# THE IMPACT OF PATHOGEN-PATHOGEN AND HOST-PATHOGEN SIGNALING ON THE VIRULENCE OF *VIBRIO HARVEYI* TOWARDS GNOTOBIOTIC BRINE SHRIMP (*ARTEMIA FRANCISCANA*) LARVAE

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De impact van pathogeen-pathogeen en gastheer-pathogeen communicatie op de virulentie van *Vibrio harveyi* tegenover gnotobiotische pekelkreeftjes (*Artemia franciscana*)

Cover picture: The double helix structure of DNA. Male brine shrimp adult, citing from http://www.warrenphotographic.co.uk/05377-brine-shrimp-adult-male. *Vibrio harveyi* cells, citing from Lambert M. Surhone *et al.*, *Vibrio harveyi.* Betascript Publishing (2010-10-01) -ISBN-13: 978-613-3-19426-7. Bioluminescence of *V. harveyi,* taking from Lab of Aquaculture and Artemia Reference Center.

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# Dedication

*To my beloved parents, for your endless love and constant support*

*And*

*To my dearest friends, for all care, patience and encouragement*

谨以此文献给我亲爱的家人及所有关心我的朋友们

**锦瑟**

锦瑟无端五十弦，一弦一柱思华年。

庄生晓梦迷蝴蝶，望帝春心托杜鹃。

沧海月明珠有泪，蓝田日暖玉生烟。

此情可待成追忆，只是当时已惘然。

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# LIST OF ABBREVIATIONS AND UNITS

| | |
|---|---|
| °C | Degree Celsius |
| % | Percentage |
| µg | Microgram |
| µl | Microliter |
| g | Relative centrifugal force for G force |
| ± | Approximately |
| / | Per |
| AHL | Acyl-homoserine lactone |
| AI-2 | Autoinducer 2 |
| CAI-1 | Cholera autoinducer 1 |
| ANOVA | Analysis of variance |
| cDNA | Complementary deoxyribonucleic acid |
| CFU | Colony forming unit |
| DNA | Deoxyribonucleic acid |
| EPSs | Exopolysaccharide production |
| FAO | Food and Agricultural Organization of the United Nations |
| FASW | Filtered and autoclaved seawater |
| g | Gram |
| g/L | Gram per liter |

| | |
|---|---|
| GenBank | Genetic sequence database of the National Institute of Health, USA |
| GRAS | Generally recognized as safe |
| h | Hour |
| HAI-1 | Harveyi autoinducer 1 |
| L | Liter |
| LB | Luria-Bertani |
| LPS | Lipopolysaccharides |
| LSI | Larval stage index |
| MA | Marine agar 2216 |
| MB | Marine broth |
| mg | Milligram |
| ml | Milliliter |
| mRNA | Messenger RNA |
| OD | Optical density |
| *P* | Statistical p-value |
| PCR | Polymerase chain reaction |
| QS | Quorum sensing |
| QSI | Quorum sensing inhibitor |
| RT-PCR | Real time-polymerase chain reaction |
| rpm | Rotations per minute |

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# CHAPTER 1

## INTRODUCTION AND THESIS OUTLINE

# 1. GENERAL REVIEW OF AQUACULTURE

## 1.1 The state of the world aquaculture production

The global human population continues to expand at a high rate and is expected to reach between 8.3 and 10.9 billion by 2050 (UN News Center, 2013), which is placing the huge challenge of feeding our planet while protecting its natural resources for future generations. FAO estimates that fish provides approximately 2.9 billion people worldwide with almost 20% of their intake of animal protein, and 4.3 billion people with about 15% of this protein (FAO, 2014). The global consumption of fish has been reported to reach a record high in 2011 (an average of 17kg per person), and is expected to reach 19.6kg per person in 2021 (FAO, 2014). However, global capture fishery production has been leveling off since the mid-1980s (Duarte *et al.*, 2009). FAO concludes that the maximum wild capture fisheries potential of most of the global oceans has been reached, and it is not likely to recover without adequate conservation strategies.

On the other hand, to meet the ever-rising demand for fish, aquaculture has become the fastest growing food-producing sector in the world, and now provides almost half of the global fish consumption **(Figure 1.1)**. According to the latest statistics of FAO (2014), world aquaculture production has attained 90.4 million tonnes in 2012, including 23.8 million tonnes of aquatic algae and 66.6 million tonnes of food fish. The food fish includes finfishes, crustaceans, molluscs, amphibians, freshwater turtles and other aquatic animals (such as sea cucumbers, sea urchins, sea squirts and edible jellyfishes) produced as food for human consumption. World food fish aquaculture production increased at an average annual rate of 6.2% during 2000-2012, while world aquaculture production volume expanded at an average rate of 8.6% per year between 1980 and 2012 (FAO, 2014). Meanwhile, the diversity of aquaculture species has also increased. In 2012, the number of species registered in FAO statistics was 567, including finfishes (345 species, with 5 hybrids), molluscs (102), crustaceans (59), amphibians and reptiles (6), aquatic invertebreates (9), and marine and freshwater algae (37). Therefore, it is now believed that aquaculture has the potential to make a significant contribution to the rising food demand and to provide safe and

high quality aquatic food.

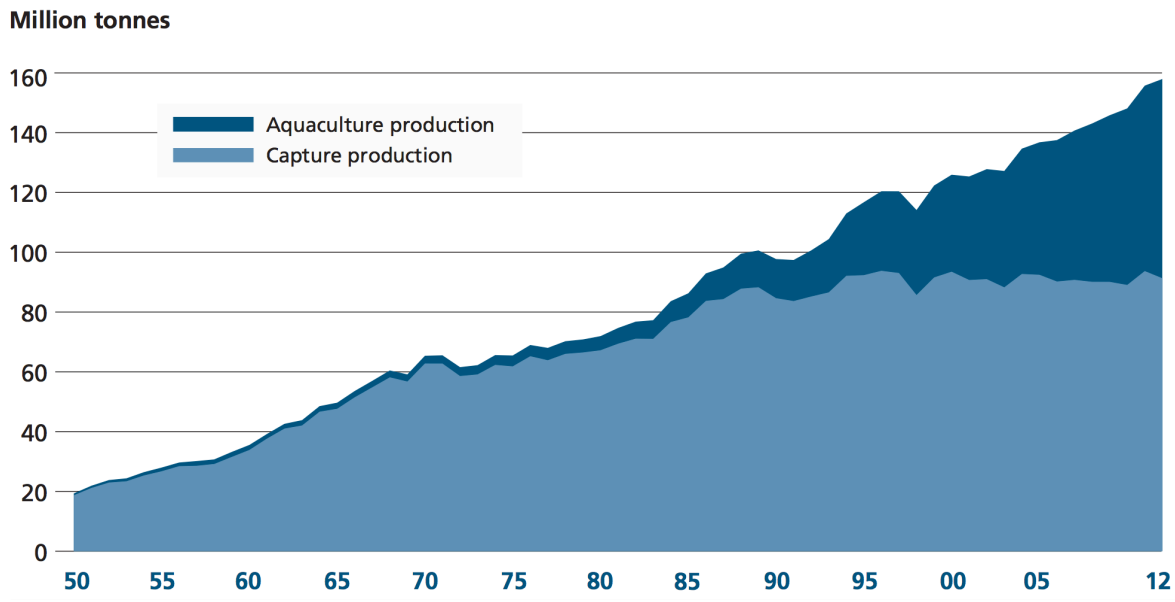



**Figure 1.1** World capture fisheries and aquaculture production (Source: FAO, 2012)

Aquaculture is a source not only of food and health but also of wealth. FAO estimates that the sector provides jobs to tens of millions and supports the livelihoods of 10-12% of the global population (FAO, 2014). Fish continues to be one of the most-traded food commodities worldwide. It is critically important for global food security and nutritional needs of people in both developing and developed countries.

## 1.2 BACTERIAL DISEASES AND ANTIBIOTIC USAGE IN AQUACULTURE

### 1.2.1 Bacterial diseases in aquaculture

The pressure for intensification and expansion of aquaculture systems has rendered most aquaculture business fragile (Piedrahita, 2003). The suboptimal oxygen levels, the accumulation of metabolic waste products and feed leftovers, injuries and wounds due to animal-to-animal interactions and animal-to-housing interactions are important factors that can significantly affect the health status of the cultured animals (Ashley, 2007). All of these factors have been shown to be related to the increasing stocking

density of the animals. When being reared in intensive systems, aquatic animals are exposed to stressful environmental conditions, which leads to decreased growth performance, reduced immune responses and increased disease susceptibility (Ashley, 2007).

Disease outbreaks have become a primary constraint to aquaculture growth and have a severe impact on both the economic and socio-economic development in many countries worldwide (Bondad-Reantaso *et al*., 2005). Economic losses in the aquaculture industry resulting from disease outbreaks have been estimated by the FAO to be in excess of US$9 billion per year, which is roughly 15% of the value of world farmed fish and shellfish production. Bacteria and viruses are the main etiological agents in aquaculture, while bacterial pathogens have been reported to cause more disease problems than all other causes combined (Meyer, 1991). Vibriosis, disease caused by bacteria belonging to the genus *Vibrio*, has become the economically most important disease in marine fish culture, affecting a large number of species (Haenen *et al*., 2014). The development and spread of bacterial diseases is the result of a complex interaction among pathogen, host and environment (Ashley, 2007). In many cases, vibrios are found to cause disease when the host organism is immune-suppressed or otherwise physiologically stressed (Peddie and Wardle, 2005). Among vibrios, strains belonging to the *Harveyi* clade (including the species *Vibrio harveyi*, *V. campbellii*, *V. parahaemolyticus* and *V. alginolyticus*) are widely accepted to be major pathogens, causing massive mortalities of cultured aquatic animals worldwide (Defoirdt *et al*., 2007; Austin and Zhang, 2006; Karunasagar *et al*., 1994).

### 1.2.2 Antibiotic use in aquaculture

Antibiotics are still critically important as the most popular and intensively used chemotherapeutic agents in the treatment of infectious diseases of humans, animals and plants. In a study by Holmström *et al*., (2003), around 74% (56 of 76) shrimp farmers being interviewed used antibiotics, and most of the farmers used the antibiotics prophylactically, even on a daily basis. All the antibiotics legally used in aquaculture must be authorized by the government agency that is responsible for veterinary medicine, such as the Food and Drug Administration (FDA) in the USA.

These regulatory agencies also set different rules for antibiotic use, including permissible routes of delivery, dose forms, withdrawal times and tolerances (Burridge *et al.*, 2010). The growing awareness that antibiotics should be used with more care has prompted more strict regulations on the use of antibiotics in aquaculture and on the presence of antibiotic residues in aquaculture products (Romero *et al.*, 2014). One notable example is the ban on the use of antibiotics as growth promoters in animal production in Europe in 2006 (European Parliament and Council Regulation No 1831/2003). An overview of common antibiotics used in aquaculture is shown in **Table 1.1**.

The use of antibiotics in aquaculture is decreasing in some countries, generally those of Northern Europe, North America and Japan, which have implied strict regulations on the use of antimicrobial agents in animal production. However, the use of antibiotics for preventive purposes is still significant in those countries with insufficient guidance. Moreover, it is difficult to determine the current levels of antibiotic use in aquaculture worldwide because different countries have different distribution and registration systems. To make things even more complicated, the data of the quantity of antibiotic usage are inadequate. As mentioned by Defoirdt *et al.*, (2011), many countries lack sufficient documentation on the quantity of antibiotics used in animal production, including aquaculture industry. Nevertheless, some early studies provided estimations of the antibiotics used in aquaculture. For example, Moriarty (1999) suggested that the quantity of antibiotics used for shrimp farming in Thailand was around 500-600 tonnes in 1994. Due to the differences of regulations regarding the use of antibiotics, the usage of antibiotics may significantly vary between countries (Burridge *et al.*, 2010). For example, the study of Smith (2008) reported that the use of antibiotics could range from 1 g per tonne in Norway to 700 g per tonne in Vietnam.

The massive use of antibiotics in the past has led to development of multiple antibiotic resistances in pathogens (**Table 1.1**). As a consequence, antibiotic treatments are becoming less effective in controlling bacterial infections in some cases at this moment. For instance, mass mortality in *Penaeus monodon* larvae caused by *Vibrio harveyi* strains with multiple resistance to cotrimoxazole, chloramphenicol, erythromycin and streptomycin has been demonstrated (Karunasagar *et al.*, 1994).

Moreover, the excessive use of antibiotics in aquaculture also presents a risk to the environment and human health, as the antibiotic resistance determinants have been found located on mobile genetic elements (Alderman and Hastings, 1998; Cabello, 2006). Therefore, these resistance determinants can be horizontally transferred to other (antibiotic-sensitive) bacteria, not only in the aquatic environment but even to bacteria from the terrestrial environment, including animal and human pathogens (Cabello *et al.*, 2013). Additionally, the application of disinfection strategies with a broad spectrum targets not only the pathogenic bacteria, but also leads to an alternation of the normal host microflora, and this might render the situation even more problematic as in this way, bacteria that compete with the pathogens for resources are killed as well (De Schryver *et al.*, 2014). Therefore, for more effective treatment of bacterial diseases, global efforts are needed to promote more judicious use of antibiotics in aquaculture and novel alternative strategies are also urgently needed for the sustainable development of aquaculture, there is an urgent need for alternatives to antibiotics in aquaculture.

**Table 1.1** The different classes of antibiotics used in aquaculture, their importance for human medicine and examples of (multi) resistant pathogenic bacteria isolated from aquaculture settings (adapted from Bondad-Reantaso *et al.,* 2005).

| Drug classes | Importance for human medicine[a] | Example | Resistant bacteria | Multiple[b] resistance | Isolated from | Reference |
|---|---|---|---|---|---|---|
| Aminoglycosides | Critically important | Streptomycin | *Edwardsiella ictulari* | Yes | Diseased striped catfish (*Pangasianodon hypophthalmus*), Vietnam | Dung *et al.*, 2008 |
| Amphenicols | Important | Florfenicol | *Enterobacter* spp. *and Pseudomonas* spp. | Yes | Freshwater salmon farms, Chile | Fernández Alarcón *et al.*, 2010 |
| Beta-lactams | Critically important | Amoxicillin | *Vibrio* spp., *Aeromonas* spp. and *Edwardsiella tarda* | Yes | Different aquaculture settings, Australia | Akinbowale *et al.*, 2006 |
| Beta-lactams | Critically important | Ampicillin | *Vibrio harveyi* | Yes | Shrimp farms and coastal waters, Indonesia | Teo *et al.*, 2000 |
| Fluoroquinolones | Critically important | Enrofloxacin | *Tenacibaculum maritimum* | Yes | Diseased turbot (*Scophthalmus maximus*) and sole (*Solea senegalensis)*, Spain and Portugal | Avendaño-Herrera *et al.*, 2008 |
| Macrolides | Critically important | Erythromycin | *Salmonella* spp | Yes | Marketed fish, China | Broughton and Walker, 2009 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Nitrofurans | Critically important | Furazolidone | *Vibrio anguillarum* | Yes | Diseased sea bass and sea bream, Greece | Smith and Christofilogiannis, 2007 |
| Nitrofurans | Important | Nitrofurantoin | *Vibrio harveyi* | Yes | Diseased penaeid shrimp, Taiwan | Liu *et al.*, 1997 |
| Quinolones | Critically important | Oxolinic acid | *Aeromonas* spp., *Pseudomonas* spp. and *Vibrio* spp. | Yes | Pond water, pond sediment and tiger shrimp (*Penaeus monodon*), Philippines | Tendencia and la Peña, 2001 |
| Sulphonamides | Important | Sulphadiazine | *Aeromonas* spp. | Yes | Diseased katla (*Catla catla*), mrigel (*Cirrhinus mrigala*) and punti (*Puntius* spp.), India | Das *et al.*, 2009 |
| Tetracyclines | Highly important | Tetracycline | *Aeromonas Hydrophila* | Yes | Water from mullet and tilapia farms, Egypt | Ishida *et al.*, 2010 |
| Tetracyclines | Highly important | Oxytetracycline | *Aeromonas Salmonicida* | Yes | Atlantic salmon (*Salmo salar*) culture facilities, Canada | McIntosh *et al.*, 2008 |

## 2 THESIS OUTLINE

The general objective of this study was to evaluate the impact of pathogen-pathogen signaling and sensing of host factors on the virulence of *V. harveyi* in a model system with gnotobiotic brine shrimp larvae. The specific objectives and the thesis outline are as following:

- **Chapter 2 (Literature review)** gives an overview of the current knowledge on *V. harveyi,* including the virulence, pathogenesis, and the regulatory mechanisms of virulence factors. This chapter also discusses antivirulence therapy as a strategy for the future treatment of bacterial infections.
- **Chapter 3 (Quorum sensing positively regulates flagellar motility in pathogenic *Vibrio harveyi*)** aims at verifying the effect of quorum sensing on swimming motility in *V. harveyi* and on the expression of selected genes involved in flagellar motility. We further investigated the importance of flagellar motility for the virulence of *V. harveyi* by applying a motility inhibitor.
- **Chapter 4 (Specific quorum sensing-disrupting activity ($A_{qsi}$) of thiophenones and their therapeutic potential in a gnotobiotic brine shrimp - *Vibrio harveyi* model system)** aims at determining quorum sensing-disrupting activity, protective effect and toxicity of 20 novel thiophenone compounds. Furthermore, we propose a new parameter to describe specific quorum sensing-inhibitory activity, $A_{QSI}$. The use of the proposed parameter $A_{QSI}$ is a straightforward and elegant way to exclude false positives by taking into account side effects related to the use of quorum sensing molecule reporters.
- **Chapter 5 (Indole and indole analogues produced by micro-algae decrease the virulence of luminescent vibrios, major pathogens of aquatic organisms)** aims at determining the impact of indole signaling on the virulence of *V. harveyi,* and at investigating whether indole analogues produced by micro-algae induce a similar response as indole.
- **Chapter 6 (Norepinephrine and dopamine increase motility, biofilm formation and virulence of *Vibrio harveyi)*** aims at investigating the impact of the catecholamines norepinephrine and dopamine (neurotransmitters produced

by higher organisms) on the growth of *V. harveyi* in serum-based medium, on the expression of various virulence-related characteristics and on virulence towards gnotobiotic brine shrimp (*Artemia franciscana*) larvae.

- **Chapter 7** summarizes the overall findings obtained in this thesis. Conclusions are drawn and possibilities for future research are proposed.

# CHAPTER II

## LITERATURE REVIEW

## ABSTRACT

*Vibrio harveyi* is amongst the most significant pathogens in the larviculture and aquaculture industry. It is able to infect a wide range of marine vertebrates and invertebrates, causing significant losses to the aquaculture industry worldwide. The pathogenicity mechanism of *V. harveyi* is not yet completely understood and is thought to involve attachment to host surfaces, biofilm formation and the production of various extracellular products. The inhibition of the production of virulence factors that are required to cause disease, i.e. antivirulence therapy, has been proposed as a novel strategy to control bacterial infections. Ideally, such a strategy does not harm the harmless and beneficial microbiota associated with the host, and is also thought to apply less selective pressure for the development of resistance than conventional antibiotics. The production of virulence factors often is under strict regulatory control, and one of the regulatory mechanisms is quorum sensing, bacterial cell-to-cell communication. Disruption of quorum sensing is the most intensively studied strategy to inhibit virulence factor production. In this review, we discuss the current knowledge with respect to pathogenicity mechanisms by which *V. harveyi* causes infection. Furthermore, an overview is given about the present knowledge with respect to antivirulence therapy.


## 2.1 *Vibrio harveyi*

### 2.1.1 Characteristics

*Vibrio harveyi* has been considered to be amongst the most significant pathogens in the larviculture and aquaculture industry (Austin and Zhang, 2006). *V. harveyi* was originally named as *Achromobacter harveyi* (Johnson and Shunk, 1936). Afterwards, it has been called *Lucibacterium harveyi*, *Beneckea harveyi* at various times (Farmer *et al*. 2005). Finally, the organism is regarded as one of the core species of the genus *Vibrio* according to the result of 16S rRNA sequence analysis (Dorsch *et al.*, 1992). *V. harveyi* is a marine Gram-negative luminous organism that is ubiquitous in the marine environment, either free-living in the sea or associated with marine organisms (Austin

and Zhang, 2006). It is rod-shaped, motile (via polar and lateral flagella), facultatively anaerobic, halophilic, and competent for both fermentative and respiratory metabolism. It does not grow below 4°C or above 35°C (Owens *et al*., 2006).

### 2.1.2 The diseases caused by *V. harveyi*

*V. harveyi* has been regarded as both a primary and opportunistic pathogen of a wide range of marine vertebrates and invertebrates, including corals, oysters, prawns, lobsters, and finfish (Austin and Zhang, 2006). Diseases caused by *V. harveyi* include eye-lesions, gastro-enteritis, vasculitis, and luminous vibriosis **(Table 2.1)**. Luminous vibriosis has been reported to cause severe losses in aquaculture worldwide, particularly in shrimp culture, and it has been considered to be a major constraint to shrimp production in South America and Asia (Austin and Zhang, 2006). Previous work in our laboratory has shown that the pathogenicity of *V. harveyi* is a strain characteristic rather than a species characteristic as some strains can be highly virulent, whereas other strains are avirulent (Ruwandeepika *et al.,* 2012). The relationship between the presence of virulence genes and the pathogenicity of bacteria is not always evident (CanoGomez *et al*. 2009). For example, it has been recently found that both highly virulent and non-virulent *V. harveyi* strains contain all virulence genes tested, including haemolysin and proteases. The regulation of virulence gene expression is critical for the virulence of these bacteria. Indeed, virulent strains showed significantly higher expression levels of virulence-related genes than non-virulent strains (Ruwandeepika *et al*. 2011).

**Table 2.1** Diseases of vertebrates and invertebrates associated with *V. harveyi*.

| Host organism | Disease characteristics | Key references |
|---|---|---|
| **Finfish** | | |
| Cobia fish (*Rachycentron canadum*) | Gastroenteritis and mass mortality | Liu *et al.*, 2003 |
| Grouper (*Epinephelus coiodes*) | Gastroenteritis and mass mortality | Lee *et al.*, 2002 |
| Red drum (*Scaienops ocellatus*) | Gastroenteritis and mass mortality | Liu *et al.*, 2003 |
| Salmonids | Up to 100% mortality | Zhang and Austin, 2000 |
| Sea horse (*Hippocampus sp.*) | Hemorrhages with more than 90% mortality | Alcaide *et al.*, 2001 |
| Summer flounder (*Paralichthys dentatus*) | Necrotizing enteritis | Soffientino *et al.*, 1999 |
| Cage-reared grouper (*Epinephelus awoara*) | Acute mortalities | Qin *et al.*, 2006 |
| Brown spotted grouper (*Epinephelus tauvina*) and silvery black porgy (*Acanthopagrus cuvieri*) | Mortalities | Saeed, 1995 |
| Milkfish (*Chanos chanos*) | Eye disease | Ishimaru and Muroga, 1997 |
| Common snook (*Centropomus undecimalis*) | Opaque white corneas | Kraxberger-Beatty *et al.*, 1990 |
| Sole (*Solea senegalensis*) | Moderate mortalities | Zorrilla *et al.*, 2003 |
| **Molluscs** | | |
| European abalone (*Haliotis tuberculate*) | Causing up to 80% mortality within a few days | Nicolas *et al.*, 2002 |
| Japanese abalone (*Sulculus diversicolor*) | Mass mortality | Nishimori *et al.*, 1998 |
| Pearl oyster (*Pinctada maxima*) | Mass mortality | Pass *et al.*, 1987 |

| | | |
|---|---|---|
| **Crustaceans** | | |
| Brine shrimp (*Artemia franciscana*) | Between 45 and 80% mortality | Soto-Rodriguez and Roque, 2003 |
| Kuruma prawn (*Penaeus japonicus*) | High mortality | Ping-Chung Liu *et al.*, 1996 |
| Ridgeback prawn (*Sicyonia ingentis*) | Detachment of mid gut epithelium resulting in up to 55% mortality | Martin *et al.*, 2004 |
| Rock lobster (*Jasus verreauxi*) | Luminescent vibriosis with up to 75% mortality in *Phyllosma* larvae | Diggles *et al.*, 2000 |
| Tiger prawn (*Panaeus monodon*) | Luminescent vibriosis resulting in mass mortality | Karunasagar *et al.*, 1994 |
| White shrimp (*Litopneaus vannamei*) | Up to 85% mortality in nauplii | Aguirre-Guzmán *et al.*, 2001 |
| *P. merguiensis* larvae (nauplii, mysis and postlarva stage) | 70-100% mortality | Sae-Oui *et al.*, 1987 |
| Black tiger shrimp | Degeneration of cells in hepatic tubules and lymphoid organs causing hepatopancreatic tubular necrosis and total mortality occured | Ruangsri *et al.*, 2004 |

### 2.1.3 Pathogenicity mechanisms

Despite its role as a serious pathogen of marine animals, the pathogenicity mechanisms of *V. harveyi* are not yet completely understood, although it is thought to involve siderophores, biofilm formation and the production of various extracellular products, such as haemolysins, proteases, (phospho)lipases and chitinases **(Table 2.2)** (Karunasagar *et al*., 1994; Austin and Zhang, 2006; Darshanee Ruwandeepika *et al*., 2012).

**Table 2.2** Overview of virulence factors produced by *Vibrio harveyi.*

| **(Putative) virulence factor** | **Key references** |
|---|---|
| Haemolysin – *vhhA, vhhB* | Rattanama *et al*., 2009 |
| | Zhang *et al*., 2001 |
| Cysteine protease | Liu *et al*., 1997; Liu *et al*., 1996 |
| Siderophore | Soto-Rodriguez and Roque, 2003; |
| | Owens *et al*., 1996 |
| Chitinase – *chi* | Abraham, 2006 |
| Phospholipase – *phlA* | Soto-Rodriguez and Roque, 2003 |
| Lipase | Teo et al., 2003b |
| Bacteriocin-like substances | Prasad *et al*., 2005 |
| Serine protease | Zhang *et al*., 2008 |
| Metalloprotease – *vhp* | Teo et al., 2003a |
| Phage related virulence (*vhml* and *vhs*) | Ruangpan *et al*., 1999; |
| | Oakey and Owens, 2000 |
| Proteinaceous exotoxin | Harris and Owens, 1999 |
| Low molecular weight lipopolysaccharides | Montero and Austin, 1999 |
| Luciferase (protection against oxidative stress) | Szpilewska *et al*., 2003 |
| Protease | Soto-Rodriguez and Roque, 2003 |

**Haemolysins.** Haemolysins are lipids and proteins that cause lysis of red blood cells by destroying their cell membrane. Bacterial haemolysins have been considered to be associated with the virulence of pathogens towards shrimp and fish by causing

hemorrhagic septicemia and diarrhea in the host (Liu *et al.*, 1996; Sun *et al.*, 2007). Haemolysin is one of the major virulence factors in *V. harveyi. V. harveyi* VIB 645, which is known to be pathogenic towards salmonids, was found to produce extracellular products with a high hemolytic activity towards fish erythrocytes, and two closely related haemolysin genes (designated *vhhA* and *vhhB*) were identified in this strain (Zhang *et al.*, 2001). Furthermore, a single residue change in *V. harveyi* haemolysin showed a loss of haemolytic activity and pathogenicity to turbot (Sun *et al.*, 2007).

**Proteases.** Proteases are enzymes that hydrolyse the peptide bonds that link amino acids together in proteins, and different classes of protease can perform the same reaction by completely different catalytic mechanisms. Proteases have been reported in different aquaculture pathogens to be linked with virulence towards both shrimp and fish (Teo *et al.*, 2003a). This group of enzymes includes metalloproteases, cysteine proteases, serine proteases, collagenases, caseinases and gelatinases (Defoirdt, 2013). Among the proteases, metalloprotease has been regarded as a virulence factor in *V. harveyi.* Teo *et al.* (2003) found that a novel metalloprotease Pap6, which was able to digest various of host proteins, including gelatin, fibronectin and type IV collagen, could play a potential role in the pathogenesis of *V. harveyi* strain AP6. Additionally, cysteine protease, lipase, phospholipase and chitinase were also considered to be major virulence factors of *V. harveyi* (Teo *et al.*, 2003b; Lee *et al.*, 1999; Soto-Rodriguez and Roque, 2003; Abraham, 2006).

**Iron acquisition and siderophores.** Iron is a vital nutrient required for several important biological cellular processes, ranging from growth and DNA replication to oxygen transport and protection against oxidative stress (Sandy and Butler, 2009). Iron is also an essential element for bacterial pathogens, as these organisms have to acquire iron within their vertebrate hosts in order to replicate and cause infection (Skaar, 2010). However, the bioavailability of iron is limited in the host, since the majority of vertebrate iron is intracellular and sequestered by lactoferrin, transferrin, and ferritin as a primary defense mechanism (Brooks *et al.*, 2004). Further, the physiological pH of serum and the aerobic environment ensures that extracellular iron is insoluble and is difficult to access by invading pathogens (Sandy and Butler, 2009).

In order to thrive within vertebrates, many pathogens have evolved iron uptake mechanisms to extract iron from their surrounding environments (Naka, 2011). These uptake systems can be divided into three main categories: siderophore-mediated iron uptake, heme-mediated iron uptake, transferrin and lactoferrin-mediated iron uptake (Sandy and Butler, 2009). *V. harveyi* can acquire iron by means of siderophores, which are low-molecular-weight iron (III) chelators secreted by the bacteria. The siderophore-iron complex is bound by a receptor at the bacterial surface. Then it is internalized into the cell and the iron is released as a nutrient source (Skaar, 2010). *V. harveyi* can produce amphiphilic enterobactin-like siderophores. Eight amphi-enterobactins have been identified in *V. harveyi* with various fatty acid appendages ranging in length ($C_{10}$-$C_{14}$), degree of unsaturation and hydroxylation (Zane *et al.*, 2014). Siderophores contribute to competitiveness in environmental bacteria and serve as virulence determinants in many vibrios such as *V. harveyi, V. cholerae, V. parahaemolyticus and V. anguillarum* (Andrus *et al.*, 1983; Owens *et al.*, 1996; Amaro *et al.*, 1990; Pybus *et al.*, 1994).

**Production of extracellular polysaccharide and biofilm formation.** Surface molecules are playing a major role during bacterial infection by enabling a complex interaction between the pathogen and its host, and these molecules include extracellular polysaccharides (EPS) and lipopolysaccharides (LPS). EPS are secreted around the cell as a capsule or as a loose slime, while LPS are components of the outer membrane in most of the Gram-negative bacteria (Costerton *et al.*, 1981). EPS are important for adhesion, nutrient sequestration, chelation of heavy metals, detoxification of toxic compounds and protection against osmotic shock (Hoagland *et al.*, 1993; Decho, 1990). Production of both EPS and LPS have been reported in *V. harveyi* as virulence factors (Bramhachari and Dubey, 2006; Montero and Austin, 1999). Capsular polysaccharides can form a capsule surrounding the bacterial cells, which is involved in attachment to host cells and play an important role in immune evasion (Chen *et al.*, 2010; Hsieh *et al.*, 2003). Another group of extracellular polysaccharides, the exopolysaccharides are able to form a loose slime outside the cell, which can form an intercellular matrix in biofilms (Mah and O'Toole, 2001). Biofilm formation is one of the important adaptive mechanisms of microorganisms to survive in the environment and within the host. The biofilm matrix enhances the

growth and survival of microorganisms by providing access to nutrients and protection from detergents and antimicrobials (Donlan and Costerton, 2002; Mah and O'Toole, 2001). The persistence and survival of *V. harveyi* in shrimp hatcheries has been attributed to the bacterium's biofilm formation ability, which is governing resistance to antibiotics and disinfectants (Karunasagar *et al.*, 1994).

**Other virulence factors.** In *V. harveyi*, it has been reported that bacteriophages are important elements in transferring virulence. A bacteriophage identified as *V. harveyi* siphoviridae-like phage (VHS1) was reported to enhance the virulence of *V. harveyi* towards black tiger shrimp (Khemayan *et al.*, 2006). Later, Munro *et al.* (2003) demonstrated that another bacteriophage, *V. harveyi* myovirus-like phage (VHML), could increase the haemolysin activity, protein secretion, and virulence towards *P. monodon* larvae. Further, Harris and Owens (1999) suggested that the protenaceaous exotoxins known as T1 and T2 encoded by a phage have a negative effect on *Penaeus monodon.*

## 2.2 VIRULENCE REGULATORY MECHANISMS

As virulence factors are often costly metabolic products, it should not be surprising that their production is under strict regulatory control. Various virulence regulatory mechanisms have been documented in pathogenic bacteria. In the following paragraphs, we will focus on quorum sensing and sensing of host stress hormones.

### 2.2.1 Quorum sensing

Quorum sensing is a cell-to-cell communication process in which bacteria control the expression of certain genes by producing, detecting and responding to small extracellular signal molecules named autoinducers (Ng and Bassler, 2009). Autoinducers are synthesized intracellularly and then passively released or actively secreted outside of the cells. The extracellular autoinducers accumulate as the bacterial population increases. When the concentration reaches the threshold level required for detection, specific cognate receptors bind the autoinducers and trigger signal transduction cascades, resulting in activation or repression of various target genes (Waters and Bassler, 2007; Ng and Bassler, 2009). This kind of mechanism

was first described in the marine bacterium *Vibrio fisheri*, and was found to exist in many other bacteria later on (Nealson *et al.*, 1970).

Originally, three main types of autoinducers were described. Acyl-homoserine lactones (AHLs) are a major class of autoinducers used by Gram-negative proteobacteria for intraspecies communication, and they also have been documented in some bacteriodetes, cyanobacteria and archaea (Sharif *et al.*, 2008; Huang *et al.*, 2008; Zhang *et al.*, 2012). AHLs consist of conserved homoserine lactone rings coupled to an acyl side chain of variable length (from $C_4$ to $C_{18}$), which can have a modification (oxo or hydroxyl) at the $C_3$ position (Fuqua and Greenberg, 2002). The second type of autoinducers is oligopeptides, which are used as signal molecules by Gram-positive bacteria. Unlike AHLs, peptide autoinducers are genetically encoded, thus each species of bacteria is capable of producing a peptide autoinducer with a unique sequence (Ng *et al.*, 2010). A third major signal molecule is autoinducer-2 (AI-2), which is synthesized by the LuxS enzyme that has been identified in over 70 species of Gram-negative and Gram-positive bacteria. AI-2 has several chemical forms derived from 4,5-dihydroxy-2,3-pentanedione (DPD), and it is proposed to allow interspecies communication (Xavier and Bassler, 2005; De Keersmaecker *et al.*, 2006; Jayaraman and Wood, 2008). However, the AI-2 biosynthesis enzyme LuxS also has an important metabolic function, as it is a key factor in the activated methyl cycle. Therefore, the role of LuxS in quorum sensing in most species needs to be further elucidated (Platt and Fuqua, 2010). In addition to these three types of signal molecules, an increasing number of autoinducers belonging to various chemical classes are still being discovered (and more are probably awaiting discovery) (for a review, see LaSarre and Federle, 2013).

Quorum sensing systems regulate a wide variety of phenotypes in bacteria, including bioluminescence, conjugal plasmid transfer, nodulation, sporulation, antibiotic resistance, antibiotic production, swarming motility and most importantly biofilm formation and virulence (**Table 2.3**; Diggle *et al.*, 2007; Fuqua and Greenberg, 2002; Miller and Bassler, 2003; Whitehead *et al.*, 2001; De Kievit and Iglewski, 2000).

**Table 2.3**. Bacterial pathogens in aquaculture and the link between virulence and quorum sensing.

| Pathogens | Signal molecules | Regulatory proteins | Phenotypes and virulence factors | Reference(s) |
|---|---|---|---|---|
| ***Aeromonas hydrophila*** | BHL[a], HHL, one AHL[b] | AhyI/AhyR | Biofilm[c,d], serine protease [c,d], glycine protease [c,d], metalloprotease gene *empA* [c,d], pigment [c,d], haemolysin [c,d], type VI [c,d], siderophores, enterotoxin | (Bi *et al.*, 2007) (Bruhn *et al.*, 2005) (Swift *et al.*, 1997) |
| ***Aeromonas salmonicida*** | BHL[a], HHL, DHL, OHHL, one AHL[b] | AsaI/AsaR | Serine protease [c,d], metalloprotease [c,d], lipase, pigment, α-haemolysin, glycero-phospholipid-cholesterol acyltransferase, siderophore, enterotoxin | (Bruhn *et al.*, 2005) (Janda and Abbott, 2010) (Swift *et al.*, 1997) |
| ***Edwardsiella tarda*** | BHL, HHL, OHHL, HeHL AI-2[a] | EdwI/EdwR LuxS homologue | Virulent-strain-specific protein [c,d], haemolysins, chondroitinase | (Han *et al.*, 2010) (Morohoshi *et al.*, 2004) |
| ***Hafnia alvei*** | OHHL | b | Siderophore production, type I and type III, fimbriae, resistance to the bactericidal effect of serum | (Padilla *et al.*, 2005) |
| ***Vibrio anguillarum*** | ODHL[a] HHL, OHdHHL[a] one AHL[b] | VanI/VanR VanM/VanN VanS/VanPQ | Biofilm [c,d], metallo-exoprotease through *epmA* expression [c,d], serine and glycine protease synthesis[c,d], | (Buchholtz *et al.*, 2006) (Milton *et al.*, 1997) (Milton *et al.*, 2001) |

| | | | | |
|---|---|---|---|---|
| | AI-2<br>CAI-1[b] | VanT master regulator | melanin pigment[c,d], siderophore, exopolysaccharide, probably haemolysin, lipase, neurotoxic acetylcholinesterase | |
| ***Vibrio alginolyticus*** | AI-2 | LuxS homologue | Flagellar biosynthesis [c,d], protease [c,d], Polysaccharide [c,e], biofilm [c,e] | (Tian *et al.*, 2014)<br>(Ye *et al.*, 2008) |
| ***Vibrio harveyi*** | OHdBHL<br>AI-2<br>CAI-1 | LuxLM/LuxN<br>LuxS/LuxPQ<br>CqsA/CqsS<br>$LuxR_{Vh}$ master regulator | Siderophore [c], type III secretion [c,e], Chitinase [c,e], exotoxin T1 [c,d], Polysaccharide[c], metalloprotease[c], Phospholipase [c,e], bioluminescence [c], Cysteine protease, caseinase, gelatinase, lipase, haemolysin | (Henke and Bassler, 2004b)<br>(Henke and Bassler, 2004a)<br>(Lilley and Bassler, 2000)<br>(Natrah et al., 2011) |
| ***Vibrio ichthyoenteri*** | Three AHLs[b],<br>AI-2 | LuxS[b] homologue | Biofilm | (Li *et al.*, 2010) |
| ***Vibrio mimicus*** | AI-2 homologue | LuxS, LuxO and LuxR homologue | Protease [c,d], haemolysin | (Sultan *et al.*, 2006) |
| ***Vibrio parahaemolyticus*** | b | LuxI/LuxR homologues | Type III secretion [c,e], opacity, protease | (Henke and Bassler, 2004a) |
| ***Vibrio salmonicida*** | OHHL, HHL | LuxI/LuxR homologues | Cryptic bioluminescence | (Nelson *et al.*, 2007) |
| ***Vibrio scophthalmi*** | OHdDDHL, | LuxS[b] homologues | b | (García-Aljaro *et al.*, |

| | | | | |
|---|---|---|---|---|
| | Two AHLs[b]<br>AI-2 | | | 2008) |
| ***Vibrio vulnificus*** | BHL, ODHL, ODDHL, minor HHL, OHL, OTHL<br>AI-2 | LuxU, LuxO, SmcR transcriptional regulator<br>LuxS/LuxPQ | Metalloprotease [c,d], cytolysin [c,e], haemolysin [c], extracellular capsular polysaccharide, siderophore, toxin RTX | (Bruhn *et al.*, 2005)<br>(Kim *et al.*, 2003) |
| ***Yersinia ruckeri*** | OOHL[a], HHL, OHHL, OHeHL, OHL, ONHL, ODHL, ODDHL | YenI/YenR | Metalloprotease, protein secretion, Siderophores, heat sensitive factors | (Bruhn *et al.*, 2005)<br>(Kastbjerg *et al.*, 2007) |

[a] Dominant signal; [b] Unknown/predicted; [c] Regulated by quorum sensing; [d] Positively regulated; [e] Negatively regulated

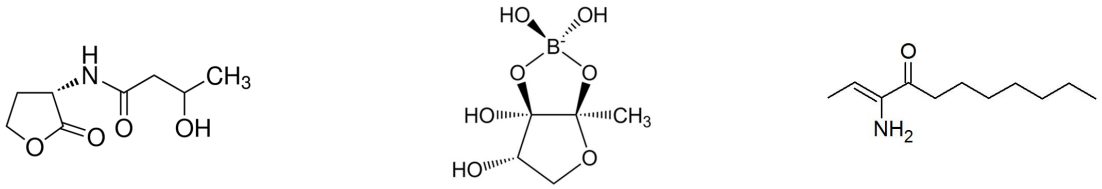
BHL: N-butanoyl-L-homoserine lactone; OHdBHL: N-(3-hydroxybutanoyl)-L-homoserine lactone; HHL: N-hexanoyl-L-homoserine lactone; OOHL: N-(3-oxohexanoyl)-L-homoserine lactone; OHdHHL: N-(3-hydroxyhexanoyl)-L-homoserine lactone; HeHL: N-heptanoyl-L-homoserine lactone; OHeHL: N-(3-oxoheptanoyl)-L-homoserine lactone; OHL: N-3-octanoyl homoserine lactone; OOHL: N-(3-oxo-octanoyl)-L-homoserine lactone; ONHL: N-3-oxononanoyl-L-homoserine lactone; DHL: N-decanoyl-homoserine lactone; ODHL: N-(3-oxodecanoyl)-L-homoserine lactone; ODDHL: N-(3-oxo-dodecanoyl)-L-homoserine lactone; OHdDDHL: N-(3-hydroxydodecanoyl)-L-homoserine lactone; OTHL: N-(3-oxo-tetradecanoyl)-L-homoserine lactone;

AI-2: Furanosyl borate diester 3A-methyl-5,6-dihydro-furo(2,3-D)(1,3,2)dioxaborole-2,2,6,6A-tetranol;

CAI-1: (Z)-3-aminoundec-2-en-4-one

### 2.2.2 The three-channel quorum sensing system of *V. harveyi*.

The quorum sensing system of *V. harveyi* has been intensively studied. *V. harveyi* produces and detects three kinds of signal molecules, harveyi autoinducer 1 (HAI-1), autoinducer 2 (AI-2) and cholerae autoinducer 1 (CAI-1) (**Figure 2.1**) (Ng and Bassler, 2009).




HAI-1
N-(3-hydroxy butanoyl)-L-homoserine lactone

AI-2
3A-methyl-5,6-dihydro-furo(2,3-D)(1,3,2)diox-aborole-2,2,6,6A-tetraol

CAI-1
(Z)-3-aminoundec-2-en-4-one

**Figure 2.1** The three types of autoinducers produced by *Vibrio harveyi.*

HAI-1 is an acyl homoserine lactone synthesized by LuxM (Cao and Meighen, 1989). To date, HAI-1 has only been found in *V. harveyi* and closely related species, such as *V. parahaemolyticus* (Bassler *et al.*, 1997). AI-2 is a furanosyl borate diester synthesized by the LuxS enzyme (Chen *et al.*, 2002). The third signal molecule, CAI-1, is (Z)-3-aminoundec-2-en-4-one and is produced by CqsA (Higgins *et al.*, 2007; Ng and Bassler, 2009). In *V. harveyi,* each of the three autoinducers is detected by a distinct membrane-bound autophosphorylating histidine sensor kinase protein, which feed a shared phosphorylation/dephosphorylation signal transduction cascade (**Figure 2.2**). LuxN recognizes HAI-1, CqsS detects CAI-1, and LuxQ responds to AI-2 via the periplasmic binding protein LuxP (Ng and Bassler, 2009).

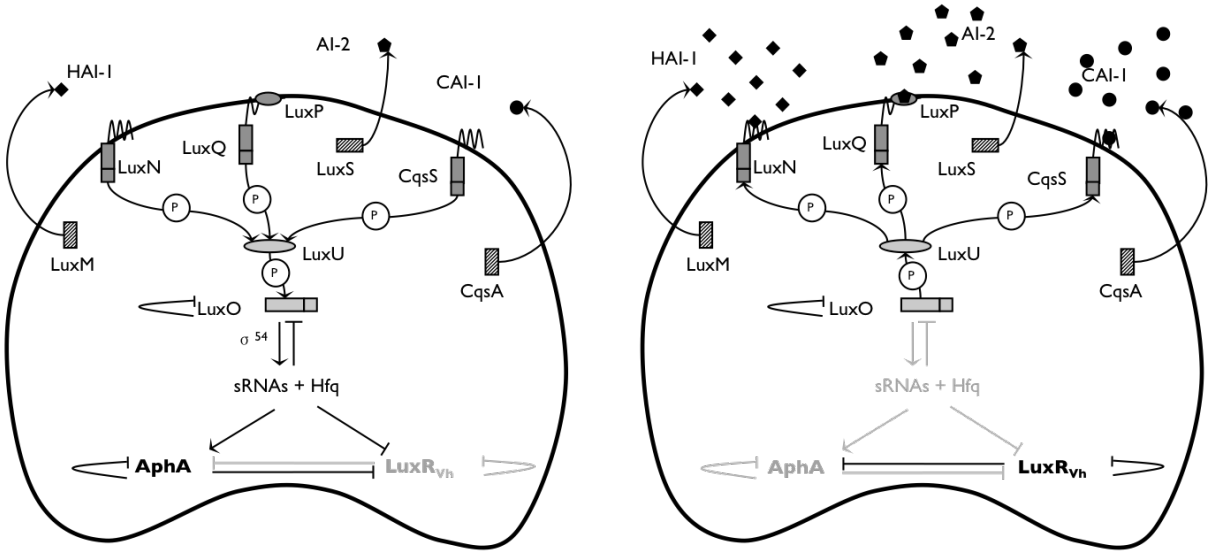



**Figure 2.2** Quorum sensing in *Vibrio harveyi*. The LuxM, LuxS and CqsA enzymes synthesise the autoinducers HAI-1, AI-2 and CAI-1, respectively. These autoinducers are detected at the cell surface by the LuxN, LuxQ and CqsS two-component receptor proteins, respectively. Detection of AI-2 by LuxQ requires the periplasmic protein LuxP. In the absence of autoinducers (left), the receptors autophosphorylate and transfer phosphate to LuxO via LuxU. Phosphorylation activates LuxO, which together with $\sigma^{54}$ activates the production of five small regulatory RNAs (sRNAs). The sRNAs promote translation of the master regulator AphA and (together with the chaperone Hfq) inhibit translation of the master regulator $LuxR_{Vh}$. Hfq mediates interactions between sRNAs and specific messenger RNA (mRNA) targets. These interactions typically alter the stability of the mRNA encoding the quorum-sensing master regulators LuxR, implicating an sRNA in the circuit. In the presence of high concentrations of the autoinducers (right), the receptor proteins switch from kinases to phosphatases, which results in dephosphorylation of LuxO. Dephosphorylated LuxO is inactive and therefore, the sRNAs are not formed, AphA is not translated and $LuxR_{Vh}$ is translated. AphA and LuxR are transcriptional regulators that (either individually or together) affect the transcription of many target genes.

In the *V. harveyi* quorum sensing circuit, two master transcription factors, AphA and LuxR, coordinate the quorum sensing response. LuxR is considered to be the major quorum sensing regulator, which controls gene expressions at both low cell density and high cell density. In contrast, AphA is absent at high cell density and maximally

produced at low cell density (Rutherford *et al.*, 2011). AphA and LuxR directly regulate expression of genes encoding the quorum sensing regulatory small RNAs, which leads to a feedback loop ensuring a well-timed transition between the individual and the group life-styles (van Kessel *et al.*, 2013). Depending on the target gene, AphA and LuxR can either activate or repress the expression to different extents. Therefore, the production of AphA and LuxR coupled with differences between target genes with respect to the sensitivity of the respective promoters for these regulators enables a myriad of gene expression patterns (van Kessel *et al.*, 2013).

A number of virulence factors has been found to be regulated by quorum sensing in *V. harveyi*, including biofilm formation (Anetzberger *et al.*, 2009), type III secretion (Henke and Bassler, 2004), production of a siderophore (Lilley and Bassler, 2000), the Vhp metalloprotease (Mok *et al.*, 2003; Ruwandeepika *et al.*, 2011), chitinase A (Defoirdt *et al.*, 2009) and three phospholipase genes (Natrah *et al.*, 2011). Interestingly, some virulence factors were found to be controlled by a subset of the autoinducers. For instance, *vhh* heamolysin gene in *V. harveyi* shows a constantly low expression in AI-2-negative mutant, however, it shows no difference in expression levels between the mutant with inactive quorum sensing system and the mutant with maximally activated quorum sensing system. This suggests that *vhh* heamolysin expression is regulated by AI-2 quorum sensing through a mechanism that is different from the currently known three-channel signal transduction pathway (Ruwandeepika *et al.*, 2011). Other virulence factors are either not controlled by quorum sensing (e.g. serine protease; Ruwandeepika *et al.*, 2011), or the link with quorum sensing has not yet been investigated (e.g. motility).

### 2.2.3 Indole signaling

A variety of both Gram-positive and Gram-negative bacteria (more than 85 species) are known to produce indole. These include many pathogenic species such as *V. vulnificus, Proteus vulgaris, Pasteurella multocida and Haemophilus influenzae* (Dalsgaard *et al.*, 1999; DeMoss and Moser, 1969; Clemons and Gadberry, 1982; Stull *et al.*, 1995). Indole is synthesized from tryptophan by tryptophanase (TnaA) through a reversible reaction, with pyruvate and ammonia as by-products (Newton

and Snell, 1965). Recently, increasing evidence suggests that indole is also used as a signaling molecule in some bacteria (Lee and Lee, 2010).

Although the indole synthesis pathway has been extensively studied, the biological functions of indole are not yet fully known. Till now, several responses to indole have been revealed (**Table 2.4**). Further, indole has also been reported to interfere with AHL-based quorum sensing in a number of Gram-negative bacteria (Hidalgo-Romano *et al.*, 2014). Some bacteria that lack tryptophanase, and therefore do not produce indole, e.g. *Pseudomonas aeruginosa,* also respond to the presence of extracellular indole (Melander *et al.*, 2014). Many non-indole-producing bacteria, such as *Pseudomonas putida* (Ensley *et al.*, 1983)*, Ralstonia pickettii* (Fishman *et al.*, 2005) and *Pseudomonas mendocina* (Tao *et al.*, 2004), can oxidize indole, and some of them utilize indole as a carbon source (Doukyu and Aono, 1997). The oxidized indole derivatives have been reported to show significant effects on gene expression, motility and biofilm formation in *E. coli* and *P. aeruginosa* (Lee *et al.*, 2007; Bansal *et al.*, 2007).

A quorum sensing property of indole was first reported in *Stigmatella aurantiaca* (Gerth *et al.*, 1993). To date, a signaling role of indole in vibrios only has been documented in *V. cholerae* and very recently in *V. anguillarum*. In *V. cholerae*, indole activated *Vibrio* polysaccharide (VPS) production and biofilm formation, whereas it decreased motility (Beyhan *et al.*, 2009). In *V. anguillarum*, by contrast, exopolysaccharide production was decreased by indole, whereas motility was not affected (Li *et al.*, 2014). Further, in *V. anguillarum*, the stationary phase sigma factor (RpoS) is involved in the production of indole as an *rpoS* deletion mutant showed elevated indole levels and increased expression of the indole synthase *tnaA* (Li *et al.*, 2014). Finally, the indole receptor has not yet been identified in any bacterium, although the transcriptional regulator SdiA has been suggested to be central in the indole signalling cascade in *E. coli* (Lee *et al*, 2007a) and the DnaK suppressor protein DksA has been hypothesised to be involved in the indole signaling cascade in *V. cholerae* (Beyhan *et al.*, 2009). In summary, indole represents a new class of signaling molecules that play an important role in interspecies communication and various biological functions.

**Table 2.4.** Phenotypic changes affected by indole (or TnaA) in microorganisms.

| Bacterium | Phenotype | Key references |
|---|---|---|
| ***Aspergillus niger*** | Inhibited cell growth | Kamath and Vaidyanathan,1990 |
| **Enteropathogenic *Escherichia coli* O127:H6** | TnaA is required for virulence against nematodes | Anyanful *et al*., 2005 |
| **Enteropathogenic *Escherichia coli* O157:H7** | Increased secretion of virulence-related EspA and EspB proteins | Hirakawa *et al*., 2009 |
| **Enteropathogenic *Escherichia coli* O157:H7** | Decreased motility, cell adherence to epithelial cells, chemotaxis, and biofilm formation | Bansal *et al*., 2007; Lee *et al*., 2007 |
| ***Escherichia coli* ATCC25404, JM109, TG1, and XL1-Blue** | Decreased biofilm formation | Lee *et al.,* 2007b |
| ***Escherichia coli* BW25113** | Enhanced plasmid stability and delayed cell division | Chant and Summers, 2007 |
| ***Escherichia coli* BW25113** | Decreased motility, cell division, biofilm formation, and acid resistance and increased drug resistance | Domka *et al*., 2006; Lee *et al*., 2007b |
| ***Escherichia coli* JM109** | Inhibited cell growth due to oxidant toxicity | Garbe *et al*., 2000 |
| ***Escherichia coli* MC1061** | Activated *astD*, *gabT*, and *tnaB* as an extracellular signaling molecule | Baca-DeLancey *et al*., 1999; Wang *et al*., 2001 |
| ***Escherichia coli* MC4100 and W3110** | Increased drug resistance via BseSR and CpxAR | Raffa and Raivio, 2002; Hirakawa *et al*., 2005; Nishino *et al*., 2005 |
| ***Escherichia coli* S17-1** | Increased biofilm formation | Di Martino *et al*., 2011 |
| ***Pseudomonas aeruginosa*** | Decreased virulence and increased antibiotic resistance and biofilm | Lee *et al*., 2009 |

| | | |
|---|---|---|
| | formation | |
| ***Salmonella enterica*** | Enhanced drug resistance via RamA | Nikaido *et al.*, 2008 |
| ***Stigmatella aurantiaca*** | Induced a spore formation | Stamm *et al.*, 2005 |
| ***Vibrio cholerae*** | Activated genes involved in polysaccharide production, increased biofilm formation and grazing resistance to phagocytic eukaryote | Mueller *et al.*, 2007; Mueller *et al.*, 2009 |
| ***Vibrio anguillarum*** | Decreased virulence towards gnotobiotic see bass (*Dicentrarchus labrax*) larvea, biofilm formation, exopolysaccharide production and expression of the exopolysacchride synthesis gene *wbfD* | Li *et al.*, 2014 |

### 2.2.4 Sensing of host factors

The metabolic condition of the host and some metabolic products has a significant impact on the success of bacterial infection. Bacteria continuously monitor the environmental cues in the host during colonization and infection, and subsequently coordinate the expression of virulence determinants at an appropriate timing (Mekalanos, 1992). Therefore, it is not surprising that bacteria have evolved a sophisticated signal transduction system to sense the presence of host cues such as mucus (Hsiao *et al.*, 2006), bile (Gotoh *et al.*, 2010), bicarbonate (Abuaita and Withey, 2009), catecholamine hormones and lipid hormones (Hughes and Sperandio, 2008). For example, mucus and bile have been reported to increase production of several virulence factors in *V. anguillarum,* including protease activity, flagellar motility, biofilm formation and exopolysaccharide production (Li *et al.*, 2014). In addition, bile has also been found to induce production of virulence factors such as type III secretion system-related proteins, haemolysins, and capsular polysaccharide in *V. parahaemolyticus* (Hsieh *et al.*, 2003). In the following paragraphs, we will focus on the bacterial sensing of host stress hormones.

*Catecholamine stress hormones*

Stress is essential for the survival of organisms, as it is the basis of a fundamental survival mechanism, the innate fight-or-flight response, which can prepare the organism to either challenge or to flee from a threat (Dhabhar, 2009). This response results in the release of several neurotransmitters, cytokines, hormones, peptides and other factors into the circulation or locally within tissues (Verbrugghe *et al.*, 2012). Among them, the major mediators of the stress response include the catecholamines norepinephrine and epinephrine, which are fast-acting mediators secreted by the sympathetic nervous system; and cortisol and corticosterone, which are slow-acting glucocorticoids released by the adrenal gland after activation of the hypothalamic–pituitary–adrenal (HPA) axis (Lundberg, 2005). Catecholamines are effector compounds derived from tyrosine and characterized by having a catecholate moiety (a benzene ring with two adjacent hydroxyl groups) and an opposing amine side chain **(Figure 2.3)**. Catecholamines affect several neuroendocrine signaling pathways in

multicellular organisms, and the catecholamine hormones epinephrine and norepinephrine are an integral part of the acute fight-or-flight stress response in higher animals (Reiche *et al.*, 2004).

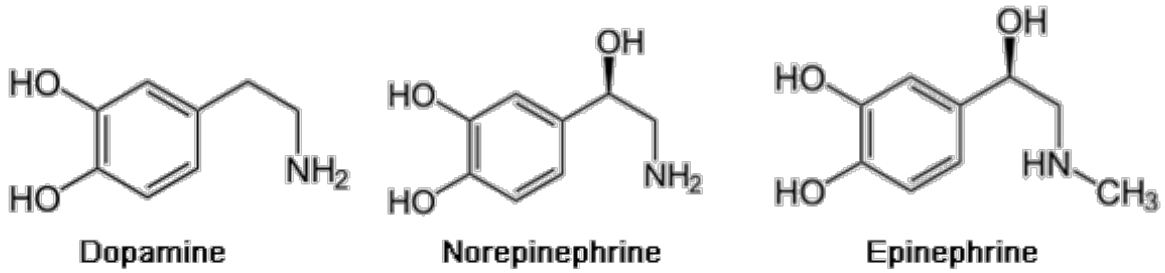



**Figure 2.3.** Chemical structures of the catecholamine stress hormones dopamine, norepinephrine and epinephrine.

Epinephrine and norepinephrine are conserved among both vertebrates (including fish) and invertebrates (including crustaceans and molluscs) (Ottaviani and Franceschi, 1996). In invertebrates, all the stress responses occur in haemocytes, i.e. cells capable of fundamental immune responses, such as graft rejection, cell motility, phagocytosis and NO production (Ottaviani and Franceschi, 1996). In mammals, norepinephrine and dopamine-containing nerve terminals are distributed throughout the body, including the intestinal tract, where they are part of the enteric nervous system. Recent work has shown that the mesenteric organs produce more than 50% of the total amount of norepinephrine in the body (Lyte, 2004). It has been reported that fish are anatomically equipped with the equivalent of all the organs and tissues that have been demonstrated to contribute to the stress response in mammals. Particularly, in addition to the hypothalamus and pituitary, interrenal tissue and cromaflin cells, which are the equivalent of the adrenal gland in mammals, are also present in fish (Ottaviani and Franceschi, 1996).

For a long time, host stress was found to be able to influence the host–microbe interactions during the course of an infection, and this has been exclusively attributed to the suppression of the host immune system or the increased susceptibility to infections (Verbrugghe *et al.*, 2012). However, more recent research has shown that

infectious bacteria have evolved specific sensing systems for detecting the stress hormones released by the host and the detection of these stress hormones can directly affect the growth and virulence of the pathogens (Lyte, 2004). These new findings led to the development of a research area named microbial endocrinology, which provides evidences of understanding the interaction between microbes and their animal host during episodes of stress.

*Catecholamines and virulence*

Most research on sensing of host stress hormones by bacteria has been conducted on the effects of the catecholamine stress hormones epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine on intestinal bacteria such as *Escherichia coli* and *Salmonella* spp. (Freestone *et al.*, 2008). Catecholamines can stimulate the growth of many Gram-negative and Gram-positive bacteria, including *Escherichia coli*, *Yersinia enterocolitica*, *Listeria monocytogenes*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus*, in minimal medium containing serum (Lyte and Ernst, 1992; Coulanges *et al.*, 1997; Kinney *et al.*, 1999; Belay *et al.*, 2003; Nakano *et al.*, 2007). Such media are iron-limited because of chelation of free iron by the high-affinity iron-binding proteins such as transferrin (Tf) and lactoferrin (Lf) that are present in serum. Sandrini *et al.* (2010) demonstrated that catecholamines can remove iron from host iron-binding proteins via direct binding of Tf/Lf-complexed iron, with a resultant reduction of the Tf/Lf-coordinated Fe (III) to Fe (II), an iron valency for which these iron-sequestering proteins have much reduced binding affinity. Subsequently, catecholamines can deliver the host-sequestered iron to bacteria through iron uptake systems, and the increased availability of iron enhances bacterial growth (Sandrini *et al.*, 2010; Sharaff and Freestone, 2011a).

In addition to the growth-stimulatory effect, catecholamines have also been reported to increase the production of virulence factors in pathogenic bacteria, including the production of Shiga toxin, chemotaxis, biofilm formation, and attachment and colonization to epithelial cells in pathogenic *E. coli*, motility and invasiveness of *Campylobacter jejuni*, motility and type III secretion in *Salmonella typhimurium*, and cytotoxic activity in *V. parahaemolyticus* (Bansal *et al.*, 2007; Cogan *et al.*, 2007;

Nakano *et al.,* 2007; Lyte *et al.*, 2011; Sharaff and Freestone, 2011b). Possibly, bacteria detect the increased concentrations of stress hormones and respond with an increased growth and enhanced potential to cause disease when the host is weakened (Freestone *et al.*, 2008).

*Specificity and receptors*

In animals, catecholamines exert their effects by binding to specific adrenergic and dopaminergic receptors. In eukaryotes, epinephrine and norepinephrine bind to adrenergic receptors, which are divided into two major families (α and β), each with a number of receptor subtypes, while dopamine binds to dopaminergic receptors with at least 5 receptor subtypes. Catecholamine binding can be prevented by antagonists specific to the catecholamine receptors (Freestone *et al.*, 2007). Interestingly, antagonists of eukaryotic adrenergic and dopaminergic receptors can also inhibit catecholamine-induced effects in bacteria (Sharaff and Freestone, 2011a). Epinephrine and norepinephrine have been found to bind to the two-component regulator sensor kinase QseC in *Escherichia coli* O157:H7, leading to the proposal that this could be a prokaryotic adrenergic receptor for these catecholamines (Clarke *et al.*, 2006). The inner membrane localized QseC receptor can autophosphorylate and transfer phosphate to the intracellular response regulator QseB in the presence of catecholamines. The roles of QseBC have been well documented in various aquaculture pathogens, such as *Aeromonas hydrophila* (Khajanchi *et al.*, 2011) and *Edwardsiella tarda* (Wang *et al.*, 2011).

Furthermore, there are increasing reports of alternative receptors for catecholamines, including QseE of the QseEF two-component signal transduction system in enterohemorrhagic *E. coli* (Reading *et al.*, 2007), BasS of the BasSR system mediating the antimicrobial peptide response in *Salmonella typhimurium* (Karavolos *et al.*, 2008), and CpxA of the CpxAR system mediating haemolytic phenotype in *S. typhimurium* (Karavolos *et al.*, 2011). However, direct binding of epinephrine or norepinephrine to BasS and CpxA remains to be demonstrated (Karavolos *et al.*, 2013).

## 2.3 ANTIVIRULENCE THERAPY

Antibiotic resistance is one of the greatest challenges of aquaculture in the 21-century. Based on the increasing knowledge of bacterial pathogenesis and intercellular communication, many potential alternative strategies have been developed to treat bacterial disease. Interference with bacterial virulence factor production, i.e. antivirulence therapy, is an especially compelling approach, as it is thought to apply less selective pressure for the development of resistance comparing with traditional antibiotic treatment (Clatworthy *et al.*, 2007). Such antivirulence therapy can consist of either specifically inhibiting a certain virulence factor or blocking several virulence factors at once through interfering with the regulation of virulence factor expression (Defoirdt, 2013).

### 2.3.1 Interfering with virulence factor regulation: quorum sensing disruption

It has been revealed that inactivating the quorum sensing system in several pathogens can result in a decreased virulence factor production and a decreased virulence (Jones *et al.*, 1993; Swift *et al.*, 1999; Wu *et al.*, 2001). Therefore, disruption of quorum sensing has been suggested as a new antivirulence strategy (Finch *et al.*, 1998). Quorum sensing systems generally offer three points of attack: the signal generator, the signal molecules and the signal receptor (Kalia, 2013; Defoirdt, 2014).

*Inhibition of quorum sensing signal production*

To date, inhibition of AHLs production is the least investigated strategy to interfere with quorum sensing, because of the fact that inhibition is difficult to measure, particularly in cell-based assays. Most work with respect to this has been conducted by application of several substrate analogues, including holo-ACP, sinefungin, L/D-*S*-adenosylhomocysterine and butyryl-*S*-adenosylmethionine, all of which were found to be able to block AHLs production *in vitro* (Parsek *et al.*, 1999). Recently, Christensen and coworkers (2013) developed and executed an enzyme-coupled high-throughput cell-free screen to discover AHLs synthase inhibitors. By screening over 12,000 compounds, they identified three strongest inhibitors, two of which showed activity in whole cells, while another one behaved as a noncompetitive

inhibitor and showed activity in a cell-based assay (Christensen *et al.,* 2013). However, none of these compounds have been tested on bacteria *in vivo.* The mechanisms by which these analogues block the AHLs production need to be further investigated in order to determine if they can affect other vital processes in prokaryotic and eukaryotic organisms.

*Enzymatic inactivation of quorum sensing signal molecules*

A second strategy to disrupt quorum sensing system is the inactivation of signal molecules through chemical inactivation or biodegradation. AHLs are chemically inactivated via alkaline hydrolysis at high pH (pH 7 or higher) (Yates *et al.*, 2002). Further, it has been demonstrated that 3-oxo-substituted AHLs were rapidly inactivated upon exposure to oxidized halogens, but the 3-oxo-unsubstituted ones retained activity (Butler and Sandy, 2009). A number of higher organisms employ this strategy to defend against invading (quorum sensing) pathogens by producing halogens, e.g. the benthic diatom *Nitzschia cf pellucida* possesses a natural haloperoxidase system that is capable of mediating the inactivation of AHLs (Syrpas *et al.*, 2014).

The inactivation of signal molecules can also be accomplished by enzymatic activity, such as AHLs biodegradation ability, which is widely distributed in the bacterial kingdom (Dong *et al.*, 2007). Four types of enzymes have been shown to be able to mediate the degradation of AHLs; i.e. AHL lactonase and decarboxylases that can hydrolyse lactone ring, while AHL acylase and deaminase that can cleave the acyl side chain (Kalia, 2013). AHL lactonases are by far the most intensively studied group of AHL degradation enzymes, and inactivate AHLs by catalysing the ring-opening reaction (Dong *et al.*, 2007). The production of the AHL lactonases named AiiA is widespread in different *Bacillus* species, and these AiiA homologues share 90% sequence identity at the peptide level (Dong *et al.*, 2002). However, the AHL lactonases production is not limited to *Bacillus* species. Several bacteria include *Pseudomonas aeruginosa* PAI-A, *Klebsiella pneumoniae*, *Agrobacterium tumefaciens*, *Arthrobacter* sp., and *Rhodococcus* sp. have been found to produce AiiA homologues (Uroz *et al.*, 2003; Carlier *et al.*, 2003; Park *et al.*, 2003; Huang *et al.*, 2003). Further,

AHL lactonases are considered to be the most specific degradation enzyme as they can hydrolyse both short- and long-chain AHLs with similar efficiency, but show no or little activity to other chemicals such as non-acyl lactones and aromatic carboxylic acid esters (Wang *et al.,* 2004).

AHL-acylases inactivate AHLs by hydrolysing the amide bond and producing corresponding fatty acids and homoserine lactone (Kalia, 2013). Furthermore, there are notable differences in the substrate specificities among AHL-acylases, such as AiiD from *Ralstonia* strain XJ12B effectively degrades long-chain AHLs and also short-chain AHLs with less efficiency (Lin *et al*., 2003), PvdQ from *P. aeruginosa* PAO1 is unable to degrade AHLs with acyl chains shorter than eight carbons (Huang *et al*., 2003), while AhlM produced by *Streptomyces* sp. shows only residue activity in degrading AHLs with acyl chains shorter than eight carbons (Park *et al*., 2005).

Paraoxonases (PONs) are a group of enzymes produced by mammals that are involved in the hydrolysis of organophosphates and lactones (Eckerson *et al.*, 1983). The paraoxonase enzyme family comprises three members, PON1, PON2, and PON3, whose genes are located adjacent to each other on the long arm of chromosome 7 in humans (Bergmeier *et al*., 2004; Li *et al*., 2003). PON1 has PON, arylesterase and lactonase activities (Billecke *et al*., 2000) and is involved in the protection against xenobiotic toxicity (Costa *et al*., 2005). PON2 and PON3 are not active against organophosphate substrates, but have lactonase activity (Draganov *et al*., 2005). PON1 has been reported to degrade 3-oxo-C12-AHL and decrease biofilm formation in *P. aeruginosa* (Ozer *et al*., 2005). Furthermore, PON2 and PON3 are also able to degrade 3-oxo-$C_{12}$-AHL, with PON2 being the most efficient with respect to this function (Ozer *et al*., 2005). Research from other groups has confirmed the fact that PONs can degrade AHLs and that PON2 has the strongest lactonase activity of the three enzymes (Draganov *et al*., 2005; Yang *et al*., 2005).

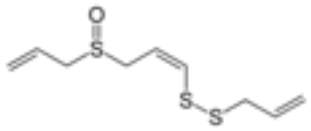
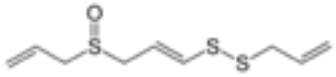
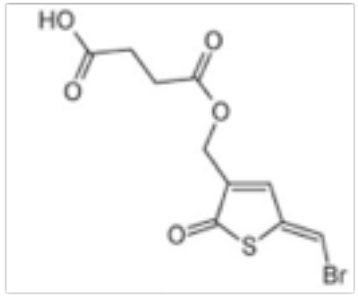
AHL-degrading enrichment cultures have been isolated from the intestinal tract of healthy shrimp and fish, and they could increase the survival of different aquaculture species, such as turbot (*Scophthalmus maximus*) larvae and giant freshwater prawn (*Macrobrachium rosenbergii*) larvae (Tinh *et al*., 2008; Nhan *et al*., 2010). More

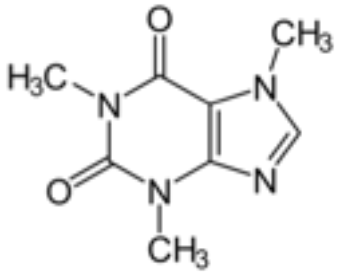
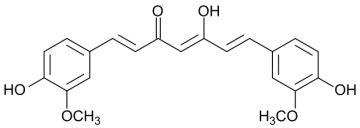
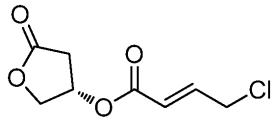
interestingly, pure strains of AHL-degrading *Bacillus* sp. have recently been isolated from these enrichment cultures (Defoirdt *et al.*, 2011), which indicates that bacteria that can degrade quorum sensing signals show promise as novel bio control agents for aquaculture.

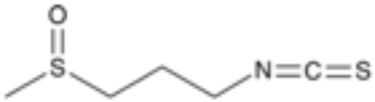
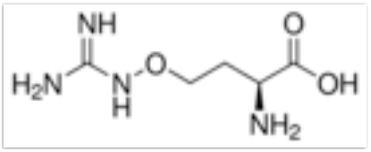
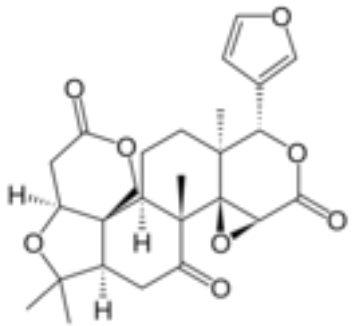
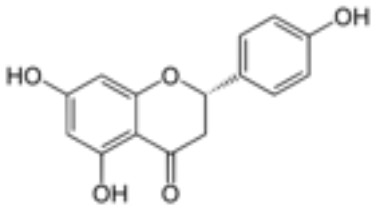
*Interference with the quorum sensing signal molecule detection and signal transduction*

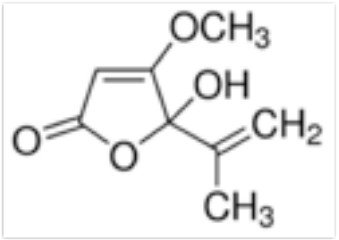
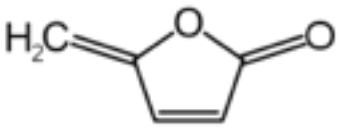
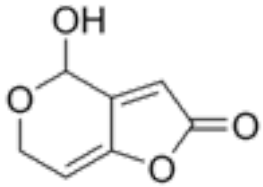
A third approach to disrupt bacterial quorum sensing is to interfere with signal molecule detection by either blockage or destruction of the receptor protein, using quorum sensing inhibitors (QSIs) (**Table 2.5)**. QSIs can be isolated from natural sources such as plants and fungi. Fungi have long been known to produce secondary metabolites which can effectively control bacterial infections, such as penicillin produced by *Penicillium* species. Recently, around 33 *Penicillium* species were found to produce QSIs, such as penicillic acid (PA) and patulin, produced by *Pe. radicicola* and *Pe. coprobium*, respectively (Rasmussen *et al.*, 2005a).

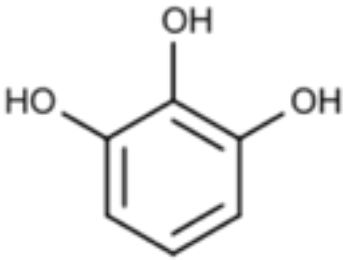
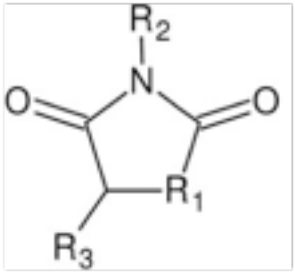
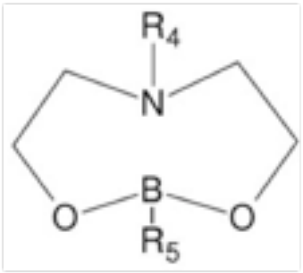
**Table 2.5.** Representative examples of published data on claimed quorum sensing (QS) inhibitors.

| QSI compounds | Chemical structure | Inhibition of signal molecule reporter [a] | Other QS-related assays | Reference(s) |
|---|---|---|---|---|
| Ajoene | Z-ajoene  E-ajoene  | Inhibition of fluorescence in *Pseudomonas aeruginosa lasB-gfp, P. aeruginosa rhlA-gfp and E.coli luxI-gfp* | Downregulation of QS-regulated genes in *P. aeruginosa.* Attenuation of rhamnolipide and C4-HSL production in *P. aeruginosa*; increase in biofilm susceptibility (*in vitro* and *in vivo*) | Jakobsen *et al.*, 2012a |
| Brominated thiophenone TF310 |  | > 2 log inhibition of bioluminescence of *V. harveyi* BB120 and different QS mutants | Inhibition of *V. harveyi* LuxR DNA binding activity, protection of brine shrimp from QS-related mortality caused by *V. harveyi* | Defoirdt *et al.*, 2012a |

| | | | | |
|---|---|---|---|---|
| Caffein |  | Inhibition of *C. violaceum* CV026 (Δ*cvil*) violacein | Inhibition of swarming in *P. aeruginosa* | Norizan *et a*l., 2013 |
| Curcumin |  | > 80% inhibition of bioluminescence in *V. harveyi* MTCC 3438 | Inhibition of caseinase activity in *V. harveyi* | Packiavathy *et al*., 2013 |
| Honaucin A |  | Inhibition of bioluminescence in *V. harveyi* BB120, and inhibition of fluorescence of *E. coli* pJBA132 (*luxR* $P_{luxI}$*::gfp*) | No | Choi *et al*., 2012 |

| | | | | |
|---|---|---|---|---|
| Iberin |  | Inhibition of fluorescence in *P. aeruginosa lasB-gfp* ($P_{lasB}$*::gfp*) and *rhlA-gfp* ($P_{rhlA}$*::gfp*) | Competition with AHLs for receptor, inhibition of rhamnolipid production in *P. aeruginosa*, transcriptomic analysis in *P. aeruginosa* | Jakobsen *et al.*, 2012b |
| L-Canavanine |  | Inhibition of violacein production in *C. violaceum* CV026 | Sin/ExpR system of *Sinorhizobium. Meliloti* | Keshavan *et al.*, 2005 |
| Limonoids (isolimonic acid and ichangin) |  | > 33% inhibition of bioluminescence in *V. harveyi* BB886 (Δ*luxPQ*) and BB170 (Δ*luxN*) | Impact on bioluminescence of other *V. harveyi* QS mutants and on expression of QS circuit genes | Vikram *et al.*, 2010 |
| Naringenin |  | 69% inhibition of *C. violaceum* CV026 (Δ*cvil*) violacein and ~50% inhibition of *E. coli* pAL101 (*rhlR rhlI::lux)* *bioluminescence* | Inhibition of *P.aeruginosa* elastase and pyocyanin; inhibition of promoter activity of QS-regulated genes in *P.aeruginosa* | Vandeputte *et al.*, 2011 |

| | | | | |
|---|---|---|---|---|
| Penicillic acid |  | Inhibition of fluorescence in *P. aeruginosa lasB-gfp* (P $_{lasB}$*::gfp*) | Las and Rhl systems of *P. aeruginosa* | Rasmussen *et al.*, 2005b |
| Protoanemonin |  | ~40% inhibition of fluorescence in *P. aeruginosa* MH602 (P $_{lasB}$*::gfp*) | Transcription analysis in *P. aeruginosa* | Bobadilla *et al.*, 2013 |
| Patulin |  | Inhibition of fluorescence in *P. aeruginosa lasB-gfp* (P $_{lasB}$*::gfp*) | Las and Rhl systems of *P. aeruginosa*; enhance biofilm susceptibility to tobramycin treatment | Rasmussen *et al.*, 2005b |

| | | | | |
|---|---|---|---|---|
| Pyrogallol |  | Complete inhibition of bioluminescence in *V. harveyi* MM32 (Δ*luxN* Δ*luxS*) | No | Ni *et al.*, 2008 |
| Thiazolidinediones and dioxazaborocanes |  Basic structure of thiazolidinediones | > 50% inhibition of bioluminescence in *V. harveyi* BB170 (Δ*luxN*) and 30-90% inhibition of bioluminescence in *V. harveyi* MM32 (Δ*luxN* Δ*luxS*) | Inhibition of bioluminescence of other *V. harveyi* QS mutants, inhibition of *V. harveyi* LuxR DNA binding activity | Brackman *et al.*, 2013 |
| |  Basic structure of dioxazaborocanes | | | |

[a] *ahyR* and *ahyI, Aeromonas hydrophila* AHL receptor and synthase; *Chromobacterium violaceum* AHL synthase; *lasB, Pseudomonas aeruginosa* QS-regulated elastase; *lasR* and *lasI, P. aeruginosa* receptor and synthase of N-3-oxododecanoyl-L-homoserine lactone; *rhlA, P. aeruginosa* QS-regulated rhamnolipid synthase; *rhlR* and *rhlI, P. aeruginosa* receptor and synthase of N-butanoyl-L-homoserine lactone; *luxR* and *luxI, Vibrio fischeri* AHL receptor and synthase; *luxN, Vibrio harveyi* AHL receptor; *luxS* and *lux PQ*, *V. harveyi* AI-2 receptor and synthase.

Additionally, a number of plants, including crown vetch, carrot, soybean, water lily, tomato, pea seedlings, habanero and garlic, have been found to produce QSIs (Teplitski, 2004; Rasmussen *et al.*, 2005).

Many plant extracts are believed to interfere with AHL signal detection by competing with them due to the similarity of their structural and/or by accelerating the degradation of the LuxR/LasR receptors of the AHL molecules (Lyon and Muir, 2003; Vattem *et al.*, 2007). For example, halogenated furanones, including both natural compounds produced by macro alga *Delisea pulchra* and synthetic derivatives, are one of the most intensively studied groups of QSIs (Janssens *et al.*, 2008). It had been assumed that furanones act by competing with AHLs for binding to LuxR-type proteins (Manefield *et al.*, 1999). The mechanism of this inhibition has received renewed attention in light of studies suggesting that, rather than displacing the AHLs from LuxR, the furanones function by accelerating LuxR turnover (Manefield *et al.*, 2002). However, the precise structural mechanism by which furanones stimulate LuxR turnover remains to be characterized (Manefield *et al.*, 2002).

Halogenated furanones were found to disrupt quorum sensing-regulated gene expression both in AHL-mediated quorum sensing systems (e.g. interfere with the AHLs receptor LuxR in *V. fischeri* ) and in multi-channel quorum sensing systems of vibrios by decreasing the DNA-binding activity of the master regulator $LuxR_{vh}$ (not homologuous to *V. fischeri* LuxR) (Manefield *et al.*, 2002; Defoirdt *et al.*, 2007). It has been demonstrated that halogenated furanones can protect both fish and crustaceans against vibriosis (Rasch *et al.*, 2004; Defoirdt *et al.*, 2006). However, these furanones are toxic to higher organisms; especially fish larvae (Natrah *et al.*, 2012), which means that they will not be safe for practical applications in aquaculture. More recently, brominated thiophenones, sulphur analogues of the brominated furanones, have been synthesized (Benneche *et al.*, 2011), and these compounds have the same effect on the quorum sensing system as brominated furanones, by decreasing the DNA-binding activity of LuxR (Defoirdt *et al.*, 2012). Thiophenones were found to be more active than the corresponding furanones (Benneche *et al.*, 2011; Lönn-Stensrud and Benneche, 2011). One of these compounds, (*Z*)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)methoxy)-4-oxobutanoic

acid (TF310) has been reported to show a promising therapeutic potential in the *V. harveyi* – brine shrimp model, with complete protection against the pathogen at 2.5 *μ*M and severe toxicity only being observed at 250 *μ*M (Defoirdt *et al*., 2012).

Interestingly, the food additive cinnamaldehyde, a non-toxic synthetic flavouring substance that is generally recognized as safe (GRAS), has been reported to have a similar activity as brominated furanones and thiophenones (Brackman *et al*., 2008). Furthermore, it has been found that cinnamaldehyde can protect giant fresh water prawn larvae and gnotobiotic brine shrimp larvae against pathogenic *V. harveyi* (Pande *et al*., 2013; Brackman et al., 2008), and blocked the virulence of both *Aeromonas hydrophila* and *Aeromonas salmonicida* towards burbot larvae (Natrah *et al*., 2012).

In addition to *D. pulchra*, production of QSIs has also been found in other macro-algae, micro-algae, corals and sponges (Skindersoe *et al*., 2008). Marine bacteria can also produce secondary metabolites with quorum sensing-inhibitory activity, such as two phenetylamide metabolites from *Halobacillus salinu*, which are able to block quorum sensing-mediated phenotypes in serveral bacteria, including *V. harveyi* (Teasdale *et al*., 2009).

### 2.3.1 Antivirulence compounds targeting other regulatory mechanisms

In addition to quorum sensing inhibition, inhibitors targeting other virulence regulatory pathways have also been demonstrated more recently. For instance, the membrane-bound QseC histidine sensor kinase, which is responsible for bacterial detection of catecholamine host stress hormones (Rasko *et al*., 2008). QseC was shown to be present in at least 25 important human and plant pathogens, and can initiate a complex phosphorylation cascade in the bacterial cells that regulates the expression of virulence genes. It has been reported that a compound, *N*-phenyl-4-[[(phenylamino)thioxomethyl]amino]- benzenesulphonamide (LED209), is able to block the binding of signaling molecules to QseC, and consequently inhibit QseC-mediated virulence gene expression (Rasko *et al*., 2008). LED209 was found to be able to protect mice and rabbits from mammalian pathogens, such as *Salmonella typhimurium* and enterohaemorrhagic *Escherichia coli* (Rasko *et al*., 2008). In addition,

the QseC-dependent signaling system shows no direct effect on bacterial growth *in vitro*, therefore, inhibition of this signaling pathway probably will not exert strong selective pressure towards development of drug resistance. These results indicate that QseC is an attractive target for novel antivirulence therapies.

Another class of virulence inhibitors interferes with the ToxR regulon in vibrios. The ToxR regulon has been studied extensively in *V. cholerae,* in which ToxR regulon was found to be responsible for expression of two crucial virlence factors: the cholera toxin and the toxin co-regulated pilus (TCP) (Matson *et al.*, 2007). Recently, one small molecule, 4-[*N*-(1,8- naphthalimide)]-n-butyric acid (also known as virstatin), was shown to directly inhibit the activity of transcriptional regulator ToxT and subsequent expression of virulence factors in *V. cholerae* (Hung *et al.*, 2005). In the same report, *in vivo* characterization in an infant-mouse model of infection showed that virstatin could reduce cholerae infectivity at 50 μM, which suggests that virstatin provides promise for the treatment of cholerae infection.

### 2.3.3 Antivirulence compounds targeting specific virulence factors

Apart from antivirulence compounds that interfere with regulatory mechanisms, antivirulence therapies could also aim at directly blocking specific virulence factors, such as toxins, adhesion factors and secretory systems, without killing or inhibiting bacterial growth (Defoirdt, 2013). Clatworthy et al. (2007) reported a group of compounds named pilicides that can block a specific virulence factor, i.e. the formation of pili. Pilicides (e.g. bicyclic 2-pyridones) might have broad-spectrum activity due to the conservation of the pathways they are targeting (Clatworthy *et al*. 2007). Another group of specific virulence factor-inhibiting compounds also show broad spectrum antivirulence activity, since they are able to block bacterial secretion systems in different bacteria. For instance, several inhibitors of type III secretion have been reported, including acylated salicylaldehyde hydrazones and thiazolidinones (Baron, 2010).

## 2.4 RESEARCH GAPS

The massive misuse of antibiotics to control infections in aquaculture has resulted in

severe problems, particularly the development of antibiotic resistance strains, which have rendered the traditional antibiotic treatment no longer effective. Therefore, alternative strategies to combat bacterial diseases are urgently needed for a sustainable further development of the aquaculture industry (Defoirdt *et al*., 2007). Antivirulence therapy is one of the alternative strategies, aiming at specifically inhibiting the function required during an infection (Clatworthy *et al.,* 2007). Antivirulence therapy is based on a thorough understanding of the mechanisms by which bacterial pathogens cause disease.

To date, the pathogenicity mechanisms of *V. harveyi* are not yet completely understood. To our knowledge, the pathogen produces a variety of virulence factors that are regulated by quorum sensing in *V. harveyi*, including biofilm formation (Anetzberger *et al*., 2009), type III secretion (Henke and Bassler, 2004), production of a siderophore (Lilley and Bassler, 2000), the Vhp metalloprotease (Mok *et al*., 2003; Ruwandeepika *et al*., 2011), chitinase A (Defoirdt *et al*., 2010) and three phospholipase genes (Natrah *et al*., 2011). Most of these virulence factors, except metalloprotease, are negatively controlled by quorum sensing. However, according to previous research that quorum sensing is required for full virulence of *V. harveyi* towards different hosts (Defoirdt and Sorgeloos, 2012; Pande *et al*., 2013), there should be some other important virulence factors that are positively regulated by quorum sensing in *V. harveyi*. Therefore, the link between quorum sensing and virulence in *V. harveyi* warrants further investigation.

The inhibition of quorum sensing is a promising alternative strategy to control disease. Comparing with strategies that specifically block a certain virulence factor, disruption of quorum sensing can interfere with several virulence factors at once. To date, a variety of quorum sensing inhibitors has been described or claimed (Kalia, 2013; Defoirdt *et al*., 2013). Brominated furanones are the most intensively studied quorum sensing inhibitors, which have been reported to disrupt quorum sensing in various Gram-negative bacteria (Manefield *et al*., 2002; Kalia, 2013). Unfortunately, these furanones are toxic to higher organisms (Natrah *et al*., 2012), which means that they will not be safe for practical applications. Therefore, finding novel quorum sensing-disrupting agents with less negative side effects is crucially important.

Most research efforts with respect to exploring new antivirulence strategies for combating bacterial infection have focused on the disruption of quorum sensing. However, this mechanism also has its limitations (e.g. technology for practical applications is not yet available). Therefore, it is important to further explore more mechanisms that might be potential targets for antivirulence therapy for aquaculture, including other signaling mechanisms (e.g. indole signaling) and sensing of host factors (e.g. catecholamine stress hormones). Different antivirulence therapies could be used synergistically to maximize the chance of success. Additionally, with less of the therapeutic agents there will be less of an opportunity for resistance development.

# CHAPTER III

## QUORUM SENSING POSITIVELY REGULATES FLAGELLAR MOTILITY IN PATHOGENIC *VIBRIO HARVEYI*

**Qian Yang and Tom Defoirdt** (2015) *Environmental microbiology 17: 960-968*

## ABSTRACT

Vibrios belonging to the *Harveyi* clade are among the major pathogens of aquatic organisms. Quorum sensing (QS) is essential for virulence of *V. harveyi* towards different hosts. However, most virulence factors reported to be controlled by QS to date are negatively regulated by QS, therefore suggesting that their impact on virulence is limited. In this study, we report that QS positively regulates flagellar motility. We found that autoinducer synthase mutants showed significantly lower swimming motility than the wild type, and the swimming motility could be restored by adding synthetic signal molecules. Further, motility of a *luxO* mutant with inactive QS (LuxO D47E) was significantly lower than that of the wild type and of a *luxO* mutant with constitutively maximal QS activity (LuxO D47A). Furthermore, we found that the expression of flagellar genes (both early, middle and late genes) was significantly lower in the *luxO* mutant with inactive QS when compared to wild type and the *luxO* mutant with maximal QS activity. Motility assays and gene expression also revealed the involvement of the quorum sensing master regulator LuxR in the QS regulation of motility. Finally, the motility inhibitor phenamil significantly decreased the virulence of *V. harveyi* towards gnotobiotic brine shrimp larvae.

**Keywords:** Quorum sensing; swimming motility; flagellum; virulence

## 3.1 INTRODUCTION

Vibrios belonging to the *Harveyi* clade (including *Vibrio harveyi* and the closely related *V. campbellii*) are gram-negative bacteria that are ubiquitous in the marine environment, either free-living in the sea or associated with marine organisms. These bacteria are considered to be amongst the most significant pathogens of aquatic organisms, which can affect a wide range of cultured marine organisms, including fish, crustaceans and mollusks (Austin and Zhang, 2006; Ruwandeepika *et al.*, 2012). Diseases caused by *Harveyi* clade vibrios include vasculitis, eye-lesions, gastro-enteritis and luminous vibriosis (Austin and Austin, 1999; Austin and Zhang, 2006). The pathogenicity mechanism is not yet completely understood, and is thought to involve attachment to host surfaces, biofilm formation, and the production of various extracellular products, such as hemolysins, proteases, (phospho)lipases and chitinases (Karunasagar *et al.*, 1994; Austin and Zhang, 2006; Ruwandeepika *et al.*, 2012).

Bacterial motility is considered as an important virulence factor in many pathogens. It is essential for pathogenic bacteria during the initial phases of infection as it helps them to overcome repulsive forces between the bacterial cell and the host tissues and hence, facilitates attachment to the host (McCarter, 2001). The most intensively investigated mode of motility in bacteria is the one governed by specialized rotating organelles, the flagella. Flagella are one of the most complex and extremely effective organelles of locomotion, and the major portion of a flagellum is the flagellar filament of 12-25 nm in diameter and up to 10 $\mu$m in length (**Figure 3.1**) (Namba and Vonderviszt, 1997). The filament has a helical shape and is rotated by a cytoplasmic membrane-embedded rotary motor at its base, which is energized by the transmembrane potential of specific ions, most commonly the proton motive force or the sodium motive force (Imae and Atsumi, 1989) (Namba and Vonderviszt, 1997). The rotation direction of the motor is considered to be reversible and to be modulated in response to signals in the environment, allowing movement towards more favorable conditions (a process called chemotaxis) (Blair, 1995).

Some *Vibrio* species (including *V. harveyi*) possess dual flagellar systems that are

suited for movement under different conditions (Hendrie et al., 1970; McCarter, 2004). A single polar flagellum is involved in swimming in liquid environments, while the numerous lateral flagella enable swarming over surfaces or movement in more viscous environments. In the whole genome sequence of *V. harveyi* BB120 (ATCC BAA-1116; recently reclassified as *Vibrio campbellii;* Lin *et al*., 2010), approximately 50 polar and 30 lateral flagellar genes have been identified to encode components of the flagellum and its export structure in *Vibrio harveyi*. However, the actual contribution of motility to virulence of *V. harveyi* thus far is unknown.

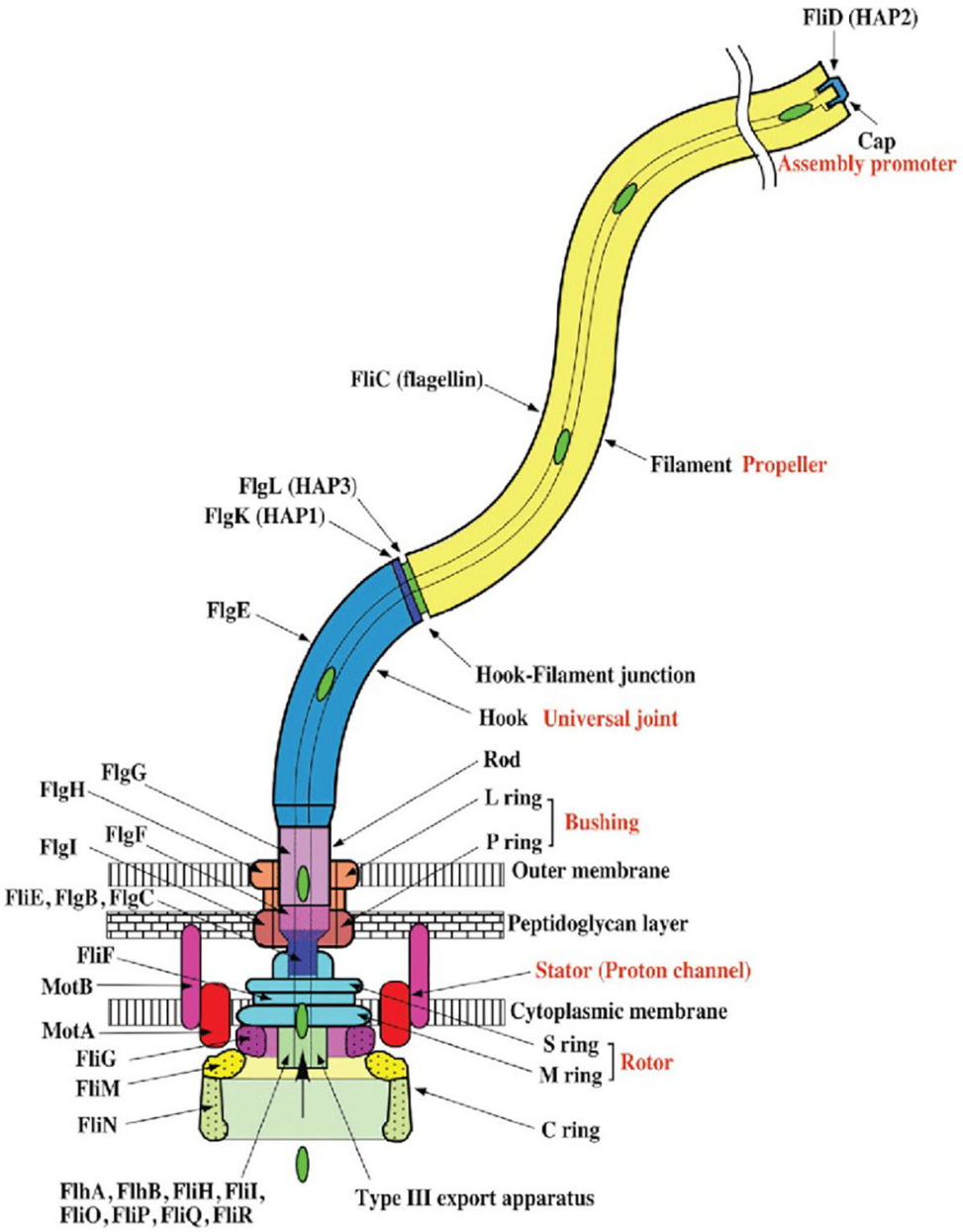



**Figure 3.1**. Schematic diagram of the flagellum. The different colours represent different protein components (adopted from Yonekura *et al.*, 2002).

As virulence factors are often costly metabolic products, it should not be surprising that their production is under strict regulatory control, and one of the regulatory mechanisms that has been found to control the expression of virulence factors in *V. harveyi*, is quorum sensing (QS), a process that allows bacteria to communicate with one another using secreted chemical signaling molecules named autoinducers (AI)

(Miller and Bassler, 2001). The QS system of *V. harveyi* BB120 has been extensively studied. The strain produces, detects and responds to three kinds of QS signal molecules, harveyi autoinducer 1 (HAI-1; Cao and Meighen, 1989), autoinducer 2 (AI-2; Chen *et al*., 2002) and cholerae autoinducer 1 (CAI-1; Higgins *et al*., 2007). These three signal molecules are detected at the cell surface by their respective two-component receptors, which feed a shared phosphorylation/ dephosphorylation signal transduction cascade (**Figure 3.2**).

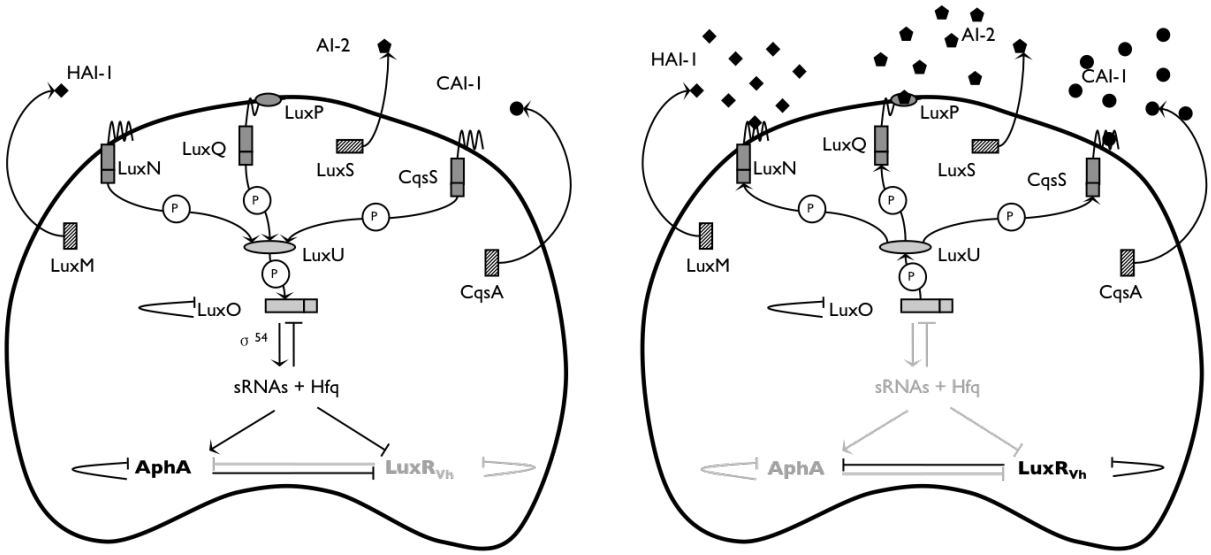



**Figure 3.2.** The *V. harveyi* quorum sensing circuit. The LuxM, LuxS and CqsA enzymes synthesize the autoinducer HAI-1, AI-2 and CAI-1, respectively. These autoinducers are detected at the cell surface by the LuxN, LuxQ and CqsS two-component receptor proteins respectively. Detection of AI-2 by LuxQ requires the periplasmic protein LuxP.

A. At low signal molecule levels the LuxN, LuxPQ and CqsS receptors function as kinases. LuxO is phosphorylated, the Qrr1-5 sRNAs are transcribed and LuxR protein is not produced.

B. At high signal molecule levels, the LuxN, LuxPQ and CqsS receptors function as phosphatases. LuxO is unphosphorylated, Qrr1-5 sRNAs are not transcribed and LuxR protein is produced. LuxR is the quorum sensing master regulator, and controls the expression of target genes by binding to their promoter regions. Solid and dotted lines denote regulatory factors that are produced and not produced respectively.

A variety of virulence factors has been found to be regulated by QS in *V. harveyi*,

including biofilm formation (Anetzberger *et al*., 2009), type III secretion (Henke and Bassler, 2004a), production of a siderophore (Lilley and Bassler, 2000), the Vhp metalloprotease (Mok *et al*., 2003; Ruwandeepika *et al*., 2011a), chitinase A (Defoirdt *et al*., 2010), and three phospholipase genes (Natrah *et al*., 2011). However, with the exception of the metalloprotease (which probably is not an essential virulence factor; Ruwandeepika *et al*., 2011b), these virulence factors are negatively regulated by QS, and this is in contrast with the fact that QS is required for full virulence of these bacteria towards different hosts (Defoirdt and Sorgeloos, 2012; Pande *et al*., 2013). Hence, in addition to the Vhp metalloprotease, there should be at least another important virulence factor that is positively regulated by QS in *V. harveyi*. Since a link between QS and motility in *V. harveyi* has thus far not been investigated, in this study, we investigated the effect of QS on swimming motility of *V. harveyi* and on the expression of selected genes involved in flagellar motility. We further investigated the importance of flagellar motility for virulence of *V. harveyi* by applying a motility inhibitor in a model system with gnotobiotic brine shrimp (*Artemia franciscana*) larvae.

## 3.2 RESULTS AND DISCUSSION

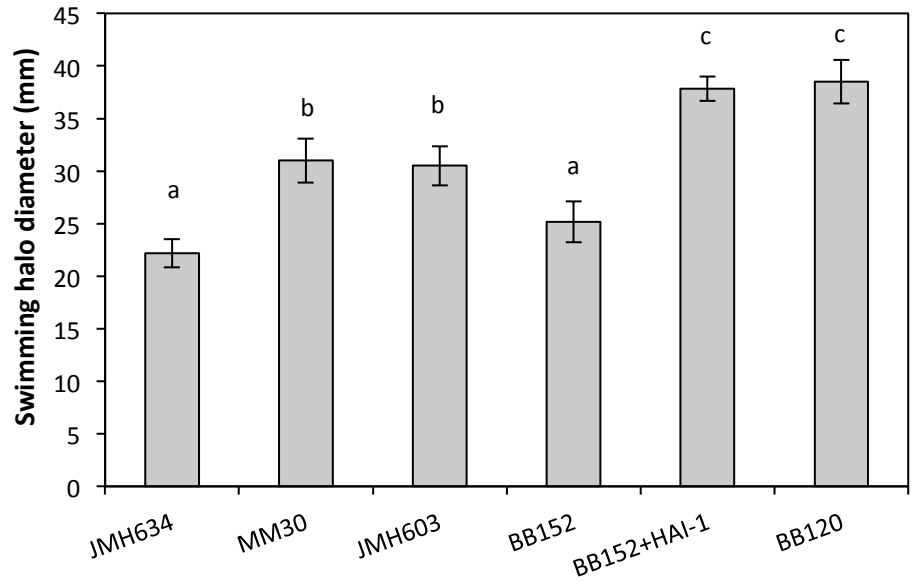
### 3.2.1 Impact of QS on the motility of *V. harveyi*

The impact of QS on the motility of *V. harveyi* was investigated using wild type *V. harveyi* BB120 and various QS mutants. The strains were spotted on soft agar plates, and zones of motility were measured after 24h of incubation. In a first experiment, we determined the impact of inactivation of signal molecule synthases and found that inactivation of all three synthases in triple mutant JMH634 resulted in a significantly decreased motility when compared to the wild type (**Figure 3.3A**). Furthermore, we investigated the impact of inactivation of single synthase mutants and found that all mutants showed lower motility than the wild type. The swimming motility of HAI-1 synthase mutant BB152 could be restored to the same level of BB120 by adding synthetic D-HAI-1. We used the synthase deletion mutants rather than testing receptor deletion mutants because the deletion of a receptor results in a condition similar to that of the receptor in the presence of its cognate signal molecule (no phosphorylation of LuxO by the deleted receptor) (Freeman and Bassler, 1999).

We further sought to confirm QS regulation of motility by using *luxO* mutants JAF483 (LuxO D47A) and JAF548 (LuxO D47E). These mutants contain a point mutation in *luxO*, resulting in LuxO proteins locked in the conformation of wild type LuxO at high and low signal molecule concentrations, respectively (Freeman and Bassler, 1999). This results in a maximally active QS system in JAF483 and a completely inactive QS system in JAF548, irrespective of cell density and signal molecule concentration. The motility of JAF548 (inactive QS), was significantly lower than that of the wild type BB120, whereas the motility of JAF483 (maximally active QS) was higher (**Figure 3.3B**). These results further substantiate positive regulation of motility by QS in *V. harveyi*.

Quorum sensing-regulated motility has been demonstrated in many *Vibrio* species, such as *V. cholerae* (Zhu *et al*., 2002) *V. fisheri* (Lupp and Ruby, 2005), *V. alginolyticus* (Tian *et al*., 2008) and *V. parahaemolyticus* (Gode-Potratz and McCarter, 2011). In contrast to *V. harveyi*, QS appears to repress motility in most of the species. However, down-regulation of motility by QS has been reported in the other important pathogenic species belonging to the Harveyi clade, *V. parahaemolyticus* (McCarter, 2004).

A.




B.

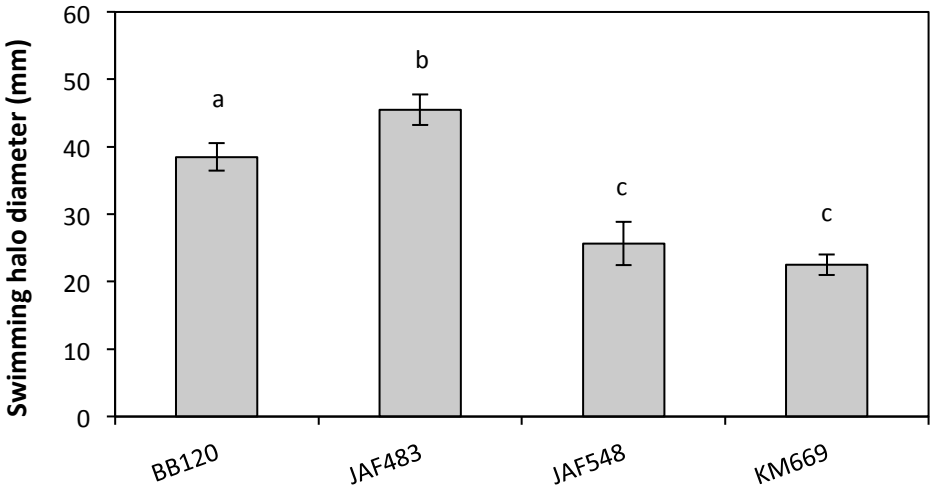



**Figure 3.3.** Swimming motility of *Vibrio harveyi* wild type and QS mutants on soft agar after 24h of incubation at 28°C.

**A.** Swimming motility of *V. harveyi* BB120 (wild type), JMH634 (autoinducer synthase triple mutant), BB152 (HAI-1 synthase mutant), MM30 (AI-2 synthase mutant), and JMH603 (CAI-1 synthase mutant). BB152 was assessed both without signal molecules and with 5 mg $l^{-1}$ HAI-1. **B.** Swimming motility of *V. harveyi* BB120 (wild type), JAF483 (maximally active QS mutant; LuxO D47A), JAF548 (inactive QS mutant; LuxO D47E) and KM669 (LuxR deletion mutant).

For both panels, the error bars indicate the standard deviation of six replicate cultures. Different letters indicate significant differences ($P < 0.01$).

### 3.2.2 Impact of QS on the expression of motility-related genes in *V. harveyi*

In order to further characterize the relation between QS and motility, we investigated the effect of QS on motility at the transcriptional level. For this experiment, we selected 10 genes involved in flagellar motility. This selection was based on the knowledge on flagellar motility in *Vibrio parahaemolyticus,* the paradigm of flagellar motility in vibrios (McCarter, 2004) and on previous reports on QS regulation of motility in other vibrios (Zhu *et al*., 2002; Lupp and Ruby, 2005; Tian *et al*., 2008). The selected genes included 6 genes involved in the synthesis of the polar flagellum (both regulators and structural genes), 2 genes involved in the synthesis of lateral flagella, and 2 genes involved in chemotaxis, which is strongly linked to flagellar motility (McCarter, 2004). The results showed that on soft agar the expression of the polar flagellum and chemotaxis genes was significantly higher in JAF483 (maximal QS activity) when compared to JAF548 (inactive QS) (**Table 3.1**). The expression levels in BB120 were intermediate between these two strains. For the two genes involved in lateral flagella synthesis, the comparison among these three strains was opposite on soft agar. In fact, the expression of lateral flagellar genes was in general low on soft agar plates, which was expected as lateral flagella are activated in more viscous environments (McCarter, 2004). If the bacteria were grown in a more viscous environment (plates with 1.5% agar), the expression levels of the two lateral flagellar genes increased and showed the same trend as observed for the polar flagellar genes in soft agar (i.e. positive regulation by QS). These findings confirmed that QS positively regulates flagellar motility.

**Table 3.1.** Relative expression of motility-related genes relative to *rpoA* mRNA in *V. harveyi* wild type and quorum sensing mutants (average ± standard deviation of three replicate cultures).

| Gene | Function | Relative expression (fold)[1] | | |
|---|---|---|---|---|
| | | **BB120 (WT)** | **JAF548 ($QS^-$)** | **JAF483 ($QS^{max}$)** |
| *flaA* | Polar flagellin | 1.0 ± 0.1[a] | 0.6 ± 0.2[b] | 1.6 ± 0.1[c] |
| *flaC* | Polar flagellin | 1.0 ± 0.4[a] | 0.4 ± 0.1[b] | 3.5 ± 0.2[c] |
| *flaK* | Polar flagellar regulator | 1.0 ± 0.2[a] | 0.8 ± 0.2[b] | 1.3 ± 0.1[c] |
| *fliA* | Polar flagellar biosynthesis sigma factor | 1.0 ± 0.2[a] | 0.4 ± 0.3[b] | 2.0 ± 0.1[c] |
| *fliS* | Polar flagellin specific chaperone | 1.0 ± 0.1[a] | 0.5 ± 0.3[b] | 1.5 ± 0.4[c] |
| *flgB* | Flagellar basal body rod | 1.0 ± 0.2[a] | 0.8 ± 0.1[b] | 1.6 ± 0.1[c] |
| *cheA* | Chemotaxis protein | 1.0 ± 0.2[a] | 0.5 ± 0.2[b] | 2.0 ± 0.2[c] |
| *cheR* | Chemotaxis protein | 1.0 ± 0.2[a] | 0.7 ± 0.1[b] | 1.8 ± 0.4[c] |
| $lafA^{SA}$ | Lateral flagellar flagellin | 1.0 ± 0.4[b] | 1.6 ± 0.3[a] | 1.0 ± 0.4[a] |
| $lafA^{HA}$ | Lateral flagellar flagellin | 1.0 ± 0.3[a] | 0.5 ± 0.6[b] | 3.4 ± 0.1[c] |
| $lafK^{SA}$ | Lateral flagellar regulator | 1.0 ± 0.2[b] | 1.6 ± 0.3[a] | 1.1 ± 0.2[a] |
| $lafK^{HA}$ | Lateral flagellar regulator | 1.0 ± 0.3[a] | 0.4 ± 0.2[b] | 1.3 ± 0.1[c] |

[1]: Values in the same row with a different letter are significantly different from each other ($p < 0.01$); [SA]: Cells were grown on soft agar (0.3% agar); [HA]: Cells were grown on hard agar (1.5% agar).
For each gene, the expression in the wild type strain BB120 was set at 1 and the expression in all other strains was normalized accordingly using the $2^{-\Delta\Delta CT}$ method.

Flagellar systems are encoded by a considerable number of genes that are tightly regulated. A hierarchy of regulation has been elucidated in many *Vibrio* species, such as *V. parahaemolyticus* (McCarter, 2001), *V. alginolyticus* (Kawagishi *et al*., 1997), *V. cholerae* (Klose and Mekalanos, 1998) and *V. anguillarum* (McGee *et al*., 1996). This hierarchy consists of three classes of genes: early, middle and late. Early genes encode the master transcriptional activators that control the entire motility regulon, middle genes encode structural components of the flagellar export system and the hook-basal body, and late genes encode the flagellar filament, motor force generator and chemotaxis signal transduction proteins (McCarter, 2001). In this study, *V. harveyi* QS was found to regulate not only the middle and late flagella genes, but also

the master regulator *flaK*, which suggests that QS regulates motility by affecting the expression of the flagellar regulators. Further, although motility has been reported to be negatively regulated by QS in *V. cholerae* and *V. fischeri* (which is in contrast to what we found in *V. harveyi*), the chemotaxis genes were shown to be up-regulated by QS in *V. cholerae* (which is the same as what we found in *V. harveyi)*, while they are down-regulated in *V. fischeri* (Lupp and Ruby, 2005; Zhu *et al*., 2002). Hence, QS regulation of flagellar motility apparently is different in different *Vibri*o species.

### 3.2.3 Impact of LuxR on the motility of *V. harveyi*

A further series of experiments aimed at determining the involvement of the QS master regulator LuxR in QS regulation of motility in *V. harveyi*. We found that the *luxR* deletion mutant KM669 showed significantly lower motility than the wild type (**Figure 3.3B**). We further determined the expression levels of the master regulator gene *flaK* and the flagellin gene *flaC* in the *luxR* deletion mutant KM669 and found that both genes showed significantly lower expression when compared to the wild type. Expression levels of *flaK* and *flaC* were 2.0 ± 0.4 ($P < 0.01$) and 1.7 ± 0.2 ($P < 0.05$) fold lower in KM669 (Δ*luxR*) than in the wild type, respectively.

### 3.2.4 Importance of swimming motility for the virulence of *V. harveyi*

In order to study the impact of motility on the virulence of *V. harveyi* to brine shrimp larvae, we followed a chemical biological approach using the motility inhibitor phenamil. Phenamil is a specific inhibitor of the sodium-driven flagellar motor of the polar flagellum (Jaques *et al*., 1999). We used this approach rather than testing mutants because some flagellar genes have pleiotropic effects, altering other polar structures and interfering with cell division (Russo *et al.,* 1992). For example, it has been reported that mutation in *flbE,* which encodes a structural component of the flagellum, also resulted in a cell division defect in *Caulobacter crescentus* (Muir and Gober, 2001). Inhibition of motility by phenamil has been reported before in pathogenic *Vibrio* species, including *V. alginolyticus* (Kawagishi et al., 1995), *V. parahaemolyticus* (Jaques et al., 1999), and *V. cholerae* (Rasmussen et al., 2011). A motility assay using soft agar showed that phenamil methanesulfonate could completely inhibit the swimming motility of *V. harveyi* BB120 at a concentration of

50$\mu$M (**Figure 3.4a**), but had no significant effect on growth rate at this concentration (**Figure 3.4b**).

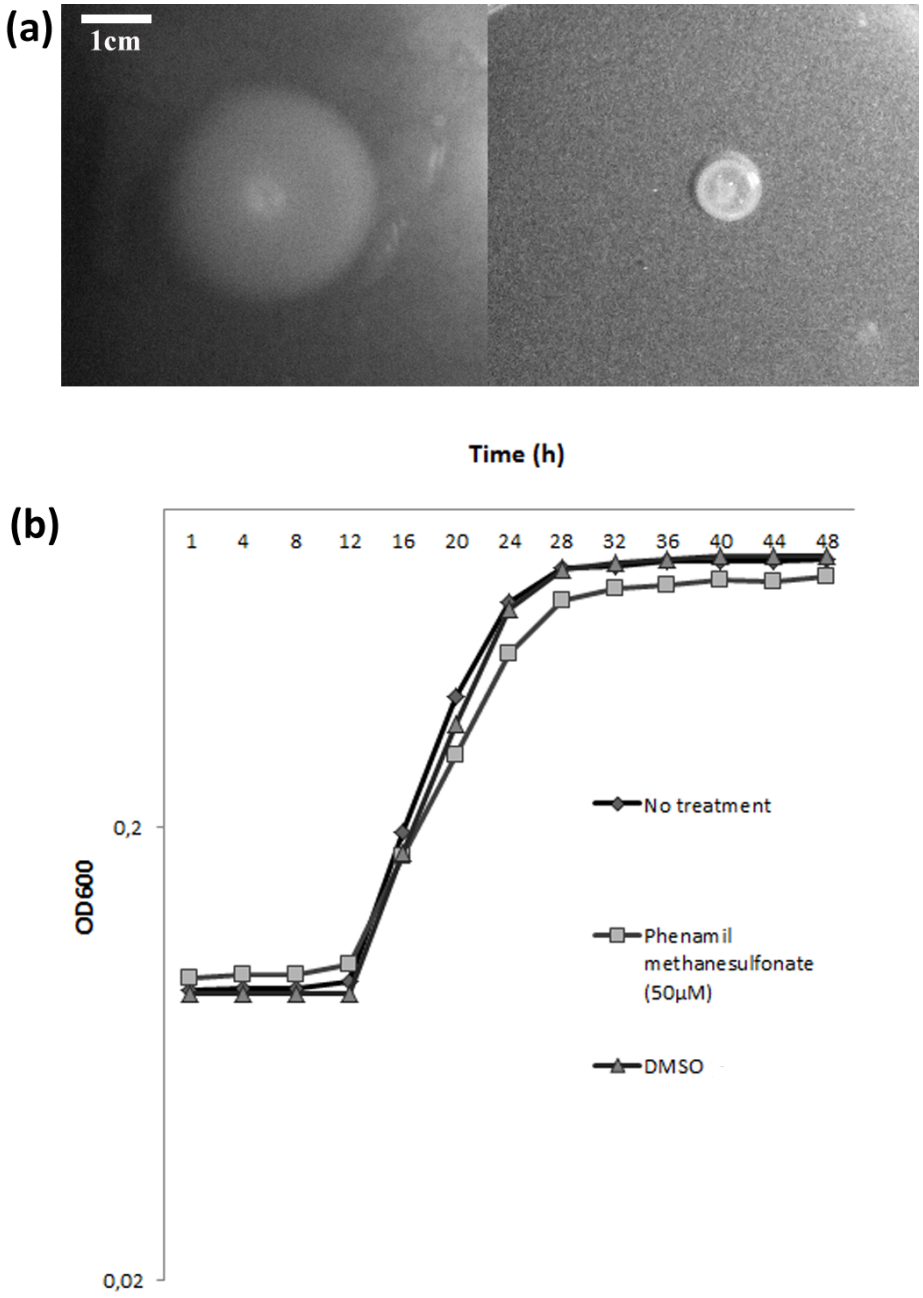



**Figure 3.4.** Effects of phenamil methanesulfonate on the swimming motility and growth rate of *V. harveyi* BB120. **(a)** Swimming motility of *V. harveyi* BB120 on soft agar after 24h of incubation at 28°C without (left) and with 50$\mu$M phenamil methanesulfonate (right). **(b)** Growth of *V. harveyi* BB120 in marine broth, in the absence and presence of phenamil methanesulfonate (50$\mu$M). As a control, the same volume of DMSO as added together with phenamil methanesulfonate was tested.

The importance of swimming motility for the virulence of *V. harveyi* was determined by measuring the effect of phenamil on the survival of brine shrimp larvae in a standardized challenge test (Defoirdt *et al*., 2005; Defoirdt and Sorgeloos, 2012). Interestingly, the addition of 50 $\mu$M phenamil methanesulfonate to the culture water significantly increased the survival of brine shrimp larvae challenged with *V. harveyi* (**Table 3.2**). Furthermore, we verified that phenamil was not toxic to the vibrios in the more harsh environmental conditions of the brine shrimp culture water (when compared to nutrient rich growth medium) by spread-plating the brine shrimp rearing water after 24h and 48h of challenge. At both time points, there were no differences in *V. harveyi* counts between control and phenamil treatment (data not shown), indicating that phenamil did not inhibit growth of *V. harveyi.* These data confirm that swimming motility plays an important role in the virulence of *V. harveyi* in the brine shrimp model. Motility has been reported to be involved in the virulence in other vibrios as well, including *Vibrio anguillarum* (O' Toole *et al*., 1996) and *Vibrio cholerae* (Merrell *et al*., 2002).

**Table 3.2.** Survival of brine shrimp larvae challenged with *V. harveyi* BB120 in the absence and presence of 50 $\mu$M phenamil methanesulfonate after 48 hours (average ± standard error of four replicate brine shrimp cultures). BB120 was added to the culture water at $10^5$ CFU/ml. Brine shrimp were fed with autoclaved LVS3 bacteria at $10^7$ cells $ml^{-1}$. *Artemia* cultures to which only autoclaved LVS3 bacteria were added as feed and that were otherwise treated in the same way as for the other treatments, were used as controls.

| Treatment | Survival (%)[1] |
|---|---|
| BB120 | $50 \pm 5^b$ |
| BB120 + phenamil | $70 \pm 5^a$ |
| Phenamil | $98 \pm 3^c$ |
| Control | $100 \pm 0^c$ |

[1]: Values with a different superscript letter are significantly different from each other ($p < 0.01$).

## 3.3 CONCLUSIONS

Although QS has been reported to be required for full virulence of *V. harveyi* towards different hosts (Defoirdt and Sorgeloos, 2012; Pande *et al*., 2013), until now, most of the virulence factors that were reported to be controlled by QS are negatively

regulated by QS. These virulence factors include chitinase A (Defoirdt *et al*., 2010), phospholipase (Natrah *et al*., 2011), siderophore (Lilley and Bassler, 2000), and components of a type III secretion system (Henke and Bassler, 2004b). As QS is essential for virulence and these virulence factors are negatively regulated by QS, their impact on virulence probably is rather limited. The only virulence factor thus far known to be positively regulated by QS in *V. harveyi* was the Vhp metalloprotease (Mok *et al*., 2003). However, this metalloprotease probably is not essential as the *V. harveyi* genome encodes several proteases and as we previously found no correlation between the expression level of *vhp* and virulence towards brine shrimp larvae (Ruwandeepika *et al*., 2011b). In this study, we further unravelled the mechanism by which QS controls the virulence of *V. harveyi* by demonstrating (1) that QS positively regulates motility by affecting flagellar biosynthesis, (2) that LuxR is involved in the regulation of motility and (3) that flagellar motility significantly affects virulence of *V. harveyi* in our gnotobiotic brine shrimp model.

## 3.4 EXPERIMENTAL PROCEDURES

### 3.4.1 Bacterial strains and growth conditions

The bacterial strains used in this study are listed in **Table 3.3**. All *V. harveyi* strains were grown at 28°C in Marine Broth (Difco) under constant agitation (100 $min^{-1}$) and the densities were measured spectrophotometrically at 600nm. Antibiotics were added to the media at the following concentrations when necessary: 20 mg/l of kanamycin (Sigma-Aldrich, Bornem, Belgium) for JAF483 and JAF548; 20 mg/l of chloramphenicol (Sigma-Aldrich) for JMH603 and JMH634.

**Table 3.3.** *Vibrio harveyi* strains used in this study.

| Strain | Relevant characteristics | References |
|---|---|---|
| BB120 | ATCC BA-1116; wild type strain from which strains JAF483, JAF548, JMH 634 and MM77 were derived | Bassler *et al*. (1997) |
| JAF483 | *luxO* D47A linked to Kan$^r$ | Freeman and Bassler (1999) |
| JAF548 | *luxO* D47E linked to Kan$^r$ | Freeman and Bassler (1999) |
| JMH634 | Δ*luxM* Δ*luxS cqsA*::Cm$^r$ | Henke and Bassler (2004a) |
| JMH603 | *cqsA*::Cmr | Henke and Bassler (2004a) |
| MM30 | *luxS*::Tn5 | Surette *et al*., 1999 |
| BB152 | *luxM*::Tn5 | Bassler *et a*l., 1994 |
| KM669 | Δ*luxR* | Henke and Bassler (2004b) |

### 3.4.2 Swimming motility assays

Swimming motility assays were performed on soft agar (Marine Agar containing 0.3% agar) as described previously (Rui *et al*., 2008). *V. harveyi* strains were grown overnight in Marine Broth, and 5 $\mu$l of the cultures (diluted to $OD_{600}$ = 1.0) were spotted in the center of the soft agar plates. Plates were incubated for 24 h, after which the diameters of the motility halos were measured.

To investigate whether signal molecules could restore the swimming motility in the HAI-1 negative mutant BB152, soft agar plates containing 5 mg $l^{-1}$ of HAI-1 were used. D-HAI-1, D-3-(hydroxyl butanoyl)-L-homoserine lactone, was purchased from University of Nottingham (UK). The signal molecules were dissolved in distilled water and stored at -20°C.

To investigate whether phenamil, an inhibitor of motility, could inhibit the swimming motility of *V. harveyi* BB120, we conducted the swimming assay on soft agar plates with phenamil methanesulfonate at a concentration of 50$\mu$M. Phenamil methanesulfonate was purchased from Sigma-Aldrich, Bornem, Belgium, and dissolved in dimethyl sulfoxide (DMSO).

### 3.4.3 RNA extraction

Three *V. harveyi* strains BB120, JAF483 and JAF548 were grown overnight in triplicate on soft agar plates (0.3 % agar) and hard agar plates (1.5% agar), respectively. Cells were harvested and RNA was extracted with the SV Total RNA Isolation System (Promega, USA) according to the manufacturer's instructions. The RNA quantity was measured spectrophotometrically (NanoDrop Technologies) and adjusted to 200 ng $\mu l^{-1}$ in all samples. The RNA integrity was checked by Agarose Gel Electrophoresis and the RNA samples were stored in -80°C for subsequent use.

### 3.4.4 Primers

The genome sequence of *V. harveyi* (Genbank) was screened for flagella-related genes and 10 genes (including regulators, structural genes and chemotaxis genes) were selected. Specific primers (**Table 3.4**) were designed using the software Primer Premier version 5.00 (Premier Biosoft International, Palo Alto, CA), with predicted product sizes in the 100 to 200 bp range. The RNA polymerase A submit (*rpoA*) mRNA was used as an endogenous control, and expression of the genes was calculated relative to the *rpoA* mRNA levels (Defoirdt *et al*., 2007).

**Table 3.4.** Specific primers used for reverse transcriptase real-time PCR.

| Gene | Primer sequences (5'-3') | Product size (bp) | Reference |
|---|---|---|---|
| *flaA* | F: CTGCGGGTCTTCAAATCTC<br>R: GTTAGTGGTCTCGTTCATTGC | 205 | VIBHAR_03173[a] |
| *flaC* | F: GCTTGATGTCGCCTTGAGAA<br>R: GCTGCCATTTGCTGCTTG | 230 | VIBHAR_01302[a] |
| *flaK* | F: ATTGCCCGTTGATGATTTG<br>R: CTTCTGTGCCCGATACTTGT | 128 | VIBHAR_03166[a] |
| *fliA* | F: CGCCGAGTGTTCAGGTAGA<br>R: CCGATGGGTCACGATTTAGT | 130 | VIBHAR_03144[a] |
| *fliS* | F: CTCCGCACAAAGTCATTCAA<br>R: CAATGTCACCACCATCTTCC | 224 | VIBHAR_03167[a] |
| *flgB* | F: AAACACGCCTGGCTACAAA<br>R: ACGCTCTAAATCCAAATCTACC | 202 | VIBHAR_01286[a] |
| *cheA* | F: AGCCTGTGATTCCTGAGCC<br>R: AGTGATGTCGCCGCTGTC | 243 | VIBHAR_03141[a] |
| *cheR* | F: ATGCGATGACGACTAACGA<br>R: ACGCTTGGCAATAAACCTG | 174 | VIBHAR_01283[a] |
| *lafA* | F: TAACTTCGCATCGCTTGTAAC<br>R: TCGTCTGCTGCTGAGTTGATA | 210 | VIBHAR_04961[a] |
| *lafK* | F: GAGCCAAATGAACACCTCG<br>R: AACAATCGCAATCACCACA | 111 | VIBHAR_04971[a] |
| *rpoA* | F: CGTAGCTGAAGGCAAAGATGA<br>R: AAGCTGGAACATAACCACGA | 197 | Defoirdt et al. (2007) |

[a]: Locus tag in the *V. harveyi* ATCC BAA-1116 (=BB120) genome sequence (GenBank).

### 3.4.5 Reverse transcription

Reverse transcription was done with the RevertAid$^{TM}$ H minus First strand cDNA synthesis kit (Fermentas Gmbh, Germany) in accordance to the manufacturer's instructions. Briefly, a mixture of 1 $\mu$g RNA and 1 $\mu$l random hexamer primer solution was mixed first. Then, 8 $\mu$l of reaction mixture containing 4 $\mu$l of 5× reaction buffer (0.25 mol$^{-1}$ Tris–HCl pH 8.3, 0.25 mol$^{-1}$ KCl, 0.02 mol$^{-1}$ $MgCl_2$, 0.05 mol$^{-1}$ DTT), 2 $\mu$l of 0.01 mol$^{-1}$ dNTP mix, 20 units of ribonuclease inhibitor, 200 units of RevertAid$^{TM}$ H minus M-MuLV Reverse Transcriptase was added. The reaction mixture was incubated for 5 min at 25°C followed by 60 min at 42°C. The reaction was terminated by heating at 70°C for 5 min and then cooled to 4°C. Complementary deoxyribonucleic acid (cDNA) samples were checked by PCR and stored at -20°C for further use.

### 3.4.6 Quantitative reverse transcriptase real-time PCR

Quantitative reverse transcriptase real-time PCR was used to quantify the expression

level of all the flagella-related genes and was performed with Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas, Cambridgeshire). The reaction was performed in an StepOne™ Real-Time PCR System thermal cycler (Applied Biosystems) in a total volume of 25 $\mu$l, containing 12.5 $\mu$l of 2× SYBR green master mix, 300 nM of forward and reverse primers and 2 $\mu$l of template cDNA. The thermal cycling consisted an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15s and primer annealing and elongation at 60°C for 1min. Dissociation curve analysis was performed to check for the amplification of untargeted fragments. Data acquisition was performed with the StepOne™ Software.

### 3.4.7 Quantitative reverse transcriptase real-time PCR data analysis ($2^{-\Delta\Delta CT}$ method)

The quantitative reverse transcriptase real-time PCR was validated by amplifying serial dilutions of cDNA synthesized from 1 $\mu$g of RNA isolated from bacterial samples. Serial dilutions of cDNA were amplified by real time PCR using gene specific primers. $\Delta C_T$ (average $C_T$ value of target-average $C_T$ value of *rpoA*) was calculated for the different dilutions and plotted against the cDNA concentration. The slope of the graph was almost equal to 0 for all of the target nine genes. Therefore, the amplification efficiency of reference and the target genes was considered to be equal. Based on this precondition, real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The expression of the target genes was normalized to the endogenous control (*rpoA*) by calculating $\Delta C_T$ :

$$\Delta C_t = C_{t\ target} - C_{t\ rpoA}$$

and expressed relative to a calibrator strain by calculating $\Delta\Delta C_t$:

$$\Delta\Delta C_t = \Delta C_t - C_{t\ calibratior}$$

Strain JAF548 (in which QS is inactive) was used as acalibrator (Ruwandeepika *et al*., 2011b). The relative expression was then calculated as

$$\text{Relative expression} = 2^{-\Delta\Delta Ct}$$

### 3.4.8 Axenic hatching of brine shrimp larvae

Two hundred milligrams of high-quality hatching cysts of *Artemia franciscana* cysts (EG® Type; INVE Aquaculture, Baasrode, Belgium) were hydrated in 18 ml of filtersterilized tap water for 1 h. Sterile cysts were obtained by decapsulation based on the method described by Marques et al. (2004). Briefly, 660 µl of NaOH (32%) and 10 ml of NaOCl (50%) were added to the hydrated cyst suspension to facilitate decapsulation. The process was stopped after 2 min by adding 14 ml of $Na_2S_2O_3$ (10 g $L^{-1}$). Filtered (0.22 µm) aeration was provided during the reaction. The decapsulated cysts were washed with filtered (passed through 0.22-µm membrane filter) and autoclaved (moist heat at 121°C for 15 min) artificle seawater (containing 35 g $l^{-1}$ of instant ocean synthetic sea salt, Aquarium Systems, Sarrebourg, France). The cysts were resuspended in a 50-ml tube containing 30 ml of filtered, autoclaved seawater and hatched for 28 h on a rotor (4 $min^{-1}$) at 28°C with constant illumination (*c.* 2000 lux). The axenity of cysts was verified by inoculating one ml of culture water into 9 ml of Marine broth and incubating at 28°C for 24 h. After 28 h of hatching, batches of 30 nauplii were counted and transferred to fresh, sterile 50-ml tubes containing 30 ml of filtered and autoclaved seawater. Finally, the tubes were returned to the rotor and kept at 28°C. All manipulations were performed in a laminar flow to maintain sterility of the cysts and nauplii.

### 3.4.9 Brine shrimp challenge test

The effect of swimming motility inhibitor on the virulence of *V. harveyi* was determined by a standardized challenge test of *Artemia*. Challenge tests were performed as described by Defoirdt *et al*. (2005) with some modifications. The animals were challenged with $10^5$ CFU $ml^{-1}$ of the vibrios per ml *Artemia* culture water. A suspension of autoclaved LVS3 bacteria (Verschuere *et al.* 1999) in filtered and autoclaved seawater was added as feed at the start of the challenge test equivalent to $10^7$ cells $ml^{-1}$ culture water. The inhibitor phenamil methanesulfonate (dissolved in DMSO at a concentration of 1mM) was added directly into the culture water at the same time at a final concentration of 50 µM. *Artemia* cultures to which only autoclaved LVS3 bacteria were added as feed, were used as controls. The survival of the larvae

was counted 48 h after the addition of the pathogens. Each treatment was carried out in quadruplicate and each experiment was repeated twice to verify the reproducibility. In each test, the sterility of the control treatments were checked at the end of the challenge by inoculating 1 ml of *Artemia* culture water to 9 ml of Marine Broth and incubating the mixture for 2 days at 28°C.

### 3.4.10 Statistical analysis

Data analysis was carried out using the SPSS statistical software (version 15). All data were compared with one-way ANOVA, followed by a Tukey's *post hoc* test.

## ACKNOWLEDGEMENT

We thank Bonnie Bassler for kindly providing us with the *V. harveyi* wild type and QS mutants. This work was funded by the China Scholarship Council, the Special Research Fund of Ghent University (BOF-UGent) and the Fund for Scientific Research Flanders (FWO project n° 1.5.013.12N). Tom Defoirdt is a postdoctoral fellow of FWO. The authors declare that no conflict of interest exists.

# CHAPTER IV

## SPECIFIC QUORUM SENSING-DISRUPTING ACTIVITY ($A_{QSI}$) OF THIOPHENONES AND THEIR THERAPEUTIC POTENTIAL IN A GNOTOBIOTIC BRINE SHRIMP - *VIBRIO HARVEYI* MODEL SYSTEM

**Qian Yang, Anne Aamdal-Scheie, Tore Benneche, Tom Defoirdt** (2015) *Scientific Reports. In press.*

## ABSTRACT

Disease caused by antibiotic resistant pathogens is becoming a serious problem, both in human and veterinary medicine. The inhibition of quorum sensing, bacterial cell-to-cell communication, is a promising alternative strategy to control disease. In this study, we determined the quorum sensing-disrupting activity of 20 thiophenones towards the quorum sensing model bacterium *V. harveyi.* In order to exclude false positives, we propose a new parameter ($A_{QSI}$) to describe specific quorum sensing activity. $A_{QSI}$ is defined as the ratio between inhibition of quorum sensing-regulated activity in a reporter strain and inhibition of the same activity when it is independent of quorum sensing. Calculation of $A_{QSI}$ allowed to exclude five false positives, whereas the six most active thiophenones inhibited quorum sensing at 0.25 $\mu$M, with $A_{QSI}$ higher than 10. Further, we determined the protective effect and toxicity of the thiophenones in a highly controlled gnotobiotic model system with brine shrimp larvae. There was a strong positive correlation between the specific quorum sensing-disrupting activity of the thiophenones and the protection of brine shrimp larvae against pathogenic *V. harveyi*. Nine quorum sensing-disrupting thiophenones (TF203, TF319, TF339, TF341, TF342, TF346, TF347, TF404 and TF405) were considered to be highly promising since they have a therapeutic potential of at least 10.

**Keywords:** **Quorum quenching, quorum sensing, antivirulence therapy**

## 4.1 INTRODUCTION

The discovery of antibiotics brought great relief from a large number of deadly illnesses in the 20$^{th}$ century, and until now, these kinds of compounds are still used as a first line therapy to treat bacterial infections in the clinic (Cheema *et al*., 2007; Defoirdt, 2013). However, excessive and non-judicious use of antibiotics has resulted in the evolution of multiple drug resistant bacterial strains. Antibiotic treatments are no longer effective in some cases at this moment, and diseases caused by antibiotic resistant bacteria are currently the second leading cause of death worldwide (WHO report, 2014). Additionally, massive use of antibiotics in animal production also constitutes a direct threat to human health and to the environment (Cabello, 2006), and this has resulted in more strict regulations with respect to antibiotic use. One notable example is the ban on the use of antibiotics as growth promoters in animal production in Europe in 2006 (European Parliament and Council Regulation No 1831/2003). However, there are good indications that this ban could result in a higher frequency of pathogenic bacteria (such as *Salmonella* spp.), which in turn could lead to a higher frequency of infections (in animals as well as consumers). As a consequence, the development of novel strategies to control bacterial diseases, both in human and veterinary medicine will be critically important in order to ensure public health in the future.

An alternative strategy to combat bacterial infections is the specific inhibition of functions required to infect the host, which has been termed antivirulence therapy (Clatworthy *et al*., 2007). This therapy consists of either inhibiting a specific virulence factor, or interfering with the regulation of virulence factor expression (Defoirdt *et al*., 2011). Quorum sensing (QS) is a mechanism by which bacteria co-ordinate the expression of certain genes in response to small signal molecules. Quorum sensing has been shown to control expression of virulence-related genes in many different pathogens, making quorum sensing-disruption an interesting strategy to control bacterial disease (Kalia, 2013; Defoirdt *et al*., 2013). *Vibrio harveyi* is one of the major pathogens of aquatic organisms, affecting a wide range of cultured marine animals, and causes significant losses in the aquaculture industry worldwide (Austin and Zhang, 2006; Defoirdt *et al*., 2007). The species is also one of the model organisms in studies

on QS in bacteria. *V. harveyi* contains a three-channel QS system, which is mediated by three types of signal molecules including HAI-1, AI-2 and CAI-1, respectively (Ng and Bassler, 2009). This QS system has been shown to be required for full virulence of the bacterium towards several aquatic hosts, including a highly controlled model system with gnotobiotic brine shrimp (*Artemia franciscana*) larvae (Defoirdt and Sorgeloos, 2012; Pande *et al*., 2013).

To date, a variety of QS inhibitors have been described or claimed (Kalia, 2013; Defoirdt *et al*., 2013). Brominated furanones are the most intensively studied QS inhibitors, and these compounds have been reported to disrupt QS in various Gram-negative bacteria (Manefield *et al*., 2002; Kalia, 2013). These compounds inhibit QS in *V. harveyi* by decreasing the DNA-binding activity of the quorum sensing master regulator LuxR (Defoirdt *et al*., 2007b). Unfortunately, these brominated furanones are toxic to higher organisms (Natrah *et al*., 2012), which means that they will not be safe for practical applications. More recently, brominated thiophenones, sulphur analogues of the brominated furanones with the same mode of action, have been synthesized (Benneche *et al*., 2011), and these compounds were found to be more active than the corresponding furanones (Benneche *et al*., 2011; Lönn-Stensrud and Benneche, 2011). One of these compounds, (*Z*)-4-((5-(bromomethylene)-2-oxo-2, 5-dihydrothiophen-3-yl) methoxy)-4-oxobutanoic acid (TF310, **Figure 4.1**) has been reported to show the highest therapeutic index of all QS-disrupting compounds tested in our *V. harveyi* – brine shrimp model thus far, with complete protection against the pathogen at 2.5 μM and severe toxicity only being observed at 250 μM (Defoirdt *et al*., 2012). Based on these promising results, in the present study, we aimed at determining quorum sensing-disrupting activity, protective effect and toxicity of 20 novel thiophenones (**Figure 4.1**). Furthermore, we propose a new parameter to describe specific quorum sensing-inhibitory activity, $A_{QSI}$, defined as the ratio between inhibition of quorum sensing-regulated activity and inhibition of the same activity when independent of quorum sensing. Most claims with respect to quorum sensing inhibition are based on experiments with quorum sensing signal molecule reporter strains. We recently argued that these experiments are prone to bias due to other effects compounds may have on reporter strains, and therefore, that good control experiments are required in order to exclude false positives (Defoirdt *et al*., 2013). The

use of the proposed parameter $A_{QSI}$ is a straightforward and elegant way to exclude false positives by taking into account (potential) bias related to the use of quorum sensing molecule reporters.

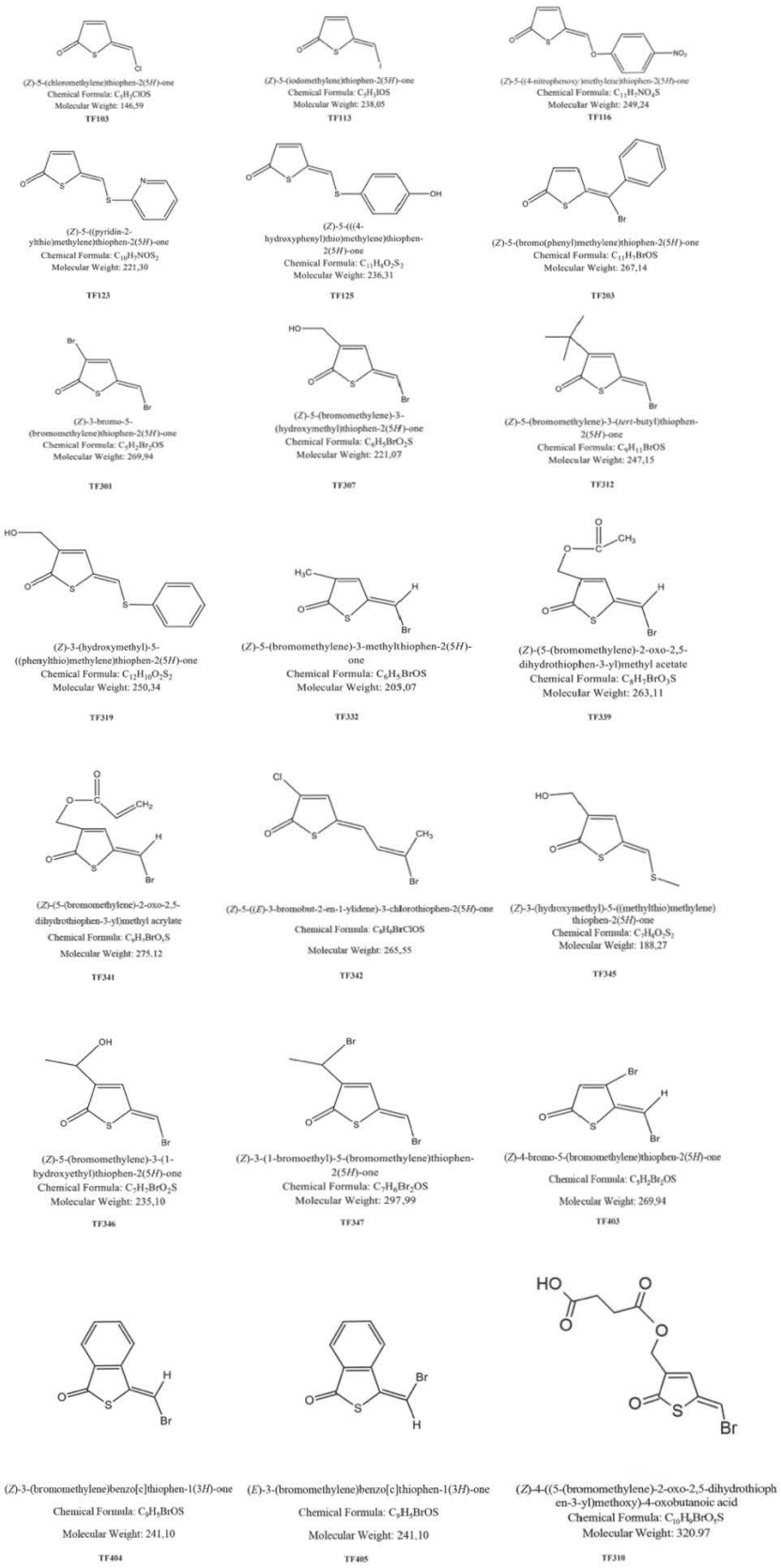



**Figure 4.1.** Structures of the thiophenones used in this study and TF310 (used in Defoirdt *et al.*, 2012a).

## 4.2 RESULTS

### 4.2.1. Impact of the thiophenones on quorum sensing-regulated bioluminescence of *V. harveyi*.

In a first experiment, we determined the impact of the thiophenones on QS-controlled bioluminescence of *V. harveyi*. Wild type *V. harveyi* was grown to high cell density in order to activate QS-controlled bioluminescence, after which the thiophenones were added at 0.25, 1, 5 and 10 $\mu$M, respectively. Bioluminescence was measured 1 h after the addition of the thiophenones and our results revealed that most of the compounds were able to block bioluminescence in wild type *V. harveyi* in a concentration-dependent way. Fifteen of the 20 compounds (TF103, TF113, TF116, TF125, TF203, TF307, TF312, TF319, TF332, TF339, TF341, TF342, TF346, TF347 and TF403) were found to inhibit bioluminescence at a concentration of 0.25 $\mu$M and higher, while TF123 and TF301 significantly reduced the bioluminescence from 5 $\mu$M onwards. Additionally, TF203 could completely inhibit the QS-regulated bioluminescence at 5 µM, and TF301, TF332 and TF341 completely blocked the bioluminescence at 10 µM. Finally, TF345, TF404 and TF405 showed no effect on the bioluminescence even at the highest concentration tested (**Figure 4.2**). Importantly, the compounds had no effect on the growth of *V. harveyi* at the concentrations used (**Figure 4.3**).

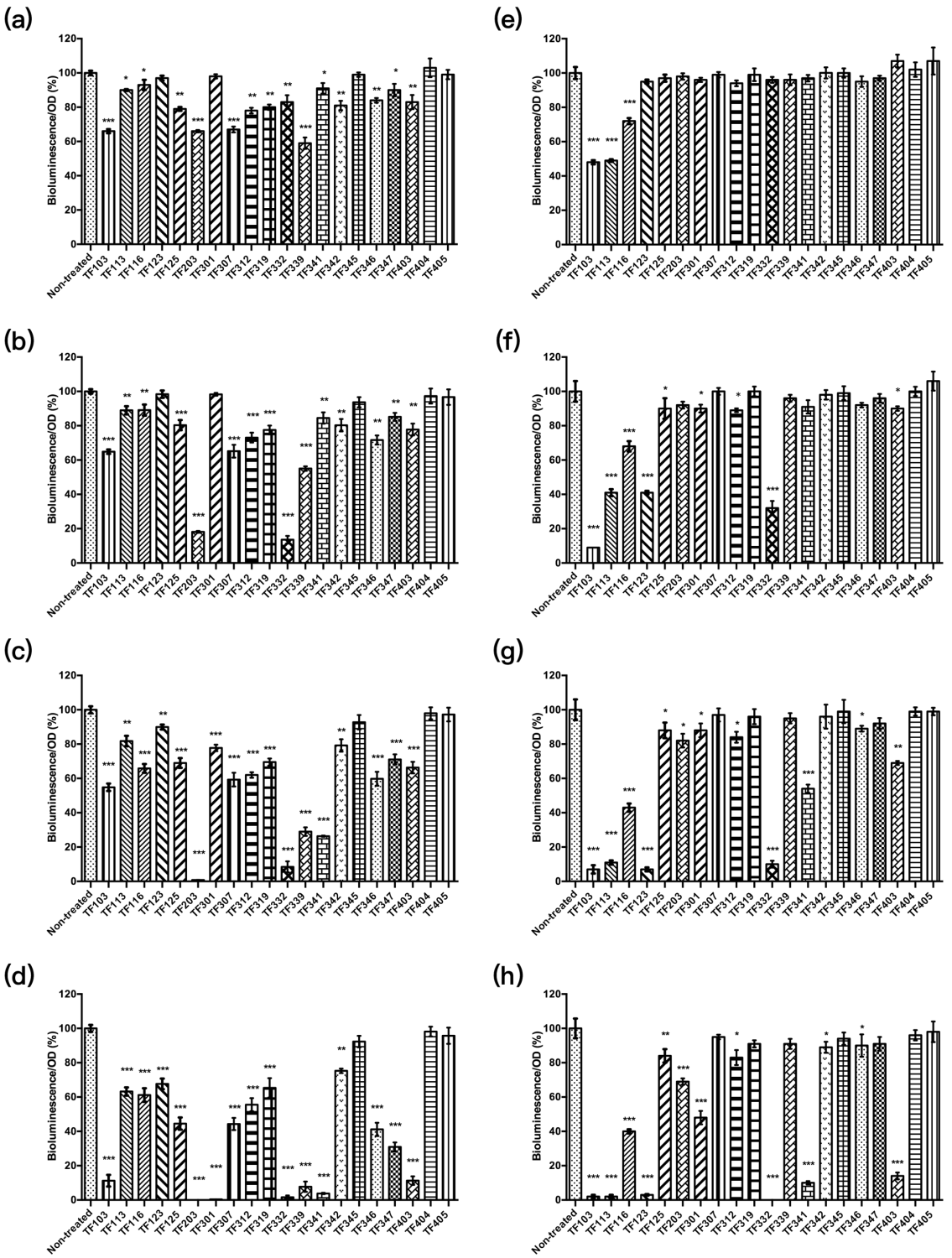



**Figure 4.2. Left**: Bioluminescence of wild type *V. harveyi* in Luria-Bertani medium containing 35g/l of sodium chloride with and without the thiophenones added at (a) 0.25 $\mu$M; (b) 1 $\mu$M; (c) 5 $\mu$M; (d) 10 $\mu$M. **Right:** Quorum sensing-independent bioluminescence of *V. harveyi* JAF548 pAK*lux*1 in Luria-Bertani medium containing 35g/l of sodium chloride with and without the thiophenones added at (e) 0.25 $\mu$M; (f) 1 $\mu$M; (g) 5 $\mu$M; (h) 10 $\mu$M. Luminescence measurements were performed 1h after the addition of the thiophenones. Bioluminescence in the control treatment was set at 100% and the other treatments were normalized accordingly. The error bars represent the standard deviation of three replicates. Asterisks indicate significant differences (independent samples T- test; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

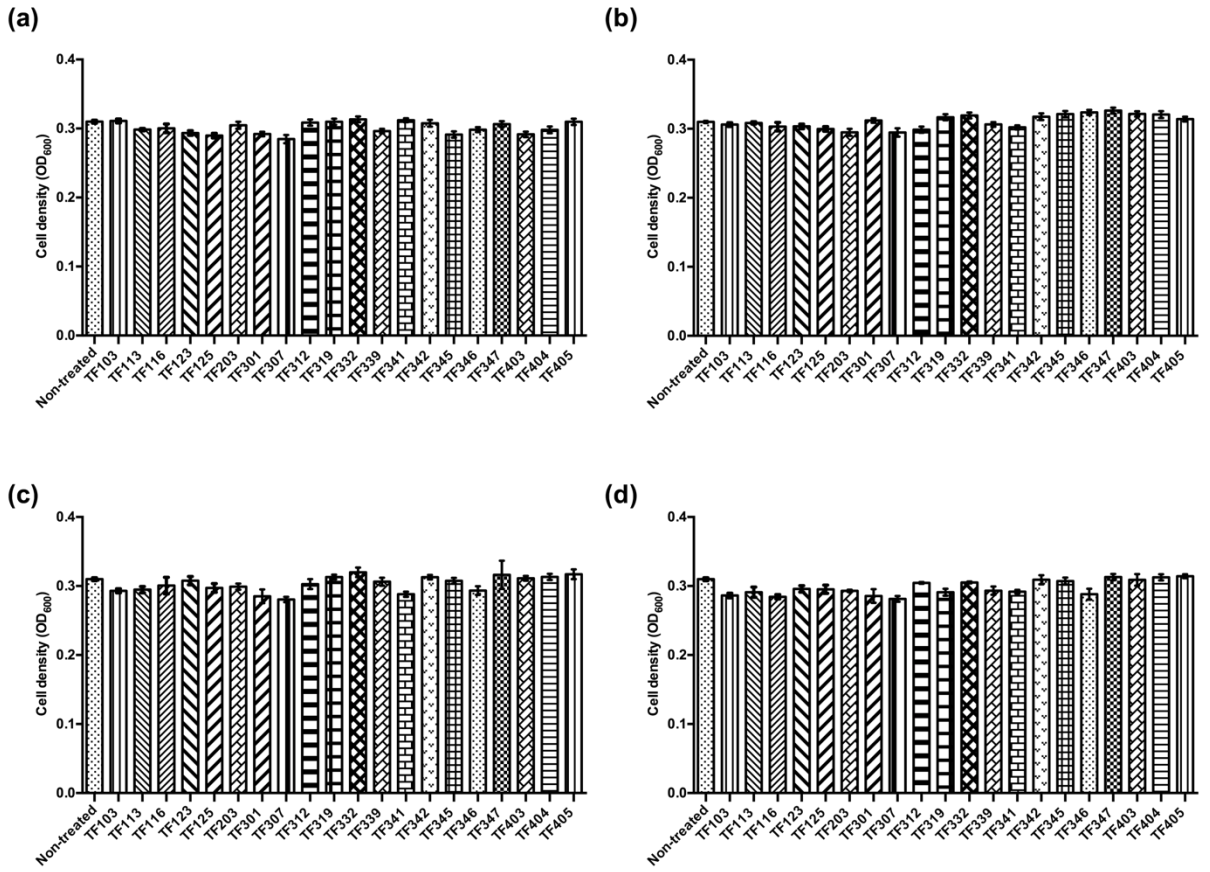



**Figure 4.3.** Growth of wild type *V. harveyi* in Luria-Bertani medium containing 35g/l of sodium chloride with and without the thiophenones added at (a) 0.25 $\mu$M; (b) 1 $\mu$M; (c) 5 $\mu$M; (d) 10 $\mu$M. Growth measurements were performed 1h after the addition of the thiophenones. The error bars represent the standard deviation of three replicates.

We previously argued that the identification of QS inhibitors using QS molecule reporter strains is prone to bias due to other effects compounds may have on the reporter strains and that therefore, adequate control experiments are required (Defoirdt *et al.*, 2013). In order to verify that the effect observed in the bioluminescence tests with wild type *V. harveyi* was not due to toxicity, we investigated the effect of the thiophenones on bioluminescence of strain JAF548 pAK*lux*1, in which bioluminescence is independent of the QS system (Defoirdt *et al.*, 2012). At 0.25 $\mu$M, none of the compounds, except for TF103, TF113 and TF116, blocked bioluminescence of JAF548 pAK*lux*1 (**Figure 4.2**), suggesting that the luminescence inhibitory effect of the three compounds was not due to a specific inhibition of QS at this concentration. Moreover, TF123, TF125, TF301, TF312, TF332 and TF403 inhibited the bioluminescence of JAF548 pAK*lux*1 at 1 $\mu$M or higher, while TF203, TF341 and TF346 blocked the bioluminescence from 5 $\mu$M onwards. TF307,

TF319, TF339, TF345, TF347, TF404 and TF405 showed no effect on the bioluminescence of JAF548 pAK*lux*1 at the highest concentration tested in this study (**Figure 4.2**), indicating that they have low toxicity.

For the compounds that showed significant inhibition of quorum sensing-regulated bioluminescence, we further calculated the specific quorum sensing-inhibitory activity $A_{QSI}$ as the ratio between the percentage inhibition of quorum sensing-regulated bioluminescence (in wild type *V. harveyi*) and the percentage inhibition of quorum sensing-independent bioluminescence (in JAF548 pAK*lux*1). The specific quorum sensing-inhibitory activity of compounds TF103, TF113, TF116, TF123 and TF301 was smaller than 2 at any of the concentrations tested (**Table 4.1**), and these compounds were therefore considered as false positives. Six compounds showed a high specific quorum sensing activity (>10) at least one of the concentrations tested: TF203, TF307, TF319, TF339, TF342 and TF403.

**Table 4.1.** Comparison of the specific quorum sensing inhibitory activity ($A_{QSI}$) and the therapeutic potential of the thiophenones used in this study.

| Compound | Specific quorum sensing inhibitory activity | | | | Therapeutic potential | | |
|---|---|---|---|---|---|---|---|
| | $A_{QSI, 0.25\mu M}$ | $A_{QSI, 1\mu M}$ | $A_{QSI, 5\mu M}$ | $A_{QSI, 10\mu M}$ | [Therapeutic][a] (*μ*M) | [Toxic][b] (*μ*M) | [Toxic]/ [Therapeutic] |
| TF103 | 0.7 | 0.4 | 0.5 | 0.9 | NO | 0.25 | - |
| TF113 | 0.2 | 0.2 | 0.2 | 0.4 | NO | 0.25 | - |
| TF116 | 0.3 | 0.3 | 0.6 | 0.7 | NO | 1 | - |
| TF123 | NS | NS | 0.1 | 0.3 | NO | 5 | - |
| TF125 | 7.0 | 2.0 | 2.7 | 3.5 | 5 | >10 | >2 |
| TF203 | 17.0 | 10.3 | 5.5 | 3.2 | 1 | 10 | 10 |
| TF301 | NS | NS | 1.9 | 1.9 | 5 | 10 | 2 |
| TF307 | 33.0 | 35.0 | 13.7 | 11.2 | 5 | >10 | >2 |
| TF312 | 3.7 | 2.5 | 2.4 | 2.6 | NO | 10 | - |
| TF319 | 20.0 | 23.0 | 7.8 | 3.9 | 1 | 10 | 10 |
| TF332 | 4.3 | 1.3 | 1.0 | 1.0 | 1 | 5 | 5 |
| TF339 | 10.3 | 11.3 | 23.4 | 10.2 | 0.25 | 10 | 40 |
| TF341 | 3.0 | 1.7 | 1.6 | 1.1 | 0.25 | 5 | 20 |
| TF342 | 19.0 | 10.0 | 5.3 | 2.3 | 1 | 10 | 10 |
| TF345 | NS | NS | NS | NS | NO | >10 | - |
| TF346 | 3.2 | 3.5 | 3.7 | 5.9 | 1 | >10 | >10 |
| TF347 | 3.3 | 3.8 | 2.9 | 7.8 | 1 | >10 | >10 |
| TF403 | 17.0 | 2.2 | 1.1 | 1.0 | 1 | 5 | 5 |
| TF404 | NS | NS | NS | NS | 1 | >10 | >10 |
| TF405 | NS | NS | NS | NS | 1 | >10 | >10 |

[a]: The lowest concentration at which the survival of challenged brine shrimp larvae increased to more than 75%.

[b]: The lowest concentration at which the compounds cause >25% mortality in axenic brine shrimp larvae.

NS: no significant inhibition of QS-regulated bioluminescence in *V.harveyi*.

NO: no therapeutic potential observed

### 4.2.2. Impact of the thiophenones on the virulence of *V. harveyi* in the gnotobiotic brine shrimp model.

Previous work in our lab showed that the virulence of *V. harveyi* in the gnotobiotic brine shrimp model system is regulated by QS (Defoirdt and Sorgeloos, 2012). Therefore, we further investigated whether the thiophenones could protect brine shrimp larvae from the pathogen in *in vivo* challenge tests. Fifteen compounds (TF113, TF125, TF203, TF307, TF312, TF319 TF332, TF339, TF341, TF342, TF346, TF347, TF403, TF404 and TF405) significantly increased the survival of brine shrimp larvae challenged to *V. harveyi* at 0.25 $\mu$M (**Figure 4.4**). Fourteen of these significantly increased the survival of challenged brine shrimp at 1 $\mu$M (TF113 induced high mortality at this concentration), and 11 of them plus TF301 significantly increased the survival of challenged brine shrimp at 5 $\mu$M (TF332, TF341 and TF403 seemed to be toxic). At 10 $\mu$M, only 6 compounds were able to increase the survival of challenged brine shrimp larvae (TF125, TF307, TF346, TF347, TF404 and TF405), whereas for most compounds, high mortality was observed (**Figure 4.5**). Seven compounds offered a complete protection to the brine shrimp larvae (no significant difference in survival when compared to unchallenged larvae): TF125 (10 $\mu$M), TF203 (1 $\mu$M), TF301 (5 $\mu$M), TF307 (5 and 10 $\mu$M), TF339 (1 $\mu$M), TF341 (0.25 and 1 $\mu$M) and TF346 (5 $\mu$M) (**Figure 4.4**).

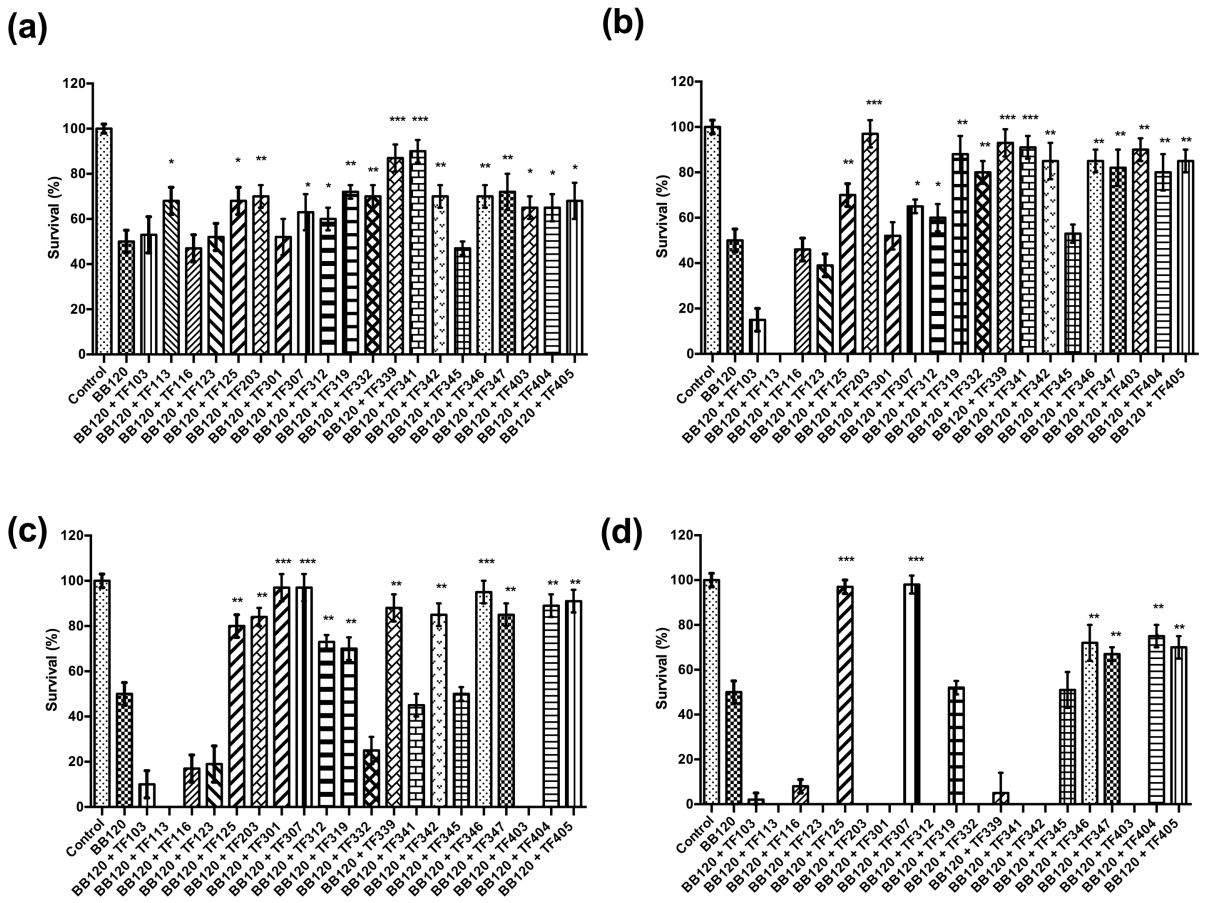



**Figure 4.4.** Relative percentage survival of brine shrimp larvae (average ± standard deviation of three replicates) after 2 days of challenge with wild type *V. harveyi*, without and with the thiophenones added to the rearing water at (a) 0.25 $\mu$M; (b) 1 $\mu$M; (c) 5 $\mu$M; (d) 10 $\mu$M. Survival of the unchallenged larvae was set at 100% and the other treatments were normalized accordingly. Asterisks indicate significant differences (independent samples T-test; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

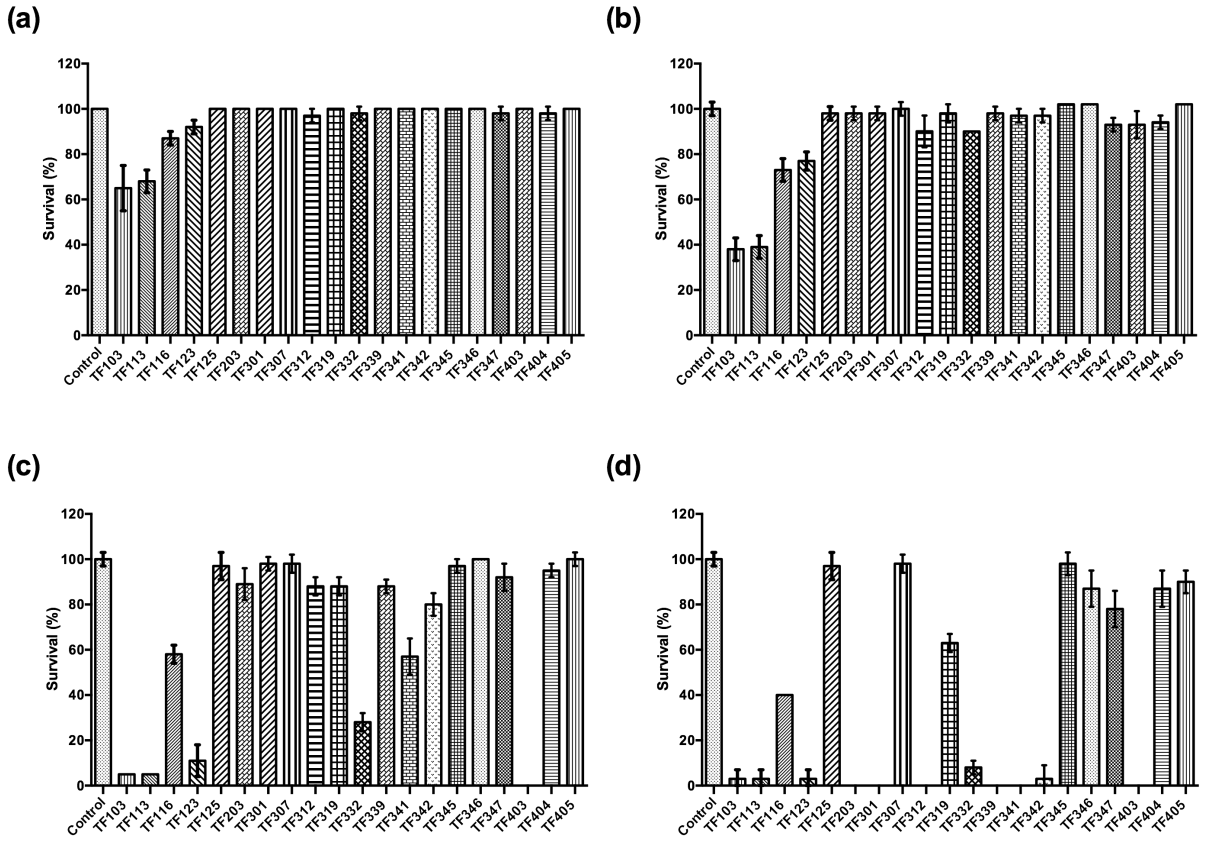



**Figure 4.5.** Relative percentage survival of axenic brine shrimp larvae (average ± standard deviation of three replicates) after 2 days without and with the thiophenones added to the rearing water at (a) 0.25 $\mu$M; (b) 1 $\mu$M; (c) 5 $\mu$M; (d) 10 $\mu$M. Survival in cultures without the addition of thiophenones was set at 100% and the other treatments were normalized accordingly.

Subsequently, in order to evaluate the relation between the inhibition of quorum sensing and the protection of brine shrimp larvae by thiophenones at different concentrations, we determined the correlation between $A_{QSI}$ and survival of brine shrimp larvae challenged with *V. harveyi* (**Figure 4.6**). The correlation is significant at all concentrations tested: 0.25 $\mu$M ($\rho = 0.481$; $P = 0.043$), 1 $\mu$M ($\rho = 0.563$; $P = 0.015$), 5 $\mu$M ($\rho = 0.726$; $P = 0.001$) and 10 $\mu$M ($\rho = 0.614$; $P = 0.007$).

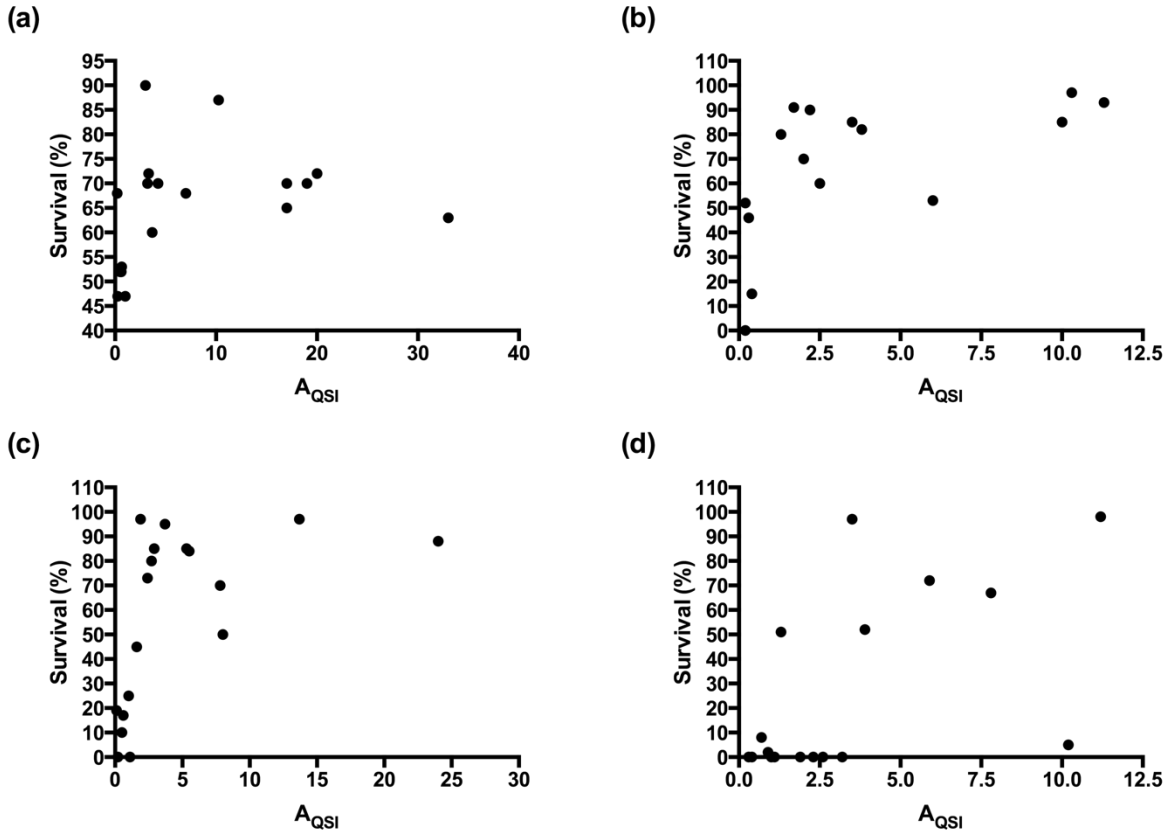



**Figure 4.6.** Scatter plots showing specific quorum sensing-inhibitory activity ($A_{QSI}$) and protection of brine shrimp larvae from *V. harveyi* for the different thiophenones at (a) 0.25 µM; (b) 1 µM; (c) 5 µM; (d) 10 µM.

### 4.2.3 Toxicity of the thiophenones to axenic brine shrimp larvae.

We determined toxicity to axenic brine shrimp larvae at 0.25, 1, 5 and 10 $\mu$M. At 0.25 $\mu$M, TF103 and TF113 showed appreciable toxicity (causing > 25 % mortality). At 1 $\mu$M, 4 compounds induced appreciable mortality, including TF103, TF113, TF116 and TF123. At 5 $\mu$M, TF332, TF341 and TF403 also started to induce appreciable mortality, and at 10 $\mu$M, only 7 compounds showed no toxic effect (TF125, TF307, TF345, TF346, TF347, TF404 and TF405), whereas all other compounds except for TF116 and TF319 induced (almost) complete mortality (**Figure 4.5**).

In addition, we also calculated correlations between inhibition of bioluminescence in JAF548 pAK*lux*1 and survival of brine shrimp larvae treated with thiophenones (**Figure 4.7**). Results showed that the correlation is significant at all the concentrations we tested in this study: 0.25 $\mu$M ($\rho = -0.676$; $P = 0.001$), 5 $\mu$M ($\rho = -$

0.762; $P = 0.000$), 5 $\mu$M ($\rho = -0.761$; $P = 0.000$) and 10 $\mu$M ($\rho = -0.635$; $P = 0.002$), indicating that the toxicity of thiophenones to pathogens is significantly related with the toxicity to brine shrimp larvae.

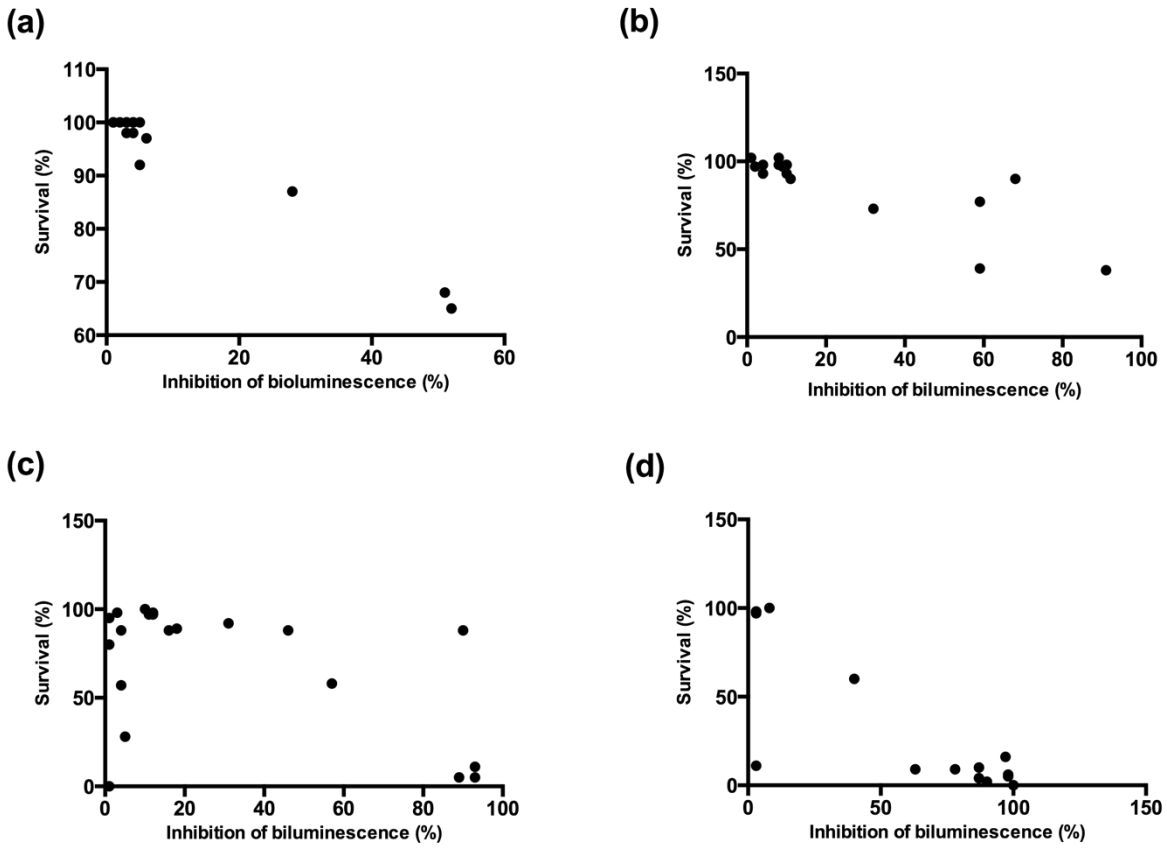



**Figure 4.7.** Correlations between toxicity towards *V. harveyi* (as determinied by inhibition of quorum sensing-independent bioluminescence in *V. harveyi* JAF548 pAK*lux*1) and toxicity towards brine shrimp (as determined by survival of brine shrimp larvae) for the different thiophenones at (a) 0.25 µM; (b) 1 µM; (c) 5 µM; (d) 10 µM.

### 4.2.4 Therapeutic potential of the thiophenones.

In order to determine the therapeutic potential of each of the compounds, we calculated the ratio between the lowest concentration at which they increased the survival of challenged brine shrimp larvae to more than 75% and the lowest concentration at which they caused more than 25% mortality in axenic brine shrimp. Nine thiophenones showed a good therapeutic potential (ratio of at least 10): TF203, TF319, TF339, TF341, TF342, TF346, TF347, TF404 and TF405 (**Table 4.1**).

## 4.3. DISCUSSION

Due to the rise of antibiotic resistance, a significant research effort currently is devoted to the development of novel methods to control bacterial disease, and quorum sensing inhibition is one of the promising alternatives that are explored. Many compounds have been claimed to be able to inhibit quorum sensing in various pathogens (Kalia, 2013). Brominated furanones are one of the most intensively studied classes of quorum sensing inhibitory compounds with a well-defined mode of action (Defoirdt *et al.*, 2007; Janssens *et al.*, 2008). However, these compounds are toxic to higher organisms, which hampers their application to control disease. Brominated thiophenones, the sulphur analogues of brominated furanones, were recently reported to be more effective and less toxic (Defoirdt *et al.*, 2012). Given the promising results obtained before with a brominated thiophenones, in this study, we investigated the quorum sensing-inhibitory activity and therapeutic potential of 20 new synthetic thiophenones in a highly controlled model system. Seventeen of the thiophenones included in this study were found to significantly decrease bioluminescence in wild type *V. harveyi* at one of the concentrations tested, and 12 of them decreased luminescence at the lowest concentration (0.25 $\mu$M).

One of the factors that have resulted in a boost of the quorum sensing research is the development of signal molecule reporter strains, which demonstrate a certain phenotype in response to quorum sensing molecules. An important limitation to the use of such reporter strains is that the quorum sensing-regulated phenotypes are often co-dependent on other factors and/or depend on the metabolic activity of the cells, leading to false positives (Defoirdt *et al.*, 2013). In order to solve this problem, we propose the use of the specific quorum sensing-inhibitory activity $A_{QSI}$, defined as the ratio between the inhibition of a quorum sensing-regulated phenotype (bioluminescence in this study) and the inhibition of the same phenotype in the same bacterium, but independent of quorum sensing, as a new parameter. We calculated the specific quorum sensing-inhibitory activity $A_{QSI}$ for the thiophenones that showed significant inhibition of bioluminescence in wild type *V. harveyi*, and this allowed us to identify 5 compounds (TF103, TF113, TF116, TF123 and TF301) as false positives. On the other hand, six thiophenones showed a high specific quorum sensing activity

($A_{QSI}$ > 10) for at least one of the concentrations tested: TF203, TF307, TF319, TF339, TF342 and TF403.

It has been proposed that the 5-bromomethylene side-chain of quorum sensing-inhibiting thiophenones enables them to bind to nucleophilic amino acid residues in LuxR, the quorum sensing master regulator in *V. harveyi* (Defoirdt *et al*., 2007). Candidate nucleophilic amino acid residues include 4 cysteine residues in the C-terminal dimerisation domain of LuxR (De Silva *et al*., 2007). Binding to one of these residues would likely decrease the ability of LuxR to form a dimer, thereby decreasing the ability to bind to target promoter DNA and to activate quorum sensing target genes (**Figure 4.6;** Defoirdt *et al*., 2007; Benneche *et al.,* 2012). Most of the thiophenones that inhibited quorum sensing *V. harveyi* (with $A_{QSI}$ > 2) also possess the bromomethylene side-chain, except for TF203, TF319 and TF342. These compounds, however contain a 5-side chain that can have the same function as the bromomethylene moiety (i.e. binding to nucleophilic amino acid residues). Some of the compounds with a 5-bromomethylene side chain (or 5-chloromethylene or 5-iodo-methylene, which have the same activity) showed no or a low specific quorum sensing inhibitory activity (i.e. TF103, TF113 and TF301). This is probably due to a low specificity of these compounds and the toxic activity that results from this (they did inhibit quorum sensing-regulated bioluminescence, but also inhibited quorum sensing-independent bioluminescence). Remarkably, the two compounds with a benzothiophenone core (TF404 and TF405) did not inhibit quorum sensing-regulated bioluminescence (despite having a bromomethylene side chain). Although the reason for this is not yet clear, we hypothesise that it might be caused by either a decreased reactivity of the bromomethylene moiety due to the presence of the benzene ring (making it less susceptible to nucleophilic attack), or steric hindrance due to the rigid benzene moiety attached to the thiophenone ring.

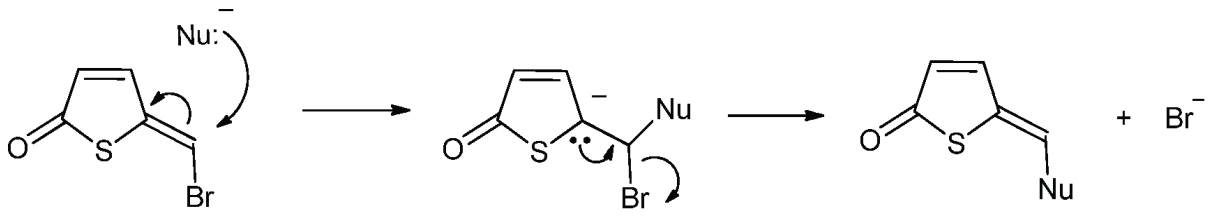



**Figure 4.8**. Proposed reaction mechanism of the thiophenones. Nu: nucleophile. Candidate nucleophiles are e.g. thio groups of cysteine residues (Adopted from Defoirdt *et al.*, 2012a).

We further determined the therapeutic potential of the compounds in a highly controlled model system with brine shrimp larvae, and found that 9 thiophenones (TF203, TF319, TF339, TF341, TF342, TF346, TF347, TF404 and TF405) showed a good therapeutic potential. Four of these (TF203, TF319, TF339 and TF342) also showed a high specific quorum sensing-inhibitory activity. One of them (TF341) showed toxicity to *V. harveyi*, which might explain the protective effect in the brine shrimp assay. The thiophenones increased the survival of challenged brine shrimp larvae at concentrations similar to those needed to block quorum sensing-regulated bioluminescence *in vitro*. Furthermore, our results also revealed a significant positive correlation between the specific quorum sensing inhibitory activity and the protection of brine shrimp larvae, suggesting that the quorum sensing-inhibitory activity largely determines the protective effect of these compounds. Remarkably, 2 of the compounds with good therapeutic potential (TF404 and TF405) showed no quorum sensing-inhibitory activity at all, nor did they show any toxicity to *V. harveyi.* This suggests that these compounds interfere with virulence gene expression by interfering with a mechanism that is distinct from the three-channel quorum sensing system.

Finally, we found that there was a significantly positive correlation between toxicity of the thiophenones towards brine shrimp larvae and toxicity towards *V. harveyi* (as revealed by the inhibition of quorum sensing-independent bioluminescence), suggesting that toxicity to *V. harveyi* could be used as a good indicator for toxicity to higher organisms. Most of the thiophenones started to cause severe mortality in brine shrimp larvae at a concentration of 10 $\mu$M. However, low toxicity was observed for TF125, TF307, TF345, TF346, TF347, TF404 and TF405 at this concentration. This might be attributed to the length and position of the side-chain since it has been

reported that the side-chain could significantly affect the toxicity of thiophenones by interfering their binding to essential proteins (Steenackers *et al.*, 2010; Defoirdt *et al.*, 2012). We indeed found that the compounds with the largest side chains at the 3-position of the thiophenone ring (e.g. TF339 and 341) showed the lowest toxicity. In addition to this, the two compounds with a benzothiophenone core (TF404 and TF405) also showed low toxicity.

In conclusion, in this study we determined the quorum sensing-inhibiting activities of 20 new synthetic thiophenones towards the quorum sensing model bacterium *V. harveyi*. We proposed the new parameter $A_{QSI}$ to determine specific quorum sensing inhibitory activity based on experiments with a quorum sensing reporter strain. We used this parameter to analyse data obtained with bioluminescence of *V. harveyi* as reporter phenotype, and it can easily be applied to any other signal molecule reporter strains. The use of $A_{QSI}$ allowed us to exclude 5 false positives out of the 17 compounds that were able to inhibit quorum sensing-regulated bioluminescence in *V. harveyi*. We identified 6 thiophenones that were able to inhibit quorum sensing at submicromolecular levels. Further, we determined the protective effect and toxicity of the thiophenones in a highly controlled gnotobiotic model system with brine shrimp larvae. There was a significantly positive correlation between the specific quorum sensing-disrupting activity of the thiophenones and the protection of brine shrimp larvae against pathogenic *V. harveyi*, and 6 quorum sensing-disrupting thiophenones were considered to be highly promising to control bacterial disease.

## 4.4 METHODS

### 4.4.1. Thiophenones

The structures of the thiophenones used in this study are shown in **Figure 4.1**. The compounds were synthesised as described before (Benneche *et al.*, 2011; Benneche *et al.*, submitted). All the thiophenones were dissolved in pure ethanol at 5 mM and stored at -20 °C.

### 4.4.2 Bacterial strains and growth conditions

*V. harveyi* wild type strain ATCC BAA-1116 (recently reclassified as *V. campbellii*; Lin *et al*., 2010) and mutant strain JAF548 pAK*lux*1 (Defoirdt *et al*., 2012) were used in this study. The latter strain is derived from wild type strain ATCC BAA-1116 (BB120). It contains a point mutantion in the *luxO* gene, rendering the LuxO protein incapable of phosphorelay, and hence the native bioluminescence operon (which is under QS control) is not activated. Then plasmid pAKlux1 was conjugated into this strain. The plasmid contains the *Photorhabdus luminescens* bioluminescence operon under the control of a constitutive promoter. Hence, strain JAF548 pAKlux1 produces bioluminescence that is independent of the quorum sensing system. This strain is used as a control in order to verify that inhibition of luminescence in *V. harveyi* is specifically caused by QS inhibition. Both strains were cultured in Luria-Bertani medium containing 35g/L of sodium chloride ($LB_{35}$) at 28 °C under constant agitation (100 $min^{-1}$). Cell densities were measured spectrophotometrically at 600 nm.

### 4.4.3. Bioluminescence assays

*V. harveyi* wild type and mutant strain were cultured overnight and diluted to an OD600 of 0.1. The thiophenones were added at different concentrations and the cultures were further incubated at 28 °C with shaking. Then bioluminescence was measured after 1h with a Tecan Infinite 200 microplate reader (Tecan, Mechelen, Belgium).

### 4.4.4. Specific quorum sensing-inhibitory activity $A_{QSI}$

The specific quorum sensing-inhibitory of the compounds at a given concentration was calculated as follows:

$$A_{QSI} = \frac{\%\text{Inhibition}_{QS-\text{regulated}}}{\%\text{Inhibition}_{QS-\text{independent}}}$$

With

% $\text{Inhibition}_{QS\text{-regulated}}$: percentage inhibition of QS-regulated bioluminescence in wild

type *V. harveyi*

% $Inhibition_{QS\text{-}independent}$: percentage inhibition of QS-independent bioluminescence of *V. harveyi* JAF548 pAKlux1

Compounds were considered as quorum sensing inhibitors if they caused a significant inhibition of quorum sensing-regulated bioluminescence and if $A_{QSI}$ was higher than 2 at one of the concentrations tested.

### 4.4.5. Axenic hatching of brine shrimp larvae

Two hundred milligrams of high-quality hatching cysts of *Artemia franciscana* (EG® Type; INVE Aquaculture, Baasrode, Belgium) were hydrated in 18 ml of filter-sterilized tap water for 1 h. Steril cysts and larvae were obtained by decapsulation according to Marques et al. (2004). Briefly, 660 *µ*l of NaOH (32%) and 10 ml of NaOCl (50%) were added to the hydrated cyst suspension to facilitate decapsulation. The process was stopped after 2 min by adding 14 ml of $Na_2S_2O_3$ (10 g $L^{-1}$). Filtered (0.22 *µ*m) aeration was provided during the reaction. The decapsulated cysts were washed with filtered (passed through 0.22-*µ*m membrane filter) and autoclaved (moist heat at 121 °C for 15 min) artificial seawater (containing 35 g l-1 of instant ocean synthetic sea salt, Aquarium Systems, Sarrebourg, France). The cysts were resuspended in a 50-ml tube containing 30 ml of filtered, autoclaved seawater and hatched for 28 h on a rotor (4 $min^{-1}$) at 28 °C with constant illumination (c. 2000 lux). The axenity of cysts was verified by inoculating one ml of culture water into 9 ml of marine broth and incubating at 28 °C for 24 h. After 28 h of hatching, batches of 30 larvae were counted and transferred to fresh, sterile 50-ml tubes containing 30 ml of filtered and autoclaved seawater. Finally, the tubes were returned to the rotor and kept at 28 °C. All manipulations were performed in a laminar flow to maintain sterility of the cysts and larvae.

### 4.4.6. Brine shrimp challenge tests

The impacts of the thiophenones on the virulence of *V. harveyi* were determined in a standardized challenge test with gnotobiotic brine shrimp larvae as described by

Defoirdt *et al*. (2005) with some modifications. A suspension of autoclaved LVS3 bacteria (Verschuere *et al*., 1999) in filtered and autoclaved seawater was added as feed at the start of the challenge test at $10^7$ cells $ml^{-1}$, and *V. harveyi* was added at $10^5$ CFU $ml^{-1}$. The thiophenones were added directly into the brine shrimp rearing water at different concentrations. Brine shrimp cultures, to which only autoclaved LVS3 bacteria were added as feed, were used as controls. The survival of the larvae was counted 48 h after the addition of the pathogens. Each treatment was carried out in triplicate and each experiment was repeated twice to verify the reproducibility. In each test, the sterility of the control treatments were checked at the end of the challenge by inoculating 1 ml of rearing water to 9 ml of marine broth and incubating the mixture for 2 days at 28 °C.

### 4.4.7. Statistics

Data analysis was carried out using the SPSS statistical software (version 21). Unless stated otherwise, all data were analysed using independent samples *t*- tests.

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# CHAPTER V

## INDOLE AND INDOLE ANALOGUES PRODUCED BY MICRO-ALGAE DECREASE THE VIRULENCE OF LUMINESCENT VIBRIOS, MAJOR PATHOGENS OF AQUATIC ORGANISMS

## ABSTRACT

Bacterial production of indole has been known for a long time. To date, around 85 species of both Gram-negative and Gram-positive bacteria have been found to produce large quantities of indole. However, diverse biological responses to indole have only recently been revealed. Most work in this respect has been conducted on *Escherichia coli*, in which indole has been reported to control several virulence-related phenotypes. To our knowledge, the role of indole in vibrios only has been documented in *V. cholerae* and very recently in *V. anguillarum*. In the present study, we investigated the impact of indole signaling on the virulence of another important pathogen *V. harveyi*, and whether indole analogues produced by micro-algae induce a similar response as indole. Results showed that indole could decrease the virulence of *V. harveyi* towards brine shrimp larvae and giant river prawn larvae. The addition of exogenous indole was found to down-regulate the production of several virulence factors, including biofilm formation, exopolysaccharide production and swimming motility. *V. harveyi* could produce indole during its growth, and the production was significantly affected by the alternative sigma factor RpoS. Finally, we found that two indole analogues, indole-3-acetic acid and indole-3-acetamide, which are produced by micro-algae, also showed a similar effect as indole except for the swimming motility. More interestingly, indole and both analogues could interfere the three-channel quorum sensing system in *V. harveyi* at different stages. Therefore, they might potentially be used for antivirulence therapies.

**Keywords:** indole, auxin, antivirulence therapy, greenwater, alga-bacteria interaction

## 5.1 INTRODUCTION

Vibrios belonging to the Harveyi clade (including *Vibrio harveyi*) are marine Gram-negative (often luminous) bacteria that can infect a wide range of wild and cultured aquatic organisms (both vertebrates and invertebrates), leading to significant losses in the aquaculture industry worldwide (Austin and Zhang, 2006; Darshanee Ruwandeepika *et al.*, 2012). Despite their role as major pathogens of marine animals, the pathogenicity mechanisms of these bacteria are currently not yet fully understood. To our knowledge, the factors that have been considered to be involved in the pathogenicity include biofilm formation, swimming motility and the production of a variety of extracellular products, including hemolysins, proteases, phospholipase and chitinases (Karunasagar *et al.*, 1994; Austin and Zhang, 2006; Darshanee Ruwandeepika *et al.*, 2012; Yang and Defoirdt, 2014).

Since virulence factors are often costly metabolic products, their expression usually is under strict regulatory control. One of the regulatory mechanisms controlling the production of virulence factors in *V. harveyi* is quorum sensing (QS), cell-to-cell communication that uses secreted signaling molecules named autoinducers (AI) (Miller and Bassler, 2003). *V. harveyi* strain ATCC BAA-1116 is a model bacterium in quorum sensing research, and the bacterium has been found to contain a three-channel quorum sensing system, which is mediated by three kinds of signal molecules, harveyi autoinducer 1 (HAI-1; Cao and Meighen, 1989), autoinducer 2 (AI-2; Chen *et al.*, 2002) and cholerae autoinducer 1 (CAI-1; Higgins *et al.*, 2007), respectively. Previous work in our lab has proven that the three-channel quorum sensing system is required for full virulence of *V. harveyi* towards different hosts (Defoirdt and Sorgeloos, 2012; Pande *et al.*, 2013).

In recent years, more and more compounds are being added to the arsenal of bacterial signaling molecules, and one of these compounds is indole (Lee and Lee, 2010). Indole is produced by tryptophanase (TnaA), which reversibly converts tryptophan into indole, pyruvate and ammonia (Lee *et al.*, 2007a; 2008). A variety of both Gram-positive and Gram-negative bacteria (more than 85 species including many pathogenic bacteria such as *V. vulnificus, Proteus vulgaris, Pasteurella*

*multocida and Haemophilus influenzae*) have been found to produce indole (Dalsgaard *et al.*, 1999; DeMoss and Moser, 1969; Clemons and Gadberry, 1982; Stull *et al.*, 1995). Diverse biological responses to indole have recently been revealed, including spore formation (Stamm *et al.*, 2005), drug resistance (Hirakawa *et al.*, 2005), virulence (Hirakawa *et al.*, 2009), plasmid stability (Chant and Summers, 2007), persister formation (Vega *et al.*, 2012), motility (Bansal *et al.*, 2007), lipopolysaccharide production (Han *et al.*, 2011) and biofilm formation (Beyhan *et al.*, 2009). Additionally, indole has been reported to interfere with AHL-based quorum sensing in a number of Gram-negative bacteria (Hidalgo-Romano *et al.*, 2014). Some bacteria that lack tryptophanase, and therefore do not produce indole, e.g. *Pseudomonas aeruginosa,* also respond to the presence of extracellular indole (Melander *et al.*, 2014). To date, a signaling role of indole in vibrios only has been documented in *V. cholerae* and very recently in *V. anguillarum*. In *V. cholerae*, indole activated *Vibrio* polysaccharide (VPS) production and biofilm formation, whereas it decreased motility (Beyhan *et al.*, 2009). In *V. anguillarum*, by contrast, exopolysaccharide production was decreased by indole, whereas motility was not affected (Li *et al.*, 2014). Further, in *V. anguillarum*, the stationary phase sigma factor (RpoS) is involved in the production of indole as an *rpoS* deletion mutant showed elevated indole levels and increased expression of the indole synthase *tnaA* (Li *et al.*, 2014). Finally, the indole receptor has not yet been identified in any bacterium, although the transcriptional regulator SdiA has been reported to be central in the indole signalling cascade in *E. coli* (Lee *et al.*, 2007a) and the DnaK suppressor protein DksA has been hypothesised to be involved in the indole signaling cascade in *V. cholerae* (Beyhan *et al.*, 2009).

In addition to indole, several bacteria have also been reported to respond to natural indole analogues (Melander *et al.*, 2014). Most notably from an ecological perspective are the auxin plant hormones, such as indole-3-acetic acid and indole-3-acetamide. These compounds are also produced by micro-algae (Stirk *et al.*, 2013), which share the aquatic environment with vibrios. Remarkably, although auxins have been reported to affect various phenotypes in a number of bacteria (including *E.coli* and *P. aeruginosa*; Spaepen and Vanderleyden, 2011), their impact on gene expression in vibrios has thus far not been investigated. In addition to the ecological significance,

unraveling micro-alga-bacteria interactions is also important from a practical point of view. Indeed, micro-algae are an important constituent of many aquaculture systems, especially the so-called greenwater systems, in which the animals are cultured in water containing high levels of micro-algae (Natrah *et al.*, 2014). Empirical evidence has suggested that when compared to conventional systems, there is a lower incidence of disease in greenwater systems (De Schryver *et al.*, 2014). However, the mechanisms behind this apparent beneficial practice are not yet understood.

Given the ecological and economical importance of *V. harveyi* as a major pathogen of aquatic organisms, the previous reports documenting the impact of indole on vibrios, and the potential impact of micro-algae on this, in the present study, we aimed at determining the impact of indole signaling on the virulence of *V. harveyi,* and at investigating whether indole analogues produced by micro-algae induce a similar response as indole.

## 5.2 RESULTS

### 5.2.1 Impact of indole on the virulence of *V. harveyi* towards gnotobiotic brine shrimp larvae and conventionally reared giant river prawn larvae

To examine whether the addition of indole could decrease the virulence of wild type *V. harveyi,* we performed a standardized challenge test with gnotobiotic brine shrimp larvae. In order to exclude any direct effects of indole on the larvae, *V. harveyi* was incubated in the presence of indole at 50 µM, 100µM and 200µM, respectively, after which the cultures were washed to remove the indole prior to inoculation into the brine shrimp rearing water. A significantly increased survival of brine shrimp larvae challenged to indole-pretreated *V. harveyi* was observed, indicating that indole indeed decreases the virulence of *V. harveyi* (**Table 5.1**). Importantly, indole at these concentrations showed no effect on the growth of *V. harveyi* (**Figure 5.1**). Moreover, all treatments received the same inoculum ($10^5$ cells per ml brine shrimp rearing water), and there were no differences between treatments with respect to the density of *V. harveyi* in the brine shrimp rearing water after 1 and 2 days of challenge (**Figure 5.2**).

**Table 5.1**. Survival of gnotobiotic brine shrimp larvae after 2 days of challenge with *V. harveyi* ATCC BAA-1116, either untreated or pretreated with indole or indole analogues prior to inoculation into the brine shrimp rearing water (average ± standard deviation of 3 shrimp cultures).

| **Treatment** | **Survival (%)[a]** |
|---|---|
| Control | 100 ± 0$^{e}$ |
| *V. harveyi* | 53 ± 6$^{a}$ |
| *V. harveyi* [50 $\mu$M Indole]$_{pretreatment}$ | 72 ± 8$^{b}$ |
| *V. harveyi* [100 $\mu$M Indole]$_{pretreatment}$ | 90 ± 5$^{d}$ |
| *V. harveyi* [200 $\mu$M Indole]$_{pretreatment}$ | 93 ± 3$^{d}$ |
| *V. harveyi* [50 $\mu$M Indole-3-acetic acid]$_{pretreatment}$ | 73 ± 8$^{b}$ |
| *V. harveyi* [100 $\mu$M Indole-3-acetic acid]$_{pretreatment}$ | 77 ± 6$^{b}$ |
| *V. harveyi* [200 $\mu$M Indole-3-acetic acid]$_{pretreatment}$ | 85 ± 5$^{c}$ |
| *V. harveyi* [50 $\mu$M Indole-3-acetamide]$_{pretreatment}$ | 72 ± 10$^{b}$ |
| *V. harveyi* [100 $\mu$M Indole-3-acetamide]$_{pretreatment}$ | 83 ± 3$^{c}$ |
| *V. harveyi* [200 $\mu$M Indole-3-acetamide]$_{pretreatment}$ | 87 ± 3$^{c}$ |

[a] Values with a different superscript letter are significantly different from each other (One way ANOVA with Tukey's *post-hoc* test; $P < 0.01$).

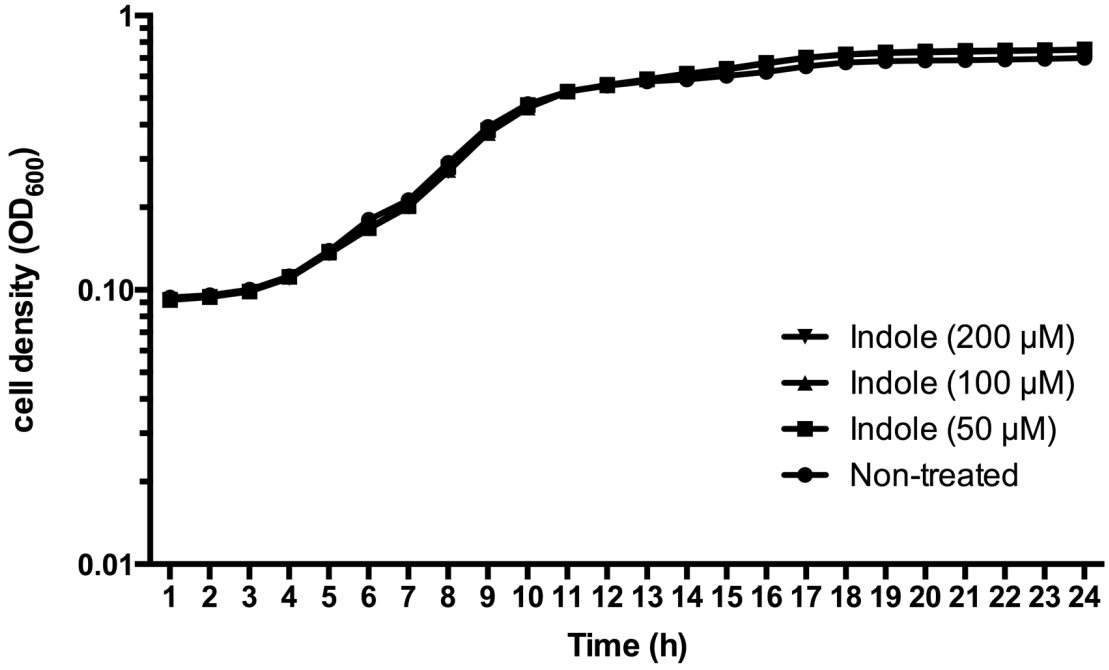


**Figure 5.1.** Growth of wild type *V. harveyi* in $LB_{35}$ medium, with and without indole.

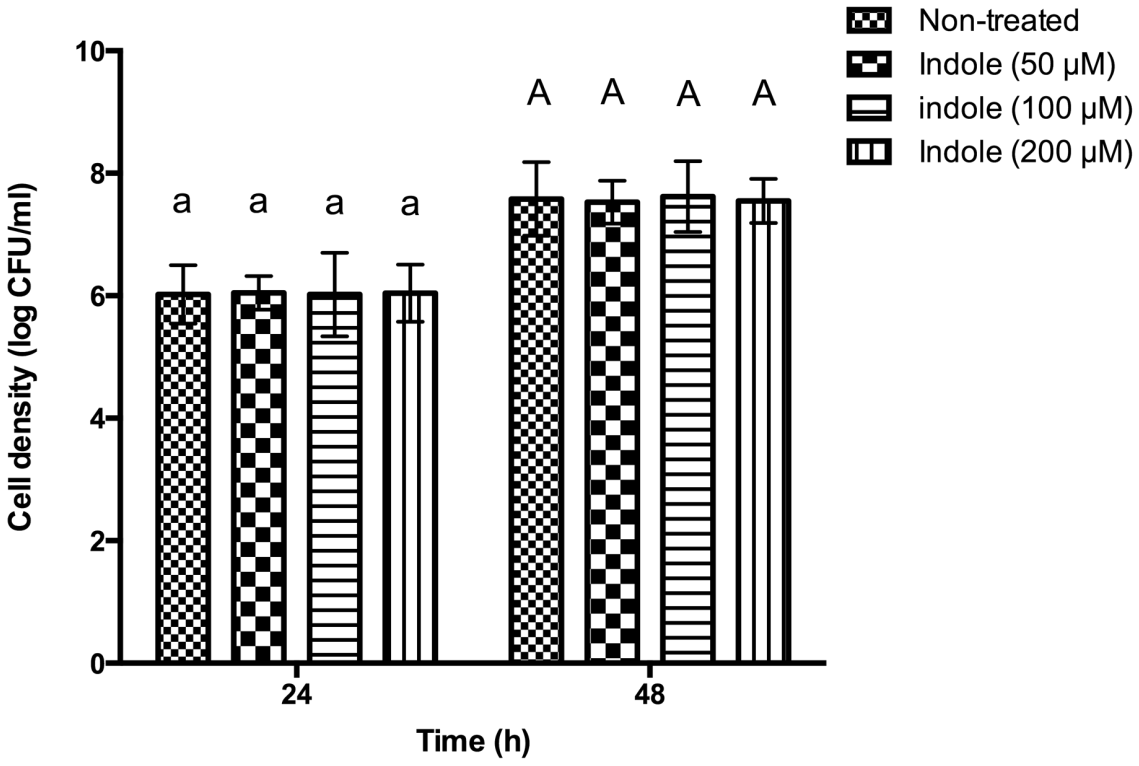


**Figure 5.2.** Density of wild type *V. harveyi,* either or not pretreated with indole prior to inoculation, in the brine shrimp rearing water after 24h and 48h of challenge.

Based on these promising results, we went further to investigate the effect of indole in giant river prawn (*Macrobrachium rosenbergii*) larvae. According to our results, the survival of giant river prawn larvae was significantly increased after 6 days of challenge with indole-pretreated (100 µM) *V. harveyi* **(Table 5.2)**.

**Table 5.2.** Survival of gnotobiotic conventionally reared giant river prawn larvae after 6 days of challenge with *V. harveyi* ATCC BAA-1116, either untreated or pretreated with indole prior to inoculation into the giant river prawn rearing water (average ± standard deviation of 3 shrimp cultures). The survival in unchallenged giant river prawn larvae was set at 100% and the survival in other treatments was normalized accordingly. This experiment was performed by G.S.J. Pande.

| **Treatments** | **Survival (%)[a]** | **LSI[b]** |
|---|---|---|
| Prawn larvae only (control) | $100 \pm 8^{c}$ | $4,4 \pm 0,5^{A}$ |
| *V. harveyi* | $51 \pm 10^{a}$ | $4,4 \pm 0,5^{A}$ |
| *V. harveyi* [100 $\mu$M Indole]$_{pretreatment}$ | $70 \pm 6^{b}$ | $4,4 \pm 0,5^{A}$ |

[a] Values with a different superscript letter are significantly different from each other (One way ANOVA with Tukey's *post-hoc* test; $P < 0.01$).
[b] LSI: The growth parameter larval stage index was determined on 5 randomly sampled larvae from each cone

### 5.2.2 Impact of indole on biofilm formation and exopolysaccharide production by *V. harveyi*

In order to determine the mechanism by which indole affects the virulence of *V. harveyi*, we investigated the impact of indole on some important virulence factors. Biofilm formation was determined by crystal violet staining, and indole was found to significantly decrease biofilm formation (**Figure 5.3a**). Since exopolysaccharide production is one of the major factors affecting biofilm formation, we subsequently investigated the impact of indole on exopolysaccharide production, and found that the addition of indole also significantly decreased exopolysaccharide production (**Figure 5.3b**).

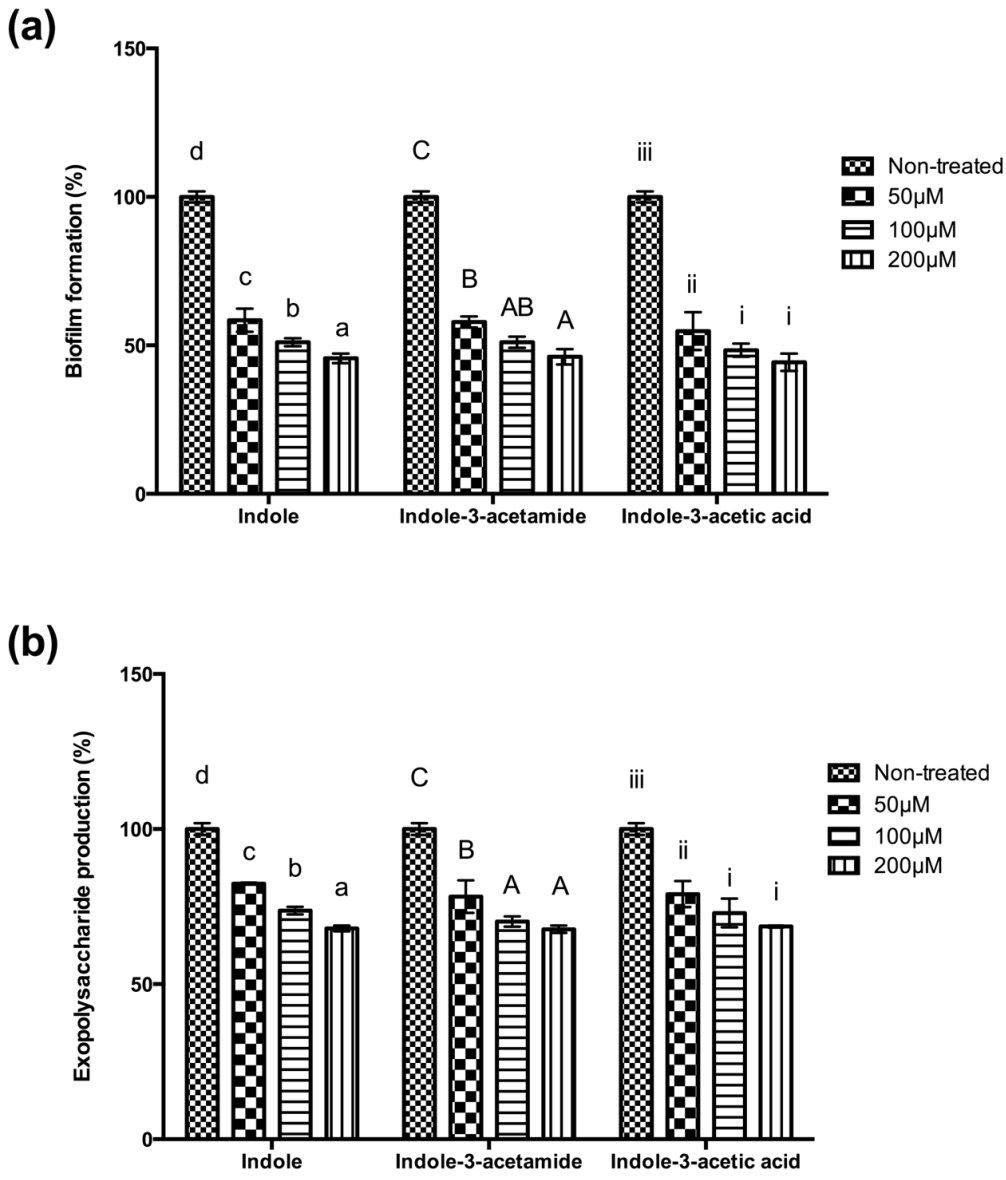


**Figure 5.3.** Impact of indole and indole analogues on (a) biofilm formation and (b) exopolysaccharide production of *V. harveyi.* Biofilm formation and exopolysaccharide production in the control treatment were set at 100% and the other treatments were normalised accordingly. The error bars represent standard deviation of three independent experiments. Different letters indicate significant differences (One way ANOVA with Tukey's *post hoc* test; $P < 0.01$).

The *Vibrio* polysaccharide (*vps*) genes are responsible for exopolysaccharide production and biofilm formation in vibrios. Therefore, to confirm the previous observations, we evaluated the impact of indole on the expression of the *vpsT* and *vpsR* genes by quantitative reverse transcriptase PCR. VpsR is the main activator of

*Vibrio* polysaccharide production in *V. cholerae*, and VpsT is a secondary activator that acts synergistically with VpsR (Yildiz *et al.*, 2001). The *V. harveyi vpsT* and *vpsR* genes show 73% and 70% nucleotide homology with the *V. cholerae* analogues, respectively. We found that the addition of indole resulted in 10-fold and 5-fold decrease in *vpsT* and *vpsR* expression, respectively, in the presence of 100 *μ*M exogenously added indole (**Table 5.3**).

**Table 5.3.** Relative expression of *Vibrio* polysaccharide production regulators, motility-related genes and the stationary phase sigma factors in *V. harveyi* after 24h of incubation in the absence and presence of 100 µM indole (average ± standard deviation of three cultures). For each gene, the expression in untreated *V. harveyi* was set at 1 and the expression in cultures supplemented with indole were normalized accordingly using the $2^{-\Delta\Delta CT}$ method. The RNA polymerase A subunit gene (*rpoA*) was used as an internal control.

| Gene | Function | Relative expression (fold)[a] | |
|---|---|---|---|
| | | **Non-treated** | **100 µM indole** |
| *Vibrio* polysaccharide (VPS) production regulators | | | |
| *vpsR*[L] | Main activator of VPS production | 1.0 ± 0.2 | 0.4 ± 0.2 *** |
| *vpsT*[L] | Secondary activator of VPS production | 1.0 ± 0.4 | 0.6 ± 0.1 *** |
| Motility genes | | | |
| *flaA*[SA] | Polar flagellin | 1.0 ± 0.3 | 0.6 ± 0.3 |
| *flaC*[SA] | Polar flagellin | 1.0 ± 0.2 | 0.5 ± 0.3 ** |
| *flaK*[SA] | Polar flagellar regulator | 1.0 ± 0.2 | 0.6 ± 0.2 |
| *fliA*[SA] | Polar flagellar biosynthesis sigma factor | 1.0 ± 0.2 | 1.0 ± 0.2 |
| *fliS*[SA] | Polar flagellin specific chaperone | 1.0 ± 0.3 | 0.9 ± 0.3 |
| *flgB*[SA] | Flagellar basal body rod | 1.0 ± 0.2 | 1.0 ± 0.3 |
| *cheA*[SA] | Chemotaxis protein | 1.0 ± 0.4 | 0.6 ± 0.4 |
| *cheR*[SA] | Chemotaxis protein | 1.0 ± 0.2 | 0.6 ± 0.2 |
| *lafA*[SA] | Lateral flagellar flagellin | 1.0 ± 0.2 | 0.4 ± 0.1 *** |
| *lafK*[SA] | Lateral flagellar regulator | 1.0 ± 0.2 | 0.4 ± 0.3 ** |
| Regulators | | | |
| *rpoS1*[L] | Stationary phase sigma factor copy 1 | 1.0 ± 0.1 | 2.5 ± 0.4 ** |
| *rpoS2*[L] | Stationary phase sigma factor copy 2 | 1.0 ± 0.3 | 2.3 ± 0.2 *** |
| *luxR*[L] | Three channel quorum sensing system master regulator | 1.0 ± 0.1 | 0.6 ± 0.1 ** |

[a]: Asterisks indicate a significant difference between untreated *V.harveyi* and *V.harveyi* in the presence of indole (an independent samples T- test; **: $P < 0.01$; ***: $P < 0.001$);
[L]: Cells were harvested from liquid cultures.
[SA]: Cells were harvested from soft agar (0.3% agar)

### 5.2.3 Impact of indole on the swimming motility of *V. harveyi*

Since motility has been reported to play a major role in the virulence of *V. harveyi*

(Yang and Defoirdt, 2014), we subsequently determined the effect of indole on the swimming motility of *V. harveyi* on soft agar. Indole was found to significantly decrease the swimming motility at all concentrations tested (**Figure 5.4**). We further investigated the effect of indole on the expression of ten selected genes involved in flagellar motility in *V. harveyi* (Yang and Defoirdt, 2014), and found that the addition of 100 μM indole had no significant impact on the genes involved in the synthesis of polar flagella, and chemotaxis, whereas the mRNA levels of genes involved in the synthesis of lateral flagella were significantly decreased in the presence of indole (**Table 5.3**). This result indicates that the inhibitory effect of indole on motility was predominantly due to a decreased expression of lateral flagella.

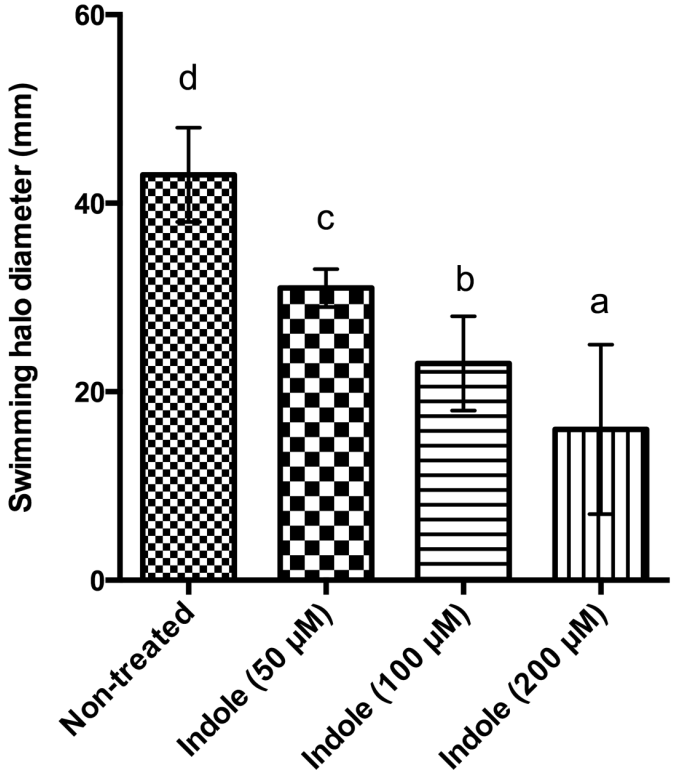


**Figure 5.4.** Impact of indole on swimming motility of *V. harveyi.* The error bars indicate the standard deviation of six replicate cultures. Different letters indicate significant differences (One way ANOVA with Tukey's *post hoc* test; $P < 0.01$).

### 5.2.4 Impact of RpoS on indole production in *V. harveyi*

We previously reported that the alternative sigma factor RpoS controls indole production in *V. anguillarum*, with an *rpoS* deletion mutant showing a significantly increased production when compared to the wild type (Li *et al.*, 2014). To determine

whether this regulation also exists in *V. harveyi*, we investigated the indole production in *V. harveyi* wild type and an *rpoS1/rpoS2* deletion mutant (the *V. harveyi* ATCC BAA-1116 genome contains 2 copies of *rpoS*). Consistent with what has been reported for *V. anguillarum,* indole production was also significantly higher in the *rpoS1/rpos2* mutant than in the wild type at 6h (exponential phase), 12h (early stationary phase) and 24h (stationary phase) of incubation (**Figure 5.5a**). Furthermore, the expression level of the indole synthase gene *tnaA* was significantly higher in the *rpoS1/rpoS2* mutant than in the wild type at all sampling points (3-fold at 6h; 4-fold at 12h and 3-fold at 24h; **Figure 5.5b**).

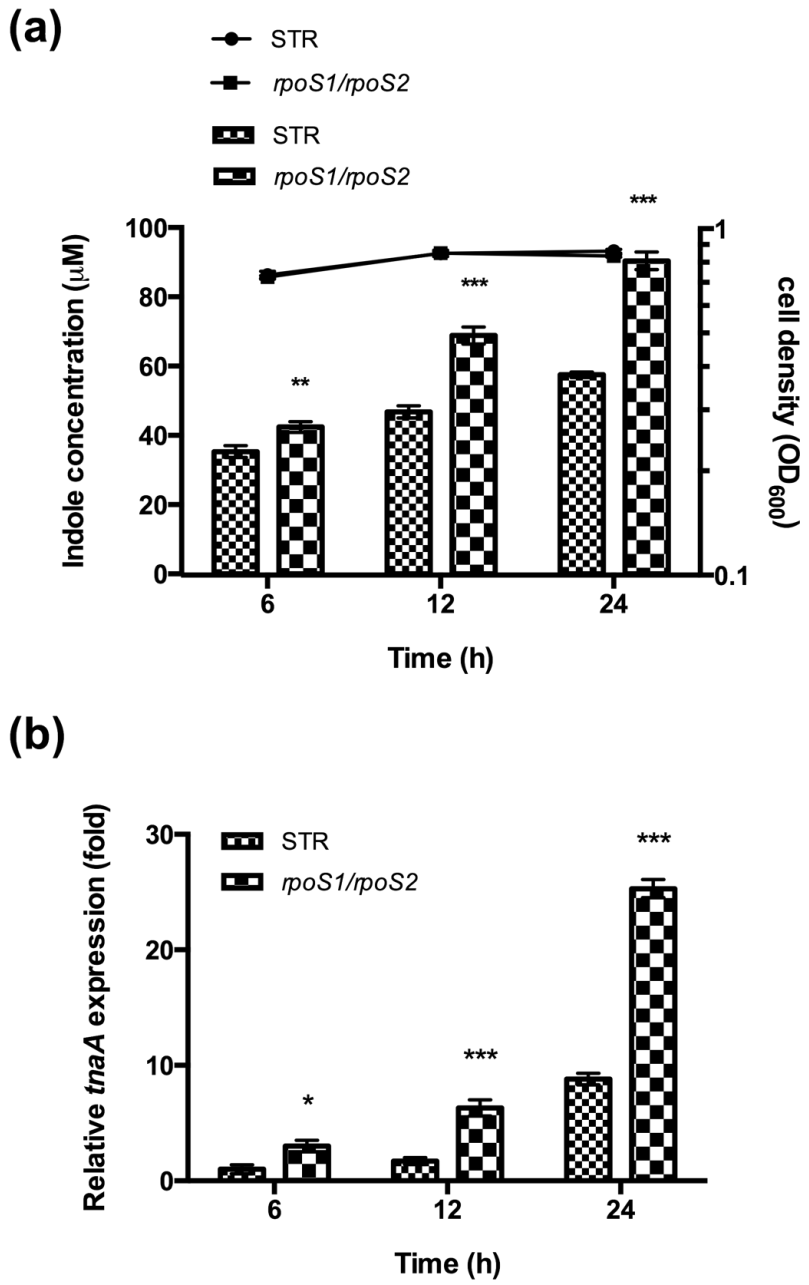


**Figure 5.5.** Indole production in *V. harveyi* spontaneous streptomycin resistant mutant (STR) and the *rpoS1/rpoS2* double deletion mutant derived from STR (*rpoS1/rpoS2*). **(a)** Indole production (bars) and cell density (lines) of *V. harveyi* STR and *rpoS1/rpoS2* during growth in $LB_{35}$ medium. **(b)** Relative expression of the indole biosynthesis gene *tnaA* in STR and *rpoS1/rpoS2*. The expression was calculated relative to the RNA polymerase A submit (*rpoA*) gene; expression in STR at 6h was set at 1 and the other data points were normalized accordingly using the $2^{-\Delta\Delta CT}$ method. For both panels, error bars represent the standard deviation of three *V. harveyi* cultures. Asterisks indicate a significant difference when compared to non-treated *V. harveyi* at the respective time point (independent samples T-test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

In addition to its impact on indole production in *V. anguillarum,* the stationary phase

sigma factor RpoS has also been reported before to be (partially) involved in the response to indole in *V. cholerae* (Mueller *et al*., 2009), and we previously found that *rpoS* mRNA levels in *V. anguillarum* are higher upon exposure to endogenous indole (Li and Defoirdt, unpublished). Consistent with this, we found that the expression levels of both *rpoS1* and *rpoS2* were also significantly higher in *V. harveyi* upon exposure to 100 *μ*M indole (**Table 5.3**).

### 5.2.5 Impact of indole on the 3-channel quorum sensing system of *V. harveyi*

Indole has been reported to interfere with other quorum sensing mechanisms in various bacteria (Kim and Park, 2013; Hidalgo-Romano *et al*., 2014), and therefore, we also evaluated whether indole interfered with the activity of the three-channel quorum sensing system of *V. harveyi.* We used bioluminescence as readout of quorum sensing activity since it is one of the phenotypes that are regulated by quorum sensing. Wild type *V. harveyi* was grown to high cell density in order to activate quorum sensing-controlled bioluminescence, after which indole was added at 50 μM, 100 μM and 200 μM, respectively, and bioluminescence was measured after 1h. Indole could indeed inhibit the quorum sensing-regulated bioluminescence in wild type *V. harveyi* (**Figure 5.6a**).

We further sought to confirm that the effect of indole on bioluminescence was due to interference with the quorum sensing system by determining the impact of indole on bioluminescence of *V. harveyi* JAF548 pAK*lux*1, in which bioluminescence is independent of the three channel quorum sensing system (Defoirdt *et al*., 2012). Indole had no effect on the bioluminescence of JAF548 pAK*lux*1 at all the tested concentrations, indicating that the inhibition of bioluminescence in wild type *V. harveyi* was caused by interference with the three-channel quorum sensing system (**Figure 5.6c**).

In order to determine at what stage in the quorum sensing signal transduction cascade indole was acting, we used mutants JAF483 (LuxO D47A) and BNL258 (*hfq::*Tn5*lacZ*). Mutant JAF483 contains a point mutation in *lux*O, that renders the LuxO protein locked in the conformation of wild type LuxO at high signal molecule concentrations (Freeman and Bassler, 1999). Strain BNL258 has a transposon

insertion in the *hfq* gene, resulting in a nonfunctional Hfq protein (Lenz *et al.*, 2004). These mutations result in a maximally active quorum sensing system and constitutive luminescence in JAF483 and BNL258, irrespective of cell density and signal molecule concentration. Blocking of luminescence in these mutants would indicate that indole interacts with a quorum sensing signal transduction component located downstream of the mutated one (Defoirdt *et al.*, 2007); a scheme of the *V. harveyi* three-channel quorum sensing system is presented in **Figure 5.7** for further information). Indole was also found to block luminescence in JAF483 and BNL258 at all the concentrations tested (**Figure 5.6 b & c**), which indicates that it interferes with signal transduction (by interacting with a signal transduction cascade component downstream of Hfq) and not by interacting with signal molecule detection (by interacting with one of the signal molecule receptors). Inversely, the three- channel quorum sensing system had no effect on the production of indole as there was no difference between the strains in indole levels (**Figure 5.8**).

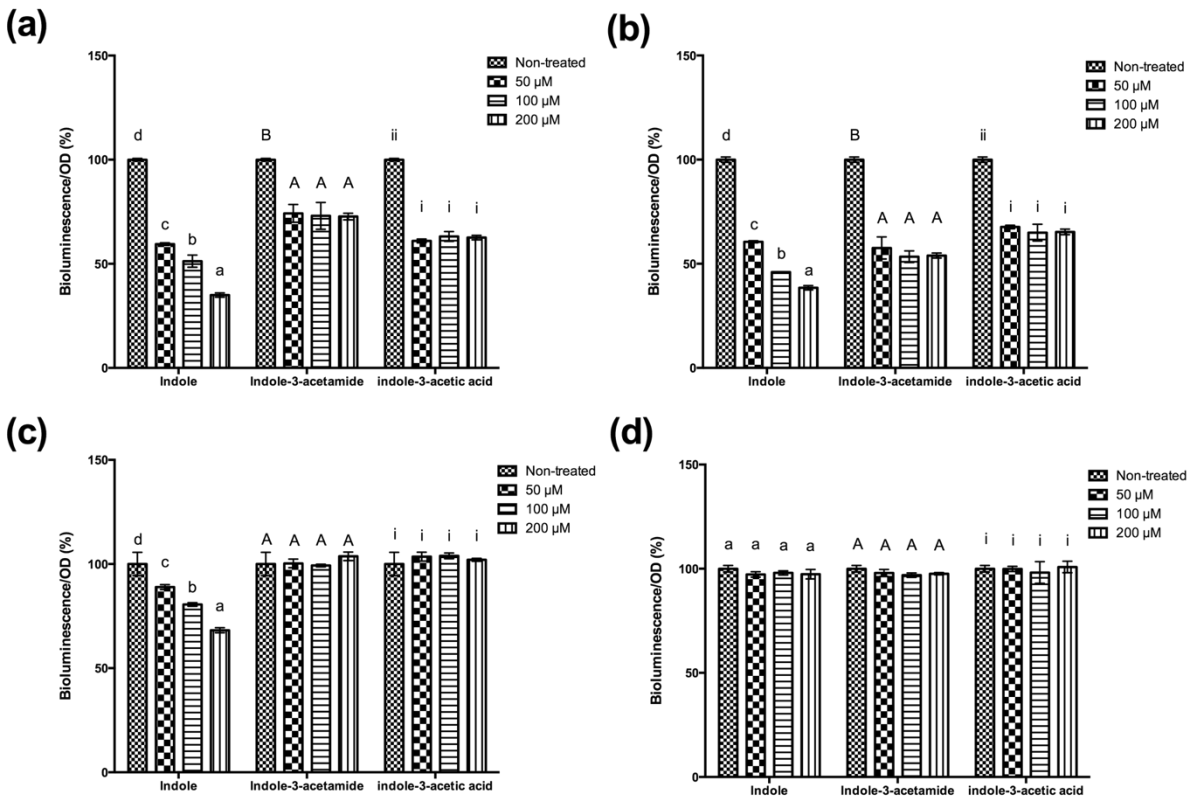


**Figure 5.6.** Impact of indole and indole analogues on bioluminescence of *V. harveyi* (a) wild type, (b) constitutively luminescent quorum sensing signal transduction mutants JAF483 (*luxO* D47A) and (c) BNL258 (*hfq* deletion) and (d) strain JAF548 pAK*lux*1, in which bioluminescence is independent of quorum sensing. Bioluminescence in the control treatment was set at 100% and the other treatments were normalized accordingly. The error bars represent the standard deviation of three replicates. Different letters indicate significant differences (One way ANOVA with Tukey's *post hoc* test; $P < 0.01$).

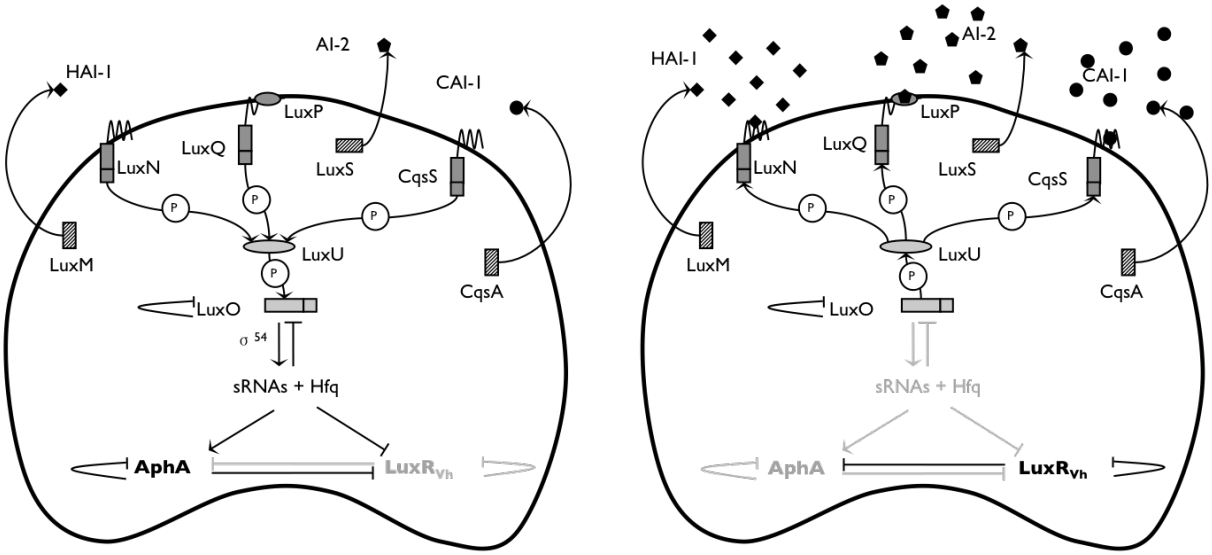


**Figure 5.7.** Quorum sensing in *Vibrio harveyi*. The LuxM, LuxS and CqsA enzymes synthesise the autoinducers HAI-1, AI-2 and CAI-1, respectively. These autoinducers are detected at the cell surface by the LuxN, LuxQ and CqsS two-component receptor proteins, respectively. Detection of AI-2 by LuxQ requires the periplasmic protein LuxP. In the absence of autoinducers (left), the receptors autophosphorylate and transfer phosphate to LuxO via LuxU. Phosphorylation activates LuxO, which together with $\sigma^{54}$ activates the production of five small regulatory RNAs (sRNAs). Hfq mediates interactions between sRNAs and specific messenger RNA (mRNA) targets. These interactions typically alter the stability of the mRNA encoding the quorum-sensing master regulators LuxR, implicating an sRNA in the circuit. The sRNAs promote translation of the master regulator AphA and inhibit translation of the master regulator $LuxR_{Vh}$. In the presence of high concentrations of the autoinducers (right), the receptor proteins switch from kinases to phosphatases, which results in dephosphorylation of LuxO. Dephosphorylated LuxO is inactive and therefore, the sRNAs are not formed, AphA is not translated and $LuxR_{Vh}$ is translated. AphA and LuxR are transcriptional regulators that (either individually or together) affect the transcription of many target genes.

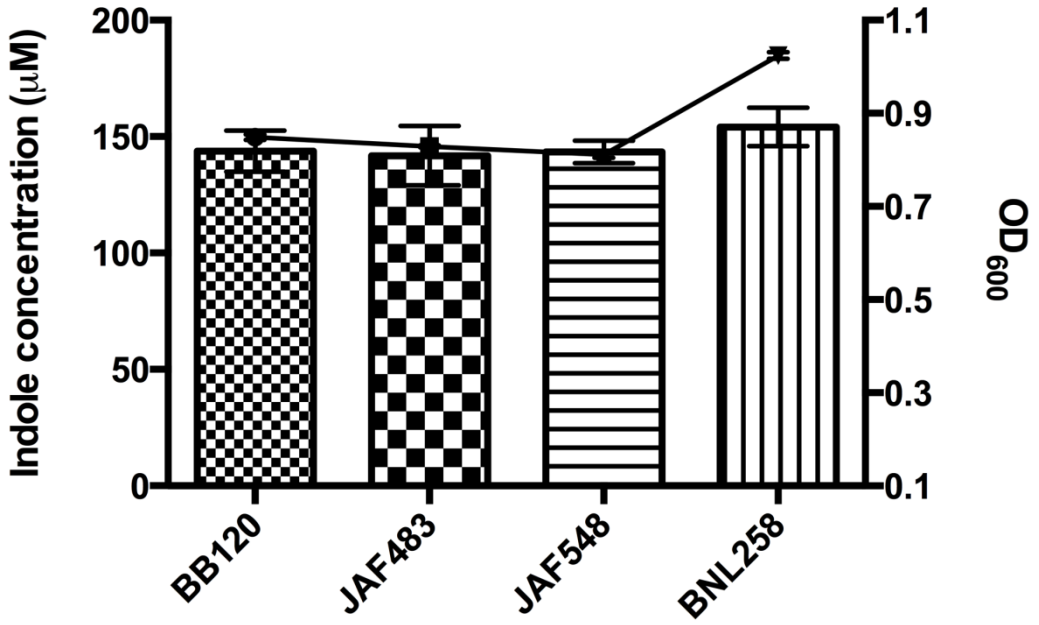


**Figure 5.8.** Indole production levels (bars) and cell density (lines) in *V. harveyi* wild type and different quorum sensing mutant strains at 24h. Error bars represent the standard deviation of three bacterial cultures.

### 5.2.6 Impact of indole analogues on virulence and virulence-related phenotypes

In a last series of experiments, we tested the impact of the indole analogues indole-3-acetic acid (IAA) and indole-3-acetamide (IAM), on the phenotypes that were affected by indole in *V. harveyi.* Both of the analogues showed a similar effect as indole, since they significantly increased the survival of brine shrimp larvae challenged with *V. harveyi* (**Table 5.1**), and decreased biofilm formation, exopolysaccharide production (**Figure 5.3**), and quorum sensing-regulated bioluminescence in wild type *V. harveyi* and mutant JAF483 (*luxO* D47A) (**Figure 5.6**). However, in contrast to what we observed for indole, neither indole-3-acetic acid nor indole-3-acetamide affected luminescence of mutant BNL258, indicating that they interact with a quorum sensing signal transduction component located downstream of LuxO, but not downstream of Hfq. Furthermore, in contract to indole, the auxins had no effect on swimming motility of *V. harveyi* (data not shown).

## 5.3 DISCUSSION

Recently, indole has been proposed as an intercellular signal molecule in bacteria, affecting various behaviors (Jin-Hyung Lee and Jintae Lee, 2010; Melander *et al.*, 2014). In this study, we demonstrated that indole controls the virulence of *V. harveyi* in our highly controlled model system with gnotobiotic brine shrimp larvae. Gnotobiotic conditions are important in this respect since many bacteria are capable of producing high levels of indole (Lee and Lee, 2010) and therefore, the microbiota that is naturally associated with the host might confuse the experiments. We observed significantly decreased virulence of *V. harveyi* in this model system when the pathogen was pretreated with indole at 50 $\mu$M or more. This concentration is similar to the concentration produced by wild type *V. harveyi* when grown in Luria-Bertani broth (hence, doubling the concentration of extracellular indole results in decreased virulence). The pathogen was pretreated with indole in order to avoid a direct effect of indole on the host, which would confuse interpretation of the results. Indeed, indole has been reported to exert a beneficial effect on higher organisms, e.g. by increasing epithelial-cell tight-junction resistance (Bansal *et al.*, 2010). The results obtained in this study are consistent with our previous observation that indole protects sea bass (*Dicentrarchus labrax*) larvae from *V. anguillarum* (Li *et al.*, 2014). Hence, indole-decreasing virulence seems to be conserved among distantly related vibrios that are pathogenic to aquatic organisms.

In order to determine the mechanism(s) by which indole reduces the virulence of *V. harveyi,* we evaluated the impact of indole on several important virulence factors that were affected by indole in other vibrios (Beyhan *et al.*, 2009; Li *et al.*, 2014). Indole decreased biofilm formation, exopolysaccharide production and motility of *V. harveyi*, and these effects were confirmed at the transcriptional level by reverse transcriptase qPCR targeting key genes involved in these phenotypes. Importantly, no effect was observed in the controls which received the same volume of DMSO as added to the other treatments. The effect on biofilm formation and exopolysaccharide production is consistent with what we observed before in *V. anguillarum* (Li *et al.*, 2014) and with what has been reported for *E. coli* (Jintae Lee, Jayaraman and Wood, 2007b; Bansal *et al.*, 2007). However, these results are in contrast with what has been reported for *V.*

*cholerae*, where deletion of the indole synthase gene *tnaA* resulted in decreased biofilm formation, and which was restored upon addition of 350 μM indole (Beyhan *et al.*, 2009). The observation that indole decreased the motility of *V. harveyi* and the expression of genes involved in bacterial flagella biosynthesis and chemotaxis, is consistent with what has been reported for *V. cholerae* (Beyhan *et al.*, 2009), *E. coli* (Lee *et al.,* 2007a), *Salmonella enterica* serovar Typhimurium (Nikaido *et al.*, 2012) and *P. aeruginosa* (Lee *et al.*, 2009). In contrast, indole had no impact on motility of *V. anguillarum* in our previous study (Li *et al.*, 2014). The decrease in flagellar motility could, together with decreased exopolysaccharide production, explain the decrease in biofilm formation induced by indole. Indeed, both phenotypes play a key role in biofilm formation in bacteria (Petrova and Sauer, 2012).

We further determined the connection of indole signaling with other key regulatory mechanisms in *V. harveyi*. The alternative sigma factor RpoS has previously been reported to regulate the production of indole in *E. coli* (Lelong *et al.*, 2007) and *V. anguillarum* (Li *et al.*, 2014). In agreement with what has been demonstrated in *V. anguillarum*, the *V. harveyi rpo*S1/*rpo*S2 double mutant produced higher indole levels than the wild type (approximately double the amount, note that this is similar to the extracellular indole levels of the wild type when adding 50 μM exogenous indole), and this was reflected in a higher expression of the indole synthase *tna*A in the *rpo*S1/*rpo*S2 mutant. However, these results are opposite to what has been revealed in *E. coli*, in which RpoS enhanced indole production (Lelong *et al.*, 2007). We also found that there is a feedback loop as addition of exogenous indole resulted in increased expression of the two *rpoS* copies in wild type *V. harveyi*. This is consistent with what we observed in *V. anguillarum* (Xuan Li and Tom Defoirdt, unpublished). We hypothesise that indole induces a stress response (by inducing expression of RpoS, which is known to be related to stress), and that down-regulation of indole production by RpoS might be a mechanism to maintain homeostasis. Furthermore, we found that indole could interfere with the three-channel quorum sensing system of *V. harveyi* by interacting with signal transduction. We previously found that motility is controlled by this three-channel quorum sensing system (Yang and Defoirdt, 2014). Hence, the decreased motility in the presence of exogenous indole might be explained by the inhibition of quorum sensing signal transduction. Inhibition of quorum sensing signal

transduction by indole will also contribute to the decreased virulence towards brine shrimp larvae since quorum sensing is required for full virulence of *V. harveyi* in this model system (Defoirdt and Sorgeloos, 2012). Inhibition of quorum sensing by indole apparently is widespread in Gram-negative bacteria, including as *Chromobacterium violaceum, Pseudomonas chlororaphis, Serratia marcescens, Pseudomonas aeruginosa* and *Acinetobacter oleivorans* (Chu *et al.*, 2012; Hidalgo-Romano *et al.*, 2014; Attila *et al.*, 2009; Kim and Park, 2013). Finally, the transcriptional regulator SdiA has been reported to be central in the indole signalling cascade in *E.coli* (Lee *et al.*, 2009), and the DnaK suppressor protein DksA has been hypothesised to be involved in the indole signaling cascade in *V. cholerae* (Beyhan *et al.*, 2009). The *V. harveyi* ATCC BAA-1116 genome does not contain a homologue of *sdiA*, but it does contain a *dksA* homologue (82% identity at nucleotide level with *V. cholerae dksA*). Unfortunately, we were not able to obtain a *dksA* deletion mutant (which would have enabled us to determine whether DksA is involved in indole sensing), and this might indicate that this gene is essential in *V. harveyi*.

*V. harveyi* shares the aquatic environment with micro-algae, which like terrestrial plants are known to produce various indole analogues as auxin hormones (Stirk *et al.*, 2013). Auxins have been reported to be produced by several unicellular and multicellular algal species, e.g. brown (*Macrocystis, Laminaria, Fucus, Ascophyllum*), red (*Botryocladia, Prionitis* and *Porphyra*), and green (*Enteromorpha*, *Chlorella*, *Cladophora* and *Caulerpa*) algae and also in cyanobacteria (*Oscillatoria* and *Chlorogloea*) (Tarakhovskaya *et al.,* 2007). The auxin concentrations measured in most of the studies are lower when compared to those in land plants, e.g. the level of free indole-3-acetic acid was 2.5 ng/g fresh weight in the red algae *Prionitis lanceolata,* while 20 ng/g fresh weight in angiosperms (Ashen *et al.*, 1999; Basu *et al.*, 2002; Han, 2006; Stirk *et al.*, 2004; 2002). Auxins can affect various phenotypes in terrestrial bacteria (Spaepen and Vanderleyden, 2011). However, despite the potential ecological significance as cross-kingdom signals, the impact of auxins produced by micro-algae on aquatic bacteria has thus far not been investigated. We found that the auxins indole-3-acetic acid and indole-3-acetamide showed similar effects as indole, except for motility (which was not affected by the auxins). Indeed, pretreatment of *V. harveyi* resulted in a similar decrease in mortality of challenged brine shrimp larvae,

and biofilm formation and exopolysaccharide production were also decreased to the same extent as was observed for indole at the same concentrations. The auxins also inhibited quorum sensing signal transduction, although the effect was slightly less pronounced than for indole and although they interact with a different three-channel quorum sensing signal transduction component. Given the fact that quorum sensing controls swimming motility, this might (in part) explain the fact that motility was not affected by the auxins.

In addition to the ecological significance of cross-communication between aquatic bacteria and micro-algae, these observations also offer an explanation for the beneficial effect that is associated with micro-algae in aquaculture. Indeed, micro-algae are an important constituent of the so-called greenwater aquaculture systems, in which the animals are cultured in water containing high levels of micro-algae (Natrah *et al.*, 2014). Empirical evidence has indicated that there is a lower incidence of disease in this kind of rearing systems (De Schryver *et al.*, 2014), which might be explained by the presence of (relatively) high levels of indole analogues in the digestive tract of animals that have ingested high levels of micro-algae. Consistent with this are several observations in our lab that feeding aquatic animals with micro-algae results in an improved survival upon challenge with a pathogen, even if the micro-algae had been autoclaved before adding them to the rearing water (e.g.) (Marques *et al.*, 2005; Natrah *et al.*, 2012). This effect was originally thought to be nutritional (Marques *et al.*, 2005). However, since many nutrients are heat-labile, this probably is not the major explanation of the beneficial effect. Given the fact that indoles are heat-stable (even resisting autoclavation), the auxins that are produced as plant hormones by micro-algae might be better candidates to explain the beneficial effect. We anticipate that the selection of micro-algae that are capable of producing high levels of auxins could be an interesting novel strategy to control bacterial disease in aquaculture. Indeed, not all algae show the same beneficial effect; there are even differences between strains belonging to the same species (Marques *et al.*, 2005), and there are also differences between micro-algae with respect to the produced levels of auxins (Stirk *et al.*, 2013).

In conclusion, our results indicate that *V. harveyi* produce large quantity of indole

during its growth, and the alternative sigma factor RpoS has significant impact on the production of indole. The indole level and the expression of indole biosynthesis gene *tna*A are found to be significantly increased in *rpo*S1/*rpo*S2, when comparing with the STR strain. Further, indole signaling can reduce the virulence of *V. harveyi* towards brine shrimp larvae and giant river prawn larvae. The addition of endogenous indole decreases the production of several virulence factors, including biofilm formation, exopolysaccharide production and swimming motility. Indole is also considered to be a potential quorum sensing inhibitor to *V. harveyi.* Additionally, two indole analogues indole-3-acetamide and indole-3-acetic acid are revealed to have a similar effect as indole on the virulence of *V. harveyi.* This is the first report on indole signaling in *V. harveyi*, and much remains to be further investigated. Understanding indole signaling will help to develop effective antivirulence strategies and biotechnology applications.

## 5.4 MATERIALS AND METHODS

### 5.4.1 Indoles

Indole, Indole-3-acetic acid and indole-3-acetamide were used in this study (**Figure 5.9**). All of them were dissolved in dimethyl sulfoxide (DMSO) at 10 mM and filter-sterilized using a 0.2 µM filter. All the chemicals were purchased from Sigma-Aldrich (Bornem, Belgium).

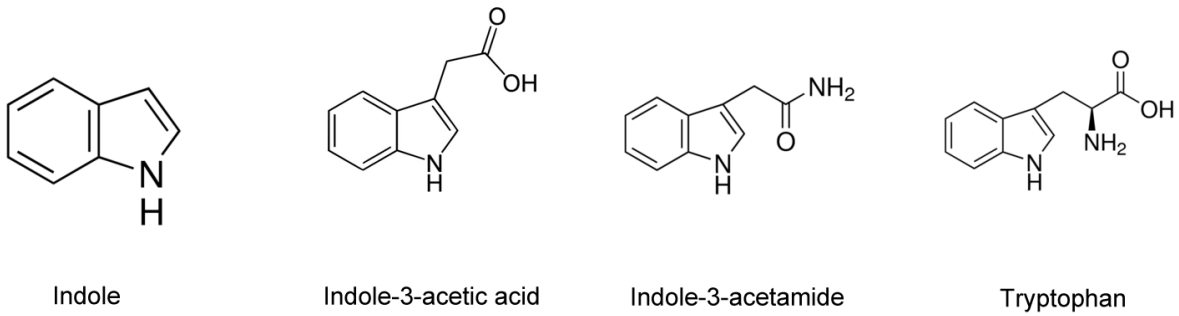


**Figure 5.9.** Structures of compounds used in this study.

### 5.4.2 Bacterial strains and growth conditions

Five *V. harveyi* strains were used in this study, all derived from wild type strain ATCC BAA-1116. Mutants JAF483 (*luxO* D47A) and mutant BNL258 (*hfq::*Tn5*lacZ*), in which the quorum sensing is maximally activated (Freeman and Bassler, 1999; Lenz *et al.*, 2004), and mutant JAF548 (*luxO* D47E) pAK*lux*1, in which bioluminescence is independent of quorum sensing (Defoirdt *et al.*, 2012) were used for bioluminescence experiments. Spontaneous streptomycin resistant mutant STR was derived from BB120 (Wang *et al.*, 2012), and the *rpoS1/rpoS2* double deletion mutant was derived from this strain (Wang *et al.*, 2012). Unless otherwise stated, all strains were cultured in Luria-Bertani medium containing 35g/L of sodium chloride ($LB_{35}$) at 28 °C under constant agitation (100 $min^{-1}$). Cell densities were measured spectrophotometrically at 600 nm.

### 5.4.3 Bacterial growth assays

For the bacterial growth assays, *V. harveyi* was grown overnight in $LB_{35}$ broth at 28°C. After that, the culture was re-inoculated at a concentration of $10^2$ CFU/ml into fresh $LB_{35}$ broth, with and without indole at 50, 100 and 200 µM, respectively. The cultures were grown in 200 µl volumes in 96-well plates at 28°C for 48 h, and the turbidity at 600 nm was monitored every hour using a Multireader machine (Infinite M200, TECAN, Austria). Growth curves were determined for three independent cultures.

### 5.4.4 Quantification of indole

*V. harveyi* cultures grown in $LB_{35}$ medium were harvested at different time points and centrifuged at 8000 x g for 5 min. The concentration of indole in the supernatants was measured as described previously (Li *et al.*, 2014) by mixing 500 µl of supernatant with 500 µl of Kovac's reagent. After vortexing, the top 200 µl were removed and the $OD_{571}$ was measured. The indole concentration in each sample was determined based on a standard curve using synthetic indole (Sigma-Aldrich). At least three different *V. harveyi* cultures were sampled for each strain at each time point.

### 5.4.5 Swimming motility assay

The swimming motility assay was performed on soft agar ($LB_{35}$ plates containing 0.2% agar) as described previously (Yang and Defoirdt, 2014). Indole was added to the autoclaved agar. *V. harveyi* was grown overnight in $LB_{35}$ broth, and 5 *µ*l aliquots ($OD_{600}$ = 1.0) were spotted in the center of the soft agar plates. Plates were incubated for 24 h, after which the diameters of the motility halos were measured. All assays were done with freshly prepared media in 6 replicates.

### 5.4.6 Biofilm formation assay

Biofilm formation was quantified by crystal violet staining, as described previously (Stepanović *et al.*, 2007). In brief, an overnight culture of *V. harveyi* was diluted to an $OD_{600}$ of 1.0 in $LB_{35}$ broth with or without indole and indole analogues, and 200 *µ*l aliquots of these suspensions were pipetted into the wells of a 96 well plate. Then the bacteria were allowed to adhere and grow without agitation for 24h at 28°C. After that, the cultures were removed and the wells were washed three times with 300 µl sterile physiological saline to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 150 µl of 99% methanol per well for 20 min, after which the methanol was removed and plates were air-dried. Then, biofilms were stained for 15 min with 150 µl per well of a 1% crystal violet solution (Pro-lab Diagnostics, Richmond Hill, ON, Canada). Excess stain was rinsed off by placing the plate under running tap water, and washing was continued until the washings were free of the stain. After the plates were air dried, the dye bound to the adherent cells was resolubilized with 150 µl of 95% ethanol per well, and absorbance was measured at 570 nm. Sterile medium served as negative control. For the quantification of exopolysaccharides, Calcofluor white staining (Sigma-Aldrich) was used. In brief, wells were rinsed after 24 h biofilm formation and 100 µl phosphate buffered saline containing 0.5 µl 5 mM Calcofluor white staining dye was added to the wells. After 60 min, fluorescence (excitation 405 nm and emission 500 nm) was measured with a Multi-reader (Infinite M200, TECAN, Austria).

### 5.4.7 Bioluminescence assays

*V. harveyi* strains were grown overnight and diluted to an $OD_{600}$ of 0.1. Indole and analogues were added at 50, 100 and 200 µM, respectively. The cultures were further incubated at 28 °C with shaking, and bioluminescence was measured after 1h with a Tecan Infinite 200 microplate reader (Tecan, Mechelen, Belgium).

### 5.4.8 RNA extraction

*V. harveyi* strains were grown in triplicate in $LB_{35}$ broth with or without 100 µM indole at 28°C, and cells were harvested after 24h (unless otherwise indicated). The samples for flagellar gene expression tests were harvested from soft (0.2 % agar) or hard agar plates (1.5% agar) after 24h incubation at 28°C. Then RNA was extracted with the SV Total RNA Isolation System (Promega, Leiden, The Netherlands) according to the manufacturer's instructions. The RNA quantity was measured spectrophotometrically (NanoDrop Technologies, Wilmington, DE, USA) and adjusted to 200 ng $\mu l^{-1}$ in all samples. The RNA integrity was checked by Agarose Gel Electrophoresis and the RNA samples were stored in -80°C.

### 5.4.9 Primers

The primers used for RT-qPCR analysis are listed in **Table 5.4.** Specific primers were designed using the software PRIMER PREMIER version 5.00 (Premier Biosoft International, Palo Alto, CA USA), with predicted product sizes in the 100 to 200 bp range. The RNA polymerase A submit (*rpoA*) mRNA was used as an endogenous control (Defoirdt *et al.*, 2007).

**Table 5.4.** Specific primers used for reverse transcriptase qPCR.

| Gene | Primer sequences (5'-3') | Product size (bp) | Reference |
|---|---|---|---|
| *flaA* | F: CTGCGGGTCTTCAAATCTC<br>R: GTTAGTGGTCTCGTTCATTGC | 205 | VIBHAR_03173[a] |
| *flaC* | F: GCTTGATGTCGCCTTGAGAA<br>R: GCTGCCATTTGCTGCTTG | 230 | VIBHAR_01302[a] |
| *flaK* | F: ATTGCCCGTTGATGATTTG<br>R: CTTCTGTGCCCGATACTTGT | 128 | VIBHAR_03166[a] |
| *fliA* | F: CGCCGAGTGTTCAGGTAGA<br>R: CCGATGGGTCACGATTTAGT | 130 | VIBHAR_03144[a] |
| *fliS* | F: CTCCGCACAAAGTCATTCAA<br>R: CAATGTCACCACCATCTTCC | 224 | VIBHAR_03167[a] |
| *flgB* | F: AAACACGCCTGGCTACAAA<br>R:ACGCTCTAAATCCAAATCTACC | 202 | VIBHAR_01286[a] |
| *cheA* | F: AGCCTGTGATTCCTGAGCC<br>R: AGTGATGTCGCCGCTGTC | 243 | VIBHAR_03141[a] |
| *cheR* | F: ATGCGATGACGACTAACGA<br>R: ACGCTTGGCAATAAACCTG | 174 | VIBHAR_01283[a] |
| *lafA* | F: TAACTTCGCATCGCTTGTAAC<br>R: TCGTCTGCTGCTGAGTTGATA | 210 | VIBHAR_04961[a] |
| *lafK* | F: GAGCCAAATGAACACCTCG<br>R: AACAATCGCAATCACCACA | 111 | VIBHAR_04971[a] |
| *tnaA* | F: GGTATGGCAGGTCGTGAT<br>R: GGCTAATGCGGTAGTGAA | 85 | VIBHAR_06709[a] |
| *vpsT* | F: TTACGGCTAAACCACATA<br>R: CGATTACAACGGAAGAGT | 127 | VIBHAR_05152[a] |
| *vpsR* | F: GCAGTTCTGATGTCTGATAGCG<br>R: CTCCAACACCACCAGCAATG | 132 | VIBHAR_00961[a] |
| *rpoS1* | F: CGCTTTACTTAGTGCCGATGA<br>R: AGAAATGTGAGACCACGCCC | 147 | VIBHAR_03010[a] |
| *rpoS2* | F: TTGGTCTGCTTGGCTATGAG<br>R: CACCTGGATCTGACGAACAC | 89 | VIBHAR_03517[a] |
| *rpoA* | F: CGTAGCTGAAGGCAAAGATGA<br>R: AAGCTGGAACATAACCACGA | 197 | Defoirdt et al. (2007) |

[a]: Locus tag in the *V. harveyi* ATCC BAA-1116 genome sequence (GenBank).

### 5.4.10 Reverse transcription

Reverse transcription was performed with the RevertAid$^{TM}$ H minus First strand cDNA synthesis kit (Fermentas Gmbh, Baden-Württemberg, Germany) in accordance to the manufacturer's instructions. Briefly, a mixture of 1 $\mu$g RNA and 1 $\mu$l random hexamer primer solution was mixed first. Then, 8 $\mu$l of reaction mixture containing 4 $\mu$l of 5× reaction buffer (0.25 $mol^{-1}$ Tris–HCl pH 8.3, 0.25 $mol^{-1}$ KCl, 0.02 $mol^{-1}$ $MgCl_2$, 0.05 $mol^{-1}$ DTT), 2 $\mu$l of 0.01 $mol^{-1}$ dNTP mix, 20 units of ribonuclease inhibitor, 200 units of RevertAid$^{TM}$ H minus M-MuLV Reverse Transcriptase was added. The reaction mixture was incubated for 5 min at 25°C followed by 60 min at 42°C. The reaction was terminated by heating at 70°C for 5 min and then cooled to 4°C. cDNA samples were checked by PCR and stored at -20°C for further use.

### 5.4.11 Real-time PCR

Real-time PCR was used to quantify the expression level of all the genes and was performed with Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas, Fisher Scientific, Erembodegem, Belgium) as described previously (Yang and Defoirdt, 2014). The reaction was performed in an StepOne™ Real-Time PCR System thermal cycler (Applied Biosystems, Gent, Belgium) in a total volume of 25 $\mu$l, containing 12.5 $\mu$l of 2× SYBR green master mix, 300 nM of forward and reverse primers and 2 $\mu$l of template cDNA. The thermal cycling consisted of an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and primer annealing and elongation at 60°C for 1 min. Dissociation curve analysis was performed to check for the amplification of untargeted fragments. Data acquisition was performed with the StepOne™ Software.

### 5.4.12 Real-time PCR data analysis ($2^{-\Delta\Delta CT}$ method)

The real-time PCR was validated by amplifying serial dilutions of cDNA synthesized from 1 $\mu$g of RNA isolated from bacterial samples. Serial dilutions of cDNA were amplified using gene specific primers. $\Delta C_T$ (average $C_T$ value of target-average $C_T$ value of *rpoA*) was calculated for the different dilutions and plotted against the cDNA concentration. The slope of the graph was almost equal to 0 for all of the target nine

genes. Therefore, the amplification efficiency of reference and the target genes was considered to be equal. Based on this precondition, real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The expression of the target genes was normalized to the endogenous control (*rpoA*) by calculating $\Delta C_T$ :

$$\Delta C_t = C_{t\ target} - C_{t\ rpoA}$$

and expressed relative to a calibrator strain by calculating $\Delta\Delta C_t$:

$$\Delta\Delta C_t = \Delta C_t - C_{t\ calibratior}$$

Unless otherwise indicated, untreated *V. harveyi* ATCC BAA-1116 was used as a calibrator, and all other treatments were normailsed accordingly. The relative expression was then calculated as

$$\text{Relative expression} = 2^{-\Delta\Delta Ct}$$

### 5.4.13 Axenic hatching of brine shrimp larvae

Two hundred milligrams of high-quality hatching cysts of *Artemia franciscana* (EG® Type; INVE Aquaculture, Baasrode, Belgium) were hydrated in 18 ml of filter-sterilized tap water for 1 h. Sterile cysts and larvae were obtained by decapsulation according to Marques et al. (2004). Briefly, 660 *µ*l of NaOH (32%) and 10 ml of NaOCl (50%) were added to the hydrated cyst suspension to facilitate decapsulation. The process was stopped after 2 min by adding 14 ml of $Na_2S_2O_3$ (10 g $L^{-1}$). Filtered (0.22 *µ*m) aeration was provided during the reaction. The decapsulated cysts were washed with filtered (passed through 0.22-*µ*m membrane filter) and autoclaved (moist heat at 121°C for 15 min) artificial seawater (containing 35 g l-1 of instant ocean synthetic sea salt, Aquarium Systems, Sarrebourg, France). The cysts were re-suspended in a 50-ml tube containing 30 ml of filtered, autoclaved seawater and hatched for 28 h on a rotor (4 $min^{-1}$) at 28°C with constant illumination (c. 2000 lux). The axenity of cysts was verified by inoculating one ml of culture water into 9 ml of marine broth and incubating at 28°C for 24 h. After 28 h of hatching, batches of 30 larvae were counted and transferred to fresh, sterile 50-ml tubes containing 30 ml of filtered and autoclaved seawater. Finally, the tubes were returned to the rotor and kept at 28°C. All

manipulations were performed in a laminar flow to maintain sterility of the cysts and larvae.

### 5.4.14 Brine shrimp challenge tests

The impacts of indole and indole analogues on the virulence of *V. harveyi* were determined in a standardized challenge test with gnotobiotic brine shrimp larvae. *V. harveyi* was incubated with or without indole and analogues at 50, 100 and 200 µM respectively, and cultures were washed with phosphate-buffered saline (pH 7.4) prior to inoculation into the brine shrimp rearing water at $10^5$ CFU $ml^{-1}$. The challenge tests were performed as described by (Defoirdt *et al.*, 2005) with some modifications. A suspension of autoclaved LVS3 bacteria (Verschuere *et al.*, 1999) in filtered and autoclaved seawater was added as feed at the start of the challenge test at $10^7$ cells $ml^{-1}$. Brine shrimp cultures, to which only autoclaved LVS3 bacteria were added as feed, were used as controls. The survival of the larvae was counted 48 h after the addition of the pathogens. Each treatment was carried out in triplicate and each experiment was repeated twice to verify the reproducibility. In each test, the sterility of the control treatments were checked at the end of the challenge by inoculating 1 ml of rearing water to 9 ml of marine broth and incubating the mixture for 2 days at 28°C.

### 5.4.15 Giant freshwater prawn experiments

Giant freshwater prawn experiments were performed as described in Pande *et al.* (2013). Briefly, prawn broodstock maintenance was performed according to Cavalli et al. (2001) and water quality parameters were adjusted according to New (2003). The larvae were obtained from a single oviparous female breeder. A matured female, which had just completed its pre-mating moult, was mated with a hard-shelled male as described before (Baruah et al., 2009). The female with fertilized eggs was then maintained for 20 to 25 days to undergo embryonic development. When fully ripe (indicated by dark grey color of the eggs), the female was transferred to a hatching tank (30 L) containing slightly brackish water (containing 6 g $L^{-1}$ Instant Ocean synthetic sea salt, Aquarium System Inc., Sarrebourg, France). The water temperature was maintained at 28 °C by a thermostat heater. After hatching, the newly hatched larvae with yolk were left for 24 h in the hatching tank. The next day,

prawn larvae with absorbed yolk were distributed in groups of 25 larvae in 200 ml glass cones containing 100 ml fresh autoclaved brackish water (12 g $L^{-1}$ synthetic sea salts). The glass cones were placed in a rectangular tank containing water maintained at 28 °C and were provided with aeration. The larvae were fed daily with 5 *Artemia* nauplii/larvae and acclimatized to the experimental conditions for 24 h. During the experiments, water quality parameters were kept at minimum 5 mg $L^{-1}$ dissolved oxygen, maximum 0.5 mg $L^{-1}$ ammonium-N and maximum 0.05 mg $L^{-1}$ nitrite-N.

Prawn larvae were challenged with wild type *V. harveyi* or pre-treated *V. harveyi* by adding the strains at $10^6$ $CFU.ml^{-1}$ to the culture water on the day after first feeding. Survival was counted daily in the treatment challenged to wild type *V. harveyi* and the challenge test was stopped when more than 50% mortality was achieved in this treatment (in order to have enough larvae remaining for the growth measurement). At this time point, larval survival was determined in all treatments by considering that only those larvae presenting movement of appendages were alive. The growth parameter larval stage index (LSI) was determined according to Maddox and Manzi (1976) on 5 randomly sampled larvae from each cone and calculated as:

$LSI = \Sigma Si/N$

Si : stage of the larva (i = 1 to 12)

N : the number of larvae examined

### 5.4.16 Statistical analyses

Data analysis was carried out using the SPSS statistical software (version 15). Log transformed gene expression data were analysed using independent samples *t*- tests. Unless stated otherwise, all other data were compared with one-way ANOVA, followed by Tukey's *post hoc* test.

## Acknowledgements

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# CHAPTER VI

## NOREPINEPHRINE AND DOPAMINE INCREASE MOTILITY, BIOFILM FORMATION AND VIRULENCE OF *VIBRIO HARVEYI*

**Qian Yang, Nguyen Duc Qyunh Anh, Peter Bossier, Tom Defoirdt** (2014) *Frontiers in Microbiology 5: 584.*

## ABSTRACT

*Vibrio harveyi* is one of the major pathogens of aquatic organisms, affecting both vertebrates and invertebrates, and causes important losses in the aquaculture industry. In order to develop novel methods to control disease caused by this pathogen, we need to obtain a better understanding of pathogenicity mechanisms. Sensing of catecholamines increases both growth and production of virulence-related factors in pathogens of terrestrial animals and humans. However, at this moment, knowledge on the impact of catecholamines on the virulence of pathogens of aquatic organisms is lacking. In the present study, we report that in *V. harveyi*, norepinephrine and dopamine increased growth in serum-supplemented medium, siderophore production, swimming motility and expression of genes involved in flagellar motility, biofilm formation, and exopolysaccharide production. Consistent with this, pretreatment of *V. harveyi* with catecholamines prior to inoculation into the rearing water resulted in significantly decreased survival of gnotobiotic brine shrimp larvae, when compared to larvae challenged with untreated *V. harveyi*. Further, norepinephrine-induced effects could be neutralized by α-adrenergic antagonists or by the bacterial catecholamine receptor antagonist LED209, but not by β-adrenergic or dopaminergic antagonists. Dopamine-induced effects could be neutralized by dopaminergic antagonists or LED209, but not by adrenergic antagonists. Together, our results indicate that catecholamine sensing increases the success of transmission of *V. harveyi* and that interfering with catecholamine sensing might be an interesting strategy to control vibriosis in aquaculture. We hypothesise that upon tissue and/or hemocyte damage during infection, pathogens come into contact with elevated catecholamine levels, and that this stimulates the expression of virulence factors that are required to colonize a new host.

**Keywords: swimming motility, flagellum, virulence, shrimp, antivirulence therapy, microbial endocrinology**

## 6.1 INTRODUCTION

*Vibrio harveyi* is a ubiquitous, bioluminescent marine bacterium which can cause luminous vibriosis in both marine vertebrates and invertebrates, leading to significant losses in the global aquaculture industry (Austin and Zhang, 2006; Ruwandeepika *et al*., 2012). For example, it has been reported to be associated with high mortalities of cultured penaeid shrimp larvae and packhorse rock lobster larvae (Soto-Rodriguez *et al*., 2003), diseased sea horses (Tendencia, 2004), and skin ulceration in juvenile sea cucumber (Becket *et al*. 2004). The pathogenicity of *V. harveyi* is considered to involve biofilm formation, swimming motility, and the production of various extracellular products, such as haemolysins, proteases, (phospho)lipases and chitinases (Karunasagar *et al*., 1994; Austin and Zhang, 2006; Ruwandeepika *et al*., 2012; Yang and Defoirdt, 2014). Conventional antibiotics are becoming increasingly ineffective to control bacterial infections in aquaculture, and consequently, alternative methods to control infections are urgently needed (Defoirdt *et al*., 2011). In this respect, inhibiting the production of virulence-related phenotypes, a strategy that has been termed antivirulence therapy, is an interesting novel approach for controlling bacterial infections (Clatworthy *et al*., 2007; Defoirdt, 2013; Defoirdt, 2014). The inhibition of specific virulence genes is possible (Baron, 2010). However, much more research effort thus far has been devoted to virulence-regulatory mechanisms because these mechanisms control the expression of (multiple) virulence factors and consequently, by targeting these mechanisms it would be possible to block several virulence factors at once.

Host stress has long been known to influence host-pathogen interactions. For a long time, the impact of stress on infection has been exclusively associated with the suppression of the immune system of the host and an increased susceptibility to infections due to elevated levels of stress hormones (Dhabhar and McEwen, 1997; Freestone *et al*., 2008). However, investigations over the past decades have introduced a new perspective which implies that infectious bacteria also respond to these stress hormones (Lyte, 2004). The catecholamine stress hormones norepinephrine and dopamine, which are an integral part of the ‘fight or flight’ stress

response in animals (Reiche *et al*., 2004), stimulate the growth of several species of bacteria (including *Escherichia coli*, *Yersinia enterocolitica*, *Listeria monocytogenes*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus*) in serum-based media (Lyte and Ernst, 1992; Coulanges *et al*., 1997; Kinney *et al*., 1999; Belay *et al*., 2003; Nakano *et al*., 2007a; 2007b). Such media are iron-limited because of chelation of free iron by high-affinity iron-binding proteins such as transferrin. According to Sharaff and Freestone (2011), catecholamines increase the availability of iron through complex formation with the iron-binding proteins, thereby decreasing their affinity for iron to a point of iron loss. Catecholamines have also been reported to increase the production of Shiga toxin, chemotaxis, biofilm formation, and attachment and colonization to epithelial cells in pathogenic *E. coli*, motility and invasiveness of *Campylobacter jejuni*, motility and type III secretion in *Salmonella typhimurium*, and cytotoxic activity in *V. parahaemolyticus* (Bansal *et al*., 2007; Cogan *et al*., 2007; Nakano *et al*., 2007b; Lyte *et al*., 2011; Sharaff and Freestone, 2011).

Catecholamines exert their effects by binding to specific receptors. In eukaryotes, epinephrine and norepinephrine bind to adrenergic receptors, which are divided into two major families (α and β), each with a number of receptor subtypes, while dopamine binds to dopaminergic receptors with at least 5 receptor subtypes; and binding of the hormones to the receptors can be prevented by specific antagonists (Freestone *et al*., 2007). Interestingly, antagonists of eukaryotic adrenergic and dopaminergic receptors can also inhibit catecholamine-induced effects in bacteria (Sharaff and Freestone, 2011). Furthermore, several bacterial catecholamine receptors have been reported as well, including the histidine sensor kinases QseC and QseE (Hughes *et al*., 2009), for which a highly active antagonist, LED209, has been identified (Rasko *et al*., 2008).

The production of catecholamines is highly conserved in the animal kingdom, both in vertebrates (including fish) and invertebrates (including mollusks and crustaceans) (Ottaviani and Franceschi, 1996). However, at this moment, knowledge on the impact of catecholamines on the virulence of major pathogens of aquatic organisms, such as *V. harveyi,* is lacking. In this study, we aimed at investigating the impact of the catecholamines norepinephrine and dopamine on the growth of *V. harveyi* in

serum-based medium, on the expression of various virulence-related characteristics and on virulence towards gnotobiotic brine shrimp (*Artemia franciscana*) larvae.

## 6.2 RESULTS

### 6.2.1 Effects of catecholamines and antagonists on the swimming motility

Motility is required for the virulence of *V. harveyi* (Yang and Defoirdt, 2014), and since catecholamines are known to affect the motility of other pathogens (Lyte *et al*., 2011), we investigated the effect of catecholamines on the swimming motility of *V. harveyi* on soft agar. Both norepinephrine and dopamine could significantly increase the swimming motility of *V. harveyi* (**Figure 6.1** and **Figure 6.2**). We investigated the effects of catecholamines on the expression of ten selected genes involved in flagellar motility in *V. harveyi*, including 6 genes involved in the synthesis of the polar flagellum (both regulators and structural genes), 2 genes involved in the synthesis of lateral flagella, and 2 genes involved in chemotaxis. Both norepinephrine and dopamine significantly up-regulated the expressions of all selected genes (**Table 6.1**), which confirmed the stimulatory effect of catecholamines on flagellar motility.

**Table 6.1.** Impact of catecholamines on the expression of flagellar motility-related genes in *V. harveyi*.

| Gene | Function | Relative expression (fold)[1] | | |
|---|---|---|---|---|
| | | Untreated | Norepinephrine (50 $\mu$M) | Dopamine (50 $\mu$M) |
| *flaA* | Polar flagellin | 1.0 ± 0.1 | 2.8 ± 0.4 ** | 1.6 ± 0.1 ** |
| *flaC* | Polar flagellin | 1.0 ± 0.1 | 1.9 ± 0.1 *** | 1.3 ± 0.2 |
| *flaK* | Polar flagellar regulator | 1.0 ± 0.3 | 2.9 ± 0.2 *** | 1.7 ± 0.3 ** |
| *fliA* | Polar flagellar biosynthesis sigma factor | 1.0 ± 0.4 | 2.9 ± 0.5 ** | 2.2 ± 0.1 *** |
| *fliS* | Polar flagellin specific chaperone | 1.0 ± 0.3 | 3.2 ± 0.5 ** | 1.7 ± 0.1 *** |
| *flgB* | Flagellar basal body rod | 1.0 ± 0.2 | 3.2 ± 0.3 ** | 1.4 ± 0.4 ** |
| *cheA* | Chemotaxis protein | 1.0 ± 0.1 | 2.5 ± 0.3 *** | 1.9 ± 0.2 ** |
| *cheR* | Chemotaxis protein | 1.0 ± 0.4 | 2.0 ± 0.1 ** | 1.4 ± 0.3 |
| *lafA* | Lateral flagellar flagellin | 1.0 ± 0.3 | 3.0 ± 0.4 ** | 1.9 ± 0.1 *** |
| *lafK* | Lateral flagellar regulator | 1.0 ± 0.2 | 3.0 ± 0.6 ** | 1.6 ± 0.4 ** |

[1] For each gene, the expression in untreated cultures was set at 1 and the expression in cultures supplemented with catecholamines was normalized accordingly using the $2^{-\Delta\Delta CT}$ method. Data are average ± standard deviation of 3 *V. harveyi* cultures. Asterisks indicate a significant difference when compared to untreated *V. harveyi* (independent samples t- test; **: $p < 0.01$; ***: $p < 0.001$).

We further evaluated whether catecholamine receptor antagonists could neutralize catecholamine-induced swimming motility. The α-adrenergic antagonists phentolamine and phenoxybenzamine, the α- and β-adrenergic antagonist labetalol and the bacterial catecholamine receptor antagonist LED209 could neutralize norepinephrine-induced swimming motility (**Figure 6.1**), whereas the non-selective β-adrenergic receptor antagonist propranolol and the dopaminergic antagonist chlorpromazine had no effect (data not shown). Further, the dopaminergic antagonist chlorpromazine and the bacterial catecholamine receptor antagonist LED209 neutralized dopamine-induced motility (**Figure 6.2**), whereas adrenergic receptor

antagonists had no effect (data not shown). Finally, we also checked the swimming motility of bacteria in the presence of only antagonists and equivalent volumes of the solvents as used in combination with the catecholamines, and no effects were observed (data not shown).

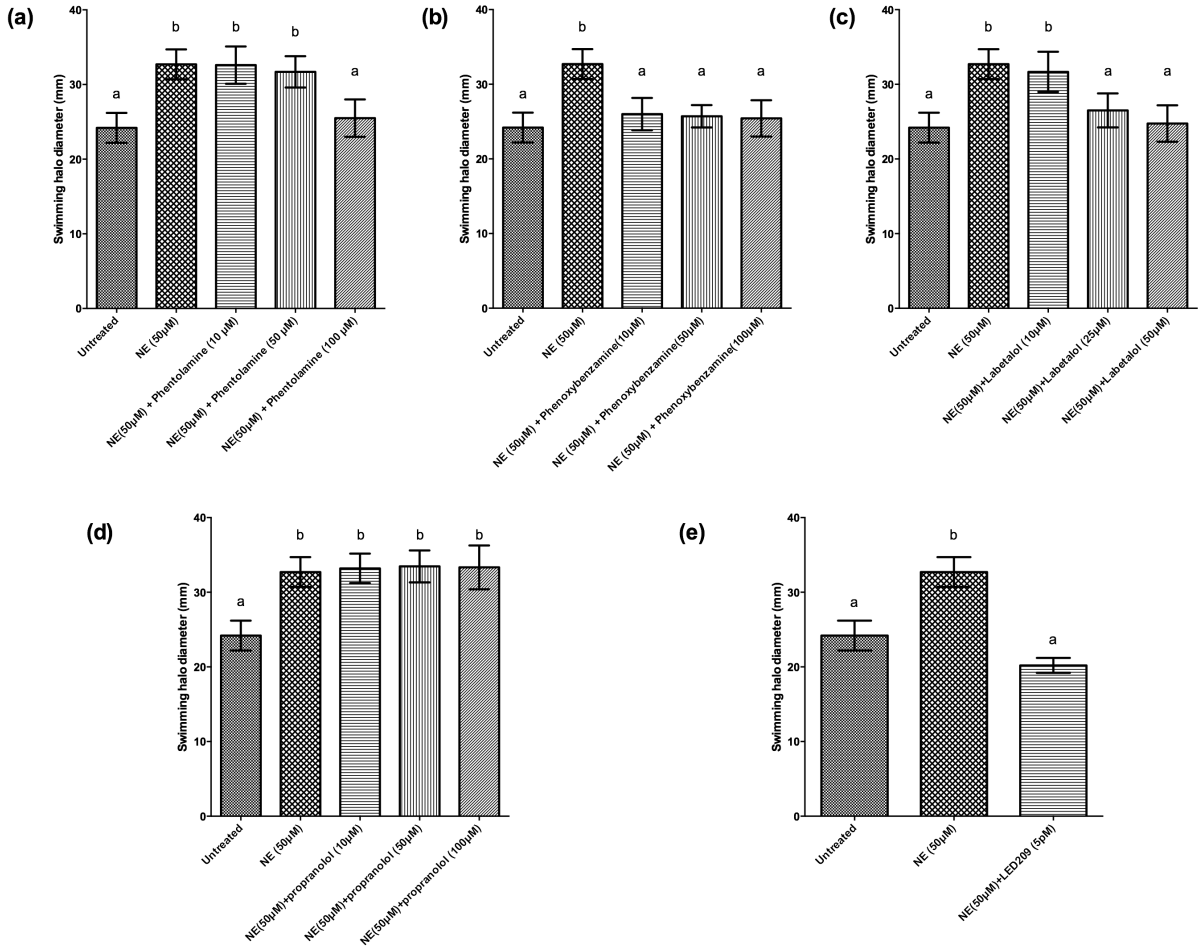


**Figure 6.1.** Impact of norepinephrine and different kinds of norepinephrine receptor antagonists on swimming motility of *V. harveyi.* Norepinephrine was added at 50 μM, and the norepinephrine receptor antagonists were added at 10μM, 50μM and 100μM, respectively. The error bars indicate the standard deviation of six replicate cultures. Different letters indicate significant differences (One way ANOVA with Tukey's post-hoc test; $P < 0.01$).

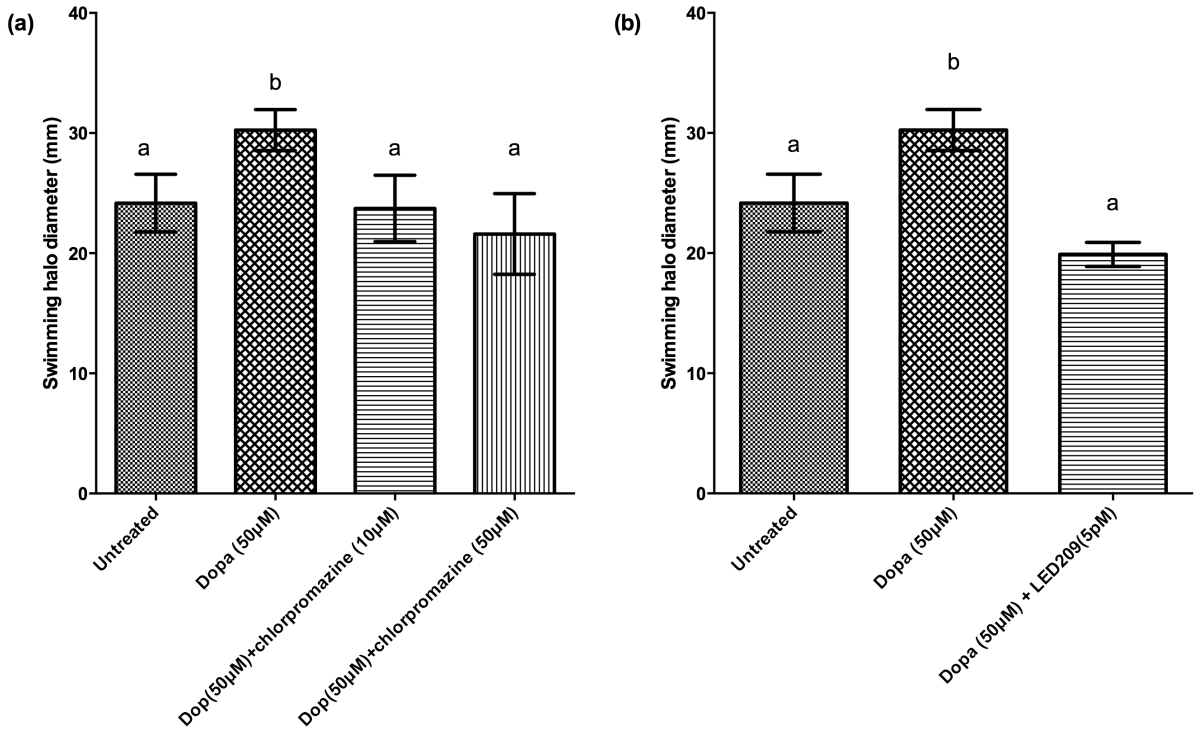


**Figure 6.2.** Impact of dopamine and different kinds of dopamine receptor antagonists on swimming motility of *V. harveyi.* Dopamine was added at 50 μM, and the dopamine receptor antagonists were added at 10μM, 50μM and 100μM, respectively. The error bars indicate the standard deviation of six replicate cultures. Different letters indicate significant differences (One way ANOVA with Tukey's post-hoc test; $P < 0.01$).

### 6.2.2 Effects of catecholamines and antagonists on the growth of *V. harveyi* in serum-supplemented medium

The addition of norepinephrine or dopamine increased the growth of *V. harveyi* in Marine Broth containing 30% (v/v) serum (**Figure 6.3**) and resulted in a 2.4- and 2.1-fold increase in the growth rate of *V. harveyi*, respectively (0.59 ± 0.05 $h^{-1}$ and 0.53 ± 0.04 $h^{-1}$ in the presence of norepinephrine and dopamine, respectively, compared to 0.25 ± 0.03 $h^{-1}$ for the untreated control). When compared to untreated cultures, the maximum turbidity was 1.6-fold higher for both norepinephrine and dopamine supplemented cultures. The catecholamines had no effect on growth of *V. harveyi* in medium without serum (data not shown).

In further experiments, we investigated whether catecholamine receptor antagonists

could neutralize the growth-stimulatory effects of the catecholamines in serum-supplemented $LB_{35}$ broth containing 50μM catecholamines. The results were consistent with what we observed in the motility assays: antagonists with α-adrenergic activity (but not antagonists with only β-adrenergic activity) were able to inhibit norepinephrine-induced growth (**Figure 6.3a**), and the adrenergic antagonists were not able to neutralize dopamine-induced growth (data not shown). Further, the dopaminergic antagonist chlorpromazine neutralized dopamine-induced growth but had no effect on norepinephrine-induced growth (**Figure 6.3b**). The prokaryotic catecholamine receptor antagonist LED209 was able to neutralize the effects of both norepinephrine and dopamine. None of the antagonists and solvents used to dissolve the antagonists affected the growth of *V. harveyi* when tested alone at the same volumes as used in combination with catecholamines (data not shown), which indicates that the growth inhibition by the antagonists was not due to toxicity, but a specific antagonism of the bacterial response to catecholamines.

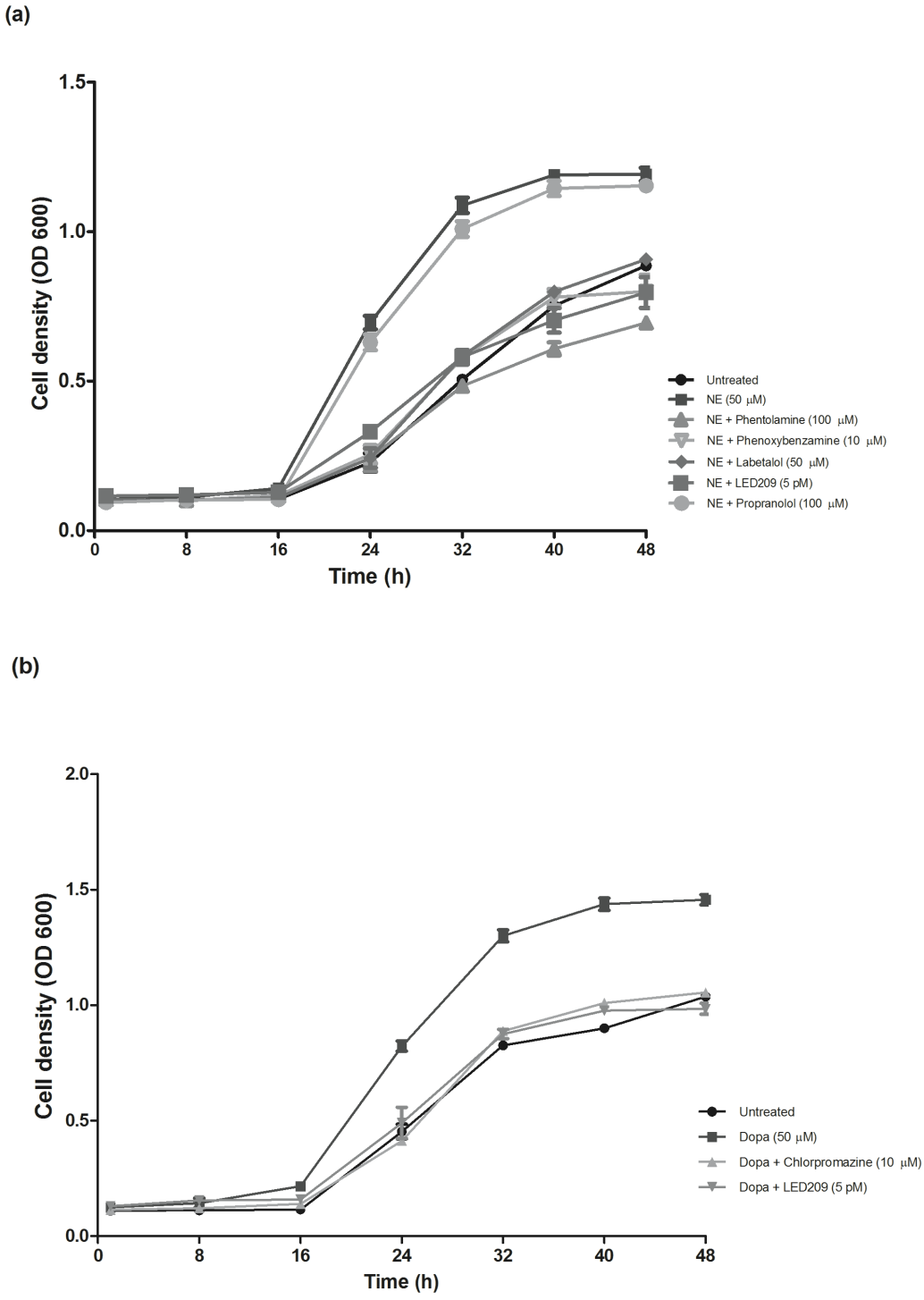


**Figure 6.3.** Impact of catecholamines and catecholamine receptor antagonists on the growth of *V. harveyi* in $LB_{35}$ broth containing 30% (v/v) serum. (a) Norepinephrine (NE) and adrenergic antagonists. **(b)** Dopamine (Dopa) and dopaminergic antagonists. The initial density of *V. harveyi* was $10^2$ CFU/ml. Error bars represent the standard deviation of three independent cultures.

### 6.2.3 Effects of catecholamines and antagonists on siderophore production

In order to further substantiate the link between iron availability and growth induction by catecholamines in serum-supplemented medium, we determined the impact of the catecholamines on siderophore production. Both norepinephrine and dopamine significantly increased the siderophore production of *V. harveyi*, and the effect could be neutralized by phentolamine and chlorpromazine at a concentration of 100 µM and 10 µM, respectively (**Figure 6.4**). This is consistent with the antagonist concentrations needed to neutralize the growth-inducing effect of the catecholamines in serum-supplemented medium.

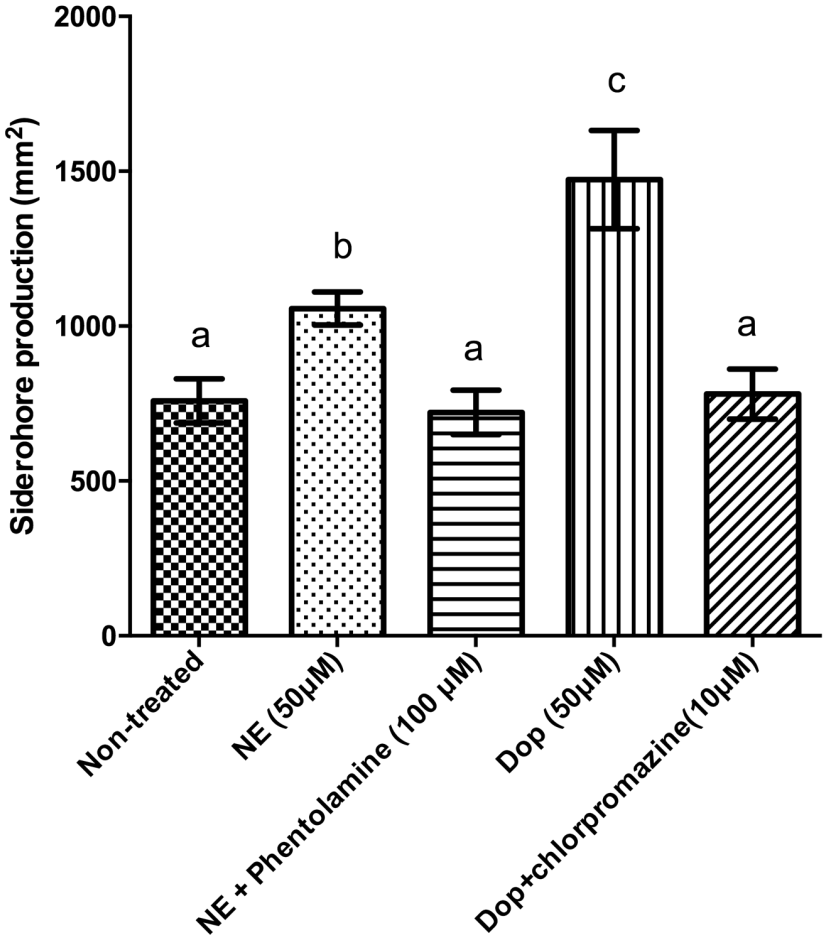


**Figure 6.4.** Impact of catecholamines and catecholamine receptor antagonists on siderophore production by *V. harveyi.* The concentration of catecholamines used is 50 $\mu$M. Phentolamine and chlorpromazine was added at a concentration of 100 $\mu$M and 10 $\mu$M, respectively. The error bars indicate the standard deviation of six replicate cultures. Different letters indicate significant differences (One way ANOVA with Tukey's post-hoc test; $P < 0.01$).

### 6.2.4 Effects of catecholamines and antagonists on biofilm formation and exopolysaccharide production

Biofilm formation of *V. harveyi* was determined by crystal violet staining. The catecholamines significantly increased biofilm formation, and the effect was blocked by the antagonists (**Figure 6.5**). Because exopolysaccharide production is one of the major factors affecting biofilm formation, we also determined the impact of the catecholamines on exopolysaccharide production by Calcofluor white staining. The catecholamines significantly increased exopolysaccharide production (2.5- and 2.0-fold increase in the presence of norepinephrine and dopamine, respectively), and this could be neutralized by the antagonists (**Figure 6.6**). Importantly, the antagonists and solvents showed no influence on biofilm formation or exopolysaccharide production in the absence of catecholamines (data not shown).

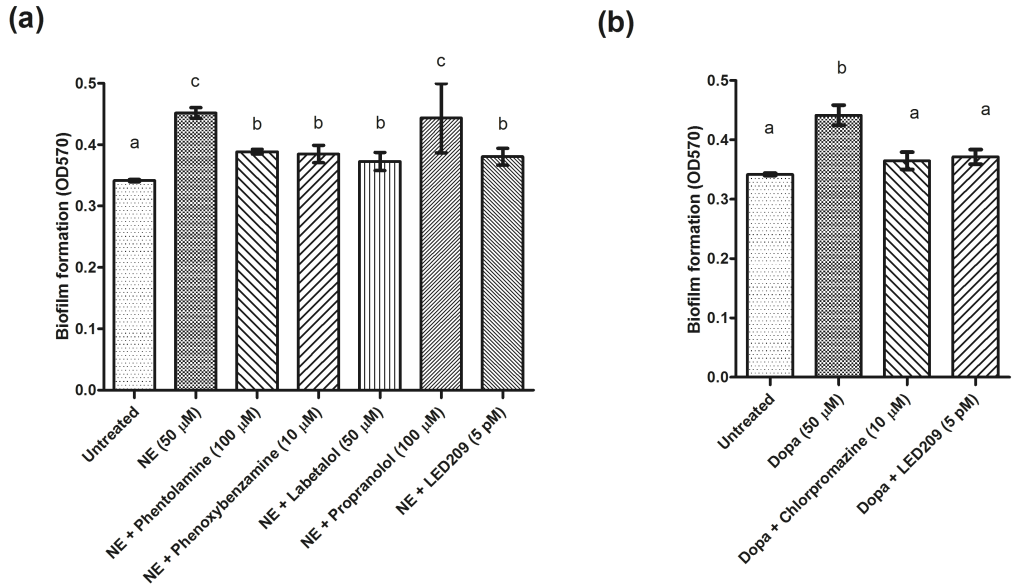


**Figure 6.5.** Impact of catecholamines and catecholamine receptor antagonists on biofilm formation by *V. harveyi*. (a) Norepinephrine (NE) and adrenergic antagonists on the biofilm formation. **(b)** Dopamine (Dopa) and dopaminergic antagonists. The error bars represent standard deviation of three independent experiments. Different letters indicate significant differences (One way ANOVA with Tukey's post-hoc test; $P < 0.01$).

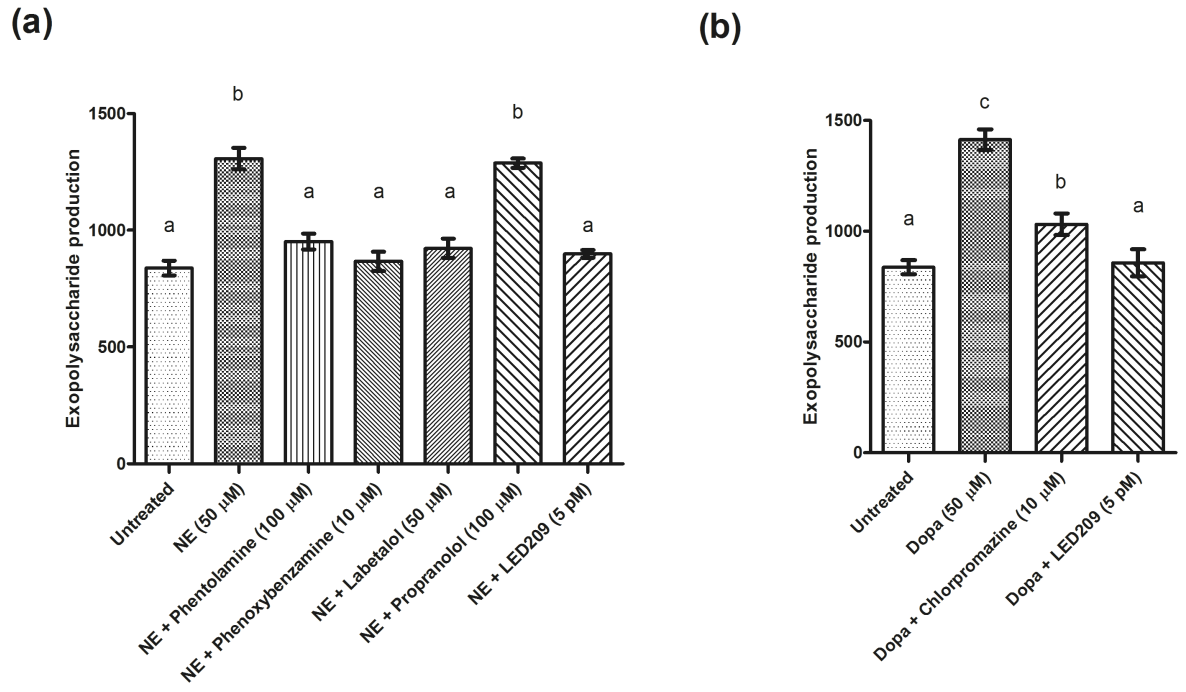


**Figure 6.6.** Impact of catecholamines and catecholamine receptor antagonists on exopolysaccharide production of *V. harveyi*. (a) Norepinephrine (NE) and adrenergic antagonists on the expopolysaccharide production. (b) Dopamine (Dopa) and dopaminergic antagonists. The error bars represent standard deviation of three independent experiments. Different letters indicate significant differences (One way ANOVA with Tukey's post-hoc test; $P < 0.01$).

### 6.2.5 Effects of catecholamines and antagonist on other virulence factors

The impacts of catecholamines on five other virulence factors (lipase, phospholipase, gelatinase, hemolysin and caseinase production) were determined as well. Norepinephrine and dopamine slightly decreased gelatinase activity (1.2- and 1.1-fold, respectively), while there was no significant effect on caseinase, lipase, phospholipase or hemolytic activity (data not shown).

### 6.2.6 Effects of catecholamines and antagonist on the virulence of *V. harveyi* towards brine shrimp larvae

*In vitro* experiments revealed that the catecholamines norepinephrine and dopamine positively regulated virulence-related phenotypes in *V. harveyi,* including growth in an

iron-limited environment, swimming motility, biofilm formation and exopolysaccharide production. To examine whether this results in an increased virulence of the bacterium *in vivo*, we performed a standardized challenge test with gnotobiotic brine shrimp larvae. In order to exclude any direct effects of the catecholamines on the host, *V. harveyi* was pretreated with the hormones, after which the pathogen was washed with phosphate-buffered saline and then inoculated into the brine shrimp rearing water. According to the results, strongest effect was observed after the pathogen was pretreated for 4h (**Table 6.1**). Therefore, *V. harveyi* was exposed to norepinephrine or dopamine for 4h prior to the challenge in the following tests, and significantly higher brine shrimp mortality rates were observed (**Table 6.2**).

We further evaluated the effects of catecholamine antagonists by pretreating *V. harveyi* with both catecholamines and antagonists. The α-adrenergic antagonists phentolamine and phenoxybenzamine, the α- and β-adrenergic antagonist labetalol and the prokaryotic catecholamine receptor antagonist LED209 were all able to neutralize the increased virulence induced by norepinephrine, whereas the dopaminergic antagonist chlorpromazine and LED209 neutralized the effect of dopamine (**Table 6.2**). Pretreatment of *V. harveyi* with the antagonists had no effect on virulence in the absence of catecholamines (data not shown).

**Table 6.2.** Impact of pretreatment of *V. harveyi* with catecholamines and on virulence of the bacterium towards gnotobiotic brine shrimp larvae at different time points.

| Pretreatment time | Survival (%)[a] |
|---|---|
| Norepinephrine | |
| 1h | $45 \pm 3^{cd}$ |
| 2h | $38 \pm 8^{bc}$ |
| 4h | $27 \pm 6^{a}$ |
| 8h | $30 \pm 8^{ab}$ |
| Control | $50 \pm 5^{d}$ |
| | |
| Dopamine | |
| 1h | $47 \pm 3^{BC}$ |
| 2h | $45 \pm 9^{B}$ |
| 4h | $35 \pm 5^{A}$ |
| 8h | $38 \pm 6^{A}$ |
| Control | $50 \pm 5^{C}$ |

[a] Survival after 2 days of challenge with *V. harveyi* ATCC BAA-1116 (average ± standard error of four replicates). Survival in the control treatment was set at 100% and the other treatments were normalized accordingly. Square brackets refer to pretreatment – *V. harveyi* was either or not pretreated with catecholamines and antagonists for 4h and washed prior to inoculation into the brine shrimp rearing water. Values with a different superscript letter are significantly different from each other (One way ANOVA with Tukey's *post-hoc* test; $P < 0.01$).

**Table 6.3.** Impact of pretreatment of *V. harveyi* with catecholamines and catecholamine receptor antagonists on virulence of the bacterium towards gnotobiotic brine shrimp larvae.

| Treatment | Survival (%)[a] |
|---|---|
| Control | 100 ± 0[c] |
| *V. harveyi* | 50 ± 5[b] |
| *V. harveyi* [50 μM norepinephrine] | 27 ± 6[a] |
| *V. harveyi* [50 μM norepinephrine + 100 μM phentolamine] | 48 ± 3[b] |
| *V. harveyi* [50 μM norepinephrine + 10 μM phenoxybenzamine] | 47 ± 3[b] |
| *V. harveyi* [50 μM norepinephrine + 50 μM labetalol] | 48 ± 3[b] |
| *V. harveyi* [50 μM norepinephrine + 100 μM propanolol] | 29 ± 4[a] |
| *V. harveyi* [50 μM norepinephrine + 5 pM LED209] | 55 ± 5[b] |
| *V. harveyi* [50 μM dopamine] | 35 ± 5[a] |
| *V. harveyi* [50 μM dopamine + 50 μM chlorpromazine] | 47 ± 3[b] |
| *V. harveyi* [50 μM dopamine + 5 pM LED209] | 52 ± 8[b] |

[a] Survival after 2 days of challenge with *V. harveyi* ATCC BAA-1116 (average ± standard error of four replicates). Survival in the control treatment was set at 100% and the other treatments were normalized accordingly. Square brackets refer to pretreatment – *V. harveyi* was either or not pretreated with catecholamines and antagonists for 4h and washed prior to inoculation into the brine shrimp rearing water. Values with a different superscript letter are significantly different from each other (One way ANOVA with Tukey's *post-hoc* test; $P < 0.01$).

## 6.3 DISCUSSION

The successful interaction between bacteria and their host depends not only on a coordinated response to population density, temperature and pH (Mekalanos, 1992; Winzers *et al*., 2001), but also on the detection of diverse host cell effector molecules such as catecholamine stress hormones (Burton *et al*., 2002). Exposure of bacteria to catecholamine stress hormones has been demonstrated to stimulate both growth and production of virulence-related factors in various pathogens of terrestrial animals and humans, such as *E. coli* (Bansal *et al*., 2007), *Campylobacter jejuni* (Cogan *et al*., 2007), *Salmonella typhimurium* (Bearson and Bearson, 2008), and *V.*

*parahaemolyticus* (Nakano *et al*., 2007b). The present study demonstrates that the catecholamines norepinephrine and dopamine could significantly increase the production of major virulence factors and growth in serum-supplemented medium of *V. harveyi*, a major pathogen of aquatic organisms with a broad host spectrum (both vertebrates such as fish and invertebrates such as crustaceans and mollusks). This result is in good agreement with, and considerably extends, the previous reports.

The catecholamines significantly increased the virulence of *V. harveyi* towards gnotobiotic brine shrimp larvae. The pathogen was pretreated with catecholamines in order to avoid a direct effect of catecholamines on the host (e.g. decreased activity of the defense system) and to ensure that any impact on survival of challenged larvae was due to increased virulence of the pathogen. This is further substantiated by our observation that in addition to their effect on growth in serum-supplemented medium and siderophore production, the catecholamines increased the production of several other phenotypes that are important for infection. Indeed, both norepinephrine and dopamine significantly stimulated biofilm formation, exopolysaccharide production, swimming motility and the expression of genes involved in flagellum synthesis (structural genes and regulators including the flagellar master regulator) in *V. harveyi*. These results agree well with previous reports documenting the impact of catecholamines on biofilm formation in *Staphylococcus epidermidis* (Lyte *et al*., 2003) and on swimming motility in *E. coli*, *C. jejuni* and *Edwardsiella tarda* (Kendall *et al*., 2007; Cogan *et al*., 2007; Wang *et al*., 2011). Furthermore, previous work in our laboratory has also confirmed the increased virulence of *V. harveyi* by catecholamines in conventionally reared giant freshwater prawn larvae (Pande *et al*., 2014).

Catecholamines are produced by hemocytes of invertebrates, and concentrations in the hemolymph of shrimp and prawn that have been reported range between 10 nM and ~3 μM (Chen *et al*., 2003; Hsieh *et al*., 2006; Pan *et al*., 2014). These concentrations are lower than the concentrations used in this study. However, local concentrations can be considerably higher. For instance, the intrasynaptic concentration of norepinephrine in the central nervous system of mammals is as high as 10 mM (versus nM levels in serum) (Lyte, 2004). Hence, upon infection, pathogens

can come into contact with local concentrations that are several orders of magnitude higher than those that are found in hemolymph (or serum in case of vertebrates). This probably is also the case when tissues and/or hemocytes are damaged during infection and hence, elevated catecholamine levels might be a cue informing the pathogen of tissue damage.

Pretreatment of *V. harveyi* with catecholamines simulated transmission of the pathogen from a host site showing elevated catecholamine levels (e.g. due to cell or tissue damage). In combination with our observation that the catecholamines increased biofilm formation, exopolysaccharide production and swimming motility of *V. harveyi* (which are all important during the initial stages of infection), our results suggest that catecholamine sensing increases the success of transmission to a new host. Hence, elevated catecholamine levels might be a cue informing the pathogen that the infection reached a final stage (cell and tissue damage) and that it is time to leave the host.

Catecholamine receptor antagonists have been used extensively to identify and characterize catecholamine receptors in mammals. The effects of various antagonists on growth and production of virulence factors in *V. harveyi* have been investigated in the present study, and the results demonstrated that α- but not β-adrenergic receptor antagonists could block responses to norepinephrine, but did not show any effect on dopamine responsiveness. On the contrary, dopaminergic receptor antagonists neutralized induction caused by dopamine, but did not neutralize induction by norepinephrine. Similar results have been reported in enteric pathogens of terrestrial animals (Freestone *et al*., 2007), and suggest that bacterial response systems for catecholamines possess a degree of specificity similar to mammalian catecholamine receptors. In contrast to the wealth of adrenergic and dopaminergic receptors described in eukaryotes, there have been only few reports examining the presence of such receptors in bacteria. Clarke *et al*. (2006) reported that norepinephrine was able to be recognized by the *E. coli* O157:H7 two-component regulator sensor kinase QseC in *in vitro* constructs, leading to the hypothesis that this is the bacterial catecholamine receptor. Later on, a QseC antagonist, LED209, has been identified in a high-throughput screen (Rasko *et al*., 2008). We found that LED209 was able to

neutralize the effects of both norepinephrine and dopamine, indicating that a similar response system for catecholamines might exist in *V. harveyi*. This is also substantiated by the fact that the *V. harveyi* ATCC BAA-1116 genome contains a QseC homolog. Alternatively, since our experiments with eukaryotic catecholamine receptor antagonists showed that dopaminergic antagonists did not neutralize norepinephrine-induced effects and vice versa, there are probably at least two different catecholamine receptors in *V. harveyi*, with LED209 being able to bind to both of them. Further research will be needed in order to identify the catecholamine receptors and the signal transduction pathways in *V. harveyi.*

Both norepinephrine and dopamine significantly stimulated the growth of *V. harveyi* in serum-supplemented medium, and this effect could be neutralized by eukaryotic catecholamine receptor antagonists. Similar results have been reported in other Gram-negative bacteria such as *E. coli* and *V. parahaemolyticus*, and the stimulation of growth in serum-supplemented medium by catecholamines has mainly been attributed to their ability to facilitate iron removal from the host iron-binding proteins transferrin and lactoferrin (Lyte, 2004; Nakano *et al*., 2007b). We used bovine serum since it was practically not feasible to obtain sufficient amounts of crustacean serum. However, it should be noted that crustaceans produce equivalents of iron-binding proteins produced by mammals (e.g. Toe *et al*., 2012). Our observations that catecholamine receptor antagonists are able to neutralize the growth-stimulatory effect of catecholamines and that adrenergic receptor antagonists were found to show no effect on dopamine-induced growth and vice versa suggest that a regulatory mechanism involving catecholamine receptors is involved as well, and this has also been reported for enteric pathogens such as *E. coli* O157:H7, *Salmonella enterica* and *Yersinia enterocolitica* (Freestone *et al*., 2007). To confirm the hypothesis that in addition to increasing iron uptake in a direct way, catecholamines induced an iron uptake mechanism, we investigated the effect of catecholamines on siderophore production, and found that both norepinephrine and dopamine could significantly induce siderophore production. Siderophores have been reported to be essential for catecholamine-induced growth in other Gram-negative bacteria such as *E. coli* and *Salmonella*, where they serve to internalize the iron removed by the catecholamines

(Freestone *et al.*, 2003; Williams *et al.*, 2006). Finally, it should be noted that in addition to limiting iron availability, serum might have other effects on the pathogens as well. Serum e.g. contains proteins (some of which might have antimicrobial activity; Zasloff, 2002). However, in view of the literature that is available on other pathogens and our observation that catecholamines increase siderophore production we think that modulation of iron availability is the most plausible explanation of growth stimulation by catecholamines in the presence of serum.

## 6.4 CONCLUSIONS

Our study has shown that the catecholamines norepinephrine and dopamine increase the virulence of *V. harveyi* by increasing swimming motility, biofilm formation, exopolysaccharide production and growth in environments with low iron availability, and the effects could be neutralized by antagonists for eukaryotic catecholamine receptors and the bacterial catecholamine receptor antagonist LED209. Different effects of the adrenergic and dopaminergic receptors antagonists indicate the presence of specific sensing systems for different catecholamines in *V. harveyi*.

## 6.5 MATERIALS AND METHODS

### 6.5.1 Bacterial strains and growth conditions

*V. harveyi* wild type strain ATCC BAA-1116 (recently reclassified as *V. campbellii*; Lin *et al.*, 2010) was used in this study. Unless otherwise stated, the strain was cultured at 28°C in Luria broth containing 35 g/L sodium chloride ($LB_{35}$) under constant agitation (100 $min^{-1}$). Cell densities were measured spectrophotometrically at 600 nm.

### 6.5.2 Catecholamines and eukaryotic catecholamine receptor antagonists

Norepinephrine was dissolved in hydrochloride acid (HCl 0.1N) at 10 mM, while dopamine was dissolved in distilled water at 10 mM. The antagonists used in this study are listed in **Table 6.3**. All the chemicals were purchased from Sigma-Aldrich (Bornem, Belgium). The reagents were sterilized using a 0.22 $\mu$m filter and stored at -20°C.

**Table 6.4.** Catecholamine receptor antagonists used in this study.

| Compound | Specificity | Solvent |
|---|---|---|
| Phentolamine hydrochloride | reversible α-adrenergic | Ethanol |
| Phenoxybenzamine hydrochloride | irreversible α-adrenergic | DMSO |
| S-propanolol hydrochloride | β-adrenergic | Ethanol |
| Labetalol hydrochloride | α- and β-adrenergic | Ethanol |
| Chlorpromazine hydrochloride | dopaminergic | Distilled water |
| LED209[1] | bacterial catecholamine receptor QseC | DMSO |

All compounds were dissolved at 10mM, except for LED209, which was dissolved at 10 $\mu$M.

[1] N-phenyl-4-[[(phenylamino)thioxomethyl]amino]-benzenesulfonamide

### 6.5.3 Bacterial growth assays

For the bacterial growth assays, *V. harveyi* was grown overnight in $LB_{35}$ broth at 28°C. After that, the culture was re-inoculated at a concentration of $10^2$ CFU/ml into fresh $LB_{35}$ broth containing 30% (v/v) adult bovine serum (Sigma-Aldrich), with and without 50 $\mu$M norepinephrine or dopamine. Additionally, different concentrations of the catecholamine receptor antagonists were added in conjunction with the catecholamines to determine whether they could neutralize catecholamine-induced growth responses. The cultures were grown in 200 $\mu$l volumes in 96-well plates at 28°C for 48 h, and the turbidity at 600 nm was monitored every hour using a Multireader machine (Infinite M200, TECAN, Austria). Growth curves were determined for three independent cultures, and the growth rate of the exponentially growing cultures was calculated. The statistical significance of specific growth rate was determined using an independent samples t-test.

### 6.5.4 Siderophore activity assay

The siderophore activity was determined by Chrome azurol S (CAS) agar diffusion

assay according to Shin *et al*. (2001), which was performed as follows. The CAS agar plates were prepared according to Schwyn and Neilands (1987); 60.5 mg Chrome azurol S (CAS)(Sigma-Aldrich) was dissolved in 50 ml deionized water, and mixed with 10 ml iron (III) solution (1 mM $FeCl_3 \cdot 6H_2O$, 10 mM HCl). Under stirring, this solution was slowly added to 72.9 mg hexadecyltrimethylammonium bromide (Sigma-Aldrich), dissolved in 40 ml water. The resultant dark blue solution was autoclaved and stored in a plastic container. Then 100 ml 10× MM9 salts, 15 g agar, 30.24 g Pipes, and 12 g of a 50% (w/v) NaOH solution (to raise the pH to 6.8) were added to 750 ml water. After autoclaving and cooling to 50°C, 30 ml of a sterile 10% casamino acids solution was added as the carbon source. The dye solution was finally added with enough agitation to achieve mixing without generation of foam. Serum (30%, v/v), catecholamines and antagonists were added directly into the agar. *V. harveyi* was cultured overnight in $LB_{35}$ broth containing 30% (v/v) serum, with or without catecholamines and antagonists. Then the CAS agar plates were punched with 5-mm-diameter holes and each hole was filled with 35 *μ*l of a *V. harveyi* culture ($OD_{600}$ = 1.0). After incubation at 28°C for 24h, the size of the orange halo formed around each hole was measured. Siderophore activity was expressed as the square value of the halo diameter.

### 6.5.5 Swimming motility assay

The swimming motility assay was performed on soft agar ($LB_{35}$ plates containing 0.3% agar) as described previously (Yang and Defoirdt, 2014). The catecholamines and antagonists were added to the autoclaved agar. *V. harveyi* was grown overnight in $LB_{35}$ broth, and 5 *μ*l aliquots (OD600 = 1.0) were spotted in the center of the soft agar plates. Plates were incubated for 24 h, after which the diameters of the motility halos were measured. All assays were done with freshly prepared media in 6 replicates.

### 6.5.6 Biofilm formation assay

Biofilm formation assay was quantified by crystal violet staining, as described previously (Stepanovic´ *et al*., 2007). In brief, an overnight culture of *V. harveyi* was diluted to an $OD_{600}$ of 1.0 in $LB_{35}$ broth with or without catecholamines and antagonists, and 200 *μ*l aliquots of these suspensions were pipetted into the wells of a 96 well plate.

Then the bacteria were allowed to adhere and grow without agitation for 24h at 28°C. After that, the cultures were removed and the wells were washed three times with 300 µl sterile physiological saline to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 150 µl of 99% methanol per well for 20 min, after which the methanol was removed and plates were air-dried. Then, biofilms were stained for 15 min with 150 µl of a 1% crystal violet solution (Pro-lab Diagnostics, Richmond Hill, ON, Canada) per well. Excess stain was rinsed off by placing the plate under running tap water, and washing was continued until the washings were free of the stain. After the plates were air dried, the dye bound to the adherent cells was resolubilized with 150 µl of 95% ethanol per well, and absorbance was measured at 570 nm. Sterile medium served as negative control. For the quantification of exopolysaccharides, Calcofluor white staining (Sigma-Aldrich) was used. In brief, wells were rinsed after 24 h biofilm formation and 100 µl phosphate buffered saline containing 0.5 µl 5 mM Calcofluor white staining dye was added to the wells. After 60 min, fluorescence (excitation 405 nm and emission 500 nm) was measured with a Multi-reader (Infinite M200, TECAN, Austria).

### 6.5.7 Lytic enzyme activity assays

All assays were conducted according to Natrah *et al.* (2011). For each assay, an overnight culture of *V. harveyi* was diluted to an OD600 of 0.5 and 5 *µ*l of the diluted culture was spotted in the middle of the test plates. The catecholamines and antagonists were added to the autoclaved agar before pouring. All assays were done at least in triplicate. Lipase and phospholipase activities were assessed on marine agar plates supplemented with 1% Tween 80 (Sigma–Aldrich) and 1% egg yolk emulsion (Sigma–Aldrich), respectively. The development of opalescent zones around the colonies was observed and the diameters of the zones were measured after 2–4 days of incubation at 28°C. Caseinase assay plates were prepared by mixing double strength Marine Agar with a 4% skim milk powder suspension (Oxoid, Basingstoke, Hampshire, UK), sterilized separately at 121°C for 5 min. Clearing zones surrounding the bacterial colonies were measured after 2 days of incubation. Gelatinase assay plates were prepared by mixing 0.5% gelatin (Sigma–Aldrich) into the agar. After

incubation for 7 days, saturated ammonium sulfate (80%) in distilled water was poured over the plates and after 2 min, the diameters of the clearing zones around the colonies were measured. Hemolytic assay plates were prepared by supplementing Marine Agar with 5% defibrinated sheep blood (Oxoid) and clearing zones were measured after 2 days of incubation.

### 6.5.8 RNA extraction

*V. harveyi* was grown overnight in triplicate on soft agar plates (0.3 % agar). Cells were harvested and RNA was extracted with the SV Total RNA Isolation System (Promega, Leiden, The Netherlands) according to the manufacturer's instructions. The RNA quantity was measured spectrophotometrically (NanoDrop Technologies, Wilmington, DE, USA) and adjusted to 200 ng $\mu l^{-1}$ in all samples. The RNA integrity was checked by Agarose Gel Electrophoresis and the RNA samples were stored in -80°C for subsequent use.

### 6.5.9 Primers

Specific primers were used for 10 selected genes involved in motility of *V. harveyi* (Yang and Defoirdt, 2014). The RNA polymerase A submit (*rpoA*) mRNA was used as an endogenous control (Defoirdt *et al.*, 2007).

### 6.5.10 Reverse transcription

Reverse transcription was performed with the RevertAid$^{TM}$ H minus First strand cDNA synthesis kit (Fermentas Gmbh, Baden-Württemberg, Germany) in accordance to the manufacturer's instructions. Briefly, a mixture of 1 $\mu$g RNA and 1 $\mu$l random hexamer primer solution was mixed first. Then, 8 $\mu$l of reaction mixture containing 4 $\mu$l of 5× reaction buffer (0.25 $mol^{-1}$ Tris–HCl pH 8.3, 0.25 $mol^{-1}$ KCl, 0.02 $mol^{-1}$ $MgCl_2$, 0.05 $mol^{-1}$ DTT), 2 $\mu$l of 0.01 $mol^{-1}$ dNTP mix, 20 units of ribonuclease inhibitor, 200 units of RevertAid$^{TM}$ H minus M-MuLV Reverse Transcriptase was added. The reaction mixture was incubated for 5 min at 25°C followed by 60 min at 42°C. The reaction was terminated by heating at 70°C for 5 min and then cooled to 4°C. cDNA samples were checked by PCR and stored at -20°C for further use.

### 6.5.11 Real-time PCR

Real-time PCR was used to quantify the expression level of all the flagella-related genes and was performed with Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas, Fisher Scientific, Erembodegem, Belgium) as described previously (Yang and Defoirdt, 2014). The reaction was performed in an StepOne™ Real-Time PCR System thermal cycler (Applied Biosystems, Gent, Belgium) in a total volume of 25 $\mu$l, containing 12.5 $\mu$l of 2× SYBR green master mix, 300 nM of forward and reverse primers and 2 $\mu$l of template cDNA. The thermal cycling consisted an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15s and primer annealing and elongation at 60°C for 1 min. Dissociation curve analysis was performed to check for the amplification of untargeted fragments. Data acquisition was performed with the StepOne™ Software.

### 6.5.12 Real-time PCR data analysis ($2^{-\Delta\Delta ct}$ method)

The real-time PCR was validated by amplifying serial dilutions of cDNA synthesized from 1 $\mu$g of RNA isolated from bacterial samples. Serial dilutions of cDNA were amplified by real time PCR using gene specific primers. $\Delta C_T$ (average $C_T$ value of target-average $C_T$ value of *rpoA*) was calculated for the different dilutions and plotted against the cDNA concentration. The slope of the graph was almost equal to 0 for all of the target nine genes. Therefore, the amplification efficiency of reference and the target genes was considered to be equal. Based on this precondition, real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). The expression of the target genes was normalized to the endogenous control (*rpoA*) by calculating $\Delta C_T$ :

$$\Delta C_t = C_{t\ target} - C_{t\ rpoA}$$

and expressed relative to a calibrator strain by calculating $\Delta\Delta C_t$:

$$\Delta\Delta C_t = \Delta C_t - C_{t\ calibratior}$$

Strain BB120 without any treatments was used as acalibrator. The relative expression

was then calculated as

$$\text{Relative expression} = 2^{-\Delta\Delta Ct}$$

### 6.5.13 Axenic hatching of brine shrimp larvae

Two hundred milligrams of high-quality hatching cysts of *Artemia franciscana* (EG® Type; INVE Aquaculture, Baasrode, Belgium) were hydrated in 18 ml of filtersterilized tap water for 1 h. Sterile cysts were obtained by decapsulation based on the method described by Marques *et al*. (2004). Briefly, 660 $\mu$l of NaOH (32%) and 10 ml of NaOCl (50%) were added to the hydrated cyst suspension to facilitate decapsulation. The process was stopped after 2 min by adding 14 ml of $Na_2S_2O_3$ (10 g $L^{-1}$). Filtered (0.22 $\mu$m) aeration was provided during the reaction. The decapsulated cysts were washed with filtered (passed through 0.22-$\mu$m membrane filter) and autoclaved (moist heat at 121°C for 15 min) artificle seawater (containing 35 g $l^{-1}$ of instant ocean synthetic sea salt, Aquarium Systems, Sarrebourg, France). The cysts were resuspended in a 50-ml tube containing 30 ml of filtered, autoclaved seawater and hatched for 28 h on a rotor (4 $min^{-1}$) at 28°C with constant illumination (*c.* 2000 lux). The axenity of cysts was verified by inoculating one ml of culture water into 9 ml of Marine broth and incubating at 28°C for 24 h. After 28 h of hatching, batches of 30 larvae were counted and transferred to fresh, sterile 50-ml tubes containing 30 ml of filtered and autoclaved seawater. Finally, the tubes were returned to the rotor and kept at 28°C. All manipulations were performed in a laminar flow to maintain sterility of the cysts and larvae.

### 6.5.14 Brine shrimp challenge test

The effects of the catecholamines and antagonists on the virulence of *V. harveyi* were determined in a standardized challenge test with gnotobiotic brine shrimp larvae. *V. harveyi* was incubated with or without norepinephrine or dopamine (50 $\mu$M) and with or without antagonists, and cultures were washed with phosphate-buffered saline (pH 7.4) prior to inoculation into the brine shrimp rearing water at $10^5$ CFU $ml^{-1}$. The challenge tests were performed as described by Defoirdt *et al*. (2005) with some modifications. A suspension of autoclaved LVS3 bacteria (Verschuere *et al*., 1999) in

filtered and autoclaved seawater was added as feed at the start of the challenge test at $10^7$ cells $ml^{-1}$ culture water. Brine shrimp cultures to which only autoclaved LVS3 bacteria were added as feed, were used as controls. The survival of the larvae was counted 48 h after the addition of the pathogens. Each treatment was carried out in quadruplicate and each experiment was repeated twice to verify the reproducibility. In each test, the sterility of the control treatments were checked at the end of the challenge by inoculating 1 ml of rearing water to 9 ml of Marine Broth and incubating the mixture for 2 days at 28°C.

### 6.5.15 Statistical analyses

Data analysis was carried out using the SPSS statistical software (version 15). Log transformed gene expression data were analysed using independent samples *t*- tests. Unless stated otherwise, all other data were compared with one-way ANOVA, followed by Tukey's *post hoc* test.

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# CHAPTER VII

## GENERAL DISCUSSION, CONCLUSION AND FUTURE PERSPECTIVES

## 7.1 INTRODUCTION

The United Nation's Food and Agriculture Organization (FAO) considers aquaculture as the fastest-growing food-producing industry worldwide. However, disease outbreaks are considered to be a significant constraint to the development of the sector (FAO, 2014). In this study, we focus on disease caused by *Vibrio harveyi*, which is an important pathogen in aquaculture that can affect almost all types of cultured animals. Due to the frequent and excessive use of antibiotics, aquaculture pathogens (including *V. harveyi*) have acquired (multiple) antibiotic resistance, rendering the antibiotic treatments less effective in some cases. Additionally, massive use of antibiotics in animal production also constitutes a threat to human health and to the environment (Cabello *et al.,* 2006), and this has resulted in more strict regulations with respect to antibiotic use. One notable example is the ban on the use of antibiotics as growth promoters in animal production in Europe in 2006 (European Parliament and Council Regulation No 1831/2003). As a consequence, the development of novel strategies to control bacterial diseases, both in human and veterinary medicine will be critically important in order to ensure public health and food security in the future. One of the novel alternative strategies to replace antibiotic use in aquaculture is preventing the pathogenic bacteria from attacking the host without the need to kill them, which is named antivirulence therapy. Consequently, there is a need for thoroughly understanding the pathogen-pathogen and host-pathogen interactions, in order to develop effective antivirulence therapies.

This study addressed the role of cell-to-cell signaling and sensing of host factors on the virulence of *V. harveyi* in the gnotobiotic brine shrimp larvae model system. We investigated the regulation of virulence-related factors by quorum sensing in *V. harveyi*, and also determined the effect of quorum sensing disruption in this host-pathogen system by the application of some novel quorum sensing inhibitors. Furthermore, this study addressed the role of indole signaling on the virulence of *V. harveyi*. In addition to bacterial cell-to-cell signaling, the present study also focused on sensing of host factors and its impact on the virulence of *V. harveyi*. In this chapter, the most important findings of this work are highlighted and discussed, and some

directions for future research are presented.

## 7.2 THE IMPACT OF QUORUM SENSING ON FLAGELLAR MOTILITY – AN ESSENTIAL VIRULENCE FACTOR IN *V. harveyi*

*V. harveyi* is one of the model organisms in studies on bacterial quorum sensing (Ng and Bassler, 2009). Unlike most other Gram-negative bacteria, *V. harveyi* has been found to use a three-channel quorum sensing system, which is mediated by three different types of signal molecules, including Harveyi Autoinducer 1 (HAI-1), Autoinducer 2 (AI-2), and Cholerae Autoinducer 1 (CAI-1). These three autoinducers are detected at the cell surface by their respective receptors that feed a shared phosphorylation/dephosphorylation signal transduction cascade. *V. harveyi* quorum sensing system has been found to control the bioluminescence (Bassler *et al.*, 1997) and the production of several virulence factors (**Figure 7.1**). However, most of these virulence factors have been reported to be negatively regulated by quorum sensing, i.e. their production decreases with increasing levels of the signal molecules (Natrah et al., 2011). This appears to be in conflict with the fact that quorum sensing is required for full virulence of *V. harveyi* towards different hosts (Defoirdt *et al.,* 2005; Pande *et al.*, 2013). In order to further characterise why quorum sensing is required for full virulence of *V. harveyi*, in **Chapter 3**, we aimed to identify more virulence factors that are positively regulated by quorum sensing in *V. harveyi.*

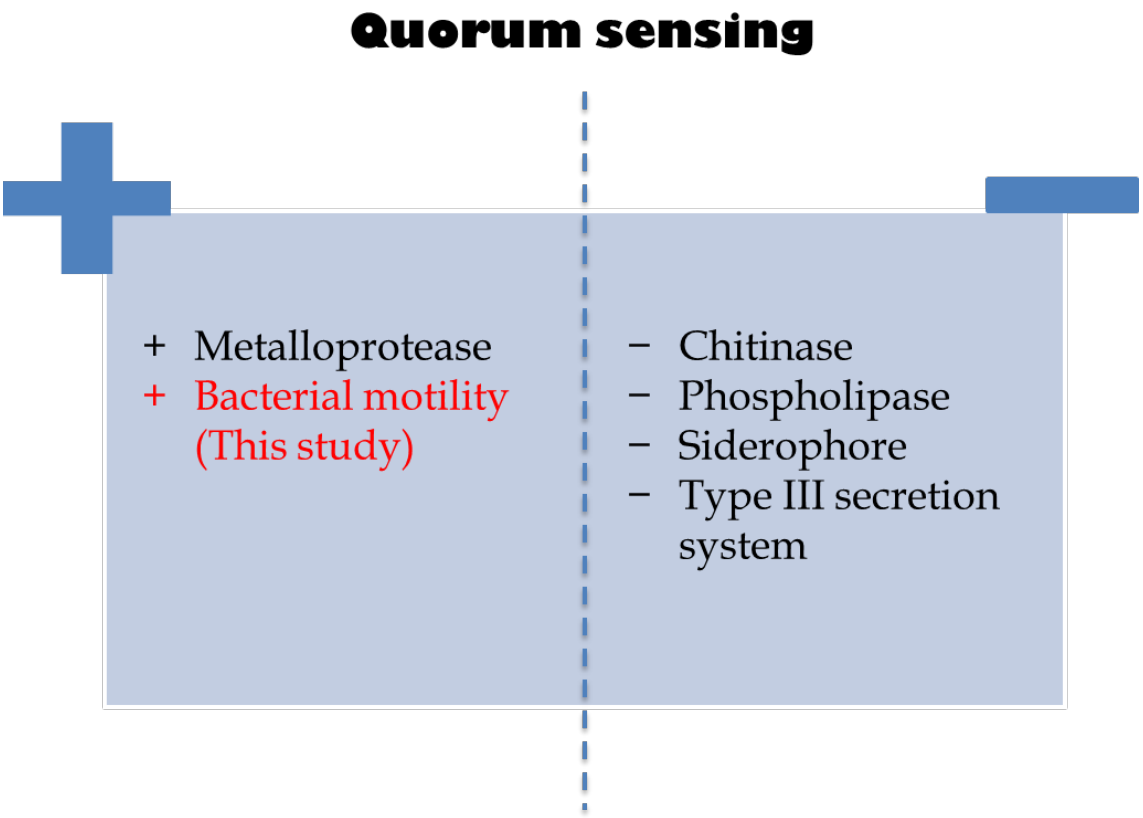


**Figure 7.1** Overview of the effect of quorum sensing on virulence factor production in *V. harveyi.* **Left**: virulence factors that are positively regulated by quorum sensing (i.e. production increases with increasing signal molecule levels); **Right**: virulence factors that are negatively regulated by quorum sensing (i.e. production decreases with increasing levels of signal molecules)

Bacterial motility is considered to be an important virulence factor in many pathogens. It plays an essential role mostly in the initial phases of infection, as it facilitates the attachment to the host by helping pathogens to overcome repulsive forces between the bacterial cell and the host tissues and enables direct contact between pathogen and host (McCarter, 2001). In **Chapter 3**, we examined the effect of quorum sensing on swimming motility of *V. harveyi*, using the wild type strain BB120 (ATCC BAA-1116) and various quorum sensing mutants derived from this strain, and we found that swimming motility is positively regulated by quorum sensing with the involvement of the quorum sensing master regulator LuxR.

Quorum sensing regulation differs for different virulence factors, with some being positively regulated, while others being either negatively regulated or independent of quorum sensing. It has also been reported in other aquaculture pathogens such as *Aeromonas* sp. and *Edwardsiella* sp. (Natrah *et al.*, 2011). The different types of

regulation probably reflect the need to express different virulence factors at different infectious stages. For example, virulence factors that are negatively regulated by quorum sensing might be predominantly required during initial infection, whereas virulence factors that are positively regulated might be also required at later stages. However, little literature data are available to support this hypothesis.

Through a further series of experiments at the transcriptional level, we confirmed that quorum sensing positively regulates motility by affecting the expression of flagellar biosynthesis genes. Flagella are the organelles that mediate bacterial motility, and a number of tightly regulated genes have been identified to encode flagellar systems (McCarter, 2001). Quorum sensing in *V. harveyi* was found to regulate not only the expression of the structural components of flagella, but also the expression of master transcriptional activators and regulators. Importantly, the quorum sensing regulation of flagellar motility apparently differs between bacterial species. For instance, in contrast to *V. harveyi*, quorum sensing was found to repress motility in other bacteria such as *V. cholerae* (Zhu *et al*., 2002) *V. fisheri* (Lupp and Ruby, 2005), and *V. parahaemolyticus* (Gode-Potratz and McCarter, 2011). These differences in the regulation of genes by quorum sensing might reflect differences with respect to the life styles of these bacteria (Hammer and Bassler, 2003).

Finally, we demonstrated that a flagellar motility inhibitor could completely inhibit the swimming motility and significantly increased the survival of brine shrimp larvae challenged with *V. harveyi.* Further, it has been reported that JAF548 (QS-), which was found to have a significantly decreased motility in our study, also showed a significantly decreased virulence towards brine shrimp larvae (Defoirdt *et al.*, 2005). The relative contribution of the three channels is dependent on the environmental conditions (Defoirdt *et al.*, 2008). All the single synthase mutants tested in this study showed a significantly lower motility compared with the wild type, with the strongest impact being observed in the HAI-1 deficient mutant. This is consistent with the relative importance of the signal molecules as observed *in vitro* grown cells (Henke and Bassler, 2004b). However, the three channels have a different impact on virulence of *V. harveyi* towards different hosts. Defoirdt *et al.* (2005; 2012) reported that inactivation of AI-2 and CAI-1 signal molecules resulted in reduced virulence of *V.*

*harveyi* towards brine shrimp larvae, whereas inactivation of HAI-1 had no effect. A recent research by Pande *et al.* (2013) revealed that HAI-1 and AI-2 deficient mutants were significantly less virulent than the wild type towards commercially important giant freshwater prawn (*Macrobrachium rosenbergii*), while CAI-1 deficient mutant has no effect on this host. This could be explained by the fact that *V. harveyi* quorum sensing system has been described as a three-way detector, with the expression of quorum sensing-regulated genes being proportional to the levels of the three signal molecules (Henke and Bassler, 2004b). The detection of two types of signal molecules results in sufficient expression levels of the quorum sensing master regulator LuxR, which allows production of the virulence factors that are essential to kill the host, whereas the LuxR expression level in the presence of only one of these two signal molecules is not (Deforidt *et al.,* 2012).

To our knowledge, this is the first report demonstrating that quorum sensing controls the virulence of *V. harveyi* by affecting swimming motility.

## 7.3 APPLICATION OF QUORUM SENSING INHIBITORS TO PROTECT AQUACULTURE ANIMALS FROM DISEASE

It is proposed that disease is the outcome of an imbalance between host, pathogen and environment. A holistic strategy, which takes into account the different aspects of the pathogen-host-environment continuum, will be the preferred approach for preventing and controlling bacterial disease in aquaculture (**Figure 7.2**).

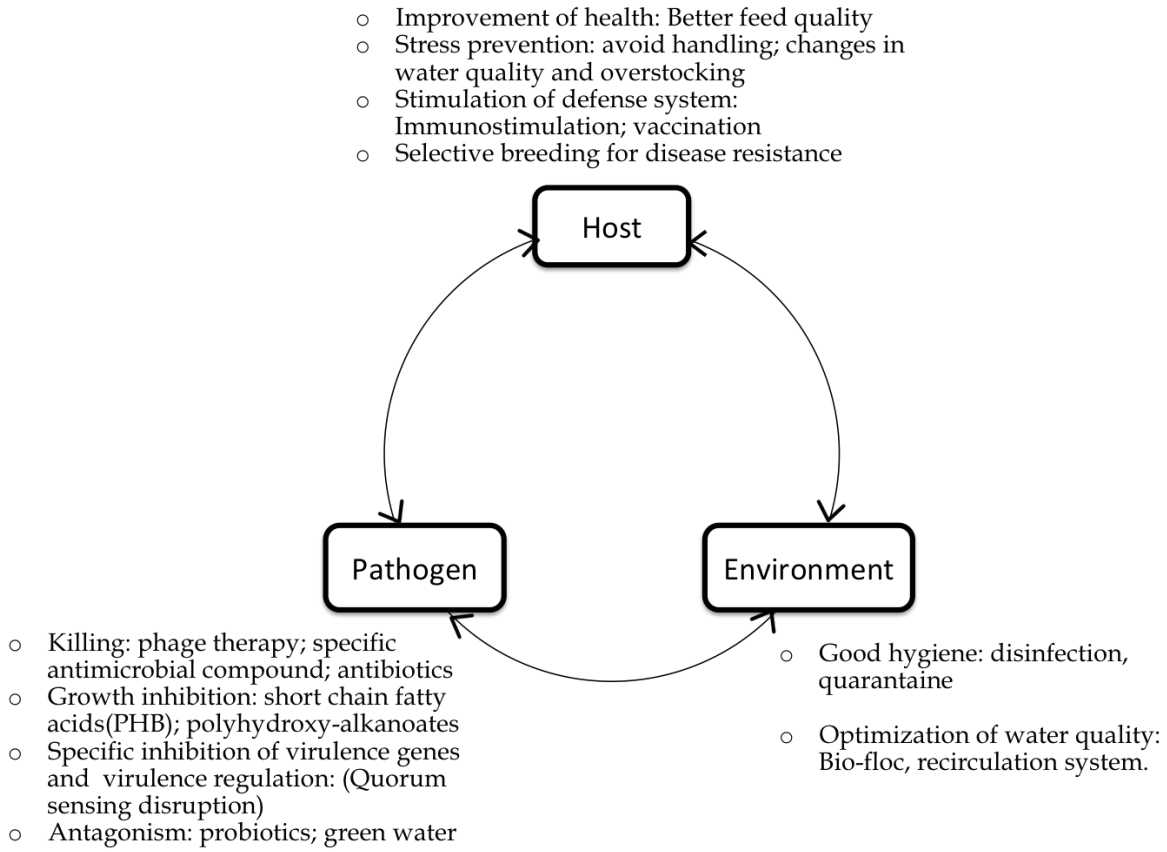


**Figure 7.2.** Schematic overview of different strategies to prevent and control bacterial disease in aquaculture. Redrawn after Defoirdt *et al*. (2011).

In this study, we focused on strategies directed towards the pathogens. Traditional approaches to combat bacterial infection mostly rely on the disruption of the growth cycle by preventing the synthesis and assembly of some key components in bacterial processes, such as cell wall synthesis, DNA replication and protein synthesis (Walsh, 2003). However, the inappropriate and indiscriminate antibiotic treatment can lead to resistant organisms in a short period of time (Rasko and Sperandio, 2010). Currently, many alternative approaches jointly termed antivirulence therapy, are being explored. These approaches are based on the inhibition of virulence rather than of bacterial growth.

In **Chapter 4**, we evaluated the potential capabilities of 20 novel thiophenones to inhibit quorum sensing in *V. harveyi* and to protect brine shrimp larvae from *V. harveyi.* Most of the compounds were able to interfere with quorum sensing-controlled

bioluminescence in *V. harveyi*. One of the factors that facilitated the development of quorum sensing research is the exploitation of signal molecule reporter strains, which demonstrate a certain phenotype responding to quorum sensing molecules. However, there is an important limitation of the use of such reporter strains, which is that the quorum sensing-controlled phenotypes usually also depend on other factors and/or on the metabolic activity of the cells and this can result in false positives (Defoirdt *et al.*, 2013). For instance, the apparent quorum sensing-inhibitory ability of pyrogallol, which is a compound previously claimed as an AI-2 quorum sensing inhibitor, was recently found to be a side effect of its toxicity rather than true quorum sensing blockage (Defoirdt *et al.*, 2013). Therefore, adequate control experiments are always required. In this study, to solve this problem of false positives, we proposed a new parameter to represent specific quorum sensing-inhibitory activity ($A_{QSI}$), which is defined as the ratio between inhibition of quorum sensing-inhibitory activity (in this case bioluminescence) and inhibition of the same activity when it is independent of quorum sensing. We set the threshold to exclude compounds at an $A_{QSI}$ of 2 (i.e. if $A_{QSI}$ was below 2, then the activity was considered to be not due to quorum sensing inhibition), and considered compounds to be strong inhibitors if $A_{QSI}$ was above 10. This approach allowed us to identify 5 compounds as false positives in the present study. Several other strategies to strengthen the evidence for quorum sensing inhibition have been proposed, such as (i) verify that the candidate QSI show no effect on the reporter phenotype when it is independent of quorum sensing; (ii) verify the impact of candidate QSI on additional phenotypes that are mediated by quorum sensing; (iii) perform a transcriptomic analysis to confirm that the candidate QSI selectively inhibits quorum sensing-regulated genes; and (iv) try to identify the molecular target of the candidate QSI compound (Defoirdt *et al.*, 2013). Another essential experiment to confirm a QSI compound is the assessment of toxicity of putative QSI towards bacterial cell. However, even the most sensitive test (e.g. determination of growth kinetics) can miss significant toxic effects (Defoirdt *et al.*, 2013). Therefore, to exclude the possibility that inhibition of quorum sensing-regulated phenotype is due to toxicity of a test compound, alternative sensitive methods to evaluate toxicity of putative QSI should be used, such as constitutive enzyme activity (Defoirdt *et al.*, 2013), metabolic activity staining (Vikram *et al.*, 2010), and viability

staining (Vandeputte *et al.*, 2011). We think that the use of the new parameter $A_{QSI}$ is a straightforward and elegant way to exclude false positives by taking into account side effects related to the use of quorum sensing molecule reporters.

We further determined the therapeutic potential of the compounds in the brine shrimp model system, and found that 9 thiophenones showed a promising therapeutic potential (at least 10 fold differences between toxic and active concentration). Correlation analysis revealed that the quorum sensing-inhibitory activity largely determines the protective effect of these compounds to brine shrimp larvae, suggesting their promising potential as antivirulence agents to combat bacterial infections. However, safety is always a concern when introducing new therapeutics. It is believed that the antivirulence therapies have reduced toxicity and less severe side effects than antibiotic treatments, as most of the antivirulence therapies are targeted to virulence factors or regulation pathways that only exist in pathogens and thus there will be less influence on the host. Nevertheless, it is possible that these compounds are degraded by the pathogens and/or produce secondary metabolites that have negative effects on the host (Rasko and Sperandio, 2010). Therefore, the potential side effects of these compounds should be carefully determined before they are released into the market. In this study, we found that there was a strong positive correlation between toxicity of the thiophenones towards brine shrimp larvae and toxicity towards *V. harveyi*, which means that the toxicity to *V. harveyi* could be used as a good indicator for toxicity to higher organisms.

Furthermore, the antivirulence therapy might also target similar pathways or factors in the normal microbiota (Defoirdt *et al.*, 2013). An interesting strategy to overcome this is to create antimicrobial peptides (AMPs) using the chemical structure of a signal molecule. A Specifically Targeted AntiMicrobial Peptide (STAMP) can be generated by the addition of a targeting peptide (e.g. antibody, signaling peptide) to an existing broad-spectrum AMP (Grandclément *et al.*, 2015). This approach is a promising way to eliminate specific pathogens while preserving the existing healthy flora. For example, *Streptococcus mutans*, which can produce competence-stimulating peptide (CSP), is considered as a primary pathogen involved in the formation of dental caries. Eckert *et al.* (2006) used CSP as a STAMP targeting domain to mediate *S.*

*mutans*-specific delivery of an antimicrobial peptide domain, and this class of STAMP is capable of selectively eliminating *S. mutans* from multispecies biofilms without affecting closly related noncariogenic oral bacteria.

For a long time, it was generally assumed that pathogens are unlikely to develop resistance to quorum sensing disruption as it engenders only a milder evolutionary pressure. Recently, Defoirdt *et al.*, (2010) challenged this assumption and argued that there is also a risk of resistance development using quorum sensing inhibitors. The authors provided an overview of data indicating that there can be variation in the expression of quorum sensing core genes among different strains of a certain species. These core genes are involved in the production of signal molecule synthases and receptors, as well as in quorum sensing signal transduction. Since the variability of core genes can be caused by horizontal gene transfer, if this variation is associated with a difference in fitness under treatment of quorum sensing inhibitors, natural selection would automatically result (Defoirdt *et al.*, 2010). Furthermore, the same report also suggested that it is critically important to correctly evaluate the effect of quorum sensing disruption on the fitness of bacteria, i.e. growth tests should be performed under conditions that are as similar as possible to the clinical situation instead of in nutrient-rich synthetic growth media (Martinez *et al.*, 2007).

The first demonstration of resistance evolvement in cells to quorum sensing disruption was by Maeda *et al.* (2012) using both realistic lab constructs and clinical strains. It was shown that cells could develop resistance to quorum sensing disruption compounds through different mechanisms, and that these mutations also occur in a clinical setting, which indicates that cells can definitely evolve resistance even in the absence of previous exposure to them (Maeda *et al.* 2012). Additional clinical evidence of the ability of resistance development in strains was provided by García-Contreras *et al.* (2013), demonstrating that Mexican clinical isolates from urine, blood and catheter tip specimens from children showed resistance to brominated furanone (C-30) and to 5-fluorouracil. More recently, quorum sensing was found to enhance the stress tolerance of *Pseudomonas aeruginosa* to oxidative stress, osmotic, thermal and heavy metals, and the link between quorum sensing and stress was shown to have important physiological and ecological consequences, including

allowing the development of quorum sensing disruption resistance in cells and preventing social cheating (García-Contreras *et al.*, 2015).

Further investigations are also required to determine the possibility of resistance to more quorum sensing disruption compounds, such as signal-degrading enzymes. Future investigations on the therapeutic development of antivirulence strategies should be proceed with more care and caution to avoid the undesired fate currently associated with antibiotic development.

## 7.4 INDOLE SIGNALING IN *V. harveyi*

Increasing evidences suggest that the bacterial metabolite indole can also act as a signal molecule in some bacteria. It has even been considered to be an interkingdom signal in commensal bacteria (Bansal *et al.*, 2010). The production of indole is observed in over 85 bacterial species including both Gram-negative and Gram-positive bacteria ( Lee and Lee, 2010). Even many bacterial species that cannot produce indole will respond to the presence of extracellular indole, and exhibit several changes in behavior (**Figure 7.3**). In addition, several indole derivatives have also been reported to affect bacterial behaviors, and indole signaling has been employed for the design of small molecules that have the potential to control bacterial virulence and lead to the potential discovery of new therapeutics (Melander *et al.*, 2014).

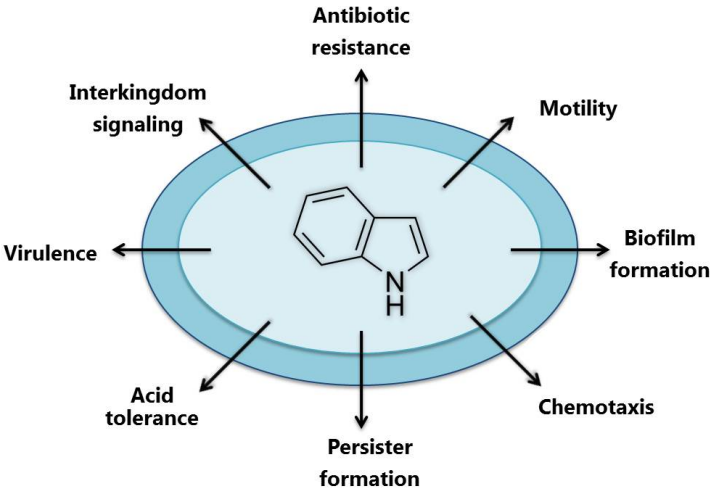


**Figure 7.3**. Overview of bacterial processes controlled by indole

In this study, we determined the impact of indole on the virulence of *V. harveyi* in **Chapter 5**. We demonstrated that addition of indole (50 μM or more) significantly decreased the virulence of *V. harveyi* towards both gnotobiotic brine shrimp larvae and conventionally reared giant river prawn larvae. This concentration is similar to the concentration produced by wild type *V. harveyi* when grown in Luria-Bertani broth, hence, doubling the concentration of extracellular indole results in decreased virulence. Since indole has been found to exert a beneficial effect on higher animals (Bansal *et al*., 2010), we pretreated the pathogen with indole in order to exclude a direct effect of indole on the host. Our findings are in accordance with previous report which has revealed that indole can protect sea bass (*Dicentrarchus labrax*) larvae from *V. anguillarum* (Li *et al*., 2014). Therefore, indole decreasing virulence seems to be conserved among distantly related vibrios that are pathogenic to aquatic organisms. Subsequently, we indicated that indole could decrease production of several major virulence factors in *V. harveyi*, including biofilm formation, exopolysaccharide production and swimming motility. These factors have been reported to play a key role in bacterial adherence and in colonization to epithelial cells that are importance for infection (Bansal *et al*., 2007).

Although indole production has been found in many species, the complex pathways by which indole exerts its effects are far from understood, even in the most intensively studied species, *E. coli.* It has been revealed that the *E. coli* LuxR-homologue SdiA is involved in indole signaling, however, there is no proof of the direct binding of indole and SdiA, and the interactions between indole and SdiA still remain to be investigated further ( Lee *et al*., 2007). In another report, Hirakawa *et al.* (2005) demonstrated that indole-induced drug resistance in *E. coli* was mediated by the two-component systems BaeSR and CpxAR. In vibrio species, indole was hypothesised to act through the RNA polymerase regulator DksA in *V. cholerae* (Beyhan *et al*., 2009). The *V. harveyi* ATCC BAA-1116 genome does not contain a homologue of *sdiA*, but it does contain a *dksA* homologue that shows 82% identity at nucleotide level with *V. cholerae dksA*. However, we were not able to obtain a *dksA* deletion mutant in several attempts for further investigation (collaboration with Gary Vora, US Naval Research Institute, Washington DC), and this might indicate that this gene is essential in *V. harveyi*.

Further, in this study, we found a link between indole sensing and other regulatory mechanisms in *V. harveyi.* According to the results, we hypothesized that indole could induce a stress response by inducing expression of the alternative sigma factor RpoS, which is known to be related to stress. Further we found that RpoS down-regulated indole production, which might be a feedback mechanism to maintain homeostasis. These results are consistent with what has been observed in another important aquaculture pathogen, *V. anguillarum* (Li *et al.*, 2014). In addition to this, indole was found to interfere with the three-channel quorum sensing system in *V. harveyi* by interacting with signal transduction. This could also in part explain the decreased virulence towards different hosts since quorum sensing is required for full virulence of *V. harveyi* in these model systems (Defoirdt and Sorgeloos, 2012).

Finally, we demonstrated that the auxin hormones indole-3-acetic acid and indole-3-acetamide, which are indole analogues produced by plants including micro-algae, showed similar effects as indole on the virulence of *V. harveyi.* To our knowledge, this is the first report about the effect of auxins produced by micro-algae on aquatic bacteria. Furthermore, it has been reported that there is a lower incidence of disease in the green water aquaculture system, which means animals are cultured in water containing high levels of micro-algae (Natrah *et al.*, 2014). According to our findings in this study, this might be explained by the presence of (relatively) high levels of indole analogues in the digestive tract of animals that have ingested high levels of micro-algae. It also could be an interesting novel strategy to control bacterial disease in aquaculture by selecting micro-algae that are capable of producing high levels of auxins.

## 7.5 SENSING OF CATECHOLAMINE STRESS HORMONES PRODUCED BY THE HOST INCREASES THE VIRULENCE OF *V. harveyi*

Host stress has long been known to influence the outcome of many bacterial infections. During a stress reaction, stress hormones such as glucocorticoids and catecholamines, are released by many higher organisms (Verbrugghe *et al.*, 2012). In addition to the effects of these stress hormones on the host immune system, research over the past decades has revealed that catecholamines can also alter the growth and

virulence of several pathogens and consequently influence the pathogen-host interactions. This new perspective leads to the development of a new research field termed microbial endocrinology, which is regarded as a means of understanding the dialogue between animals and their microflora (Sharaff and Freestone, 2011).

In **Chapter 6**, we evaluated the impact of two kinds of catecholamines, norepinephrine and dopamine, on the growth and virulence of *V. harveyi.* Results indicated that both catecholamines could significantly increase the growth of *V. harveyi* in serum-supplemented medium, which might be attributed to the capacity of catecholamines to supply the pathogen of iron. The increased availability of iron that is supplied through the intervention of catecholamines probably plays a key role in the effects of stress on the outcome of an infection (Verbrugghe *et al.*, 2012). In addition to increase iron uptake in a direct way, we also found that both norepinephrine and dopamine could significantly induce siderophore production, which has been reported to be able to internalize the iron removed by catecholamines (Freestone *et al.*, 2003).

Further, we found that both norepinephrine and dopamine can enhance the production of some virulence factors in *V. harveyi* that are essential for colonization and adhesion to the host surfaces*,* such as swimming motility, exopolysaccharide production and biofilm formation. As part of the 'fight or flight' response, stress in a host will increase the systemic catecholamine levels (Freestone *et al.*, 2008). Our results in this study suggested that the increasing concentration of catecholamines might be indicator informing the pathogen that the host is less fit and it is time to relocate to a suitable one.

In animals, catecholamines have been found to exert their effects by binding to specific adrenergic and dopaminergic receptors, and the binding can be blocked by antagonists specific to the catecholamine receptors (Freestone *et al.*, 1999). In the present study, the results demonstrated that α- but not β-adrenergic receptor antagonists could block responses to norepinephrine, but did not show any effects on dopamine responsiveness. On the contrary, dopaminergic receptor antagonists neutralized induction caused by dopamine, but did not neutralize induction by norepinephrine. This suggests that bacterial response system for catecholamine

recognition shows a degree of specificity similar to that demonstrated for catecholamine receptors in animals.

In terms of a bacterial catecholamine receptor, there are so far only few reports examining the existence of such receptors. It has also been revealed that these receptors show large difference depending on the type of pathogen, the host or the type of stress hormone (Sharaff and Freestone, 2011). For instance, *Escherichia coli* O157:H7 and *Salmonella enterica* have been reported to sense norepinephrine and epinephrine as quorum sensing signals through histidine sensor kinases (QseC and QseE), while norepinephrine was found to enhance the growth and virulence of *Pseudomonas aeruginosa* through the *las* quorum sensing pathway (Hughes *et al.*, 2009; Pullinger *et al.*, 2010; Hegde *et al.*, 2009). In this study, a QseC antagonist, LED209 was found to be able to neutralize the effects of both norepinephrine and dopamine, indicating that a similar response system for catecholamines might exist in *V. harveyi*. This is also substantiated by the fact that the *V. harveyi* ATCC BAA-1116 genome contains a QseC homolog. Furthermore, catecholamines have been found to regulate virulence gene expression and enhance the potential to cause disease in all these species mentioned above. Therefore, these receptors could be considered as regulators of multiple virulence factors, making them a possible target for antivirulence therapy to combat bacterial infections.

The fact that stress hormones can influence the outcome of an infection has also been confirmed by *in vivo* test. However, such studies are scarce, and contradictory results have been reported (Cray *et al.*, 1998; Kudahl *et al.*, 2007; Dutta *et al.*, 2009; Wesley *et al.*, 2009). In order to obtain a better understanding of the interactions between the host immune system and pathogen, it is of utmost importance that (highly controlled) animal models are created and more *in vivo* tests are conducted to investigate the effects of stress hormones on bacterial infections in different hosts. In this study, we investigated the impact of norepinephrine and dopamine and the respective receptor antagonists on the virulence of *V. harveyi* in our highly controlled brine shrimp larvae system. Significantly higher brine shrimp mortality was observed when exposing *V. harveyi* to norepinephrine or dopamine prior to the challenge, and the induced virulence could be neutralized by specific antagonists. However, pretreatment of *V.*

*harveyi* with the antagonists had no effect on virulence in the absence of catecholamines. Furthermore, previous work in our lab has also confirmed the increased virulence of *V. harveyi* by catecholamines in giant freshwater prawn larvae (Pande *et al.*, 2014).

## 7.6 CONCLUSIONS

- This study revealed an important mechanism by which quorum sensing controls the virulence of *V. harveyi* by demonstrating (i) that quorum sensing positively regulates motility by affecting flagellar biosynthesis, (ii) that LuxR is involved in the regulation of motility and (iii) that flagellar motility significantly affects virulence of *V. harveyi* in our gnotobiotic brine shrimp model.
- In this study we determined the quorum sensing-inhibiting activities of 20 new synthetic thiophenones towards the quorum sensing model bacterium *V. harveyi*, and 6 thiophenones were identified to be able to inhibit quorum sensing at submicromolecular levels. There was a strong positive correlation between the specific quorum sensing-disrupting activity of the thiophenones and the protection of brine shrimp larvae against pathogenic *V. harveyi*, and 6 quorum sensing-disrupting thiophenones were considered to be highly promising.
- We proposed a new parameter, $A_{QSI}$, to determine specific quorum sensing inhibitory activity based on experiments with a quorum sensing reporter strain. This parameter allowed us to exclude 5 false positives out of the 17 thiophenone compounds that were able to inhibit bioluminescence in *V. harveyi.* We think that the use of this new parameter is a straightforward and elegant way to exclude false positives by taking into account side effects related to the use of quorum sensing molecule reporters.
- This study demonstrated for the first time that indole sensing could reduce the virulence of *V. harveyi* towards gnotobiotic brine shrimp larvae and conventionally reared giant river prawn larvae. Indole decreased the production of several virulence factors in *V. harveyi*, including biofilm formation, exopolysaccharide production and swimming motility.
- The alternative sigma factor RpoS was found to be involved in the production of indole in *V. harveyi*, and indole could interfere with the three-channel quorum

sensing system of *V. harveyi.*

- Auxin hormones that are produced by micro-algae showed similar effects as indole, suggesting that the selection of micro-algae that can produce high levels of auxins could be an interesting novel strategy to control bacterial disease in aquaculture.
- The catecholamines norepinephrine and dopamine increased the virulence of *V. harveyi* by increasing swimming motility, biofilm formation, exopolysaccharide production and growth in environments with low iron availability. These effects could be neutralized by antagonists for eukaryotic catecholamine receptors and the bacterial catecholamine receptor antagonist LED209.
- Different effects of the adrenergic and dopaminergic receptors antagonists indicate the presence of specific sensing systems for different catecholamines in *V. harveyi*.

## 7.7 FUTURE PERSPECTIVES

The present study demonstrated that the flagellar motility significantly affects virulence of *V. harveyi* in our gnotobiotic brine shrimp model. Additionally, flagellar motility has also been considered as an important virulence factor in numerous pathogens. Can flagellar motility serve as a therapeutic or vaccine target? To our knowledge, little practical work has been done to evaluate motility as a therapeutic target. Tsutsui *et al*., (2000) reported that a proton pump inhibitor (PPI), which could markedly inhibit the motility of *Helicobacter pylori,* has already been widely used for treatment of ulcers in humans. In another report, flagellin proved to be a specific and efficient antigen to inhibit infection of *V. cholerae* towards rabbits (Yancey *et al*., 1979). In addition, flagellar preparations of *Pseudomonas aeruginosa* were used for the development of a vaccine formula for cystic fibrosis patients, which has been proven to be effective and safe both in animal models and human volunteers (Holder and Naglich, 1986; Doring and Dorner, 1997). However, flagella and motility have not been intensively studied as therapeutic target in aquaculture so far. Due to the apparent role of bacterial flagellin as an immunomodulatory agent, it might represent one promising ingredient in composite subunit vaccines (Josenhans and Suerbaum, 2002).

To date, most research with respect to exploring novel antivirulence strategies to combat bacterial infections in aquaculture have focused on the pathogen-pathogen signaling. Quorum sensing disruption has been considered to be an effective strategy to control diseases caused by various pathogens. Application of compounds with quorum sensing-inhibitory activity are one of the promising tools for quorum sensing disruption. However, several questions remain to be answered, e.g. whether it is possible to control the pathogen without affecting the normal microbiota. Additionally, although most of the antivirulence agents have been tested both *in vitro* and *in vivo*, large-scale trials are still required to prove their safety in real aquaculture environments before bringing them into practical applications. Furthermore, increasing evidence suggests that pathogens will probably develop resistance to antivirulence agent as well. Therefore, it is important to further develop different strategies that could be used synergistically to maximize the chance of successfully protecting the animals.

The development of alternative antivirulence therapies will require continued investigations to obtain a better understanding of the bacterial virulence strategies, signaling pathways and potential ways to exploit them. In addition to quorum sensing, more pathogenicity mechanisms in bacterial pathogens have been unraveled to be potential targets for antivirulence therapy. For example, host-pathogen signaling mediated by catecholamine stress hormones. In this study, we demonstrated that catecholamines norepinephrine and dopamine could significantly enhance the virulence of *V. harveyi,* and specific sensing systems for different catecholamines seem to be present in *V. harveyi*. In the future, there is a need to identify bacterial adrenergic receptors and improve our understanding on why there are multiple sensors responding to one or more signals. Perhaps there is a specialized need for certain receptors in specific niches.

Finally, it would also be interesting to search new interkingdom communication mechanisms, in order to provide not only new biological insights into pathogenicity but also novel strategy for antivirulence research. For example, *V. anguillarum* was found to be affected by sensing of several host factors (e.g. mucin, bile salts and cholesterol) and subsequently showed a decreased virulence towards see bass larvae.

# Appendix A

## REFERENCES

# REFERENCES

## A

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# Appendix B

## SUMMARY/SAMENVATTING

## SUMMARY

*Vibrio harveyi* is amongst the most significant pathogens in the larviculture and aquaculture industry. It is able to infect a wide range of marine vertebrates and invertebrates, causing significant losses to the aquaculture industry worldwide. The pathogenicity mechanism of *V. harveyi* is not yet completely understood. The inhibition of the production of virulence factors that are required to cause disease, i.e. antivirulence therapy, has been proposed as a novel strategy to control bacterial infections. The production of virulence factors in *V. harveyi* is under strict regulatory control, and one of the regulatory mechanisms is quorum sensing, bacterial cell-to-cell communication. Disruption of quorum sensing is the most intensively studied strategy to inhibit virulence factor production. In this work, we evaluated the impact of pathogen-pathogen signaling and sensing of host factors on the virulence of *V. harveyi* in a model system with gnotobiotic brine shrimp larvae.

First, a literature research was done on the current knowledge on *V. harveyi*, including the virulence, pathogenesis, and the regulatory mechanisms of virulence factors (**Chapter 2**). This chapter also discusses antivirulence therapy as a strategy for the future treatment of bacterial infections.

In **Chapter 3**, we verified the effect of quorum sensing on swimming motility in *V. harveyi* and on the expression of selected genes involved in flagellar motility. We further investigated the importance of flagellar motility for the virulence of *V. harveyi* by applying a motility inhibitor. Our results suggested an important mechanism by which quorum sensing controls the virulence of *V. harveyi* by demonstrating (i) that quorum sensing positively regulates motility by affecting flagellar biosynthesis, (ii) that LuxR is involved in the regulation of motility and (iii) that flagellar motility significantly affects virulence of *V. harveyi* in our gnotobiotic brine shrimp model.

In **Chapter 4**, the quorum sensing-disrupting activity, protective effect and toxicity of 20 novel thiophenone compounds were determined. Six thiophenones were identified to be able to inhibit quorum sensing at submicromolecular levels. There was a strong positive correlation between the specific quorum sensing-disrupting activity of the

thiophenones and the protection of brine shrimp larvae against pathogenic *V. harveyi*, and 6 quorum sensing-disrupting thiophenones were considered to be highly promising. We also found that there was a strong positive correlation between toxicity of the thiophenones towards brine shrimp larvae and toxicity towards *V. harveyi*, which means that the toxicity to *V. harveyi* could be used as a good indicator for toxicity to higher organisms. Furthermore, in this chapter, we proposed a new parameter, $A_{QSI}$, to determine specific quorum sensing inhibitory activity based on experiments with a quorum sensing reporter strain. This parameter allowed us to exclude 5 false positives out of the 17 thiophenone compounds that were able to inhibit bioluminescence in *V. harveyi*. We think that the use of this new parameter is a straightforward and elegant way to exclude false positives by taking into account side effects related to the use of quorum sensing molecule reporters.

In **Chapter 5**, we aimed at determining the impact of indole sensing on the virulence of *V. harveyi,* and at investigating whether indole analogues produced by micro-algae induce a similar response as indole. The results demonstrated for the first time that indole sensing could reduce the virulence of *V. harveyi* towards gnotobiotic brine shrimp larvae and conventionally reared giant river prawn larvae. Indole also decreased the production of several virulence factors in *V. harveyi*, including biofilm formation, exopolysaccharide production and swimming motility. The alternative sigma factor RpoS was found to be involved in the production of indole in *V. harveyi*, and indole could interfere with the three-channel quorum sensing system of *V. harveyi*. Further, we also demonstrated that auxin hormones that are produced by micro-algae showed similar effects as indole, suggesting that the selection of micro-algea that can produce high levels of auxins could be an interesting novel strategy to control bacterial disease in aquaculture.

In **Chapter 6**, we investigated the impact of the catecholamines norepinephrine and dopamine (neurotransmitters produced by higher organisms) on the growth of *V. harveyi* in serum-based medium, on the expression of various virulence-related characteristics and on virulence towards gnotobiotic brine larvae. According to the results, the catecholamines norepinephrine and dopamine increased the virulence of *V. harveyi* by increasing swimming motility, biofilm formation, exopolysaccharide

production and growth in environments with low iron availability. These effects could be neutralized by antagonists for eukaryotic catecholamine receptors and the bacterial catecholamine receptor antagonist LED209. Moreover, different effects of the adrenergic and dopaminergic receptors antagonists indicate the presence of specific sensing systems for different catecholamines in *V. harveyi*.

Finally, in **Chapter 7**, the most important results obtained in this thesis are highlighted and discussed. Suggestions for further research are proposed, including perspectives on exploring novel antivirulence strategies.

In conclusion, the work presented in this thesis indicates the complexity of the virulence mechanisms in *V. harveyi* and proposes a novel mechanism by which indole and host factors regulate the virulence of *V. harveyi*. Additionally, thiophenones are found to be promising antivirulence agent for infections caused by *V. harveyi*, and we suggest that the use of new parameter $A_{QSI}$ is a straightforward and elegant way to exclude false positives in screening quorum sensing inhibitors by taking into account side effects related to the use of quorum sensing molecule reporters.

# SAMENVATTING

*Vibrio harveyi* is één van de belangrijkste ziekteverwekkers in de larvicultuur en aquacultuur industrie. Deze bacterie kan een brede waaier van mariene vertebraten en invertebraten infecteren, en dit leidt wereldwijd tot ernstige verliezen in de aquacultuurindustrie. Het mechanisme waarmee *V. harveyi* zijn gastheren infecteert is nog niet volledig gekend. Men heeft antivirulentie therapie, het blokkeren van de productie van virulentiefactoren die nodig zijn om ziekte te veroorzaken, voorgesteld als een nieuwe strategie om bacteriële infecties te bestrijden. De productie van virulentiefactoren in *V. harveyi* is zeer sterk gereguleerd, en één van de regulatiemechanismen is quorum sensing, bacteriële cel tot celcommunicatie. Het verstoren van quorum sensing is de meest intensief bestudeerde strategie om de productie van virulentiefactoren te blokkeren. In dit werk hebben we de invloed van pathogeen-pathogeen communicatie en het reageren op gastheerfactoren op de virulentie van *V. harveyi* bestudeerd in een modelsysteem met gnotobiotische pekelkreeftlarven.

Vooreerst werd een literatuurstudie uitgevoerd i.v.m. de huidige kennis over *V. harveyi*, met inbegrip van virulentie, pathogenese en regulatiemechanismen die de productie van virulentiefactoren regelen (**Chapter 2**). Bovendien wordt in dit hoofdstuk antivirulentie therapie besproken als een strategie voor ziektebestrijding in de toekomst.

Vervolgens (**Chapter 3**) onderzochten we de invloed van quorum sensing op de zwembeweging van *V. harveyi* en op de expressive van een aantal geselecteerde genen die betrokken zijn bij deze beweging. Vervolgens onderzochten we het belang van de zwembeweging voor de virulentie van *V. harveyi* door gebruik te maken van een specifieke inhibitor. Onze resultaten toonden aan dat (i) quorum sensing de zwembeweging reguleert door de aanmaak van flagellen te regulatie, (ii) dat de quorum sensing regulator LuxR betrokken is bij deze regualtie en (iii) dat de zwembeweging een belangrijke invloed heeft op de virulentie van *V. harveyi* in ons gnotobiotisch model.

In het vierde hoofdstuk (**Chapter 4**) bepaalden we de quorum sensing verstorende capaciteit van 20 nieuwe synthetische thiofenonen. Zes thiofenonen waren in staat om quorum sensing te blokkeren aan submicromolaire concentraties. Er was een sterke positieve correlatie tussen de specifieke quorum sensing verstorende activiteit van de thiofenonen en de bescherming die ze boden tegen *V. harveyi* in het gnotobiotisch model met pekelkreeftjes. Er was eveneens een sterke positieve correlatie tussen de toxiciteit van de componenten t.o.v. steriele pekelkreeftjes en toxiciteit t.o.v. *V. harveyi*, wat betekent dat toxiciteit t.o.v. *V. harveyi* een goede indicator is voor toxiciteit t.o.v. hogere organismen. Bovendien stelden we in dit hoofdstuk een nieuwe parameter, $A_{QSI}$, voor om specifieke quorum sensing verstorende activiteit te bepalen gebaseerd op experimenten met een quorum sensing reporter stam. Het bepalen van deze parameter liet ons toe om 5 vals positieven uit te sluiten. Het gebruik van deze parameter is een eenvoudige en elegante manier om vals positieven uit te sluiten door rekening te houden met neveneffecten gerelateerd aan het gebruik van reporterstammen.

In het vijfde hoofdstuk (**Chapter 5**) onderzochten we de invloed van indool op de virulentie van *V. harveyi* en onderzochten we of indoolanalogen geproduceerd door micro-algen een gelijkaardige respons veroorzaakten. De resultaten toonden aan dat indool de virulentie van *V. harveyi* verlaagt, zowel t.o.v. gnotobiotische pekelkreeftjes als t.o.v. conventioneel gekweekte reuzen zoetwatergarnalen. Indool verlaagde eveneens de productie van verschillende virulentiefactoren zoals biofilm vorming, eopolysaccharide productie en zwembeweging. De alternatieve sigma factor RpoS bleek betrokken te zijn bij de productie van indool in *V. harveyi*, en indool interfereerde met het driewegs quorum sensing system van *V. harveyi.* Tenslotte toonden we aan dat auxine plantenhormonen die o.a. geproduceerd worden door micro-algen gelijkaardige effecten teweeg brachten. Dit wijst erop dat de selectie van micro-algen die in staat zijn om hoge concentratie auxines te produceren een interessante nieuwe strategie zou kunnen zijn om ziekten te bestrijden in de aquacultuur.

In het zesde hoofdstuk (**Chapter 6**) onderzochten we de invloed van de catecholamines noradrenaline en dopamine (neurotransmitters geproduceerd door hogere organismen) op de groei van *V. harveyi* in medium met serum, op de productie

van verschillende virulentiefactoren en op virulentie t.o.v. gnotobiotische pekelkreeftjes. Beide catecholamines verhoogden de virulentie van *V. harveyi* door de zwembeweging, biofilmvorming, exopolysaccharide vorming en groei in een omgeving met lage beschikbaarheid van ijzer te bevorderen. Deze effecten konden geneutraliseerd worden door antagonisten van eukaryote catecholamine receptoren en door een antagonist van de bacteriële catecholamine receptor QseC (LED209). Bovendien suggereren de verschillende activiteiten van de verschillende antagonisten dat er een bepaalde mate van specificiteit bestaat wat betreft de detectie van de verschillende catecholamines.

In het laatste hoofdstuk (**Chapter 7**) worden de belangrijkste resultaten van dit onderzoek benadrukt en besproken. Daarenboven worden suggesties naar voor gebracht voor verder onderzoek, zoals het verkennen van nieuwe strategieën voor antivirulentie therapie.

# Appendix C

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# Appendix D

## CURRICULUM VITAE

# CURRICULUM VITAE


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Quorum sensing in *Vibrio harveyi* positively regulates flagellar motility, an essential virulence factor *(**Oral presentation**)*

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