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PHENOTYPIC VARIATION IN *VIBRIO HARVEYI*: VIRULENCE FACTORS AND IMMUNE RESPONSE IN *ARTEMIA FRANCISCANA*

Thesis submitted in fulfillment of the requirements for the degree of Doctor in Applied Biological Sciences (PhD)

Dutch translation of the title:

Fenotypische variatie in *Vibrio harveyi*: virulentie factoren en immuunreactie bij *Artemia franciscana.*

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DEDICATION

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

And

To my little family: my husband and my little boy for all care, patience, and encouragement

LIST OF ABBREVIATIONS AND UNITS

°C	Degree Celsius
%	Percentage
µg	Microgram
µl	Microliter
g	Relative centrifugal force for G force
±	Approximately
$^{-1}$	Per
min	Minute
s	second
h	Hour
AHL	Acyl-homoserine lactone
AI-2	Autoinducer 2
CAI-1	Cholera autoinducer 1
ANOVA	Analysis of variance
CFU	Colony forming unit
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
HAI-1	Harveyi autoinducer 1
L	Liter
LB	Luria-Bertani
LPS	Lipopolysaccharides
MA	Marine agar 2216
MB	Marine broth
mg	Milligram
ml	Milliliter
OD	Optical density
P	Statistical p-value
QS	Quorum sensing
RT-PCR	Real time-polymerase chain reaction
rpm	Rotations per minute
AMP	Anti-microbial peptides
bp	base pairs
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
Dscam	Down syndrome cell adhesion molecule
Hsp70	Heat shock protein 70
Hmb1	High mobility group box-1
lgbp	lipopolysaccharide and β-1, 3-glucan binding protein
LPS	Lipopolysaccharide
LVS 3	*Aeromonas hydrophila* Strain 3 isolated by Laurent Verschuere
mRNA	Messenger ribonucleic acid
PAMP	Pathogen associated molecular pattern
DAMP	Damage associated molecular pattern
PCR	Polymerase Chain Reaction
pxn	Peroxinectin

spn	Serpin
PGN	Peptidoglycans
pH	Measure of the acidity of solution
ProPO	Prophenoloxidase
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPS	Relative percentage survival
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
tgase	Transglutaminase
ftn	Ferritin
TLR	Toll-like receptor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PVDF	Polyvinylidene fluoride membranes
L	Luminescence
NL	Non-luminescence
RACE	Rapid Amplification of cDNA ends
UTR	Untranslated region

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

Chapter 1

Introduction & thesis outline

1.1 Aquaculture – the current status

Aquaculture plays an important role for generating lasting benefits for global food security, rural development and poverty alleviation in many countries. In addition, about 58.3 million people were engaged in 2012 in the primary sector of capture fisheries and aquaculture, and about 10 – 12% of the world's population depends on fisheries and aquaculture for their livelihoods.

Today, aquaculture is one of the fastest growing food-production sectors in the world. According to FAO statistics, the world total production of aquaculture rapidly increased from 13.1 million tons in 1990 to 66.6 million tons in 2012 (Table 1). From those, two-thirds (44.2 million tons) were finfish species from inland aquaculture [1] (38.6 million tons) and mariculture[2] (5.6 million tons) (Figure 1.1). In the last five decades (1960 – 2012) food fish supply has increased at an average annual rate of 3.2 percent outpacing world population growth which amounts to 1.6 percent. The average fish consumption per capita increased from 9.9 kg in 1960s to 19.2 kg in 2012.

On the other hand, the global capture fishery production remained stable in the last five decades (1970 – 2012), at an average of 91.3 million tons in 2012. The major fish capture countries such as China, Indonesia, United States of America and Peru have seen their fisheries reaching their maximum potential. Therefore, total fish capture from nature is anticipated not to reach the global demand of aquatic food. FAO estimated that the global aquaculture production would need to reach 80 million tons by 2050 to guarantee fish supply.

In addition, overfishing not only causes negative ecological consequences but also reduces fish production, further leading to negative social and economic consequences. Therefore aquaculture will play a critical role to meet the increasing global demand contributing to safe and quality food fish.

[1] inland aquaculture: freshwater aquaculture

[2] mariculture: coastal and brackishwater aquaculture

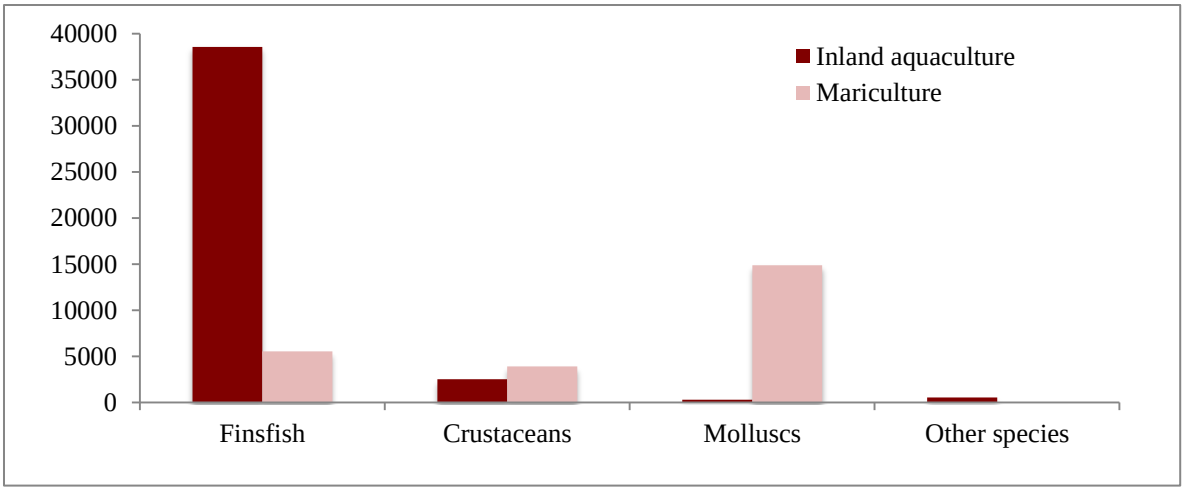

Figure 1.1 World production (million tonnes) of farmed species groups from inland aquaculture and mariculture in 2012 (Source: [1]).

Inland aquaculture growth has outpaced marine culture growth, with average annual growth rates of 9.2 and 7.6 percent, respectively. Consequently, inland aquaculture steadily increased its contribution to total farmed food fish production from 50% in 1980 to 63% in 2012.

Aquaculture development is imbalanced among the world regions. Asia holds the top position in world aquaculture production, accounting for 88% of global aquaculture production by volume in 2012, in which China alone supplied 61.7% of world aquaculture production by volume (Table 1.1). Generally, the world aquaculture production in different regions remains stable since 2010.

In 2012, the farmed crustaceans accounted for 9.7% (6.4 million tons) of food fish aquaculture production by volume but 22.4% (US$30.9 billion) by value. Mollusc production (15.2 million tons) was more than double that of crustaceans, but its value was only half that of crustaceans (Table 1.2).

Table 1.1 Production by region: quantity (tons) and percentage of world total production 2012 (Source: [1]).

Regions	**Quantity (tonnes)**	**Percentage (%)**
Africa	1485367	2.23
Americas	3187319	4.78
Asia	58895736	88.39
Europe	2880641	4.32
Oceania	184191	0.28
World	66633253	

- Data exclude aquatic plants

Aquaculture remains a critical food producing sector that can create a significant contribution to the increasing demand for safe and high quality products. However, currently the aquaculture sector is facing issues such as biological threats and infectious diseases mainly due to the intensive aquaculture conditions. It is also anticipated that aquaculture will face a shortage of fish protein and fish oil. Moreover, depending on use of chemicals, such as antibiotics, still remains a problem. Although more than 88% of the world's aquaculture production was from Asian countries such as Indonesia, Philippines and Vietnam [1], aquaculture production is mostly relying on the conventional and empirical approaches (with a strong evolution ongoing) in theses countries. In addition, management and regulations (and their enforcement) from the local authority and government is often insufficient. Despite aquaculture production in Asia being successful so far, in order to develop a more predictable, reliable, cost-effective and ultimately more sustainable industry, development of more knowledge-based procedures based on the understanding of the underlying mechanism of all biological processes are becoming increasingly indispensable [2].

Table 1.2 World production of aquaculture species from inland aquaculture and mariculture (Source [1]).

	Inland aquaculture	**Mariculture**	**Quantity subtotal**		**Value subtotal**	
	(Million tons)	*(Million tons)*	*(Million tons)*	*(% by volume)*	*(US$million)*	*(% by value)*
Finfish	38.599	5.56	44.151	60.30	87.499	63.5
Crustacean	2.530	3.917	6.447	9.70	30.864	22.4
Molluscs	0.287	14.89	15.171	22.80	15.857	11.5
Other species	0.530	0.335	0.865	1.30	3.512	2.5
Total	41.95	24.69	66.63	100	137.732	100

1.2 Vibriosis in aquaculture

Disease outbreaks are major threats to the aquaculture sector, affecting the economic development of the sector in many countries [3]. 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 major etiological agents in aquaculture. Bacterial

pathogens have been reported to cause more disease problems than all other causes combined [4].

Vibrio spp. such as *V. anguillarum, V. alginolyticus, V. campbellii, V. splendidus, V. coralliilyticus* and *V. harveyi,* are the common and serious pathogens in fish and selfish marine aquaculture worldwide. *Vibrios* are Gram-negative, ubiquitous in marine and estuarine ecosystems as well as aquaculture farms. Many *vibrios* are serious pathogens for animals reared in aquaculture. Vibriosis, caused by infection by *Vibrio* spp, is one of the most prevalent diseases in fishes and other aquaculture-raised organisms, and is widely responsible for mortality in culture aquaculture systems worldwide. Vibriosis is a disease which occurs rapidly, induces weak muscular movement, red discoloration, partial tail cramping, tissue and appendage necrosis, slow metamorphosis and growth, body malformation, bioluminescence. Resulting in high mortality for many aquatic species such as shrimp, fin fish and mollusks [5].

Recently, *V. parahaemolyticus* has been received more attention as one of the agents that causes early mortality in shrimp cultivation ponds. Early mortality syndrome (EMS) refers to unusual, acute mortality in shrimp approximately within the first 35 days after stocking cultivation ponds. Such outbreaks in farmed pacific white shrimp or whiteleg shrimp (*Panaeus vannamei*) were first reported in Thailand from farms in 2012. The Thai Department of Fisheries reported that outbreaks of EMS in cultivated shrimp were responsible for a 33% drop in shrimp production during the first quarter of 2013. Currently, it has been reported that unlike other fish-pathogenic *Vibrio* species, in which the plasmids contribute to virulence via a plasmid-encoded iron acquisition system or confer resistance against the serum killing effect to overcome host defenses. In *V. parahaemolyticus,* the virulence plasmids produce toxins that immediately destroy the host cells [6].

V. harveyi is associated with luminescent vibriosis in shrimps for instance, *Litopenaeus vannamei* and *Penaeus monodon,* and it has been considered as a major constraint to shrimp production by causing mass mortality in South America and Asia [7]. Moreover, *V. harveyi* is one of the most significant pathogens in larviculture. It has been regarded as both a primary and opportunistic pathogen for a wide range of marine species, including corals, finfish, oysters, shrimp, prawns and lobsters. Accumulating evidence have suggested that *V. harveyi* causes diseases in the host through several steps, which include penetration of the host tissues by chemotactic motility, sequestration of iron from the host in order to replicate and cause disease [8], and infliction of damage by producing a extracellular virulence factors, such as hemolysins, phospholipases, proteases, and biofilm formation [7, 9].

As virulence factors are often costly metabolic products, their expression usually is controlled by regulatory mechanism such as quorum sensing, bacterial cell-to-cell communication with small signal molecules [10]. In *V. harveyi*, virulence factors have been found to be quorum sensing-regulated include biofilm formation, the production of siderophores, chitinase, caseinase, gelatinase, metalloprotease, phospholipase, extracellular toxins, type III secretion and motility [11-13]. In addition, quorum sensing has also been reported to control the virulence of luminescent *V. harveyi* towards different hosts *in vivo* [14, 15].

V. harveyi is also one of the model organisms in studies on quorum sensing in bacteria. This bacterium contains a three-channel quorum sensing system, with three different types of signal molecules feeding a shared signal transduction cascade. Central in the signal transduction cascade is the LuxO protein. Phosphorylated LuxO indirectly inhibits production of the transcriptional regulator protein LuxR, whereas unphosphorylated LuxO is incapable of exerting this activity because of a conformational change. LuxR directly activates the *lux* operon and directly or indirectly controls many other quorum sensing target genes [16, 17].

Despite extensive studies on this species, the pathogenicity mechanisms of *V. harveyi* are not completely understood. A good understandings of the behavior of bacterial pathogens or how do they perform to adapt under alterations of the natural ecosystems and within the host is urgently needed for vaccine development and for sustainable development of aquaculture.

Research objectives and thesis outline

This PhD study consists of two main parts: the first part of this study focuses on producing the non-luminescent variants from the luminescent of the wild type *V. harveyi* and its quorum sensing mutant strains. Subsequently, differences in virulence factors such as caseinase, haemolysin, elastase, biofilm formation between the luminescent and its non-luminescent variant of the *V. harveyi* are investigated. In the second part, the GART system is used in order to verify how the host responds to luminescent and non-luminescent variants of *V. harveyi* at both transcriptional level for instance, the expression profile of some immune related genes, and translational level of heat shock protein 70 and high-mobility group box-1 proteins. Finally, the molecular characterization and expression of hmgb1 in *Escherichia coli* is solved for further applications.

The general objective of this study was to evaluate phenotypic alterations in *Vibrio* (e.g. in term of virulence factors production) and their effect on host immune responses using brine shrimp *Artemia* as a model.

The specific objectives and the thesis outline were as follows:

Chapter 2 (Review of literature) aimed at giving an overview of the literature on phenotypic variation in bacteria, their role in natural ecosystems, and consequence of these variations for host microbial interactions for instance in relation to the immune response.

Chapter 3 (Characterization of phenotype variation of luminescent and non-luminescent variants of *V. harveyi* wild type and quorum sensing mutants) aimed at studying phenotype variation with respect to haemolysis, elastase, caseinase and biofilm formation of luminescent and non-luminescent variants of *Vibrio harveyi* wild type and its quorum sensing mutants.

Chapter 4 (Expression profile of immune-related genes in *Artemia franciscana* challenged with luminescent and non-luminescent variants of *V. harveyi* wild type and quorum sensing mutants) aimed at investigating the expression profile of immune-related genes in *Artemia franciscana* challenged with luminescent and non-luminescent variants of *Vibrio harveyi* wild type and its quorum sensing mutants.

Chapter 5 (Transcriptional and translational analysis of expression profiles of heat shock protein 70 and high-mobility group box-1 protein in gnotobiotic brine shrimp challenged with isogenic *V. harveyi* with different levels of virulence) aimed at investigating the transcriptional and translational analysis of expression profiles of heat shock protein 70 and high-mobility group box-1 protein in gnotobiotic brine shrimp challenged with *Vibrio harveyi*.

Chapter 6 (Molecular characterization and expression of the *Artemia franciscana hmgb1* gene) aimed at cloning and sequencing the full-length cDNA of the high-mobility group box-1 gene and its recombinant production in *E. coli*.

Chapter 7 (General discussion and conclusion) highlights and discusses the most important findings of this work and provides some direction for future research.

Chapter 2

Review of literature

2.1. Phenotypic variation in bacteria: an opportunity for thriving, persisting and evading the host immune system

The potential of bacteria to thrive in a variety of ecological niches strongly depends on their genetic content. However, bacteria are influenced within these niches by fluctuating environmental factors, such as pH, salinity, temperature and the availability of nutrients [18]. In addition, pathogenic bacteria may encounter interspecies competition, such as the immune defense system from the host during infection. Bacteria adapt to survive these stressors by sensing the changes and responding appropriately, changing phenotype to meet fluctuating environmental conditions [19]. For example, *E. coli* can switch spontaneously and reversibly to a phenotype that can slow down its growth, reducing its sensitivity to antibiotics [20].

Phenotypic (phase) variation (Figure 2.1) is defined as a process of reversible, high frequency phenotypic variation that is mediated by DNA mutations, reorganization or modification [21]. Phenotypic variation was first described in *Candida albicans* over 20 years ago and later this phenomenon was observed in fungal and bacterial species, such as *Cryptococcus neoformans*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp and *Neisseria* spp [22-24]. Phenotypic variation has been observed in both pathogenic and non-pathogenic bacteria. The switch involves changes in the polysaccharide capsule and cell wall that affect the pathogen's ability to resist phagocytosis. In addition, the phenotypic switch variants elicit qualitatively different inflammatory responses in the host [22]. Moreover, phenotypic variation can alter the host-pathogen relationship. For example, pathogenic *Neisseria* spp. possess 12 different phase-variable Opa proteins that mediate various pathogen-host cell interaction, including bacterial engulfment by epithelial cells and opsonin-independent phagocytosis by phagocytes [25].

Phenotypic variation is used by several bacterial species to generate population diversity that enhances bacterial fitness under certain conditions and is important in niche adaptation and host immune evasion. However, a fraction of the population preserves its phenotypic unaltered status ensuring the ability of the population to overcome future and new adverse conditions that may occur [26]. Phenotypic variation can be modulated by a variety of external factors (responsive switching) [23, 27]. For example, studies on the capsular polysaccharide (CPS) phase variation in *Vibrio vulnificus* revealed that under the alteration of environmental conditions, such as variation in aeration, temperature, and incubation time, the

amount of CPS expressed on the cell surface was significantly changed, and the colony phenotype converted from an opaque to a translucent colony [28].

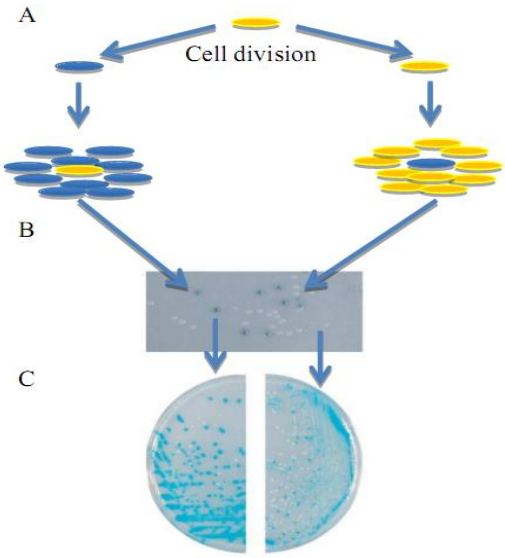

Figure 2. 1 Phenotypic variation refers to a reversible switch between two phenotypic states, analogue to a mechanism ON/OFF (Source [29]).

(A) When the expression state of one of the cells of an "off" cell has switched, and both grow to a colony. "ON" cells are depicted blue and "OFF" cells white. (B) Colonies from a streak of a single white colony. Combination of phenotypes: colonies with predominantly "ON" cells are blue and colonies with predominantly "OFF" cells are white. (C) Typical result of streaking an "ON" (blue) colony and an "OFF" (white) colony for single colonies. The plates clearly illustrate the bias in progeny phenotypes as a result of parental phenotype. The number of generations during which this bias exists depends on the frequency of switching.

Phase variation has been intensively studied in *Pseudomonas* [23, 24, 30] and *Vibrio* bacteria [31, 32]. The studies on *Pseudomonas* showed that phenotypic variation is involved in influencing the production of exo-enzymes, secondary metabolites, colonization behavior, and biocontrol activity of rhizosphere bacteria [33, 34]. In addition, phenotypic variation may contribute both to pathogenicity of *V. vulnificus* and to its survival under adverse environmental conditions [35] or enhance predation-driven persistence of *V. cholera* from the predation of protozoa in the environment [31]. Recently, Srivastava *et al.* [32] showed that the virulence of *V. vulnificus* was related to phenotypic variation in colony morphology, for example, opaque (encapsulated, virulent), translucent (reduced encapsulation, avirulent) and rugose (wrinkled, biofilm-enhanced) colony morphology. Results from this study also suggested that variable colony types reflect differences in the capsular polysaccharide

composition or level of expression and provide adaptive strategies for survival in different environments.

2.1.1. Role of phenotypic variation in bacteria

In general, the role of phenotypic variation is to provide the bacterium with a strategy for adapting to more than one particular environment [36]. The existence of single cells in the clonal population may ensure the survival of the population as a whole. Reversible phase variation (ON-OFF) continuously generates cells with varied phenotypes, leading to a mixed population. This phenotypic diversity ensures that, a percentage of the bacterial cells will express the phenotypic variation necessary for survival, regardless of the environmental changes[37]. These environments may be inside or outside the host, or may comprise more than one site within that host or even different steps in a pathogenic progress. *Paenibacillus dendritiformis*, a gram-positive, spore-forming bacterium can overcome competition and nutrient limitation by switching between two distinct vegetative phenotypes: motile rods and immotile cocci. Growing colonies of the rod-shaped bacteria can produce a toxic protein, or "sibling lethal factor" (Slf), which reduce the population density by killing cells of encroaching sibling colonies. However, sublethal concentrations of Slf induce the switch from motile, rod-shaped cells to non-motile, Slf-resistance, cocci in a small proportion of the cells. These cocci replicate, unlike dormant spores of *P. dendritiformis*. When over population is reduced and nutrients are no longer limiting, bacteria produce a signal that induces cocci to switch back to motile rods, allowing the population to disseminate [38].

Phenotypic variation has been intensively studied in *Pseudomonas*, highlighting the important roles of phenotypic variation in *Pseudomonas.* Different morphology variants were achieved as a result of phenotypic variation. For instance, thick, small and opaque versus thin translucent colonies [39-41]. However, the determinants of these differences in colony morphology in *Pseudomonas* are not known. These variants showed differences in swimming and swarming. Switching motility in *P. aeruginosa* via regulation of type IV pili gene expression [41] can regulate root colonization by *P. fluorescens* WCS365 and *P. fluorescens* F113 in tomato rhizosphere [42]. Phenotypic variation is also suggested to be an effective strategy for increasing the colonization ability in *P. brassicacearum* [43]. In addition, pathogenicity was affected by phenotypic variation in *Pseudomonas* species. Weiser *et al.* [44] suggested that pathogenicity of in *P. aeruginosa* may be associated with the expression of the phosphorylcholine epitope of a 43 kDa protein through phase variation [44].

Previous studies have proposed that phase variation is a mechanism by which pathogenic bacteria evade the host immune responses [45]. Surface components of the bacteria are highly immunogenic and therefore prime targets for protective antibodies. Recent studies showed that fimbrial phase variation in *Salmonella* is a mechanism by which the microorganism evade cross-immunity between different serotypes, thereby allowing their co-existence within a host [36]. In addition, phase variation of adhesion may assist bacteria to detach from colonized surfaces, to disseminate within the host, to shed back into the environment and/or to transfer to another host [46].

Recent work has shown that phenotypic variation is a phenomenon that is not only relevant in host-pathogen interactions but also influences colonization, biocontrol activity and the expression of co-enzymes and secondary metabolites (6).

2.1.2. Implication of phenotypic variation

Colony morphology variants

The generation of heterogeneous and dynamic populations as a result of phenotypic variation can overcome stressful factors. The most visible feature of phenotypic variation is colony morphology variation, first described in 1964 by Zierdt *et al.* [47]. Colony macroscopic visualization, allows for predicting bacterial traits probably altered and their relationship with external factors. Moreover, the relationship between colony morphology changes, virulence, antimicrobial resistance and persistence can be established as well, which will be extremely useful in the design of biotechnological and therapeutic approaches.

Several different colony morphologies have been identified and described for several bacteria, such as the small colonies variants (SCVs), rugose colony, and the mucoid morphotypes. Some of these morphotypes were associated with antimicrobial resistance, altered metabolism and reduced immunogenicity, contributing to increased bacterial pathogenicity and persistence. SCVs have been involved in persistent and device-related infections [48]. Deziel *et al*. [41] have revealed that SCVs are hyperpiliated with increased ability to form biofilm in comparison with the fast growing wild type morphotypes. Besides SCVs, the mucoid phenotype has also been addressed and was associated with the overproduction of exopolysaccharides (EPS). The overproduction of EPS protects bacteria from host defense and antibiotic therapy, extending the length of the infection [26].

In addition, phenotypic variation can be visualized in some cases by color variation in colonies grown on specific media including a dye. For example, Phuoc *et al.* [49] reported that phase variation in *V. campbellii* leads to variable colony color when grown on Congo red agar, for example, non-luminescent colonies were black while its luminescent variant was red (Figure 2.2). The same phenomenon was also observed in *Staphylococcus epidermidis* under phase variation process of a polysaccharide adhesion [50].

A **B**

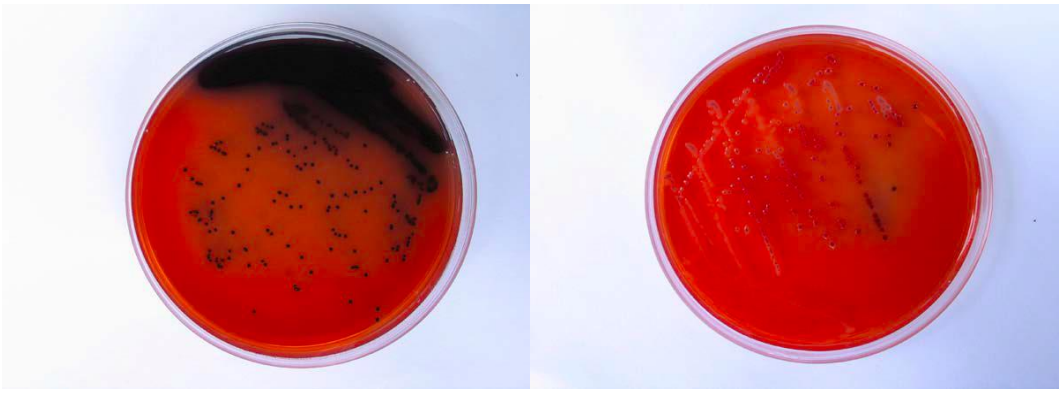

Figure 2. 2 Non-luminescent (A) and luminescent (B) colonies of *V. campbellii* in Marine Agar with Congo red [49].

In addition, the capsule can influence interaction with the host cells and host environment, including invasion, adhesion, and serum sensitivity, and is well known as virulence factor. Phase variation of capsule synthesis can involve a classic ON/OFF but also has been used to describe modulation in the level of capsule production; thereby it can change colony morphology [51]. For example, a study using encapsulating bacterium, *Neisseria meningitidis*, showed that phenotypic phase variation controls the expression of the capsule gene in this bacterium. This finding indicated that bacteria could escape vaccine-induced or natural protective immunity by switching of capsule phenotype. Capsule switching may be an important virulence mechanism in other encapsulated bacterial pathogens. As vaccine development progresses and broader immunization with capsular polysaccharide conjugate vaccines becomes a reality, the ability to switch capsular types may have importance for the impact of these vaccines [52].

Phenotypic persisters

In medicine and technology, the existence of subpopulation of cells highly tolerant to conventional treatments has a huge impact on the control and elimination of the infected bacteria and contaminations.

Persistence is one of the most remarkable features of bacteria; it is the ability of bacteria to thrive after exposure to severe stressful conditions. Consequently, a rapid killing of the vast majority of cells is observed, followed by a more complex and slow killing of the remaining cells, called persister cells. Persisters are not resistant cells and do not have genetically-acquired antimicrobial resistance. They are dormant cells, *i.e.,* they remain metabolically inactive in stressful conditions, in a "non-dividing state". After removal of the lethal conditions, persister cells can switch back to normal cells and generate a new population that is equally susceptible. The ability of persister cells to revert the to original population is supposed to be a sign of phenotypic variation [53].

Kussell *et al.* [54] suggested persistence as a bet-hedging strategy because under optimal conditions, the normal cells grow quickly and a small subpopulation suppresses growth. During time of stress, such as the presence of antibiotics, these persistent cells can prevent extinction of the entire population by their slow proliferation rate under growth condition leading to lower population fitness against antibiotic encounters [54]. Persisters show ability to reduce translation and topoisomerase activity, and/or reduce cell wall biosynthesis. As a result the target of many bactericidal antibiotics are blocked, and therefore these cells cannot be easily killed [53]. The molecular mechanism underlying persistence remained unclear until recently.

Biofilm phenotypic switching

The ability of biofilm formation in bacteria is an important virulence factor. Biofilm are assemblages of microorganisms, encased in a matrix [55]. Biofilm formation is an efficient microbial strategy to persist adverse conditions. Biofilm is developed through several stages. It is initiated when planktonic cells are released, migrate to a new surface and subsequently attach irreversibly. Later, bacteria grow and divide forming a mature biofilm. In the mature biofilm, cells are encased in an extracellular matrix composed of protein, exopolysaccharides and extracellular DNA. In addition, to immobilizing the bacteria, the matrix is a scaffold that traps nutrients and various biologically active molecules. The matrix works as an external

digestion system, as it can accumulate enzymes that can degrade various matrix components as well as any nutrients or other substrates. Subsequently, the degraded products are in close proximity to the cell and therefore facilitate uptake (Figure 2.3) [55]. Moreover, the matrix acts as a shield therefore bacteria in a biofilm exhibit various advantages over their planktonic counterparts, such as enhanced antimicrobial resistance, protection against predation, dehydration and phagocytosis [56]. During biofilm growth, bacteria receive a biofilm-specific phenotype that is distinct from their corresponding planktonic cells.

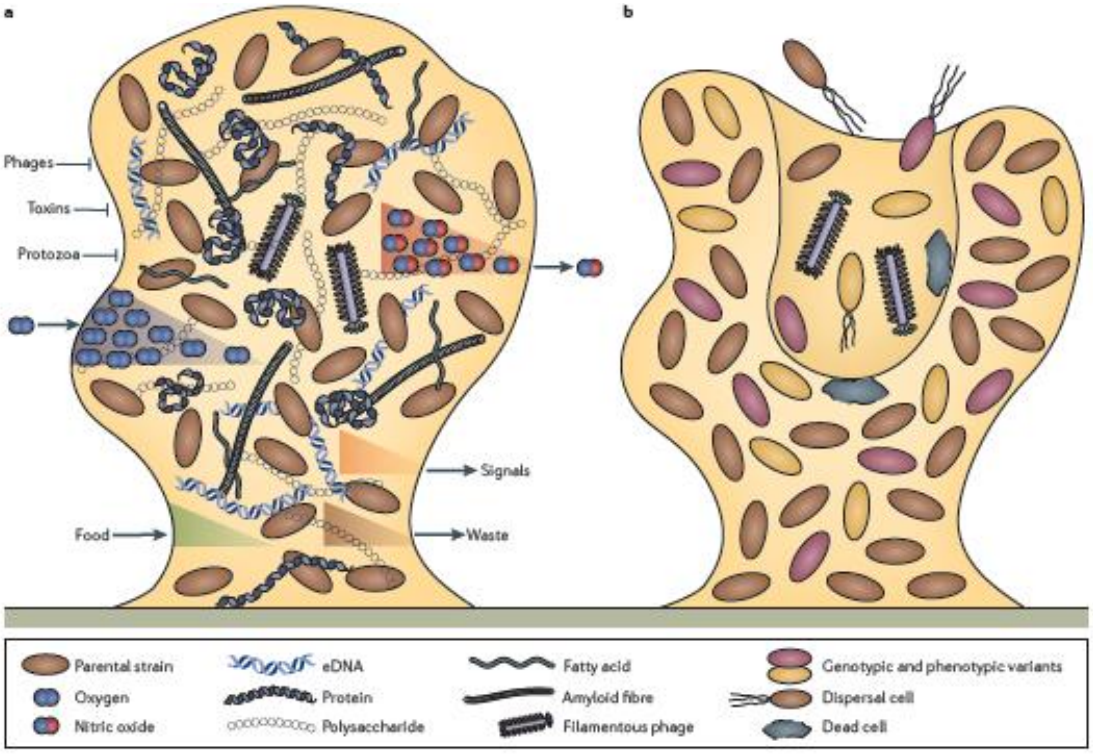

Figure 2. 3 The complex structure of bacterial biofilm (Source [55])

a. Microcolonies in the mature biofilm are characterized by an extracellular polymerix substances (EPS) matrix, composed of extracellular DNA, polysaccharides, proteins, amyloid fibres and bacteriophages. The matrix functions as a shield to protect the bacterial community or population from predator, such as protozoa or lytic phages, as well as from chemical toxins. The EPS matrix may help to sequester nutrients, and along with the underlying bacteria, is also responsible for the establishment of gradients (for example, oxygen and nutrients diffusing inwards, and waste products as well as signals, such as nitric oxide diffusing outwards).
b. At the time of dispersal, microcolonies undergo cell death and lysis along with appearance of superinfective bacteriophases as well as morphotypic and genetic variants in the biofilm effluent.

Bacteria in a mature biofilm undergo specific changes in physiological and protein regulation, especially those related with proteins involved in resistance to oxidative damage, exopolysaccharide production, phospholipids synthesis and membrane transport [24]. In fact, gene expression, morphology, phenotype, stage of differentiation and development are different between the cells in a mature biofilm [55]. For example, genes encoding for pili and flagella synthesis are down regulated because these elements are no longer required in a mature biofilm. Switching to biofilm-specific phenotypes can trigger mechanisms that are responsible for antimicrobial resistance, enhanced virulence and persistence [24]. The heterogeneous nature of the mature biofilm may be a consequence of differential gene expression by cells across different regions in the biofilm structure, as a result of nutrient gradients, electron acceptors and waste products or mutations that lead to a diversity of genotypic and phenotypic variants [55]. In addition, biofilms have been documented to reduce activation of complement systems, and as biofilm-growing bacteria are encased within exopolysaccharide matrix, they are less susceptible to phagocytosis. In some cases, biofilms are resistant even to humoral response [57]. For example, it has been shown that there is a link between antibiotic resistant and ability to form biofilms in *P. aeruginosa* [58]. In addition, another study also suggested that the increased resistance of *Staphylococcus aureus* biofilms to amikacin is largely dependent on the presence of persister cells. Biofilms harbor a greater number of persisters than the planktonic culture. In addition, planktonic persisters, even if they exist, are liable to be eliminated by the immune system *in vivo*, whereas biofilm persisters are protected by the exopolysaccharide matrix [59]. 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 [60].

Virulence phenotypic variants

As stated above, phenotypic variation assists bacteria to escape the host's immune recognition during their colonization and infection, affecting host-pathogen interaction [27]. Deitsch *et al*. [61] revealed that the subpopulation generated by present different sets of antigens and the immune system only reacts against those antigens that they can recognize. Therefore the phenotypic variants become a problem when the host immune system does not respond effectively.

The relationship between phenotypic variation and virulence has been documented in many pathogenic bacteria. For example, in a study carried out on *Streptococcus pneumoniae*, it was

shown that opaque colony variants exhibited higher virulence than translucent variants [62]. Similar trends were observed for *Helicobacter pylori* [63], *Salmonella enterica* and *Pseudomonas fluorescens.* Phuoc *et al.* [64] have reported that non-luminescent variants of *V. harveyi* obtained by culturing luminescent variants under static conditions, showed colonies with different morphology compared with those of their parental luminescent strain. The difference in colony appearance was determined by differential staining on Marine Agar Congo red and it was found that the non-luminescent colonies were less round shaped on Marine Agar compared to the luminescent ones. It was also shown that non-luminescent strains exhibited less pathogenicity as verified in a challenge test carried out using the gnotobiotic *Artemia franciscana* model organism (GART) system. Following this study, Hong *et al.*[65] showed that there was a significant difference in virulence factors production such as swimming motility, biofilm formation, hemolysis and caseinase activities in non-luminescent variants compared to its luminescent counterparts.

Extracellular proteases play vital role in the virulence of many vibrios [66]. Serine protease and metalloprotease seem to be the major proteases in *V. harveyi* [67, 68]. Ruwandeepika *et al.* [69] reported that metalloprotease levels were found to be lower in the non-luminescent variants of *V. harveyi* when compared with the corresponding luminescent strains. The lower level of metalloprotease in the non-luminescent form was accompanied with the lower level of *luxR* mRNA – a virulence gene regulator. Moreover, differences in the metalloprotease expression might explain the lower pathogenicity of the non-luminescent variants towards the host *Artemia* in the challenge test. Recently, Zhou *et al.* [70] have reported that the non-luminescent *V. harveyi* HBL0905 is highly pathogenic and generally causes epizootics with high mortalities in pond-cultivated penaeid shrimp population, especially following stressful events. The parental luminescent strain could be extremely virulent in comparison with its non-luminescent one, although, the luminescent strain was not investigated in this study. These results indicate that there may be a link between the phenotypic variation and pathogenicity in *V. harveyi*.

Viable but non-culturable state

The viable but non-culturable state (VBNC) state is a unique survival strategy that bacteria employ in response to adverse environmental conditions. The VBNC state of bacteria was previously referred as dormancy state but VBNC has been currently accepted as a distinct survival state of bacteria [71]. Cells in the VBNC state exhibit very low, but detectable,

metabolic activity compared to actively growing cells. Such dormant cells maintain the integrity of their cell membranes and continue to express genes at low levels. However, when they are transferred to solid nutrient media, they fail to form colonies, as opposed to active cells (Figure 2.4)

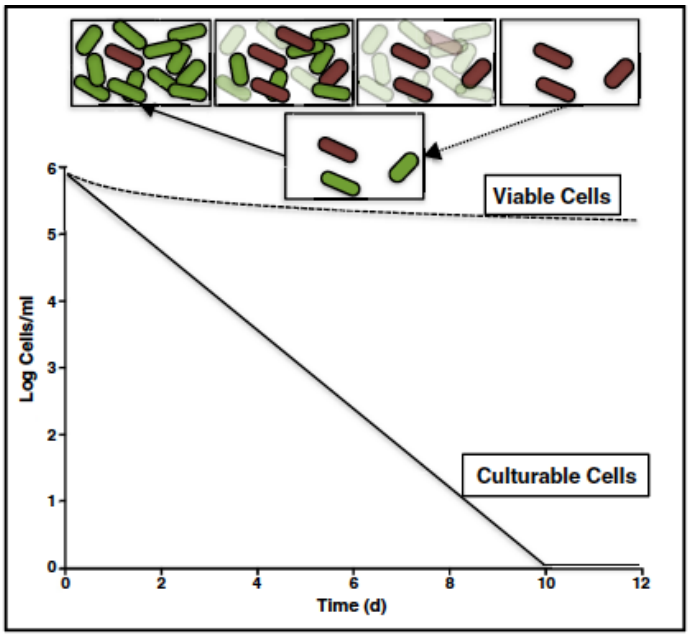

Figure 2. 4 Classic VBNC induction experiment (Source [72]).

VBNC cells (dark red) are induced by incubating a cell suspension (green) in a stressful environment (for example at low temperature). Over time, cells become undetectable on nutrient media (solid line); however, a large portion of the population remains viable (dashed curve). When the inducing stress is removed, the population becomes active after a resuscitation period (broken arrow). When cells are provided with nutrients they give rise to a population that is similarly tolerant to the inducing stress as was the original population (unbroken arrow).

VBNC cells can be induced by variety of stressful conditions such as starvation, growth-inhibiting temperature, and pH. For example, *V. vulnificus* can withstand a variety of stresses while dormant, including high dose of antibiotics, toxic heavy metals, high temperature, high salinity, ethanol, and acids [73]. In addition, in *V. parahaemolyticus*, a halophilic Gram-negative bacterium is frequently isolated from fish, clams and crustaceans. *V. parahaemolyticus* may be present in a VBNC state during the winter, and could not be cultured on agar medium. It has been shown that *V. parahaemolyticus* can reach the VBNC state in 50 to 80 days during starvation at 3.5°C. Moreover, the VBNC cells of *V. parahaemolyticus* were highly resistant to thermal (42, 47°C), low salinity (0% NaCl), or acid

(pH 4.0) inactivation. These results suggested that the VBNC state is part of a survival strategy in an adverse environment of *V. parahaemolyticus* [74].

The physiological consequence of the VBNC state could be an adaptive effect that supports long-term survival under unfavorable conditions or an effect of cellular deterioration, which conserves specific features of viable cells but results in a loss of cultivability with available techniques. This state can be thought of as an inactive form of life waiting for revival under suitable conditions [75].

The luminous vibrio species such as *V. harveyi, V. fisheri, V. cholera* are capable of transforming into a VBNC state and undergo alteration in cell morphology. In this state, *V. cholera* cells become smaller in size and change from rod to ovoid or coccoid morphology, with the central region of the cells becoming compressed and surrounded by denser cytoplasm by using transmission and scanning electron microscopy [76].

V. harveyi and *V. fisheri* entered into the VBNC state upon salinity and nutrient limitation of the artificial sea water. Adaptation of these bacteria to low nutrient conditions or to other environmental parameters was demonstrated by the rapid switching off of bioluminescence in nutrient-free microcosms. The ability of these luminous bacteria to recover from VBNC state was observed after addition of a low concentration of several nutrients [77].

2.1.3 Mechanism of phenotypic variation

Phenotypic variation can be mediated by specific genetic alterations or by epigenetic mechanisms. Hence, it has been difficult to demonstrate whether the mechanism attributed to phenotypic variation *in vitro* are the same at play *in vivo.* The following are the (proposed) molecular mechanisms that may be involved in phenotypic variation in bacteria.

Slipped-strand mispairing

Slipped-strand mispairing uses short sequence repeats to regulate gene expression at the transcriptional or translational level. Repeats associated with a single locus, present in the promoter region or within the coding region, can alter gene expression as a result of changes in the number of repeats. The number of repeats is varied via a RecA-independent mechanism through the formation of heteroduplex DNA (H-DNA), which is induced by superhelical coiling [78]. This H-DNA consists of a triple-stranded region, based on the formation of triple residue bonds within the repeat region, with as a result a single-stranded region, which will

stimulate slipped-strand mispairing [36]. Change in number of repeats will result in an incomplete gene product due to a shift in reading frames (Figure 2.5). Examples of traits regulated via slipped-strand mispairing are presented in Table 2.1.

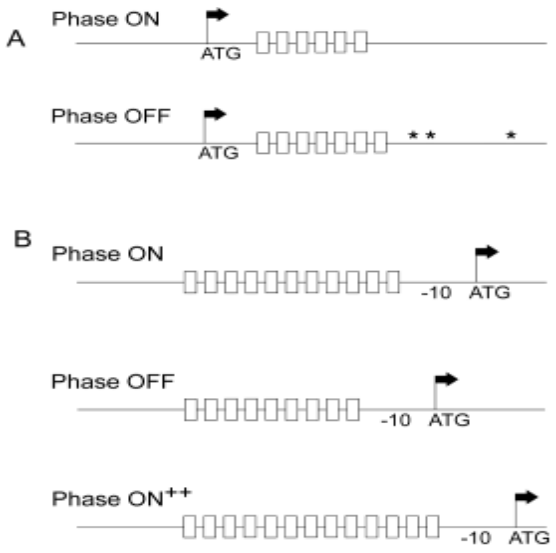

Figure 2. 5 Model for phase variation via slipped-strand mispairing (Source [21])

A. Model of ON and OFF switching of traits via slipped-strand mispairing. Variation in the number of repeats within the coding region of the gene results in a shift of reading frame in or out of the frame. A shift out of frame will introduce the premature stop codon (*).

B. Model for volume control via slipped-strand mispairing. Variations in the number of repeats within the promoter region of the gene will vary promoter -10 and -35 spacing, thereby increasing (ON^{++}) or decreasing (ON or OFF) promoter efficiency [21].

The alteration of slipped-strand mispairing mechanism regulates gene expression at the level of transcription [79]. This regulation is mediated by the presence of the repeats upstream of the encoding gene, which upon variation of the number of repeats, results in an increase or decrease in expression by varying the promoter spacing. Slipped-strand mispairing as a regulator mechanism is present in a wide range of bacteria and regulates various traits.

Genomic rearrangement

Genomic rearrangements combine a wide range of processes involved in phase variation, including inversions, deletions, gene duplication and gene transfer using silent copies (recombinational deletion) [80]. For example, control of expression of type 1 fimbriae in

Escherichia coli is based on the presence of inverted repeats and the action of site-specific recombinases. The presence of inverted repeats within the promoter region facilitates the inversion of the promoter switching expression ON or OFF. Alternatively, when the promoter itself is flanked by inverted repeats, different sets of genes can be expressed.

Table 2.1 Examples of phase variation traits (Source [21])

Mechanism	Locus	Species	Property affected	Reference
Slipped-strand mispairing ON↔OFF	*fuc*T2 (AF076779)	*Helicobacter pylori*	LPS antigenicity, Lewis Y antigen	[81]
	*lic*1A, 2A, 3A (M37912-14)	*Haemophilus influenza*	LPS antigenicity	[82]
	*pil*C (Z49120)	*Neisseria gonorhoeae*	Fimlbrial expression	[83]
	*sia*D (M95053)	*N. meningitides*	Capsular polysaccharides	[84]
	flhB (AF031418)	*Pseudomonas putida*	Flagellum export	[43]
	opa (P11296)	*N. gonorhoeae/N. meninggitides*	Adhension/invation/neutrophil interaction	[85]
	bvgS (M25401)	*Bordetella spp.*	Two component sensing	[86]
	lob1 (U94833)	*Haemophilus somnus*	Antigenicity of LOS	[87]
	p78 (AF100324)	*Mycoplasma fermantans*	ABC transporter	[88]
	ctpH (X74730)	*Vibrio cholera*	ToxR regulon	[89]
	hpuA (AF031495)	*N. gonorrhoeae*	Hemoglobin binding outer membrane proteins	[90]
	hmbR, hpuAB (AF105339, U73112)	*N. meningitidis*	Hemoglobin receptors	[91]
Genomic rearrangement				
Site-specific inversion ON↔OFF	*fimA* (Z37500)	*Escherichia coli*	Fimbrial expression	[92]
	omp1 (U02462)	*Dichelobacter nodusus*	Major outer membrane protein	[93]
	vspA (L81118)	*Moraxella bovis*	Surface lipoprotein antigens	[94]
	hsd1 (AF003541)	*Mycoplasma pulmonis*	DNA restriction and modification properties	[95]
$ON_a/OFF_b \leftrightarrow ON_b/OFF_a$	*Hin* (V01370)	*Salmonella* spp.	Flagellar expression	[96]
	piv (M34367)	*Maraxella lacunata*	Type IV fimbriae	[97]
Recombinational deletion ON ↔ OFF	*pilE* (AF043652)	*N. gonorrhoeae/N. meningitides*	Fimbrial expression	[98]
	vsg genes	*Trypanosoma* spp.	Variable surface glycoproteins	[99]
	vsp (AF396970 and AH008162)	*M. bovis*	Surface lipoproteins	[94]
	vsp/vlp (AF049852)	*Borrelia* spp.	Surface proteins	[100]
Spontaneous duplications ON↔OFF	*cap3, cap8, tts* (Z12159, AJ239004, AJ131985)	*Streptococcus pneumonia*	Capsule production	[101]
	pheN (U95300)	*Pseudomonas tolaasii*	Secondary metabolism/morphology	[39]
Differential methylation **ON↔ OFF**	*agn43* (U24429)	*E. coli*	Autoaggregation	[102]
	pap (X03391)	*E. coli*	Pilus expression	[103]
	pef (AB041905)	*Salmonella typhimurium*	Filmbrial expression	[104]
Unprogrammed variation				
Spontaneous mutation ON↔OFF	*vir* locus	*Bordetella bronchiseptica*	Virulence factors	[105]
	gacA/S (AY236957)	*Pseudomonas* sp. PCL1171	Secondary metabolism and exo-enzymes	[21]
Mechanism unknown		*Pseudomonas aeruginosa*	Motility/flagella/biofilm formation	[41]
		Pseudomonas brassicacearum	Root colonization/flagella/exo-enzymes	[40]
		Pseudomonas fluorescens WCS365	Root colonization	[42]
		P. fluorescens F113	Root colonization/motility/biofilm formation	[106]
		Azospirillum lipoferum	Motility and assimilation of certain sugars	[107]

LPS: lipopoly saccharide; LOS: lipo-oligo saccharide; ABC: ATP-binding cassette.

Gene conversion is the most versatile of all mechanisms of recombinational forms of antigenic variation. A second form of variation based on genomic rearrangement, uses deletion for example, when regulating variation of type IV pili in *N.gonorrhoeae* [108] and the expression of the surface protein in *Borrelia* spp. [100]. Gene duplication and gene transfer further create a large number of potential proteins to express. These combinations can enable bacteria to produce up to 10^7 variant proteins [109]. Examples of traits regulated via genomic rearrangement are presented in Table 2.1.

Differential methylation

Variation by differential methylation is described as an epigenetic phenomenon because phase variation occurs in the absence of a change in DNA sequence. It involves differentially methylated sequences in the regulatory regions of the phase-varying gene or operon. Since the expression state is heritable but reversible and since regulation is not caused by a DNA sequence change, it is epigenetic by definition. The essential feature of the DNA methylation-dependent phase variation system is that the methylation state of a target sequence at a specific site in the chromosome affects the DNA binding of a regulatory protein that directly regulates transcription.

The mechanism of differential methylation is extensively studied in the regulation of *P. fimbrial* (pyelonephritis-associated pili and Pap-related fimbriae) expression in uropathogenic *E. coli.* Fimbriae undergo reversible phase variation (ON-OFF) at 37°C, while at the temperatures below 26°C, expression is shut off [110]. The phase variation event at 37°C is controlled at the transcriptional level by differential methylation of two GATC sites (GATC-I and GATC-II). This methylation is regulated by the competitive action of deoxyadenosine methyltransferase (Dam) and leucine-responsive regulatory protein (Lrp): when GATC-I is methylated and GATC-II is unmethylated, the cells are phase OFF and in phase ON cells, GATC-I is unmethylated and GATC-II is methylated [110]. Examples of genes controlled via differential methylation are presented in Table 2.1.

Unprogrammed variation

Random unprogrammed variation is based on the introduction of mutation due to imperfect replication and the subsequent removal of these mutations, coinciding with a switch back to the wild-type situation. One of the drawbacks of a mechanism stimulating diversification

based on imperfect replication is a high mutation load. Higher organisms had to evolve a mechanism of mutation while controlling the mutation rate using mechanisms of mutation like mismatch repair pathways [80]. Most organisms use this strategy to create diversity. Examples of spontaneous mutations in phase variation, switching genes ON and OFF are presented in Table 2.1.

2.1.4 Significance for aquaculture

The mechanisms and ability of phase variation in bacteria under *in vitro* and *in vivo* conditions indicated that bacteria could alter their phenotypic traits, such as morphologies, antigenic and virulence factors for their thrive, survival and protection against biotic and abiotic stressors. Understanding phenotypic variation involved in bacterial diseases in aquaculture animals might have potential implications in the management of diseases in this sector. Until now, studies on the importance of phase variation in vibriosis in aquatic animals such as shrimp and fish are scarce. However, understanding the mechanism of phenotypic variation is vital in identifying and foreseeing the species that may cause future outbreaks. Recognizing the role of phenotypic diversity may also prevent potential harmful invading species and aid in the development of control measures against infectious diseases caused by bacteria in aquaculture.

Moreover, understanding processes of phenotypic variation and the response of the bacterial population to environmental stresses are fundamental to develop adequate disinfection protocols and therapeutic treatments. The knowledge of the determinant of phenotypic variation can be of great significance to solve real problems, such as multi-antimicrobial resistance, to design therapeutic approaches and for vaccine development for clinical diseases [26].

2.2 The innate immune system in invertebrates

In an aquatic ecosystem, crustaceans share their environment together with invading parasites and pathogens. Therefore, crustaceans must have an efficient immune system to defend themselves against invading pathogens.

The defense system of crustaceans includes a physical barrier namely the exoskeleton and the alimentary tract, and cellular and humoral innate defense reactions. The physical barriers are the first line of defense to combat pathogenic microorganisms. Once a pathogen overcomes

the physicochemical barrier of the cuticle, the cellular and the humoral innate defense system reactions occur instantly, allowing elimination or decrease of invading pathogens (Figure 2.6) [111]. The cellular immune reactions are mediated by circulating blood cells (which in crustaceans are known as hemocytes) that are capable of neutralizing and/or eliminating pathogens by phagocytosis, or entrapping them in hemocyte aggregates, nodules or encapsulation, whereas the humoral responses involve the synthesis and release of several immune proteins, such as antimicrobial peptides (AMPs), proteinase inhibitor, production of reactive oxygen and nitrogen metabolites, complex enzymatic cascades leading to melanization and clotting proteins [112].

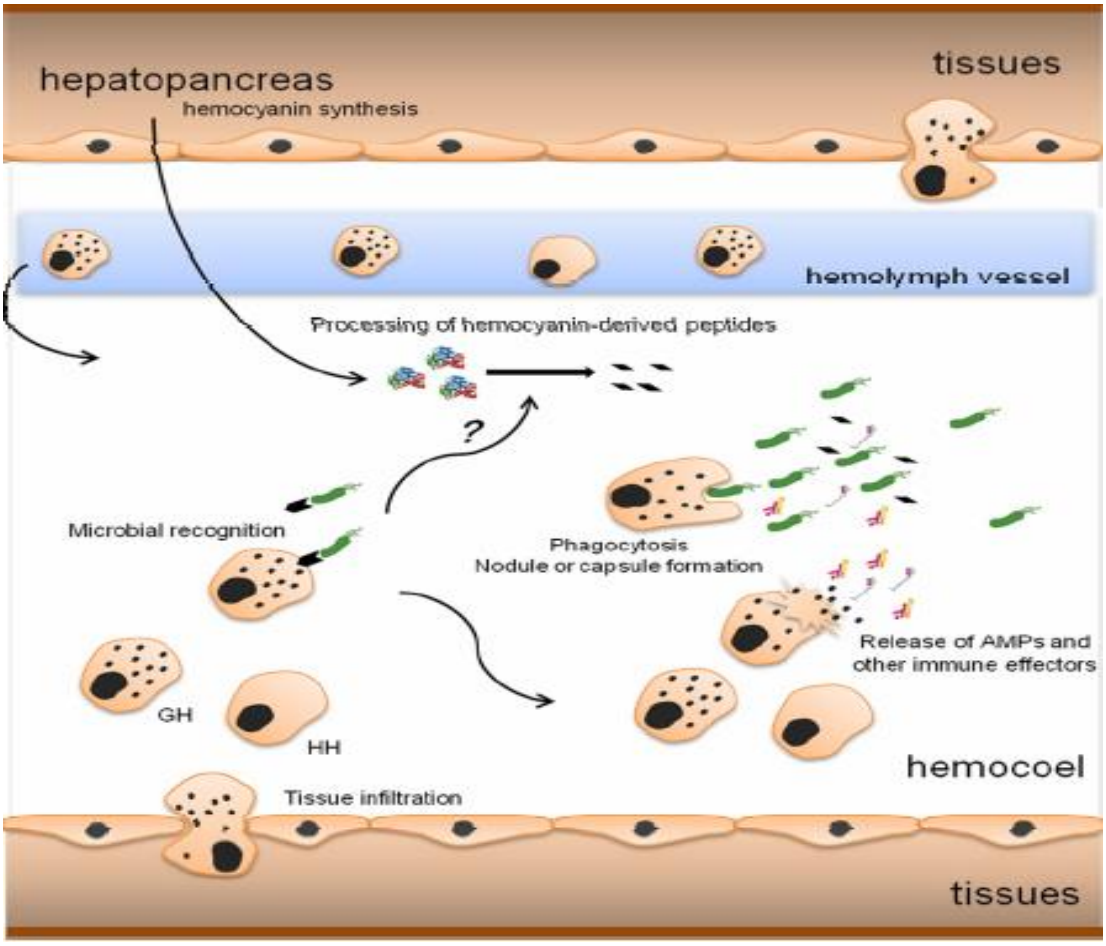

Figure 2. 6 Crustacean cellular and humoral immune-reactions after a microbial challenge (Source [111]).

HH= hyaline hemocytes, GH = granular hemocytes

2.2.1 Pattern recognition receptors (PRRs) in crustacean

PRRs recognize the presence of pathogens by binding to molecular patterns rather than to a specific component of a given pathogen. These molecular patterns, called pathogen-associated molecular patterns (PAMPs) include lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria, peptidoglycan (PGC) and lipoteichoic acid (LTA) from Gram-positive bacteria, and glucans from fungal cells [113]. Moreover, the patterns can be polynucleotides, such as bacterial and viral unmethylated CpG DNA, single-strand and double-strand RNA from viruses [114]. The recognition process can trigger the innate immune response in crustaceans. To date, 11 types of PRRs have been identified in shrimp, for instance, β-1,3-glucanase-related proteins, β-1,3-glucan-binding proteins, C-type lectins, scavenger receptors, galectins, fibrinogen-related proteins, thioester-containing proteins, down syndrome cell adhesion molecules, serine protease homologs, trans-activation response RNA-binding protein and Toll like receptors [115]. The Down syndrome cell adhesion molecule (dscam) is also involved in neural development [116]. The typical domain structure of dscam is highly conserved within arthropods and vertebrates. Previous studies based on the genomic analysis showed that *dscam* gene of *Daphnia pulex* and *P. leniusculus* can generate up to 13,000 and 22,000 different unique isoforms, respectively, by alternative splicing of variable exons [117, 118]. Obviously, *dscam* can produce pathogen-specific splice form variants with specific binding capabilities after immune challenge or infection resulting in actively recognized and ingested of this foreign agent by phagocytic hemocytes [119]. In *Drosophila*, dscam has been confirmed as a receptor involved in phagocytosis through its ability to bind foreign cells. Dong *et al.* [119] reported that transient silencing of *Anopheles gambiae dscam* depleted the mosquito resistance to bacterial infection. In addition, recently another important PRR which received more attention is the lipopolysaccharide and β-1, 3-glucan binding protein (*lgbp*). It is a pattern recognition receptor for lipopolysaccharide and β-1, 3-glucan, the latter being present in Gram-negative bacteria and fungi. In crustacean, the binding of LGBP to lipopolysaccharide or β-1, 3-glucan has been documented to activate the proPO system of the freshwater crayfish, *P. leniusculus* [120]. Recently, Amparyup *et al.* [121] found that *PmLGBP* transcripts are expressed in the hemocytes of *Penaeus monodon* after *V. harveyi* infection. The recombinant protein (r*Pm*LGBP) could enhance the phenoloxidase activity of a hemocyte suspension in the presence of LPS and β-1, 3-glucan.

In addition, the innate immune system is activated by endogenous substances released by damaged or dying cells, called damage-associated molecular patterns (DAMPs). High-mobility group box 1 (hmgb1) and heat shock protein 70 (hsp70) are DNA and protein chaperons respectively. These molecules are considered as extracellular signaling molecule and damage associated molecular proteins during inflammation and various cellular processes. Extracellular Hmgb1 and hsp70s have been documented to be involved in activation of cell surface innate immune receptors, through Toll-like receptors upon contact with pathogenic biotic stressors, thereby affecting many aspects of the host immune responses [122]. Hsp70 is an immune modulator and can be up-regulated after infection. Heat shock proteins are involved in the folding and unfolding of other proteins and are induced when the host is exposed to high temperatures or other stress factors. Hsp70 can be both intracellular providing cytoprotection through protein folding, and extracellular, facilitating immune responses [123]. Hsp70 is known to be a molecular chaperon but it seems also to mediate humoral and cellular innate immune responses [124].

2.2.2 Melanization and prophenoloxidase activating system (proPO system)

The prophenoloxidase (proPO) activating system plays an important role in the crustacean immune system as it is considered to be one of the major immune defense mechanisms against foreign particles or molecules for invertebrate animals [125]. The recognition of the microbial PAMPs (elicitor) by PRRs activates the serine proteinase cascade and eventually culminates in the proteolytic cleavage of inactive proPO to the active PO enzyme, this results in the production of the polymeric melanin and its accumulation at the site of infection or around the surface of foreign microorganisms.

Melanization plays crucial role in defense reactions, such as wound healing encapsulation, sequestration of microbes, and the production of toxin intermediates, that are may kill invading microorganisms [126]. The melanization reaction can be observed at the site of cuticle injury or on the surface of invading parasite or pathogens. This melanotic reaction occurs due to the synthesis and deposition of melanin mediated by proPO activation (Figure 2.7) [127].

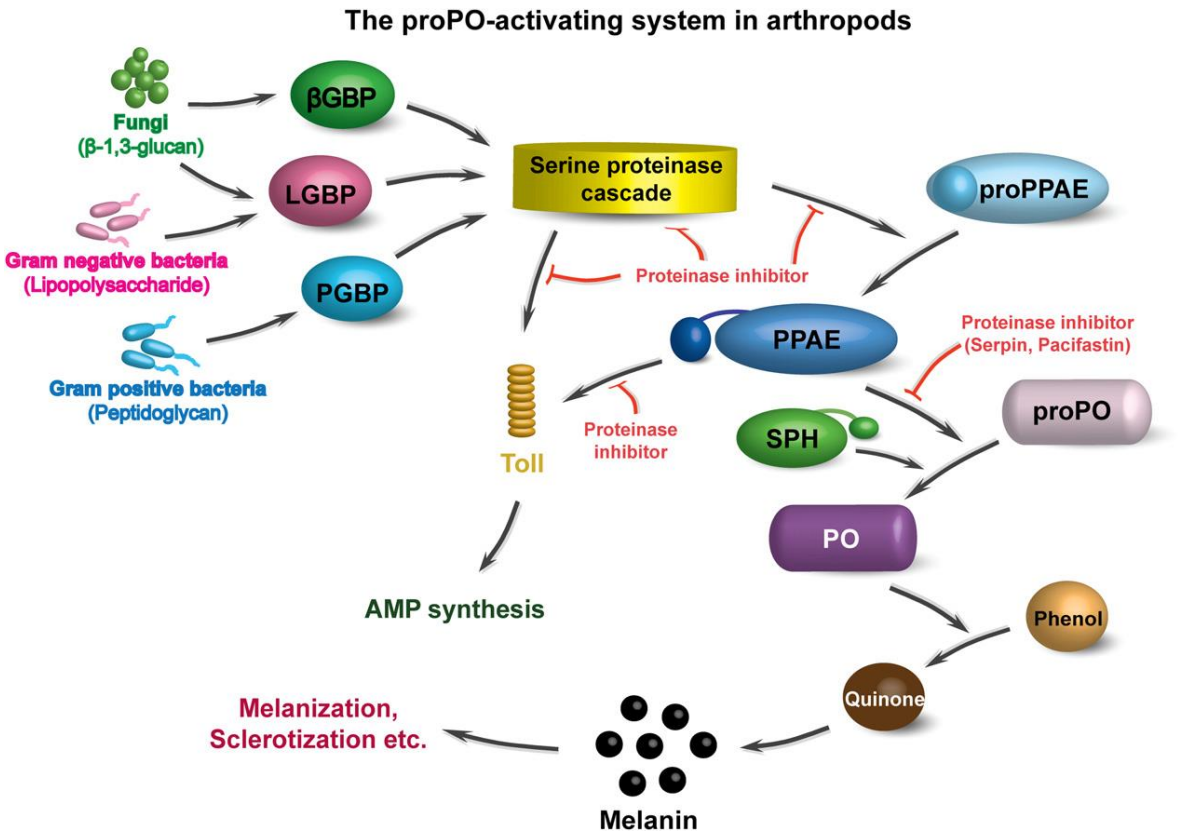

Figure 2. 7 Schematic view of the prophenoloxidase (proPO)-activating system in arthropods (Source [128]).

During microbial infection, non-self molecules that act as PAMPs are recognized by appropriate PRRs, leading to the activation of the serine proteinase cascade, and resulting in a final clip-domain serine proteinase designated as proPo-activating enzyme (PPAE). Subsequently, the inactive proPO zymogen is converted to active phenoloxidase (PO), by PPAE to produce quinones, which can cross-link neighboring molecules to form melanin around invading microorganisms.

In crayfish, the PO has been suggested to be involved in the defense of the host against *Aeromonas hydrophila* infection. Thus, silencing of crayfish proPO leads to increased bacterial growth, lower phagocytosis, lower PO activity, lower nodule formation, and higher mortality when the animals are infected with *Aeromonas*. In contrast, depletion of pacifastin, an inhibitor of the crayfish proPO activation cascade, resulted in lower bacterial growth, increased phagocytosis, increased nodule formation, higher phenoloxidase activity and delayed mortality [129].

The melanization cascade (the proPO activating system) was indicated to be associated with factors stimulating cellular defense by aiding phagocytosis [127]. Mavrouli *et al,* [130] reported that proPO can bind to the outer membrane of some hemocytes, enhancing the

melanization response. Another study indicated that PO might be involved in the blood clotting process, promoting the cellular defense reaction and enhancing the efficiency of plasma coagulation following *Anopheles gambiae* [131].

Upon the activation of the proPo system in crayfish, peroxinectin, a cell adhesion factor that functions as peroxidase and opsonin are generated [132]. Peroxinectin is produced in the blood cells, stored in granules and released during degranulation [133]. The formation of peroxinection is activated by lipopolysaccharide and β-glucan. It activates the proPO cascade and promotes phagocytosis or encapsulation [116]. It was reported that peroxinectin might produce hypohalic acid from hydrogen peroxide produced by SOD and consequently, it functions as an efficient microbial attack system to invading microorganisms [134]. Recently, Sivakamavalii *et al.* [135] suggested that peroxinectin was involved in the regulatory mechanism of PO enzyme synthesis in the shrimp *Fenneropenaeus indicus.*

Melanization induced by activation of the proPO system plays an important role in the immune defense. However, the production of quinone in the melanization process can be harmful to the animal [136]. Therefore, this process requires melanization inhibitors, for example, melanization inhibitor protein (mip) to regulate unwanted production of quinones [137]. The study of Angthong *et al. [138]* on *Penaeus monodom* suggested that upon *V. harveyi* infection the *Pm*mip protein is first released from the tissue into hemolymph and then degraded to allow melanization to occur for eliminating the bacteria. In addition, serpin - another proteinase inhibitor that functions as a negative regulator of the proPO cascade by inhibiting the serine proteinases has been reported to occur in insects and crustaceans [127].

2.2.3 Antimicrobial peptides (AMPs)

Antimicrobial peptides or proteins (AMPs) are one of the major components of the innate immune defense and are widespread in nature, from bacteria to mammals. AMPs are primarily known as “natural antibiotics” because of their rapid and efficient antimicrobial effects against a broad range of microorganism, including Gram-positive and Gram-negative bacteria, yeast, fungi, protozoa and viruses [111, 139].

Basically, AMPs predominantly act by disrupting the membrane integrity of the cell target. The cationic portion of the peptide is first attracted to the negatively-charged bacterial and fungal cell walls and/or membranes. Subsequently, the peptide inserts into and permeabilizes the microbial cell membranes through its hydrophobic portion. This results in the destruction

of the microorganism via membrane destabilization and/or pore formation [140]. In addition, other evidence suggested that AMPs could be translocated into the cytoplasm of the microorganism, where they act on specific intracellular targets. Once inside, the peptides interfere with several essential metabolic functions, such as protein, nucleic acid and cell wall syntheses, leading to bacterial death [140, 141].

During the past few years, AMPs have been intensively studied in crustaceans, including astacidin 1, astacidin 2 and crustin1-3. In freshwater crayfish, these peptides were shown to markedly inhibit the growth of both Gram-positive and Gram-negative bacteria [142]. Anti-lipopolysaccharide factors (ALFs) is one type of the AMPs in penaeid shrimps [143]. AFLs were first isolated from the hemocytes of horsehoe crabs (*Tachlypleus tridentatus, Limulus polyphemus)* and from the shrimp *P. monodon.* Being one of the six ALFs in *P. monodon,* AFL*Pm3* is the most abundant isoform and it showed bactericidal effect on Gram-negative and Gram-positive bacteria and it can bind to the main bacterial cell wall components of Gram-negative and Gram-positive bacteria, i.e. LPS and lipoteichoic acid respectively during a *V. harveyi* infection. These results suggested that AMP*Pm3* performs its antibacterial activity by binding to components of the bacterial cell wall with a high affinity [144]. Jaree *et al.* [145] showed that the recombinant (rALF*Pm3)* could bind to *V. harveyi*, inhibit growth and effectively kill *V. harveyi* through altering the bacterial membrane permeability.

2.2.4 Coagulation

A coagulation system is the first line of defense and it is considered as part of the whole invertebrate immune system. Clotting plays an important role in prevention of blood loss and in the initiation of the wound healing process in crustaceans. It is also critical in forming a secondary barrier to infection by immobilizing bacteria/fungi and hence promoting their killing.

Two different coagulation mechanisms have been characterized in invertebrates: 1) the hemocyte-derived clotting cascade in the horseshoe crab (*Tachpleus tridentatus*) [146] and 2) the transglutaminase (TGase)-dependent clotting reaction in freshwater crayfish, (*Pacifastacus leniusculus*) [147]. The proteins participating in the horseshoe crab clotting system are all stored in the hemocytes and upon activation they are released form the cytoplasmic L-granules into the hemolymph through rapid exocytosis. The horseshoe crab clotting system is a proteolytic cascade and it is activated by microbial cell wall components,

subsequently leading to the activation of the proclotting enzyme and resulting in conversion of a soluble protein (coagulogen) into an insoluble aggregate (coagulin) [146]. In crustacean, clotting works through the polymerization of the clotting protein catalyzed by a Ca^{2+} ion dependent transglutaminase (TGase), which is released from hemocytes upon activation by foreign microorganism or tissue damage [147] (Figure 2.8). TGase and clottable protein have been suggested to be involved in the blood coagulation system of many crustaceans, such as shrimps, lobsters and freshwater giant prawns. A higher mortality rate was observed in TGase and clotting protein depleted infected shrimps, illustrating the crucial role of these two molecules in shrimp immune protection against microbial challenge [148].

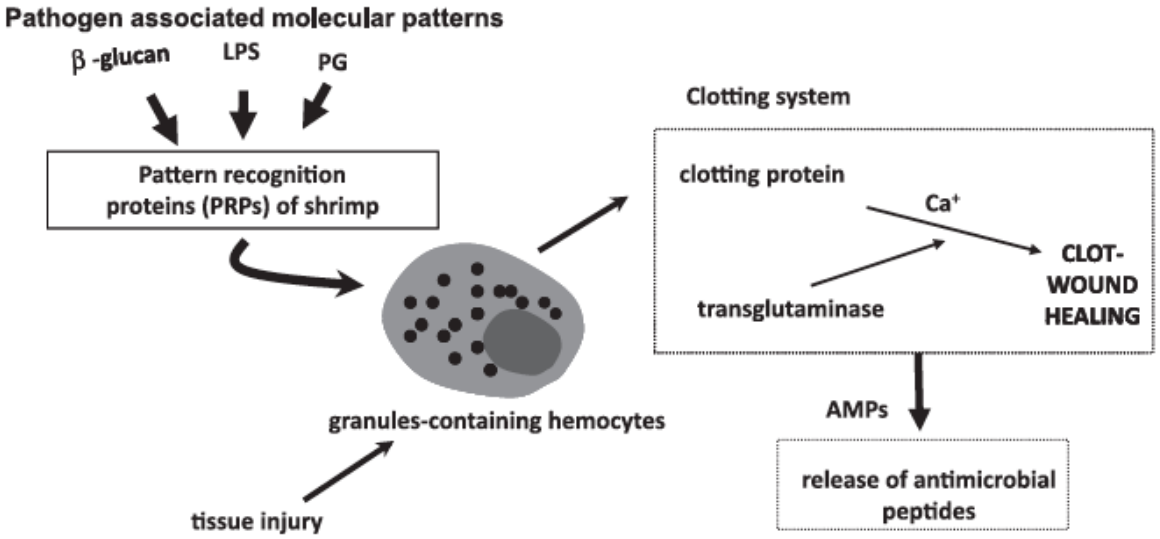

Figure 2. 8 Proposed clotting system in shrimp and its association with the activation of AMPs (Source [149]).

2.2.5 Cellular immune response

Cellular immune responses are regulated by circulating blood cells or hemocytes that are capable of neutralizing and/or eliminating pathogens by phagocytosis or entrapment in hemocyte aggregates or nodules, or by encapsulating larger microorganisms such as certain parasites [150]. Nodule and capsule formation become melanized, through the action of phenoloxidase [125]. Phagocytosis is one of the earlier immune responses in invertebrates. It is initiated by the attachment of the phagocyte to the target particle, followed by cytoskeleton modification, and finally internalization and destruction of the engulfed target within phagosomes.

In crustaceans, the hemocytes originate from the hematopoietic tissue and mature hemocytes can be divided into three cell types based on their structural and functional features: hyaline cells (HCs), semigranular cells (SGCs) and granular cells (GCs). Each of them has different biological functions (Table 2.2). Recently, hemocytes of penaeid shrimp (*Litopenaeus vannamei*) were separated into five subpopulations (Figure2.9) [151].

Table 2. 2 Hemocyte types and known biological functions (source: [152])

Hemocyte type	**Function in immunity**
Hyaline cell	Phagocytosis
Semigranular cell	Encapsulation Phagocytosis (limited) Storage and release of the proPO system Cytotoxicity
Granular cell	Storage and release of the proPO system Cytotoxicity

Phagocytosis plays an important role in host defense against invading pathogens and in the removal of apoptotic cells produced during development. Several receptor proteins involved in phagocytosis have been well documented such as members of the scavenger receptor family [153], the epidermal growth factor (EGF)-domain protein Eater [154], complement-like opsonins, and the immunoglobulin super family (IgSF)-domain protein down syndrome cell adhesion molecule (Dscam) [155]. In *Drosophila*, it was observed that suppression of NimC1 – a putative phagocytosis receptor known to be present in *Drosophila* plasmatocytes would inhibit the phagocytosis of *Staphylococcus aureus* [154]. Recently, scavenger receptors (SRs) that are known to be involved in innate immunity through recognizing pathogen-associated molecular patterns (PAMPs) and in pathogenesis of diseases through interaction with damage-association molecular patterns (DAMPs). It has been shown that after knockdown of B scavenger receptor from kuruma shrimp, *Marsupenaeus japonicus* (MjSR-B1), both bacterial clearance and phagocytotic ability of *M. japonicas* against *V. anguillarum* were impaired, and several phagocytosis-related genes were down-regulated. Overexpression of MjSR-B1led to enhanced bacterial clearance, phagocytosis rate and up-regulation of phagocytosis-related and antimicrobial peptide genes [156].

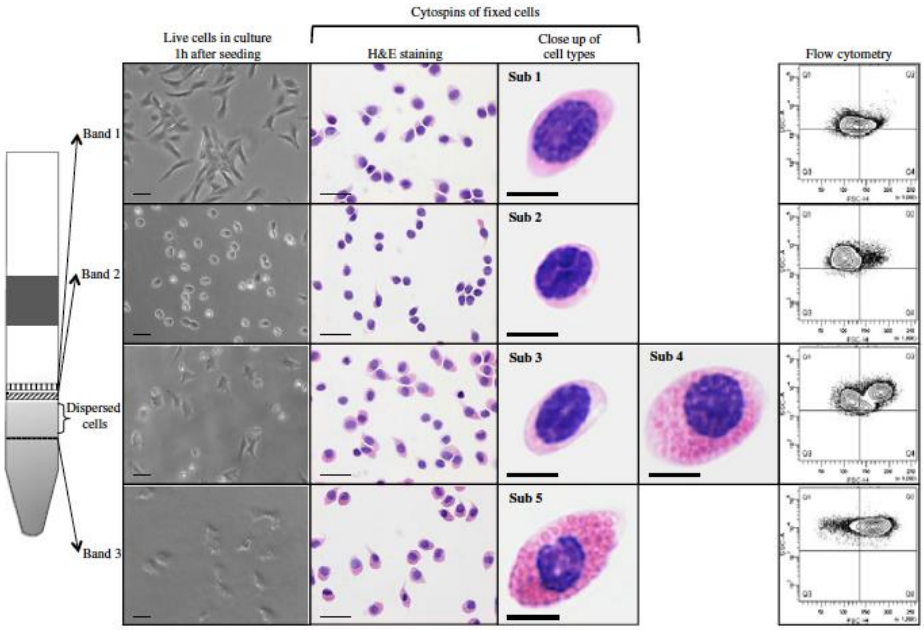

Figure 2. 9 Analysis of individual *P. vannamei* hemocyte band by *in vitro* culture, H&E staining and flow cytometry (Source [151]).

Subpopulation 1 (sub 1) showed cells that attached strongly to glass, had an average diameter size, containing little or no basophilic granules in the cytoplasm and high nucleus: cytoplasmatic ratio (N:C). Subpopulation 2 (sub 2) enclosed cells that did not attach or attached very weakly to glass, had a small diameter size, contained few small basophilic cytoplasmatic granules and had a very high N:C ratio. The dispersed cell fraction enclosed two cells types, cells of subpopulation 3 (sub 3) and cells of subpopulation 4 (sub 4). Sub 3 cells behaved similarly to sub 2 cells but had a higher N:C ratio and diameter. Sub 4 cells behaved similarly to subpopulation 5 (sub 5) cells but the granularity was lower and the diameter was the biggest.

2.3. Gnotobiotic *Artemia* culture (GART) system

Artemia franciscana was used as a model organism throughout this PhD study. *Artemia franciscana* is a small branchiopod crustacean found almost worldwide in nature saline lakes and solar salt works. They are used worldwide as live feed in aquaculture production. It has been used as a model organism for host-microbial studies among others. They are unique organism that can produce dormant cysts or encapsulated embryos, called cysts, when conditions are unfavorable. These cysts can be stored during a long period of time in a dry state, making them readily available on the market. When brought back into favorable conditions, the cyst, if out of diapause, will hatch within 24 h and can used as live feed for culturing other species or for scientific purposes [157].

The rational for using a GART system is to eliminate the possibility of microbial interference in mechanistic studies [158]. In GART system, axenic *Artemia* were obtained following decapsulation and hatching (section 4.2.2). GART system offers a unique way to study host-

microbe interactions without interference of unknown microbial communities that are naturally present in the rearing environment. Being small animals with a short life cycle, they allow for an easy integration of experimental approaches and molecular analysis [159].

Chapter 3

Characterization of phenotype variations of luminescent and non-luminescent variants of *Vibrio harveyi* wild type and quorum sensing mutants

Redrafted after:

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ABSTRACT

Vibrio harveyi, a luminescent Gram-negative motile marine bacterium, is an important pathogen responsible for causing severe diseases in shrimp, finfish and molluscs leading to severe economic losses. Non-luminescent *V. harveyi* obtained by culturing luminescent strains under static and dark condition were reported to alter the levels of virulence factors and *metalloprotease* gene and *luxR* expression when compared to their luminescent variants. We conducted an *in vitro* study aiming at the characterization of virulence-related phenotypic traits of the wild type *V. harveyi* BB120 strain and its isogenic quorum sensing mutants before and after switching to the non-luminescent status. We measured the production of caseinase, haemolysin and elastase and examined swimming motility and biofilm formation. Our results showed that switching from the luminescent to the non-luminescent state changed the phenotypic physiology or behavior of *V. harveyi* resulting in alterations in caseinase and hemolytic activities, swimming motility, and biofilm formation. The switching capacity was to a large extent independent from the quorum sensing status, because quorum sensing mutants were equally capable of making the phenotypic switch.

Keywords: phenotype switch, virulence factor, *Vibrio harveyi*, luminescent, non-luminescent.

3.1 Introduction

Vibrio harveyi is a luminescent Gram-negative motile marine bacterium widely distributed in the marine environment [7]. This bacterium is a major pathogen responsible for causing life-threatening vibriosis in the wild and in cultured aquatic animals such as shrimps, finfish and molluscs. In particular, at early live stages, the infection leads to severe economic losses and marked impacts on biodiversity [7, 160]. *V. harveyi* is known to exist as free-swimming single cells, adhered to abiotic surfaces as a constituent of biofilm consortia, and in association with a host, as a pathogen of marine animals [161].

Several studies have shown that the pathogenicity of *V. harveyi* is associated with extracellular compounds, and the production of some of these compounds is regulated by quorum sensing [11, 162, 163]. By definition, quorum sensing or cell-to-cell communication between bacteria refers to a mechanism by which bacteria coordinate certain phenotypic behaviors (such as virulence factor production) by secreting, detecting and responding to small signal molecules [164]. *V. harveyi* uses a multi-channel quorum sensing system with three quorum sensing signal molecules: harveyi autoinducer 1 (HAI-1), autoinducer 2 (AI-2) and cholerae autoinducer 1 (CAI-1), which act synergistically in virulence gene regulation [165-167]. The signal molecules are detected at the cell surface by distinct membrane-bound receptors feeding a shared phosphorylation/dephosphorylation signal transduction cascade. Central in this signal transduction cascade is the LuxO protein. At low concentrations of the signal molecules, LuxO is phosphorylated and indirectly inhibits the production of the transcriptional regulator protein LuxR. Conversely, LuxO becomes dephosphorylated in the presence of high concentrations of signal molecules, leading to the translation of the *luxR* mRNA. The LuxR protein, finally, regulates the transcription of quorum sensing target genes [168].

Bioluminescence in *V. harveyi* is another phenotypic characteristic that is regulated by quorum sensing [169]. The biochemistry and genetics of bioluminescence have been extensively investigated [170, 171]. However, its biological role in non-symbiotic bacteria remains elusive. Bioluminescence consumes 20% of the cellular energy [172], and consequently, it must confer a considerable selective advantage on non-symbiotic bacteria or else it would have been lost during evolution. One of the assumptions is that *V. harveyi* emits light to stimulate DNA repair [170]. Interestingly, *V. harveyi* bioluminescence has also been shown to be involved in the detoxification of reactive oxygen species such as H_2O_2, thus playing a protective role against oxidative stress [173]. As H_2O_2 is an important part of the

innate immune defense mechanism in eukaryotic hosts against infections [174], bioluminescence could also be considered as a virulence factor or innate immune evasion strategy. In a previous study, we obtained non-luminescent variants of luminescent *V. harveyi* strains by culturing the latter under static conditions in the dark and found that these non-luminescent variants were less virulent towards brine shrimp (*Artemia franciscana*) larvae than their luminescent counterparts [49]. In a second study, we found that the non-luminescent variants produced lower mRNA levels of the quorum sensing master regulator *luxR*, and the *vhp* metalloprotease (known to be regulated by quorum sensing) [69].

Virulence factors are utilized by bacteria to evade the host immune system, to cause diseases and resist to antibiotics. Here we aimed at characterizing the production of virulence factors, including caseinase, haemolysin, elastase, as well as the swimming motility, and biofilm formation in isogenic luminescent and non-luminescent variants of *V. harveyi* wild type and quorum sensing mutants. These virulence factors have often been studied in *Vibrio* spp. and serve as credible biomarkers to detect and describe virulence of *Vibrio* [175], and their associated lethality towards different hosts *in vivo* [11, 176].

3.2. Bacteria strains and growth conditions

3.2.1 Bacterial strains and growth conditions

V. harveyi strains used in this study are shown in Table 3.1. The strains were stored in 20% glycerol at -80 °C. Rifampicin resistant (RR) strains were produced by inoculating one fresh single colony from Marine Agar (MA, Difco laboratories, MI, USA) plates (28 ^{o}C for 24 h) in a 100 mL Erlenmeyer flask containing 10 mL Marine Broth (MB) 2216 (Difco laboratories, MI, USA) and incubating the flask at 28 ^{o}C for 24 h. Bacterial cultures (50 μL) were transferred to a 10 mL MB containing 0.5 mg L^{-1} of rifampicin (R3501, Sigma-Aldrich, USA). The culture was incubated at 28 ^{o}C for 24 - 48 h. The following days, sub-cultures were made in MB with increasing concentration of rifampicin (50-100 mg L^{-1}). When the bacteria were grown in the final rifampicin concentration of 100 mg L^{-1}, bacterial supernatants were inoculated on MA containing 100 mg L^{-1} of rifampicin, and incubated overnight at 28 ^{o}C for obtaining single colonies.

Autoclaved *Aeromonas* sp. LVS3 bacteria were used as feed source for *Artemia* [158]. LVS3 was grown in MB. Overnight grown LVS3 in MB was centrifuged at 2200 x *g* for 15 min. The supernatant was discarded, and the pellet was re-suspended in filtered autoclaved

seawater (FASW). The bacterial densities were determined spectrophotometrically at an optical density (OD) of 550 nm assuming that an OD of 1.0 corresponds to 1.2 x 10^9 cells mL^{-1} (McFarland standard; BioMerieux, France). Subsequently, the cell suspension was autoclaved at 121 °C for 20 min.

3.2.2 Selection of non-luminescent variants

Non-luminescent variants (RR-NL) were selected as described previously [49]. Briefly, one single freshly grown luminescent colony (RR-L) was inoculated in a 250 mL Erlenmeyer flask containing 5 mL MB. The culture was kept in the dark under static conditions for 2 or 3 days. Subsequently, the medium was discarded and the Erlenmeyer flask was rinsed with FASW to remove the motile cells. The cells attached to the bottom of the flask were collected by adding 1 mL FASW, and collected by shaking. Re-suspended cells were inoculated on MA plates supplemented with 100 mg L^{-1} of rifampicin, and incubated for 24 h at 28 °C to check for the presence of the dark colonies. In case no non-luminescent colonies were detected, fresh MB was added to the Erlenmeyer and the above-described procedure was repeated until a non-luminescent strain was found.

3.2.3 Measurement of bioluminescence

Overnight grown bacterial cultures were diluted to an OD of 0.2 at 600 nm. Subsequently, 200 µL aliquots were transferred into the wells of a black 96-well, flat-bottomed sterile polystyrene microtiter plate. The bioluminescence was measured by using a Tecan Infinite 200 microplate reader (Tecan, Switzerland)[14].

Table 3. 1 Bacterial strains used in this study

Strain	Relevant feature	References/Sources
BB120	Wild type, luminescent	[177]
BB120 RR-L	Rifampicin-resistant luminescent	This study
BB120 RR-NL	Rifampicin-resistant nonluminescent	This study
BB152	Mutation in LuxM (AI-1 synthase), luminescent	[169]
BB152 RR-L	Rifampicin–resistant luminescent	This study
BB152 RR-NL	Rifampicin-resistant nonluminescent	This study
MM30	Mutation in LuxS (AI-2 synthase), luminescent	[178]
MM30 RR-L	Rifampicin–resistant luminescent.	This study
MM30 RR-NL	Rifampicin-resistant nonluminescent	This study
MM77	Mutation in LuxM (AI-1 synthase) and LuxS (AI-2 synthase), luminescent	[179]
MM77 RR-L	Rifampicin-resistant luminescent.	This study
MM77 RR-NL	Rifampicin-resistant nonluminescent.	This study
JMH603	Mutation in CqsA (CAI-1), luminescent	[161]
JMH603 RR-L	Rifampicin resistant luminescent.	This study
JMH603 RR-NL	Rifampicin-resistant nonluminescent	This study
JMH606	Mutation in LuxM (AI-1 synthase) and CqsA (CAI-1)	[161]
JMH606 RR-L	Rifampicin-resistant luminescent.	This study
JMH606 RR-NL	Rifampicin-resistant nonluminescent.	This study
JMH634	Mutation in LuxM (AI-1 synthase), LuxS (AI-2 synthase) and CqsA (CAI-1), luminescent	[161]
JMH634 RR-L	Rifampicin-resistant luminescent.	This study
JMH634 RR-NL	Rifampicin-resistant nonluminescent	This study

3.2.4 Caseinase and haemolysin activities, and swimming motility

The caseinase and haemolysin activities were measured as described by Natrah *et al.* [11]. The caseinase agar plates were prepared by mixing double strength MA with 4% skim milk powder suspension (Oxoid, Basingstoke, Hampshire, UK), each sterilized separately at 121°C for 5 min prior to mixing. Hemolytic assay plates were prepared by supplementing MA with 5% defibrinated sheep blood (Oxoid, Basingstoke, Hampshire, UK). Swimming motility assay plates were prepared by supplementing MB with 0.3% agar [180]. Overnight grown bacterial cultures were diluted to an OD_{600} of 0.5. Subsequently 10 µL was spotted in the middle of each test plate. For caseinase and haemolysis activities, clearing zones surrounding the bacterial colonies were measured after 2 days of incubation. Swimming motility zones were measured after 24 h of incubation.

3.2.5 Elastase activity

Elastolytic activity in all bacterial strains was determined as described by Visca *et al.* [181], utilizing elastin Congo red (Sigma-Aldrich, USA) as substrate. The bacterial cultures were centrifuged, supernatants filter sterilized (0.22 µm), and then, the sterile filtrates (150 µL) were transferred to Eppendorf tubes. Subsequently, 20 mg of elastin Congo red and 850 µL of 100m M Tris-Cl/1 mM $CaCl_2$ solution (pH 7.5) were added. The mixtures were incubated overnight at room temperature with continuous shaking. Finally, insoluble particles were removed by centrifugation at 13 000 x *g* for 1 min and the supernatant was used for measuring the absorbance at 495 nm.

3.2.6 Quantification of biofilm formation

Biofilm formation was quantified as described previously [182] with some modifications. Briefly, strains were grown in MB overnight at 28 °C until an OD_{600} of 1 and then diluted in fresh MB to an OD_{600} of approximately 0.1. Aliquots (200 µL) were transferred, in triplicate, into the wells of a sterile polystyrene microtiter plate. Non-inoculated wells receiving only MB were used as negative controls. The plate was covered with a lid and incubated at 28 °C for 24 h under static conditions. The bacterial suspensions were aspirated and each well was rinsed three times with 300 µL of sterilized distilled water. After that, the biofilms were fixed with 150 µL of methanol for 20 min. The plate was air-dried, and the biofilms were stained with 150 µL of crystal violet solution (1%) for 15 min at room temperature, washed in running water and dried at room temperature. One hundred and fifty µL of 95% ethanol was

gently added to each well and then incubated at room temperature for 30 min without shaking. The OD_{570} was measured using a Tecan Infinite 200 microplate reader.

3.2.7 Protein extraction and DnaK detection

Bacterial strains were cultured overnight in MB-medium containing 100 mg L^{-1} of rifampicin at 28 °C under constant shaking (150 min^{-1}). The cultures (OD_{600}: 0.8-1) were centrifuged at 4000 x *g* for 15 min. The pellets obtained were rinsed once with FASW (35 g L^{-1}). Bacteria were homogenized by rapid agitation with 0.1 mm diameter glass beads in cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 40 mM HEPES, pH 7.4) containing a protease inhibitor cocktail (Sigma-Aldrich, Inc. USA). Subsequent to centrifugation at 2200 x *g* for 1 min at 4 °C, supernatant protein concentrations were determined by the Bradford method [183]. Supernatants (30 µg protein) were then combined with loading buffer, vortexed, heated at 95 °C for 5 min, centrifuged at 4000 x *g* for 1 min and then electrophoresed in 10% SDS-PAGE gels. HeLa cells (Enzo Life Sciences, USA) (12 µg) served as a positive control and for calculating the amount of DnaK in each sample. Proteins were transferred to polyvinylidene fluoride membranes (BioRad Immun-BlotTM PVDF). After incubation in blocking buffer (phosphate-buffer saline containing 0.2% *(v/v)* Tween-20 and 5% (*w/v*) bovine serum albumin) for 1 h, the membrane was incubated with rabbit polyclonal antibody raised against the ATPase domain of *E. coli* DnaK (a generous gift from Dr. Bernd Bukau, ZMBH) and then with horseradish peroxidase conjugated goat anti-rabbit IgG at recommended dilutions of 1:2500 and 1:5000, respectively. Membranes were treated with Immune-star™ WesternC™ chemiluminescence reagent (Biorad, Belgium), and analyzed by the ChemiDoc MP Imaging System (Biorad, Belgium). The relative signal intensity was quantified by densitometry using Biorad Image Lab™ Software version 4.1.

3.2.8 Brine shrimp *Artemia* challenge tests

After 28 h of incubation, groups of 20 nauplii were transferred to sterilized 40 mL glass tubes containing 20 mL of FASW (35 g L^{-1}). *Artemia* were fed once with 10^7 cells mL^{-1} autoclaved LVS3. Simultaneously, they were challenged with different vibrio species at 10^7 cells mL^{-1}. The survival of *Artemia* was scored at 48 h post challenge. The relative percentage of survival (RPS) of *Artemia* was calculated as described by Amend [184]. Each treatment was carried out in quadruplicate. All manipulations were performed under a laminar flow hood in order to maintain the axenity of cyst and nauplii. The axenity of the control (to which no live bacteria

added) was checked at the end of the experiment by inoculating 100 µL of the culture water on fresh MA plate. The plates were incubated at 28 °C for 3 days.

3.2.9 Statistical analysis

Significant differences in the phenotypes between the luminescent and non-luminescent variants and between the luminescent wild type BB120 with the other luminescent quorum sensing mutant strains were analyzed by independent sample student's *t*-tests. Differences were considered statistically significant when $P < 0.05$.

3.3 Results

3.3.1 Luminescence

The luminescence of the luminescent (L) and non-luminescent variants (NL) of wild type *V. harveyi* and its quorum sensing mutants was verified prior to the analysis of additional phenotypic characteristics. The luminescence of the non-luminescent variants was significantly low compared to the respective luminescent variants (Table 3.2).

Table 3. 2 Luminescence (counts/sec) of the isogenic luminescent and non-luminescent variants of *V. harveyi* wild type and quorum sensing mutants

Bacterial strains	Features	Luminescent	Non-luminescent
BB120	Wild type	28×10^5	17
BB152	Inactive HAI-1 synthase	19×10^5	21
MM30	Inactive AI-2 synthase	17×10^5	26
MM77	Inactive HAI-1 & AI-2 synthase	21×10^2	22
JMH603	Inactive CAI-1 synthase	15×10^4	215
JMH606	Inactive HAI-1 & CAI-1 synthase	60×10^2	31
JMH634	Inactive HAI-1, AI-2 & CAI-1 synthase	34×10^3	202

3.3.2 Caseinase activities

Among the luminescent variants, the wild type *V. harveyi* BB120 and the mutant BB152 showed the highest caseinase activity and the activity of their caseinase was statistically the same. The mutants JMH603, JMH606 and JMH634 showed a significantly lower caseinase activity which also differed significantly from the caseinase activity of the wild-type *V. harveyi* BB120 (Table 3.3). In addition, there was also a significant difference in the caseinase activity between the luminescent wild type BB120 and the luminescent mutants MM30 and MM77.

Comparison between the luminescent and non-luminescent variants showed that the caseinase activity of the luminescent variants of BB120, BB152, MM77 and JMH634 was significantly higher than their respective non-luminescent variants, whereas the luminescent variants JMH603 and JMH606 exhibited significantly lower caseinase activity than their corresponding non-luminescent variants. Among all the bacterial strains, the non-luminescent variant of the signal molecule synthase triple mutant JMH634 showed the lowest caseinase activity.

3.3.3 Hemolytic activity

In the luminescent group, most of the mutants of the wild-type BB120 strain had hemolytic activity significantly lower from the wild-type BB120 strain. Maximum activity was recorded in the luminescent BB152 (inactive HAI-1 synthase) (Table 3.4). The other luminescent strains MM30, MM77, JMH603, JMH606 and JMH634 showed a significantly lower hemolytic activity than the one of the wild type BB120. Result also showed that the hemolytic activity in all the non-luminescent variants was significantly inactivated ($P < 0.05$).

Table 3.3 Caseinase activities of the luminescent and non-luminescent variants of *V. harveyi* wild type and quorum sensing mutants

Strain	Features	Luminescent variants			Non-luminescent variants		
		Clearing zone (mm)	Colony diameter (mm)	Ratio	Clearing zone (mm)	Colony diameter (mm)	Ratio
BB120	Wild type	22.0 ± 1.0	12.0 ± 0.0	1.8 ± 0.1	18.0 ± 0.5	12.0 ± 0.0	1.5 ± 0.0♦
BB152	Inactive HAI-1 synthase	22.0 ± 0.6	12.0 ± 0.0	1.8 ± 0.0	20.0 ± 0.0	12.0 ± 0.0	1.7 ± 0.0♦
MM30	Inactive AI-2 synthase	20.0 ± 0.7	12.0 ± 0.0	1.7 ± 0.1*	20.0 ± 0.4	12.0 ± 0.0	1.7 ± 0.0
MM77	Inactive HAI-1 & AI-2 synthase	18.0 ± 0.4	12.0 ± 0.0	1.5 ± 0.0*****	16.0 ± 0.0	12.0 ± 0.0	1.3 ± 0.0♦
JMH603	Inactive CAI-1 synthase	16.0 ± 0.8	12.0 ± 0.0	1.3 ± 0.1*	20.0 ± 0.6	12.0 ± 0.0	1.6 ± 0.0♦
JMH606	Inactive HAI-1 & CAI-1 synthase	15.0 ± 0.0	12.0 ± 0.0	1.3 ± 0.0*	17.0 ± 0.4	12.0 ± 0.0	1.4 ± 0.0♦
JMH634	Inactive HAI-1, AI-2 & CAI-1 synthase	16.0 ± 0.0	12.0 ± 0.0	1.3 ± 0.0*	12.0 ± 0.0	12.0 ± 0.0	1.0 ± 0.0♦

Data are expressed as mean ± standard deviation (SD) of four replicates. Values are presented with one digit after the comma, not as an indication of the accuracy of the measurement, but in order to be able to see the actual SD. Values in the same column with (*) are significantly different from the wild type luminescent BB120 and diamonds (♦) indicate significant difference between luminescent variant and its respective non-luminescent variant (independent-samples t-test, $P < 0.05$).

Table 3.4 Hemolytic activities of the luminescent and non-luminescent variants of *V. harveyi* wild type and quorum sensing mutants

Strain	Features	Luminescent variants			Non-luminescent variants		
		Clearing zone (mm)	Colony diameter (mm)	Ratio	Clearing zone (mm)	Colony diameter (mm)	Ratio
BB120	Wild type	17.0 ± 0.5	10.0 ± 0.0	1.7 ± 0.1	10.0 ± 0.5	10.0 ± 0.0	1.0 ± 0.0♦
BB152	Inactive HAI-1 synthase	18.0 ± 0.6	10.0 ± 0.0	1.8 ± 0.1*	10.0 ± 0.0	10.0 ± 0.0	1.0 ± 0.0♦
MM30	Inactive AI-2 synthase	16.0 ± 0.5	10.0 ± 0.0	1.6 ± 0.1*	10.0 ± 0.0	10.0 ± 0.0	1.0 ± 0.0♦
MM77	Inactive HAI-1 & AI-2 synthase	16.0 ± 0.5	10.0 ± 0.0	1.6 ± 0.1*	10.0 ± 0.0	10.0 ± 0.0	1.0 ± 0.0♦
JMH603	Inactive CAI-1 synthase	16.0 ± 0.8	10.0 ± 0.0	1.6 ± 0.0*	10.0 ± 0.6	10.0 ± 0.0	1.0 ± 0.0♦
JMH606	Inactive HAI-1 & CAI-1 synthase	15.0 ± 0.0	10.0 ± 0.0	1.5 ± 0.1*	10.0 ± 0.0	10.0 ± 0.0	1.0 ± 0.0♦
JMH634	Inactive HAI-1, AI-2 & CAI-1 synthase	15.0 ± 0.0	10.0 ± 0.0	1.5 ± 0.1*	10.0 ± 0.0	10.0 ± 0.0	1.0 ± 0.0♦

Data are expressed as mean ± standard deviation (SD) of four replicates. Values are presented with one digit after the comma, not as an indication of the accuracy of the measurement, but in order to be able to see the actual SD. Values in the same column with (*) are significantly different from the wild type luminescent BB120 and diamonds (♦) indicate significant difference between luminescent variant and its respective non-luminescent variant (independent-samples t-test, $P < 0.05$).

3.3.4 Elastase activity

Elastase activity was observed in both the luminescent and non-luminescent strains of the wild-type BB120 and its isogenic mutants. In the luminescent group, the elastase activity of the wild-type BB120 appeared to be highest and was not significantly different from the BB152 (Figure 3.1). The luminescent mutants MM30, MM77, JMH603, JMH606 and JMH634 had significant lower elastase activity than the wild-type BB120. Lowest elastase activity in the luminescent group was recorded in the mutant MM77. Results also showed that there was no significant difference in the elastase activity between the luminescent variants and the corresponding non-luminescent variants except for the mutants BB152 (inactive HAI-1 synthase) and MM77 (inactive HAI-1 and AI-2 synthase) (Figure 3.1).

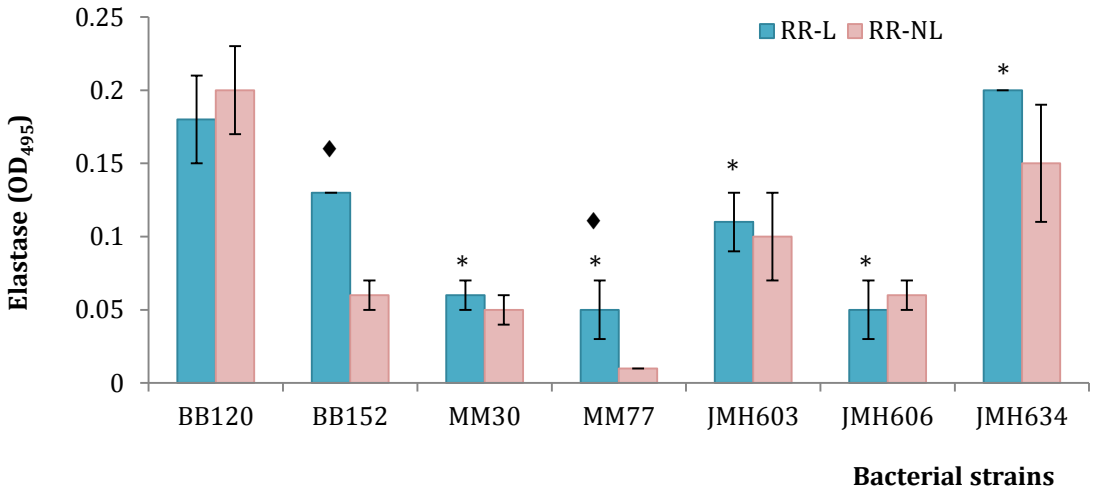

Figure 3. 1 Elastase activity of the luminescent and non-luminescent variants of *Vibrio harveyi* wild type and quorum sensing mutants.

Data are expressed as mean ± standard deviation of three replicates. Values in the same column with (*) are significantly different from the wild-type luminescent BB120, and diamonds (♦) indicate significant difference between luminescent variant and its respective non-luminescent variants (independent-samples *t*-test, $P < 0.05$).

3.3.5 Swimming motility

The swimming motility of all the non-luminescent variants was significantly higher than of their corresponding luminescent variants (Table 3.5).

Table 3.5 Relative percentage of swimming motility of the non-luminescent variants of *V. harveyi* wild type and quorum sensing mutants

Strain	Feature	Non-luminescent variants (%)
BB120	Wild type	130.0 ± 0.6
BB152	Inactive HAI-1 synthase	180.0 ± 1.5
MM30	Inactive AI-2 synthase	200.0 ± 3.8
MM77	Inactive HAI-1 & AI-2 synthase	380.0 ± 3.6
JMH603	Inactive CAI-1 synthase	150.0 ± 0.8
JMH606	Inactive HAI-1 & CAI-1 synthase	120.0 ± 5.5
JMH634	Inactive HAI-1, AI-2 & CAI-1 synthase	110.0 ± 0.6

The value of the swimming motility (in %) (mean of four replicates) of each non-luminescent (NL) variant was expressed relative to the swimming motility of the luminescent variant. Motility values in the NL strains were all significantly higher (independent-samples *t*-test, $P < 0.05$).

3.3.6 Biofilm formation

In the luminescent group, the wild type BB120 exhibited the highest level of biofilm formation (Figure 3.2). However, it was not significantly different from biofilm formation by the mutants MM30, JMH603, JMH606 and JMH634. The non-luminescent BB152, MM30, MM77 and JMH603 variants had a significantly ($P < 0.05$) higher capacity to form biofilms than their corresponding luminescent variants.

3.3.7 Induction of DnaK

Result showed the presence of DnaK (bacterial equivalent heat shock protein 70) in all the bacterial strains but at different levels (Figure 3.3). Both the luminescent and non-luminescent variants of the JMH634 (inactive HAI-1, AI-2 and CAI-1 synthase) had the highest level of DnaK whereas the inactive AI-2 synthase MM30 strain (both luminescent and non-luminescent

variants) had the lowest level (Figure 3.3). The DnaK level in the luminescent mutants BB152 (inactive HAI-1 synthase) and MM77 (inactive HAI-1 and AI-2 synthase) was significantly lower than in their corresponding non-luminescent variants. In contrast, the luminescent JMH603 mutant (inactive CAI-1 synthase) had a significantly higher DnaK level than its corresponding non-luminescent variant.

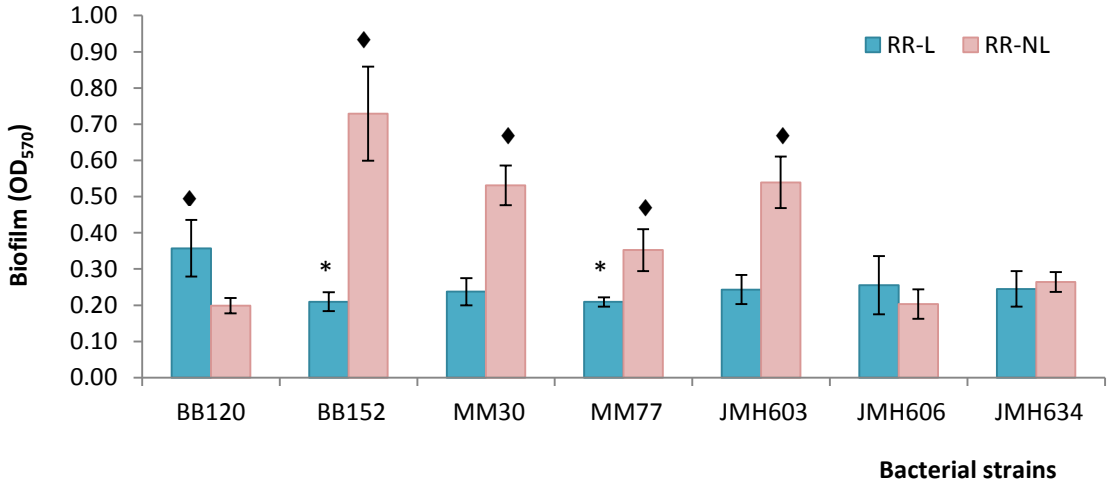

Figure 3. 2 Biofilm formation of the luminescent and non-luminescent variants of *Vibrio harveyi* wild type and quorum sensing mutants. Data are expressed as mean ± standard deviation of three replicates. Values in the same column with (*) are significantly different from the wild-type luminescent BB120, and diamonds (♦) indicate significant difference between luminescent variant and its respective non-luminescent variants (independent-samples *t*-test, $P < 0.05$).

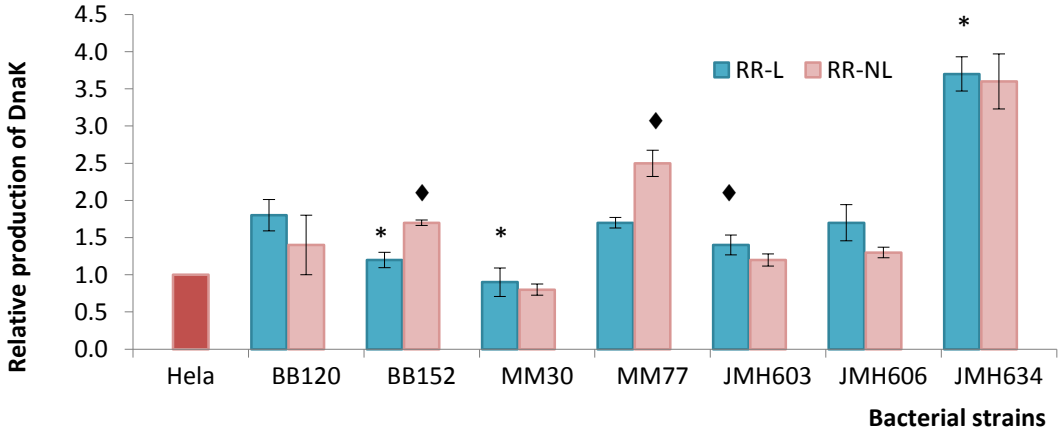

Figure 3. 3 Induction of DnaK of the luminescent and non-luminescent variants of *Vibrio harveyi* wild type and quorum sensing mutants. Data are expressed as mean ± standard deviation of three replicates. Values in the same column with (*) are significantly different from the wild-

type luminescent BB120, and diamonds (♦) indicate significant difference between luminescent variant and its respective non-luminescent variants (independent-samples *t*-test, $P < 0.05$).

3.3.8 Survival of *Vibrio*-challenged *Artemia*

The virulence of luminescent and non-luminescent variants of the wild type *V. harveyi* BB120 and its quorum sensing mutant strains was confirmed by an experimental infection in gnotobiotic brine shrimp (*Artemia franciscana*). *Artemia* challenged with the wild type BB120 (luminescent or non-luminescent variants) had the lowest survival. The survival of *Artemia* challenged by luminescent variants of the wild type strain BB120 and its isogenic mutants was lower than the survival of the *Artemia* challenged by their non-luminescent counterparts, but significant differences were not observed except for the mutant strain BB152 (inactive HAI-1 synthase) (Table 3.6). The survival rates in *Artemia* challenged with the non-luminescent BB152 were 4.5 times greater than in their corresponding luminescent variants.

Table 3.6 Survival of *Artemia* nauplii at 48h post challenge with wild type BB120 and its isogenic quorum sensing mutant strains

Bacterial strains	Features	Luminescent (%)	Non-luminescent (%)
BB120	Wild type	14 ± 3	21 ± 5
BB152	Inactive HAI-1 synthase	15 ± 2♦	68 ± 7
MM30	Inactive AI-2 synthase	73 ± 11*	87 ± 9
MM77	Inactive HAI-1 & AI-2 synthase	70 ± 6*	87 ± 7
JMH603	Inactive CAI-1 synthase	72 ± 7*	80 ± 5
JMH606	Inactive HAI-1 & CAI-1 synthase	87 ± 10*	96 ± 6
JMH634	Inactive HAI-1, AI-2 and CAI-1 synthase	91 ± 10*	97 ± 6

Data are expressed as mean ± standard deviation of five replicates. Values in the same column with (*) are significantly different from the wild type luminescent BB120 and diamonds (♦) indicate significant difference between luminescent variant and its respective non-luminescent variant (independent-samples t-test, $P < 0.05$).

3.4 Discussion and conclusion

Microorganisms can adapt to environmental changes, such as variation in chemical composition, local temperature, or illumination by sensing the changes and responding appropriately, for example, by switching phenotype or behavior [51, 54, 185, 186]. In this study, we showed that luminescent phenotypes of wild type *V. harveyi* strain and their quorum sensing mutants switch to non-luminescence when cultured in the dark under static condition. The underlying cause(s) behind such a non-luminescent phenotype in the non-luminescent variants remains unresolved. Similar phenotypic shifts have been described previously [11, 49, 69]. In a previous study, Phuoc *et al.* [49], aimed to verify whether the phenomenon of the non-luminescent phenotypes in *V. harveyi* and *V. campbellii* cultured under similar static conditions was linked to quorum sensing molecule production. To verify this assumption, the authors used wild type *V. harveyi* and mutant strains (with mutations in the HAI-1 and AI-2 quorum sensing pathway) and found that the luminescent mutant variants were still able to switch to non-luminescent variants. In fact, the quorum sensing molecules (HAI-1 and AI-2) were still present in cultures of non-luminescent *V. harveyi* BB120 and *V. campbellii*, indicating that the non-luminescent phenotype was not caused by inability of the strains to produce these quorum sensing molecules. Perhaps a mutation or gene rearrangement of the quorum sensing system or in the regulatory protein (such as quorum sensing master regulator *LuxR*) might be involved in the creation of the non-luminescent status [69].

Having observed such a phenotypic switch in response to change in the culture conditions, we next verified whether any changes occur in the production of virulence factors in all tested variants. Our results indicated that switching from luminescent variant of the wild type *V. harveyi* and its quorum sensing mutants to their respective non-luminescent variants significantly influenced multiple aspects of their phenotypic behavior. The type of influence (positive or negative) differed depending on the virulence factor and also on the mutation in quorum sensing signal molecules. Caseinase activity in both the luminescent and non-luminescent variants of the wild type *V. harveyi* strain and its quorum sensing mutants was positively regulated by quorum sensing, possible by CAI-1, being the main quorum sensing signal molecules influencing the production of caseinase, as the inactive CAI-1 synthase strains showed a significant lower caseinase activity in comparison to other quorum sensing mutant strains. This result coincides with the finding of Natra *et al.* [11] who showed that caseinase activity in *V. harveyi* is quorum

sensing dependent. Our result also indicated that most of the luminescent variants had a significant higher caseinase activity than their corresponding non-luminescent variants. Similar results were obtained for the hemolytic activity: non-luminescent variants showed a significant reduced hemolytic activity relative to their luminescent variants.

Motility and biofilm formation are important virulence factors in bacterial pathogens. In some species, motility is only important in the initial phase of the infection as it promotes contact dependent adhesion to host cells. In other bacteria motility needs to be established and maintained during the infection. Biofilm formation is known to be mediated by several factors, such as quorum sensing, cyclic di-GMP, pilli, flagella and exopolysaccharide. The ability to create a biofilm could assist bacteria to become more resistant to environmental stresses, such as bacteriophage infection, antibiotic and other antimicrobial agents [55, 187]. We found a relationship between motility and biofilm formation in most of the non-luminescent variants. In fact, most of the non-luminescent strains showed a significant higher capacity of biofilm formation and a significantly improved swimming motility. These results are consistent with the finding of Watnick & Kolter [188], who showed that mobility is the major contributor to biofilm formation in *V. cholerae* EI Tor. Perhaps the motile non-luminescent variants spend a significant part of their cellular energy on the formation of the motile apparatus and for its rotation [189], leaving less energy for the production of other virulence factors, such as caseinase and hemolysin. Josenhans & Suerbaum [189], stated that motile pathogenic bacteria have the ability to switch motility on and off as required and are even able to alter between a motile or sessile life style in the same host and habitat, depending on their growth condition. In *Vibrio*, conditions inducing a shift from the non-luminescent stage back to luminescence have not been described yet.

Several studies demonstrated the important role of Hsps in regulating the virulence of pathogens [190, 191]. For instance, Hoffman & Garduno [192], reported that DnaK of the ulcer-causing bacterium, *Helicobacter pylori*, mediates attachment to host gastric epithelial cells. The increased expression of this DnaK following acid shock correlates with both increased bacterial adhesion and inflammation of the gastric mucosa. Chakrabarti *et al.* [193], described a possible interference of DnaK with virulence factor production by *V. cholera*. Our results showed a phenotypic shift from bioluminescence to non-luminescence was not associated with a clear alteration in the DnaK level. Additionally, DnaK production by the wild type BB120 and its

quorum sensing mutants was quorum sensing independent, indicating that different unknown factor(s) might be regulating the production of DnaK in these strains.

This study also confirmed that concomitant with the loss of luminescence, the non-luminescent variants appeared to be less virulent towards *Artemia* (Table 3.5). The lower production level of various virulence factors might explain the lower virulence of the non-luminescent variants in our challenge test. Results of the *in vivo* test also revealed that AI-2 and CAI-1 signal molecules were the crucial signaling factors affecting the virulence of *V. harveyi* towards *Artemia*. This is in line with the results of an earlier study of our group [14].

Overall results indicated that switching to the non-luminescent state could change the phenotypic physiology or behavior of *V. harveyi* resulting in altered caseinase and hemolysin activities and in improved swimming motility and biofilm formation. The phenotype switching capacity was to a large extent independent from the quorum sensing status, because quorum sensing mutants were equally capable of performing the phenotypic switch.

Acknowledgement

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Chapter 4

Expression profile of immune-related genes in *Artemia franciscana* challenged with luminescent and non-luminescent variants of *Vibrio harveyi* wild type and quorum sensing mutants

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4.1 Introduction

Vibrio harveyi, a marine bacterium that belongs to the *Vibrio* clade, is considered as one of the most important bacterial pathogens in aquaculture, affecting a wide range of cultured marine organisms, including fish, crustaceans and molluscs [7]. Many studies carried out on crustaceans have reported that the expression levels of immune-related genes in these organisms were markedly elevated in response to *V. harveyi* infection [138, 194-196]. The invertebrate crustaceans, unlike vertebrate, rely solely on their only defense system i.e. the innate immunity, which is essential for host defense to eliminate the invader. The innate immune system has two principle components: the cellular and humoral, which are activated upon immune challenge. These two components work synergistically to detect, eliminate and combat foreign organisms. The cellular component involves those processes mediated by hemocytes, such as encapsulation, nodule formation and phagocytosis. Whereas the humoral components involve the cell free components of the hemolymph including clotting cascade, anti-oxidant defense enzymes like super-oxidase dismutase, peroxidase, catalase and nitric oxide synthase, defensive enzymes like lysozyme, acid phosphatase and alkaline phosphatase, reactive oxygen and nitrogen intermediates and antimicrobial peptides [128].

The innate immune system recognizes self and non-self molecules by a limited number of germline-encoded receptors called pattern recognition receptors (PRRs). PRRs are proteins that are present on the cell membrane or in the cytoplasm as sensor for any type of pathogens. PRRs recognize pathogens by binding to molecular patterns/structures rather than to a specific component of a given pathogen. These molecular patterns, called pathogen-associated molecular patterns (PAMPs) are usually the polysaccharides and glycoproteins on the surface of microbes, such as lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycan (PGN) and lipotechoic acid (LTA) from Gram-positive bacteria, and glucans from fungal cells. The recognition process leads to the rapid induction of cellular and humoral responses by the host [115]. However, Vance *et al.* [197] reported that the response of the host towards pathogens is not only limited to recognition of microbial structures but also to recognition of distinct pathogen-induced processes that contribute to the progression of disease. The authors also proposed that recognition of pathogen-induced events would provide the host with strategies for distinguishing a virulent organism from one that has lower disease-causing potential. For

example, it has been suggested that the host could sense translocated PAMPs as a signal for cytosolic access. In fact, as an example, translocated PAMPs sensed in the cytosol are flagellin. Since deliver of flagellin to the host cell is strictly dependent on type III or IV secretion systems, the cytosolic presence of flagellin is a strong signal to the immune system that a pathogen (as opposite to the commensal) is present [197].

Recently, a number of genes involved in the shrimp innate immune response have been identified and characterized, including those of pattern recognition receptors [115, 198], proteins associated with cell adhesion activity [199] and proteins involved in blood coagulation [200] like proteinase inhibitors, proPO activation or cytokine activation [138, 201]. Our previous study showed that the non-luminescent variants of the wild type *V. harveyi* BB120 and its quorum sensing mutant JMH634 respond differently in terms of virulence factor production and virulence towards *Artemia* compared to its parental luminescent strains [65]. In this study, the gnotobiotic *Artemia* model organism (GART) system is used to verify the hypothesis that phenotypic variation in *V. harveyi,* resulting in differences *in vitro* virulence factors production and virulence to *Artemia* in luminescent and non-luminescent of the wild type *V. harveyi* and a quorum sensing mutant, affect the defense system of *Artemia.* For that, germ-free *Artemia* were challenged with the luminescent and non-luminescent variants of the *V. harveyi* wild type strain and a triple quorum sensing mutant and the transcript levels of key genes related to innate immunity in *Artemia* were determined. The following genes were studied: lipopolysaccharide and β-1,3-glucan binding protein (*lgbp*), down syndrome cell adhesion molecule (*dscam*), peroxinectin (*pxn),* prophenoloxidase (*proPO*), transglutaminase (*tgase*), *ferritin (ftn),* melanin inhibition protein (*mip*) and *serpin* (*spn*).

4.2 Material and methods

4.2.1 Bacterial strains and growth conditions

V. harveyi strains used in this study are shown in Table 4.1. The strains were stored in 20% glycerol at -80 °C. Rifampicin resistant (RR) strains were produced as described by Hong *et al*. [65].

Autoclaved *Aeromonas* sp. LVS3 bacteria were used as feed for *Artemia* [158]. LVS3 were grown in Marine Broth (MB) 2216 (Difco laboratories, MI, USA), centrifuged at 2200 x g for 15

min. The supernatant was discarded and the pellet was re-suspended in filtered autoclaved seawater (FASW; 35g/L, Aquarium Systems, Sarrebourg, France). Subsequently, the cell suspension was autoclaved at 121 °C for 20 min. The bacterial densities were determined spectrophotometrically at an optical density (OD) of 600 nm assuming that an OD of 1.0 corresponds to 1.2 x 10^9 cells mL^{-1} (McFarland standard; BioMerieux, France).

Table 4.1 Bacterial strains and their relevant feature

Strain	Relevant feature	References
BB120 RR-L	Rifampicin-resistant luminescent	[65]
BB120 RR-NL	Rifampicin-resistant non-luminescent	[65]
JMH634 RR-L	Rifampicin-resistant luminescent.	[65]
JMH634 RR-NL	Rifampicin-resistant non-luminescent	[65]

4.2.2 Axenic hatching of *Artemia*

Axenic hatching of *Artemia* cysts was performed following decapsulation and hatching procedures as described previously [202]. Briefly, 4 g of *Artemia* cysts originating from the Great Salt Lake, Utah, USA (EG® Type, batch 21452, INVE Aquaculture, Dendermonde, Belgium) were hydrated in 89 ml of sterile distilled water for 1 h. Sterile cysts and nauplii were obtained after decapsulation via using 3.5 mL NaOH (32%) and 50 mL NaOCl (50%). The reaction was stopped after 2 min by adding 70 mL $Na_2S_2O_3$ (10 g/L). Thereafter the decapsulated cysts were washed with sterile FASW artificial seawater and then suspended in 1 L glass bottles containing FASW, and then incubated at 28°C for 28 h with constant illumination of approximately 27 $\mu E/m^2$.sec for hatching. After 28 h of incubation, the axenicity of the hatched *Artemia* nauplii was verified both by spread plating (100 ml) and by adding (500 µl) hatching water on Marine Agar and in Marine Broth (Difco, Detroit, USA), respectively followed by incubation at 28°C for 5 days [202]. Bottles containing non-sterile nauplii were excluded from the experiment.

4.2.3 *Artemia* challenge test

After 28 h of incubation at 28 °C, swimming nauplii at the instar II stage were collected, counted volumetrically and then transferred into 1 L glass bottles containing sterile artificial seawater.

The bottles were placed in a rectangular tank containing water maintained at 28^0 C using a thermostatic heater with constant illumination (approximately 27 μE/m2.sec.) and aeration. The nauplii were fed once with 10^7 cells mL^{-1} of autoclaved LVS3. Simultaneously, they were challenged with luminescent (group 1) or nonluminescent (group 2) variants of *Vibrio* at 10^6 cells mL^{-1}. *Artemia* nauplii that were non-challenged (group 3) were used as control. Three replicates were maintained for each group. Samples containing 0.1 g of live nauplii were harvested from each glass bottle at 6 and 12 h post challenge, rinsed in sterile distilled water, immediately frozen in liquid nitrogen and preserved at -80 °C for further analysis.

4.2.4 Quantitative real-time reverse transcription PCR

Total RNA was extracted from different *Artemia* samples using 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 500 ng μL^{-1} in all samples and the RNA samples were stored in -80 °C for subsequent use.

Reverse transcription was done with the RevertAid^{TM} H minus First strand cDNA synthesis kit (Fermentas, Fisher Scientific, Erembodegem, Belgium) according to manufacturer's instructions. Briefly, 9 μL of reaction mixture containing 4 μL of 5x reaction buffer (0.25 mol^{-1} of Tris –HCl pH 8.3, 0.25 mol^{-1} of KCl, 0.02 mol^{-1} of $MgCl_2$, 0.05 mol^{-1} of DTT), 2 μL of 0.01 mol^{-1} of dNTP mix, 20 units of ribonuclease inhibitor, 200 units of RevertAid H minus M-MuLV Reverse Transcriptase (Fisher Sciencetific, Erembodegem, Belgium), 1 μL of random hexamer primer. Then, 500 ng of RNA was added to the reaction mixture. Subsequently, 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 polymerase chain reaction (PCR) and stored at -20 °C for further use.

The primers used for analyses of *proPO, tgase* and *ftn* genes were previously described by Niu *et al.* [203]. For the genes *pxn, dsam, lgbp, cal, spn, ef*$_{\alpha 1}$, specific primers were designed using the *Artemia* Genome database (Sony, not published). Amplification products were re-sequenced to confirm homology with the genes identified in *Pacifastacus* and/or other invertebrate. Evidence

for their functionality awaits further research. Primers were designed using the online software Primer Express® Software v3.0.1 (Primer 3, IDT) and obtained from Eurogentec (Seraing, Belgium).

Quantitative reverse transcription real-time PCR was used to quantify the gene expression level in *Artemia*. The reaction was carried out with Maximal® SYBR Green/ROX qPCR master Mix (Fisher Scientific, Erembodegem, Belgium) and was carried out in an StepOne Real-Time PCR System thermal cycler (Applied Biosystems, Gent, Belgium) in a total volume of 25 µL, containing 12.5 µL of 2 x SYBR green master mix, primer (concentration see Table 4.2) and 2 µL 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 15 s and primer annealing and elongation at 60 °C for 1 min. Melting curve analysis was done to check for the amplification of untargeted fragments. Data acquisition was done with the STEPONE software.

Based on the result of the optimalisation experiment, the amplification efficiency of the reference and target genes were found to be equal allowing to analyze real-time PCR data with the $2^{-\Delta\Delta CT}$ method [204]. The expression was normalized to the endogenous control ($EF_{\alpha 1}$) by calculating ΔC_T:

$$\Delta C_t = C_{t\ target} - C_{t\ EF\alpha 1}$$

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

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

The sample of unchallenged *Artemia* at 6 h was used as calibrator. The relative expression was then calculated as

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

4.2.5 Statistical analysis

All statistical analyses were performed using the Statistical package for the Social Sciences (SPSS) version 20.0 using a significant level of 5%. Homogeneity of variances and normality of data were not fulfilled for an ANOVA analysis. The data was log transformed prior subject to statistical analysis. All treatments were compared to the control and to each of the other

treatments individually using independent samples t-test for each time point. Per time point, a matrix was obtained with an overview of all the results from the independent samples t-test.

Table 4.2 Real-time quantitative RT-PCR primers

Gene	Efficiency	Primer concentration	Annealing temperature	Strand	Primer sequence (5'-3')
ef$_{\alpha}$1	2.0	0.20μM	60°C	F	GGTCGGGGTTGAAACTGGTAT
				R	AGGGATTCGTGGGCATTT
ftn	2.2	0.20μM	60°C	F	TCCAAGGATTATCCGATGAACA
				R	ATGACCAAGTGAGTGCTTCTTCT
tgase	2.2	0.20μM	60°C	F	CCCCACAAGAACCATCTGAAG
				R	TCTCTCCGTGTCTCTCCAAAAG
proPO	2.2	0.20μM	60°C	F	TCTGCAAGGAGGATTTAAGGA
				R	TGACTGAAAGGAGATGGGAC
lgbp	2.0	0.20μM	50°C	F	GTGGACTGATGCTGAATG
				R	TGGTGGTCCAGATGATAC
pxn	2.0	0.20μM	60°C	F	GAGCTACCGATGAAGATCCAG
				R	CGTTTCCTGAACAGCGAATAAA
dscam	1.9	0.25μM	51°C	F	CATAGTCCAAGAGTGAATG
				R	GCCACATATTCAGTTAGAA
mip	2.0	0.20μM	54°C	F	CGTGGTAATGCTAGTGA
				R	CACCATCCACCTTCATA
spn	2.0	0.20μM	62°C	F	AAACCAGGCTTGCCGTT
				R	GCCGTGATTATTGTCCTTTGC

4.3 Results and discussion

This study aimed to verify that differences in *in vitro* virulence factors production or virulence to *Artemia* in luminescent and non-luminescent *V. harveyi* as a result from phenotypic variation that affects the defense system of *Artemia*. To verify the proposed hypothesis, germ-free *Artemia* and virulent/non-virulent strains of *V. harveyi* (for details, see Table 4.1) were used as host-pathogen model system. As readouts of host immune responses, a battery of key immune-related genes, including the *lgbp, dscam, proPo, ftn, tgase, pxn, mip,* and *spn* were analyzed. The germ-free *Artemia* culture system was employed for such test because it is a crucial tool that allows eliminating the possible interference of different microbial communities that exists in

environment and furthermore facilitates in studying the effects of interaction between the host and the tested bacteria. Under this experimental condition, it was observed that there was a significant alteration in the transcription levels of the immune receptors and a few downstream genes known to be involved in the humoral and cellular innate immune response in *Artemia* in response to challenge with luminescent or non-luminescent variants of the wild type *V. harveyi* and their corresponding mutants (Table 4.3 & 4.4).

Table 4.3 Overview of the statistically significant results for the tested immune-related genes in *Artemia* larvae. The effects of a challenge with L or NL *V. harveyi* on gene transcription are shown.

	120L			**120NL**			**634L**			**634NL**		
Gene	6 h	12 h	24 h	6 h	12 h	24 h	6 h	12 h	24 h	6 h	12 h	24 h
lgbp	↓	↑	↑	↓	↓	-	-	↓	-	-	↓	-
dscam	↓	-	-	↓	-	-	-	-	-	-	-	-
proPO	-	-	-	↓	-	-	↓	-	-	-	-	-
fnt	↓	↑	-	↓	-	-	-	-	-	-	↑	-
tgase	-	-	-	↓	-	-	-	-	-	-	-	-
pxn	-	-	-	↓	-	-	-	↑	-	↓	↑	-
mip	↓	↓	-	↓	↓	-	↑	↓	-	↑	↓	-
spn	-	↓	-	↓	↓	-	-	↓	↓	↑	↓	-

↑: significant up-regulation of the gene compared to the control at this time point
↓: significant down-regulation
-: no significant difference between the treatment and the control

PRRs are involved in the first step of immune responses in invertebrates by binding to highly conserved pathogen structures, such as peptidoglycan and lipopolysaccharide (LPS) from bacteria or to danger associated molecular patterns, such as Hsp70 and Hmgb1 [122]. In this study we investigated the transcriptional level of two important PRRs in invertebrates i.e. lipopolysaccharide and β-1,3-glucan binding protein (*lgbp*) and down syndrome cell adhesion molecule (*dscam).*

The lipopolysaccharide and β-1,3-glucan binding protein *(lgbp),* also known as Gram-negative bacteria-binding protein (GNBP), is a pattern recognition receptor that can recognize and bind lipopolysaccharide (LPS) and β-1,3-glucan. In invertebrates, *lgbp* plays vital roles in the innate immune defense against Gram-negative bacteria and fungi. It induces the cell and humoral mediated immune responses like encapsulation, phagocytosis, nodule formation, clotting, synthesis of antimicrobial peptides and activation of the prophenoloxidase (proPO) system. A

recent study showed that the expression of *lgbp* in shrimp *Penaeus monodon* increased at 24 h post injection with pathogenic bacterium *Vibrio harveyi* [121]. Another PRR is *dscam*, which is a hypervariable protein created by alternative splicing, involved in both general innate immunity and pathogen-specific immune response in invertebrate [205].

Table 4.4 Summary of significant differences in immune-related genes expression

Gene	120L vs. 120NL			120L vs. 634L			634L vs. 634NL		
	6 h	12 h	24 h	6 h	12 h	24 h	6 h	12 h	24 h
lgbp	↑	↑	↑	↓	↑	↑	-	-	-
dscam	↑	-	-	-	-	-	-	-	-
proPO	↑	-	-	↑	-	-	-	-	-
ftn	↑	-	-	↓	-	-	-	-	-
tgase	↑	-	-	-	-	-	-	-	-
pxn	↑	-	-	-	↓	-	-	-	-
mip	↑	-	-	↓	-	-	-	-	-
spn	↑	↑	-	↓	↑	↑	↓	-	↓

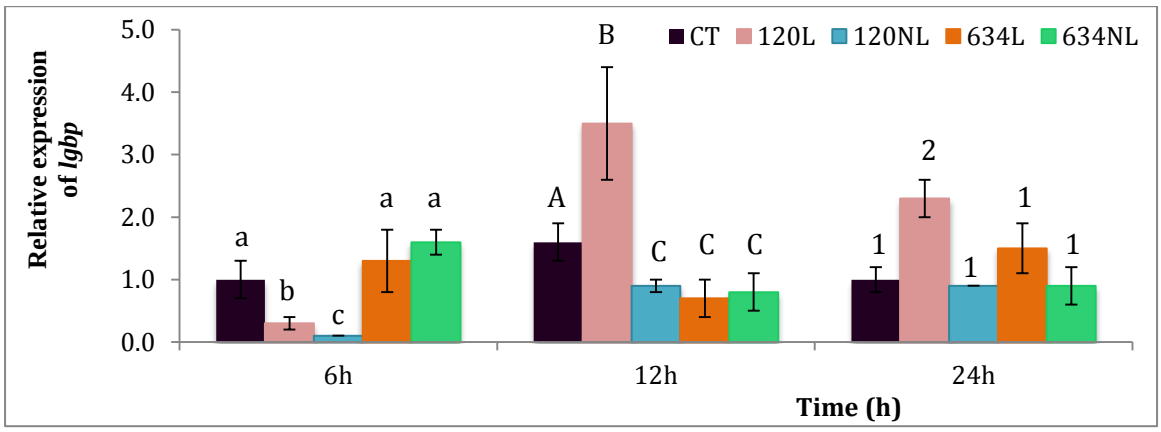
↑: significant up-regulation
↓: significant down-regulation
-: no significant difference

Significant differences in the expression levels of *lgbp* among the different groups were observed at different time points post challenge (Figure 4.1A). At 6 h post challenge, the expression level in the group exposed to luminescent or non-luminescent wild type *V. harveyi* BB120 was significantly down-regulated compared to the control. There was a significant difference in *lgbp* expression level between the *Artemia* challenged with luminescent BB120 and its non-luminescent variant (BB120NL) or luminescent JMH634. However, at 12 h, expression level of *lgbp* was significantly up-regulated in the BB120L-challenged group compared to the BB120NL and JMH634L-challenged groups, and control group, whereas in all the other (treated) groups, there was a significant down-regulation in the expression level. At 24 h, the BB120L-challenged group exhibited significant increase in the expression level of *lgbp* compared to all other groups.

The expression pattern of *dscam* showed significant decrease at 6 h post challenge in the BB120L and BB120NL compared to the JMH634-exposed group and control group. However, the expression level of *dscam* in the group exposed to either luminescent or non-luminescent variants of the mutant JMH634 was not significantly different from that of the control. No significant differences among the groups were observed either at both 12 and 24 h time points (Figure 4.1B).

Next, we analyzed the expression of a set of four genes related to humoral and cellular immune system: *proPO, ftn*, *tgase* and *pero*, as these immune components were previously reported to be involved in inducing resistance in invertebrate against bacterial infection [206]. As shown in Figure 4.2A, at 6 h post challenge, the group challenged with BB120NL exhibited a significant decrease in the *proPO* expression level compared to the un-challenged control group and to the group challenged with either luminescent BB120 strain (Figure 2A). The expression level of *proPO* significant down-regulated in the JMH634-exposed groups relative to the BB120L, and there is no significant difference in gene expression in these treatment groups (JMH634L and JMH634NL). No significant differences were observed among the groups at both 12 and 24 h time points (Figure 4.2A).

(A)



(B)

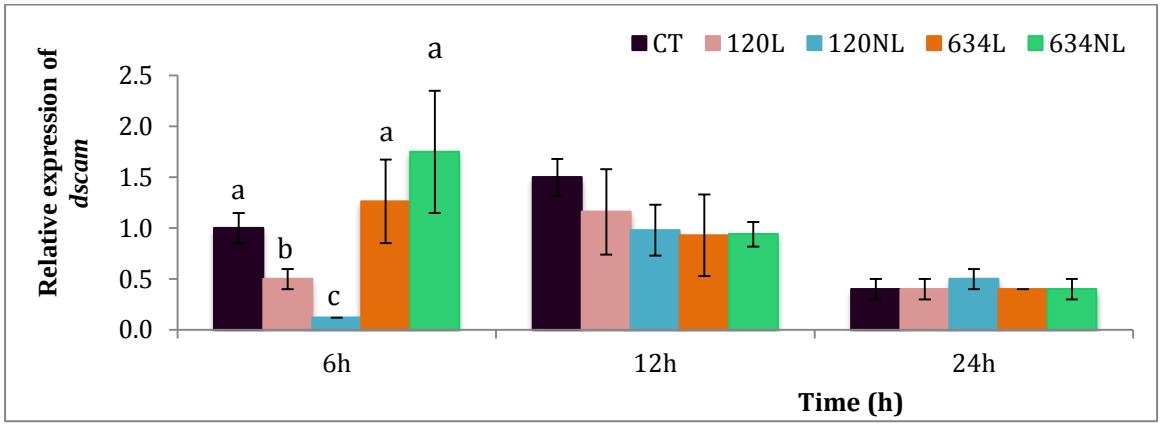


Figure 4. 1 Relative expression of *lgbp* (A) and *dscam* (B) genes in *Artemia* larvae.

The larvae were challenged with luminescent (L) or non-luminescent (NL) of the wild type BB120 and its quorum sensing mutant JMH634 or not. The unchallenged *Artemia* larvae served as control. Sample was collected for *lgbp* and *dscam* gene expression at 6, 12 and 24 h post challenge. The expression was calculated relative to the *ef$_{\alpha}$1* gene; expression in unchallenged *Artemia* at 6 h was set at 1 and the other data points were normalized accordingly. Error bars indicated the standard deviation. Different letters/numbers (small and capital for 6 and 12 h, respectively and number for 24 h) were indicated for significant differences among groups ($P<0.05$).

The *ftn* gene showed different expression pattern at different time points post challenge (Figure 4.2B). At 6 h post challenge, the expression level of *ftn* was significant decreased in the *Artemia* exposed to either BB120L or BB120NL relative to the control and JMHL634-exposed groups. There was a significant difference in *ftn* gene expression between the BB120L and BB120NL-exposed group while no significant difference between the JMH634L and JMH634NL was

observed. However, at 12 h post challenge, there was no significant difference in *ftn* gene expression among *Vibrio*-exposed groups. At 24 h post challenge, no significant differences among the groups were observed (Figure 4.2B). This result is accordance with the finding of Ong *et al* [207], they reported that mRNA expression of the horseshoe crab *ftn* was up-regulated 3 h after challenged with *Pseudomonas aeruginosa*, and the plasma ferritin disappeared between 6-48 h post challenge. This result suggested that during infection, ferritin might be concealed intracellularly as it withholds iron from the invading pathogen.

Similar to what was observed for *ftn* at 6 h post challenge, the expression level of *tgase* in *Artemia* challenged with BB120NL was significantly low compared to the control and to the BB120L-exposed group (Figure 2C). No significant differences were observed between BB120L and JMH634 treatment groups. No significant differences were observed among the groups at both 12 and 24 h time points (Figure 4.2C).

Pxn-encoding gene-*pxn* is a molecule first isolated and described from the fresh water crayfish *P. leniusculus* and this protein combines being a cell adhesion ligand and a peroxidase. In addition, crayfish pxn is also an opsonin, a degranulation and an encapsulation-promoting factor [133]. Pxn is a proPO system-associated protein and plays a crucial role in cellular defense for encapsulation enhancement in crustacean. For example when a foreign particle enters the hemolymph, hemocytes recognize the foreign intruder as non-self and change from non-adhesive to adhesive cells, strongly adhering to the foreign target, and subsequently form a multilayer sheath of cells during encapsulation [208, 209]. Previous studies observed that *pxn* gene was up-regulated significantly in white shrimp *Litopenaeus vannamei* and *Fenneropenaeus indicus* when the shrimp was infected through injection by *V. alginolyticus* or *V. harveyi*, respectively [195, 209].

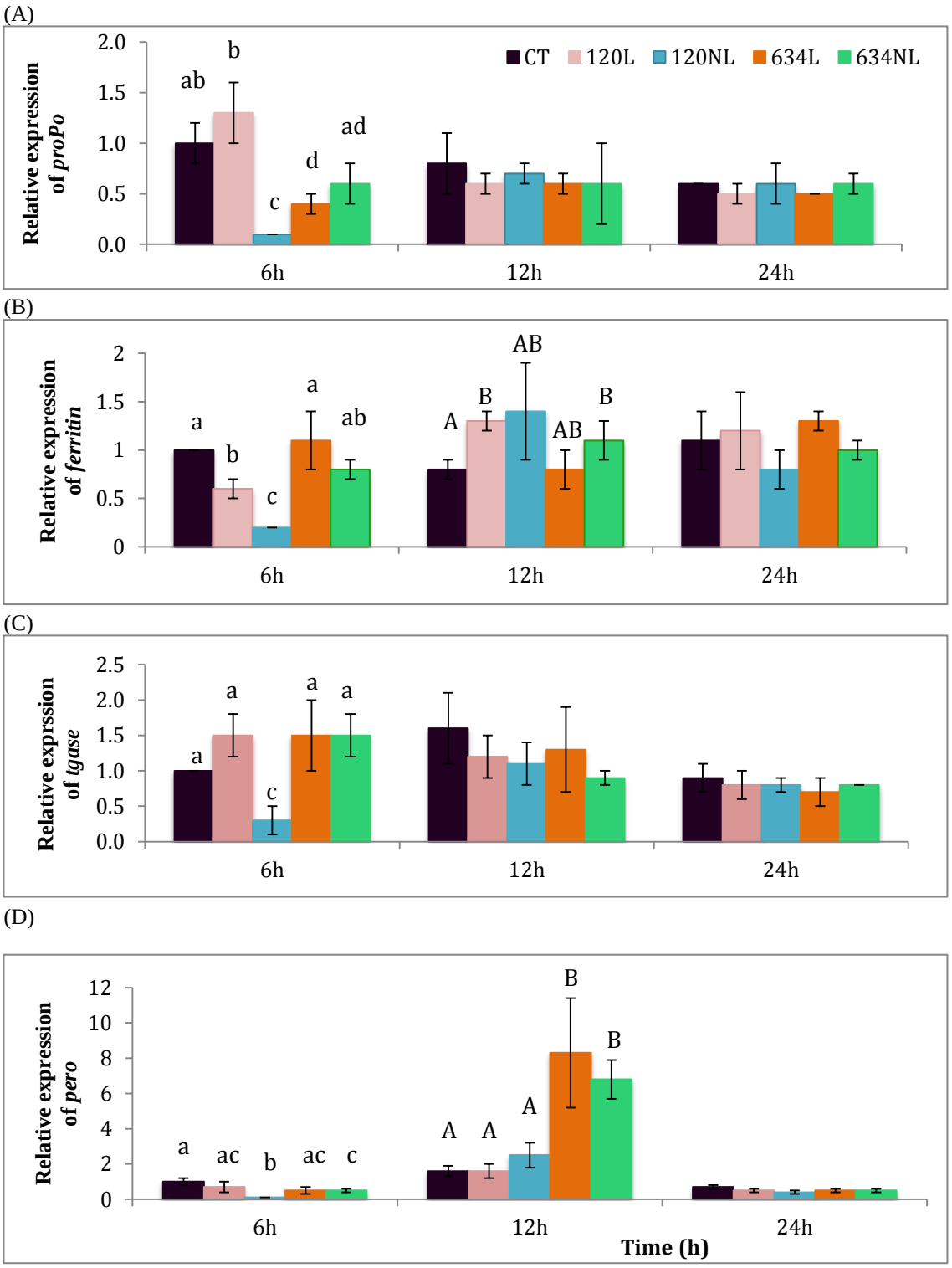


Figure 4.2 Relative expression of *proPo* (A), *ferritin* (B), *tagse* (C) and *pero* (D) genes in *Artemia* larvae. The larvae were challenged with luminescent (L) or non-luminescent (NL) of the wild type BB120 and its quorum sensing mutant JMH634 or not. The unchallenged *Artemia* larvae served as control. Sample was collected for *proPo, ferritin, tagse* and *pero* gene expression at 6, 12 and 24 h post challenge. The expression was calculated relative to the $ef_{\alpha}1$ gene; expression in unchallenged *Artemia* at

6 h was set at 1 and the other data points were normalized accordingly. Error bars indicated the standard deviation. Different letters/numbers (small and capital for 6 and 12 h, respectively and number for 24 h) were indicated for significant differences among groups ($P<0.05$).

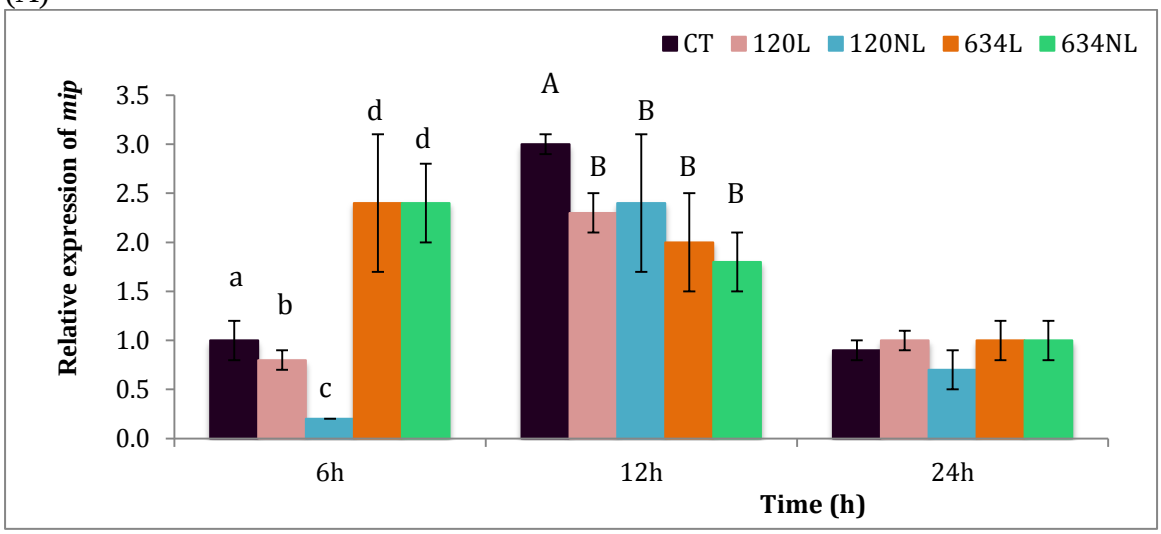
As showed in Figure 4.2D, 6 h post infection, expression level of *pxn* was significantly lower in the *Artemia* challenged with BB120NL compared to its luminescent variant (BB120L) and to the control. The *pxn* expression level was not significant different among BB120L, JMH634L and JMH634NL-exposed groups (Figure 4.2D). 12 h post challenge, expression of *pxn* by the BB120L and BB120NL remained at the same level compared to the control. However, in the mutant JMH634-challenged group, expression levels of *pxn* were significantly increased compared to the BB120L-challenge group and to the control group but no significant difference was observed among these treatments (Figure 4.2D).

Melanization plays an crucial role in defense reactions, such as wound healing encapsulation, sequestration of microbes, and the production of toxin intermediates, that are speculated to kill invading microorganism [126]. Melanin synthesis is essential for defense and development but must be tightly controlled because systemic hyperactivation of the proPO system, excessive formation of quinones, and inappropriate excessive melanin synthesis are also deleterious to the hosts [137]. The melanization inhibition protein (mip) is involved in regulation of excessive production of quinones [138].

A study carried out by Angthong *et al.* [138] showed that *mip* was expressed in all the examined tissues in *Penaeus monodon* except hemocytes. The expression of this gene was very low during the larval stages and hardly present in eggs and at the nauplii stage upon *V. harveyi* challenge.

In the present study, we found that a significant increase in the expression level of *mip* was detected in the JMH634L and JMH634NL-exposed groups relative to the BB120L and to the control group but no significant difference among the latter treatments was observed (at 6 h) (Figure 4.3A). Subsequently, the expression level of this gene was down-regulated significantly in all groups compared to the control at 12 h. Our result supports the findings of Angthong *et al.* [138], who suggested that upon *V. harveyi* infection the mip protein in *Penaeus monodon* (PmMIP) is first released from the tissues into the hemolymph whereafter the mip protein is being degraded during the bacterial infection to allow melanization.

(A)



(B)

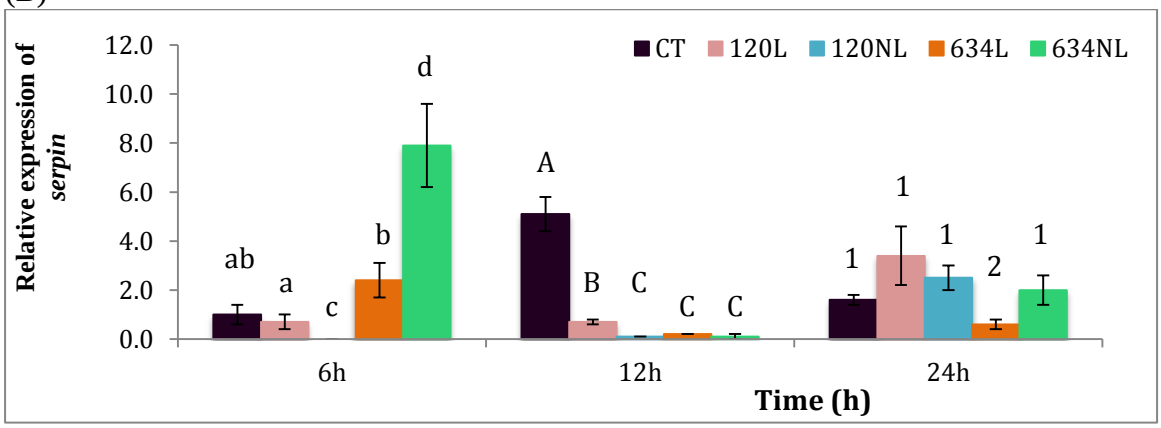


Figure 4.3 Relative expression of *mip* (A) and *serpin* (B) genes in *Artemia* larvae.

The larvae were challenged with luminescent (L) or non-luminescent (NL) of the wild type BB120 and its quorum sensing mutant JMH634 or not. The unchallenged *Artemia* larvae served as control. Sample was collected for *mip* and *serpin* gene expression at 6, 12 and 24 h post challenge. The expression was calculated relative to the $ef_{\alpha}1$ gene; expression in unchallenged *Artemia* at 6 h was set at 1 and the other data points were normalized accordingly. Error bars indicated the standard deviation. Different letters/numbers (small and capital for 6 and 12 h, respectively and number for 24 h) were indicated for significant differences among groups ($P<0.05$).

The *spn* gene encoding the protein spn is involved in a number of biological processes, such as blood coagulation, complement activation, fibrinolysis, angiogenesis, inflammation, tumor suppression and hormone transport [210]. The spn protein in arthropod from hemolymph are likely to function in protecting their hosts from infection by pathogens or parasites through inhibiting fungal or bacterial proteinases or regulating endogenous proteinases involved in

coagulation, proPO activation, or cytokine activation [211]. In this study, the pattern of *spn* gene expression was different in different challenge groups (at 6 h) (Figure 4.3B). The decrease of *spn* expression gene at 6 h post challenge with either BB120L or BB120NL might indicate that low synthesis of spn and high expression of relevant serine proteinase occurred during this period. This phenomenon might be caused by the involvement of the serine proteinases in wound healing, proPO activity, phagocytosis and other defense responses after bacterial challenge [129]. Then the expression of *spn* gene remained significantly low at 12 h in these treatment groups compared to the control after that it slightly increased towards the end of the experiment. It corresponds to the re-increased synthesis of spn to inhibit over-expressed serine proteinase, as observed in *Fenneropenaeus chinensis* [201]. In contrast, for the *Artemia* challenged with JMH634 groups, the trend of the *spn* gene expression was inversely.

Overall, different patterns of gene expression were observed in different challenge groups at different time points. , BB120L and BB120NL are virulent to *Artemia*, and switching from L to NL status reduced a number of virulence factors in the wild type BB120 strain *in vitro* (Chapter 3) (but increased swimming) and *in vivo* condition [49, 65, 69]. In the present study, exposure to the BB120NL variant significantly decreased the mRNA level of *Artemia* in all tested genes at 6 h in comparison to its parental luminescent strain (BB120L). Based on the previous results and on this study, it is suggested that the higher production of virulence factors such as luminescence, caseinase and haemolytic activity as observed in the BB120L, might be involved in the induction at the transcriptional level of these genes in *Artemia*. But apparently the increased expression of all these genes does not need to be sustained except for the *lgbp* gene. It is unclear why sustained expression of the *lgbp* gene is needed in the BB120L challenge in the absence of higher expression of genes downstream in the defense.

The strain BB120L and JMH634L appeared to be virulent and non-virulent towards *Artemia* in the challenge test, respectively (Chapter 3). In addition, production of some virulence factors was higher *in vitro* in the BB120L relative to JMH634L (Chapter 3). It has been shown that bacterial pathogens have co-evolved with their host, displaying remarkable refined molecular mechanism, often ending in highly specialized bio-chemical reactions between the microbe and its target cells. As a result, bacteria manipulate diverse host cellular processes for the benefit of the microbe but not for the host [212]. In many cases bacterial virulence factors require complicated secretion systems allowing the toxins or adhesins to be presented in the right context and at the

accurate time on the bacterial cell [213]. Interestingly, T3SS is known to be a potent virulence mechanism that involved in invasion, proliferation, and causing disease. T3SS is shared by a broad spectrum of pathogenic Gram-negative bacteria that interact with host by injecting effector proteins into the cytosol of host cells [212]. It has been reported that in *V. harveyi*, T3SS is regulated by quorum sensing [214], so probably, in the triple quorum sensing mutant JMH634L the T3SS is inactivated and no cellular damage can be induced by JMH634L exposure.

Also here (yet not at 6h) expression of the *lgbp* gene is sustained in the challenge with a more virulent strain. Early upon BB120L exposure, *proPO* expression is upregulated relative to JMH634L, which might be logic for a more virulent strain. Yet other effector genes such *as ftn* and *pxn* are down-regulated later on. Hence a dynamic differential regulation is displayed here. *Mip* and *spn* are upregulated later on, which might be needed to regulate for instance phenoloxidase activity induced by the virulent strain. For the case JMH634L and JMH634NL, both strains are non-pathogenic to *Artemia* in the challenge test although some differences in *in vitro* virulence factors was noticed between these strains (Chapter 3). Most of the tested genes (except the *spn* gene) exhibited no significant difference in gene expression between these treatment groups at either time point. This might be the consequence of the fact that both strains are non-pathogenic and might not be able to inflict cellular damage or they are subject to host phagocytosis without further negative consequences.

It has not been possible yet to demonstrate that non-luminescent strains are capable to switch back to luminescent strains, neither *in vitro* nor *in vivo*. Yet it is possible that this is happening as the BB120L strains produces more virulent factors which could be beneficial in the infection process. Since BB120L and BB120NL are not significantly different in virulence towards *Artemia* (Chapter 3) such a back switch from non-luminescent to luminescent could be assumed on that basis. It is difficult to imagine what the effect could be of such a back-switch at the level of the host. One can assume that there would be little difference in host response or merely a delay in response because of a slower infection process. This could explain why the BB120L strain induces a more rapid response only in the beginning and little difference in expression level is found later upon exposure. Confirmation that a back-switch in *vivo* is present needs to be addressed.

In conclusion, virulence factors are an integrated multifactor system or products expressed by a pathogen that are essential for allowing the pathogen to replicate in the host and cause clinical symptoms [213]. Virulence factors are not constitutively expressed and their production tightly depends on the environmental conditions faced by the bacterium. Depending on the stage of infection, in the intestinal lumen, inside epithelial cells or professional phagocytes, or in the bloodstream, the set of virulence factors expressed in these different conditions has to vary accordingly. Exposure to the virulent or non-virulent of the luminescent and non-luminescent wild type BB120 and its quorum sensing mutant significantly altered the host defense response in term of immune-related gene expression in *Artemia.* Results of this study provide evidence that alternation of *in vitro* virulence factors such as luminescence and the enzyme activities caused by phenotypic variation might contribute to the pathogen-induced processes and host-pathogen interaction, by which bacteria can modulate host defense reaction according to the pathogenic invader. Virulent strains or strains producing more virulent factors induce *proPO* expression only early on upon exposure while *spn*/mip are upregulated later on, probably to regulate phenoloxidase activity. The *lbgp* gene was overexpressed within the experimental time frame. This is unexpected in view of the fact that most of the test effector genes are down-regulated towards the end of the experiment. Difference in gene expression dynamics of the BB120L and B120NL strains leads to the assumption that the non-luminescent strains may switch back to the luminescent strains after infection, but that remains speculative.

Chapter 5

Transcriptional and translational analysis of expression profiles of heat shock protein 70 and high-mobility group box-1 protein in gnotobiotic brine shrimp challenged with isogenic *Vibrio harveyi* strains with different levels of virulence

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5.1 Introduction

Vibrio harveyi is a ubiquitous, bioluminescent marine bacterium which causes vibriosis in both marine vertebrates and invertebrates, leading to significant losses in the global aquaculture industry [7, 215]. Bacterial infection process includes three steps: colonization, adhesion and initial multiplication of the pathogen to the host surface, and then its penetration inside the body through one or several portals. Then, invasion of the pathogen inside the host organs and/or the circulatory system concurs with the expression of virulence factors, followed by exit of the pathogen and transmission of the disease [216]. The main mode of virulence of *Vibrio spp*. is the production of extracellular products that damage the cuticle and organs of the animals. The extracellular products include gelatinase, caseinase, phospholipase, haemolysin, elastase, chitinase, alkaline proteases, cysteine proteases, alkaline metal chelator-sensitive proteases, serine proteases, metalloproteases and siderophores [217, 218].

In response to a *Vibrio* infection, the host employs protective immune, repair and stress mechanisms to maintain cellular homeostasis and integrity, and to limit pathogenicity [219]. However, pathogenic *Vibrio* spp. evade host defensive mechanisms by using different strategies. For instance, *Vibrio* spp. evade stress caused by the host antimicrobial peptides (AMPs) by releasing their outer membrane vesicles to create a protective membranous shield. In *V. cholerae*, as another example, the resistance-nodulation-cell-division superfamily (RND) efflux pumps are particularly important in transporting AMPs out of the cytoplasm[220]. In *V. vulnificus*, evasion was done through surface expression of capsule polysaccharides that help bacteria to avoid phagocytosis by macrophages [221].

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 [222]. 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. In *V. vulnificus*, a novel iron acquisition system has been found i.e. *vep20.* It is a host specific virulence gene that encodes vep20, an iron-regulated outer membrane protein that is involved in iron acquisition not only in eel but also in human [223] . These strategies enable the bacteria to survive, and thus to colonize skin and internal tissues [224, 225].

Hsp70 and hmgb1 proteins are molecular chaperone for protein and DNA, respectively. As part of the innate immunity response, hmgb1 can be actively released from multiple cell types including macrophages, monocytes, and endothelial cells [226]. Hmgb1 can also be passively released from necrotic or damaged cells [227]. Hmgb1 released by activated immune cells and injured or necrotic cells, plays an important role in host responses to both types of threat: infection and sterile injury. Thus, it is a critical mediator in a final common pathway to morbidity and mortality during infection and sterile injury. Presently, hmgb1 has been considered as a therapeutic target for inflammatory diseases caused by injury or infection [228]. Novel methods for enhancing the availability of heat shock proteins (hsps) within the host have been developed either by supplying exogenous prokaryotic hsps derived from bacteria or by supplying exogenous eukaryotic hsp. Sung *et al.* 2008 [159] demonstrated that up-regulation of endogenous *hsp70* in brine shrimp *Artemia franciscana* was associated with enhanced resistance to *Vibrio* infection. Moreover, similar results were observed by feeding the *Artemia franciscana* with *E. coli*, overproducing prokaryotic hsps or by feeding bacteria that overproduce heat shock proteins by exposure to a non-lethal heat shock [229]. These studies suggested that feeding hsp70 might be a viable alternative to use of antibiotics to control vibriosis and other infections in *Artemia franciscana.*

Recently, a few studies have indicated that pathogenic bacteria evade host defense mechanisms during infection by limiting translation of host mRNAs which encode proteins that mediate defense responses [230, 231]. In particular, it has been suggested that pathogenic bacteria produce virulence factors such as type III secretion system (T3SS) effector proteins, which are important components of the infection process, and by introducing these effectors directly into the cytoplasm of the host, bacteria suppress host's protein translation. For example, *Pseudomonas entomophila* induces a significant transcription of the gene encoding for the host AMP daptomycin, but it blocks the translation of daptomycin mRNA to a functional protein [193, 232].

V. harveyi have been reported to regulate their pathogenicity by producing a wide variety of virulence factors, and the production of these factors are regulated by quorum sensing signal molecules [11, 163]. *V. harveyi* uses a multichannel quorum sensing system with three quorum sensing signal molecules viz. Harveyi Autoinducer 1 (HAI-1), Autoinducer 2 (AI-2) and

Cholerae Autoinducer 1 (CAI-1), which act synergistically in regulating the production of virulence factors [165, 166]. Among others, luminescence in *V. harveyi* is an important virulence factor that is regulated by quorum sensing [169]. *V. harveyi* luminescence has also been shown to be involved in the detoxification of reactive oxygen species such as H_2O_2 produced by the host, thus playing a protective role against oxidative stress [173]. As H_2O_2 is an important part of the immune mechanisms in eukaryotic hosts against infections [174], bioluminescence is considered as an immune-evasive strategy. In a previous study, we obtained non-luminescent variants of luminescent *V. harveyi* strains by culturing the latter under dark and static conditions and found that some of these non-luminescent variants were less pathogenic towards gnotobiotically-cultured brine shrimp (*Artemia franciscana*) than their luminescent counterparts [49, 65].

In this study, using the gnotobiotic *Artemia* model organism (GART) system, we aimed to determine whether isogenic *V. harveyi* strains with different levels of virulence or in vitro virulence factor production inflict damages and cause disease in *Artemia* by interfering with the mRNA translational machineries of the host defense system. We used the GART system to conduct this study since here *Artemia* can be cultured under germ free environment and a controlled population of pathogens with different virulence levels can be added. This system is a crucial tool for such studies because it allows eliminating the interference of the microbiota that is naturally present in any type of (aquatic) environment and facilitates in determining exclusively the action of the pathogen of interest on the host [158, 233].

5.2 Material and methods

5.2.1 Bacterial strains and growth conditions

Both luminescent and non-luminescent variants of *V. harveyi* strains were used (Table 1). These strains are rifampicin-resistant (RR) and were produced as previously described [65]. Briefly, single colonies of each strain picked from Marine Agar (MA, Difco laboratories) plates were cultured in 10 mL Marine Broth (MB) 2216 (Difco laboratories) in a 100 mL Erlenmeyer flask. After incubation at 28 °C for 24 h, 50 µL of the each culture was taken by micropipette and transferred to a 10 mL MB containing 0.5 mg L^{-1} of rifampicin (Sigma-Aldrich, USA). The cultures were incubated at 28 °C for 24-48 h. In the following days, subcultures were made in MB, increasing the rifampicin concentration gradually from 1, 2, 4, 8, 16, 32, 64 to finally 100

mg L^{-1}. When bacteria cultures were growing well in the final concentration of rifampicin (100 mg L^{-1}), they were inoculated on MA plates containing 100 mg L^{-1} rifampicin for obtaining single colonies. For long-term storage, the stock was preserved in 20% glycerol at -80 °C.

Autoclaved *Aeromonas* sp. (LVS3) was used as feed for *Artemia* (Marques et al. 2006). LVS3 were grown by inoculating one colony from a MA plate (28 °C for 24 h) in a 50 mL Erlenmeyer flask containing 20 mL MB and incubating the flask at 28 °C for 24 h. The grown LVS3 was centrifuged at 2200 x *g* for 15 min, supernatant discarded, and the pellet was washed and resuspended in filtered autoclaved sea water (FASW). Bacterial densities were determined spectrophotometrically as described previously [233]. Subsequently, the cell suspension was autoclaved at 121 °C for 20 min.

Table 5. 1 *Vibrio harveyi* strains.

Strain	Relevant feature	References
BB120 RR-L	Wild type, rifampicin-resistant luminescent	[65]
BB120 RR-NL	Wild type, rifampicin-resistant non-luminescent	[65]
JMH634 RR-L	Mutation in LuxM (AI-1 synthase), LuxS (AI-2) synthase) and CqsA (CAI-1), rifampicin-resistant luminescent.	[65]
JMH634 RR-NL	Mutation in LuxM (AI-1 synthase), LuxS (AI-2) synthase) and CqsA (CAI-1), rifampicin-resistant non-luminescent	[65]

5.2.2 Axenic hatching of *Artemia*

Axenic hatching of *Artemia* cysts was carried out following decapsulation and hatching procedures as described previously [202]. Briefly, 4 g of *Artemia* cysts originating from the Great Salt Lake, Utah, USA (EG® Type, batch 21452, INVE Aquaculture, Dendermonde, Belgium) were hydrated in 89 ml of sterile distilled water for 1 h. Sterile cysts and nauplii were obtained after decapsulation using 35 mL NaOH (32%) and 50 mL NaOCl (50%). The reaction was stopped after 2 min by adding 70 mL $Na_2S_2O_3$ (10 g/L). Thereafter the decapsulated cysts were

washed with sterile artificial seawater (35g/L, Aquarium Systems, Sarrebourg, France) and then suspended in 1 L glass bottles containing sterile artificial seawater, and then incubated at 28°C for 28 h with constant illumination of approximately 27 µE/m^2.sec for hatching. After 28 h of incubation, the axenicity of the hatched *Artemia* nauplii was verified both by spread plating (100 ml) and by adding (500 µl) hatching water on Marine Agar and in Marine Broth (Difco, Detroit, USA), respectively followed by incubation at 28°C for 5 days [202]. Bottles containing non-sterile nauplii were excluded from the experiment.

5.2.3 *Artemia* challenge test

Live axenic nauplii at the instar II stage were collected, counted volumetrically and then distributed into 1-L glass bottles containing sterile artificial seawater. The bottles were placed in a rectangular tank containing water maintained at 28°C using a thermostatic heater with constant illumination (approximately 27 µE/m2.sec.) and aeration. The nauplii were fed once with 10^7 cells mL^{-1} of autoclaved LVS3. The nauplii were challenged with luminescent (group 1) or non-luminescent (group 2) variants of *Vibrio* at 10^6 cells mL^{-1}. *Artemia* nauplii that were non-challenged (group 3) were used as control. Three replicates were used for each group. Samples containing 0.1 g of live nauplii were harvested from each glass bottle at 6 and 12 h post challenge, rinsed in sterile distilled water, immediately frozen in liquid nitrogen and preserved at -80°C for further analysis.

5.2.4 Analysis of heat shock protein 70 (*hsp70*) and high mobility group box 1 (*hmgb1*) gene expression by quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the *Artemia* nauplii (0.1 g) using the SV Total RNA Isolation System (Promega, Leiden, The Netherlands) according to manufacturer's instruction. First strand cDNA was synthesized from 1 µg total RNA using the RevertAid™ H minus First strand cDNA synthesis kit (Fermentas Gmbh, Germany) following the manufacturer's guidelines. The expression of *hsp70* and *hmgb1* genes was analyzed by qRT-PCR using the following two primer pairs (*hsp70*: forward - CGATAAAGGCCGTCTCTCCA, reverse - CAGCTTCAGGTAACTTGTCCTTG; *hmgb1*: forward- GGATGAAAGCAAACCCCGTg, reverse - GTGCTCTTCTCTGCAAGTCTG). qRT-PCR was carried out in a total volume of 25 µl, containing 9.8 µl of nuclease free water, 0.2 µM of each primer, 12.5 µl of Maxima SYBR

Green qPCR Master mix (Fermentas, USA) and 2 µl (1000 ng) of cDNA template. The qRT-PCR was performed using a One Step qRT-PCR instrument (Applied Biosystems) and the following protocol: denaturation (10 min at 95°C); 40 cycles of amplification and quantification (15 s at 95°C, 30 s at 60°C, and 30 s at 72°C); melt curve analysis (55-95°C with a heating rate of 0.10°C s-1 and a continuous fluorescence measurement) and finally cooling to 4°C. Melt curve analysis was done to check for the application of untargeted fragments. Data acquisition was done with the STEPONE software**.** Upon optimization, the amplification efficiency of the reference gene (elongation factor, *EF$_\alpha$1*) and target genes were found to be equal. Therefore, qRT-PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method for calculation of relative gene expression (fold-changes) (Livad and Schmittgen, 2001). The expression was normalized to the endogenous control (elongation factor, *EF$_\alpha$1*: forward – GGTCGGGTTGAAACTGGTAT, reverse - AGGGATTCGTGGTGCATTT) by calculating ΔC_T (= $C_{t\ target} - C_{t\ EF\alpha1}$), and expressed relative to calibrator sample by calculating $\Delta\Delta C_t$ (= ΔC_t - $\Delta C_{t\ calibrator}$). A sample of non-challenged *Artemia* at 6 h was used as calibrator. The relative expression was then calculated as Relative expression = $2^{-\Delta\Delta CT}$.

5.2.5 Protein extraction and analysis

Sampled *Artemia* nauplii (0.1 g) were homogenized in cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5mM $MgCl_2$, 5 mM NaH_2PO_4, 40 mM HEPES, pH 7.4) (Clegg *et al*. 2000) supplemented with a protease inhibitor cocktail (Sigma-Aldrich®, USA) as recommended by the manufacturer. After centrifugation at 2200 x g for 1 min at 4°C, the supernatant was collected and the protein concentration was determined as previously described (Bradford, 1976). Loading buffer was added to the supernatants which were subsequently vortexed, heated at 95°C for 5 min and finally electrophoretically separated by SDS-PAGE (10% and 8-16% polyacrylamide gel for hsp70 and hmgb1, respectively) using 30 µg of protein per lane. Heat shocked HeLa cells (human cervical adenocarcinoma cell line; Enzo Life Sciences, USA) (12 µg) were loaded on to one well to serve as a positive control and for calculating the relative amount of target proteins in the sample. Proteins were then transferred to a polyvinylidene fluoride membrane (BioRad Immun-Blot™ PVDF) using a semi-dry blotting device (Biorad). The membranes were incubated with blocking buffer [50 ml of 1x phosphate buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin] for 60 min at room temperature followed by a primary cross-

reactive antibody for human hsp70 (1:5000; Affinity BioReagents Inc., USA) [123] or a primary cross-reactive antibody for human hmgb1 (1:2500; Affinity Abcam, ab18256, United Kingdom). The membranes were then incubated with horseradish peroxidase conjugated donkey anti-mouse IgG (1: 2500; Affinity BioReagents Inc., USA) and horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibodies (1:10000; Sigma, USA) for hsp70 and hmgb1, respectively. The membranes were then treated with enhanced chemiluminescence reagent (GE healthcare, UK) and the signals were detected by a ChemiDoc MP Imaging System (Biorad, Belgium). The signal intensity as quantified for Hela cells was set as 1 and the signal intensity in the samples as determined by densitometry, was expressed relative to the Hela cell signal (Biorad Image Lab™ Software version 4.1, USA).

5.2.6 Statistical analysis

All statistical analyses were performed using the Statistical package for the Social Sciences (SPSS) version 20.0 using a significant level of 5%. Homogeneity of variances and normality of gene expression data were not fulfilled for an ANOVA analysis. The data were log transformed prior subject to statistical analysis. All treatments were compared to the control and to each of the other treatments individually using independent samples t-test for each time point respectively. Per time point, a matrix was obtained with an overview of all the results from the independent samples t-test.

Protein analysis data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple range tests using the statistical software Statistical Package for the Social Sciences (SPSS) version 20.0.

5.3 Results and discussion

When challenged with a pathogen, the host tries to maintain cellular integrity by triggering an immune response to restrict pathogen growth while repairing any tissue damage [234]. Previous studies suggested that pathogenic microorganisms (a bacteria, viruses) prevent both of these processes by arresting the protein synthesis in the host. For instance, in response to *Pseudomonas entomophila* infection, *Drosophila* significantly increased in the expression of antimicrobial peptides and repair pathways such as epithelium renewal and gut structure at transcriptional level.

However, *P. entomophila* evade the protective responses of *Drosophila* by blocking the synthesis of the protective molecules at the translational level [193]. In this study, using the germ-free *Artemia* as the host, we investigated whether pathogenic *V. harveyi* strains cause disease in *Artemia* by interfering with the host protective mechanisms. To this end, we exposed the germ-free *Artemia* to luminescent and non-luminescent wild type *V. harveyi* BB120 and its isogenic quorum sensing mutant JMH634 and then analyzed the transcription and translation profiles of heat shock protein 70 (hsp70) and the high-mobility group box-1 protein (hmgb1) in *Artemia*. We focused on hsp70 and hmgb1 proteins because these two proteins are associated with both repair and immune mechanisms to fight against pathogenic stressors [219, 235] (and also because antibodies are available by now). More specifically, hsp70 and hmgb1 proteins upon induction play key roles in defining the resistance of organisms against stressors [235] by performing multifaceted functions, such as acting as molecular chaperone for protein and DNA, respectively, functioning as danger associated molecular pattern (DAMP) during inflammation and various cellular processes [236, 237], and/or participating in the activation of cell surface innate immune receptors, thereby modulating many aspects of host's immune responses [238, 239].

Strain JMH634L is less virulent to *Artemia*, compared to BB120L. This coincides with reduced *in vitro* production of some of the listed virulence factors. At 6 h post exposure *hsp70* gene expression is up-regulated by JMH634L, while protein production is down-regulated. At 12 h post exposure there are no significant differences in production level of Hsp70 protein between the BB120L-exposed group and the JMH634-exposed group (Figure 5.1 & 5.2). Next, using these 2 strains to expose *Artemia* nauplii, results showed that there is no significant difference in *Hmgb1*expression but production of this protein is up-regulated by JMH634L relative to BB120L at both time points (Figure 5.3 & 5.4). The latter is an indication for the fact that a virulent strain BB120L would be able to down-regulate protein production. Yet this result is not equaled by the analysis of the gene expression and protein production profile of hsp70.

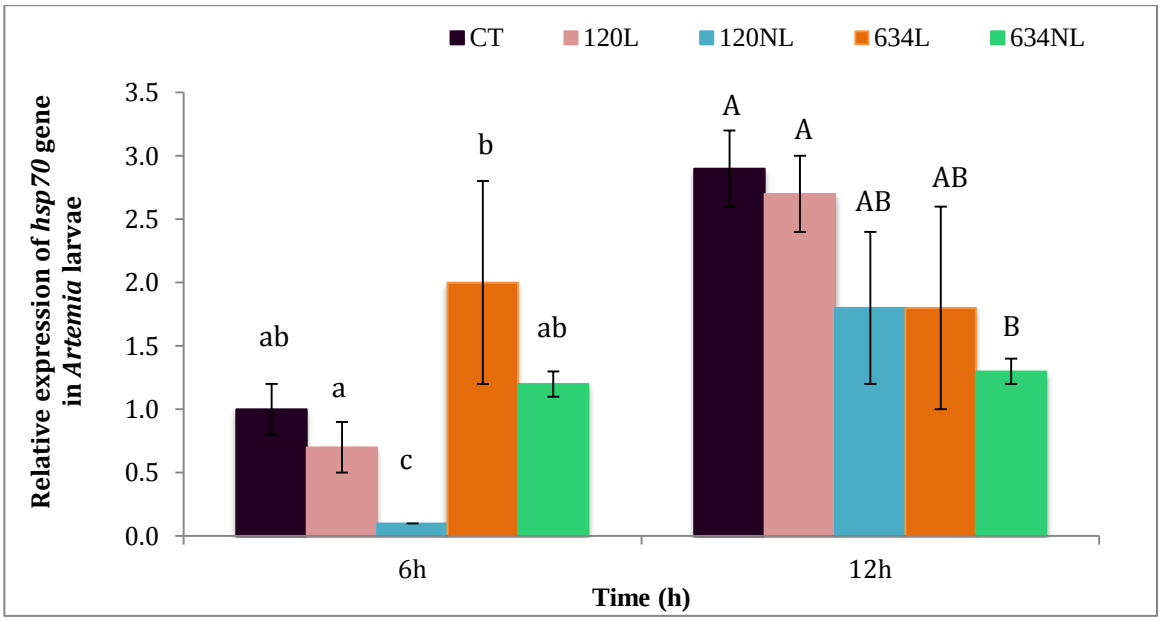

Figure 5. 1 Relative expression of the *Hsp70* gene in *Artemia* larvae.

The larvae were either challenged with luminescent (L) or non-luminescent (NL) wild type *V. harveyi* BB120 (120) or its isogenic quorum sensing mutant JMH634 (634). Unchallenged *Artemia* larvae sampled at 6 h served as control. Samples were collected for *Hsp70* gene expression at 6 and 12 h post challenge. The expression level of the *Hsp70* gene at 6 h in the control group was set as 1. Results, which are mean of 3 biological replicates, are presented relative to the expression of the *Artemia* elongation factor α 1 (*efα1*) gene. Bars indicate standard deviation of 3 biological replicates. Different letters (small and capital letters for 6 and 12 h, respectively) were used for significant differences among groups ($P<0.05$).

In addition, strain BB120NL is as virulent as BB120L in the *Artemia* challenge test. Most of the virulence factors are down-regulated *in vitro* by the L to NL switch, except for swimming motility which is very strongly up-regulated in BB120NL (Table 5.2). *Hsp70* gene expression in *Artemia* challenged with BB120NL is down-regulated only at 6 h post exposure whereas the hsp70 protein production is not affected. As observed in Figure 5.3 & 5.4, *hmgb1* gene expression is down-regulated at 6 h only, while its protein production is up-regulated at 12h by BB120NL-exposure.

In Table 5.2, data previously published on phenotypic differences among these 4 strains are summarized.

Table 5. 2 Summary of differences in *in vitro* virulence factors production and expression and production of hsp70 and hmgb1 in *Artemia* challenged with luminescent and non-luminescent BB120 and JMH634 strains [65].

	120L vs. 634L	**120L vs. 120NL**	**634L vs. 634 NL**
Caseinase	↑	↑	↑
Heamolysin	↑	↑	↑
Elastase	↑	-	-
Biofilm formation	-	↑	-
Swimming motility	-	↓	↓
DnaK content	↓	-	-
Luminescence	↑	↑	↑
Artemia survival	↓	-	-
Hsp70 gene (6 h)	↓	↑	-
Hsp70 protein (6 h)	↑	-	-
Hsp70 gene (12 h)	-	-	-
Hsp70 protein (12 h)	-	-	-
Hmgb1 gene (6 h)	-	↑	-
Hmgb1 protein (6 h)	↓	-	-
Hmgb1 gene (12h)			
Hmgb1 protein (12 h)	↓	↓	-

↑: significant increase
↓: significant decrease
-: no significant difference

Moreover, strain JMH634NL is just as strain JMH634L non-virulent to *Artemia*. *In vitro* most of the virulence factors are down-regulated in JMH634NL relative to JMH634L, except for swimming motility which is strongly up-regulated (Table 5.2). There are no significant differences in hsp70 expression and protein production at either time point. A similar result was obtained for hmgb1.

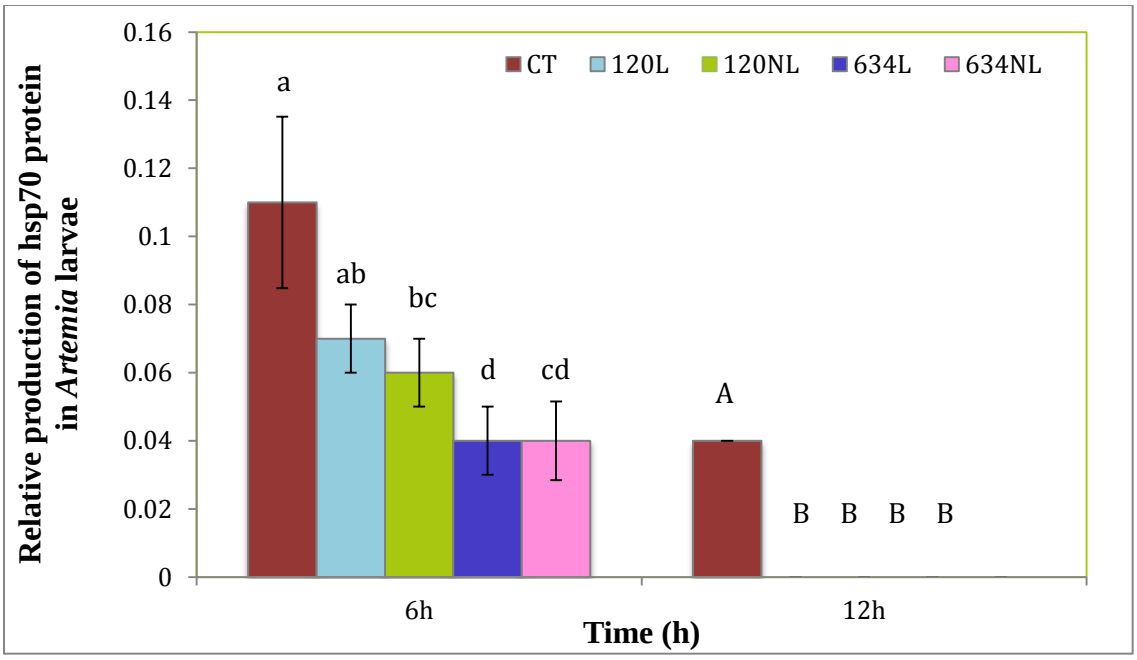

Figure 5. 2 Relative production of Hsp70 protein in *Artemia* larvae at 6 h and 12 h post challenge. Bars indicate standard deviation of 3 biological replicates. Different letters (small and capital letters for 6 and 12 h, respectively) were used for significant differences among groups ($P<0.05$).

In case of JMH634NL and JMH634L, both having a low virulence (limited *Artemia* mortality), *Artemia* exposed to either JMH634L or JMH634NL did not display a difference in gene expression level or protein production. So most likely these 2 strains do not impose infection pressure and hence no cellular damage is induced, resulting in an absence of regulation for these 2 genes.

Interpretation of the differences observed in the BB120L and BB120NL-exposed groups comparison is less straightforward. Little is known about the importance and dynamics of *in vivo* expression of the virulence factors. Yet, the BB120L strain seems to up-regulate expression of both *hsp70* and *hmgb1* genes with no associated increase in protein. Again this observation indicates that exposure to a more virulent strain will induce gene expression, while not necessarily resulting in increased protein production.

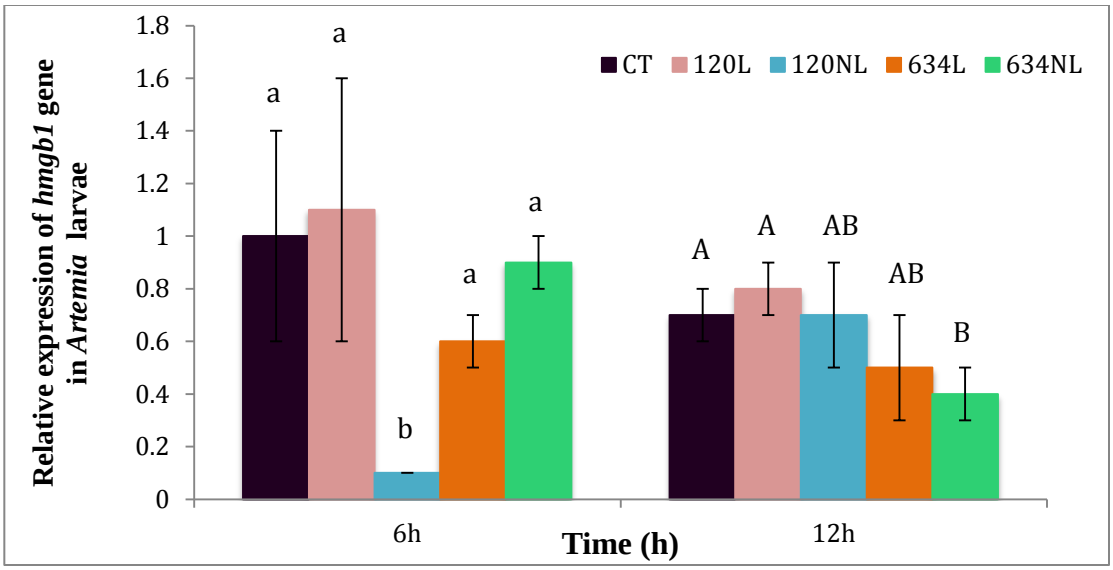

Figure 5. 3 Relative expression of *Hmgb1* gene in *Artemia* larvae.

The larvae were either challenged with luminescent (L) or non-luminescent (NL) wild type *V. harveyi* BB120 (120) or its isogenic quorum sensing mutant JMH634 (634). Unchallenged *Artemia* larvae sampled at 6 h served as control (CT). Samples were collected for *hmgb1* gene expression at 6 and 12 h post challenge. The expression level of the *hmgb1*gene at 6 h in the control group was set as 1. Results, which are the mean of 3 biological replicates, are presented relative to the expression of *Artemia* elongation factor α 1(efα1) gene. Bars indicate standard deviations of 3 biological replicates. Different letters (small and capital letters for 6 and 12 h, respectively) were used for significant differences among groups ($P<0.05$).

In addition, it is possible that just before or during infection non-luminescent cells switch back to luminescent cells. This is a speculation as we could never isolate luminescent cells from non-luminescent culture (Hong *et al.* unpublished). Yet phenotypic switches are possible *in vivo* during the infection process. Recently *V. campbellii* cells with different morphology were observed after phagocytosis by shrimp hemocytes (35). Assuming that such a phenotypic switch would indeed be necessary for infection, then non-luminescent might infect *Artemia* with an unknown amount of delay (relative to luminescent cells) because of the (short) time needed to make that switch. Yet at least *in vitro* their motility is much higher. So that might facilitate them to reach the locus of infection faster than luminescent cells. Such a scenario might have a complex impact on the dynamics of gene expression and protein production.

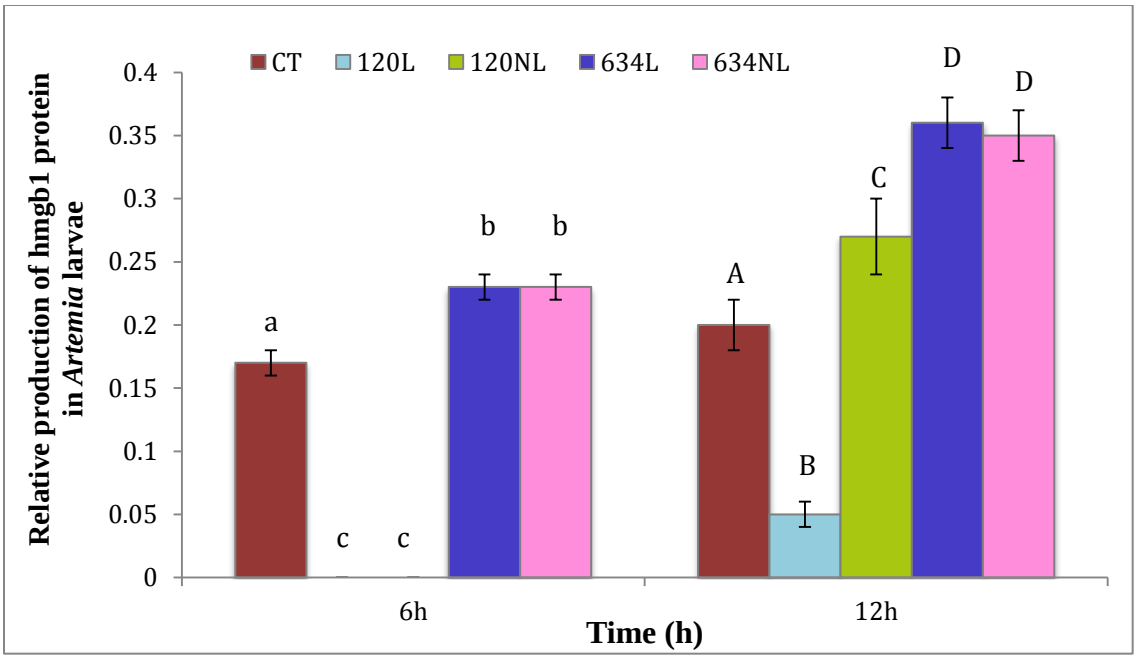

Figure 5.4 Relative production of Hmgb1 protein in *Artemia* larvae at 6 h and 12 h post challenge. Bars indicate standard deviation of 3 biological replicates. Different letters (small and capital letters for 6 and 12 h, respectively) were used for significant differences among groups ($P<0.05$).

In another case, BB120L and JMH634L are virulent and non-virulent to *Artemia*, respectively. *Artemia* exposed to BB120L seems to reduce the protein production of hmgb1 while the gene expression not affected. Probably, inhibition of host protein synthesis might be one of the virulence strategies of pathogenic bacteria to survive and cause disease after infection. This phenomenon has also been revealed in many recent studies [193, 231]. For example, the secreted ligand, Upd3 was not produced in *Drosophila* infected with *P. entomophila* despites the strong induction of the *upd3* gene. This study indicated that the uncoupling between protein and messenger RNA (mRNA) amount was indeed due to an inhibition of translation and not a general decrease in protein stability since it mainly affected proteins synthesized de novo upon infection (Upd3) but not protein produced prior to infection [193].

In conclusion, exposure of *Artemia* to pathogenic *V. harveyi* might be associated with modulation of the synthesis of hsp70 and hmgb1 proteins. At 12 h post infection either with BB120L or BB120NL, a significant down-regulation in the level of hsp70 and hmgb1 production was observed despite of the equal expression level of these genes compared to the control. This down-

regulation of hsp70 and hmgb1 protein levels might link to the decreased resistance of *Artemia* against *V. harveyi*. Owing to the fact that a battery of immune factors are involved in inducing resistance in the host against *V. harveyi*, further studies are warranted to determine the effect of pathogenic factors on the translational machineries of the host defense system and to confirm our findings with additional strains. Also, because the results point towards a strong dynamic response (eventually further confounded by the possibility of a back-switch by NL strains) it might be needed in the future to perform a much more regular and sustained sampling.

Acknowledgments

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Chapter 6

Molecular characterization and expression of the *Artemia franciscana hmgb1* gene

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6.1 Introduction

High mobility group box 1 (hmgb1) is an ancient, highly conserved and ubiquitous protein present in the nuclei and cytoplasm of nearly all cell types. The name of this protein is based on its ability to migrate quickly during electrophoresis. Hmgb family contains hmgb1, 2 and 3, from which hmgb1 is the most abundant (more than 1 million molecules/cell). Hmgb1 protein is virtually identical (> 99%) for all mamals, mostly present in the nucleus, where it bends DNA and promotes protein assembly on a specific DNA target [240]. Hmgb1 is a non-histone DNA-binding protein. During cellular stress, it localizes to the cell cytosol and can exit the cell through loss of membrane integrity or active secretion. Once it is free from the cells, it acts as damage-associated molecular pattern (DAMP) molecule to activate innate immune receptors and drive inflammatory responses [241].

Generally, hmgb1 is not tightly bound to chromatin therefore it passively diffuses from necrotic cells. During apoptotic cell death, under-acetylation of histones and chromatin condensation cause irreversible attachment of Hmgb1 to the chromatin. In contrast, the acetylation status of Hmgb1 itself is not changed during apoptosis. Consequently, hmgb1 is sequestrated inside the nucleus in apoptosis cells, thus contributing to the anti-inflammatory response exerted by these cells under physiological conditions [242]. Hmgb1 consists of two fairly rigid, L-shaped DNA-binding domains, each referred to as an 'HMG box' [243] and unstructured tail that ends with 30 consecutive negatively charged amino acids. The tail interacts with the HMG boxes and may modulate their intermolecular interaction [244]. In nucleus, hmgb1 support the structure of the chromatin and is involved in the transcriptional regulation of genes. Recently, intracellular hmgb1 has been supposed to be involved in autophagy and in inflammation activation [245]. Extracellular hmgb1 is involved in immune responses and it is considered as a prototypic alarm signal [246]. This is the first report on full sequence of the *hmgb1* gene in *Artemia franciscana* and overexpression of hmgb1 recombinant protein in *E. coli*. Results of this study can be used for further studies on functionality of *hmgb1* gene and protein in *Artemia*.

6.2 Material and methods

6.2.1 RNA isolation and cDNA synthesis

Total RNA was extracted from *Artemia franciscana* larvae using the SV Total RNA Isolation System (Promega, Leiden, The Netherlands) according to manufacturer's instruction. The RNA quantity was measured spectrophotometrically (NanoDrop Technologies, Wilmington, DE, USA) and adjusted to 500 ng μL^{-1} in all samples and the RNA samples were stored in -80 °C for subsequent use.

Reverse transcription was done with the RevertAid^{TM} H minus First strand cDNA synthesis kit (Fermentas, Fisher Scientific, Erembodegem, Belgium) in accordance to the manufacturer's instructions. Briefly, a 20 μL of reaction mixture containing 4 μL of 5x reaction buffer (0.25 mol^{-1} of Tris –HCl pH 8.3, 0.25 mol^{-1} of KCl, 0.02 mol^{-1} of $MgCl_2$, 0.05 mol^{-1} of DTT), 2 μL of 0.01 mol^{-1} of dNTP mix, 20 units of ribonuclease inhibitor, 200 units of RevertAid H minus M-MuLV Reverse Transcriptase (Fisher Sciencetific, Erembodegem, Belgium), 1 μL of Oligo-dT primer (Table 1) and 500 ng of total RNA. Subsequently, 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) was then used as template for isolating Hmgb1 using RACE method.

6.2.2 Isolating of the full coding sequence of *Art*Hmgb1

The 5'UTR and HMGB1 3'UTR sequence of *ArtHmgb1* were obtained via RACE method (Rapid Amplification of cDNA ends). The gene-specific primers (Hmgb1F and Hmgb1R) were designed from cDNA database of *Artemia franciscana* genome project (The project of Artemia Reference Center, Ghent University) (Table 6.1). The HMGB1F primer was combined with adaptor primer to amplify the 3'UTR sequence of *Hmgb1* by PCR reaction from cDNA sample. The HMGB1R primer was used to amplify the 5'UTR sequence as instruction of SMARTer RACE 5'/3' Kit from Clontech, USA.

Briefly, 2 μL of the first strand cDNA were used as template for 50μl PCR reaction, containing 0.5μM HMGB1F primer and 0.5μM adaptor primer, 10 μL of 5X Reaction buffer; 1 μL of 10 mM dNTP; 1 μL of Taq DNA polymerase (EP0701, Thermo Fisher Scientific, USA). DNA free

water were added to the reaction mixture to the final volume of 50 µL. The PCR thermal cycle was followed by denaturation for 3 minutes s at 95 °C subsequently, 30 cycles of annealing step were carried out for 30 s at 95 °C; 30 s at 58 °C; 60 s at 72 °C. The reaction was extended for 10 min at 72 °C and then cooled to 4 °C.

The HMGB1R primer was used as gene specific primer to amplify the 5'UTR sequence as instruction of SMARTer RACE Kit (Clontech, USA). Briefly, the cDNA for isolating of 5'UTR sequence was prepared by SMARTer RACE 5'/3': The 20 µl cDNA reaction contains 4 µL 5X First-Strand Buffer, 0.5 µL DTT (100 mM) 1 µL dNTPs (20 mM) 5'-CDS Primer A, 10 µL total RNA, 1 µl of the SMARTer II A Oligonucleotide, 0.5 µl RNase Inhibitor (40 U/µl), 2.0 µl SMARTScribe Reverse Transcriptase (100 U) flowing by incubation at 42°C for 90 minutes. Subsequently, PCR reaction was carried out to amplify 5'UTR sequence: 1 µl cDNA, 5 µl PCR buffer, 1 µl dNTPs (10mM), 5.0 µl 10X UMP primer (Clontech Kit), 0.5mM HMGB1R primer and 0.25 µl Taq DNA polymerase. The PCR thermal cycle was followed by denaturation for 3 minutes s at 95 °C subsequently, 30 cycles of annealing step was carried out for 30 s at 95 °C; 30 s at 55 °C; 60 s at 72 °C. The reaction was extended for 10 min at 72 °C and then cooled to 4 °C.

The PCR products of 3'UTR and 5'UTR sequences were purified from agarose gel using Wizard® SV gel and PCR Clean-up System (Promega) and then ligated into the pGEM®-T easy vector (Promega) using T4 ligase (LigaFast™ Rapid DNA Ligation System, Promega). The recombinant vectors were transformed into the TOP10 chemically competent *E. coli* (ThermoFisher Scientific, Belgium), the positive clones were selected through ampicillin resistance selection and PCR cloning analysis using gene specific primer combining with vector primer (T7 and SP6 primer, Table 6.1). The recombinant vector bearing the 5'UTR and 3'UTR sequence from positive transformant clones were purified by Wizard® Plus SV Minipreps DNA Purification System kit (Promega) for sequencing both strands, using T7 and SP6 primer. The full length of hmgb gene was assembled by Vector NTI software (Invitrogen, USA) from the obtained 5'UTR and 3'UTR sequences.

6.2.3 Cloning of the full-length *ArtHmgb1* cDNA

The PCR products were purified from agarose gel using Wizard® SV gel and PCR Clean-up System (Promega, Belgium) and then ligated into the pGEM®-T easy vector (Promega, Belgium).

The recombinant vectors were transformed into the TOP10 chemically competent *E. coli* (ThermoFisher Scientific, USA), the positive clones were selected through ampicillin resistance selection and PCR cloning analysis using gene specific primer combining with vector primer (T7 and SP6 primer, Table 6.1). The recombinant vector bearing the 5'UTR and 3'UTR sequence from positive transformant clones were purified by Wizard® Plus SV Minipreps DNA Purification System kit (Promega) for sequencing both strands, using T7 and SP6 primer. The full length of *ArtHmgb1* gene was assembled by Vector NTI software (Invitrogen, USA) from the obtained 5'UTR and 3'UTR sequences.

Table 6.1 Primer sequences used in this study

Name	Primer sequence (5'-3')	Brief information
HMGB1R	GTGCTCTTCTCTGCAAGTCTG	Gene specific primer for RACE
HMGB1F	GGATGAAAGCAAACCCCGTG	Gene specific primer for RACE
Adapter primer	CTGACCTGAAGTTCATCTGCTTTTTTTTTTTTTTTT	Oligo (dT) for cDNA synthesizing
T7	TAATACGACTCACTATAGGG	Phage promotor primer on the vector for sequencing
SP6	TATTTAGGTGACACTATAG	Phage promotor primer on the vector for sequencing
HMGB1-BamHI-F	GCACGGATCCATGCCACGCTCTAAGGATG	Gene-specific primer for recombinant protein
HMGB1-SalI-R	GCGCTCGTCGACTTACTCGTCATCCTCTTCCTCC	Gene-specific primer for recombinant protein

6.2.4 Sequence characterization and multiple sequences alignment

The amino acid sequence information for homology analysis is shown in Table 6.2. The nucleotide and protein sequence similarity were conducted with Blast algorithm at the National Center Biotechnology Information (NCBI, http://www.ncbi.nlm.gov/blast/). Multiple sequence alignment of Hmgb1 protein was performed with ClustalW multiple alignment program 2.1

(http://www.ch.embnet.org/software /ClustalW.html). The protein phylogeny analysis was done on web platform http://www.phylogeny.fr.

Table 6. 2 Species and accession number of *Hmgb1* genes used in the homology analysis.

Species	**Accession number**
Homo sapiens	CAG33144
Bos Taurus	NP_788785
Gallus gallus	NP_990233
Sus scrofa	NP_999228
Salmo salar	ACM09402
Danio rerio	CAX12897
Lampetra fluviatilis	CAA67363
Lipopenaeus vannamei	ADQ43366
Litopenaeus vannamei	ADQ43367

6.2.5 The recombinant expression of *Art*Hmgb1

Base on the obtained full length of *ArtemiaHmgb1*, two primers HMGB1-BamHI-F and HMGB1-SalI-R (Table 6.) were designed to allow amplifying the entire ORF of *Art*Hmgb1 and cloned into pET28a(+) expression vector at the BamHI/SalI sites (Table 6.1). The open reading frame (ORF) of *Art*Hmgb1 was amplified by PCR using Phusion High-Fidelity DNA Polymerase (F-530S, Thermal scientific, USA). Briefly, 50 µL PCR reaction contains 5 µL of 5X Phusion HF buffer, 1 µL of dNTPs (10mM), 0.5 µL of Phusion DNA polymerase (2U/µL), 0.5 µM of each gene-specific primer and cDNA sample as template. The thermal cycling conditions were : 1 cycle of denaturation for 30 seconds at 98 °C; 35 cycles of 10 seconds each at 98 °C, 20 seconds at 60 °C, 2 minutes at 72 °C; 1 cycle of extension for 10 minutes at 72 °C. Finally, the reaction was cooled at 4 °C. The PCR product was gel-purified with Wizard® SV Gel and PCR Clean-up System (Promega, Belgium) and then digested with BamHI (# R0136S, NEB, USA) and SalI (# R0138S, NEB, USA) subsequently cloned into the expression vector pET28a(+) at BamHI/SalI sites (Novagen, Gemany). The recombinant expression vector (pER28a-artHMGB1) was

transformed into TOP10 chemically competent cells. The positive transformants were screened by PCR with primers T7 and HMGB1-SalI-R (Table 6.1) and then confirmed by further nucleotide sequencing. Finally, the purified pER28a-*ArtHmgb1* was transformed into Rosetta^{TM}(DE3) chemically competent cells (#70954-3, Novagen, Gemany) for protein expression. The pET28a(+) vector without insert fragment was designed as A(-) and used as negative control. The negative transformants were designed as A1 and the positive transformants were designed as A2 and incubated in liquid Luria-Bertani (LB) broth medium containing 50mgmL^{-1} Kanamycin (K1377, Sigma, USA) and 34 mg mL^{-1} Chloramphenicol (C0378, Sigma, USA) at 37°C with shaking at 220 rpm overnight. The bacterial supernatant were upscaled in the fresh LB broth containing 50mg mL^{-1} Kanamycin (K1377, Sigma, USA) and 34 mg mL^{-1} Chloramphenicol (C0378, Sigma, USA) at 37°C with shaking at 220 rpm for 2 more h. When the culture media reached OD_{600} of approximately 0.5-0.7, the cell were induced with isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma-Aldrich, Inc, USA) at 0.5 mmol L^{-1} and incubated for an additional 4 h. The bacterial supernatant were collected by centrifugation at 4000 x g, rinsed twice with sterile demi water and then stored at -80 °C for further experiment.

6.2.6 Protein extraction and detection

The induced and non-induced A2 bacterial strain were homogenized by rapid agitation with 0.1 mm diameter glass bead in cold buffer K (150mM $MgCl_2$, 5 mM NaH_2PO_4, 40 mM HEPES, pH 7.4) containing a Protease inhibitor cocktail (Sigma-Aldrich, Inc, USA). Subsequently to centrifugation at 2200 g for 1 min at 4 °C, supernatant protein concentrations were determined by the Branford method (Bradford 1976). Supernatant (30 μg protein) were then combined with loading buffer, vortexed, heated at 95 °C for 5 min, centrifuged at 4000 g for 1 min and then electrophoresed in 8 -16 % SDS-PAGE gels. HeLa (heat shocked) cells (Enzo Life Sciences, USA) (12 μg) served as a positive control and for calculating the relative amount of hmgb1 protein in each sample. Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (BioRad Immune-Blot^{TM} PVDF) for antibody probing. After incubation in blocking buffer (50 mL of 1 x phosphate buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin) for 1 h at room temperature followed by incubation with primary antibodies for hmgb1 either "HMGB1 (1:2500; Affinity Abcam, ab18256, United Kingdom) or ARP59013-P5050 (1:1000; Holzel diagnostika, Germany). The membranes were then incubated

with horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (1:10000; Gentaur BVBA, Belgium). The membranes were then treated with enhanced chemiluminescence reagent (GE healcare, UK) and the signals were detected by a ChemiDoc MP Imaging System (Biorad, Belgium). The relative signal intensity was quantified by densitometry with Biorad Image lab™ Software version 4.1.

6.3 Results and discussion

6.3.1 Cloning of *ArtHmgb1*

ArtHmgb1 was a 912 bp fragment, with an open reading frame (ORF) of 603 bp (Figure 1.6). The gene encoded a protein of 200 amino acids with a calculated molecular weight of 26.7 kDa. The alignment of the amino acid sequence of *Art*Hmgb1 with other known Hmgb1s revealed a conserved acid tail in the amino acid sequence of *Art*Hmgb1 and confirmed that Hmgb1 proteins are highly conserved between vertebrates and invertebrates (Figure 2.6)

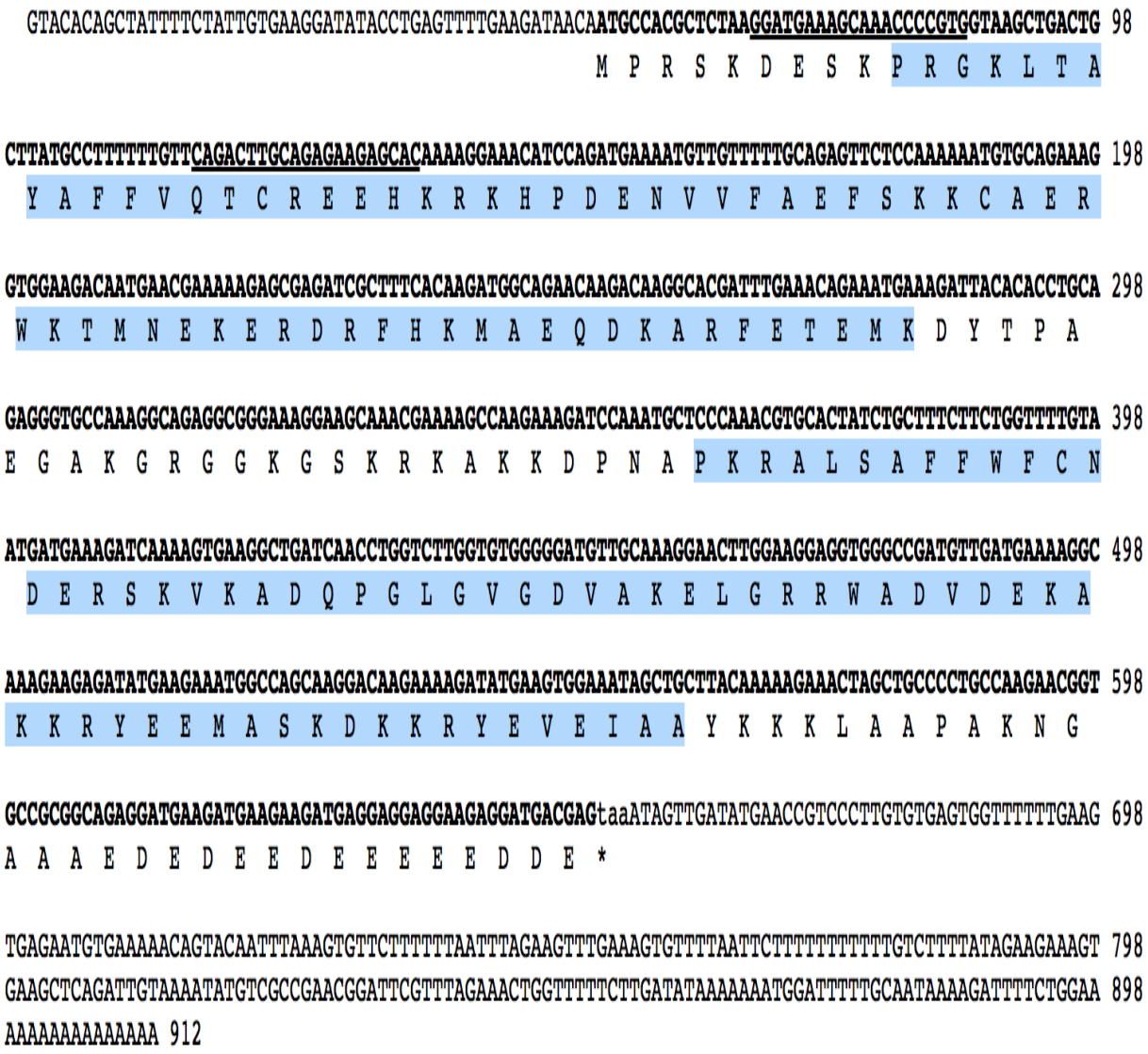

Figure 6.1 Nucleotide and deduced amino acid sequences of *Art*Hmgb1.

The nucleotides are numbered along the right margin. The ORF is shown in bold letters, the 5´ and 3´-UTRs are shown in un-bold letters. The two HMG domains are shaded. The gene-specific primers HMGB1F and HMGB1R for the RACE method are underlined.

```
Artemia                 MPRSK----DESKPRGKLTAYAFFVQTCREEHKRKHPDENVVFAEFSKKC
                        * :         **:*: ::**:****** ***: :*  .* *:***::*

Human                   SERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGETK--------K
Cow                     SERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGETK--------K
Chicken                 SERWKTMSSKEKGKFEDMAKADKLRYEKEMKNYVPPKGETK--------K
Pig                     SERWKTMSAKEKSKFEDMAKSDKARYDREMKNYVPPKGDKK--------G
Salmon                  SERWRTMSAKEKGKFEDLAKLDKMRYEREMRSYIPPKGEKK--------K
Zebrafish               SERWRTMSSKEKGKFEEMAKTDKVRYDREMKNYVPPKGAKGG-------K
Lamprey                 SERWKTMSEKEKTRFEDMAKVDKVRYDREMKTYVPPKGERGS-------R
L.vannamei              SERWKTMSDKEKKKFHDMAEDDKKRYDEEMKDFVPSPGAGRRGRRARGRR
L.vannameib             AERWKTMTDKEKDRFYDMADKDKARYDTEMKGYR-GPRTPRVSRKRRNR-
Artemia                 AERWKTMNEKERDRFHKMAEQDKARFETEMKDYTPAEGAKGRGGKGSKRK
                        :***:**. **: :* .:*. ** *:: **: :

Human                   KFKDPNAPKRPPSAFFLFCSEYRPKIKGEHPGLSIGDVAKKLGEMWNNTA
Cow                     KFKDPNAPKRPPSAFFLFCSEYRPKIKGEHPGLSIGDVAKKLGEMWNNTA
Chicken                 KFKDPNAPKRPPSAFFLFCSEFRPKIKGEHPGLSIGDVAKKLGEMWNNTA
Pig                     KKKDPNAPKRPPSAFFLFCSEHRPKIKSEHPGLSIGDTAKKLGEMWSEQS
Salmon                  RFKDPNAPKRPSSAFFIFCADFRPQVKGETPGLSIGDVAKKLGEKWNNLT
Zebrafish               KKKDPNAPKRPPSAFFVFCSDHRPKVKGDNPGISIGDIAKKLGEMWSKLS
Lamprey                 KKKDPNAPKRPPSAFFIYCAEYRSKVRAENPGLTIGSIAKKLGEMWNNAP
L.vannamei              P-KDPNKPKRALSAFFYYANDERPKVRAANPDFSVGEVAKELGRQWNELG
L.vannameib             --KDPNAPKRALSAFFWFCNDERAKVRAANPDMGVGDVAKQLGAAWSNTP
Artemia                 AKKDPNAPKRALSAFFWFCNDERSKVKADQPGLGVGDVAKELGRRWADVD
                          **** ***. **** :. : *.:::.  *.: :*. **:**  * .

Human                   ADDKQPYEKKAAKLKEKYEKDIAAYRAK--GKPDAAKKGVVKAEKSKKKK
Cow                     ADDKQPYEKKAAKLKEKYEKDIAAYRAK--GKPDAAKKGVVKAEKSKKKK
Chicken                 ADDKQPYEKKAAKLKEKYEKDIAAYRAK--GKVDAGKKVVAKAEKSKKKK
Pig                     AKDKQPYEQKAAKLKEKYEKDIAAYRAK--GKGEAGKKGPGRPTGSKKKN
Salmon                  AEDKVPYEKKAARLKEKYEKDITAYRNK--GKVP-------VSMPAKAAA
Zebrafish               PKEKSPYEQKAMKLKEKYEKDVAAYRAK--GGKPDGAKKGGPGRPAGKKA
Lamprey                 ADEKSIYERKTAKLKEKYDKDMASYRSK--GKVET------SKVASKPAS
L.vannamei              EDEKVKYEKLAEEDRARYDREMTAYKFG--GASPQ------KKMKASNGH
L.vannameib             PEAKAKYEALAASDKERYEKEMKAFKEGNFGAKKH------KTMNAPN--
Artemia                 EKAKKRYEEMASKDKKRYEVEIAAYKKK--LAAPA------KNGAAAE--
                         . *  **  :   : :*: :: :::                   :

Human                   EEEEDEEDEEDEEEEEDEEDEDEEEDDDDD- 215
Cow                     EEEEDEEDEEDEEEEEDEEDEEEEEDDDDE- 215
Chicken                 EEEEDEDEDEEDEEDEEEEEEEEEDDDDDE- 215
Pig                     EPE-DEEEEEEEEEDEDEEEEDEDEE----- 210
Salmon                  PAKDDDDDDDDDEDEDDDDDDDEDDE----- 203
Zebrafish               EADDDDDEDEDEEDEEEEDEEDEDDDDE--- 213
Lamprey                 KQRDDDDDEDDDEEDDEDEDEDDDDDDE--- 208
L.vannameia             PVDADPEDDEDEVGGEEDDEDDVSDEGSEDE 222
L.vannameib             --EDDDEEEESESEEEEEEEDDE-------- 206
Artemia                 -----DEDEEDE----EEEEDDE-------- 200
                              :::::.:    ::::::
```

Figure 6.2. Multiple sequence alignment of *Art*Hmgb1 protein with other Hmgb1s deposited in GenBank

The (*) indicated positions where all sequences share the same amino acid residue. Similar amino acids are shared by (: and .) .Gaps are indicated by dashes to improve the alignment.

6.3.2 Recombinant protein expression in *E.coli*

The recombinant vector pET28(+)/*Art*Hmgb1 was transformed in *E. coli* strain Rosetta^{TM}(DE3) competent cells. After IPTG induction, the whole-cell lysate was separated by SDS-PAGE, and a distinct band of *Art*Hmgb1 was revealed with a molecular weight of appropriately 26.7 kDa (Figure 3.6). Different antibodies were used to detect the *Art*Hmgb1 recombinant protein. The result of Western blotting showed that the *Art*Hmgb*1* gene was up-regulated in the ITPG-induced A2 strain, yielding a polypeptide of approximately 26.7 kDa visible on the PVDF membrane probed with either HMGB1 (Figure 4.6 A) or ARP59013-P5050 (Figure 4.6 B) antibodies.

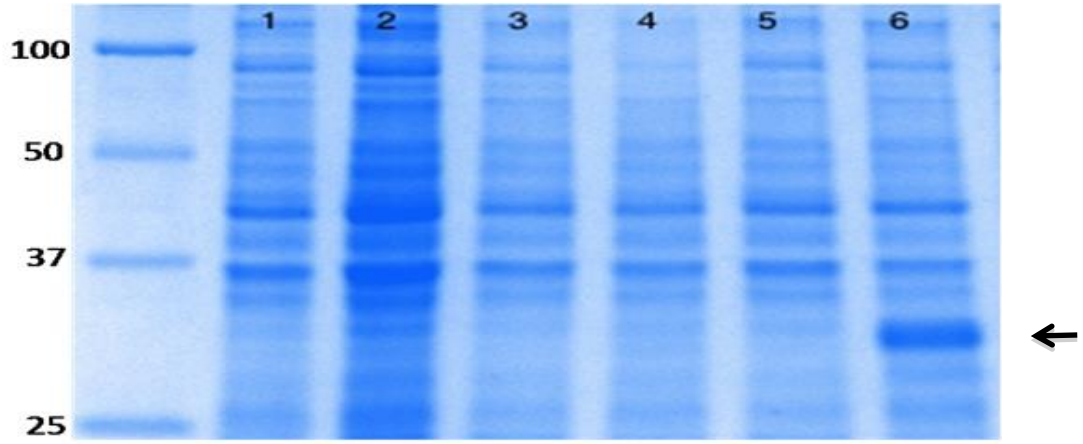

Figure 6.3 ITPG induced overproduction of *Artemia* Hmgb1 protein.

Protein extracted from *E. coli* strain was resolved in SDS-PAGE gel and then stained with Coomassie blue. Molecular mass standards in kDa are on the left. 1: non-induced of vector without insert fragment A(-); 2: induced of vector without insert fragment A(-); 3: non-induced of negative colony (A1); 4: induced of the negative colony (A1); 5: non-induced of positive colony (A2); 6: induced of positive colony (A2).

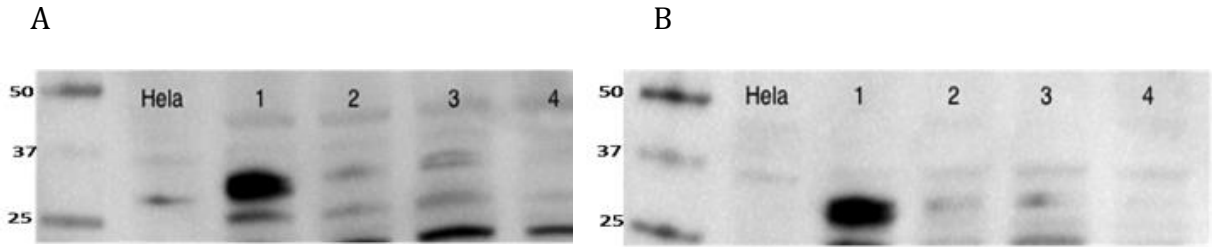

Figure 6.4 ITPG induced overproduction of *Artemia* Hmgb1 protein.

Protein extracted from *E. coli* strain A2 induced with ITPG was transferred to polyvinylidene fluoride membrane and either probed with HMGB1 (A) or ARP59013-P5050 (B) antibody.

Thirty microgram of bacteria protein was loaded in each land. Molecular mass standards in kDa are on the left. 1: induced positive colony (A2); 2: non-induced positive colony (A2); 3: induced negative colony (A1); 4: non-induced negative colony (A1).

As show in Figure 6.5 Hmgb1 protein from *Artemia* samples either challenged with JMH634L or JHM634NL was detected by the same antibody HMGB1 that also worked with the *Art*Hmgb1 recombinant protein overexpressed in *E.coli*. In all protein samples from *Artemia* Hmgb1 were detected on the PVDF membrane at an approximate size of 38.5 kDa.

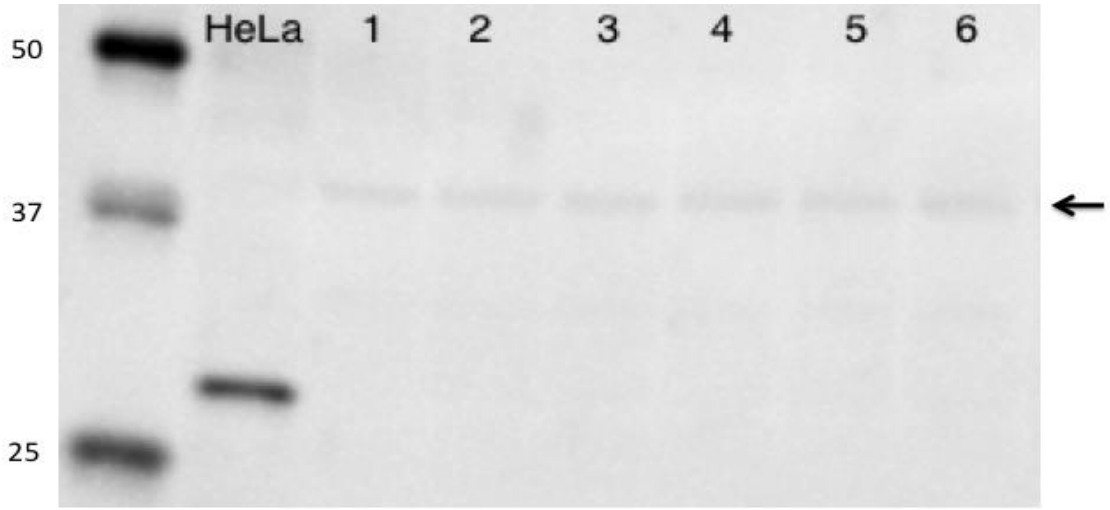

Figure 6.5 Expression of Hmgb1 protein in *Artemia. Artemia* either challenged with JMH634 luminescent (1, 2, and 3) or JMH634 non-luminescent variant (4, 5, 6) for 6 h.

Protein extract from different groups was transferred to polyvinylidene fluoride membrane and probed with HMGB1 antibody to *Artemia* Hmgb1. Thirty microgram of *Artemia* protein was loaded in each lane. Molecular standards in kDa are on the left.

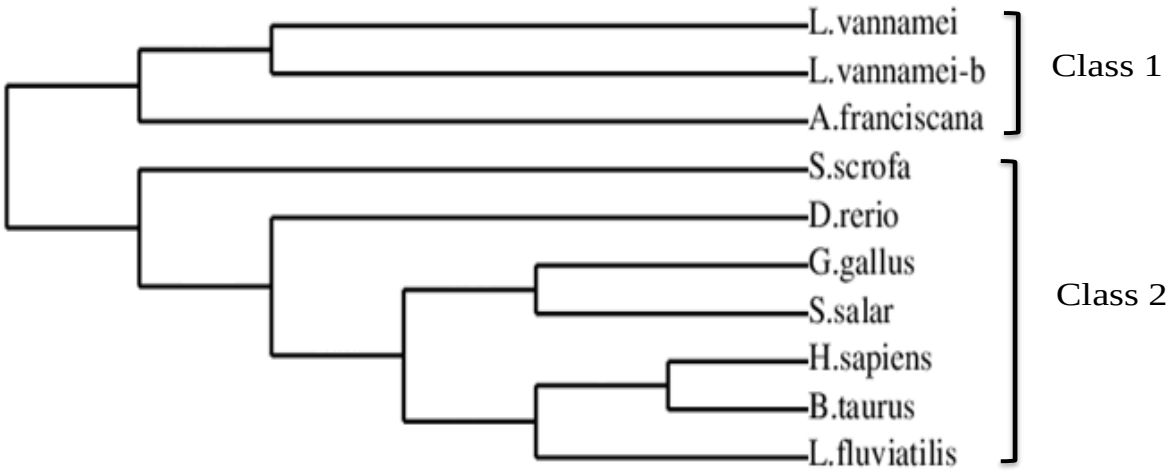

Figure 6.6 Phylogenetic tree of Hmgb1 protein from invertebrates and vertebrates.

The tree was constructed by an NJ algorithm using the http://www.phylogeny.fr website based on the multiple sequence alignment by ClusterX.

Hmgb1 proteins were subjected to phylogenetic analysis using the neighbor-joining (NJ) method by http://www.phylogeny.fr website. The phylogenetic tree illustrates that using available Hmgb1 proteins, they can be divided into 2 classes, namely invertebrates and vertebrates.

In conclusion, Hmgb1 plays an important role in maintaining nucleosome structures, transcription, and inflammation. They have been identified in many vertebrates and invertebrates [247]. In this study, the full-length cDNA of Hmgb1 was cloned from *Artemia franciscana*. The deduced polypeptide of *Art*Hmgb1 consisted of 200 amino acids, and it calculated molecular weight was 26.7 kDa, which is highly conserved to those from vertebrates and invertebrates.

The conserved function domains of *Art*Hmgb1 showed high similarity with other identified Hmgb1s and the phylogenetic relationship collectively suggested that *Art*Hmgb1 was a novel member of invertebrate Hmgb1 family, and it could have similar functions to those from vertebrates and other invertebrates. In *Artemia* a protein with a higher apparent molecular mass of 38.5 kDa was detected. At least according to the website of one manufacturer this is a probability and may be the consequence of differential glycosylation (http://www.abcam.com/recombinant-human-hmgb1-protein-ab167718.html). It is not known why glycosylation of Hmgb1 would be so different in HeLa cells relative to *Artemia.*

Chapter 7

General discussion and conclusions

7.1 Phenotypic variation *in vitro* conditions

Bacteria have to survive in different niches and in the presence of various stresses, such as starvation, temperature shocks, pH alterations, UV exposure, predation, antimicrobial agents challenge (antibiotics, biocides and disinfectants) and immune host defences [248]. The ability of a bacterium to survive and accommodate sudden changes in the conditions is termed as adaptive response and strongly depends on its genetic content. However, these survival strategies are often only used by part of a bacterial population. These responses heterogeneity could help bacteria to utilize different niches within an ecosystem, and even has potential to increase the overall fitness of the species.

Phenotypic variation mostly involves colony morphology change, biofilm phenotypic variation, virulence and persistence of bacteria.

One of the most important roles of phenotypic variation is to cope with dynamic environments. In fact, the conventional perspective on microbial adaptation to fluctuating environments is that individuals sense environmental cues and respond to them through signal transduction and the regulation of gene expression, thereby attaining a phenotype that performs well in the current environmental condition. However, it is likely that this strategy – sensing and responding – is not always possible, such as when the number of the possible environmental conditions that an organism encounters is so large or when environmental impact arises so rapidly that organisms can not express phenotypic features that would accommodate the impact fast enough [249]. In these cases, organisms can evolve to express these protective features probabilistically and with a low probability, independently of environmental cues. At any point in time, the majority of the population will not express the features; these individuals will grow well in the absence of environmental impacts but poorly during impacts. A minority of the individuals in a clonal population will express these features, and they will potentially grow more slowly in the absence of environmental impacts if these features are costly to express but will survive environmental impacts (Figure 7.1). Through this process, phenotypic variants are produced, and allows organism to persist in fluctuating environments [249]. For example, growth rate heterogeneity in a population of *E. coli* contributes to survival on exposure to antibiotics. A small subpopulation of the cells (known as persisters) in clonal populations grows very slowly or not at all, and these cells have a higher chance of surviving sudden exposure to antibiotics. The slow growth that

confers tolerance to antibiotics can be caused by a range of different exterior and interior factors, including nutrient shifts or expression of virulence genes [250].

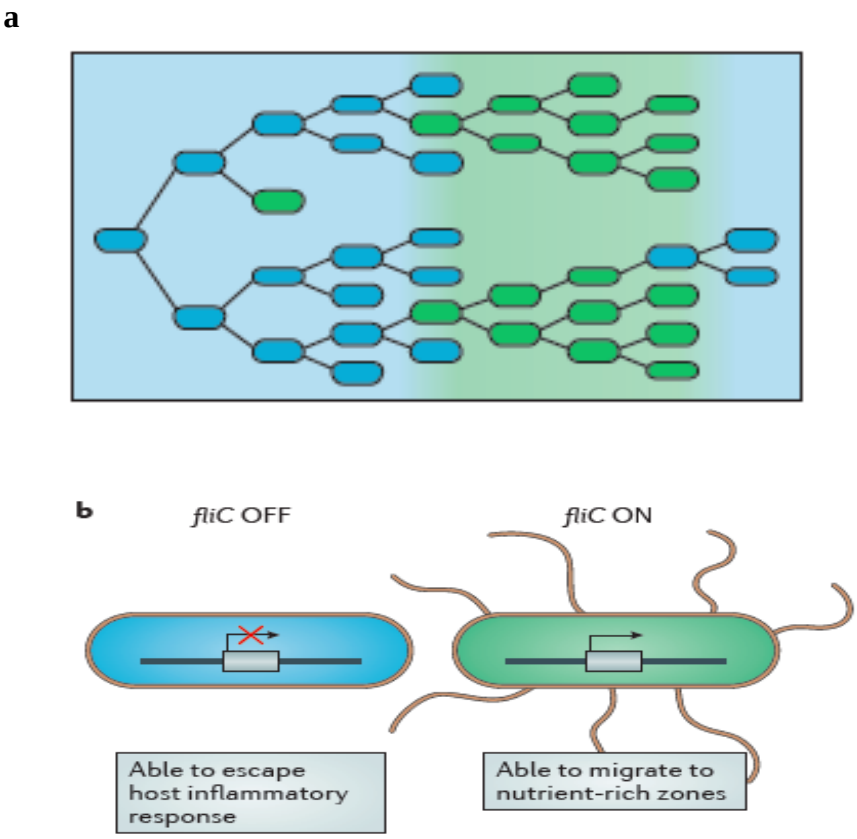

Figure 7.1 Persistence in fluctuating environments (Source [249]).

a. A genotype that expresses two different phenotypes (blue and green cells) can persist in an environment that fluctuates between two states (light blue and light green) in which only individuals that express that matching phenotype can survive.
b. *Salmonella typhimurium* exhibit two different phenotypic variants in the expression of the flagellin gene *flicC*, and this allows *S. typhimurium* to persist in an environment that fluctuates between favouring flagellation (*flicC* ON) and selecting against it (*flicC* OFF) [251].

Phenotypic variation has been shown to influence bacterial virulence and also microbial resistance. The small colony variants (SCVs) that has been isolated from several types of respiratory and urinary infections are the best example of how phenotypic variation can alter the antimicrobial resistance profile and virulence ability and their great adaptability in such different environments [252, 253]. In a previous study, non-luminescent variants of luminescent *V. harveyi*

strains were obtained by culturing the luminescent variant under static condition in the dark, and it was found that these non-luminescent variants were less virulent towards brine shrimp (*Artemia franciscana*) larvae than their luminescent counterparts [49]. In another study, it was found that the non-luminescent variants had lower mRNA transcript levels of the quorum sensing master regulator *luxR,* and the *vhp* metalloprotease (known to be regulated by quorum sensing)[69]. Chapter 3 of this thesis is an extension of the above studies wherein it was aimed to investigate the association in terms of virulence factors expression between the non-luminescent variants and their "parental" luminescent strains of *V. harveyi* and their quorum sensing mutant strains. In the present study (Chapter 3) the production of virulence factors, including caseinase, haemolysin and elastase, as well as the swimming motility and biofilm formation were characterized. Our results indicated that switching from luminescent variant of the wild-type *V. harveyi* and its quorum sensing mutants to their respective non-luminescent variants significantly influenced multiple aspects of their phenotypic behaviour resulting in altered caseinase and haemolytic activities and improved swimming motility and biofilm formation. The phenotypic variation capacity was to a large extent independent from the quorum sensing status, because quorum sensing mutants were equally capable of making the phenotypic switch. These findings suggest that *V. harveyi* could change its cell morphology, virulence factors and persistence in the natural ecosystem as well to adapt the fluctuation in the environment. However, the environmental conditions and mechanisms that regulate this switching are not elucidated. In addition, whether these non-luminescent variants would be recognized by host and how the host would respond to the exposure and subsequent infection caused by the different variants of the *V. harveyi* need to be verified. Experiments were conducted to try to answer these questions in the subsequent chapters (Chapter 4 & Chapter 5).

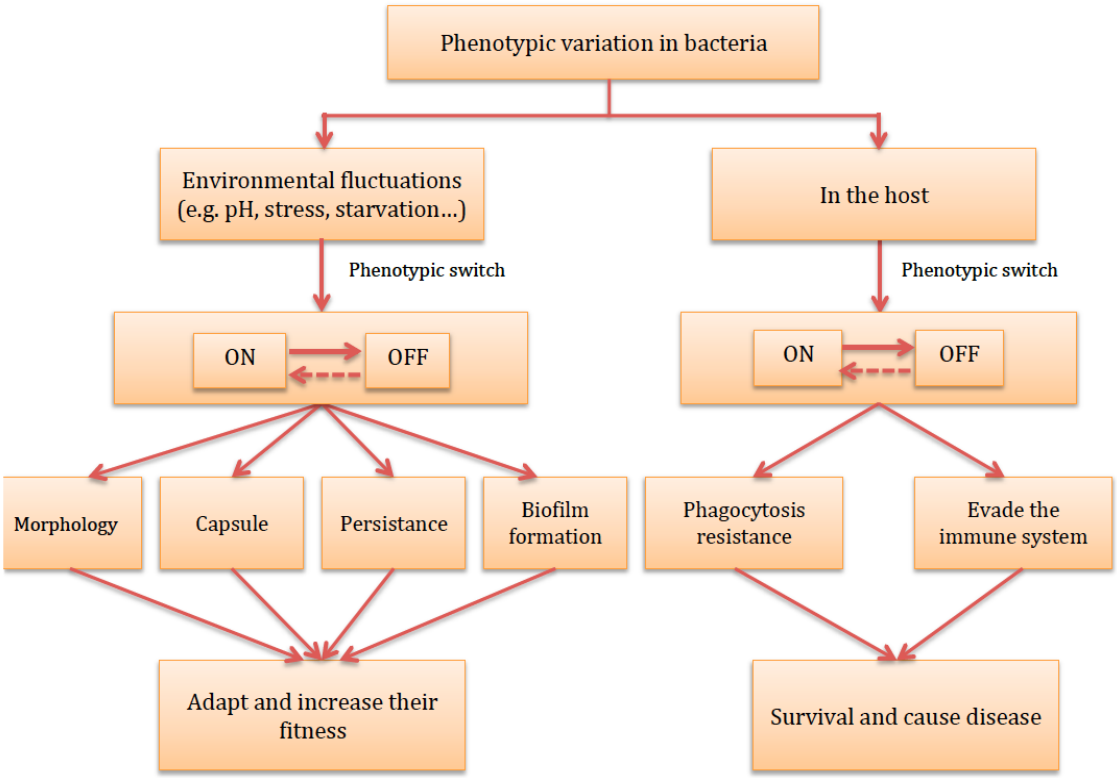

Figure 7.2 Phenotypic variation in bacteria

Infections caused by pathogenic bacteria that have the ability to form biofilms (biofilm infection) are difficult to treat, and *in vitro* susceptibility tests have shown that biofilms are considerably more resistant than planktonic cells to the action of antibiotics [252]. In addition, the enhanced antibiotic resistance of biofilms has been attributed to the presence of an exopolysaccharide matrix, a slow growth rate, spatial heterogeneity and biofilm-specific drug-resistant or drug-tolerant physiologies including the presence of persister cells and small colony variants. In the present study (Chapter 3), the biofilm formation was higher in the luminescent wild type BB120 compared to that of its luminescent quorum sensing mutant strains. Switching from luminescent to non-luminescent variants seemed to enhance the biofilm formation in most of the non-luminescent variants of the *V. harveyi*. Also in some quorum sensing mutants switching towards the NL status reduced the virulence towards *Artemia franciscana*. Although in this study the topic of persisters was not studied, it is conceivable that in the NL population more persisters are around making this population of NL variants more resistant to antibiotics [65]. However this is hypothetical and could be the topic of further investigation.

The notion of phenotypic variation has changed how we look at microbial populations. Microbial cells are individuals that differ from each other in terms of their behavior and their properties. Phenotypic variants can have important functions and can provide individuals and groups with new functionality. Several studies have suggested that phenotypic variation is an important aspect of the biology of microorganism in their natural environment and that it contributes to functionally relevant biological diversity in microbial populations. It hence contributes to the concept of differential properties and behaviors of individual cells [254, 255]. An overview of different aspects influenced by phenotypic variation of bacteria in general is illustrated in Figure 7.2.

7.2 Phenotypic variation in the host

Pathogens try to evade host's immunity to survive after infection. There are various potential strategies. Bacteria can switch hosts and become free-living some of the time, or they can find sites within a host that are "privileged" or hidden from host immunity. Moreover, they can try to alter antigen display to avoid recognition. Pathogens commonly interact with hosts through proteins or other macromolecules in their cell surface; those that elicit immune response within the host are considered as antigens. [255]. These surface-exposed proteins can have a variety of functions including transporter, porins, receptors, colonizing factors, enzymes, which in addition might contribute to evading the host immunity [51]. For example, in *Neisseria* spp., the expression of outer membrane proteins that are involved in iron acquisition, including the siderophore receptor FetA in *N. gonorrhoeae* [256] and two hemoglobin receptors in *N. meningitides* [257] contribute to the bacterial evasion of immune system.

The innate immune response is assumed to be activated by pathogen-associated molecular patterns (PAMPs), for instance, lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycan (PGN) and lipotechoic acid (LTA) from Gram-positive bacteria, and glucans from fungal cells. However, it has been suggested that the innate immune response can also be stimulated by host signals commonly associated with infection, such as damage-associated molecular patters (DAMPs), for instance, DNA, ATP, uric acid, DNA binding proteins (hmgb1), alarmins or endogenous adjuvants. In fact, damage-induced responses play a significant role in the host response to pathogens, keeping in mind that the inflicted damage might in part by due to the host response rather than the pathogen. This self-inflicted damage may amplify the immune

response. Therefore, many pathogens appear to go to considerable lengths to avoid host damage [197]. For example, pathogens are able to manipulate downstream signaling pathways to prevent responses downstream of PAMP recognition [258] and several bacterial pathogens have efficient mechanisms for down-regulating flagellin expression within the host.

Over the last decade, many studies reported that the response to invading microorganisms is stimulated by recognition of PAMPs. Recently, Vance *et al.* [197] suggested that beside PAMPs hosts may recognize distinct pathogen-induced processes that contribute to the progression of disease. This recognition would provide the host different strategies for distinguishing a virulent organism from one that has lower disease-causing potential. The host could then escalate immune responses to a level commensurate with the attack being mounted. Based on these evidences, in the present study (Chapter 4) we investigated the mRNA expression level of a few important immune-related genes in order to verify the hypothesis that change in the production of virulence factors in *V. harveyi* by phenotypic variation plays a role in host's recognition, consequently modulating the host's immunity. Based on the result of a previous study (Chapter 3) four bacterial strains: luminescent and non-luminescent of the wild type BB120 (120L; 120NL) that were virulent towards *Artemia* and the triple mutant JMH634 (is the strain lacks three quorum sensing systems) and both the luminescent and non-luminescent variants of this strain that previously shown to cause no pathogenic effect on *Artemia* [49, 65]. In this study, we found that exposure with the virulent strain (BB120L) could significantly affect the expression level of most tested genes: *lgbp, dsacm, proPo, ferritin, mip, serpin* in comparison with that in the *Artemia* exposed to the non-virulent strain JMH634L (Table 4.3). As observed in the previous study (Chapter 3), the production of virulence factors such as caseinase, heamolysis, elastase, luminescent, DnaK content was significant different between the virulent bacterial strain (BB120L) and the non-virulent strain (JMH634L) (Table 5.3). In addition, the production of extracellular enzymes, for example, proteases, phospholipase has been considered as another pattern of pathogenesis that may liberate host ligands that are sensed by the innate immune system or produced cellular damage that trigger host responses [197]. Taking all together, we suggested that these pattern of pathogenesis might have the potential to allow the immune system to distinguish the pathogenic strain (BB120L) from the non-pathogenic strain (JMH634L).

In Gram-negative bacteria, LPS is the main constituent of the outer membrane. LPS consist of a lipid A moiety, a core of polysaccharide, and an O antigen. LPS variability among species and

serotypes occurs mainly in the O antigen, especially in the identity and number of sugars in the polysaccharide chain. LPS, also referred as endotoxin, is a powerful stimulant of the immune system due to the lipid A moiety, which is a pathogen-associated molecular pattern recognized specifically by toll-like receptor 4 (TLR4) [259]. The chemical identity of LPS is defined by the addition of side groups, for example as a result of the activity of glycosyltransferases or by the addition of phosphorylcholine (ChoP). These traits can vary within a clonal population as a result of phase variation of one or more enzymes involved in the modification [51]. For example, the pathogen *Porphyromonas gingivalis* can produce a variety of at least 12 different lipid A molecules, resulting in stimulatory, invisible or antagonistic varieties to TLR4 [260].

Phagocytosis plays a key role in the innate immune response to restrict the spread of infectious diseases. Microorganisms internalized by phagocytosis are sequestered in phagosomes, which are initially inapt at killing and degrading pathogens. These phagosomes acquire their microbicidal properties through a complex maturation process involving sequential fusion with endocytic organelles, leading to the formation of phagolysosomes [261]. Several bacteria have evolved specific strategies to adapt and survive in their hosts by avoiding the harsh environment of phagolysosomes [262, 263].

Like many other bacterial processes, intracellular replication is heterogeneous: infection of tissue-cultured host cells with a clonal population of bacteria frequently results in variable numbers of bacteria in each host cell. For example, study on intracellular trafficking undertaken by *Leptospira interrrogans* (pathogenic strain) and *L. biflexa* (non-pathogenic strain) in mouse bone marrow-derived macrophages showed that the delayed clearance of *L. interrogans* in murine macrophages was observed, indicating that *L. interrogans* has a capability to survive and replicate within these cells. Although bone marrow-derived macrophages could degrade *L. biflexa* as well as *L. interrogans*, a population of *L. interrogans* was able to survive and replicate. Furthermore, *L. biflexa* were completely degraded at 24 h post infection but *L. interrogans* were observed releasing in the extracellular medium. Results of this study suggested that pathogenic *L. interrogans* are able to survive, replicate and exit from mouse macrophages, enabling their eventually spread to target organs [264]. The heterogeneity of intracellular replication of bacterial pathogens might be caused by bacterial and host factors, as well as the variable intracellular microenvironments inhabited by these pathogens [265].

Similarly, during the infection of *Salmonella typhimurium* bacteria in mice, Saicedo *et al.* found that most bacteria were found in intracellular location 3 days after inoculation. Using a panel of antibodies that bound to cells of different lineages, the authors have shown that the vast majority of *S. typhimurium* bacteria reside within macrophages. They suggested that in *Salmonella* SPI-2 type III secretion system is required for replication within splenic macrophages, and sifA(-) and SPI-2 are involved in maintenance of the vacuolar membrane and intracellular replication *in vivo* [266].

A primary characteristic of many pathogens is the ability to deliver microbial molecules into the host cells cytosol by which bacteria can access, replicate in the cytosol and cause disease in the host. One of the best examples of this mechanism is the type III secretion system (T3SSs) that is encoded by numerous of Gram-negative bacterial pathogens [267].

T3SSs form needle-like pores through the inner and outer membranes of bacteria that deliver bacterial proteins, usually enzymes from the cytoplasm of bacteria directly into their target eukaryotic host cells. The secreted proteins are referred as "effectors". Once in the host cells, the effectors perform a variety of functions, including modifying host signaling and immune responses that contribute to pathogenesis [268]. *V. harveyi* possesses a T3SS and it is known to be regulated by quorum sensing [214]. The T3SS-secreted effector Vopz that is essential for *V. parahaemolyticus* pathogenicity. Vopz plays distinct, genetically separate roles in enabling intestinal colonization and cause disease [269]. Extracellular pathogens, such as *Yersinia* can utilize T3SSs to deliver effectors that block the uptake of bacteria into cells; conversely, intracellular pathogen such as *Samonella* can utilize T3SSs to enter cells and establish intracellular compartments that support replication [267]. It is worth noting that while the apparatus of the T3SSs itself is conserved across species, the effectors and their activities from each system differ widely in their mechanism of action. These effectors are typically potent proteins that mimic or capture an endogenous eukaryotic activity to disrupt the cellular response to infection [270]. Bacterial pathogens lacking T3SSs often encode evolutionarily unrelated systems that nevertheless fulfill the same basic function. Moreover, in the case of Gram-positive bacteria, some pore-forming toxins such as streptolysin O may serve as portals for the injection of bacterial molecules into the host cell cytosol (Figure 7.3). Some pore-forming toxins deliver not just a few effectors to the cytosol but are also involved in phagosome disruption, allowing an entire pathogen to access the cytosol. For example, in the case of *Listeria monocytogenes* a pore-

forming toxin named listeriolysin O, was required to escape the phagosome, replicate in the cytosol, and cause disease in hosts [271]. Therefore secretion or pore forming systems are sensed by host cells via detection of the physical damage associated with the outer membrane compositions of the bacterium penetrating the plasma or phagosomal membranes. Difference in the production of the virulence factors that was observed in the non-luminescent variants and its luminescent parental strain might cause distinct levels of physical damage when bacteria insert into host membranes. Upon damage, hosts may mount appropriate responses for distinguishing a virulent organism (BB120) from one that has lower disease-causing potential (JMH634). This might explain the different patterns of gene expression that were observed in most tested genes in the BB120L-exposed group versus the JMH634L-exposed group (Chapter 4, Table 4.4). Thus, host immune responses may be shaped not only in response to PAMPs but also in response to contextual cues for instance, pattern of pathogenesis such as adherence of pathogen to host cell, production of extracellular enzymes (such as proteases, phospholipases, elastase) provided by infected bacteria as part of their virulence strategy [197].

Based on this type of interpretation and conceptualization, it can be assumed that the switching from the L to NL variant, and differences in term of virulence factors production that were observed between the L and NL of the BB120 (Table 5.3) as a result from phase variation, may as well have an effect on host responses (Table 4.3 & 4.4).

This differential level of virulence factor expression might lead to a distinct host response via gene expression for instance. However, at 12 h post infection, the gene expression levels of most tested genes in the *Artemia* challenged with the non-luminescent variant (BB120NL) did not significantly differ in comparison with its luminescent strain (BB120L) (Table 4.4). This result might be subject to 2 different interpretations. The first being, that host responses at the transcription level happen mostly with the first 6 h upon exposure. Or alternatively, there is a possibility that the non-luminescent strain might switch back to the luminescent phenotype, upon initial contact with the host (as they seem to swim faster) or upon initial infection. Consistent with the idea that *in vivo* switching is possible, a recent study on differences on uptake and killing of pathogenic (*Vibrio)* and non-pathogenic (*E. coli*) by hemocyte subpopulations of penaeid shrimp was conducted by Tuan *et al.* [272]. The authors observed that both pathogenic and non-pathogenic bacteria were phagocytosed by penaeid shrimp hemocytes, however, only non-pathogenic bacteria were destroyed upon phagocytosis. When these bacteria were mixed with

shrimp hemocyte subpopulations and incubated for 180 min, the percentage of viable intracellular *V. campbellii* recovered was significantly higher than the percentage recovered from *V. harveyi* group treated in the same manner. Interestingly, and related to the idea of phenotype switching, during the uptake experiment the morphology of the majority of *V. campbellii* cells changed to rod shaped immediately after ingestion but converted into a coccal form after 2 h of incubation. Results of this study also indicated that these pathogenic bacteria: *V. campbellii* and *V. harveyi* most probably inhibited phagosome maturation and phagosome-lysosome fusion.

Host pathogenic bacteria could undergo physiological changes to adapt within the severe environment, and avoid host recognition, host cell damage as long as possible. Thus, host immune responses wil be evaded through PAMPs modification, resistance to antimicrobial peptides or escape from phagocyte responses [260, 272]. Alteration of these biological processes might be as a result of phenotypic variation or it is one of the mechanisms that are used by different phenotypic variants for surviving and cause disease.

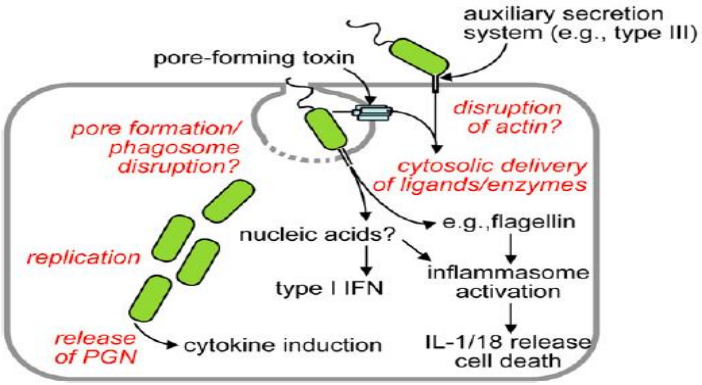

Figure 7.3 Pattern of pathogenesis – a pore-forming toxin as proposed for mammal (Source [197])

To survive and develop in a dynamic environment, invertebrates need to have an efficient defense mechanism to control the resident microflora and prevent invasive microbial disease. However, it appears that microbial organisms have coevolved with their host to overcome protective host barriers and innate host responses. Many bacterial pathogens avoid host recognition or suppress the subsequent immune activation through sophisticated interactions with the host responses, but some pathogens benefit from the stimulation of inflammatory reactions [273]. Pathogenic *Vibrio* spp. evade host defensive mechanism by using different strategies, for example, *Vibrio spp.* avoid the host antimicrobial peptides (AMPs) by releasing their outer membrane vesicles to

create a protective membranous shield. In *V. cholerae*, the resistance-nodulation-cell-division superfamily (RND) efflux pumps are used to transport AMPs out of their cytoplasmid space [220] or through surface expression of capsule polysaccharides *V. vulnificus* can avoid host phagocytosis by macrophages [223]. Moreover, pathogenic microbes must acquire essential nutrients, including iron, from the host in order to proliferate and cause infections. In *V. vulnificus* as a result of phase variation, the bacteria shifted their yellow cells from producing antibiotic and pigment production to producing components involved in acquisition of iron, which may increase fitness during periods of iron limitation. Another strategy that was used by pathogenic bacteria to evade the host immunity is their involvement in host's inhibitory immune pathways that are the ways of controlling over host immune responses, such as overamplification (of DAMPs) or inflammation. Some bacterial pathogens such as *Moraxella catarrhalis, Neisseria meningitidis* can take advantage of host inhibitory signaling by using the surface ligands to directly engage the immunoreceptor tyrosine-based inhibitory motif (ITIM)-bearing receptors, which they can co-ligate with activated PPRs (such as TLRs) to suppress cellular activation and increase bacterial survival [274].

Recently, many studies have shown that pathogenic bacteria evade host defense mechanisms during infection by limiting translation of host mRNAs that encode for proteins that mediate defense responses [193, 230]. Study of Fontana *et al.* [230] showed that upon infection of macrophages with virulent *Legionella pneumophila,* secretion of five effector proteins caused a global decrease in host translation. The *lgt1,2,3* genes encode related glucosyltransferases that modify eEF1A, si*dl* encodes a protein that binds to eEF1A and eEF1βγ, and *sidL* is toxic to mammalian cells and inhibits translation *in vitro* by an unknown mechanism. In case of *Pseudomonas entomophila* – a bacterial pathogen of *Drosophila,* to identify how the gut response to lethal and nonlethal bacterial pathogen differs, three Gram-negative bacterial strains: *P. entomophia* (lethal), *E. carotovora* (nonlethal, but pathogenic) and *P. entomophila gacA* mutant (avirulent) were investigated [193]. Results of this study showed that either injection with the virulent or avirulent bacterial strains induced an up-regulation of 142 genes involved in antimicrobial and oxidative stress in *Drosophila.* Although infection with both *P. entomophila* and *Ecc15* caused a high induction of *Dpt-lacZ* transcription (a marker fusion gene), the corresponding increase in LacZ activity was low in infected *P. entomophila* compared to *Ecc15*-

infected guts. In addition infection with *P. entomophila* also caused a protein inhibition of L-azidohomoalanine (AHA), a methionine analog upon infection.

Microbial infection impacts host translation in a variety of ways. Not only can infection modify the translational capacity of the host, but regulating mRNA translation can influence microbial pathogenesis by limiting or promoting translation of host mRNAs encoding the effector proteins that mediate innate responses. Damage to epithelia by bacteria can suppress host translation via cell signaling pathways, alerting the host to danger. By introducing the effector directly into the cytoplasm, bacteria can directly suppress host translation [275].

In the present study (Chapter 5), we aimed to determine whether luminescent *V. harveyi* inflict damage and cause disease in *Artemia* by interfering with the mRNA translational machineries of the host defense system. To this end, we exposed the germ-free *Artemia* to luminescent and non-luminescent wild type *V. harveyi* BB120 and its isogenic quorum sensing mutant JMH634 and then analyzed the transcription and translation profiles of heat shock protein 70 (Hsp70) and the high mobility group box 1 protein (Hmgb1) in *Artemia.*

Hsp70 and Hmgb1 proteins are associated with both repair and immune mechanisms to fight against pathogenic stressors [219, 235]. More specifically, Hsp70 and Hmgb1 proteins upon induction play key roles in defining the resistance of organisms against stressors [235] by performing multifaceted functions, such as acting as molecular chaperone for protein and DNA, respectively, functioning as danger associated molecular pattern (DAMP) during inflammation and various cellular processes [236, 237], and/or participating in the activation of cell surface innate immune receptors, thereby modulating many aspects of the host's immune responses [238, 239]. Results of this study (Chapter 5) showed that the Hsp70 protein level in *Artemia* exposed to luminescent variant of the wild type BB120 did not differ significantly from those exposed to either the non-luminescent variant of the wild type BB120 or to the (non-) luminescent variants of the mutant JMH634. Our findings were in line with the previous study on *P. entomophila* showing that Hsp70 proteins were not detectable in the gut of *Drosophila* infected with *P. entomophila*, although the Hsp70 gene was induced transcriptionally [193]. From these results, it appeared less likely that this virulence factor (luminescent) contributed to the inhibition of the hsp70 synthesis in BB120. However, at this point we also can not exclude the possibility that *V. harveyi* BB120 modulates the Hsp70 production level independent of their virulence factors.

In addition, a *Vibrio* infection significantly changed the Hmgb1 level in *Artemia* (Chapter 5). In response to exposure to BB120L, a significant decrease in the production of Hmgb1 protein was observed at 6 h and 12 h relative to the JMH634L while no significant difference in *Hmgb1* gene expression in both treatment groups was observed (Table 5.3). The latter is an indication for the fact that a virulent strain BB120L would be able to down-regulate protein production. Our results are in agreement with our earlier study that showed that in response to *V. campbellii* challenge, a significant reduction of hmgb1 was observed at the protein level but not at the mRNA level [219]. Probably, inhibition of host protein synthesis might be one of the virulence strategies of pathogenic bacteria to survive and cause disease after infection. This phenomenon has also been revealed in many recent studies [193, 231]. It could also be possible that upon the level of cellular damage inflicted by *V. harveyi* infection, the differences between nonlethal and lethal infections could be due to the severity of cellular damages determining different ranges of host responses [193].

Chakrabarti *et al* [193] also supposed that the rate of protein synthesis could act as direct sensor of pathogen-induced processes, modulating the immune response. Such sensor would reflect the global level of cellular stress and therefore act in response to any type of infectious damage, rather than recognizing a specific pathogen effector. Presumably, bacterial pathogens that disrupt host translation drive benefit from this activity, perhaps by increasing availability of amino acid nutrients or by dampening production of the host response. Therefore there is a need to have meticulous data on the dynamics of transcription and translation in the host, together with evidence for the infection process to determine the effect of pathogenic factors on the machineries of the host defense system.

In conclusion, pathogenic bacteria *V. harveyi* might alter the physiological processes, such as virulence factors production, and the bacteria–host interaction when they undergo the phenotypic variation by which bacteria might improve their persistence, or evade the host immune system, or affects host's response, and eventually influence on the survival of host (*Artemia)* (Figure 7.4). Evidence in relation to the ecological significance of the described phenotypic switch remains to be gathered, especially because no environmental condition has been pinpointed that facilitates a switch from a non-luminescent strains back to a luminescent strain.

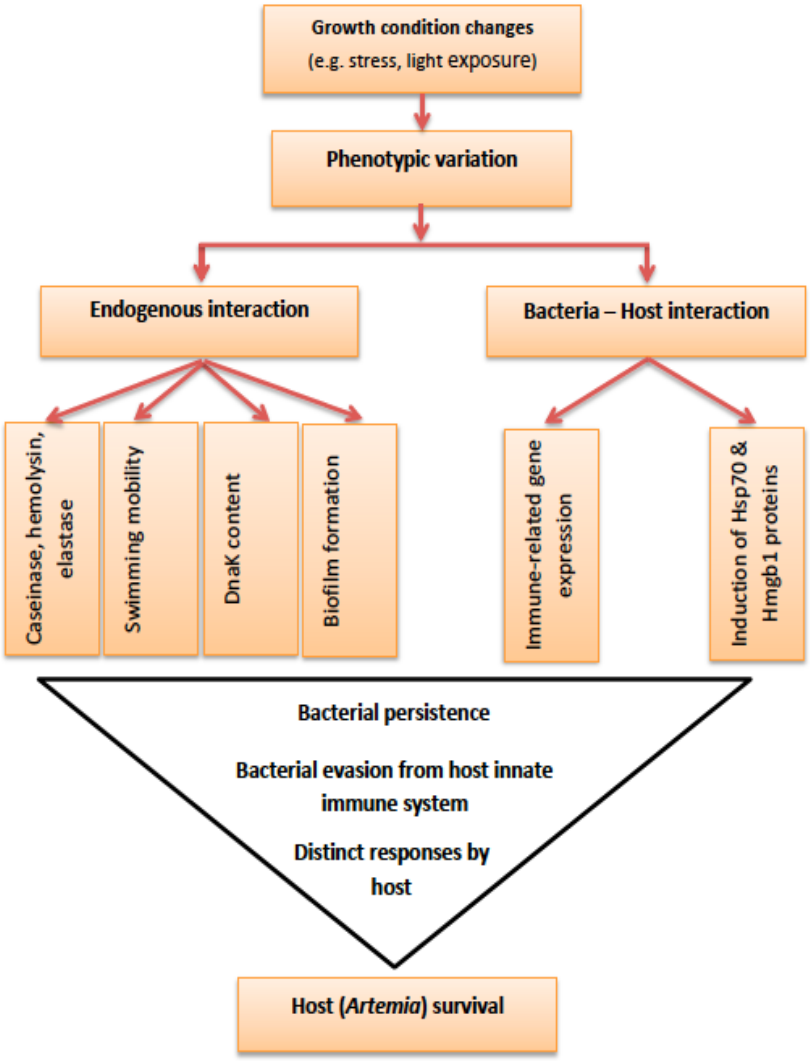

Figure 7.4 Detailed scheme of the thesis

Ability to switch to another phenotype of *V. harveyi* and its association in changing virulence factors *in vitro* and *in vivo* may attract attention to design therapeutic approaches and/or to develop improved vaccine for this or related pathogens in aquaculture.

7.3 Future perspectives

Based on the results obtained in this PhD study, the question can be raised whether the non-luminescent variant of the wild type *V. harveyi* can switch back to the luminescent variety once in the host. In the future, attempts could be made to reisolate the L variety from infected *Artemia*.

For that the difference in *in vitro* expression of virulence factors could be used. For instance one could verify for the reoccurrence of luminescent colonies or hemolytic colonies.

Variation of surface proteins and/or LPSs in many bacteria is important, resulting in antigenic changes. Thus, further study on genes and proteins variation due to phenotypic variation in *V. harveyi* may be one of the subjects for study.

Moreover, how the different mechanisms, environmental regulation, and the gene products each contribute to the success of the bacterial population largely remains to be determined. In addition, identification of environmental factors and regulatory networks that influence phenotypic variation in *V. harveyi,* will probably contribute to our understanding of the global changes that allow bacteria to adapt and survive in a changing environment.

Research on inducing the VBNC status in luminescent and non-luminescent variants in *V. harveyi* can be a subject for further study. This might allow gaining insight into how the L and NL status relates to the capacity to acquire the VBNC status.

A molecular method that can be used to verify the phenotypic status in *V. harveyi* and develop such method into a marker to test the status of newly isolated strains and/or verify the phenotypic status of strains in vibrio population present in aquaculture facilities could be of interest in the future.

In addition, antiphagocytosis is being used by many bacterial pathogens to avoid host recognition ensuring their survival and causing disease in human [276, 277] . For example, *Yersinia* species, which cause gastrointestinal symptoms and are responsible for plague, are among the better-studied antiphagocytic pathogens. After ingestion by the host, *Yersinia* translocates several effectors into the host cells that mediate antiphagocytosis. The first antiphagocytic effector described was the protein tyrosine phosphatase (PTP) YopH [276]. YopH is localized to focal complexes during phagocytosis where its carboxy-terminal tyrosine-phosphatase activity specifically targets and dephosphorylates host cell proteins [278]. YopE is another *Yersinia* effector implicated in the avoidance of phagocytosis by disrupting actin filaments that are essential for phagocytosis [279]. In *V. harveyi,* T3SS genes have been identified and their expression is regulated by quorum sensing system [214]. However, the functionality of T3SS genes, for instance in relation to effectors probably being translocated into the host by this system and their involvement in antiphagocytosis during infection has not studied yet in *V. harveyi.*

Further research on other immune-related genes at transcriptional level and translational level is recommended. With the *Artemia* genome now being available it will become possible to clone and express the cDNA of much more immune related genes. This will allow in turn to generate antibodies, which can be used to verify the production of immune-related proteins.

Result of *ArtHmgb1* gene isolation and *Art*Hmgb1 recombinant protein can be used to facilitate the study on the functionality of this *hmgb1* gene in *Artemia*. For instance it can allow to verify if feeding hmgb1, through *E. coli* cells overproducing them, can contribute to the protection of *Artemia* against *Vibrio*, as has been established by feeding *E. coli* overproducing Hsp70.

Appendices

A- REFERENCES

B- SUMMARY/SAMENVATTING

C- ACKNOWLEDGEMENTS

D- CURRICULUM VITAE

APPENDICES

A

REFERENCE

1. FAO. The State of World Fisheries and Aquaculture. FAO Fisheries Department Foodand Agriculture Organization of the United Nation, Rome, Italy. 2014.
2. Sorgeloos P. Aquaculture: The blue biotechnology of the future. World Aquaculture This article based on the plenary talk "Resources, technologies and services for future aquaculture: a need assessment for sustainable development" by the author at the FAO Global on Aquaculture (September 2010, Phuket, Thailand). 2013.
3. Bondad-Reantaso MG, Subasinghe RP, Arthur JR, Ogawa K, Chinabut S, Adlard R, et al. Disease and health management in Asia aquaculture. Vet Parasitol. 2005 132:249-72.
4. Haldar S, Chatterjee S. Vibrio related diseases in aquaculture and development of rapid and accurate identification methods. Journal of Marine Science: Research & Development. 2012 2013.
5. Adams D, Boopathy R. Use of formic acid to control vibriosis in shrimp aquaculture. Section Cellular and Molecular Biology. 2013 68:1017-21.
6. Lee CT, Chen IT, Yang YT, Ko TP, Huang YT, Huang JY, et al. The opportunistic marine pathogen *Vibrio parahaemolyticu*s becomes virulent by acquiring a plasmid that expresses a deadly toxin. Proceedings of the National Academy of Sciences. 2015 112:10798-803.
7. Austin B, Zhang XH. *Vibrio harveyi:* a significant pathogen of marine vertebrates and invertebrates. Lett Appl Microbiol. 2006 43:119-24.
8. Skaar EP. The Battle for Iron between Bacterial Pathogens and Their Vertebrate Hosts. PLoS pathogens. 2010 6(8). p.e1000949.
9. Ruwandeepika HAD, Jayaweera TSP, Bhowmick PP, Karunasagar I, Bossier P, Defoirdt T. Pathogenesis, virulence factors and virulence regulation of vibrios belonging to the *Harveyi* clade. Rev Aquacult. 2012 4:497-502.
10. Defoirdt T. Virulence mechanisms of bacterial aquaculture pathogens and antivirulence therapy for aquaculture. Reviews in Aquaculture. 2014 6:100-14.
11. Natrah FMI, Ruwandeepika HDA, Pawar S, Karunasagar I, Sorgeloos P, Bossier P, et al. Regulation of virulence factors by quorum sensing in *Vibrio harveyi*. Vet Microbiol. 2011 154:124-9.
12. Defoirdt T, Boon N, Sorgeloos P, Verstraete W, Bossier P. Quorum sensing and quorum quenching in *Vibrio harveyi*: lessons learned from *in vivo* work. ISME journal. 2008 2:19-26.
13. Yang Q, Defoirdt T. Quorum sensing positively regulates flagellar motility in pathogenic *Vibrio harveyi*. Environ Microbiol. 2014 17:960-8.

14. Defoirdt T, Sorgeloos P. Monitoring of *Vibrio harveyi* quorum sensing activity in real time during infection of brine shrimp larvae. The ISME journal. 2012 6:2314-9.
15. Pande GS, Natrah FM, Sorgeloos P, Bossier P, Defoirdt T. The V*ibrio campbellii* quorum sensing signals have a different impact on virulence of the bacterium towards different crustacean hosts. Vet Microbiol. 2013 167:540-5.
16. Henke JM, Bassler BL. Three parallel quorum-sensing systems regulate gene expression in Vibrio harveyi. Journal of bacteriology. 2004 186:6902-14.
17. Tu KC, Bassler BL. Multiple small RNAs act additively to integrate sensory information and control quo- rum sensing in *Vibrio harveyi.* Genes & development. 2007 21:221-33.
18. Dubnau D, Losick R. Bistability in bacteria. Mol Microbiol. 2006 61:564-72.
19. Smits KW, Veening WJ, Kuipers PO. Phenotypic variation and bistable switching in bacteria. Bacterial Physiology: A Molecular Approach. 2008:339-65.
20. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. Bacterial persistence as a phenotypic switch. Science. 2004 305:1622-5.
21. Broek DVD, Bloemberg GV, Lugtenberg B. The role of phenotypic variation in rhizosphere *Pseudomonas* bacteria. Environ Microbiol. 2005 7:1686-97.
22. Guerrero A, Jain N, Goldman DL, Fries BC. Phenotypic switching in *Cryptococcus neoformans.* Microbiology. 2006 152:3-9.
23. Rossignol G, Sperandio D, Guerillon J, Duclairoir PC, Soum-Soutera E, Orange N, et al. Phenotypic variation in the *Pseudomonas fluorescens* clinical strain MFN1032. Res Microbiol. 2009 160:337-44.
24. Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. *Pseudomonas aeruginosa* Displays Multiple Phenotypes during Development as a Biofilm. J Bacteriol. 2002 184:1140-54.
25. Dehio C, Gray-Owen SD, Meyer TF. The role of neisserial Opa proteins in interactions with host cells. Trends in microbiology. 1998 6:489-95.
26. Sausa AM, Machado I, Pereira MO. Phenotypic switching an opportunity to bacteria thrive. In Méndez-Vilas, A (ed): Science Against Microbial Pathogens: Communicating Current Research and Technological Advances. 2012:252-62.
27. van der Woude MW. Re-examining the role and random nature of phase variation. FEMS Microbiol Lett. 2006 254:190-7.
28. Hilton T, Rosche T, Froelich B, Smith B, Oliver J. Capsular polysaccharide phase variation in *Vibrio vulnificus.* Appl Environ Microbiol. 2006 72:6986-93.
29. van der Woude MW. Some Types of Bacterial Phase Variation Are Epigenetic. Microbe. 2008 3:21-6.
30. Nelson LK, Stanton MM, Elphinstone RE, Helwerda J, Turner RJ, Ceri H. Phenotypic diversification *in vivo*: *Pseudomonas aeruginosa* gacS- strains generate small colony variants *in vivo t*hat are distinct from *in vitro* variants. Microbiology. 2010 156:3699-709.
31. Matz C, McDougald D, Moreno AM, Yung PY, Yildiz FH, Kjelleberg S. Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. Proc Natl Acad Sci USA. 2005 102:16819-24.
32. Srivastava M, Tucker MS, Gulig PA, Wright AC. Phage variation, capsular polysaccharide, pilus and flagella contribute to uptake of *Vibrio vulnificus* by the Eastern oyster (*Crassostrea virginica*). Environ Microbiol. 2009 11:1934-44.

33. Chabeaud P, de Groot A, Bitter W, Tommassen J, Heulin T, Achouak W. Phase-variable expression of an operon encoding extracellular alkaline protease, a serine protease homolog, and lipase in Pseudomonas brassicacearum. J Bacteriol. 2001 183:2117-20.
34. Chancey ST, Wood DW, Pierson EA, Pierson LS. Survival of GacS/GacA Mutants of the Biological Control Bacterium *Pseudomonas aureofaciens* 30-84 in the Wheat *Rhizosphere*. App Environ Microbiol. 2002 68:3308-14.
35. Grau BL, Henk MC, Pettis GS. High-frequency phase variation of *Vibrio vulnificus* 1003: isolation and characterization of a rugose phenotypic variant. J Bacteriol. 2005 187:2519-25.
36. Henderson IR, Owen P, Nataro JP. Molecular switches - the ON and OFF of bacterial phase variation. Mol Microbiol. 1999 33:919-32.
37. Dybvig K. DNA rearrangement and phenotyic switching in prokaryotes. Mol Microbiol. 1993 10:465-71.
38. Be'er A, Florin EL, Fisher CR, Swinney HL, Payne SM. Surviving baterial sibling rivalry: inducible and reversible phenotypic swiching in *Paenibacillus dendritiformis*. Microbiology. 2011 2:1-6.
39. Han B, Pain A, Johnstone K. Spontaneous duplication of a 661 bp element within a two-component sensor regulator gene causes phenotypic switching in colonies of *Pseudomonas tolaasii,* cause of brown blotch disease of mushrooms. Mol Microbiol. 1997 25.
40. Chabeaud P, de Groot A, Bitter W, Tommassen J, Heulin T, Achouak W. Phase-variable expression of an operon encoding extracellular alkaline protease, a serine protease homolog, and lipase in *Pseudomonas brassicacearum.* J Bacteriol. 2001 183:2117-20.
41. Deziel E, Comeau Y, Villemur R. Initiation of Biofilm Formation by *Pseudomonas aeruginosa* 57RP Correlates with Emergence of Hyperpiliated and Highly Adherent Phenotypic Variants Deficient in Swimming, Swarming, and Twitching Motilities. J Bacteriol. 2001 183:1195-04.
42. Dekkers LC, Phoelich CC, van der Fits L, Lugtenberg BJ. A site-specific recombinase is required for competitive root colonization by *Pseudomonas fluorescens* WCS365. Proc Natl Acad Sci USA. 1998 95:7051-6.
43. Segura A, Hurtado A, Duque E, Ramos JL. Transcriptional phase variation at the *flhB* gene of *Pseudomonas putida* DOT-T1E is involved in response to environmental changes and suggests the participation of the flagellar export system in solvent tolerance. J Bacteriol. 2004 186:1905-9.
44. Weiser JN, Goldberg JB, Pan N, Wilson L, Virji M. The phosphorylcholine epitope undergoes phase variation on a 43-kilodalton protein in *Pseudomonas aeruginosa* and on pili of *Neisseria meningitidis* and *Neisseria gonorrhoeae.* Infect Immunol. 1998 66:4263-7.
45. Tuchscherr L, Medina E, Hussaun M, Volker W, Heitmnn V, Niemann S, et al. *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol Med. 2010 3:129-41.
46. Nassif X, Lowy J, Stenberg P, O'Gaora P, Ganji A, So M. Antigenic variation of pilin regulates adhesion of *Neisseria meningitidis* to human epithelial cells. Mol Microbiol. 1993 8:719-25.
47. Zierdt CH, Schmidt PJ. Dissociation in *Pseudomonas aeruginosa.* J Bacteriol. 1964 87:1003-10.

48. Proctor RA, von Eiff C, Kahl BC, Becker M, McNamara P, Herrmann M, et al. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol. 2006 4:295-305.
49. Phuoc LH, Defoirdt T, Sorgeloos P, Bossier P. Virulence of luminescent and non-luminescent isogenic vibrios towards gnotobiotic *Artemia franciscana* larvae and specific pathogen-free *Litopenaeus vannamei* shrimp. J Appl Microbiol. 2009 106:1388-96.
50. Ziebuhe W, Krimmer V, Rachid S, Lossner I, Gotz F, Hacker J. A novel mechanism of phase variation of virulence in *Staphylococcus epidermidis:* evidence for control of the polysaccharide intercellular adhesin synthesis by alternating insertion and excision of the insertion sequence element. Mol Microbiol. 1999 32:345-433.
51. van der Woude MW, Bäumler AJ. Phase and Antigenic Variation in Bacteria Clin Microbiol Rev. 2004 17:581-611.
52. Swartley JS, Marfin AA, Edupuganti S, Liu LJ, Cieslak P, Perkins B, et al. Capsule switching of *Neisseria meningitidis*. Microbiology. 1997 94:271-6.
53. Lewis K. Persister cells. Annu Rev Microbiol. 2010 64:357-72.
54. Kussell E, Kishony R, Balaban NQ, Leibler S. Bacterial persistence: A model of survival in changing environments. Genetics. 2005 169:1807-14.
55. McDougald D, Rice S, Barraud N, Steinberg PD, Kjelleberg S. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Nat Rev Microbiol. 2012 10:39-50.
56. Dunne WM. Bacterial Adhesion: Seen Any Good Biofilms Lately? Clin Microbiol Rev. 2002 15:155-66.
57. Davies D. Understanding biofilm resistance to antibacterial agents. Nature reviews Drug discovery. 2003 2:114-22.
58. Drenkard E, Ausubel FM. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature. 2002 416:740-3.
59. Lewis K. Persister cells, dormancy and infectious disease. Nat Rev Microbiol. 2007 5:48-56.
60. Karunasagar I, Pai R, Malathi GR, Karunasagar I. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. Aquaculture. 1994 128:203-9.
61. Deitsch KW, Moxon ER, Wellems T. Shared Themes of Antigenic Variation and Virulence in Bacterial, Protozoal, and Fungal Infections. Microbiol Mol Biol Rev. 1997 61:281-93.
62. Kim JO, Weiser JN. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae.* J Infect Dis. 1998 177:368-77.
63. Tannaes T, Grav HJ, Bukholm G. Lipid profiles of Helicobacter pylori colony variants. APMIS. 2000 108:349-56.
64. Phuoc LH, Defoirdt T, Sorgeloos P, Bossier P. Virulence of luminescent and non-luminescent isogenic vibrios towards gnotobiotic *Artemia franciscana* larvae and specific pathogen-free *Litopenaeus vannamei* shrimp. Journal of Applied Microbiology. 2009 106:1388-96.
65. Hong NTX, Baruah K, Vanrompay D, Bossier P. Characterization of phenotype variations of luminescent and non-luminescent variants of *Vibrio harveyi* wild type and quorum sensing mutants. J Fish Dis. 2015.

66. Häse CC, Finkelstein RA. Bacterial Extracellular Zinc-Containing Metalloproteases. Microbiol Rev. 1993 57:823-37.
67. Won KM, Park SI. Pathogenicity of *Vibrio harveyi* to cultured marine fishes in Korea. Aquaculture. 2008 285:8-13.
68. Zhang WW, Sun K, Cheng S, Sun L. Characterization of DegQVh, a serine protease and a protective immunogen from a pathogenic *Vibrio harveyi* strain. App Environ Microbiol. 2008 74:6254-62.
69. Ruwandeepika HAD, Defoirdt T, Bhowmick PP, Karunasagar I, Bossier P. Expression of virulence genes in luminescent and nonluminescent isogenic vibrios and virulence towards gnotobiotic brine shrimp (*Artemia franciscana*). J App Microbiol. 2010 110:399-406.
70. Zhou J, Fang W, Yang X, Zhou S, Hu L, Li X, et al. A nonluminescent and highly virulent *Vibrio harveyi* strain is associated with "bacterial white tail disease" of *Litopenaeus vannamei* shrimp. PloS one. 2012 7(2) p.e29961.
71. Oliver JD. Recent findings on the viable but nonculture able state in pathogenic bacteria. FEMS Microbiol Rev. 2010 34:415-25.
72. Ayrapetyan M, Oliver JD. The viabe but nonculturable state and its relevance in food safety. Current Opinion in Food Science. 2016 8:127-33.
73. Wong HC, Wang P. Induction of viable but nonculturable state in *Vibrio parahaemolyticus* ans its susceptibility to environmental stresses. J App Microbiol. 2004 96:359-66.
74. Wong HC, Wang P. Induction of viable but nonculturable state in Vibrio parahaemolyticus and its susceptibility to environmental stresses. Journal of Applied Microbiology. 2004 96:359-66.
75. Ramamurthy T, Ghosh A, Pazhani GP, Shinodo S. Current perspectives on viable but nonculture able (VBNC) pathogenic bacteria. Front Public Health. 2014 2:1-9.
76. Chaiyanan S, Chaiyanan S, Huq A, Maugel T, Colwell RR. Viability of the nonculture able *Vibrio cholerae* 01 and 0139. Syst Appl Microbiol. 2001 24:331-41.
77. Ramaiah N, Ravel J, Straube WT, Hill RT, Cowell RR. Entry of *Vibrio harveyi* and *Vibrio fishcheri* into the viable but nonculture able state. J Appl Microbiol. 2002 93:108-16.
78. Lovett ST, Feschenko VV. Stabilization of diverged tandem repeats by mismatch repair: evidence for deletion formation via a misaligned replication intermediate. Proc Natl Acad Sci USA. 1996 14:7120-4.
79. Sarkari J, Pandit N, Moxon ER, Achtman M. Variable expression of the Opc outer membrane protein in *Neisseria meningitidis* is caused by size variation of a promoter containing poly-cytidine. Mol Microbiol. 1994 13:207-17.
80. Borst P. Mechanism of antigenic variation: an overview. pp 1-15 In: Antigenic variation. 2003. Craig, A and Scherf, A (eds). London, UK: Elsevier Academic Press. pp. 1-15

81. Wang G, Rasko DA, Sherburne R, Taylor DE. Molecular genetic basis for the variable expression of Lewis Y antigen in *Helicobacter pylori:* analysis of the alpha (1,2) fucosyltransferase gene. Mol Microbiol. 1999 31:1265-74.
82. Hood DW, Moxon ER. *Haemophilus influenzae.* In: Antigenic variation. 2003. Craig, A, and Scherf, A (eds). London, UK: Elsevier Academic Press. pp. 102-41.

83. Jonsson AB, Nyberg G, Normark S. Phase variation of gonococcal pili by frameshift mutation in *pilC,* a novel gene for pilus assembly. EMBO Journal. 1991 10:477-88.
84. Hammerschmidt S, Muller A, Sillmann H, Muhlenhoff M, Borrow R, Fox A. Capsule phase variation in *Neisseria meningitidis* serogroup B by slipped-strand mispairing in the polysialyltransferase gene (siaD): correlation with bacterial invasion and the outbreak of meningococcal disease. Mol Microbiol. 1996 20 1211-20.
85. Stern A, Brown M, Nickel P, Meyer TF. Opacity genes in *Neisseria gonorrhoeae:* control of phase and antigenic variation. Cell. 1986 47:61-71.
86. Stibitz S, Aaronson W, Monack D, Falkow S. Phase variation in *Borderella pertussis* by frameshift mutation in a gene for a novel two-component system. Nature. 1989 338:266-9.
87. Inzana TJ, Hensley J, McQuiston J, Lesse AJ, Campafnari AA, Boyle SM, et al. Phase variation and conservation of lipooligosaccharide epitopes in *Haemophilus somnus.* Infect Immunol. 1997 65:4675-81.
88. Thesis P, Wise KS. Localized frameshift mutation generates selective, high-frequency phase variation of a surface lipoprotein encoded by a mycoplasma ABC transporter operon. J Bacteriol. 1997 179:4013-22.
89. Carroll PA, Tashima KT, Rogers MB, DiRita VJ, Calderwood SB. Phase variation in *tcpH* modulates expression of the ToxR regulon in *Vibrio cholerae*. Mol Microbiol. 1997 25:1099-111.
90. Chen CJ, Elkins C, Sparling PF. Phase variation of hemoglobin itilization in *Neisseria gonorrhoeae*. Infect Immunol. 1998 66:987-93.
91. Lewis LA, Gipson M, Hartman K, Ownbey T, Vaughn J, Dyer DW. Phase variation of HpuAB and HmbR, two distinct haemoglobin receptors of *Neisseria meningitidis* DNM2. Mol Microbiol. 1999 32:977-89.
92. Abraham J, M., Freitag CS, Clements JR, Eisenstein BI. An invertible element of DNA controls phase variation of type 1 fimbriae of *Escherichia coli.* Proc Natl Acad Sci USA. 1985 82:5724-7.
93. K. ME, Good RT, Sinistaj M, Billington SJ, J. lC, Rood JI. A multiple site-specific DNA inversion model for the control of Omp1 phase and antigenic variation in *Dichelobacter nodosus*. Mol Microbiol. 1995 17:183-96.
94. Lysnyansky I, Sachse K, Rosenbush R, Levisohn S, Yogev D. The *vsp* locus of *Mycoplasma bovis:* gene organization and structural features. Journal of Bacteriology. 1999 181:5734-41.
95. Dybvig K, Yu H. Regulation of a restriction and modification system via DNA inversion in *Mycoplasma pulmonis*. Mol Microbiol. 1994 12:547-60.
96. Zieg J, Silverman M, Hilmen M, Simon M. Recombinational switch for gene expression. Science. 1977 196:170-2.
97. Marrs CF, Rozsa FW, Hackel M, Stevens SP, Glasgow AC. Identification, cloning and sequencing of *piv,* a new gene involved in inverting the pilin genes of *Moraxella lacunata.* J Bacteriol. 1990 172:4370-7.
98. Haas R, Meyer TF. The repertoire of silent pillus genes in *Neisseria gonorrhoeae:* evidence for gene conversion. Cell. 1992 44:107-15.
99. Agur Z, Abiri D, Van der Ploeg H. Oderred apparance of antigenic variants of Afican trypanosomes explained in a mathematical model based on a stochastic switch process and

immune-selection against putative switch intermediates. Proc Natl Acad Sci USA. 1989 86:9626-30.
100. Barbour AG. Antigenic variation in Borrelia: relapsing fever and lyme borreliosis. In: Antigenic variation. Craig, A, and Scherf, A (eds). London, UK: Elsevier Academic Press. pp. 319-56.
101. Waite RD, Penfold DW, Struthers JK, Dowson CG. Spontaneous sequence duplications within capsule genes *cap8E* and *tts* control phase variation in *Streptococcus pneumoniae* serotypes 8 and 37. Microbiology. 2003 149:497-504.
102. Owen P, Meehan M, Loughry-Doherty H, Henderson I. Phase-variable outer membrane proteinsin *Escherichia coli.* FEMS Immunol Med Microbiol. 1996 16:63-76.
103. Blyn LN, Braaten BA, Low DA. Regulation of *pap* pili phase variation by a mechanism involving differential *dam* methylation states. EMBO Journal. 1990 9:4045-54.
104. Nicholson B, Low D. DNA methylation-dependent regulation of *per* expression in *Salmonella typhimurium*. Mol Microbiol. 2000 35.
105. Monack DM, Arico B, Rappuoli R, Falkow S. Phase variation of *Bodetella bronchiseptica* arise by spontaneous deletions in the *vir* locus. Mol Microbiol. 1989 3:1719-28.
106. Sanchez-Contreras M, Martin M, Villacieros M, O'Gara F, Bonilla I, Rivilla R. Phenotypic selection and phase variation occur during alfalfa root colonization by *Pseudomonas fluorescens* F113. J Bacteriol. 2001 184:1587-96.
107. Vial L, Pothier JF, Normand P, Moenne-Loccuz Y, Bally R, Wisniewski-Dye F. Construction of a *recA* mutant of *Asospirillum lipoferum* and involvement of *recA* in phase variation. FEMS Microbiol Lett. 2004 236:291-9.
108. Seifert HS. Questions about gonococcal pilus phase-and antigenic variation. Mol Microbiol. 1996 21:433-40.
109. Haas R, Veit S, Meyer TF. Silent pilin genes of *Neisseria gonorrhoeae* MS11 and the occurence of related hypervariant sequences among other gonococcal isolates. Mol Microbiol. 1992 6:197-208.
110. Woude MVD, Braaten B, Low D. Epigenetic phase variation of the pap operon in *Escherichia coli.* Trends Microbiol. 1996 4:5-9.
111. Rosa RD, Barracco MA. Antimicrobial peptides in crustaceans. Information System Journal. 2010 7:262-84.
112. Tassanakajon A, Somboonwiwat K, Supungul P, Tang S. Discovery of immune molecules and their crucial functions in shrimp immunity. Fish Shellfish Immunol. 2013 34:954-67.
113. Christophides GK, Vlachou D, Kafatos FC. Comparative and finctional genomics of the innate immune system in the malaria vector *Anopheles gambiae.* Immunol Rev. 2004 198:127-48.
114. Jensen S, Thomsen A, R. Sensing of RNA viruses - a review on innate immune receptors involved in recognizing RNA virus invasion. J Virol. 2012 86:2900-10.
115. Wang XW, Wang JX. Pattern recognition receptors acting in innate immune system of shrimp against pathogen infections. Fish Shellfish Immunol. 2013 34:981-9.
116. Hauton C. The scope of the crustacean immune system for disease control. J Invertbr Pathol. 2012 110:251-60.

117. Brites D, McTaggart S, Morris K, Anderson J, Thomas K, Colson I, et al. The Dscam homologue of the crustacean *Daphnia* is diversified by alternative splicing like in insects. Methods Enzymol. 2008 25:1429-39.
118. Watthanasurorot A, Jiravanichpaisal P, Liu H, Söderhäll I, Söderhäll K. Bacterial-induced Dscam isoforms of the crustacean, *Pacifastacus leniusculus.* PLoS pathogens. 2011 7(6) p.e1002062.
119. Yassine H, Osta MA. *Anopheles gambiae* innate immunity. Cell Microbiol. 2010 12:1-9.
120. Lee SY, Wang R, Söderhäll K. A lipopolysaccharide- and beta-1,3-glucan-binding protein from hemocytes of the freshwater crayfish *Pacifastacus leniusculus.* Purification, characterization, and cDNA cloning. J Biol Chem. 2000 275:1337-43.
121. Amparyup P, Sutthangkul J, Charoensapsri W, Tassanakajon A. Pattern Recognition Protein Binds to Lipopolysaccharide and β-1,3-Glucan and Activates Shrimp Prophenoloxiadase System. J Biol Chem. 2012 287:10060-9.
122. Harris H, E., Anderson U, Pisetsky D, S. HMGB1: a mulitfunctional alarmin driving autoimmune and inflammatory disease. . Nat Rev Rhe. 2012 8:195-202.
123. Baruah K, Norouzitallab P, Roberts RJ, Sorgeloos P, Bossier P. A novel heat-shock protein inducer trigger heat shock protein 70 production and protects *Artemia franciscana* nauplii against abiotic stressors. Aquaculture. 2012 334-337:152-8.
124. Sung YY, Macrae TH, Sorgeloos P, Bossier P. Stress response for disease control in aquaculture. Rev Aquacult. 2011 3:120-37.
125. Cerenius L, Söderhäll K. The prophenoloxidase-activatingsystem in invertebrates. Immunol Rev. 2004 198:116-26.
126. Söderhäll K, Cerenius L. Role of the prophenoloxidase-activating system in invertebrate immunity. Curr Opin Immunol. 1998:23-8.
127. Cerenius L, Lee BL, Söderhäll K. The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol. 2008 29:263-71.
128. Amparyup P, Charoensapsri W, Tassanakajon A. Prophenoloxidase system and its role in shrimp immune responses against major pathogens. Fish Shellfish Immunol. 2013 34:990-1001.
129. Liu H, Jiravanichpaisal P, Cerenius L, Lee BL, Söderhäll I, Söderhäll K. Phenoloxidase is an important component of the defense against *Aeromonas hydrophila* Infection in a crustacean, *Pacifastacus leniusculus*. J Biol Chem. 2007 282:33593-8.
130. Mavrouli M, D., Tsakas S, Theodorou G, L., Lampropoulou M, Marmaras V, J. MAP kinase mediate phagocytosis and melanization via prophenoloxidase activation in medfly hemocytes. Biochem Biophys Acta. 2005 1744:145-56.
131. Agianian B, Lesch C, Loseva O, Dushay MS. Preliminary characterization of hemolymph coagulation in *Anppheles gambiae* larvae. Dev Comp Immunol. 2007 31:879-88.
132. Johansson MW, Söderhäll K. Isolation and Purification of a Cell Adhesion Factor from Crayfish Blood Cells. JCB. 1988 106:1795-803.
133. Johansson MW, Lind MI, Holmblad T, Thörnqvist PO, Söderhäll K. Peroxinectin, a novel cell adhesion protein from crayfsh blood. Biochem Biophys Res Commun. 1995 216:1079-87.
134. Holmblad T, Söderhäll K. Cell adhesion molecules and antioxidative enzymes in a crustacean, possible role in immunity. Aquaculture. 1999 172:111-23.

135. Sivakamavalli J, Selvaraj C, Singh SK, Vaseeharan B. In vitro and in silicon studies on cell adhesion protein peroxinectin from *Fenneropenaeus indicus* and screening of heme blockers against activity. J Mol Recogn. 2016 29:186-98.
136. Gillespie J, Kanost MR, Trenczek T. Biological mediator of insect immunity. Annu Rev Entomol. 1997 42:611-43.
137. Zhao M, Söderhäll I, Park JW, Ma YG, Osaki T, Ha NC, et al. A novel 43-kDa protein as a negative regulatory component of phenoloxidase-induced melanin synthesis. J Biol Chem. 2005 280:24744-51.
138. Angthong P, Watthanasurorot A, Klinbunga S, Ruangdej U, Söderhäll I, Jiravanichpaisal P. Cloning and characterization of a melanization inhibition protein (PmMIP) of the black tiger shrimp, *Penaeus monodon*. Fish Shellfish Immunol. 2010 29:464-8.
139. Bulet P, Stöcklin R, Menin L. Anti-microbial peptides: from invertebrates to vertebrates. Immunol Rev. 2004 198:169-84.
140. Yount NY, Bayer AS, Xiong YQ, Yeaman MR. Advances in antimicrobial peptide immunobiology. Biopolymer. 2006 84.
141. Nicolas P. Multifunctional host defense peptides: intercellular-targeting antimicrobial peptides. FEBS Journal. 2009 276:6483-96.
142. Jiravanichpaisal P, Lee SY, Kim YA, Andren T, Söderhäll I. Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Pacifastacus leniusculus:* characterization and expression pattern. Dev Comp Immunol. 2007 31:441-55.
143. Tassanakajon A, Amparyup P, Somboonwiwat K, Supugul P. Cationic antimicrobial peptides in penaeid shrimp. Mar Biotechnol. 2011 13:639-57.
144. Somboonwiwat K, Bachere E, Rimphanichayakit V, Tassanakajon A. Localization of anti-lipopolysaccharide factor (ALF*Pm3)* in tissues of the black tiger shrimp, *Penaeus monodon,* and characterization of its binding properties. Dev Comp Immunol. 2008 32:1170-6.
145. Jaree P, Tassanakajon A, Somboonwiwat K. Effect of the anti-lipoplolysaccharide factor isoform 3 (ALF*Pm3*) from *Penaeus monodon* on *Vibrio harveyi* cells. Dev Comp Immunol. 2012 38:554-60.
146. Kawabata SI, Muta T, Iwanaga S. The clotting cascade and defense molecules found in the hemolymph of the horseshoe crab. In: New Dirrection in Invertebrate Immunology. Söderhäll, K, Iwanaga, S, Vasta, G, R (Eds). SOS Publications, Fair Haven. 1996. pp.255-83.
147. Hall M, Wang R, Antwerpen RK, Sottrup-Jensen L, Söderhäll K. The crayfish plasma clotting: a vitellogenin-related protein resposible for clot formation in crustacean blood. Proc Nati Acad Sci USA. 1999 96:1965-70.
148. Manigas MB, Kondo H, Hirono I, Saito-Taki T, Aoki T. Essential function of transglutaminase and clotting protein. Mol Immunol. 2008 45:1269-75.
149. Maningas MB, Kondo H, Hirono I. Molecular mechanisms of the shrimp clotting system. Fish Shellfish Immunol. 2013 34:968-72.
150. Jiravanichpaisal P, Lee BL, Söderhäll K. Cel-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology. 2006 211.
151. Dantas-Lima JJ, Tuan VV, Corteel M, Grauwet K, An NTT, Sorgeloos P, et al. Separation of *Penaeus vannamei* haemocyte subpopulations by iodixanol density gradient centrifugation. Aquaculture. 2013 408-409:128-35.

152. Söderhäll K, Cerenius L. Crustacean immunity. Annu Rev Fish Dis. 1992:3-23.
153. Yamada Y, Doi T, Hamakubo T, Kodama T. Scavenger receptor family proteins: roles for atherosclerosis, host defence and disorders of the central nerous system. Cell Mol Life Sci. 1998 54:628-40.
154. Kurucz E, Markus R, Zsamboki J, Folkl-Medzihradszky K, Darula Z, Lukacsovich T, et al. Nimrod, a putative phagocytosis receptor with EGF repeats in *Drosophila* plasmatocytes. Curr Biol. 2007 17:649-54.
155. Watson FL, Puttmann-Holgado R, Thomas F, Lammar DL, Hughes M, Kondo M, et al. Extensive diveristy of Ig-superfamily proteins inthe immune system of insects. Science. 2005 309:1874-8.
156. Bi W-J, Li D-X, Xu Y-H, Xu S, Li J, Zhao X-F, et al. Scavenger receptor B protects shrimp from bacteria by enhancing phagocytosis and regulating expression of antimicrobial peptides. Developmental & Comparative Immunology. 2015 51:10-21.
157. Sorgeloos P, Lavens P, Leger P, Tackaert W, Versichele D. Manual for the culture and use of brine shrimp *Artemia* in aquaculture. Belgium: Artemia Reference Center, Faculty of Agriculture, State University of Ghent. 1986:91-5.
158. Marques A, Dhont J, Sorgeloos P, Bossier P. Evaluation of different yeast cell wall mutants and microalgae strains as feed for gnotobiotically grown brine shrimp *Artemia franciscana.* J Exp Mar Biol Ecol. 2004 312:115-36.
159. Sung YY, Pineda C, MacRae TH, Sorgeloos P, Bossier P. Exposure of gnotobiotic *Artemia franciscana* larvae to abiotic stress promotes heat shock protein 70 synthesis and enhances resistance to pathogenic *Vibrio campbellii.* Cell Stress Chaperon. 2008 13:59-66.
160. FAO. The state of World Fisheries and Aquaculture. FAO Fisheries Department, Food and Agriculture Orgnization of the United Nations, Rome, Italy. 2010.
161. Henke JM, Bassler BL. Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi.* J Bacteriol. 2004 186:6902-14.
162. Aguirre-Guzman G, Ruiz MH, Ascencio F. A review of extracellular virulence product of *Vibrio* species important in diseases of cultivated shrimp. Aquacult Res. 2004 35:1395-404.
163. Defoirdt T, Ruwandeepika HAD, Karunasagar I, Boon N, Bossier P. Quorum sensing negatively regulates chitinase in *Vibrio harveyi*. Environ Microbiol Rep. 2010 2:44-9.
164. Defoirdt T, Ruwandeepika HAD, Karunasagar I, Boon N, Bossier P. Quorum sensing negatively regulates chitinase in *Vibrio harveyi.* Environ Microbiol Rep. 2010 2:44-9.
165. Cao JG, Meighen EA. Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. J Biol Chem. 1989 264:21670-6.
166. Higgins DA, Pomianek ME, Kraml CM, Taylor RK, Semmelhack MF, Bassler BL. The major *Vibrio cholerae* autoinducer and its role in virulence factor production. Nature. 2007 450:883-6.
167. Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelczer I, Bassler BL, et al. Structural identification of a bacterial quorum sensing containing boron. Nature. 2002 415:545-9.
168. Tu KC, Bassler BL. Multiple small RNAs additively to integrate sensory information and control quorum sensing in *Vibrio harvey.* Genes Dev. 2007 21:221-33.
169. Bassler BL, Wright M, Silverman MR. Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. Mol Microbiol. 1994 13:273-86.

170. Wegrzyn G, Czyz A. How do marine bacteria produce light, why are they luminescent, and can we employ bacterial bioluminescence in quatic biotechnology? . Ocenological. 2002 44:291-305.
171. Nealson KH, Hastings JW. Bacterial bioluminescence: its control and ecological significance. Microbiology. 1979 43:496-518.
172. Makemson JC. Luciferase - dependent oxygen consumption by bioluminescent *Vibrio.* J Bacteriol. 1986 165:461-6.
173. Czyz A, Wegrzyn G. On the function and evolution of bacterial luminescence. Bioluminescence and Chemiluminescence. 2001:31-4.
174. Murray HW, Cohn ZA. Macrophase oxygen-dependent antimicrobial activity. III. Enhanced oxidation metabolism as a expression of macrophage activation. J Exp Med. 1980 152:1596-609.
175. Al-Assafi MMK, Mutalib SA, Ghani MA, Aldulaimi M. A Review of Important Virulence Factors of" Vibrio vulnificus". Current Research Journal of Biological Sciences. 2014 6:76-88.
176. Hung NV, De Schryver P, Tam TT, Garcia-Gonzalez L, Bossier P, Nevejan N. Application of poly-β-hydroxybutyrate (PHB) in mussel larviculture. Aquaculture. 2015 446:318-24.
177. Bassler BL, Greenberg EP, Stevens AM. Cross species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. J Bacteriol. 1997 179:4043-5.
178. Surette MG, Miller MB, Bassler BL. Quorum sensing in *Escherichia coli, Salmonella typhimurium*, and *Vibrio harveyi:* A new family of genes responsible for autoinducer production. Proc Natl Acad Sci USA. 1999 96:1639-44.
179. Mok KC, Wingreen NS, Bassler BL. *Vibrio harveyi* quorum sensing: a coincidence detector for two autoinducers controls gene expression. EMBO J. 2003 22:870-81.
180. Rashid MH, Kornberg A. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa.* Proc Natl Acad Sci USA. 2000 97:4885-90.
181. Visca P, Chiarini F, Mansi A, Vetriani C, Serino L, Orsi N. Virulence determinant is *Pseudomonas aeruginosa* strains from urinary tract infection. Epidemiol Infect. 1992 108:323-36.
182. Stepanovic S, Veronika D, Hola V, Bonaventura G, Djukic S, Cirkovic I, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by *staphylococci*. APMIS. 2007 115:891-9.
183. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 1976 72:248-54.
184. Amend D. Petency testing of fish vaccines. Dev Boil Stand. 1981 49:119-24.
185. Bossier P, Verstraete W. Comamonas testosteroni Colony Phenotype Influences Exopolysaccharide Production and Coaggregation with Yeast Cells. App Environ Microbiol. 1996 62:2687-91.
186. Moxon E, R., Rainey PB, Nowak MA, Lenski RE. Adaptive evolution of highly mutable loci in pathogenic bacteria. Cell Biology. 1994 4:24-33.

187. Kokare CR, Chakraborty S, Khopade AN, Mahadik KR. Biofilm : Importance and applications. Indian Journal of Biotechnology. 2009 8:159-68.
188. Watnick PI, Kolter R. Steps in the development of a *Vibrio cholerae* EI Tor biofilm. Mol Microbiol. 1999 34:586-95.
189. Josenhans C, Suerbaum S. The role of motility as a virulence factor in bacteria. IJMM. 2002 291:605-14.
190. Chakrabarti S, Sengupta N, Chowdhury R. Role of DnaK in In Vitro and In Vivo Expression of Virulence Factors of *Vibrio cholerae*. Infect Immun. 1999 67:1025-33.
191. Gophna U, Ron EZ, Graur D. Bacterial type III secretion systems are ancient and evolved by multiple horizontal-transfer events. Gene. 2003 312:151-63.
192. Hoffman PS, Garduno RA. Surface-associated heat shock proteins of *Legionella pneumophila* and *Helicobacter pylori*: roles in pathogenesis and immunity. Infect Dis Obstet Gynecol. 1999 7:58-63.
193. Chakrabarti S, Liehl P, Buchon N, Lemaitre B. Infection-induced host translational blockage inhibits immune responses and epithelial renewal in the *Drosophila* gut. Cell Host Microbe. 2012 12:60-70.
194. Liu Y, Liu T, Hou F, Wang X, Liu X. Lvserpin3 is involved in shrimp innate immunity via the inhibition of bacterial proteases and proteases involved in prophenoloxidase system. Fish Shellfish Immunol. 2016 48:128-35.
195. Shanthi S, Manju S, Rajakumaran P, Vaseeharan B. Molecular cloning of peroxinectin gene and its expression in response to peptidoglycan and *Vibrio harveyi* in Indian white shrimp *Fenneropenaeus indicus.* Cell Commun Adhes. 2014 21:281-9.
196. Amparyup P, Sutthangkul J, Charoensapsri W, Tassanakajon A. Pattern recognition protein binds to lipopolysaccharide and β-1,3-glucan and activates shrimp prophenoloxidase system. J Biol Chem. 2012 287:10060-9.
197. Vance RE, Isberg RR, Portnoy DA. Patterns of pathogenesis: discrimination of pathogenic and non-pathogenic microbes by the innate immune system. Cell Host Microbe. 2009:10-21.
198. Akira S. Innate immunity to pathogens: diversity in receptors for microbial recognition. Immunol Rev. 2009 227:5-8.
199. Liu CH, Cheng W, Kuo CM, Chen JC. Molecular cloning and characterisation of a cell adhesion molecule, peroxinectin from the white shrimp *Litopenaeus vannamei.* Fish Shellfish Immunol. 2004 17:13-26.
200. Fagutao FF, Maningas MB, Kondo H, Aoki T, Hirono I. Transglutaminase regulates immune-related genes in shrimp. Fish Shellfish Immunol. 2012 32:711-5.
201. Liu Y, Li F, Wang B, Dong B, Zhang X, Xiang J. A serpin from Chinese shrimp *Fenneropenaeus chinensis* is responsive to bacteria and WSSV challenge. Fish Shellfish Immunol. 2008 26:345-51.
202. Baruah K, Ranjan J, Sorgeloos P, Bossier P. Efficacy of heterologous and homologous heat shock protein 70s as protective agents to *Artemia franciscana* challenged with *Vibrio campbellii.* Fish Shellfish Immunol. 2010 29:733-9.
203. Niu Y, Defoirdt T, Baruah K, Van de Wiele T, Dong S, Bossier P. *Bacillus* sp. LT3 improves the survival of gnotobiotic brine shrimp (*Artemia franciscana*) larvae challenged with *Vibrio campbellii* by enhancing the innate immune response and by decreasing the activity of shrimp-associated vibrios. Vet Microbiol. 2014 173:279-88.

204. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 25:402-8.
205. Ng TH, Chiang YA, Yeh YC, Wang HC. Review of Dscam-mediated immunity in shrimp and other arthopods. Dev Comp Immunol. 2014 46:129-38.
206. Park J, Stanley D, Kim Y. Roles of Peroxinectin in PGE2-Mediated Cellular Immunity in *Spodoptera exigua.* PLOS One. 2014 9(9). p.e105717.
207. Ong DST, Wang LH, Zhu Y, Ho B, Ding JL. The response of ferritin to LPS and acute phase of *Pseudomonas* infection. J Endotoxin Res. 2005 11:267-80.
208. Sritunyalucksana K, Wongsuebsantati K, Johansson M, .W., Söderhäll K. Peroxinectin, a cell adhesive protein associates with the ProPO system from the black tiger shrimp. Dev Comp Immunol. 2001 25:353-63.
209. Liu CH, Cheng W, Chen JC. The peroxinectin of white shrimp *Litopenaeus vannamei* is synthesised in the semi-granular and granular cells, and its transcription is up-regulated with *Vibrio alginolyticus* infection. Fish Shellfish Immunol. 2005 18:431-44.
210. Potempa J, Korzus E, Travis J. The serpin superfamily of proteinase inhibitors: structure, function and regulation. J Biol Chem. 1994 269:15957-60.
211. Kanost MR. Serine proteinase inhibitors in arthropod immunity. Dev Comp Immunol. 1999 23:291-301.
212. Keyser P, Elofsson M, Rosell S, Wolf-Watz H. Virulence blockers as alternatives to antibiotics: type III secretion inhibitors against Gram-negative bacteria. J Intern Med. 2008 264:17-29.
213. Salyers AA, Whitt DD, Wilson BA and Winkler ME (eds). Bacterial pathogenesis: a molecular approach. 2002. American Society for Microbiology.
214. Waters CM, Wu JT, Ramsey ME, Harris RC, Bassler BL. Control of the type 3 secretion system in *Vibrio harveyi* by quorum sensing through repression of ExsA. App Environ Microbiol. 2010 76:4996-5004.
215. Ruwandeepika HAD, Defoirdt T, Bhowmick PP, Karunasagar I, Karunasagar I, Bossier P. *In vitro* and in *vivo* expression of virulence genes in *Vibrio* isolates belonging to the *Harveyi* clade in relation to their virulence towards gnotobiotic brine shrimp (*Artemia franciscana).* Environ Microbiol. 2011 13:506-17.
216. Finlay BB, Falkow S. Common themes in microbial pathogenicity revisited. Mirobiol Mol Biol Rev. 1997 61:136-69.
217. Owens L, Austin D, A., Austin B. Effect of strain origin on siderophore production in *Vibrio harveyi* isolates. Dis Aquat Organ. 1996 27:157-60.
218. Colquhoun DJ, Sorum H. Temperature dependent siderophore production in *Vibrio salmonicida*. Microbial pathogenesis. 2001 31:213-9.
219. Norouzitallab P, Baruah K, Muthappa DM, Bossier P. Non-lethal heat shock induces HSP70 and HMGB1 protein production sequentially to protect *Artemia franciscana* against *Vibrio campbellii*. Fish Shellfish Immunol. 2015 42:395-9.
220. Destoumieux-Garzón D, Duperthuy M, Vanhove A, Schmitt P, Wai S. Resistance to Antimicrobial Peptides in Vibrios. Antibiotics. 2014 3:540-63.
221. Kashimoto T, Ueno S, Hanajima M, Hayashi H, Akeda Y, Miyoshi S, et al. *Vibrio vulnificus* induces macrophage apoptosis in *vitro* and *in vivo.* Infect Immunol. 2003 71:533-5.

222. Sandy M, Butler A. Microbial iron acquisition: marine and terrestrial siderophores. Chemical reviews. 2009 109:4580-95.
223. Pajuelo D, Lee CT, Roig FJ, Hor LI, Amaro C. Novel host-specific iron acquisition system in the zoonotic pathogen *Vibrio vulnificus.* Environ Microbiol. 2015 17:2076-89.
224. Finlay BB, McFadden G. Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. Cell. 2006 124:767-82.
225. Armstrong PB. Proteases and protease inhibitors: a balance of activities in host-pathogen interaction. Immunobiology. 2006 211:263-81.
226. Harris HE, Andersson U, Pisetsky DS. HMGB1: a multifunctional alarmin driving autoimmune and inflammatory disease. Nature Reviews Rheumatology. 2012 8:195-202.
227. Bell CW, Jiang W, Reich CF, Pisetsky DS. The extracellular release of HMGB1 during apoptotic cell death. American Journal of Physiology-Cell Physiology. 2006 291:C1318-C25.
228. Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. Annual review of immunology. 2011 29:139.
229. Sung YY, Ashame MF, Chen S, Macrae TH, Sorgeloos P, Bossier P. Feeding Artemia franciscana (Kellogg) larvae with bacterial heat shock protein, protects from *Vibrio campbellii* infection. J Fish Dis. 2009 32:675-85.
230. Fontana MF, Banga S, Barry KC, Shen X, Tan Y, Luo ZQ, et al. Secreted bacterial effectors that inhibit host protein synthesis are critical for induction of the innate immune response to virulent *Legionella pneumophila*. PLoS Pathog. 2011 7(2). p.e1001289.
231. McEwan DL, Kirienko NV, Ausubel FM. Host translational inhibition by *Pseudomonas aeruginosa* Exotoxin A Triggers an immune response in *Caenorhabditis elegans.* Cell Host Microbe. 2012 11:364-74.
232. Rajamuthiah R, Mylonakis E. Effector triggered immunity. Virulence. 2014 5:697-702.
233. Baruah K, Duy Phong HP, Norouzitallab P, Defoirdt T, Bossier P. The gnotobiotic brine shrimp (*Artemia franciscana*) model system reveals that the phenolic compound pyrogallol protects against infection through its prooxidant activity. Free Radic Bio Med. 2015 89:593-601.
234. Molloy MJ, Bouladoux N, Belkaid Y. Intestinal microbiota: shaping local and systemic immune responses. Semin Immunol. 2012 24:58-66.
235. Chen T, Cao X. Stress for maintaining memory: HSP70 as a mobile messenger for innate and adaptive immunity. Eur J Immunol. 2010 40:1541-4.
236. Norouzitallab P, Baruah K, Vandegehuchte M, Van Stappen G, Catania F, Vanden Bussche J, et al. Environmental heat stress induces epigenetic transgenerational inheritance of robustness in parthenogenetic *Artemia* model. FASEB journal. 2014 28:3552-63.
237. Baruah K, Ranjan J, Sorgeloos P, Macrae TH, Bossier P. Priming the prophenoloxidase system of *Artemia franciscana* by heat shock proteins protects against *Vibrio campbellii* challenge. Fish Shellfish Immunol. 2011 31:134-41.
238. Baruah K, Norouzitallab P, Shihao L, Sorgeloos P, Bossier P. Feeding truncated heat shock protein 70s protect *Artemia franciscana* against virulent *Vibrio campbellii* challenge. Fish Shellfish Immunol. 2013 34:183-91.
239. Almeida MB, do Nascimento JL, Herculano AM, Crespo-Lopez ME. Molecular chaperones: toward new therapeutic tools. Biomed Pharmacother. 2011 65:239-43.

240. Agresti A, Bianchi ME. HMGB proteins and gene expression. Curr Opin Genet Dev. 2003 13:170-8.
241. Janko C, Filipovic M, Munoz LE, Schorn C, Schett G, Ivanovic-Burmazovic I, et al. Redox modulation of HMGB1-related signaling. Antioxidants & redox signaling. 2014 20:1075-85.
242. Scaffidi P, Misteli T, Blanchi M, E. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature. 2002 418:191-5.
243. Thomas J, O., Travers A, A. HMGB1 and 2, and related 'architectural' DNA-binding proteins. Trends Biochem Sci. 2001 26:167-74.
244. Wang Q, Zeng M, Wang W, Tang J. The HMGB1 acidic tail regulates HMGB1 DNA binding specificity by a unique mechanism. Biochem Biophys Res Commun. 2007 360:14-9.
245. Lu B, Nakamura T, Inouye K, Li J, Tang Y, Lundback P, et al. Novel role of PKR in inflammasome activation and HMGB1 release. Nature. 2012 488:670-4.
246. Venereau E, Schiraldi M, Uguccioni M, Bianchi ME. HMGB1 and leukocyte migration during trauma and sterile inflammation. Mol Immunol. 2013 55:76-82.
247. Yanai H, Taniguchi T. Essential role of hig-mobiity group box proteins in nucleic acid-mediated innate immune responses. J Intern Med. 2011 270:301-8.
248. Costerton JW, Cheng KJ, Geesey G, G, Ladd TI, Nickel JC, Dasgupta M, et al. Bacterial biofilms in nature and disease. Annu Rev Microbiol. 1987 41:435-64.
249. Ackermann M. A functional perspective on phenotypic heterogeneity in microorganism. Nat Rev Microbiol. 2015 13:496-508.
250. Arnoldini M, Vizcarra IA, Pena-Miller R, Stocker N, Diard M, Volgel V. Bistable expression of virlence genes in *Salmonella* leads to the formation of an antibiotic-tolerant subpopulation. PLoS biology. 2014 12(8). p.e1001928.
251. Stewart MK, Cummiings LA, Johnson ML, Berezow AB, Cookson BT. Regulation of phenotypic heterogeneity permits *Salmonella* evasion of the host caspase-1 inflammatory response. Proc Natl Acad Sci USA. 2011 108:20742-7.
252. Singh R, Ray P, Das A, Sharma M. Role of persisters and small-colonyvariants in antibitic resistance of plantonic and biofilm-associated *Staphylococcus aureus:* an *in vitro* study. J Med Microbiol. 2009 58:1067-73.
253. Mirani ZA, Aziz M, Khan SI. Small colony variants have a mojor role in stability and persistence of *Staphylococcus aureus* biofilms. J Antibiot. 2015 68:98-105.
254. Smits WK, Kuipers OP, Veening JW. Phenotypic variation in bacteria: the role of feedback regulation. Nat Rev Microbiol. 2006 4:259-71.
255. Foley J. Mini-review: Strategies for Variation and Evolution of Bacterial Antigens. Computational and structural biotechnology journal. 2015 13:407-16.
256. Carson SD, Stone B, Beucher M, Fu J, Sparling PF. Phase variation of the gonococcal siderophore receptor FetA. Mol Microbiol. 2000 36:585-93.
257. Lewis LA, Gipson M, Hartman K, Ownbey T, Vaughn J, Dyer D, W. Phase variation of HpuAB and HmbR, two distinct haemoglobin receptors of *Neisseria meningitidis* DNM2. Mol Mcrobiol. 1999 32:977-89.
258. Ashida H, Kim M, Sasakawa C. Manipulation of the host cell death pathway by *Shigella*. Cell Microbiol. 2014 16:1757-66.
259. Beutler B. TLR4 as the mamalian endotoxin sensor. Curr Top Microbiol Immunol. 2002 270:109-20.

260. Reife RA, Coast SR, Al-Qutub M, Braham PA, Billharz RJ, Howald WN, et al. Porphyromonas gingivalis lipopolysaccharide lipid A heterogeneity: differential activities of tetra and penta-acylated lipid A structures on E-selection expression and TLR4 recognition. Cell Microbiol. 2006 8.
261. Stuart LM, Ezekowitz RA. Phagocytosis: elegant complexity. Immunity. 2005 22:539-50.
262. Meena S, Rajni. Survival mechanism of pathogenic *Mycobacterium tuberculosis* H37Rv. FEBS journal. 2010 277:2416-27.
263. Jantsch J, Chikkaballi D, Hesel M. Cellular aspects of immunity to intracellular *Salmonella enterica.* Immunol Rev. 2011 240:185-95.
264. Toma C, Okura N, Takayama C, Suzuki T. Characteristic features of intracellular pathogenic *Leptospira* in infected murine macrophages. Cellular microbiology. 2011 13:1783-92.
265. Helaine S, Holden DW. Heterogeneity of intracellular replication of bacterial pathogens. Current opinion in microbiology. 2013 16:184-91.
266. Salcedo SP, Noursadeghi M, Cohen J, Holden DW. Intracellular replication of *Salmonella typhimurium* strains in specific subsets of splenic macrophages *in vivo.* Cell Microbiol. 2001 3:587-97.
267. Galán JE, Wolf-Watz H. Protein delivery into eukaryotic cells by type III secretion machines. Nature. 2006 444:567-73.
268. Viboud GI, Bliska JB. *Yersinia* outer proteins: role in modulation of host cell signaling reponses and pathogenesis. Annu Rev Microbiol. 2005 59:69-89.
269. Zhou X, Gewurz B, Ritchie JM, Takasaki K, Greenfeld H, Davis BM, et al. A *Vibrio parahaemolyticus* T3SS effector mediates pathogenesis by independently enabling intestinal colonization and inhibiting TAK1 activation. Cell Rep. 2013 3:1690-702.
270. Ham H, Sreelatha A, Orth K. Manipulation of host membranes by bacterial effectors. Nat Rev Microbiol. 2011 9:635-46.
271. Schnupf P, Portnoy D, A. Listeriolysin O: a phagosome-specific lysin. Microbes Infect. 2007 9:1176-87.
272. Tuan VV, Dantas-Lima JJ, Thuong KV, Li W, Grauwet K, Bossier P, et al. Differences in uptake and killing of pathogenic and non-pathogenic bacteria by haemocyte subpopulations of penaeid shrimp, *Litopenaeus vannamei,* (Boone). J Fish Dis. 2016 39:163-74.
273. Hornef MW, Wick MJ, Rhen M, Normark S. Bacterial strategies for overcoming host innate and adaptive immune responses. Nat Immunol. 2002 3:1033-40.
274. Van Avondt K, van Sorge NM, Meyaard L. Bacterial immune evasion through manipulation of host inhibitory immune signaling. PLoS Pathog. 2015 11:e1004644.
275. Mohr I, Sonenberg N. Host translation at the nexus of infection and immunity. Cell Host Microbe. 2012 12:470-83.
276. Rosqvist R, Bölin I, Wolf-Watz H. Inhibition of phagocytosis in *Yersinia* pseudotuberculosis: a virulence plasmid-encoded ability involving the Yop2b protein. Infection and immunity. 1988 56:2139-43.
277. Celli J, Finlay BB. Bacterial avoidance of phagocytosis. Trends in microbiology. 2002 10:232-7.

278. Persson C, Nordfelth R, Andersson K, Forsberg Å, Wolf‐Watz H, Fällman M. Localization of the *Yersinia* PTPase to focal complexes is an important virulence mechanism. Molecular microbiology. 1999 33:828-38.
279. Rosqvist R, Forsberg A, Wolf-Watz H. Intracellular targeting of the *Yersinia* YopE cytotoxin in mammalian cells induces actin microfilament disruption. Infection and immunity. 1991 59:4562-9.

APPENDICES

B

SUMMARY/SAMENVATING

Phenotypic (phase) variation or phenotypic switching is defined as a process of reversible, high frequency phenotypic variation that is mediated by DNA mutations, reorganization or modification [21]. Phenotypic variation is used by several bacterial species to generate population diversity that enhances bacterial fitness under certain conditions and is important in niche adaptation and host immune evasion. Phenotypic variation was intensively studied in *Pseudomonas* spp. or in *E. coli*. However, little is known about phenotypic variation in *Vibrio* especially in luminous vibriosis diseases for many aquaculture species.

Vibrio harveyi, a luminescent Gram-negative motile marine bacterium, is an important pathogen responsible for causing severe diseases in shrimp, finfish and molluscs leading to severe economic losses. Non-luminescent *V. harveyi* obtained by culturing luminescent strains under static and dark conditions were reported to alter the levels of virulence factors and *metalloprotease* gene and *luxR* expression when compared to their luminescent variants. Presently, our results (Chapter 3) showed that switching from the luminescent to the non-luminescent state changed the phenotypic physiology or behavior of *V. harveyi*, resulting in alterations in caseinase and hemolytic activities, swimming motility, and biofilm formation. The switching capacity was to a large extent independent from the quorum sensing status, because quorum sensing mutants were equally capable of preforming the phenotypic switch.

In the next chapter (Chapter 4 & 5) the gnotobiotic *Artemia* model organism (GART) system was used to investigate the influence of virulent and non-virulent luminescent and non-luminescent of wild type *V. harveyi* (BB120) and its quorum sensing mutant (JMH634) on immune defense of *Artemia franciscana.*

The mRNA transcriptional level of eight immune-related genes including lipopolysaccharide and β-1,3-glucan binding protein (*lgbp*), down syndrome cell adhesion molecules (*dscam*), peroxinectin (*pxn*), prophenoloxidase (*proPO*), transglutaminase (*tgase*), ferritin (*ftn*), melanin inhibition protein (*mip*) and serpin (*spn*) was investigated by challenge with these bacterial strains to verify the hypothesis that changes in the production of virulence factors in *V. harveyi* by phenotypic variation might play a role in host's recognition, consequently stimulating the host immune response in a different pattern of gene expression in gnotobiotic *Artemia* (Chapter 4). Results of this study provide evidence that alternation of *in vitro* virulence factors production such as luminescence and the enzyme activities caused by phenotypic variation might contribute to the pathogen-induced processes and host-pathogen interaction, by which bacteria can modulate host defense reaction commensurate with the attack being mounted. Virulent strains or strains producing more virulent factors induce *proPO* expression only early on upon pathogen exposure while *spn*/mip are up-regulated later on, probably to regulate phenoloxidase activity. The *lbgp* gene is overexpressed more constitutively within the experimental time frame. This is unexpected in view of the fact that most of the tested effector genes are down-regulated towards the end of the experiment. Assuming the non-luminescent (NL) strains could switch back to luminescent (L) strains, the difference in gene expression dynamics could be explained in the experiment with the BB120L and BB120NL strains, but that remains speculative.

In response to a *Vibrio* infection, the host employs protective immune, and repair and stress mechanisms to maintain cellular homeostasis and integrity, and to limit pathogenicity. However, pathogenic *Vibrio spp*. evade host defensive mechanisms by using different strategies. Recently, a few studies have indicated that pathogenic bacteria evade host defense mechanisms during infection by limiting translation of host mRNAs which encode proteins that mediate defense responses. In this study (Chapter 5), we aimed to determine whether luminescent *V. harveyi* inflict damages and cause disease in *Artemia* by interfering with the mRNA translational machineries of the host defense system. Result of this study showed that there was a non-corresponding response at transcriptional level and translational level of hsp70 and hmgb1 in *Artemia* at 12 h post infection, indicating that transcription of these 2 genes is still ongoing, but translation tends to be limited

The full-length sequence of Hmgb1 of *Artemia franciscana* was isolated for the first time in this thesis (Chapter 6). Results show that the deduced polypeptide of *Art*Hmgb1 consistent of 200 amino acids, and it calculated molecular weight was 26.7 kDa, which was highly conserved to those from vertebrates and invertebrates. The conserved functional domains of *Art*Hmgb1 were high similar to previously characterized *Hmgb1* genes, suggesting that *Art*Hmgb1 is a novel member of the invertebrate Hmgb1 family, and it could have similar function to those from vertebrates and other invertebrates.

In conclusion, the work presented in this thesis indicates that *in vitro* virulence factors are changed as a result of phenotypic variation in *V. harveyi.* These alterations in the luminescent and non-luminescent variants of the wild type BB120 and its quorum sensing mutant JMH634 might modulate the innate immune response, and consequently influence the survival of *Artemia*. Evidence in relation to the ecological significance of the described phenotypic switch remains to be gathered, especially because no environmental condition has been pinpointed that facilitates a switch from a non-luminescent strains back to a luminescent strain.

SUMMARY/SAMENVATING

Fenotypische variatie of alternatie bij bacteriën wordt gedefinieerd als een proces van reversibel hoge frequentie fenotypische alternatie dat wordt bewerkstelligd door DNA mutaties, reorganisaties of modificaties. Fenotypische variatie wordt gebruikt door verschillende bacteriën om diversiteit op populatie niveau te genereren, om op dit manier hun "fitness" onder bepaalde omstandigheden te verhogen. Het is een belangrijk proces in niche adaptatie en ontwijking van het gastheer immuun systeem. Fenotypische variatie is intens bestudeerd in bvb *Pseudomonas* spp. of in *E. coli*. Bij luminescente *Vibrio* is het fenomeen echter veel minder bestudeerd, alhoewel vibriosis een zeer belangrijke ziekte is in aquacultuur.

Vibrio harveyi, een luminescente Gram negatieve mobiele marine bacterie, is een belangrijke pathogeen verantwoordelijk voor ziekten bij garnalen, mariene vissen en mollusken met zware economische consequenties. Niet-luminescente *V. harveyi* (NL) kunnen bekomen worden door luminescente (L) stammen te kweken onder statische en donkere omstandigheden. Deze switch gaat gepaard met veranderingen op het niveau van de productie van virulentie factoren zoals metalloprotease en luxR. Hier wordt aangetoond (Hfst 3) dat de switch van de L naar de NL status gepaard gaat met een verandering in fysiologische karakteristieken, zoals productie van caseinase, hemolytische activiteit, mobiliteit zoals zwermen, en biofilm aanmaak. De capaciteit om van status te veranderen is voor een groot deel onafhankelijk van de quorum sensing (QS) status, aangezien QS mutanten ook in staat waren deze switch te maken.

In de volgende hoofdstukken (4 en 5) werd het gnotobiotisch Artemia model systeem (GART) gebruikt om de invloed van virulente en niet-virulent luminescente en niet-luminescente stammen van de wild type stam *V. harveyi* (BB120) en zijn QS triple-mutant (JMH634) op het immuun systeem te bestuderen.

Het transcriptie niveau van acht verschillende immuun-gerelateerde genen, met onder andere "lipopolysaccharide en β-1,3-glucan binding protein" (*lgbp*), "down syndrome cell adhesion" molecules (*dscam*), peroxinectine (*pxn*), prophenoloxidase (*proPO*), transglutaminase (*tgase*), ferritine (*ftn*), "melanin inhibition protein" (*mip*) and serpin (*spn*) werden onderzocht onder

blootstelling met deze bacteriële stammen om de hypothese te verifiëren dat veranderingen in de productie van virulentie factoren bij *V. harveyi* door fenotypische switch een rol zou kunnen spelen in de herkenning door de gastheer, met een verschillend patroon van gen expressie als gevolg. (Hfst 4). De resultaten van deze studie tonen aan dat de verandering in fenotype door een switch kan bijdragen tot andere patronen van gen expressie die waarschijnlijk verbonden zijn met een andere dynamiek van infectie. Virulente stammen of stammen die meer virulentie factoren produceren, induceren proPO expressie vroeg na blootstelling aan de pathogeen, terwijl *spn*/mip genen (genen betrokken in het moderatie van phenoloxidase activiteit) pas later opgereguleerd worden, waarschijnlijk om proPO activiteit te controleren. Het *lbgp* gene komt constitutief tot expressie na blootstelling aan virulente stammen binnen de experimentele periode. Dit is eerder onverwacht aangezien genen lager in de "cascade" betrokken in het uitschakelen van bacteriën eerder neerwaarts gereguleerd worden naar het einde van het experiment. In de veronderstelling dat NL stammen tijdens de infectie terug kunnen "switchen" naar een L stam, zou het verschil in dynamiek van gen expressie na blootstelling aan BB120L en BB120NL kunnen verklaard worden. Een NL naar L switch werd echter nooit vastgesteld en interpretatie van de data blijft dus speculatief.

Als antwoord op *Vibrio* infectie, ontwikkelt de gastheer een immunologische response, en herstel om cellulaire homeostasis en integriteit te bewaren en pathogeniteit te limiteren. Nochtans kan verwacht worden dat *Vibrio* in staat is om het immuun systeem te ontwijken via verschillende mogelijke strategieën. Onlangs toonden verschillende studies aan dat pathogene bacteriën in staat zijn om het gastheer immuun systeem te ontwijken door de gastheer translatie van mRNA te beïnvloeden. In deze studie (Hfst5) werd een poging ondernomen om aan te tonen dat *V. harveyi* in staat is translatie in de gastheer te beïnvloeden. De resultaten van de studie tonen dat bij HSP70 en hmgb1 er een discrepantie is tussen gen expressie en eiwit productie, 12 uur na blootstelling aan de pathogeen, waarbij er in de aanwezigheid van gen expressie er een gelimiteerde translatie plaatsgrijpt.

De cDNA sequentie van Hmgb1 van *Artemia franciscana* werd geïsoleerd voor de eerste maal (Hfst 6). De resultaten tonen aan het afgeleide eiwit *Art*Hmgb1 200 aminozuren bevat met een voorspeld MW van 26, 7 kDa. Er is een hoge graad aan aminozuur conservatie in vergelijking

met Hmgb1 van andere vertebraten en invertebraten. De geconserveerde domeinen van het gen hebben een hoge gelijkenis met de andere Hmgb1 genen, wat er op wijst dat *Art*Hmgb1 een nieuw lid is van de Hmgb familie, met gelijke functie.

In conclusie, de resultaten van dit werk tonen aan dat *in vitro* productie van virulentie factoren en/of virulentie beïnvloed wordt door een fenotypische switch bij *V. harveyi*. Deze veranderingen tussen luminescente en niet-luminescente stammen van het wild type BB120 en de triple mutant JMH634 moduleren de response van het aangeboren immuun systeem met effecten op het niveau van *Artemia* overleving. Er is op dit ogenblik geen bewijs voor de ecologische relevantie van de beschreven fenotypen aangezien een switch van NL naar L stammen nooit is kunnen aangetoond worden, door het gebrek aan omgevingsomstandigheden die een dergelijke switch kan induceren.

APPENDICES

C

The Ct value of the house keeping gene (*efα1*) and target genes (*hmgb1* and *hsp70*).

	Code		EFα1	HMGB1	HSP70
Sample 1	NHS	1a 0h	20.5	26.6	27.8
Sample 2	NHS	1b 0h	19.7	26.2	26.5
Sample 3	NHS	1c 0h	19.3	25.4	28.0
Sample 4	NHS	2a 6h	20.7	26.9	29.7
Sample 5	NHS	2b 6h	19.5	25.1	28.2
Sample 6	NHS	2c 6h	20.4	27.0	29.0
Sample 7	NHS+120L	3a 6h	21.6	27.2	29.2
Sample 8	NHS+120L	3b 6h	20.4	27.4	28.4
Sample 9	NHS+120L	3c 6h	21.7	27.5	28.6
Sample 10	NHS+120NL	4a 6h	18.9	28.3	24.8
Sample 11	NHS+120NL	4b 6h	18.3	27.9	24.7
Sample 12	NHS+120NL	4c 6h	18.4	28.1	24.7
Sample 13	NHS+634L	5a 6h	18.4	25.4	25.5
Sample 14	NHS+634L	5b 6h	18.0	25.3	25.6
Sample 15	NHS+634L	5c 6h	19.0	25.7	25.8
Sample 16	NHS+634NL	6a 6h	18.6	24.7	25.7
Sample 17	NHS+634NL	6b 6h	18.6	25.3	25.7
Sample 18	NHS+634NL	6c 6h	18.9	25.0	25.7
Sample 19	NHS	7a 12h	18.9	25.5	24.9
Sample 20	NHS	7b 12h	18.5	25.4	24.8
Sample 21	NHS	7c 12h	18.6	25.0	24.8
Sample 22	NHS+120L	8a 12h	18.7	25.2	25.2
Sample 23	NHS+120L	8b 12h	18.8	25.1	24.7
Sample 24	NHS+120L	8c 12h	18.8	25.3	25.2
Sample 25	NHS+120NL	9a 12h	18.5	26.0	24.8
Sample 26	NHS+120NL	9b 12h	18.5	24.9	25.0
Sample 27	NHS+120NL	9c 12h	18.6	25.2	25.2
Sample 28	NHS+634L	10a 12h	18.4	25.2	26.0
Sample 29	NHS+634L	10b 12h	18.8	25.8	26.5
Sample 30	NHS+634L	10c 12h	18.2	26.2	25.6
Sample 31	NHS+634NL	11a 12h	18.7	26.1	28.1
Sample 32	NHS+634NL	11b 12h	18.3	25.9	27.2
Sample 33	NHS+634NL	11c 12h	18.3	25.3	27.3

APPENDICES

D

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APPENDICES

E

CURRICULUM VITAE

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EDUCATION

1997 - 2001	Aquaculture Engineering, Hue University of Agriculture and Forestry, Vietnam
2004 - 2006	Master of Science in Aquaculture, University of Ghent, Belgium
2011 - 2016	PhD student – Laboratory of Aquaculture & Artemia Reference Centre, Faculty of Bioscience Engineering, Ghent University, Belgium.
2011 - 2016	PhD scholarship of Lotus Project, Erasmus Mundus program of the European Union and Vietnamese Scholarship.

WORKSHOP & TRAINING

2002	Training course (1 month) on Parasite in aquatic animals, RIA . 3, Nha Trang, Vietnam
2003	Training course (3 months) at Major University, Chiang Mai, Thailand.
2005	Participant of Larvi conference in Ghent University, Belgium.
2012	Training on Microbial community management in aquaculture organized by the EU promicrobe project and LARVANET, Ghent University, Belgium.
2013	Poster presentation in Larvi 2013 conference in Ghent University, Belgium.
2013	Oral presentation in Asian Pacific Aquaculture Conference, Ho Chi Minh city, Vietnam.
2014	Training on basic of biology for engineers organized by IMEC, Leuven University Belgium.

2014	Training on Chemical tool for biology researcher organized by VIB, Antwerpen University Belgium.

PROFESSIONAL ACTIVITIES

2002 to present	Lecturer at Faculty of Fishery, Hue University of Agriculture and Forestry.
2007 - 2010	Scientific consultant of IMOLA project, Hue, Vietnam.
2007 - 2011	Part-time member of CARD center, Hue, Vietnam
2007 - 2011	Scientific consultant of JICA Project, Hue, Vietnam.

PUBLICATIONS

Nguyen Thi Xuan Hong, Nguyen Van Nam, 2009. Development of Aquaculture trial model for disaster preparedness and environmental protection at Huong Phong commune, Thua Thien Hue province. GSGES Asia Platform, Annual Report.

Nguyen Thi Xuan Hong, Nguyen Van Nam, 2010. Changes in fish resources in upland area, Thua Thien Hue Province, Vietnam. Agriculture Publishing House.

Hong NTX, Baruah K, Vanrompay D and Bossier P, 2015. Characterization of luminescent and nonluminescent variants of *Vibrio harveyi* wild type and quorum sensing mutants. Journal of Fish Disease.

MSc and PhD THESIS TITLE

MSc Title: "Interference of bacteriophage and quorum sensing sensitivity in *Vibrio harveyi* ''

PhD title: "Phenotypic variation in *Vibrio harveyi*: virulence factors and immune response in *Artemia franciscana*".

I hereby declare on my word of honor that this information is correct and complete.