs was difficult because their short chain made them more water soluble as compared to other AHLs.

4.4. AHL stability

There are several factors that can affect the stability of AHL and the two most important are described below.

4.4.1. pH

In the very first report on extraction of AHLs, it was revealed that acid stabilizes AHL since AHLs undergoes a lactone ring opening reaction in alkaline media (Eberhard et al., 1981). Hence acid was added to all the culture samples as soon as they were harvested. Acid was also added to all types of the standards stock solution and we found no hydrolysis taking place even after storing them for 1-2 years.

4.4.2. Solvent

Acetonitrile was chosen as the eluent for the reverse phase chromatography. In earlier reports methanol was used as eluent, however it was proved later that methanol forms more adducts as compared to acetonitrile (Cataldi et al., 2008). Moreover, we also realized that the adduct formation was not uniform between sample and standards (due to unknown reasons) and methanol adducts further formed hydrogen and sodium adducts. Hence, methanol was discarded as solvent since the analysis became more complicated by keeping track of multiple species formed.

4.5. Has *A. salmonicida* a novel LuxR type of regulator?

It has been reported that the order of the structural genes of the *lux* operon in *A. salmonicida* is similar to that of *lux* operons from other luminous bacterial species, except additional regulator LuxR2 followed by LuxI outside the *lux* operon (Nelson et al., 2007). LuxR from *A. fischeri* is

known to bind C8-HSL and 3-oxo-C6-HSL at different densities. LuxR in *A.fischeri* forms homo-dimer like TraR (Vannini et al., 2002) and LasR (Bottomley et al., 2007). Our results indicate that the transcriptional activators LuxR1 and LuxR2 are both necessary for activation of LuxI. This may implicate that the two LuxRs form a hetro-dimer in order to activate LuxI. It is also reported that *lux* operon is vertically transferred into the *Vibrionaceae* family (Kasai et al., 2007, Urbanczyk et al., 2008). LuxR1 and LuxR2 are ~60% identical to each other and to the LuxR of *A. fischeri*, which indicate that the duplication has taken place. Moreover, the LuxRs are regulating gene expression in both directions as sense and antisense products of some genes of *lux* operon (Nelson et al., 2007). Two Lux boxes also have been reported similar to that of *A. fischeri*, one box is in front of the *lux* operon and the other in front of the *luxI* gene (Nelson et al., 2007). In a recent review (Stevens et al., 2011), LuxR type of proteins were classified into 5 categories (Figure 13). Type 1 is TraR receiver from *A. tumefaciens* where the AHL is not only required for receiver protein folding but also for dimerization of AHL binding domains which leads to development of DNA binding capabilities of the C-terminal DNA binding domain. In this type the AHL binding is very tight and not reversible and in absence of AHL, TraR is rapidly degraded by proteases (Zhu and Winans, 2001, Zhu and Winans, 1999). Type 2 is LuxR of *A. fischeri* where the AHL is required for receiver protein folding and stability, but the binding of AHL is reversible leading to inactive dimer formation (Urbanowski et al., 2004). Type 3 is MrtR type of receivers from *Mesorhizobium tianshanense* which do not require AHL for receiver protein folding and remain as inactive monomers. When they receive an AHL molecule they dimerize and become functional, however AHL binding to receiver protein is weak and reversible (Yang et al., 2009). Type 4 is EsaR type of receiver from *Pantoea stewartii* which does not require AHL for receiver protein folding and is also functional dimer without AHL binding, but in an opposite way becomes un-functional upon binding to AHL molecules. However binding of AHL is still reversible which could lead to an active dimer. Type 5 is SdiA type of receiver from *E. coli* does not require AHL for protein folding but can receive multiple AHL to become functional and still remain as a monomer. Binding of AHL to the SdiA protein is reversible. In general, single AHLs could bind to several receivers or multiple AHLs bind to the same receiver under different conditions. This is seen in *A. fischeri* where AinS product C8-HSL binds to both AinR as well as LuxR at intermediate cell density. The same LuxR also binds 3-oxo-C6-HSL at high cell density (Lupp et al., 2003). The above discussion it can be extrapolated that the LuxR1 and LuxR2 may form a heterodimer and bind several AHLs produced

by the LuxI synthase and may form a new type of LuxR recievers. However, this has to be further investigated.

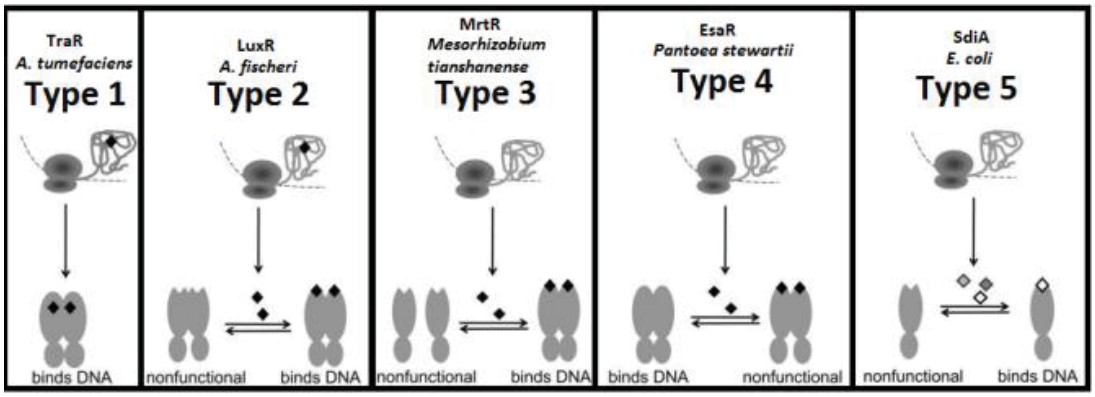


Figure 13: Classification of LuxR type receivers based on the way they bind AHL and their properties of forming functional or non-functional dimers. Figure adapted from (Stevens et al., 2011).

4.6. Why do bacteria produce different AHLs and at different concentrations?

Most aliivibrios seems to produce multiple AHLs at different concentrations. However, out of 57 strains screened many of them namely *Aliivibrio* R8-63, R8-67, *V. tapetis*, and *Photobacterium* (SP001, SP002, SP004, SP005, SP044) did not produce AHL. This could mean they do not have an AHL-based QS system or that the AHLs are not expressed under the growth condition used. Some bacteria has been shown to be able to receive signals, but not able to produce AHLs. Such strains are also sometimes referred to as cheaters (Hense et al., 2007). The above-mentioned strains could also belong to this class of bacteria.

The different concentration of the AHLs could also be explained in the terms of Hamilton rule, which has been recently applied on the QS system (Diggle et al., 2007, Strassmann et al., 2011). It can be interpreted as, favoring of beneficial cooperation takes place when the difference between the product of the distance of genetically related species (r) and the fitness cost to beneficiary (b), subtracted by fitness cost to the altruist is a positive value (i.e $rb-c > 0$). The mutual benefits could be like exo-protease, siderophore and biofilm production. AHLs present in lower amount could be less for QS but more for blocking other bacteria's QS systems or to confuse unrelated species of bacteria. For example QS in *Aeromonas* are found to be blocked by longer chain length AHLs (Swift et al., 1999).

4.7. LitR is temperature regulated and effects AHL synthesis

In paper 2 we found that deletion of *litR* resulted in decresed production of 3-OH-C10-HSL (AinS product) at lower temperature and we also saw that AHL production are higher at lower temperature (6°C) as compared to higher temperatures (16°C). This suggests that LitR is regulating synthesis of this AHL by controlling *ainS* gene expression. Moreover, it also suggests that LitR could be itself temperature regulated. The AHLs produced by LuxI were also to some extend, reduced in the LitR mutant which could indicate an indirect regulation of *luxI* gene expression.

4.8. Is the regulation of the *A. salmonicida* bioluminescence operon unique?

The bioluminiscence operon in *A. salmonicida* is unique in many ways. Firstly the cryptic bioluminescence reported by Fidopiastis et al. (Fidopiastis et al., 1999) and secondly the unique arrangement of the *lux* operon (Nelson et al., 2007). The cryptic bioluminescence has been explained by a defect in the *luxD* gene leading to a truncated acyl-ACP transferase that supplies the requisite aldehyde substrate for the bioluminescent reaction (Manukhov et al., 2011). When deleting either LuxR1 or LuxR2, no generation of AHLs was observed indicating a direct regulation of *luxI* gene expression. We speculate in paper 2 that both LuxR1 and LuxR2 are nesessary for the LuxI signal generation, which suggests a hetero-dimer formation between the two LuxRs. Hence we belive that *A. salmonicida* LFI1238 has a unique bioluminescence system compared to other known lux operons.

4.9. Is it possible to predict AHL producing QS systems from experimental identified AHLs?

There are at the moment three different types of AHL producing enzymes LuxI, HdtS and LuxM identified. The majority of AHL synthases identified show either formation of 3-OH-HSLs or 3-oxo-HSLs along with or without un-substituted AHLs of the same or similar acyl chain lengths (Churchill and Chen, 2011). In paper 1 we have been able to identify 3-OH-C8-HSL in *A. fischeri* ES114 at very low concentration, which has not been reported earlier. This indicates that both 3-OH-C8-HSL and C8-HSL can be produced by LuxM type proteins. On the other hand we were able to detect 3-oxo-C8-HSL and C8-HSL by LuxI type enzyme in *A. salmonicida* LFI1238 (see paper 2). This indicates C8-HSL can be produced by both LuxM and LuxI type of enzymes and thus by simply looking at the AHL it will be misleading to predict the system producing it. Another point that can mislead is that in almost all cases LuxM homologues have been involved in the production

of 3-OH-HSL. Some LuxI family members are also known to produce 3-OH-HSL like PhzI from *Pseudomonas fluorescens* (Khan et al., 2007). Hence it will not be sufficient to predict the AHL producing QS system simply by analyzing the AHLs.

4.10. Purification and stabilization of VanM enzyme

VanM is an AHL synthase from *V. anguillarum* NB10 responsible for production of C6-HSL and 3-OH-C6-HSL (Milton et al., 2001). VanM belongs to the LuxM family of AHL synthases. The homologues proteins AinS from *A. fischeri* was reported to be purified with maltose binding protein as a fusion tag to aid solubility, as the AinS on its own precipitated very quickly (Hanzelka et al., 1999). In our lab AinS from *A. salmonicida* LFI1238 was successfully expressed in Artic express cell line at 8°C. However we were not able to purify it to homogeneity. We believe the reason is that the cold adapted chaperone from Arctic express cell line used in the expression was bound to the N terminal of AinS enzyme. Such a situation also happened while expressing and purifying VanM as *E. coli* chaperone was bound to the VanM protein (confirmed by MS). The only way to improve the yield of VanM purification was to use 750 mM sodium chloride in the sonication step, which broke the chaperone and VanM interaction.

VanM was finally expressed as a fusion protein with intein and chitin binding protein. However, VanM could not be concentrated above 0.3 mg/ml. Glycerol, K_2SO_4, 2-mercaptoethanol, and EDTA was added to the buffer to enhance stability of VanM. Change in pH (from 9.2 to 8) and reducing the sodium chloride (to 200 mM) also improved the protein stability. Although the protein stabilized, real improvement was seen only by the addition of SAM and the protein could be concentrated to 10 mg/ml. SAM is not the preferred stabilizer as it has a very short half-life after which it gets converted into an inhibitor SAH (Hanzelka et al., 1999) and kinetic studies is not possible. Hence a screening of different components was conducted to identify molecules that could stabilize VanM. Different concentrations of glycerol, trehalose, and ethylene glycol were mixed with a fixed amount of enzyme and stored at different temperature (4, -20 and -80°C). Trehalose was the only additive that was able to stabilize the VanM.

4.11. Prediction of the active site in LuxM family

The primary structure of VanM is not similar to the LuxI family of AHLs synthases. This indicates structural differences and prediction of the active site in VanM are extremely difficult. However, as the substrates, products and the chemical reaction are similar, it motivates us to look for highly

conserved active site residues in LuxM and LuxI families. As described in the introduction section, there are three protein structures of LuxI homologue been reported, Table 10 shows the identities among the sequences and Figure 14 shows the structural alignment along with the conserved residues.

Table 10: LuxI homologues with 3D structures available in the protein data bank.

Enzymes	Name	% identity with As_LuxI	PDB	RMSD CA (Å) Consensus scores[a]	RMSD CA (Å) Compared to LasI[a]	Z score[a]
LasI *P. aeruginosa*	LasI	30%	1RO5	1.0986	------	------
VanI *V.anguillarum* NB10	Va_VanI	46%	------	------	------	------
LuxI *A.salmonicida* LFI1238	As_LuxI	100%	------	------	------	------
LuxI *A.fischeri* ES114	Af_LuxI	80%	------	------	------	------
TofI *Burkholderia glumae*	TofI	29%	3P2H	1.2003	1.39	11.0
EsaI *Pantoea stewartii*	EsaI	7%	1K4J	1.4528	1.99	6.4

[a] Data generated from http://www.ebi.ac.uk/msd-srv/ssm/ssmstart.html

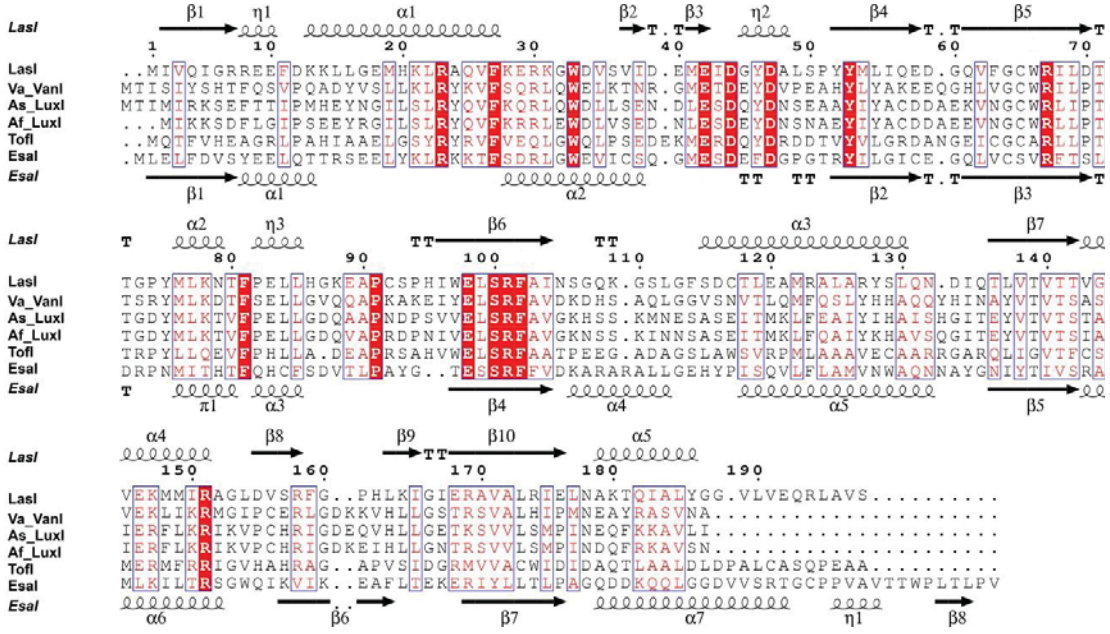


Figure 14: Sequence and structural alignment of selected LuxI and VanI homologues.
Alpha stands for Alpha helices and beta stands for beta sheets. Sequence alignment done in ClustalW (Thompson et al., 1994) and Structural alignment done in ESPript 2.2 (Gouet et al., 1999).

Enzymes of the LuxI family usually have a V shaped cleft with a deep cavity/tunnel formed by mixed alpha-beta-alpha sandwich type of protein folding. This V-shaped cleft is formed by

antiparallel beta sheet. The active site was recently revealed in this V-shaped cleft with the structure of TofI bound with inhibitors (Chung et al., 2011). One of the inhibitor was a homoserine lactone analog and the second inhibitor was MTA (see Figure 15 (A)). The residues in this site one are commonly associated with both the inhibitors and they are highly conserved (ELSRF, residue ~100 in the alignment shown in Figure 14). Other important residues associated with the MTA part were TRP33, PHE83, VAL82, GLN34, LEU31 and ARG104. For the acyl chain SER148, PHE153, MET149, PHE105, LEU102 and THR145 were found interacting (see Figure 15(B)). According to Chung et al (Chung et al., 2011) Asp44 was found to be highly conserve and important for stability whereas ARG104 was found to be conserve, interacting with both the inhibitors and important for activity. The acyl chain aligns itself along the beta sheet such that the acylation and lactonization reaction takes place at the top of the V-shaped cleft (see site 1 in Figure 15(C)). Based on this knowledge a Swiss models (Arnold et al., 2006) was prepared for both *V. anguillarum* VanI and *A. salmonicida* LuxI using LasI PDB 1RO5 as template. An inhibitor (same as shown in figure 15 (B)) was also modeled into the overlapped structure of VaVanI and AsLuxI using Pymol (see Figure 15(D)), indicating that this inhibitor could be used for crystallography or kinetic experiments.

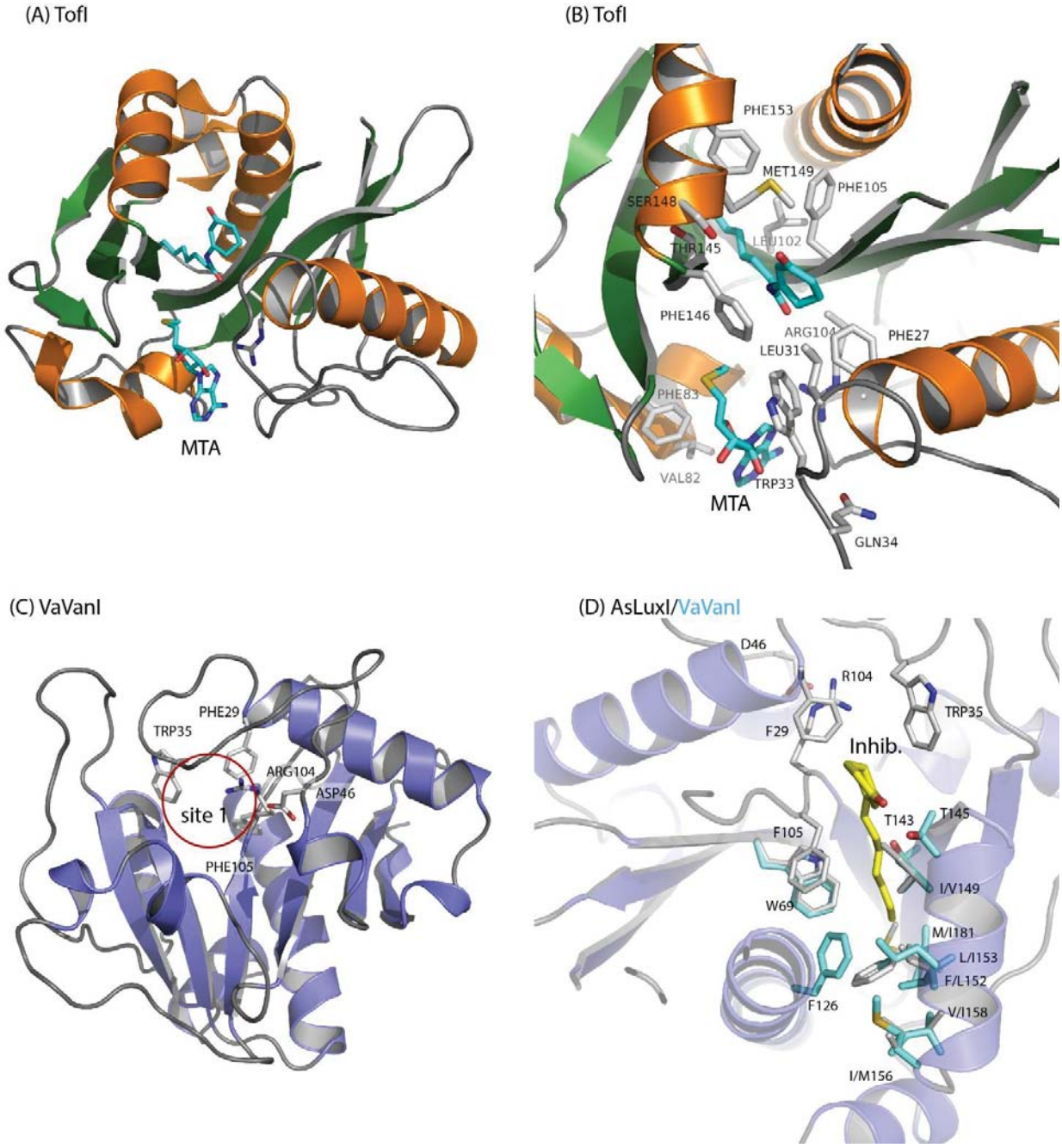
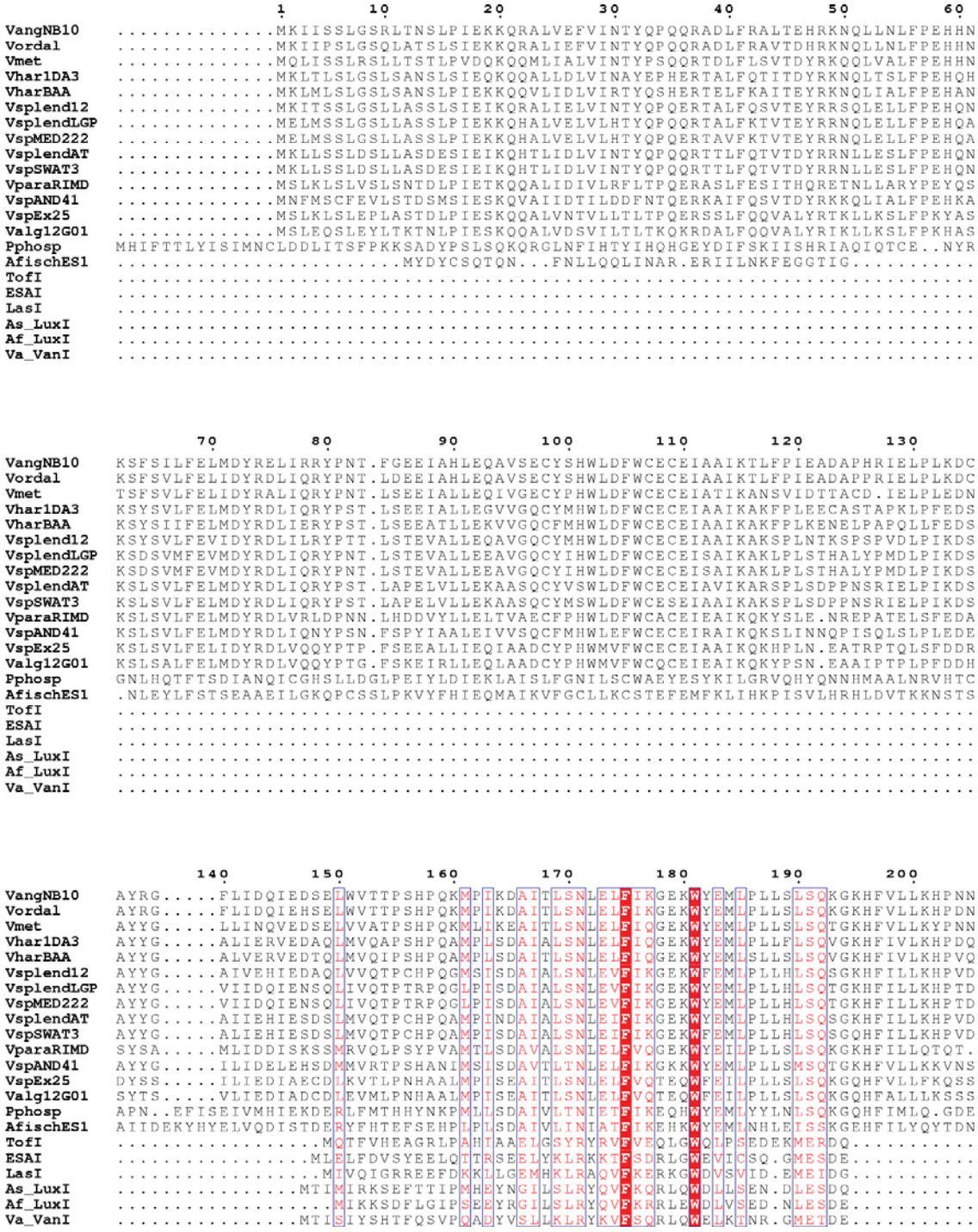


Figure 15: Over all ribbon structure of (A) TofI PDB 3P2H (Chung et al., 2011) (B) TofI active site residues bound with inhibitors (AHL analog and MTA). (C) Va_VanI model, site 1 is probable site of reaction. (D) As_LuxI model overlapped with Va_VanI and the AHL analog inhibitor was modeled in using Pymol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.)

Using ClustalW we tried to align all the three groups of LuxM family members (presented in paper 3 phylogenetic tree) along with LuxI family members from Figure 14 alignment, but it failed to show any conserved residues around the LuxI family conserved active site. Then we took some selected sequences from group 1 and group 2 of the LuxM family members and aligned it with LuxI family members in ClustalW which gave us the alignment shown in Figure 16. This alignment shows us the exact site 1 conserved residue seen previously in Figure 14 (ELSRF) and Trp33 (from Figure 14) also aligns itself with Trp181 (Figure 16) along with a most conserved area of the LuxM family. The N-terminal of VanM (first 150 residues) do not show any similarity with LuxI proteins, indicating a secondary or dual function of the enzyme. In our opinion the N-terminal of the enzyme may have a chaperone binding site, as the GroEL co-eluted with VanM during purification (see paper 3). We also had problems with purifying AinS (from *A. salmonicida* LFI1238) which was expressed in Arctic express cell line at lower temperature with N-terminal His-tag but we failed to purify (data not shown).



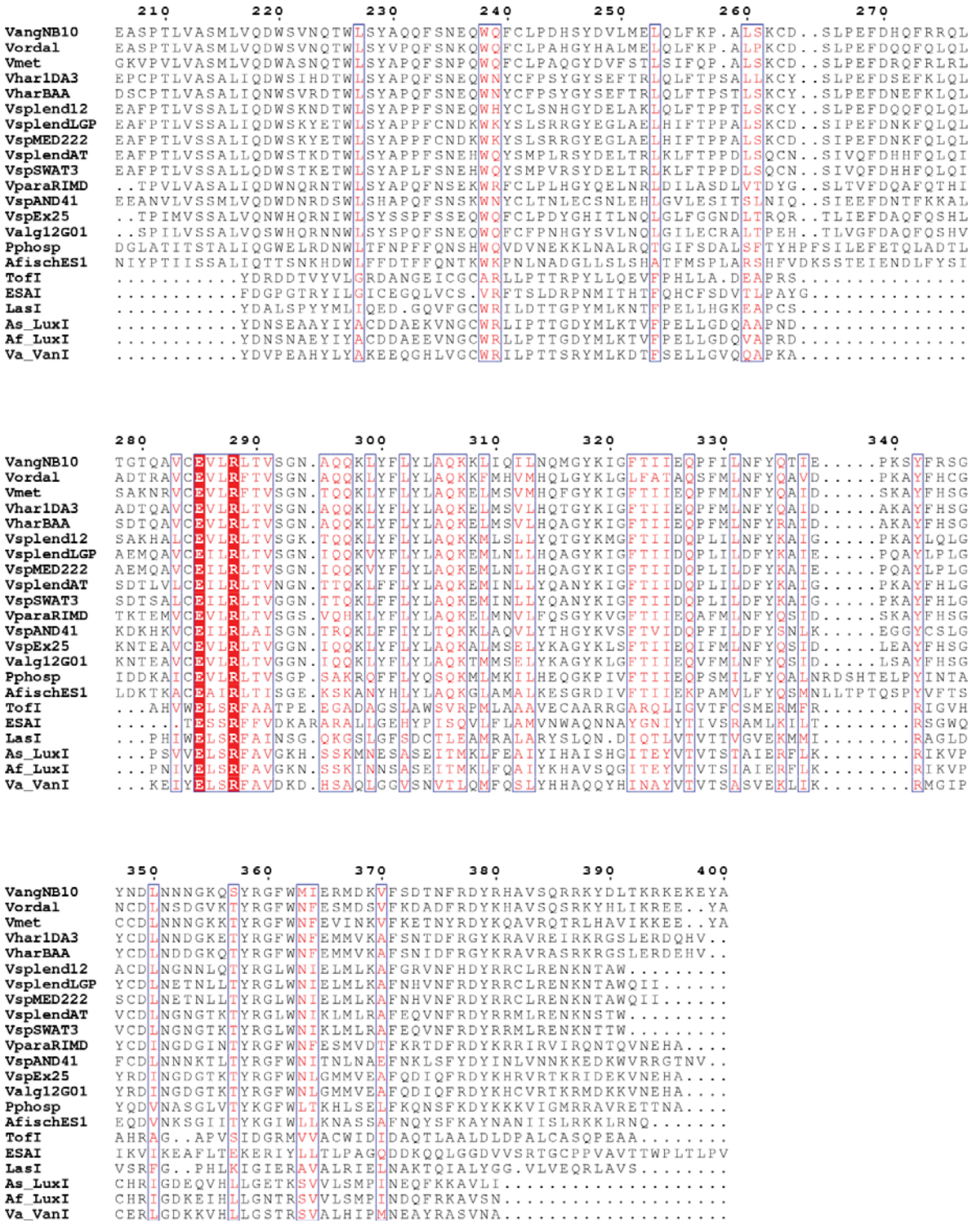


Figure 16: Sequence alignment of LuxM and LuxI family members.
Sequence alignment done in ClustalW (Thompson et al., 1994) and Structural alignment done in ESPript 2.2 (Gouet et al., 1999).

V. Conclusion

We have modified and improved an existing method for detecting AHLs using HPLC-MS. The sample requirement has been reduced to 75 µL. Different extraction solvents were tried and ethyl acetate was found to be the best for 15 different AHLs standards with varying chain length and substitution. Limit of quantification and detection were defined for each standard. Different environmental stains of *Aliivibrio*, *Photobacterium* and *Vibrio* genus were tested for the presence of AHLs and it was found that strains producing similar AHLs had an evolutionary relationship.

Several mutants of *A. salmonicida* were constructed to study the QS systems by measuring the AHLs concentration produced by the bacteria. LuxI was responsible for production of 7 different AHLs namely 3-oxo-C4-HSL, C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL, 3-oxo-C8-HSL, and 3-oxo-C10-HSL. On the other hand AinS is responsible for producing 3-OH-C10-HSL. It was indicated that LuxR1 and LuxR2 work as a heterdimers and also seems as if AinS product was reducing the activity of LuxI product formation. It can be concluded that *A. salmoncida* has a temperature dependent QS system.

VanM belongs to LuxM family of enzymes responsible for the production of C6-HSL and 3-OH-C6-HSL in *V. anguillarum*. We successfully expressed and purified the enzyme. We were able to optimize a stabilizing condition and also conduct enzyme kinetics. Reaction rate kinetics was measured to evaluate the substrate specificity with respect to chain length and 3rd substitution. It was revealed that C6 was the optimum chain length and 3-OH was the most preferred substitution for the VanM enzyme.

VI. Future perspectives

A sensitive method has been reported in this thesis for detection of AHL. This method could be further developed to included simultaneous detection of other signals, similar to a recent report where both AHLs and quinolone signaling molecules were detected (Ortori et al., 2011). AHLs can further get converted into other compounds such as tetramic acids which form important parts of many naturally occuring compounds (Ueda et al., 2010, Lowery et al., 2009). By feeding the bacteria with isotope labeled SAM, it could be possible to detect other derivative of biological importance. In Paper 2 many mutants of QS genes were constructed and they could be used to study indept metabolomic changes taking place due to the absence or presence of certain QS genes. Since information about VanM stability has been gained. Crystallization trials have been largly

unsuccessful, a possible reason could be due many lysines (12%, 48 of 400 amino acid residues) in the protein. Methylating the lysines increase the chances of crystallization of proteins (Walter et al., 2006). Moreover in paper 3 we have shown that many homologous sequences do exist. Hence it could be possible to find a more stable VanM homologue for crystallization. The enzymatic reaction mechanism for LuxI family has been elusidated but not for the LuxM family of synthases. More thorough analysis of VanM has to be perforemd in order to resolve the reaction mechanism.

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PAPER-I

PAPER-II

PAPER-III

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