The impact of cell-to-cell signaling and host cues on the virulence of Vibrio anguillarum towards gnotobiotic sea bass (Dicentrarchus labrax) larvae

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Cover page image: Photograph of newly hatched sea bass larvae (Dicentrarchus labrax). (http://www.larvalbase.org/PhotoArchive/Photothumbnails/PhotoArchieve.htm)

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# List of abbreviations and units

- **°C**: Degree Celsius
- **%**: Percentage
- **µg**: Microgram
- **µl**: Microliter
- **g**: Relative centrifugal force or G force
- **±**: Approximately
- **/**: Per
- **AHL**: Acyl homoserine lactone
- **ANOVA**: Analysis of variance
- **cDNA**: Complementary deoxyribonucleic acid
- **CFU**: Colony forming unit
- **DAH**: Day after hatching
- **DNA**: Deoxyribonucleic acid
- **EPSs**: Exopolysaccharide production
- **FAO**: Food and agricultural organization of the United Nations
- **FASW**: Filtered and autoclaved seawater
- **g**: Gram
- **g/L**: Gram per liter
- **h**: Hour
- **L**: Liter
- **LB**: Luria-Bertani
- **LPSs**: Lipopolysaccharides
- **mg**: Milligram
- **mRNA**: Messenger RNA
- **OD**: Optical density
- **OMPs**: Outer membrane proteins
- **P**: Statistical p-value
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<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time-polymerase chain reaction</td>
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<td>rpm</td>
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Chapter 1

General introduction and thesis outline
Chapter 1

1. General introduction to aquaculture

1.1 The status of world aquaculture

Each day 200,000 people are added to the world food demand (UN population Division, 2007). To meet this demand, aquaculture became one of the fastest growing food production industries. According to FAO statistics, global fish production has grown steadily in the last five decades (Figure 1.1). World capture production in 2000 was more than twice that of aquaculture, but it has already decreased by 1% compared to 1997, whereas aquaculture increased by 59%. In 2012, global capture production reached 91.3 million tonnes, a decrease of 2.6 percent compared with the previous year. Meanwhile, over-fishing is claimed to put marine ecology in danger (FAO, 2012). FAO concludes that the maximum wild capture fisheries potential from the world's oceans has probably been reached. On the other hand, aquaculture production has continued to show strong growth, increasing at an average annual growth rate of 6.1 percent from 36.8 million tonnes in 2002 to 66.6 million tonnes in 2012. By 2030, capture production and aquaculture are projected to be equal (FAO, 2002). It is now believed that aquaculture has the potential to make a significant contribution to the increasing food demand and to provide safe and high quality aquatic food.

![Figure 1.1 World capture fisheries and aquaculture production (Source: FAO, 2012).](image)
In addition to the total production, the diversity of aquaculture species has also increased. As in 2012, the number of species registered in FAO statistics was 567, including finfishes (354 species with 5 hybrids), molluscs (102), crustaceans (59), amphibians and reptiles (6), aquatic invertebrates (9), and marine and freshwater algae (37). It is estimated that more than 600 aquatic species are cultured worldwide for production in a variety of farming systems and facilities with different input intensities and technological sophistication, using either freshwater, brackish water or marine water. For most farmed aquatic species, hatchery and nursery technology have been established.

Fish and fishery products play a critical role in global food security and nutritional needs of people in developing and developed countries. Fish is a key source of protein, essential amino-acids and minerals, especially in some densely populated countries where total protein intake may be low (Rice and Garcia, 2011; FAO, 2012). There are 100 million tonnes of fish eaten world-wide each year, providing two and a half billion people with at least 20 percent of their animal protein intake (FAO, 2012).

1.2 European sea bass (Dicentrarchus labrax)

European sea bass is a widely distributed species in Northeast Atlantic shelf waters from southern Norway, through the North Sea, the Irish Sea, the Bay of Biscay, Atlantic Iberian Waters to Northwest Africa, the Mediterranean, and the Black Sea. Sea bass was the second fish species (after salmon) to be farmed commercially in Europe (FAO, 2014). One of the largest success stories in European aquaculture is the sea bass industry, which in 20 years grew from a few thousand tonnes to 150 000 tonnes in 2010 (Figure 1.2).

The sea bass is a marine fish that undergoes metamorphosis (Govoni et al., 1986). At the moment of hatching, its entire gut consists of an undifferentiated straight tube, which is less developed than that of adults. The gut is closed and depending on the rearing temperature, the mouth and anus open at about 2−5 days after hatching (DAH) (Hernández et al., 2001; Sucré et al., 2009). After hatching, sea bass larvae are kept in
complete darkness at 16°C and the illumination, temperature and salinity are increased in the course of time. During the absence of feed, sea bass larvae can grow in darkness and in relatively low temperatures (16°C) relying solely on their yolk. The feeding starts with Artemia Instar I from DAH 8-9 until DAH 20-25, followed by enriched Artemia Instar II until DAH 35-40 (Chatain, 1997). The production cycle in intensive systems is shown in Figure 1.3. Larval rearing is a major bottleneck in the sea bass culture industry due to the high number of malformations and diseases that could lead to unpredictable and high mortality rates (Darias et al., 2008). Most of these mortalities have been associated with opportunistic pathogens (nonobligate and/or non-specialist pathogen of a focal host). If the classic assumptions (obligate pathogen and specialist on one host) of virulence evolution theory fail, we have an opportunistic pathogen such as Vibrio spp. (Frans et al., 2011; Brown et al., 2012).

Figure 1.2 Global Aquaculture Production for European sea bass (Source: FAO, 2014).
Figure 1.3 Overview of the production cycle of European sea bass (intensive system) (based on FAO, 2011).

1.3 Disease and disease control

Although a sturdy species, sea bass are subject to a wide range of diseases under rearing conditions. Viral encephalo-retinopathy, vibriosis, photobacteriosis or pseudotuberculosis, myxobacteriosis, mycobacteriosis, epitheliocystis, amyloodiniasis, cryptocaryoniasis, scuticociliatosis, myxosporidiosis, microsporidiosis, gill fluke infections, anisakis infection, and isopodiasis are the most commonly found diseases in sea bass farming according to FAO (2014). Vibriosis is a disease caused by an infection with bacteria of the Vibrio genus that can cause skin infections, and/or blood infections, most commonly Vibrio anguillarum, Vibrio parahaemolyticus, Vibrio alginolyticus and Vibrio ordali in sea bass.
Bacterial diseases in fish generally occur as a result of the complex interactions between pathogen, fish and environmental stress that affect the susceptibility of the fish to diseases. Environmental stresses can affect the homeostatic mechanism of fish thus reducing their resistance to disease-causing organisms. Fish reared in intensive systems are exposed to sub-optimal environmental conditions, and they may be more sensitive to bacterial pathogens than wild populations (Ashley, 2007). Hence, effective control of diseases is one of the most critical elements in successful aquaculture. Below is an evaluation of several means by which virulence therapy may be exerted.

The most common and traditional disease control method is the use of antibiotics. Current levels of antibiotics used in aquaculture worldwide are not easy to determine because different countries have different distribution and registration systems. Burridge et al. (2010) reported that the amount of antibiotics and other compounds used in aquaculture differed significantly between countries. Defoirdt et al., (2011) reported the large variation between different countries, with antibiotic use ranging from 1 g per metric ton of production in Norway to 700 g per metric ton in Vietnam. However, problems occur when antibiotics are used routinely and excessively, especially when there are no apparent diseases. Heavy use of antibiotics has resulted in resistance development in aquaculture pathogens (Cabello, 2006). Moreover, in some cases, antibiotic resistance can be transmitted by horizontal gene transfer to bacteria in the vicinity, including animal and human pathogens (Defoirdt et al., 2011). Another problem created by the excessive use of antibiotics is the presence of residual antibiotics in commercialized fish products, which can affect normal microflora and generate problems of allergy and intoxication (Alderman and Hastings, 1998; Angulo et al., 2004; Cabello, 2004).

As a result of the growing awareness that antibiotics should be used with more care, novel therapeutics is being developed and tested (Defoirdt, 2013). Treatments focusing on the aquaculture animals, aiming at increasing their resistance for pathogens are being investigated and applied, such as vaccines (Sommerset et al., 2005), immunostimulants (Bricknell and Dalmo, 2005) and heat shock protein inducers (Baruah...
et al., 2011). These treatments can enhance host resistance by stimulation of the non-specific or innate immune system and/or by triggering the specific immune response. Applications of probiotics or beneficial bacteria, which control pathogens through a variety of mechanisms, are currently a popular alternative technique to antibiotic treatment. Verschuere et al. (2000) suggested the following definition for probiotics ‘a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment’. Another alternative to antibiotics is preventing the pathogens from attacking the host, without the need to kill them. This strategy has been termed antivirulence therapy (Clatworthy et al., 2007) and such antivirulence therapy can consist of either interfering with the regulation of virulence factor expression (thereby often affecting several virulence factors at once) or specifically inhibiting a certain virulence factor.

To prevent and control vibriosis, it is necessary to maintain a good culture environment and prevent the deterioration of water environment, and minimize the chance of introducing pathogens to the water body. Kocylowski (1963) recommended transfer fish to cold, well-aerated water to alleviate problems with vibriosis. *V. anguillarum* has been one of the few successful candidates for vaccine development. Complete protection is recorded (Relative percentage survival = 100 %) in sea bass (Angelidis et al., 2006) when a vaccine is applied by bathing prior to challenge. Recently, *V. anguillarum* has also been the target of modern approaches for vaccine development, i.e. DNA vaccine comprising a mutated *empA* gene (Yang et al., 2009) and multivalent vector vaccine based on secretary antigen-delivery system (Zhou et al., 2010). Moreover, probiotics, such as a non-pathogenic isolate of *V. alginolyticus* (Austin et al., 1995a) and *Pseudomonas fluorescens* AH2 (Gram et al., 1999), and quorum sensing inhibitor such as furanone C-30 (Rasch et al., 2004) are determined to be beneficial at reducing mortalities caused by *V. anguillarum*. 
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2. Vibrio anguillarum

2.1 Characteristics

Vibriosis caused by *V. anguillarum* is one of the most prevalent fish diseases. It has been reported in more than 50 freshwater and marine fish species including various species of economic importance to the larviculture and aquaculture industry (Toranzo et al., 2005). *V. anguillarum* is first isolated by Canestrini (1893), who designated the causal agent of ‘red-pest’ in eels as *Bacterium anguillarum*. A few years later, Bergman proposed the name *Vibrio anguillarum* for the aetiological agent of the ‘red-pest’ in the Baltic Sea (Bergman, 1909). Because of the high similarity between the disease signs and characteristics of the causal bacterium described in both reports, it was suggested that both concerned the same causative agent.

*V. anguillarum* is a Gram-negative, comma-shaped rod bacterium, belonging to the family Vibrionaceae. It is polarly flagellated, non-sporeforming, halophilic and facultatively anaerobic (Austin and Austin, 2012). The bacterium grows rapidly at temperatures between 25 and 30 °C on rich media containing 1.5–2 % sodium chloride (NaCl), forming cream-colored and round-shaped colonies (Frans et al., 2011). At present, 23 O serotypes (O1–O23) within *V. anguillarum* are distinguished on the basis of an O-antigen neutralization test, each displaying a different pathogenicity and host specificity. It has been demonstrated that serotypes O1 and O2, and to a lesser extent serotype O3, are associated with vibriosis in fish (Pedersen et al., 1999). The other *V. anguillarum* serotypes represent environmental isolates from sediment, plankton or sea water that are mostly non-pathogenic. In previous studies at our laboratory, 11 out of 16 *V. anguillarum* strains (representing the major pathogenic serotypes O1, O2 and O3) showed to be virulent towards sea bass larvae (Dierckens et al., 2009; Frans et al., 2013; Li et al., 2014). However, no relation could be found among virulence and serotype in these *V. anguillarum* strains.

2.2 Epizootiology
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*V. anguillarum* constitutes part of the normal microflora of the aquatic environment (West and Lee, 1982; Muroga et al., 1986). Experiments have suggested that *V. anguillarum* survives for >50 months in seawater (Hoff, 1989; Prol-García et al., 2010). When the immune system of fish is compromised or when the mucosal surfaces of the fish are damaged, *V. anguillarum* causes a fatal hemorrhagic septicemia in marine fish. For sea bass larvae (and also for juvenile and adult sea bass), the presence of *V. anguillarum* in the rearing environment can be considered as a threat, especially when rearing conditions favor fast-growing bacteria. This is similar to what De Schryver et al. (2014) discussed for early mortality syndrome (EMS) outbreaks in shrimp. Indeed, it has also been suggested that disease outbreaks can occur when *V. anguillarum* dominates in the larval intestine (Grisez et al., 1997). The infection route of *V. anguillarum* is still a major point of discussion in the literature. Typically, infected fish show skin discoloration, the presence of red necrotic lesions in the abdominal muscle, and erythema (bloody blotches) at the base of the fins, around the vent, and within the mouth. The gut and rectum may be distended, and filled with clear viscous fluid. Exophthalmia may be evident. Nevertheless, in acute epizootics, the infection spreads so rapidly that most of the infected fish die without showing any external signs (Anderson and Conroy, 1970; Toranzo et al., 2005; Actis et al., 2011; Austin and Austin, 2012).

2.3 Pathogenicity

*V. anguillarum* can invade fish epithelium at more than one site, including the skin and the intestinal tract (Grisez et al., 1996; Spanggaard et al., 2000; Rekecki et al., 2013). The exact mode of infection is unclear, but undoubtedly involves colonization of (attachment to) the host, and hence penetration of the tissues. Several (putative) virulence factors have been identified in *V. anguillarum*, including the iron uptake system, chemotaxis and motility, lipopolysaccharides (LPSs), exopolysaccharides (EPSs), haemolysin, proteases, outer membrane proteins (OMPs), and secretion systems.
The iron uptake system is one of the most important virulence factors in many pathogenic bacteria. In the host, iron is not freely available, and the concentration of free iron is much lower than what a bacterium needs for growth. Bacteria need to overcome this iron limitation to survive and establish an infection (Skaar, 2010). Most of the V. anguillarum serotype O1 pathogenic strains contain a virulence plasmid pJM1, which is associated with iron uptake system under iron limited conditions (Actis et al., 1986; Stork et al., 2002; Di Lorenzo et al., 2003). It is demonstrated that possession of the pJM1 iron uptake genetic determinants is essential to cause disease (Walter et al., 1983). Different from the plasmid-mediated system, many V. anguillarum strains belonging to serotype O2 and O3 express a chromosomally encoded iron uptake system (Lemos et al., 1988; Muiño et al., 2001).

Chemotaxis and motility are necessary for virulence of V. anguillarum (Larsen et al., 2004), particularly invasion of the host. Chemotaxis is an important first step in infection. V. anguillarum is attracted to amino acids and carbohydrates particularly in intestinal and to a lesser extent to skin mucus (O’Toole et al., 1999). A chemotaxis mutant of V. anguillarum is deficient in host colonization and avirulent towards fish in an immersion challenge, while no decreased virulence is observed when fish are infected intraperitoneally (O’Toole et al., 1996). It is suggested that chemotaxis plays an important role in the movement of the pathogen towards the host, but not in infection and persistence in the host. Evidence points to a flagellin A protein (encoded by the flaA gene) being essential for virulence (Milton et al., 1996). Thus, loss of flagella by transposon mutagenesis led to a 500-fold reduction in virulence following an immersion challenge (O’Toole et al., 1996).

Lipopolysaccharides (LPSs), found in the outer membrane of some Gram negative bacteria, play an important role in the pathogenesis of certain bacterial infections because they are capable of acting as immunostimulators and immunomodulators in the host animals (Moran et al., 1996). LPSs are large molecules consisting of a lipid and a polysaccharide composed of O-antigen, outer core and inner core joined by a covalent bond. The O-antigen polysaccharide of V. anguillarum is
shown to be essential for virulence in fish animal models, for resistance to complement-mediated killing found in fish blood serum (Boesen et al., 1999), and for iron uptake system (Welch and Crosa, 2005). Moreover, mutants of V. anguillarum defective in O-antigen polysaccharide transport failed to colonize rainbow trout skin (Lindell et al., 2012).

Exopolysaccharides (EPSs) are important for bacterial biofilm formation (Karatan and Watnick, 2009). A biofilm is a polymeric conglomeration generally composed of extracellular DNA, proteins, and polysaccharides. EPS production by biofilm bacteria serves many functions, including the facilitation of the initial attachment of bacteria to surfaces, the formation and maintenance of micro colony and biofilm structure, enhanced biofilm resistance to environmental stress and antimicrobial agents, protection of the biofilm from protozoan grazing, and biofilm nutrition (Costerton et al., 1995). EPSs are required for V. anguillarum in order to be able to attach to fish tissues (Croxatto et al., 2007). A V. anguillarum mutant defective for EPS export is attenuated for virulence towards fish (Weber et al., 2010).

Haemolysins are exotoxins that cause lysis of blood cells in the host. They are considered to be responsible for hemorrhagic septicemia during infection (Frans et al., 2011). Two haemolysin gene clusters, vah1-plp and rtxACHBDE, have been previously identified and described in V. anguillarum (Hirono et al., 1996; Li et al., 2008). Loss of rtxA function results in avirulence of V. anguillarum towards juvenile Atlantic salmon (Li et al., 2008). Strains with mutations in plp or in both plp and vah1 exhibited no significant reduction in virulence towards rainbow trout (Li et al., 2013). Furthermore, V. anguillarum mutant defective for hns gene, which acts as a regulator of both gene clusters, exhibits significantly attenuated virulence towards rainbow trout (Mou et al., 2013).

Proteases are considered as (putative) virulence factors in several pathogens. Extracellular metalloprotease EmpA has been shown to account for nearly 90 % of the total extracellular proteolytic activity of V. anguillarum (Han et al., 2011a). It has been reported to be involved in mucus degradation and penetration (Norqvist et al., 1990;
Denkin and Nelson, 1999). Induction of this protease activity varies between different V. anguillarum strains (Staroscik et al., 2005). Loss of empA function results in avirulence of V. anguillarum against Atlantic salmon and turbot (Denkin and Nelson, 2004; Han et al., 2011a). Despite of the reports about obvious contribution of EmpA to virulence of V. anguillarum, the relationship between production of EmpA and pathogenesis is still controversial (Denkin and Nelson, 2004).

**Outer Membrane Proteins (OMPs)** are abundant in the bacterial outer membrane and are suggested to play an important role in bile resistance (Wang et al., 2003). The ability to resist bile is important for survival in and colonization of the intestine. Bacteria regulate resistance to bile by altering their membrane permeability. In many vibrios such as V. cholerae, V. splendidus, V. alginolyticus, a major OMP OmpU is involved in virulence (Chakrabarti et al., 1996; Cai et al., 2010; Duperthuy et al., 2010). In V. anguillarum, OmpU is required for bile resistance, but not for virulence (Wang et al., 2003).

V. anguillarum has been reported to contain a **type VI secretion system** (T6SS) (Weber et al., 2009). T6SS was first characterized in V. cholerae (Pukatzki et al., 2006). Since its discovery, T6SSs have been shown to be required for several phenotypes in pathogenic bacteria: virulence of Burkholderia cenocepacia toward mice, survival of Aeromonas hydrophila in macrophages, resistance of V. cholerae to amoeba predation, biofilm formation by enteroaggregative Escherichia coli (Aschtgen et al., 2008; Hunt et al., 2004; Pukatzki et al., 2006; Suarez et al., 2008). T6SS have been reported to positively regulate expression of the stress-response regulator RpoS and the quorum sensing regulator VanT in V. anguillarum. The T6SS proteins also inversely regulate expression of two extracellular proteases, EmpA and PrtV (Weber et al., 2009). V. anguillarum also encodes components of Type I and Type II secretion. However, Type I and Type II secretion systems have not yet been identified in V. anguillarum.

3. **Regulation of virulence factor production**
Bacteria scan diverse small molecules to access information about both their extracellular environment and their intracellular physiological status, and based on this information, they continuously interpret their circumstances and mount appropriate responses to environmental changes (Camilli and Bassler, 2006). The phenotypic response to signaling molecules often leads to the regulation of virulence related gene expression. Interfering with the regulation of virulence factor expression (thereby often affecting several virulence factors at once) and specifically inhibiting a certain virulence factor are alternative strategies to control disease caused by pathogenic bacteria. Because antivirulence therapy does not aim at killing the pathogen and many virulence factors are organism-specific, a major advantage of this therapy is that it will not affect the commensal (harmless and/or beneficial) microbiota (Barczak and Hung 2009).

3.1 Quorum sensing

One major role of bacterial extracellular small-molecule signaling is in cell to cell communication (quorum sensing, QS), which involves the production, release, and community-wide detection of molecules called autoinducers (Waters and Bassler, 2005). *V. anguillarum* has been documented to contain two quorum sensing systems, a ‘classical’ acyl homoserine lactone (AHL) system involving the signal synthase/receptor pair VanI/VanR, and a three-channel system as found in many vibrios (Milton, 2006). Figure 1.4 depicts the high cell density quorum-sensing model in *V. anguillarum*.

3.1.1. Acyl homoserine lactone (AHL) quorum sensing

AHLs consist of a common hydrophilic homoserine lactone ring moiety and a hydrophobic acyl side chain of variable length, allowing the water soluble AHL to freely pass cell membranes. AHL signal molecules, which have been recognized in *V. anguillarum*, may well have a role in the expression of virulence factors, i.e. biofilm formation and protease production (Buchholtz et al., 2006). *V. anguillarum* produces two dominant AHLs, i.e. N-(3-oxodecanoyl)-L-homoserine lactone (synthase/receptor pair VanI/R) and N-(3-hydroxy-hexanoyl)-L-homoserine lactone (synthase/receptor pair
**Figure 1.4** Model of *V. anguillarum* quorum-sensing systems (detailed information in the text). Solid lines indicate gene regulation. Double arrowhead indicates phosphorelay, and a single arrowhead indicates gene activation. Line with cross bar indicates gene inhibition. H1, H2, D1 and D2 are conserved histidine and aspartate residues in the hybrid two-component systems (adapted from Lindell, 2012, PhD thesis).
VanM/N). Smaller amounts of other molecules are also present. Similar to V. fischeri, a link between the VanI/R system and VanM/N system is found since VanM regulates signal production via VanI (Milton et al., 2001).

### 3.1.2. Three-channel quorum sensing systems in vibrios

The three-channel system in V. anguillarum involves the production of three different types of signal molecules, which are detected by membrane bound receptors VanN, VanQ, and CqsS. These receptors feed a shared phosphorylation/dephosphorylation signal transduction cascade controlling the production of the quorum sensing master regulator VanT. VanT positively regulates metalloproteases, pigment production, biofilm production, and negatively regulates type IV secretion system (Croxatto et al., 2002). One of the central components of the signal transduction cascade is VanO, which is phosphorylated in the absence of signal molecules, whereas it is dephosphorylated in the presence of high levels of signal molecules (Weber et al., 2011). The phosphorylation status of VanO is activated and together with the alternative sigma factor RpoN (σ^N), induces expression of small regulatory RNA Qrr1 (quorum regulatory RNA) and determines the production of the master regulator VanT (Croxatto et al., 2002; Weber et al., 2008). A second response regulator, RR-2, belongs to the NtrC protein family and may be phosphorylated by VanU. Active and phosphorylated RR-2 is predicted to inhibit Qrr1-4 expression (Weber et al., 2011). VanM is directly regulated by VanT which binds vanM promoter and activates transcription, but negatively regulated by the RNA chaperone Hfq (Lindell et al., 2012, PhD thesis). Moreover, the alternative sigma factor RpoS (σ^S) has been reported to be an additional factor controlling the production of VanT (Weber et al., 2008) and important in V. anguillarum pathogenicity (Ma et al., 2009).

### 3.1.3. Indole signaling

Indole is a relatively recent addition to the list of signaling molecules used by bacteria (Lee and Lee, 2010). Indole is produced as a by-product of the breakdown of
tryptophan by the enzyme tryptophanase (TnaA) (Newton and Snell, 1965) and is widespread in the natural environment. By far, at least 85 bacterial species have been shown to produce large quantities of this molecule, including both Gram-positive and Gram-negative bacteria of which many are pathogens (Lee and Lee, 2010). Most work in this respect has been done mainly on E. coli, in which indole and TnaA have been reported to be responsible for virulence-related phenotypes such as biofilm formation, motility, chemotaxis and adherence to epithelial cells (Lee et al., 2007a; Bansal et al., 2010) and necessary for virulence to nematodes (Anyanful et al., 2005). The only published report in vibrios documents that in V. cholerae, indole affects polysaccharide production, biofilm formation and grazing resistance (Mueller et al., 2009). Grisez et al. (1991) has investigated that most of the V. anguillarum strains (63 out of 68 strains) produce indole by using API 20E.

3.2 Alternative sigma factors

A sigma factor (σ factor) is a protein needed only for initiation of RNA synthesis. It is a bacterial transcription initiation factor that enables specific binding of RNA polymerase to gene promoters (Gruber and Gross, 2003). In addition to housekeeping sigma factors that control transcription of essential genes, bacteria also possess alternative sigma factors that recognize the promoters of a specific set of genes. Alternative sigma factors can directly regulate expression of virulence genes or indirectly affect virulence by enhancing survival against host defense and other stress conditions (Kazmierczak et al., 2005). Alternative sigma factors can be classified into two structurally unrelated families: the σ\(^{70}\) (including RpoS) and the σ\(^{54}\) (including RpoN) families. RpoS plays a key role in the survival of bacteria during starvation or exposure to stress conditions and is required for the expression of many genes in the stationary phase of growth (Lacour and Landini, 2004). RpoS is required for virulence in many pathogens, such as Borrelia burgdorferi, V. cholerae, V. anguillarum, Burkholderia plantarii, and Salmonella enterica (Hengge-Aronis, 2002; Dong and Schellhorn, 2010). In some pathogens including Erwinia carotovora and Pseudomonas aeruginosa, mutants
defective for RpoS are more virulent (Dong and Schellhorn, 2010). However, the virulence of \textit{V. anguillarum} \textit{rpoS} mutant is severely reduced in zebrafish (\textit{Danio rerio}) (Ma et al., 2009). This mutant is also impaired in production of extracellular enzymes, including phospholipase, diastase, lipase, caseinase, haemolysin, catalase, and protease.

3.3 Sensing of host factors

To be successful pathogens, bacteria must often restrict the expression of virulence genes to host environments. This requires a bacterial system to sense for the presence of a host in order to appropriately time the activation of virulence factors.

3.3.1 Mucus

Fish mucus is considered to play important roles in the survival of fish (Shephard, 1994). Mucus contains many biochemical agents (lectins, proteases, mucins etc.) capable of reaction against infective organisms and thus providing the host with an immediate or a first line defence mechanism (Ingram, 1980). Several studies indicate that the initiation of \textit{V. anguillarum} infection occurs after the attachment of the pathogen to the mucosal surfaces (skin and intestine) of fish (Spanggaard et al., 2000; O’Toole et al., 2004). Therefore, the response to the host factor mucus is an essential requirement for the infection by \textit{V. anguillarum}. Mucus from skin and intestine has been reported to enhance growth and the production of virulence factors such as metalloprotease and chemotactic motility in \textit{V. anguillarum} (Garcia et al., 1997; Denkin and Nelson, 1999; O’Toole et al., 1999; Larsen et al., 2001). The main constituents of mucus are heavily glycosylated proteins known as mucins (Forstner and Forstner, 1994). These are glycoproteins secreted by the mucosal and submucosal glands. The mucin molecule consists of a polypeptide core with branched oligosaccharide side chains (o-linked glycan) (Thornton et al., 1996). Mucin has been reported to promote motility, biofilm formation and antibiotic resistance in \textit{P. aeruginosa} (Landry et al., 2006; Yeung et al., 2012).
3.3.2 Bile

Bile, a digestive fluid produced by the liver of most vertebrates, is a heterogeneous mixture of bile acids, inorganic salts, cholesterol and other components (Begley et al., 2005). Bile has been reported to induce the expression of virulence genes involved in adhesion, capsule production, haemolysin production and type III secretion system in V. parahaemolyticus (Pace et al., 1997; Osawa et al., 2002; Gotoh et al., 2010). Although bile has been reported to repress the expression of genes encoding cholera toxin and toxin-coregulated pili (Gupta and Chowdhury, 1997; Schuhmacher and Klose, 1999), V. cholerae recognizes a set of bile salts as signals to activate the virulence regulator TcpP (Yang et al., 2013). Resistance to bile has been reported to be critical for V. anguillarum to colonize the intestines of the fish host (Horne and Baxendale, 1983; Olsson et al., 1996; Olsson et al., 1998).

3.3.3 Catecholamine stress hormones and cholesterol

Under rearing conditions, fish are exposed to various stressful conditions. Stress can lead to changes in the level of various hormones. There are three broad categories of mammalian hormones: proteins (or peptides), steroids (a subclass of lipidic hormones including cholesterol) and amino-acid derivatives (or amines including adrenaline, noradrenaline and dopamine) (Hughes and Sperandio, 2008). Noradrenaline stimulates the growth of V. parahaemolyticus and V. mimicus, but not V. cholerae and V. vulnificus in the presence of transferrin (Nakano et al., 2007b). It also modulates V. parahaemolyticus pathogenicity via type III secretion systems (Nakano et al., 2007a). Norepinephrine and dopamine increase the growth and motility of pathogenic V. campbellii and V. anguillarum, and significantly increase the virulence of V. campbellii toward giant freshwater prawn larvae (Macrobrachium rosenbergii) (Pande et al., 2014). Injection of noradrenaline or adrenocorticotropic hormone causes higher mortality and accumulation of V. splendidus in challenged oysters (Lacoste et al., 2001). Cholesterol has been reported to serve as a binding site of V. vulnificus on the plasma membrane of target cells (Kim and Kim, 2002). Cholesterol increases the motility of V. cholerae while it
has no effect on growth or the expression of the major virulence genes of *V. cholerae*, *ctxAB* and *tcpA* (Chatterjee et al., 2007).

### 3.4 Knowledge gaps

The pathogenicity mechanisms of *V. anguillarum* are not yet completely understood. To our knowledge, the bacterium produces a number of (putative) virulence factors. Three factors that have been reported to be essential for pathogenicity include the iron uptake system (Crosa, 1980; Stork et al., 2007), chemotactic motility (McGee et al., 1996; O'Toole et al., 1996) and exopolysaccharide (Croxatto et al., 2007). The bacterium produces a number of other (putative) virulence factors, including haemolysin, lipase and protease (Norqvist et al., 1990; Rock and Nelson, 2006; Yang et al., 2007). However, whether or not these factors are really essential for pathogenicity is currently not clear. In this dissertation, several virulence-related phenotypes (chemotaxis, motility, EPSs, haemolysin and proteases) were used as virulence indicators. Quorum sensing regulates a number of target genes, including a few virulence genes in *V. anguillarum*. However, further research on the role of quorum sensing in the pathogenesis of *V. anguillarum* is needed. Moreover, the impact of other regulatory mechanisms (including signaling mechanisms) as well as sensing of and responding to host factors (mucin, bile salts and cholesterol) is currently unknown or not completely clear.

### 4. Thesis outline

The general objective of this study is to evaluate the impact of quorum sensing systems and the host factors on the virulence of *V. anguillarum* in a model system with gnotobiotic sea bass larvae. The specific objectives and the thesis outline are as following:
- **Chapter 2** aims at verifying the effect of dead hosts (either homogenised sea bass larvae or brine shrimp) on the virulence of *V. anguillarum* by using a model system with gnotobiotic sea bass larvae. In order to accommodate for this dead hosts’ effect, an improved challenge set-up of the sea bass model was developed for later experiments.

- **Chapter 3** aims at investigating the impact of quorum sensing and the alternative sigma factor RpoS on the virulence of *V. anguillarum* towards gnotobiotic sea bass larvae. Mutants defective for the quorum sensing system or *rpoS* gene were tested in the model system improved in chapter 2. The role of indole in the virulence of *V. anguillarum* was also evaluated.

- **Chapter 4** aims at verifying the effect of the host factors mucin, bile salts and cholesterol on several virulence-related phenotypes in *V. anguillarum* and on the virulence of the bacterium towards gnotobiotic sea bass larvae.

- **Chapter 5** aims at examining the response of 15 different wild type *V. anguillarum* strains to the host factors mucin, bile salts and cholesterol and determining correlations between this response and the virulence to sea bass larvae in order to determine whether the capability to respond to host factors is linked to virulence.

- **Chapter 6** summarizes the overall findings obtained in this thesis and provides some directions for future research.
Chapter 2

Host-induced increase in larval sea bass mortality in a gnotobiotic challenge test with *Vibrio anguillarum*

Abstract

*Vibrio anguillarum* is the major cause of haemorrhagic septicaemia, vibriosis, which is a severe disease affecting marine fish. In this work, it was found that the mortality of gnotobiotic sea bass larvae challenged with *V. anguillarum* was dependent on the number of dead fish in the vials at the moment of challenge. Based on this finding, the effect of dead hosts (homogenised sea bass larvae or brine shrimp) on the virulence of *V. anguillarum* towards sea bass larvae was further investigated. Addition of homogenised hosts led to significantly increased larval mortality of challenged larvae, and this was observed for 3 different *V. anguillarum* strains, i.e. 43, NB10 and HI610. In contrast, the addition of similar levels of tryptone had no effect on mortality. In line with this, the motility of all 3 *V. anguillarum* strains was significantly increased by the addition of homogenised hosts but not by tryptone. These results suggest that dead hosts increase infectivity of *V. anguillarum*, not merely by offering nutrients to the bacteria, but also by increasing virulence-associated activities such as motility.
Chapter 2

Introduction

*Vibrio anguillarum* is the causative agent of vibriosis, a fatal haemorrhagic septicaemia affecting a variety of fish species and other aquatic animals, and causing large economic losses in the aquaculture industry worldwide (Austin and Austin, 2012). This is especially significant for European sea bass *Dicentrarchus labrax*, a species that is very sensitive to stressors and pathogens, and infections are often the major limiting factor for successful rearing (Torrecillas et al., 2007). A few virulence-related factors and genes have been identified in *V. anguillarum* (Frans et al., 2011), including genes affecting chemotaxis and motility (McGee et al., 1996; O’Toole et al., 1996), an iron uptake system (Crosa, 1980; Stork et al., 2007), haemolytic activity (Rock and Nelson, 2006), extracellular metalloprotease (Norqvist et al., 1990; Yang et al., 2007), lipopolysaccharides (Milton et al., 1995; Welch and Crosa, 2005) and exopolysaccharides (Croxatto et al., 2007). However, the mechanisms of pathogenicity behind the disease caused by the bacterium are not yet completely understood.

As virulence factors produced by vibrios are often costly metabolic products, their production is generally tightly regulated (Ruwandeepika et al., 2012). Regulatory mechanisms that have been reported to control virulence gene expression in vibrios include quorum sensing and the ToxR regulon. However, neither of these has been found to affect the virulence of *V. anguillarum* (Wang et al., 2002; Milton, 2006). In addition to these regulatory mechanisms, host cues such as mucus (Hsiao et al., 2006), bile (Gotoh et al., 2010), bicarbonate (Abuaita and Withey, 2009), catecholamine hormones and lipid hormones (Hughes and Sperandio, 2008) are also known to affect virulence gene expression in pathogenic bacteria. However, to the best of our knowledge, the role of host cues in the pathogenicity of *V. anguillarum* has not yet been investigated.

In this study, we aimed at investigating whether the presence of dead hosts has an effect on the virulence of *V. anguillarum* towards sea bass larvae cultured under highly controlled gnotobiotic conditions.
Materials and methods

Bacterial strains and culture conditions

V. anguillarum strains 43 (B. Austin, Heriot-Watt University, U.K.), NB10 (Norqvist et al., 1989) and HI610 (O. Bergh, Institute of Marine Research, Norway) were used in this study. These strains have also been tested in a previous study with gnotobiotic sea bass larvae at our laboratory (Dierckens et al., 2009). All strains were made rifampicin resistant by natural selection, allowing enumeration after introduction into the culture water of the axenic sea bass larvae. The bacteria were grown in 10 % marine broth (Difco Laboratories, U.K.) with the addition of Instant Ocean artificial sea salt (Aquarium Systems, Sarrebourg, France) to obtain a salinity of 36 g l⁻¹ on a horizontal shaker (150 rpm) at 16 °C for 48 h. The density of the bacterial suspensions was determined with a spectrophotometer (Genesys 20, Thermo Scientific, U.S.A.) at 550 nm according to the McFahrland standard (BioMérieux, Marcy L’Etoile, France).

Challenge tests with V. anguillarum and gnotobiotic sea bass larvae

The disinfection of sea bass eggs, hatching and tests for axenity were performed according to Dierckens et al. (2009). On DAH 0, sea bass larvae were stocked in groups of 12 animals in 10 ml sterile screw cap vials with the addition of 10 mg l⁻¹ rifampicin. Ten replicates were prepared for each treatment. V. anguillarum strain HI610 was added to the sterile culture water on DAH 3 or DAH 7 at 10⁵ CFU ml⁻¹ (bath challenge). The survival was checked on DAH 3, 5, 7, 9 and 11 by counting the living sea bass larvae (transparent and swimming) under a dissecting microscope. Survival was monitored in the same vials for all the sampling points. The larvae were not fed during the experiment. Each experiment was repeated 3 times with a different batch of sea bass larvae. All tests in this study were approved by the ethical committee of Ghent University (EC2012/071).

Preparation of homogenised larvae
Chapter 2

The axenic live sea bass larvae were killed using an overdose of benzocaine, rinsed 3 times with sterile artificial sea water (36 g l⁻¹) and homogenised using a sterile ground-glass tissue minihomogeniser. The homogenised fish suspension was kept frozen at –20 °C until use. The homogenised brine shrimp were prepared in the same way.

Impact of homogenised hosts on the virulence of *V.anguillarum* towards gnotobiotic sea bass larvae

Axenic larvae were stocked immediately before challenging (both on DAH 3). *V.anguillarum* strains 43, NB10 and Hi610 were added to the culture water at 10⁵ CFU ml⁻¹ with and without the addition of 1 or 10 mg l⁻¹ tryptone, or 3 mg l⁻¹ homogenised sea bass larvae or brine shrimp *Artemia franciscana* (dry weight). Ten replicates were prepared for each treatment. The survival was checked on DAH 11. The larvae were not fed during the experiment.

Determination of the bacterial density associated with the fish larvae and the culture water

The bacterial density associated with the fish larvae and the culture water (in the experiment with the addition of 3 mg l⁻¹ fish larvae or *Artemia*) was determined on DAH 7, 9 and 11. The sampling of live sea bass larvae and the culture water and the subsequent quantification of the bacterial density was performed according to Dierckens et al. (2009) with some modifications. Living sea bass larvae contaminated with *V.anguillarum*, with and without the addition of 3 mg l⁻¹ homogenised larvae (dry weight), were killed using an overdose of benzocaine, rinsed 3 times with sterile artificial sea water (36 g l⁻¹) and homogenised. Subsequently, the homogenised fish suspension and culture water were plated on 10 % marine agar (10 % marine broth and 1.5 % agar with the addition of Instant Ocean artificial sea salt to obtain a salinity of 36 g l⁻¹) and incubated for 48 h at 28 °C.

Virulence factor assays
Lipase, phospholipase, caseinase, gelatinase and haemolysin activities were assessed according to Natrah et al. (2011). Motility was assessed as described previously (Wang et al., 2011) on marine broth with 0.3 % agar. Briefly, 2 μl of each *V. anguillarum* overnight culture (OD$_{550}$ = 0.5) was point inoculated in the middle of soft agar plates. After incubation for 24 h at 28 °C, motility halos were measured. The impact of homogenised hosts was studied by adding the same density of homogenised fish or brine shrimp (3 mg l$^{-1}$ dry matter) to the agar as used in the challenge tests. In an additional treatment, 6 mg l$^{-1}$ tryptone (i.e. double the amount of dry matter added through addition of homogenised hosts) was used to test the effect of the overall nutrient level.

**Statistics**

The data were analyzed using one-way ANOVA followed by Tukey’s post-hoc test or by independent samples t-tests. For the comparison of the survival of the sea bass larvae, data were arcsin transformed before one-way ANOVA analysis. All statistical analyses were done using the Statistical Package for the Social Sciences (SPSS) software, version 19.

**Results and discussion**

A challenge test with *V. anguillarum* strain HI610 and gnotobiotic sea bass larvae was performed according to Dierckens et al. (2009). In this set-up, variable mortality of sea bass larvae was observed in the vials between stocking (DAH 0) and the day of *V. anguillarum* bath challenge (DAH 7). Interestingly, these ‘dead before challenge’ larvae significantly affected the survival of sea bass larvae after challenge with strain HI610. The mortality of the challenged sea bass larvae increased with increasing numbers of ‘dead before challenge’ larvae in the vials (Figure 2.1A). In contrast, there was no relation between the mortality of non-challenged larvae between DAH 7 and DAH 11, and the number of dead larvae present in the vials on DAH 7 (Figure 2.1B). These data are representative for 3 independent experiments. This observation was confirmed by
another independent sea bass challenge test. In this test, the larvae were stocked on DAH 0 and bath-challenged with strain HI610 on DAH 3. There was a positive correlation between the mortality of challenged sea bass larvae and the number of dead larvae before challenge (Figure 2.1C), while there was no such relation in the non-challenged group (Figure 2.1D). This increase in larvae mortality was significantly higher when the number of dead larvae before challenge was equal to or higher than 3.

Based on this finding, we set up a new challenge protocol to investigate the effect of dead hosts on the mortality of sea bass larvae due to *V. anguillarum*. The sea bass larvae were stocked on DAH 3 and immediately challenged to avoid the uncontrolled presence of dead larvae before challenge. Three homogenised fish larvae per vial (3 mg l\(^{-1}\) dry matter) were added to simulate the presence of dead larvae. *V. anguillarum* has been reported to be pathogenic to brine shrimp *Artemia franciscana* (Defoirdt et al., 2005), and therefore homogenised brine shrimp (3 mg l\(^{-1}\) dry matter) was used as a second host potentially influencing the outcome of a *V. anguillarum* challenge. Tryptone, at different concentrations, was used as a control. Neither homogenised hosts nor different concentrations of tryptone had any effect on the survival of unchallenged sea bass larvae. Strains 43, NB10 and HI610 did not cause significant mortality when added to the culture water at 10\(^5\) CFU ml\(^{-1}\) in the absence of any other addition. However, in the presence of homogenised fish or brine shrimp, all 3 strains caused significant mortality (Table 2.1). Strain 43 was defined as avirulent towards sea bass larvae in a previous study (Dierckens et al. 2009). However, the addition of homogenised fish together with strain 43 resulted in significant mortality of the sea bass larvae.
Figure 2.1 Sea bass larvae challenged with *V. anguillarum*. (A, B) Mortality between Day 7 after hatching (DAH 7) and DAH 11 (A) of gnotobiotic sea bass larvae challenged with *V. anguillarum* HI610 on DAH 7 and (B) of axenic larvae, as a function of the number of dead larvae present in the vial on DAH 7, and (C,D) mortality between DAH 3 and DAH 11 (C) of gnotobiotic larvae challenged with *V. anguillarum* HI610 on DAH 3 and (D) of axenic larvae, as a function of the number of dead larvae present in the vial on DAH 3. Error bars represent the standard error. These data are representative for 3 independent experiments.
**Table 2.1** Relative percentage survival of gnotobiotic sea bass larvae. Relative percentage survival of gnotobiotic sea bass larvae on Day 11 after hatching (DAH 11) challenged with *V. anguillarum* strains (mean ± SE of 10 replicates). Relative percentage survival = 1 (% mortality in the challenge with treatment group/% mortality in the challenge group) × 100. **P < 0.01** as compared with corresponding challenge control value.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative percentage survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>1 mg l⁻¹ tryptone</td>
<td>104 ± 5</td>
</tr>
<tr>
<td>10 mg l⁻¹ tryptone</td>
<td>109 ± 11</td>
</tr>
<tr>
<td>Homogenised fish</td>
<td>89 ± 4</td>
</tr>
<tr>
<td>Homogenised brine shrimp</td>
<td>99 ± 3</td>
</tr>
<tr>
<td>NB10</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>NB10 + 1 mg l⁻¹ tryptone</td>
<td>103 ± 8</td>
</tr>
<tr>
<td>NB10 + 10 mg l⁻¹ tryptone</td>
<td>82 ± 9</td>
</tr>
<tr>
<td>NB10 + homogenised fish</td>
<td>24 ± 5**</td>
</tr>
<tr>
<td>NB10 + homogenised brine shrimp</td>
<td>35 ± 2**</td>
</tr>
<tr>
<td>HI610</td>
<td>100 ± 11</td>
</tr>
<tr>
<td>HI610 + 1 mg l⁻¹ tryptone</td>
<td>80 ± 13</td>
</tr>
<tr>
<td>HI610 + 10 mg l⁻¹ tryptone</td>
<td>75 ± 13</td>
</tr>
<tr>
<td>HI610 + homogenised fish</td>
<td>18 ± 12**</td>
</tr>
<tr>
<td>HI 610 + homogenised brine shrimp</td>
<td>0 ± 0**</td>
</tr>
<tr>
<td>43</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>43 + homogenised fish</td>
<td>41 ± 13**</td>
</tr>
</tbody>
</table>
Together, these experiments (Figure 2.1, Table 2.1) demonstrate that sea bass larval mortality after bath challenge with *V. anguillarum* is affected by the presence of host substances, as none of the strains was virulent in the absence of homogenised or dead larvae, whereas they were virulent in their presence. Hence, the addition of homogenised fish at the moment of challenge constitutes an experimental improvement, as it makes the outcome of the challenge independent from the presence of dead larvae.

The addition of 3 mg l⁻¹ organic matter in the form of homogenised fish larvae or brine shrimp constitutes a source of nutrients for the added pathogens. This was clearly confirmed by the increase in *Vibrio*-CFU in the sea bass culture water (Table 2.2). At the majority of sampling points (6 out of 7 cases), independently of the strain used, the bacterial density was significantly higher in the treatments receiving homogenised fish or brine shrimp. The increase in bacterial density in the treatments receiving homogenised fish or brine shrimp was less strong for the fish larvae-associated bacteria (only 2 out of 7 cases).

With the addition of homogenised host (3 mg l⁻¹ dry matter) in sterile artificial sea water, *V. anguillarum* strains 43, NB10 and Hi610 grew from an initial density of 10⁵ CFU ml⁻¹ to approximately 10⁶ CFU ml⁻¹. A similar increase in *V. anguillarum* density was observed after addition of 10 mg l⁻¹ tryptone. Thus, a higher number of bacteria can be ruled out as the sole reason for the higher mortality.
Chapter 2

Table 2.2 *V. anguillarum* infecting sea bass larvae. Density of the *V. anguillarum* strains 43, NB10 and HI610 associated with live sea bass larvae and in the larvae culture water (average ± SE of 3 fish cultures), with and without addition of homogenised fish or brine shrimp to the culture water on Day 3 after hatching (DAH 3). The larvae were challenged on DAH 3 by adding the strains to the culture water at $10^5$ CFU ml$^{-1}$. The samples were from the experiment reported in Table 1. HF: homogenised fish (3 mg l$^{-1}$ dry matter), NA: not available. * Significantly different from the corresponding treatment without homogenised fish ($P < 0.05$).

<table>
<thead>
<tr>
<th>Time point (DAH)</th>
<th>Larvae log(CFU larva$^{-1}$)</th>
<th>Culture water log(CFU ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control + HF</td>
<td>Control + HF</td>
</tr>
<tr>
<td><em>V. anguillarum</em> 43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>NA 4.5 ± 0.1</td>
<td>NA 6.1 ± 0.1</td>
</tr>
<tr>
<td>9</td>
<td>4.4 ± 0.0</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>11</td>
<td>4.4 ± 0.4</td>
<td>5.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>3.5 ± 0.3</td>
<td>6.3 ± 0.3*</td>
</tr>
<tr>
<td><em>V. anguillarum</em> NB10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4.1 ± 0.1</td>
<td>5.9 ± 0.4</td>
</tr>
<tr>
<td>9</td>
<td>4.5 ± 0.5</td>
<td>7.3 ± 0.0</td>
</tr>
<tr>
<td>11</td>
<td>4.8 ± 0.1</td>
<td>6.7 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>4.8 ± 0.5</td>
<td>7.1 ± 0.2*</td>
</tr>
<tr>
<td><em>V. anguillarum</em> HI610</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>NA 5.4 ± 0.1</td>
<td>4.6 ± 0.0</td>
</tr>
<tr>
<td>9</td>
<td>3.6 ± 0.0</td>
<td>NA 6.7 ± 0.1</td>
</tr>
<tr>
<td>11</td>
<td>3.6 ± 0.3</td>
<td>6.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>4.1 ± 0.2</td>
<td>7.1 ± 0.1*</td>
</tr>
</tbody>
</table>
Table 2.3 Motility of *V. anguillarum* strains 43, NB10 and HI610, with and without the addition of tryptone, homogenised fish or homogenised brine shrimp (average ± SE of 3 replicates). Values in the same column with different superscript letters are significantly different (*P* < 0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>43</th>
<th>NB10</th>
<th>HI610</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29.7 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.0 ± 2.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>48.7 ± 1.9&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tryptone</td>
<td>30.0 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.3 ± 1.8&lt;sup&gt;A&lt;/sup&gt;</td>
<td>49.7 ± 0.9&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>Homogenised fish</td>
<td>56.3 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59.0 ± 0.6&lt;sup&gt;B&lt;/sup&gt;</td>
<td>68.0 ± 1.5&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td>Homogenised brine shrimp</td>
<td>59.0 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>63.3 ± 1.7&lt;sup&gt;B&lt;/sup&gt;</td>
<td>69.3 ± 1.2&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

As host cues are also known to affect virulence gene expression in pathogenic bacteria, in a final series of experiments, we investigated whether homogenised hosts had an effect on virulence factor activities of the vibrios. The addition of homogenised fish or brine shrimp significantly increased the motility of all 3 *V. anguillarum* strains on soft agar plates (Table 2.3). The addition of 6 mg l<sup>−1</sup> tryptone (i.e. double the amount of dry matter when compared to the homogenised host treatments), in contrast, did not affect the motility. This result suggests that the increase in motility on plates with homogenised hosts was not due to increased growth of the bacteria caused by the extra nutrients added through the homogenised hosts. However, other virulence factors (including lipase, phospholipase, caseinase, gelatinase and haemolysin activities) were not affected by the addition of homogenised hosts (data not shown).

Studies have shown that for many pathogens, motility is essential in some phases of their life cycle and that virulence and motility are often intimately linked by complex regulatory networks (Josenhans and Suerbaum, 2002). *V. anguillarum* uses motility to colonize its aquatic hosts, which is essential for this bacterium to cause disease (Weber et al., 2010). Non-motile *V. anguillarum* mutants have been constructed and were found to be avirulent in a bath challenge, whereas infection via intraperitoneal
injection showed no loss in virulence (O’Toole et al., 1996). The importance of motility for transmission and invasion has been corroborated using mutants defective in producing the major flagellin FlaA (Milton et al., 1996). Ormonde et al. (2000) showed that motility aids invasion by *V. anguillarum*, both *in vitro* and *in vivo*. Therefore, our results suggest that the larval mortality in treatments receiving homogenised fish or brine shrimp was not only due to an increased bacterial density, but also to increased virulence-associated activity of the *V. anguillarum* strains. Similar results have been reported for *V. cholerae*, where cells shed from the human gastrointestinal tract showed greatly enhanced infectivity, which led the authors to hypothesise that epidemics may be propagated by the human host (Merrell et al., 2002). This increased infectivity was linked to high expression levels of genes required for nutrient acquisition and motility. Substances present in homogenised fish or brine shrimp that have been previously reported to enhance motility in pathogenic bacteria include mucus (Burghoff et al., 1993; Hsiao et al., 2006), catecholamine hormones, i.e. norepinephrine (Hegde et al., 2009), and lipid hormones, i.e. cholesterol (Chatterjee et al., 2007; Gilk et al., 2013). These factors might be responsible for the increased virulence of the strains in the treatments receiving homogenised hosts.

**Conclusion**

We have shown that under highly controlled conditions, the virulence of *V. anguillarum* strains HI610, NB10 and 43, and thereby the outcome of a gnotobiotic *V. anguillarum* bath challenge, are dependent on the presence of host substances. In order to accommodate for this, an improved experimental set-up was developed in which the vibrios were added to the culture water at $10^5$ CFU ml$^{-1}$ on DAH 3 together with homogenised fish (added at 3 mg l$^{-1}$, equivalent to 3 larvae per 10 ml). In vitro observations have shown that specific, but as yet uncharacterised, host cues present in the homogenised hosts strongly increase motility of *V. anguillarum*, suggesting that the increased mortality is (at least partly) due to a host-induced increase in virulence. Our
study indicates that, in practice, weak fish and live feed not only serve as a vector for pathogens, but can actually make pathogens more virulent.

**Acknowledgements**

This work was funded by The European Community’s Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 227197 Promicrobe ‘Microbes as positive actors for more sustainable aquaculture’, by the Special Research Fund of Ghent University GOA project BOF12/GOA/022 ‘Host microbial interactions in aquatic production’, the China Scholarship Council and a Special Research Grant (BOF-Ugent) of Ghent University. T.D. is a postdoctoral fellow of FWO-Vlaanderen. We thank Professor Dr. O. Vadstein, Professor Dr. D. Milton and Professor Dr. B. Austin for kindly providing the *V. anguillarum* strains. We thank T. Baelemans, J. Desmyter and B. Van Moffaert for their excellent technical support.
RpoS and indole control the virulence of *Vibrio anguillarum* towards gnotobiotic sea bass (*Dicentrarchus labrax*) larvae

Abstract

Quorum sensing, bacterial cell-to-cell communication with small signal molecules, controls the virulence of many pathogens. In contrast to other vibrios, neither the VanI/VanR acyl homoserine lactone quorum sensing system, nor the three-channel quorum sensing system affects virulence of the economically important aquatic pathogen *Vibrio anguillarum*. Indole is another molecule that recently gained attention as a putative signal molecule. The data presented in this study indicate that indole signaling and the alternative sigma factor RpoS have a significant impact on the virulence of *V. anguillarum*. Deletion of rpoS resulted in increased expression of the indole biosynthesis gene tnaA and in increased production of indole. Both rpoS deletion and the addition of exogenous indole (50-100 µM) resulted in decreased biofilm formation, exopolysaccharide production (a phenotype that is required for pathogenicity) and expression of the exopolysaccharide synthesis gene wbfD. Further, indole inhibitors increased the virulence of the rpoS deletion mutant, suggesting that indole acts downstream of RpoS. Finally, in addition to the phenotypes found to be affected by indole, the rpoS deletion mutant also showed increased motility and decreased sensitivity to oxidative stress.
Introduction

*Vibrio anguillarum* is the causative agent of vibriosis, a fatal haemorrhagic septicaemia affecting many aquatic organisms (fish, crustaceans as well as molluscs) (Frans et al., 2011). The bacterium is a major pathogen of aquaculture organisms, causing significant economic losses in the aquaculture industry (Austin and Austin, 1985). Several (putative) virulence factors have been identified, although for many of these factors, the specific role in disease is not yet known. Three factors that have been reported to be essential for pathogenicity include the iron uptake system involving the siderophore anguibactin (Crosa, 1980; Stork et al., 2007), chemotactic motility (which is required for entry into the host) (McGee et al., 1996; O’Toole et al., 1996) and exopolysaccharide production (which is required for attachment to the host) (Croxatto et al., 2007). The bacterium produces a number of other (putative) virulence factors, including haemolysin, lipase and protease (Norqvist et al., 1990; Rock and Nelson, 2006; Yang et al., 2007). However, whether or not these factors are really essential for pathogenicity is currently not clear.

As virulence factors are often costly metabolic products, their expression usually is tightly regulated. Quorum sensing, a type of bacterial cell-to-cell communication that uses small signal molecules, is one of the regulatory mechanisms controlling the expression of virulence genes in many bacteria (Jayaraman and Wood, 2008). *V. anguillarum* has been documented to contain two quorum sensing systems, a ‘classical’ acyl homoserine lactone (AHL) system involving the signal synthase/receptor pair VanI/VanR, and a three-channel system as found in many vibrios (Milton, 2006). The latter system involves the production of three different types of signal molecules, which are detected by membrane-bound receptors. These receptors feed a shared phosphorylation/dephosphorylation signal transduction cascade controlling the production of the quorum sensing master regulator VanT (Croxatto et al., 2002). One of the central components of the signal transduction cascade is VanO, which is phosphorylated in the absence of signal molecules, whereas it is dephosphorylated in the presence of high levels of signal molecules (Weber et al., 2011). The
phosphorylation status of VanO determines the production of the master regulator VanT. Unlike other vibrios, reports published to date indicate that quorum sensing is not involved in regulating the virulence of *V. anguillarum*. More recently, the alternative sigma factor RpoS has been reported to be an additional factor controlling the production of VanT (Weber et al., 2008), and an *rpoS* insertion mutant has been documented to have lower virulence to zebrafish in an injection model (Ma et al., 2009).

Indole is another molecule that recently gained attention as a putative quorum-sensing signal molecule (Lee and Lee, 2010). Indole is produced by tryptophanase (encoded by the *tnaA* gene), which reversibly converts tryptophan into indole, pyruvate and ammonia (Newton and Snell, 1965). Despite the fact that many bacteria (including several vibrios) have been known for a long time to produce substantial amounts of indole, its biological role as a signal molecule has only recently been revealed (Lee and Lee, 2010). Most work in this respect has been done on enteric bacteria, mainly *E. coli*, in which indole has been reported to control virulence-related phenotypes such as biofilm formation, motility, chemotaxis and adherence to epithelial cells (Lee et al., 2007a; Bansal et al., 2010). In enteropathogenic *E. coli*, the indole biosynthase TnaA, has been reported to be required for virulence to nematodes (Anyanful et al., 2005). Thus far, very little is known on the role of indole in vibrios and the only report published to date documents that indole affects polysaccharide production, biofilm formation and grazing resistance in *V. cholerae* (Mueller et al., 2009).

In the present study, we aimed to investigate the impact of indole signaling and RpoS on the virulence of *V. anguillarum* in a highly controlled model system with gnotobiotic european sea bass (*Dicentrarchus labrax*) larvae and on the production of several important virulence factors.

**Materials and methods**

**Bacterial strains and culture conditions**
V. anguillarum strains used in this study are listed in Table 3.1. The bacteria were cultured in LB20 medium (Luria-Bertani medium plus 2 % NaCl) at 28 °C for 24 h. The bacteria used for challenge tests were grown in 10 % of LB20 medium with the addition of Instant Ocean artificial sea salt (Aquarium Systems, Sarrebourg, France) to obtain a salinity of 36 g l⁻¹ on a horizontal shaker (150 rpm) at 16 °C for 48 h. The density of the bacterial suspensions was determined with a spectrophotometer (Genesys 20, Thermo Scientific, U.S.A.) at 550 nm according to the McFahrland standard (BioMérieux, Marcy L’Etoile, France).

Table 3.1 V. anguillarum strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or relevant markers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB10</td>
<td>Wild type from which all other strains were derived, serotype O1, clinical isolate from the Gulf of Bothnia</td>
<td>Norqvist et al., 1989</td>
</tr>
<tr>
<td>DM21</td>
<td>Deletion of vanI (AHL synthase that produces ODHLᵃ and to a lesser extend OHLᵇ and OOHLᶜ)</td>
<td>Milton et al., 1997</td>
</tr>
<tr>
<td>DM25</td>
<td>Deletion of vanR (AHL receptor that detects the AHLs produced by VanI)</td>
<td>Milton et al., 1997</td>
</tr>
<tr>
<td>DM26</td>
<td>Deletion of both vanI and vanR</td>
<td>Milton et al., 1997</td>
</tr>
<tr>
<td>DM88</td>
<td>Deletion of vanO that encodes an alanine instead of an aspartate at position 56 (D56A)</td>
<td>Weber et al., 2011</td>
</tr>
<tr>
<td>DM89</td>
<td>Deletion of vanO that encodes a glutamate instead of an aspartate at position 56 (D56E)</td>
<td>Weber et al., 2011</td>
</tr>
<tr>
<td>AC10</td>
<td>Deletion of vanT</td>
<td>Croxatto et al., 2002</td>
</tr>
<tr>
<td>AC12</td>
<td>Deletion of rpoS</td>
<td>Weber et al., 2008</td>
</tr>
</tbody>
</table>

a. N-(3-oxodecanoyl)-L-homoserine lactone.
b. N-octanoyl-L-homoserine lactone.
c. N-(3-oxooctanoyl)-L-homoserine lactone.
Challenge tests

The disinfection of sea bass eggs, hatching and axenity tests were performed according to Dierckens et al. (Dierckens et al., 2009) and the challenge tests were performed according to Li et al. (Li et al., 2014). Axenic larvae were stocked immediately before challenging (at Day After Hatching 3 –DAH3). No rifampicin was used in the challenge tests performed in this study. Ten replicate fish cultures were used per treatment. *V. anguillarum* strains were added to the culture water at $10^5$ CFU ml$^{-1}$. The survival of sea bass larvae was checked at DAH 5, 7, 9 and 11. The larvae were not fed during the experiment. All the challenge experiments were approved by the ethical committee of Ghent University (no. EC2014/13 and no. EC2014/59).

Stress sensitivity test

*V. anguillarum* strains were suspended at $10^7$ CFU ml$^{-1}$ in synthetic sea water (36 g l$^{-1}$ Instant Ocean), with or without pyrogallol (10 mg l$^{-1}$; Sigma-Aldrich, U.S.A.) and with or without catalase from bovine liver (10 mg l$^{-1}$; Sigma-Aldrich, U.S.A.) as described previously (Defoirdt et al., 2013). After 6h incubation at 28°C, the suspensions were spread-plated on LB$_{20}$ agar.

Virulence factor assays

Lipase, phospholipase, caseinase, gelatinase and haemolysin activity were assessed according to Natrah et al. (2011). Activity zones were corrected by colony diameter. Motility was assessed as described previously (Wang et al., 2011) on LB$_{20}$ medium with 0.3% agar. Two microliter volumes of overnight grown cultures (set at OD$_{550}$ = 0.5) were inoculated in the middle of the soft agar plates. After incubation for 24 h at 28°C, motility halos were measured. All assays were done at least in triplicate.

Quantification of indole
V. anguillarum cultures grown in LB20 medium were harvested at different time points and centrifuged at 8000 x g for 5 min. The concentration of indole in the supernatants was measured by mixing 500 μl of supernatant with 500 μl of Kovac’s reagent (Sigma-Aldrich, U.S.A.). After vortexing, the top 200 μl were removed and the absorbance at 571 nm was measured. The indole concentration in each sample was determined based on a standard curve using synthetic indole (Sigma-Aldrich, U.S.A.). At least three different V. anguillarum cultures were sampled for each treatment at each time point.

Biofilm formation assay and quantification of exopolysaccharides

The biofilm formation assay was performed in 96-well polystyrene microtiter-plates (SPL life sciences, Korea), as previously described (Brackman et al., 2008) with some modifications. Overnight cultures in LB20 were diluted with fresh LB20 medium to OD$_{550}$ = 0.1 and inoculated into a 96-well plate (200 μl per well). The plate was incubated at 28 °C for 48 hours, after which wells were washed three times with 300 μl sterile physiological saline to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 200 μl of 99% methanol per well for 2 hours, and the plate was emptied and left to air dry overnight. Then, the plate was stained for 20 min with 200 μl of 1% crystal violet per well. Excess stain was rinsed off by placing the plate under running tap water. After the plate was air dried, the dye bound to the adherent cells was resolubilized with 200 μl of 95% ethanol per well. The absorbance of each well was measured at 571 nm. For the quantification of exopolysaccharides, a Calcofluor white staining (Sigma-Aldrich, U.S.A.) was used as previously described (Brackman et al., 2008). For each assay, a minimum of three different V. anguillarum cultures were used for each treatment. The reported data are representative of three independent experiments.

Quantitative reverse transcriptase PCR (qRT-PCR)
Gene expression was determined with qRT-PCR as described previously (Ruwandeepika et al., 2011). *V. anguillarum* cultures grown in LB20 medium were collected at 6 h, 12 h and 24 h. Three different *V. anguillarum* cultures were sampled for each treatment. Total RNA from culture samples was extracted using the Total RNA Isolation Kit (Promega, U.S.A.) according to the manufacturer’s instructions. The cDNA was synthesized by using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, U.S.A.). The qRT-PCR was performed in an StepOne™ Real-Time PCR System thermal cycler (Applied Biosystems). Data acquisition was performed with the StepOne™ Software. Expression of the genes encoding tryptophanase *tnaA*, lipoprotein (exopolysaccharide export) *wza*, and polysaccharides (EPS) biosynthesis *wbfD* was determined using the ΔΔC₇ method (Livak and Schmittgen, 2001) using the RNA polymerase A subunit (*rpoA*) gene as reference gene. Specific Primer sequences are presented in Table 3.2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene function</th>
<th>Primer sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rpoA</em></td>
<td>RNA polymerase A</td>
<td>F: AGATTAGACGACACACGCAGCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGTTACGACACAACCTGGCA</td>
</tr>
<tr>
<td><em>tnaA</em></td>
<td>Tryptophanase (biosynthesis of indole)</td>
<td>F: ACTGCTGTGTGGCGAAAAAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GCGATAGAGACAGGCTGACC</td>
</tr>
<tr>
<td><em>wza</em></td>
<td>Exopolysaccharide export</td>
<td>F: GCCGATAGGCGCTCATCTTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGAGCACAGTGCCGGCATT</td>
</tr>
<tr>
<td><em>wbfD</em></td>
<td>Exopolysaccharide biosynthesis</td>
<td>F: CCTGATCCTCTAGCGATTGGTTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGATTGACGTGATATTGGGT</td>
</tr>
</tbody>
</table>

**Table 3.2 Primers used for quantitative RT-PCR**

**Statistics**

The data were analyzed using one-way ANOVA followed by Tukey’s post-hoc test or by independent samples t-tests. For the comparison of the survival of the sea bass
larvae, data were arcsin transformed before one-way ANOVA analysis. All statistical analyses were done using the Statistical Package for the Social Sciences (SPSS) software, version 19.

**Results**

**Impact of the three-channel quorum sensing system, the AHL quorum sensing system and the alternative sigma factor RpoS on the virulence of V. anguillarum towards gnotobiotic sea bass larvae**

Gnotobiotic sea bass larvae were challenged with *V. anguillarum* wild type and quorum sensing mutants using a standardised protocol (Li et al., 2014). None of the quorum sensing mutants showed a consistently lower virulence than the wild type (Table 3.3).

Further, there was no difference in virulence between strains DM88 and DM89, which have their three channel quorum sensing systems locked in the “on” and “off” configuration, respectively. Both strains were less virulent than the wild type in the first experiment, whereas they were not in the second. Furthermore, the mutant with a deletion in *vanT*, the master regulator of the three channel quorum sensing system, also showed no difference in virulence when compared to the wild type. Mutants with inactivated components of the VanI/VanR AHL quorum sensing system tended to be more virulent than the wild type. Indeed, single mutants with deleted *vanI* or *vanR* showed higher virulence than the wild type in one of the two independent experiments, whereas the double mutant showed higher virulence than the wild type in both experiments.
Table 3.3 Survival of gnotobiotic sea bass larvae at DAH 11 challenged with *V. anguillarum* wild type and mutants (average ± standard error of ten replicate fish cultures). Test 1, 2 and 3 represent three independent challenge tests with larvae hatched at different dates. Values in the same column with different superscript letters are significantly different (ANOVA with Tukey’s post-hoc test; *P* < 0.01).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mutation in</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Test 1</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>73 ± 4</td>
</tr>
<tr>
<td>NB10</td>
<td>Wild type</td>
<td>4 ± 4 b</td>
</tr>
<tr>
<td>DM88</td>
<td><em>vanO</em> D56A</td>
<td>29 ± 8 c</td>
</tr>
<tr>
<td>DM89</td>
<td><em>vanO</em> D56E</td>
<td>29 ± 8 c</td>
</tr>
<tr>
<td>AC10</td>
<td><em>vanT</em></td>
<td>NT</td>
</tr>
<tr>
<td>DM21</td>
<td><em>vanI</em></td>
<td>NT</td>
</tr>
<tr>
<td>DM25</td>
<td><em>vanR</em></td>
<td>NT</td>
</tr>
<tr>
<td>DM26</td>
<td><em>vanI</em> <em>vanR</em></td>
<td>NT</td>
</tr>
<tr>
<td>AC12</td>
<td>rpoS</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT: not tested.

In contrast to the quorum sensing mutants, the *rpoS* deletion mutant showed a significantly decreased virulence in all experiments, with no significant difference in survival when compared to unchallenged larvae (Table 3.3, Figure 3.1). This indicates that the alternative sigma factor RpoS plays an important role in the pathogenicity of *V. anguillarum*.

**Impact of RpoS on indole production**

RpoS had previously been reported to affect indole production in *E. coli* by inducing the expression of the tryptophanase gene *tnaA* (Lelong et al., 2007) and consequently, we investigated the impact of the *rpoS* deletion on indole production in *V.*
anguillarum. In contrast to what has been reported for E. coli, indole production was significantly increased in the rpoS mutant when compared to wild-type V. anguillarum (Figure 3.2A). The difference between the two strains in indole levels was two-fold in late exponential phase (12 h) and three-fold in stationary phase (24 h). In addition, we determined the relative expression levels of the indole biosynthesis gene tnaA in the wild type and rpoS mutant by quantitative reverse transcriptase PCR, and found that the expression was significantly higher in the rpoS mutant at all sampling points, with between 3- and 12-fold differences between both strains (Figure 3.2B).

**Impact of indole on the virulence of V. anguillarum towards sea bass larvae**

As the rpoS deletion mutant showed reduced virulence and increased indole production, we hypothesised that the effect of RpoS might (at least in part) be mediated by indole and consequently, we investigated whether the addition of indole could decrease the virulence of wild-type V. anguillarum. Direct addition of indole to the sea bass rearing water resulted in a significantly increased survival at 50 µM indole or more (Figure 3.1). However, we noticed that indole also affected the sea bass larvae since they were clearly more active in the indole treatments (especially the 100 µM treatment). To exclude any effect of indole on the larvae, wild-type V. anguillarum was grown in the presence of indole, and cultures were washed to remove the indole prior to inoculation into the sea bass rearing water. Pretreatment with indole also resulted in a significantly decreased mortality of sea bass larvae challenged with wild-type V. anguillarum (Figure 3.1), indicating that the decrease in mortality is at least partially due to decreased virulence of V. anguillarum. Importantly, 100 µM indole has no effect on growth of V. anguillarum, nor does it affect its survival in sea water (Figure 3.3 and Table 3.4).
Figure 3.1 Survival of gnotobiotic sea bass larvae challenged with *V. anguillarum* wild type (WT), with or without indole (either added to the sea bass rearing water or added to *V. anguillarum* cultures and removed prior to inoculation into the rearing water), and the *rpoS* deletion mutant (ΔrpoS), with or without the indole inhibitors isatin and acetyl-tryptophan (both added to the rearing water at 50 μM) at DAH 11. Error bars represent the standard error of 10 fish cultures. Different letters denote significant differences (ANOVA with Tukey’s post-hoc test; *P* < 0.01). “Control” refers to unchallenged larvae that were otherwise treated in the same way as in all other treatments.
Figure 3.2 (A) Indole production by *V. anguillarum* wild type (WT) and rpoS mutant (ΔrpoS) during growth in LB20 medium. (B) Relative expression of the indole biosynthesis gene *tnaA* in the wild type and the rpoS mutant. The expression was calculated relative to the RNA polymerase A subunit (*rpoA*) gene, expression in the wild type at the 6h time point was set at 1 and the other data points were normalised accordingly. For both panels, error bars represent the standard error of three *V. anguillarum* cultures. ** indicates a significant difference when compared to the wild type at the respective time point (independent samples t-test; *P* < 0.01).
Table 3.4. Survival of wild type \textit{V. anguillarum} (WT) after 6 h incubation in sea water without indole and with 100 μM indole (average ± standard error of three \textit{V. anguillarum} cultures).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>WT + 100 μM indole</td>
<td>98 ± 4</td>
</tr>
</tbody>
</table>

\textbf{Figure 3.3} Growth of wild type \textit{V. anguillarum} in LB20 medium with and without indole. Error bars represent the standard deviation of three \textit{V. anguillarum} cultures.

Furthermore, we investigated whether the addition of the indole inhibitors isatin and acetyl-tryptophan could increase the virulence of the \textit{rpoS} mutant, which would confirm that the impact of RpoS on virulence is (partly) mediated by indole signaling. Isatin has been described before to decrease the production of indole in \textit{E.coli} by decreasing \textit{tnaA} expression (Lee and Lee, 2010) whereas acetyl-tryptophan has been described as a noncompetitive inhibitor of tryptophanase (Scherzer et al., 2009). Both inhibitors (added to the rearing water at 50 μM) decreased the survival of sea bass larvae challenged to the \textit{V. anguillarum rpoS} mutant (\textbf{Figure 3.1}), but the difference was not significant for isatin.
Impact of RpoS and indole on sensitivity to oxidative stress

We subsequently investigated the mechanism by which RpoS and indole affect the virulence of *V. anguillarum*. RpoS has been reported to affect stress sensitivity in many bacteria, including *V. anguillarum* (Dong and Schellhorn, 2010). In a first experiment, we tested the impact of RpoS and indole on the resistance of *V. anguillarum* to reactive oxygen, which is part of the defence system of vertebrates. We have previously reported that the polyphenol compound pyrogallol inactivates vibrios by releasing peroxide, and that peroxide is neutralised by the addition of catalase (Defoirdt et al., 2013). Therefore, to assess the impact of RpoS on resistance to oxidative stress, we exposed wild-type *V. anguillarum* and the rpoS mutant to pyrogallol, with and without catalase. We found that the addition of pyrogallol resulted in >70% reduction of cell counts in wild-type *V. anguillarum* and that catalase could neutralise this effect (Figure 3.4). The rpoS mutant was more sensitive than the wild type, with approximately 90% reduction in cell counts. Again, the effect of pyrogallol could be neutralized by the addition of catalase. Finally, the addition of 100 µM indole did not affect resistance to oxidative stress of wild-type *V. anguillarum*, indicating that increased sensitivity to reactive oxygen is not the mechanism responsible for the decreased virulence of the bacterium in the presence of elevated indole levels.
Figure 3.4 Survival of *V. anguillarum* wild type (WT) and *rpoS* mutant (Δ*rpoS*) after 6h incubation in sea water, with or without pyrogallol (10 mg l⁻¹), and with or without catalase (10 mg l⁻¹). Survival was determined by plate counting on LB20 agar. Error bars represent the standard error of three independent experiments. ** denotes a significant difference in survival of Δ*rpoS* when compared to WT (independent samples t-test; *P* < 0.01).

**Impact of RpoS and indole on virulence factor production**

We went further to investigate the impact of RpoS on the production of various virulence factors in *V. anguillarum*, including motility, lipase, phospholipase, caseinase, gelatinase and haemolysin activities. Of these, only motility showed to be significantly different between wild-type and *rpoS* mutant. Remarkably, the motility of the *rpoS* mutant was almost two-fold higher than that of the wild-type, with motility zones of 25.0 ± 0.9 and 52.2 ± 0.7 for wild-type and *rpoS* mutant, respectively. Finally, the addition of 100 μM indole to wild-type *V. anguillarum* did not affect any of these virulence factors.
Impact of RpoS and indole on biofilm formation and exopolysaccharide production

In a last series of experiments, we investigated the impact of RpoS and indole on exopolysaccharide production and biofilm formation. These two phenotypes are linked with each other (i.e. exopolysaccharide production contributes to biofilm formation) and are also required for pathogenicity of *V. anguillarum* (Croxatto et al., 2007). We found that the *rpoS* mutant produced significantly less biofilm (in fact, the mutant hardly produced any biofilm) and exopolysaccharides than the wild type ([Table 3.5](#)). Furthermore, the addition of indole also decreased biofilm formation and exopolysaccharide production in wild-type *V. anguillarum*.

Finally, we determined the impact of RpoS and indole on the expression of the *wbfD* and *wza* genes, which are involved in exopolysaccharide biosynthesis and export in *V. anguillarum*, respectively (Croxatto et al., 2007). The expression of the exopolysaccharide synthesis gene *wbfD* was significantly lower in the *rpoS* mutant than in the wild type at all sampling points ([Figure 3.5A](#)), whereas the expression of the exopolysaccharide export gene *wza* was higher in the *rpoS* mutant at all time points ([Figure 3.5B](#)). Furthermore, the addition of indole (both 50 and 100 µM) to the wild type resulted in an over 10-fold decrease in *wbfD* expression, whereas there was no effect on the expression of *wza*. 

---

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Table 3.5 Biofilm formation and exopolysaccharide production of *V. anguillarum* wild type (WT) and rpoS mutant (ΔrpoS) (average ± standard error of three independent replicates). Different superscript letters denote significant differences (ANOVA with Tukey’s post-hoc test; \( P < 0.01 \)).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Biofilm formation(^1)</th>
<th>Exopolysaccharide production(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.23 ± 0.01(^a)</td>
<td>1414 ± 178(^A)</td>
</tr>
<tr>
<td>WT + 50 μM Indole</td>
<td>0.15 ± 0.02(^b)</td>
<td>1197 ± 32(^B)</td>
</tr>
<tr>
<td>WT + 100 μM Indole</td>
<td>0.14 ± 0.01(^b)</td>
<td>899 ± 37(^C)</td>
</tr>
<tr>
<td>ΔrpoS</td>
<td>0.09 ± 0.01(^c)</td>
<td>697 ± 70(^C)</td>
</tr>
</tbody>
</table>

\(^1\) Absorbance of Crystal Violet stained biofilm at 571nm

\(^2\) Fluorescence intensity (excitation at 405 nm, emission at 500 nm) of calcofluor white stained cultures
Figure 3.5 Relative expression of the exopolysaccharide biosynthesis gene *wbfD* (A) and the exopolysaccharide export gene *wza* (B) in wild type *V. anguillarum* and the *rpoS* mutant (*ΔrpoS*). The expression was calculated relative to the RNA polymerase A subunit (*rpoA*) gene, expression in the wild type at the 6h time point was set at 1 and the other datapoints were normalised accordingly. Error bars represent the standard error of three different *V. Anguillarum* cultures. ** denotes a significant difference when compared to the wild type strain without indole at the respective time point (independent samples t-test; *P* < 0.01).
Discussion

In this study, we investigated the impact of various quorum sensing signaling pathways in V. anguillarum on the virulence of the bacterium towards gnotobiotic sea bass (Dicentrarchus labrax) larvae. Inactivation of neither the VanI/VanR AHL quorum sensing system, nor the three-channel system as typically found in vibrios resulted in decreased virulence, which is consistent with what had been reported previously in other fish hosts (Milton, 2006). In fact, inactivation of the VanI/VanR system resulted in a slightly increased virulence. Based on previously published reports, we further investigated the impact of the alternative sigma factor RpoS on the virulence of V. anguillarum towards sea bass larvae and found that an rpoS deletion mutant was significantly less virulent than the wild type. This result is consistent with what Ma et al. (2009) reported based on an injection model in zebrafish, although the difference was more pronounced in our immersion challenge model.

We went further to explain the lower virulence of the rpoS mutant and found that the mutant was significantly more sensitive to oxidative stress than wild type V. anguillarum, which is also consistent with what has been reported before for V. anguillarum and various other species, as RpoS is generally known to be a key response regulator to stress conditions in proteobacteria (Dong and Schellhorn, 2010). Since the production of reactive oxygen species is one of the components of the innate immune defence of fish (Ellis, 2001), higher sensitivity to oxidative stress might be a key factor explaining the decreased virulence of the rpoS mutant. We furthermore found that the rpoS mutant was deficient in biofilm formation and that it produced lower exopolysaccharide levels than the wild type. Furthermore, the mutant showed lower expression levels of the exopolysaccharide synthase wbfD, whereas the expression levels of the exopolysaccharide transport protein Wza were higher in the mutant than in the wild type. Since exopolysaccharide production has been reported to be required for pathogenicity of V. anguillarum (Croxatto et al., 2007), the lower exopolysaccharide production might be another key factor explaining the lower virulence of the rpoS mutant. Further, we found no difference between wild type and rpoS mutant in lipase,
phospholipase, protease and haemolysin activities, which is in contrast to what Ma et al. (2009) reported. This might reflect differences in the wild type strain (W-1 vs. NB10 in our study) or differences in the mutation type (insertion vs. in-frame deletion in our study). Finally, we also found that the rpoS mutant was more motile than the wild type, which is somewhat surprising as motility is also linked to virulence.

Because RpoS had previously been reported to affect the production of the signaling molecule indole, we determined indole production in V. anguillarum wild type and rpoS mutant. In contrast to what has been reported before in E. coli (Lelong et al., 2007), the rpoS mutant showed higher expression of the indole synthase tnaA than the wild-type, and approximately three-fold higher indole levels were detected in cultures of the rpoS mutant, when compared to wild type cultures. To the best of our knowledge, this is the first report demonstrating that RpoS regulates indole production in vibrios. Furthermore, the addition of exogenous indole to similar levels as those produced by the rpoS mutant decreased biofilm formation, exopolysaccharide levels and expression of the exopolysaccharide synthesis gene wbfD in wild type V. anguillarum, whereas the exopolysaccharide transport gene wza was not affected. These results are opposite to what has been reported for V. cholerae, where indole increases biofilm formation, exopolysaccharide levels and activates the expression of polysaccharide synthesis genes (Mueller et al., 2009). Furthermore, addition of indole to the sea bass rearing water of larvae challenged with wild-type V. anguillarum or challenge with wild-type that had been pretreated with indole before challenge resulted in decreased mortality when compared to the larvae that were challenged with untreated wild-type V. anguillarum. Finally, the addition of the indole inhibitor acetyltryptophan to the rearing water of sea bass larvae challenged to the rpoS mutant resulted in increased mortality. In contrast to what we found for the rpoS mutant, indole did not affect motility or sensitivity to oxidative stress.

Together, these data indicate that RpoS has a strong impact on the virulence of V. anguillarum to sea bass larvae, which might be due to increased resistance to reactive oxygen and to negative regulation of indole production. Indole in turn negatively affects
exopolysaccharide production (which is required for pathogenicity). Indole has been reported to have a positive effect on the intestinal epithelial barrier function in mice (Bansal et al., 2010; Shimada et al., 2013). However, to the best of our knowledge, this is the first report showing the involvement of indole in bacterial infection of a vertebrate host, thereby broadening the repertoire of phenotypes that are regulated by indole in bacteria.

Acknowledgements

We thank the Eclosérie Marine de Gravelines (Gravelines, France) for providing us with sea bass eggs. This work was financially supported by the Scientific Research Fund of Flanders (FWO-Vlaanderen project n° 1.5.013.12N), the Special Research Fund of Ghent University (GOA project n° BOF12/GOA/022). XL and QY are doctoral researchers funded by a China Scholarship Council grant and a Special Research Grant (BOF-UGent) of Ghent University. TD is a postdoctoral fellow of FWO-Vlaanderen. The authors have no conflict of interest to declare.
Chapter 4

Impact of mucin, bile salts and cholesterol on the virulence of *Vibrio anguillarum* NB10 towards gnotobiotic sea bass (*Dicentrarchus labrax*) larvae

Abstract

In this study, we investigated the impact of the host factors mucin, bile salts and cholesterol on the virulence of the economically important aquatic pathogen *Vibrio anguillarum* strain NB10 towards sea bass larvae. Pretreatment of *V. anguillarum* with either one of the host factors (at 10 mg l⁻¹) prior to inoculation into the sea bass rearing water increased virulence of the bacterium, although the effect of cholesterol was not significant. Each of the three host factors significantly increased several virulence-related phenotypes in *V. anguillarum*, i.e. protease activity, flagellar motility, biofilm formation and exopolysaccharide production, whereas there was no effect on growth of the bacterium under these conditions. Furthermore, the host factors increased expression of the metalloprotease *empA*, the flagellar transcriptional regulator *fleQ*, the flagellin gene *flaA*, the chemotaxis methyltransferase gene *cheR*, the exopolysaccharide biosynthesis gene *wbfD* and the exopolysaccharide export gene *wza*. Our results indicate that *V. anguillarum* uses host mucin, bile salts, and cholesterol as cues to promote the expression of several important virulence traits that enhance the success of transmission from one host to another.
Introduction

*Vibrio anguillarum* is the causative agent of vibriosis, a fatal haemorrhagic septicaemia in many marine organisms (fish, crustaceans as well as mollusks) (Frans et al., 2011). The bacterium is a major pathogen of aquatic organisms, causing significant economic losses in the aquaculture industry (Austin and Austin, 2012). The pathogenicity mechanisms behind the disease caused by the bacterium are not yet completely understood. We previously reported that homogenised animals (either fish or brine shrimp larvae) increase the mortality of sea bass larvae challenged with different *V. anguillarum* strains (Li et al., 2014), suggesting that the bacterium responds to one or more host factors.

Host factors are known to affect growth and virulence gene expression in pathogenic bacteria. Given the increasing evidence which implicates that *V. anguillarum* can invade fish epithelium at more than one site, including the skin and the intestinal tract (Grisez et al., 1996; Spanggaard et al., 2000), host factors which are produced at these sites were chosen for further study. Mucus from skin and intestine has been reported to enhance growth and the production of virulence factors such as metalloprotease and chemotactic motility in *V. anguillarum* (Garcia et al., 1997; Denkin and Nelson, 1999; O’Toole et al., 1999; Larsen et al., 2001). However, the impact of the main constituents of mucus, heavily glycosylated proteins known as mucins (Forstner and Forstner, 1994), has not yet been determined in *V. anguillarum*. Bile, a digestive fluid produced by the liver of most vertebrates, is a heterogeneous mixture of bile acids, inorganic salts, cholesterol and other components (Begley et al., 2005). In *V. parahaemolyticus*, bile acids and bile salts have been reported to increase the production of virulence factors such as capsule production, adherence to epithelial cells, production of thermostable direct haemolysin and production of a type III secretion system (Pace et al., 1997; Osawa et al., 2002; Gotoh et al., 2010). In *V. cholerae*, crude bile has been reported to repress the expression of virulence genes such as the genes encoding cholera toxin and toxin-coregulated pili (Gupta and Chowdhury, 1997; Schuhmacher and Klose,
1999), whereas motility was increased. In contrast, it has been reported that *V. cholerae* recognizes a set of bile salts including taurocholate as signals to activate the transmembrane transcription activator TcpP (Yang et al., 2013). Interestingly, Wang et al. (2003) reported that bile induced the expression of the outer membrane protein OmpU in *V. anguillarum* and that an *ompU* mutant showed slightly decreased motility and bile resistance, and increased biofilm formation. Cholesterol, another constituent of bile, is essential for proper membrane structure and functioning in eukaryotic cells. It has been reported to serve as a binding site of *V. vulnificus* on the plasma membrane of target cells (Kim and Kim, 2002). Cholesterol has been reported to increase the motility of *V. cholerae* while having no effect on growth or the expression of the major virulence genes of *V. cholerae*, ctxAB and tcpA (Chatterjee et al., 2007). To the best of our knowledge, the impact of bile salts and cholesterol on virulence factor production in *V. anguillarum* currently is unknown.

In this study, we aimed at investigating the effect of mucin (type II mucin), bile salts (glycocholate and taurocholate) and cholesterol on several virulence-related phenotypes in *V. anguillarum* and on the virulence of the bacterium towards gnotobiotic sea bass larvae.

**Materials and methods**

**Bacterial strains and culture conditions**

*V. anguillarum* strain NB10 (Norqvist et al., 1989) was used in this study. The bacterium was cultured in LB$_{20}$ medium (Luria-Bertani medium plus 2% NaCl) at 28°C for 24 h. Natural rifampicin resistant mutants were selected as described before (Pande et al., 2013) and these mutants were used in the challenge tests. The inoculum used for challenge tests was grown in 10% of LB$_{20}$ medium with the addition of Instant Ocean artificial sea salt (Aquarium Systems, Sarrebourg, France) to obtain a salinity of 36 g l$^{-1}$ on a horizontal shaker (150 rpm) at 16 °C for 48 h. The density of the bacterial suspensions was determined with a spectrophotometer
(Genesys 20, Thermo Scientific, U.S.A.) at 550 nm and cell densities were calculated according to the McFahrland standard (BioMérieux, Marcy l’Etoile, France), assuming that OD<sub>550</sub> of 1 corresponds to 1.2 x 10⁹ cells ml⁻¹.

**Challenge tests**

Sea bass eggs were obtained from Ecloserie Marine in Gravelines, France. The disinfection of sea bass eggs, hatching and axenity tests were performed according to Dierckens et al. (2009) and the challenge tests were performed according to Li et al. (2014), without the addition of homogenized fish larvae. Axenic larvae were stocked immediately before challenging (at Day After Hatching 3 – DAH3) with the addition of 10 mg l⁻¹ rifampicin. *V. anguillarum* NB10 was cultured overnight with or without the addition of 10 mg l⁻¹ mucin (type II mucin from porcine stomach, Sigma-Aldrich, U.S.A.), 10 mg l⁻¹ bile salts (50 % sodium glycocholate and 50 % sodium taurocholate, Oxoid, U.K.) or 10 mg l⁻¹ cholesterol (Sigma-Aldrich, U.S.A.). The cultures were centrifuged to remove the supernatants and resuspended in sterile artificial sea water (36 g l⁻¹ Instant Ocean sea salts). Then the bacteria were added to the culture water of sea bass larvae at 10⁵ CFU ml⁻¹. Five replicate fish cultures were used per treatment. The survival of sea bass larvae was checked at DAH 11. The larvae were not fed during the experiment. The experiment was approved by the ethical committee of Ghent University (no. EC2014/87).

**Protease activity assays**

*V. anguillarum* NB10 was cultured as described above and at different time points (6, 12 and 24h), cell-free culture supernatants were collected. Protease activity was determined using azocasein (Sigma-Aldrich, U.S.A.) according to the manufacturer’s instructions with slight modifications. Briefly, 100 µl of the culture supernatant was added to 250 µl of azocasein solution (2.5 % azocasein in 0.5 % Sodium Bicarbonate Buffer) with 150 µl 0.5 % Sodium Bicarbonate Buffer (pH 8.0). The mixture was incubated at 37 °C for 30 min. One hundred micro litre of reaction
mixture was taken and the reaction was terminated by adding 400 μl of 5.0 % trichloroacetic acid solution. The mixture was centrifuged at 12000 x g for 5 min, 500 μl of the supernatant was mixed with 150 μl of 500 mM NaOH, and the absorbance was determined at 440 nm. One unit of protease activity was defined as the amount of enzyme producing an increase of 0.01 absorbance units under the specified conditions. At least three independent samples were analyzed for each treatment at each time point.

Swimming motility assays

Swimming motility assays were performed on soft agar (LB20 Agar containing 0.3% agar) as described previously (Li et al., 2014). *V. anguillarum* NB10 was grown overnight in LB20 medium, and 2 μl of the cultures (diluted to OD550 = 1.0) were spotted in the center of soft agar plates with or without 10 mg l⁻¹ mucin, bile salts or cholesterol. Plates were incubated for 24 h at 28°C, after which the diameters of the motility halos were measured. The assays were done at least in triplicate.

Determination of biofilm formation and quantification of exopolysaccharides

The biofilm formation assay was performed in 96-well polystyrene microtiter-plates (SPL life sciences, Korea), as previously described (Brackman et al., 2008) with some modifications. Overnight cultures in LB20 were diluted with fresh LB20 medium to OD550 = 0.1 and inoculated into a 96-well plate (200 μl per well). The plate was incubated at 28 °C for 48 hours, after which wells were washed three times with 300 μl sterile physiological saline to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 200 μl of 99% methanol per well for 2 hours, and the plate was emptied and left to air dry overnight. Then, the plate was stained for 20 min with 200 μl of 1% crystal violet per well. Excess stain was rinsed off by placing the plate under running tap water. After the plate was air dried, the dye bound to the adherent cells was resolubilized with 200 μl of 95% ethanol.
per well. The absorbance of each well was measured at 571 nm. For the quantification of exopolysaccharides, a Calcofluor white staining (Sigma-Aldrich, U.S.A.) was used as previously described (Brackman et al., 2008). For each assay, a minimum of three different V. anguillarum cultures were used for each treatment. The reported data are representative of three independent experiments.

Quantitative reverse transcriptase PCR (qRT-PCR)

Gene expression was determined with qRT-PCR as described previously (Ruwandeepika et al., 2011). V. anguillarum cultures grown in LB20 medium were collected at 24 h in triplicate. Total RNA from culture samples was extracted using the Total RNA Isolation Kit (Promega, U.S.A.) according to the manufacturer’s instructions. The cDNA was synthesized by using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, U.S.A.). The qRT-PCR was performed in a StepOne™ Real-Time PCR System thermal cycler (Applied Biosystems). The temperature program consisted an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15s and primer annealing and elongation at 60°C for 1min. Data acquisition was performed with the StepOne™ Software. Relative expression of virulence-related genes was determined using the ΔΔCt method (Livak and Schmittgen, 2001). The RNA polymerase A subunit (rpoA) gene was used as housekeeping gene. Specific Primer sequences are presented in Table 4.1.

Statistics

The data were analyzed using one-way ANOVA followed by Tukey’s post-hoc test or by independent samples t-tests. For the comparison of the survival of the sea bass larvae, data were arcsin transformed before one-way ANOVA analysis. All statistical analyses were done using the Statistical Package for the Social Sciences (SPSS) software, version 19.
Table 4.1. Primers used for quantitative RT-PCR and PCR efficiencies.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene function</th>
<th>Primer sequence (5′ → 3′)</th>
<th>PCR Efficiencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoA</td>
<td>RNA polymerase A</td>
<td>F: AGATTAGCAGACACACACGCA</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGTTACAGCACAACCTGGCA</td>
<td></td>
</tr>
<tr>
<td>fleQ</td>
<td>Flagellar master regulator</td>
<td>F: GCAAGTCTCAAGGCACAGAAG</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ACAGTAACTCGCACAACAGG</td>
<td></td>
</tr>
<tr>
<td>flaA</td>
<td>Flagellin</td>
<td>F: GGATTCAACAGGGCGAG</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TCGTCATTTTCGCTGGTTTC</td>
<td></td>
</tr>
<tr>
<td>cheR</td>
<td>Chemotaxis methyl transferase</td>
<td>F: GCCATGACCACCAACGAA</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GCTTGCCGCTGACCAA</td>
<td></td>
</tr>
<tr>
<td>empA</td>
<td>Extracellular metalloprotease</td>
<td>F: CTGGACCTGGTGAAATCGAAGAC</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTGCGTCATTAGCGGTGAATAAGC</td>
<td></td>
</tr>
<tr>
<td>wza</td>
<td>Exopolysaccharide export</td>
<td>F: GCCGATAGGGTCATCTTGGT</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGACACATGTCGCGGCGATT</td>
<td></td>
</tr>
<tr>
<td>wbfD</td>
<td>Exopolysaccharide biosynthesis</td>
<td>F: CCTGATCCTCTAGCGATTGGTTT</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGATTGACGTGATATTGGGTGT</td>
<td></td>
</tr>
</tbody>
</table>

Results

Impact of the host factors on the virulence of *V. anguillarum* towards gnotobiotic sea bass larvae

Gnotobiotic sea bass larvae were challenged with *V. anguillarum* NB10 using a standardised protocol (Li et al., 2014). The pathogen was grown in the presence of host factors and washed prior to the challenge. Untreated and washed *V. anguillarum* was used as a control. The mortality of the larvae was significantly higher when the pathogen was pretreated with mucin or bile salts (Table 4.2). Pretreatment with cholesterol did not result in a significant difference in survival, although there was a trend towards higher mortality.
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Table 4.2 Survival of gnotobiotic sea bass larvae challenged with *V. anguillarum* NB10, after eight days of challenge (average ± standard error of five replicate fish cultures). The pathogen was grown in the presence of host factors and washed prior to the challenge. *V. anguillarum* grown in medium without host factors and otherwise treated in the same way was used as a control.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival (%)¹</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test 1</td>
<td>Test 2</td>
</tr>
<tr>
<td>Control</td>
<td>77 ± 3 a</td>
<td>70 ± 4 A</td>
</tr>
<tr>
<td>NB10</td>
<td>49 ± 3 b</td>
<td>62 ± 5 AB</td>
</tr>
<tr>
<td>NB10 [Mucin 10mg/l]</td>
<td>18 ± 3 c</td>
<td>33 ± 4 C</td>
</tr>
<tr>
<td>NB10 [Bile salts 10mg/l]</td>
<td>16 ± 3 c</td>
<td>38 ± 3 C</td>
</tr>
<tr>
<td>NB10 [Cholesterol 10mg/l]</td>
<td>43 ± 4 b</td>
<td>55 ± 3 B</td>
</tr>
</tbody>
</table>

¹Values in the same column with different superscript letters are significantly different (p < 0.01). Test 1 and Test 2 refer to two independent experiments performed with larvae hatched on different dates. Square brackets refer to pretreatment.

Impact of the host factors on protease activity

We further investigated the impact of the host factors on protease (caseinase) activity and on expression of the metalloprotease *empA*. The addition of mucin, bile salts or cholesterol resulted in an approximately 1.6 fold increase in protease activity at 12 h and a 2 fold increase at 24 h (Figure 4.1). Importantly, the addition of mucin, bile salts or cholesterol had no effect on growth under these conditions (Figure 4.2). The extracellular metalloprotease EmpA has been shown to account for nearly 90 % of the total extracellular proteolytic activity of *V. anguillarum* (Han et al., 2011). Therefore, to further confirm these results, we determined the impact of the host factors on the expression of *empA*. We found that *empA* mRNA levels were significantly increased in the presence of 10 mg l⁻¹ mucin (3.5 fold) and even more in the presence of bile salts or cholesterol (5.8 fold) (Table 4.3).
Figure 4.1 Protease activity of *V. anguillarum* NB10 with and without the host factors mucin, bile salts or cholesterol (average ± standard error of three replicates). One unit of protease activity was defined as the amount of enzyme producing an increase of 0.01 absorbance units (OD$_{440}$) under the specified conditions. Asterisks indicate significant differences between the control and the respective treatment at different time points (*$P < 0.01$).
Figure 4.2 Growth of *V. anguillarum* NB10 in LB20 medium with and without mucin, bile salts or cholesterol (average ± standard error of three replicate cultures).
Table 4.3 Impact of the host factors cholesterol, bile salts and mucin (10 mg l⁻¹) on the expression of the flagellar motility and chemotaxis genes fleQ, flaA and cheR, the exopolysaccharide export and synthesis genes wza and wbfD and the metalloprotease gene empA (average ± standard error of three replicate V. anguillarum cultures).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Relative expression (fold)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NB10</td>
</tr>
<tr>
<td>fleQ</td>
<td>Flagellar master regulator</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>flaA</td>
<td>Flagellin</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>cheR</td>
<td>Chemotaxis methyl transferase</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>empA</td>
<td>Extracellular metalloprotease</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>wza</td>
<td>Exopolysaccharide export</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>wbfD</td>
<td>Exopolysaccharide biosynthesis</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

¹mRNAs were isolated from three independent V. anguillarum cultures (grown to stationary phase; OD₅₅₀ 1.5) for each treatment. mRNA levels were normalized to the rpoA mRNA levels. For each gene, the expression in the untreated control was set at 1 and the expression in all other treatments was normalized accordingly using the 2⁻ΔΔCT method. Asterisks indicate significant differences between untreated NB10 and NB10 with the respective treatment (* P<0.05; ** P<0.01).

Impact of the host factors on flagellar motility

We assessed the impact of the host factors on the motility of V. anguillarum NB10 on soft agar. The motility in the presence of 10 mg l⁻¹ mucin, bile salts or cholesterol was approximately 2 fold higher than in the untreated control (Table 4.4). In order to further substantiate this observation, we determined the impact of
the host factors on the expression of genes involved in flagellar motility. Flagellar motility requires many genes in vibrios (e.g. approximately 90 genes in \textit{V. parahaemolyticus}, the hallmark of flagellar motility in vibrios; McCarter, 2004). We selected the flagellar master regulator \textit{fleQ}, the flagellin gene \textit{flaA} and the chemotaxis methyltransferase gene \textit{cheR} for our study. Each of the host factors significantly increased expression of all three genes (between 2.4 and 4.5 fold increase) (Table 4.3).

Table 4.4 Motility of \textit{V. anguillarum} NB10 on soft agar with and without the host factors mucin, bile salts or cholesterol (average ± standard error of three replicates).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Motility halo (mm)(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB10</td>
<td>35 ± 0(^a)</td>
</tr>
<tr>
<td>NB10 + 10 mg l(^-1) Mucin</td>
<td>67 ± 1(^b)</td>
</tr>
<tr>
<td>NB10 + 10 mg l(^-1) Bile salts</td>
<td>67 ± 2(^b)</td>
</tr>
<tr>
<td>NB10 + 10 mg l(^-1) Cholesterol</td>
<td>65 ± 0(^b)</td>
</tr>
</tbody>
</table>

\(^1\)Different superscript letters denote significant differences (P < 0.01).

Impact of the host factors on biofilm formation and exopolysaccharide production

We determined the impact of the host factors on biofilm formation by Crystal violet staining and on exopolysaccharide production by Calcofluor white staining, and found that mucin, bile salts and cholesterol all significantly increased both phenotypes (1.5-1.8 fold and 1.3-1.9 fold, respectively) (Table 4.5). Croxatto et al. (2007) identified genes involved in exopolysaccharide biosynthesis and export, i.e. \textit{wbfD} and \textit{wza}, respectively. To further substantiate our findings, we determined the impact of the host factors on the expression of these two genes. mRNA levels of both genes were significantly increased by all three host factors (1.8-4.9 and 2.5-4.9 fold for \textit{wbfD} and \textit{wza}, respectively) (Table 4.3).
Table 4.5 Biofilm formation and exopolysaccharide production of *V. anguillarum* NB10, with and without the host factors mucin, bile salts or cholesterol (average ± standard error of three replicates). Different superscript letters denote significant differences ($P < 0.01$). These results are representative of three independent experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Biofilm formation</th>
<th>Exopolysaccharide production</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB10</td>
<td>0.24 ± 0.02a</td>
<td>4562 ± 71A</td>
</tr>
<tr>
<td>NB10 + 10 mg l$^{-1}$ Mucin</td>
<td>0.42 ± 0.01b</td>
<td>6079 ± 938B</td>
</tr>
<tr>
<td>NB10 + 10 mg l$^{-1}$ Bile salts</td>
<td>0.36 ± 0.03b</td>
<td>8637 ± 834B</td>
</tr>
<tr>
<td>NB10 + 10 mg l$^{-1}$ Cholesterol</td>
<td>0.36 ± 0.02b</td>
<td>8817 ± 707B</td>
</tr>
</tbody>
</table>

$^1$ Absorbance of Crystal Violet stained biofilm at 571nm

$^2$ Fluorescence intensity (excitation at 405 nm, emission at 500 nm) of calcofluor white stained cultures

**Discussion**

Our previous study showed that the presence of homogenised fish led to significantly increased mortality of sea bass challenged to different *V. anguillarum* strains (Li et al., 2014). The results presented in the current study showed that the host factors mucin and the bile salts glycocholate and taurocholate increased the virulence of *V. anguillarum* towards sea bass larvae (approx. 2-3 fold). Cholesterol also tended to increase the virulence of the bacterium, although the effect was not significant ($P > 0.01$). Furthermore, mucin, cholesterol and the bile salts also increased several phenotypes that are linked to the virulence of *V. anguillarum*, including protease activity, flagellar motility, biofilm formation and exopolysaccharide production. Additionally, mRNA levels of genes involved in these phenotypes were significantly increased by these host factors. These observations
can explain the increased virulence of *V. anguillarum* to sea bass larvae in the presence of these host factors. Mucus has been reported to enhance growth (Garcia et al., 1997), protease activity (Denkin and Nelson, 1999) and chemotactic motility in *V. anguillarum* (Larsen et al., 2001). However, the component responsible for this effect had not been identified. Bile has been proposed to be a cue to enteric pathogens having entered the small intestine (Gunn, 2000). Bile and bile salts have been reported before to affect virulence gene expression in vibrios, including the human pathogens *V. cholerae* (Yang et al., 2013) and *V. parahaemolyticus* (Pace et al., 1997; Osawa et al., 2002; Gotoh et al., 2010). Cholesterol has been reported to affect the production of virulence factors in human-pathogenic vibrios such as *V. vulnificus* (Kim and Kim, 2002) and *V. cholerae* (Chatterjee et al., 2007). To the best of our knowledge, this is the first report demonstrating the effect of mucin, the bile salts glycocholate and taurocholate, and cholesterol on the virulence of *V. anguillarum*.

Many of the virulence traits that are affected by the host factors are needed during the early stages of infection. Flagellar motility mutants (e.g. a flagellin A mutant) and chemotaxis mutants of *V. anguillarum* are deficient in host colonization and are avirulent towards fish (Milton et al., 1996; O'Toole et al., 1996). Exopolysaccharide production is important with respect to bacterial biofilm formation (Karatan and Watnick, 2009) and is required for *V. anguillarum* in order to be able to attach to fish tissues (Croxatto et al., 2007; Weber et al., 2010). A *V. anguillarum* mutant defective for exopolysaccharide export is attenuated for virulence towards fish (Croxatto et al., 2007; Weber et al., 2010). The results presented in the current study lead us to hypothesize that the host factors might “prime” the pathogen for a successful transmission from one host to another. Indeed, *V. anguillarum* that had been in contact with a host (simulated by pretreatment with host factors) are more successful in infection of a new host (as manifested by increased mortality in sea bass larvae).
Chapter 4

In previous studies, mucin has been tested to affect adherence in *V. cholerae* at the range of 5-40 g l\(^{-1}\) (Bhowmick et al., 2008). Mucin, as well as bile, mediated chemotactic response in *V. anguillarum* at the range of 0.1-10 g l\(^{-1}\) (O’Toole et al., 1999). Bile acid deoxycholate at 1 g l\(^{-1}\) (Pace et al., 1997) and taurocholic acid at 2.5 g l\(^{-1}\) (Osawa et al., 2002) have been tested to affect virulence gene expression in *V. parahaemolyticus*. Bile salts have also been reported affecting virulence gene expression in *V. cholerae* at 50 mg l\(^{-1}\) (Yang et al., 2013). Gotoh et al. (2010) showed activity of bile salts in the range 5-50 mg l\(^{-1}\) in *V. parahaemolyticus*. Cholesterol has been reported to affect virulence factors at 0.4 g l\(^{-1}\) in *V. cholerae* (Chatterjee et al., 2007). In this study, we determined the impact of selected host factors (mucin, bile salts and cholesterol) on the virulence of *V. anguillarum* at a lower concentration of 10 mg l\(^{-1}\). No mucin was visible in the midgut and hindgut of sea bass larvae in the first 15 days after hatching (Rekecki et al., 2012). However, the level of mucin in the skin of sea bass larvae is unknown. The synthesis of biliary products has been observed from the beginning of the trophic life (mouth opening, around DAH 5) in sea bass (Diaz et al., 2002), but little is known about the qualitative and quantitative aspects. Bile salts are approximately 1 μg per 100 grams fish in larval stage sea lamprey (Yeh et al., 2012) but unknown in sea bass. Cholesterol in sea bass ranged from 55 up to 66 mg per 100 grams fish (Zotos and Vouzanidou, 2012). The local levels of bile salts and cholesterol in the gut will be higher.

In conclusion, our results indicate that *V. anguillarum* uses host mucin, bile salts, and cholesterol as cues to promote the expression of several important virulence traits. These observations extend the current knowledge on host-pathogen interactions by demonstrating specific mechanisms by which the fish pathogen *V. anguillarum* NB10 senses its host and responds to interactions with a host, and emphasise the importance of isolating diseased animals from the production units. Indeed, our results indicate that diseased animals release pathogens with increased infectivity. In our further research we plan to determine whether this phenomenon is common for different *V. anguillarum* strains (both pathogenic and non-pathogenic.
strains). We hope that this kind of studies will fuel future research aiming to further unravel the mechanism(s) by which pathogens respond to host factors, which can ultimately lead to the development of novel methods to control diseases (i.e. by interfering with these sensory mechanisms).

**Acknowledgements**

We thank the Eclosérie Marine de Gravelines (Gravelines, France) for providing us with sea bass eggs. This work was financially supported by the Scientific Research Fund of Flanders (FWO-Vlaanderen project n° 1.5.013.12N), the Special Research Fund of Ghent University (GOA project n° BOF12/GOA/022). XL is a doctoral researcher funded by a China Scholarship Council grant and a Special Research Grant (BOF-UGent) of Ghent University. TD is a postdoctoral fellow of FWO-Vlaanderen.
Chapter 5

Relation between the virulence of *Vibrio anguillarum* strains and their response to the host factors mucin, bile salts and cholesterol

Li, X., Defoirdt, T., Bossier, P.
Abstract

In this study, we investigated three virulence factors (motility, biofilm formation and exopolysaccharide production) of 15 V. anguillarum strains to three host factors: mucin, bile salts and cholesterol. At 48 hours, the swimming motility of almost all V. anguillarum strains significantly increased in the presence of either of the three host factors, except for JLL237 (responding to only bile salts). Biofilm formation significantly increased with the addition of host factors for five V. anguillarum strains: 43 (with all three host factors), VIB 160 (with mucin and bile salts), VIB103 (with mucin), VIB 15 and JLL237 (with bile salts), respectively. Exopolysaccharide production of three V. anguillarum strains (43, VIB103 and VIB160) significantly increased in the presence of either one of the three host factors. The capabilities of the strains to react on different host factors (percentage increase of motility, biofilm formation and exopolysaccharide production in the presence of either of the three host factors) were significantly correlated with each other. Our results indicate strong linkages between the phenotype responses to three host factors. However, no significant correlations were obtained between three phenotypes and virulence to the sea bass larvae.
Introduction

*Vibrio anguillarum* is the causative agent of vibriosis, a fatal haemorrhagic septicaemia in many marine organisms (fish, crustaceans as well as molluscs) (Frans et al., 2011). The bacterium is a major pathogen of aquaculture organisms, causing significant economic losses in the aquaculture industry (Austin and Austin, 2012). *V. anguillarum* is part of the microflora of marine fish as well as the aquatic environment (Hansen and Olafsen, 1999). Out of 23 identified O-serotypes (O1–O23), only serotypes O1 and O2, and to a lesser extent serotype O3, have been linked to vibriosis in fish (Pedersen et al., 1999). Frans et al. (2013) has demonstrated that no relation could be found among virulence and serotype, genotypic or virulence factors in 15 *V. anguillarum* strains belonging to 3 different serotypes. However, the reason for this is not yet known.

Although the mechanism of pathogenesis is not completely understood, a few virulence-related factors and genes have been identified in *V. anguillarum*, including genes affecting chemotaxis and motility (Milton et al., 1996; O'Toole et al., 1996), iron uptake system (Crosa, 1980; Stork et al., 2007), exopolysaccharide production (Croxatto et al., 2007) and extracellular products with proteolytic (Denkin and Nelson, 2004) or haemolytic activity (Rock and Nelson, 2006). Many of the virulence traits in pathogenic bacteria are affected by the host factors such as mucin, bile salts and cholesterol. Mucus has been reported to enhance growth (Garcia et al., 1997), protease activity (Denkin and Nelson, 1999) and chemotactic motility in *V. anguillarum* (Larsen et al., 2001). Bile and bile salts have been reported before to affect virulence gene expression in vibrios, including the human pathogens *V. cholerae* (Yang et al., 2013) and *V. parahaemolyticus* (Pace et al., 1997; Osawa et al., 2002; Gotoh et al., 2010). Cholesterol has been reported to affect the production of virulence factors in human-pathogenic vibrios such as *V. vulnificus* (Kim and Kim, 2002) and *V. cholerae* (Chatterjee et al., 2007).

Our previous study has shown that the host factors mucin and bile salts increased the virulence of *V. anguillarum* to sea bass larvae (Li et al., 2014, in press). Mucin, bile salts and cholesterol also increased swimming motility, biofilm formation
and exopolysaccharide production, all of which are essential for virulence of the bacterium. In this study, we examined the response of 15 \textit{V. anguillarum} strains to the host factors mucin, bile salts and cholesterol and calculated correlations between this response and the virulence to sea bass larvae in order to determine whether the capability to respond to host cues is linked to virulence.

**Materials and Methods**

**Bacterial strains and culture conditions**

Fifteen \textit{V. anguillarum} strains, representing the major pathogenic serotypes O1, O2 and O3, were used in this study (Table 5.1). The bacteria were cultured in LB\textsubscript{20} medium (Luria-Bertani medium plus 2 % NaCl) at 28°C for 24 h. The density of the bacterial suspensions was determined with a spectrophotometer (Genesys 20, Thermo Scientific, U.S.A.) at 550 nm according to the McFahrland standard (BioMérieux, Marcy L’Etoile, France).

**Swimming motility assays**

Swimming motility assays were performed on soft agar (LB\textsubscript{20} Agar containing 0.3% agar) as described previously (Li et al., 2014). \textit{V. anguillarum} strains were grown overnight in LB\textsubscript{20} medium, and 2 \textmu l of the cultures (diluted to OD\textsubscript{550} = 1.0) were spotted in the centre of soft agar plates with or without 10 mg l\textsuperscript{-1} mucin (from porcine stomach, Sigma-Aldrich, U.S.A.), bile salts (50 % sodium glycocholate and 50 % sodium taurocholate, Oxoid, U.K.) or cholesterol (Sigma-Aldrich, U.S.A.). After incubation at 28°C, motility halos were measured at 24 h and 48 h. The assays were done at least in triplicate.
Table 5.1 V. anguillarum strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Virulence towards sea bass</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIB15</td>
<td>O1</td>
<td>+</td>
<td>Austin et al., 1995b</td>
</tr>
<tr>
<td>VIB93</td>
<td>O1</td>
<td>-</td>
<td>Austin et al., 1995b</td>
</tr>
<tr>
<td>87-9-116</td>
<td>O1</td>
<td>-</td>
<td>Skov et al., 1995</td>
</tr>
<tr>
<td>87-9-117</td>
<td>O1</td>
<td>+</td>
<td>Skov et al., 1995</td>
</tr>
<tr>
<td>VaNT1</td>
<td>O1</td>
<td>-</td>
<td>Pedersen et al., 1999</td>
</tr>
<tr>
<td>S3 4/9</td>
<td>O1</td>
<td>-</td>
<td>Pedersen and Larsen, 1995</td>
</tr>
<tr>
<td>JLL237</td>
<td>O1</td>
<td>+</td>
<td>Bay et al., 2007</td>
</tr>
<tr>
<td>43</td>
<td>O1</td>
<td>+</td>
<td>Grisez et al., 1996</td>
</tr>
<tr>
<td>VIB12</td>
<td>O2</td>
<td>-</td>
<td>Austin et al., 1995b</td>
</tr>
<tr>
<td>VIB103</td>
<td>O2</td>
<td>+</td>
<td>Austin et al., 1995b</td>
</tr>
<tr>
<td>VIB160</td>
<td>O2</td>
<td>+</td>
<td>Austin et al., 1995b</td>
</tr>
<tr>
<td>JLL143</td>
<td>O2</td>
<td>+</td>
<td>Bay et al., 2007</td>
</tr>
<tr>
<td>HI610</td>
<td>O2</td>
<td>+</td>
<td>Samuelsen et al., 2003</td>
</tr>
<tr>
<td>VIB113</td>
<td>O3</td>
<td>+</td>
<td>Austin et al., 1995b</td>
</tr>
<tr>
<td>CNEVA NB11008</td>
<td>O3</td>
<td>+</td>
<td>Tiainen et al., 1997</td>
</tr>
</tbody>
</table>

*a + = Virulent; - = Avirulent (according to Frans et al., 2013).

Biofilm formation assay and quantification of exopolysaccharides

The biofilm formation assay was performed in 96-well polystyrene microtiter-plates (SPL life sciences, Korea), as previously described (Brackman et al., 2008) with some modifications. Overnight cultures in LB20 were diluted with fresh LB20 medium to OD550 = 0.1 and inoculated into a 96-well plate (200 μl per well). The plate was incubated at 28 °C for 48 hours, after which wells were washed three times with 300 μl sterile physiological saline to remove all non-adherent bacteria. The remaining attached
bacteria were fixed with 200 μl of 99% methanol per well for 2 hours, and the plate was emptied and left to air dry overnight. Then, the plate was stained for 20 min with 200 μl of 1% crystal violet per well. Excess stain was rinsed off by placing the plate under running tap water. After the plate was air dried, the dye bound to the adherent cells was resolubilized with 200 μl of 95% ethanol per well. The absorbance of each well was measured at 571 nm. For the quantification of exopolysaccharides, a Calcofluor white staining (Sigma-Aldrich, U.S.A.) was used as previously described (Brackman et al., 2008). For each assay, a minimum of three different V. anguillarum cultures were used for each treatment. The reported data are representative of three independent experiments.

Statistics

The data were analyzed using one-way ANOVA followed by Tukey’s post-hoc test or by independent samples t-tests. All statistical analyses were done using the SPSS software, version 19. Correlations were calculated using the Pearson correlation coefficient.

Results

Impact of the host factors on swimming motility

All strains showed increased swimming motility in the presence of either of the three host factors, although the difference was not significant for strains VIB15 and JLL237 (for all three host factors) and for strain VIB93 (for cholesterol) at 24 hours (Table 5.2). Remarkably, strains 87-9-116, 87-9-117, VIB12, VIB103, VIB160, JLL143, VIB113 and CNEVA NB11008 showed almost no swimming motility at 24 hours in the absence of host factors, but they did in the presence of either of the three host factors. At 48 hours, all the strains showed large motility halo in the absence of host factors, except for VIB113 (Table 5.3). Moreover, all the strains showed a significantly increased
motility in the presence of either of the three host factors, except for JLL237 (for bile salts).

**Table 5.2** Motility of *V. anguillarum* strains with and without the addition of host factors (average ± standard error of three replicates) after 24 hours. Asterisks indicate significant differences between the control and the respective treatment (*P* < 0.05; **P* < 0.01).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Control</th>
<th>Mucin</th>
<th>Bile salts</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIB15</td>
<td>27 ± 1</td>
<td>33 ± 2</td>
<td>33 ± 1</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>VIB93</td>
<td>20 ± 2</td>
<td>28 ± 1*</td>
<td>30 ± 2**</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>87-9-116</td>
<td>4 ± 0</td>
<td>24 ± 1**</td>
<td>19 ± 1**</td>
<td>17 ± 0**</td>
</tr>
<tr>
<td>87-9-117</td>
<td>4 ± 0</td>
<td>25 ± 1**</td>
<td>18 ± 1**</td>
<td>26 ± 1**</td>
</tr>
<tr>
<td>VaNT1</td>
<td>35 ± 2</td>
<td>44 ± 1**</td>
<td>45 ± 1**</td>
<td>49 ± 2**</td>
</tr>
<tr>
<td>S3 4/9</td>
<td>28 ± 2</td>
<td>42 ± 1**</td>
<td>42 ± 1**</td>
<td>46 ± 1**</td>
</tr>
<tr>
<td>JLL237</td>
<td>29 ± 1</td>
<td>30 ± 2</td>
<td>34 ± 1</td>
<td>33 ± 0</td>
</tr>
<tr>
<td>43</td>
<td>32 ± 1</td>
<td>45 ± 1**</td>
<td>43 ± 1**</td>
<td>42 ± 1**</td>
</tr>
<tr>
<td>VIB12</td>
<td>15 ± 3</td>
<td>24 ± 0**</td>
<td>27 ± 1**</td>
<td>31 ± 2**</td>
</tr>
<tr>
<td>VIB103</td>
<td>4 ± 0</td>
<td>19 ± 0**</td>
<td>17 ± 2**</td>
<td>24 ± 1**</td>
</tr>
<tr>
<td>VIB160</td>
<td>4 ± 0</td>
<td>14 ± 0**</td>
<td>13 ± 1**</td>
<td>9 ± 0**</td>
</tr>
<tr>
<td>JLL143</td>
<td>4 ± 0</td>
<td>16 ± 1**</td>
<td>16 ± 0**</td>
<td>22 ± 2**</td>
</tr>
<tr>
<td>HI610</td>
<td>36 ± 1</td>
<td>55 ± 2**</td>
<td>56 ± 1**</td>
<td>52 ± 1**</td>
</tr>
<tr>
<td>VIB113</td>
<td>4 ± 0</td>
<td>11 ± 1**</td>
<td>10 ± 1**</td>
<td>11 ± 1**</td>
</tr>
<tr>
<td>CNEVA NB11008</td>
<td>4 ± 0</td>
<td>11 ± 1**</td>
<td>13 ± 1**</td>
<td>13 ± 1**</td>
</tr>
</tbody>
</table>
Table 5.3 Motility of *V. anguillarum* strains with and without the addition of host factors (average ± standard error of three replicates) after 48 hours. Asterisks indicate significant differences between the control and the respective treatment (* P < 0.05; ** P < 0.01).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Motility halo (mm)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Mucin</td>
<td>Bile salts</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>VIB15</td>
<td>49 ± 1</td>
<td>59 ± 1 **</td>
<td>60 ± 1 **</td>
<td>65 ± 0 **</td>
</tr>
<tr>
<td>VIB93</td>
<td>50 ± 0</td>
<td>60 ± 0 **</td>
<td>60 ± 0 **</td>
<td>60 ± 0 **</td>
</tr>
<tr>
<td>87-9-116</td>
<td>56 ± 1</td>
<td>61 ± 1 **</td>
<td>61 ± 1 **</td>
<td>65 ± 1 **</td>
</tr>
<tr>
<td>87-9-117</td>
<td>53 ± 2</td>
<td>63 ± 2 **</td>
<td>60 ± 0 *</td>
<td>61 ± 1 **</td>
</tr>
<tr>
<td>VaNT1</td>
<td>54 ± 2</td>
<td>63 ± 2 **</td>
<td>62 ± 2 **</td>
<td>69 ± 1 **</td>
</tr>
<tr>
<td>S3 4/9</td>
<td>50 ± 0</td>
<td>62 ± 2 **</td>
<td>61 ± 1 **</td>
<td>66 ± 1 **</td>
</tr>
<tr>
<td>JLL237</td>
<td>49 ± 1</td>
<td>58 ± 2 *</td>
<td>53 ± 1</td>
<td>58 ± 1 **</td>
</tr>
<tr>
<td>43</td>
<td>48 ± 1</td>
<td>68 ± 2 **</td>
<td>66 ± 3 **</td>
<td>64 ± 2 **</td>
</tr>
<tr>
<td>VIB12</td>
<td>63 ± 2</td>
<td>77 ± 2 **</td>
<td>74 ± 2 **</td>
<td>73 ± 2 **</td>
</tr>
<tr>
<td>VIB103</td>
<td>38 ± 1</td>
<td>70 ± 1 **</td>
<td>65 ± 3 **</td>
<td>67 ± 1 **</td>
</tr>
<tr>
<td>VIB160</td>
<td>60 ± 3</td>
<td>72 ± 2 **</td>
<td>73 ± 1 **</td>
<td>72 ± 2 **</td>
</tr>
<tr>
<td>JLL143</td>
<td>60 ± 0</td>
<td>71 ± 1 **</td>
<td>70 ± 0 **</td>
<td>71 ± 1 **</td>
</tr>
<tr>
<td>HI610</td>
<td>58 ± 2</td>
<td>64 ± 1 **</td>
<td>65 ± 0 **</td>
<td>71 ± 1 **</td>
</tr>
<tr>
<td>VIB113</td>
<td>8 ± 2</td>
<td>29 ± 2 **</td>
<td>26 ± 1 **</td>
<td>28 ± 1 **</td>
</tr>
<tr>
<td>CNEVA NB11008</td>
<td>58 ± 2</td>
<td>70 ± 3 **</td>
<td>67 ± 2 **</td>
<td>77 ± 2 **</td>
</tr>
</tbody>
</table>
Table 5.4 Biofilm formation of *V. anguillarum* strains with and without the addition of host factors (average ± standard error of three replicates) after 48h. Values in the control column with different superscript letters are significantly different from each other (*P* < 0.01). For each strain, asterisks indicate significant differences between the control treatment and the treatment with the respective host factor (** *P* < 0.01).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Biofilm formation†</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Mucin</td>
<td>Bile salts</td>
<td>Cholesterol</td>
<td></td>
</tr>
<tr>
<td>VIB15</td>
<td>0.16 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.20 ± 0.01 **</td>
<td>0.14 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>VIB93</td>
<td>0.14 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>87-9-116</td>
<td>0.03 ± 0.03</td>
<td>0.00 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>87-9-117</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>VaNT1</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>S3 4/9</td>
<td>0.02 ± 0.00</td>
<td>0.03 ± 0.00</td>
<td>0.03 ± 0.00</td>
<td>0.02 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>JLL237</td>
<td>0.03 ± 0.00</td>
<td>0.03 ± 0.00</td>
<td>0.05 ± 0.00 **</td>
<td>0.03 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>0.15 ± 0.01</td>
<td>0.23 ± 0.01 **</td>
<td>0.19 ± 0.01 **</td>
<td>0.19 ± 0.02 **</td>
<td></td>
</tr>
<tr>
<td>VIB12</td>
<td>0.07 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>VIB103</td>
<td>0.05 ± 0.01</td>
<td>0.09 ± 0.01 **</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>VIB160</td>
<td>0.29 ± 0.02</td>
<td>0.50 ± 0.02 **</td>
<td>0.50 ± 0.03 **</td>
<td>0.33 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>JLL143</td>
<td>0.22 ± 0.06</td>
<td>0.17 ± 0.05</td>
<td>0.22 ± 0.02</td>
<td>0.19 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>HI610</td>
<td>0.05 ± 0.01</td>
<td>0.00 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>VIB113</td>
<td>0.05 ± 0.00</td>
<td>0.07 ± 0.02</td>
<td>0.08 ± 0.02</td>
<td>0.07 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>CNEVA</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.00</td>
<td>0.06 ± 0.00</td>
<td>0.07 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>NB11008</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† Absorbance of Crystal Violet stained biofilm at 571nm with subtraction of a blank (medium only).
Table 5.5 Exopolysaccharide production of *V. anguillarum* strains with and without the addition of host factors (average ± standard error of three replicates) after 48h. Asterisks indicate significant differences between the control and the respective treatment (** *P* < 0.01).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Control</th>
<th>Mucin</th>
<th>Bile salts</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIB15</td>
<td>3920 ± 425</td>
<td>3716 ± 596</td>
<td>3380 ± 586</td>
<td>3726 ± 439</td>
</tr>
<tr>
<td>VIB93</td>
<td>2895 ± 410</td>
<td>2763 ± 147</td>
<td>3162 ± 841</td>
<td>2691 ± 69</td>
</tr>
<tr>
<td>87-9-116</td>
<td>960 ± 103</td>
<td>1339 ± 273</td>
<td>723 ± 91</td>
<td>1022 ± 288</td>
</tr>
<tr>
<td>87-9-117</td>
<td>1405 ± 602</td>
<td>1239 ± 559</td>
<td>1303 ± 234</td>
<td>1903 ± 229</td>
</tr>
<tr>
<td>VaNT1</td>
<td>1455 ± 186</td>
<td>2240 ± 175</td>
<td>1425 ± 509</td>
<td>1974 ± 158</td>
</tr>
<tr>
<td>S3 4/9</td>
<td>1160 ± 436</td>
<td>1499 ± 437</td>
<td>1690 ± 164</td>
<td>2017 ± 297</td>
</tr>
<tr>
<td>JLL237</td>
<td>2153 ± 491</td>
<td>1835 ± 257</td>
<td>1793 ± 387</td>
<td>2158 ± 307</td>
</tr>
<tr>
<td>43</td>
<td>731 ± 142</td>
<td>1484 ± 162 **</td>
<td>1781 ± 73 **</td>
<td>1645 ± 294 **</td>
</tr>
<tr>
<td>VIB12</td>
<td>2895 ± 110</td>
<td>3378 ± 333</td>
<td>2615 ± 289</td>
<td>3408 ± 50</td>
</tr>
<tr>
<td>VIB103</td>
<td>1036 ± 249</td>
<td>2042 ± 126 **</td>
<td>1819 ± 251 **</td>
<td>2867 ± 222 **</td>
</tr>
<tr>
<td>VIB160</td>
<td>3337 ± 242</td>
<td>4201 ± 196 **</td>
<td>5193 ± 286 **</td>
<td>4455 ± 255 **</td>
</tr>
<tr>
<td>JLL143</td>
<td>5062 ± 187</td>
<td>4236 ± 162</td>
<td>4905 ± 235</td>
<td>5437 ± 327</td>
</tr>
<tr>
<td>HI610</td>
<td>2485 ± 326</td>
<td>3308 ± 220</td>
<td>2984 ± 481</td>
<td>2804 ± 400</td>
</tr>
<tr>
<td>VIB113</td>
<td>4086 ± 190</td>
<td>3370 ± 384</td>
<td>3057 ± 390</td>
<td>3456 ± 573</td>
</tr>
<tr>
<td>CNEVA NB11008</td>
<td>2132 ± 453</td>
<td>2108 ± 480</td>
<td>1579 ± 303</td>
<td>2941 ± 913</td>
</tr>
</tbody>
</table>

1 Fluorescence intensity values (excitation at 405 nm, emission at 500 nm) of calcofluor white stained cultures with subtraction of the fluorescence intensity of a blank (medium only).
Impact of the host factors on biofilm formation and exopolysaccharide production

In general, biofilm formation was low for most of the strains (OD of stained biofilms < 0.1), except for strains VIB15, VIB93, 43, VIB160 and JLL143 (Table 5.4). The host factors had no effect on biofilm formation of the strains that showed low biofilm formation in the absence of host factors, except for JLL237 (in case of bile salts) and VIB103 (in case of mucin). Two of the five strains showing clear biofilm formation showed increased biofilm formation in the presence of mucin (43 and VIB160), 3/5 showed increased biofilm formation in the presence of bile salts (VIB15, 43 and VIB160), and 1/5 showed increased biofilm formation in the presence of cholesterol (43).

Exopolysaccharide production is one of the factors that are involved in biofilm formation. We determined exopolysaccharide production by Calcofluor white staining and found that only 3 of the strains showed increased exopolysaccharide production in the presence of host factors; the fluorescence intensity of stained exopolysaccharides of strains 43, VIB103 and VIB160 was significantly increased in the presence of either one of the three host factors (Table 5.5).

Correlation analyses between different phenotypes and virulence to sea bass larvae

In order to determine whether there is a relation between the phenotypes determined in this study (motility, biofilm formation and exopolysaccharide production) and virulence to the sea bass larvae, Pearson correlation coefficients were calculated. No significant correlations were obtained (Table 6). Further, a second series of correlations was calculated in order to determine whether there is a relation between the capability of the strains to react on host factors (i.e. percentage increase in the relevant phenotype in the presence of the host factor of interest) and virulence to sea bass larvae. For none of the host factors, a significant correlation was obtained (data not shown).
We also calculated correlation coefficients between the phenotypes in the absence of host factors. As shown in Table 5.6, no significant correlations were obtained, except for biofilm and exopolysaccharide production which were relatively strongly correlated ($r = 0.58$). Finally, we determined the correlations between the capabilities of the strains to react on the host factors (percentage increase of motility, biofilm formation and exopolysaccharide production in the presence of either of the three host factors) and found that all of them were significantly correlated (Table 5.7). Correlations were very strong, with coefficients ranging between 0.66 and 0.99.

**Table 5.6** Pearson correlations between virulence, motility, biofilm formation and exopolysaccharide production in the absence of host factors. *P* values are shown between brackets.

<table>
<thead>
<tr>
<th>Characteristic 1</th>
<th>Virulence 1</th>
<th>Motility</th>
<th>Biofilm formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility</td>
<td>0.24 (0.38)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Biofilm formation</td>
<td>-0.27 (0.33)</td>
<td>0.24 (0.39)</td>
<td>-</td>
</tr>
<tr>
<td>Exopolysaccharide production</td>
<td>-0.30 (0.28)</td>
<td>-0.11 (0.70)</td>
<td>0.58 (0.02)</td>
</tr>
</tbody>
</table>

1Mortality caused in sea bass larvae (data obtained from Frans et al., 2013)
Table 5.7 Pearson correlations between the effects of the host factors (percentage increase) on motility, biofilm formation and exopolysaccharide production. P values are shown between brackets.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Host factor 1</th>
<th>Host factor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mucin</td>
<td>Bile salts</td>
</tr>
<tr>
<td>Motility</td>
<td>Bile salts</td>
<td>0.99 (0.00)</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>0.99 (0.00)</td>
</tr>
<tr>
<td>Biofilm formation</td>
<td>Bile salts</td>
<td>0.83 (0.00)</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>0.78 (0.00)</td>
</tr>
<tr>
<td>Exopolysaccharide production</td>
<td>Bile salts</td>
<td>0.78 (0.00)</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>0.85 (0.00)</td>
</tr>
</tbody>
</table>

Discussion

Host factors are known to affect virulence in various pathogenic bacteria in general and vibrios in specific. Mucus has been reported to enhance growth (Garcia et al., 1997), protease activity (Denkin and Nelson, 1999) and chemotactic motility in *V. anguillarum* (Larsen et al., 2001). Bile and bile salts have been reported to affect virulence gene expression such as cholera toxin and toxin-coregulated pili in *V. cholerae* (Yang et al., 2013) and adherence to epithelial cells, production of thermostable direct haemolysin and type III secretion system in *V. parahaemolyticus* (Pace et al., 1997; Osawa et al., 2002; Gotoh et al., 2010). Cholesterol has been reported to inactivate *V. vulnificus* cytolsin (Kim and Kim, 2002) and increase the motility of *V. cholerae* (Chatterjee et al., 2007). Many of the virulence traits affected by host factors are needed during the early stages of infection. Flagellar motility mutants and chemotaxis mutants of *V. anguillarum* are deficient in host colonization and are avirulent towards fish (Milton et al., 1996; O'Toole et al., 1996). Exopolysaccharide production is required for *V. anguillarum* in order to be able to attach to fish tissues (Croxatto et al., 2007). A V.
**anguillarum** mutant defective for exopolysaccharide export is attenuated for virulence towards fish (Weber et al., 2010).

Our previous study showed that mucin, bile salts and cholesterol increase the virulence of *V. anguillarum* strain NB10 towards sea bass larvae, and several virulence-related phenotypes (including protease activity, swimming motility, biofilm formation and exopolysaccharide production) as well as the mRNA levels of selected genes that are involved in these phenotypes, were also increased (Li et al., 2014, in press). In the current study, we examined the virulence-related phenotypic responses of 15 *V. anguillarum* strains to these host factors. Almost all strains showed significantly increased motility in the presence of either of the three host factors except for strain JLL237 (response only for bile salts). Biofilm formation increased significantly with the addition of host factors for five *V. anguillarum* strains: 43 (with all three host factors), VIB160 (with mucin and bile salts), VIB103 (with mucin), VIB15 and JLL237 (with bile salts), respectively. Exopolysaccharide production of *V. anguillarum* strains 43, VIB103 and VIB160 was significantly increased in the presence of either one of the three host factors. These results indicate that not all *V. anguillarum* strains show the same response to the host factors. Only for strains 43, VIB103, VIB160 (in this study) and NB10 (Li et al., 2014, in press) an increased activity of all virulence factors in response to all three host factors was observed. These four strains are all virulent strains, but belong to different serotypes (O1 and O2). Although the addition of host factors increased the virulence-related phenotypic responses in some *V. anguillarum* strains, no relation could be found between the responses and virulence of the strains without previous contact with the host factors. However, there were significant correlations between the responses of the strains (expressed as percentage increase in virulence factor activity) to the host factors. This result suggests that the responses of *V. anguillarum* to the three host factors might be linked. It indicates that if *V. anguillarum* responds to one of the host factor, it will probably also respond to the other ones. It has been demonstrated that pretreatment with host factors could increase the virulence of *V. anguillarum* strain NB10 towards sea bass (Li et al., 2014, in press). Further research is necessary to
elucidate whether the virulence of the *V. anguillarum* strains included in this study is affected by previous contact with host factors, i.e. whether previous contact with a host ‘primes’ the virulence of the strains.

In this study, no significant correlations were obtained between the three virulence-related phenotypes (motility, biofilm formation and exopolysaccharide production) on one hand and virulence to the sea bass larvae on the other hand. This conclusion is consistent with observations reported in a previous study (Frans et al., 2013), where no direct relation between virulence and genotypic or phenotypic characteristics (such as serotype, the production of extracellular enzymes) was reported. These findings illustrate the complexity of the virulence mechanisms in *V. anguillarum*. Biofilm formation was significantly correlated with exopolysaccharide production (*p* < 0.05). This result is consistent with the fact that exopolysaccharide production is important with respect to bacterial biofilm formation (Karatan and Watnick, 2009).

In conclusion, this study illustrates that all 15 *V. anguillarum* strains showed increased swimming motility but only 5 strains showed increased biofilm formation and 3 strains increased exopolysaccharide production in the presence of host factors. Our results indicate that not all the *V. anguillarum* strains respond to host factors and the responses are not related to the virulence of these *V. anguillarum* strains towards sea bass larvae. Interestingly, we found strong and significant correlations between the virulence-related phenotypic responses of *V. anguillarum* to three host factors.

**Acknowledgements**

We thank Prof. Hans Rediers for providing us with *V. anguillarum* strains. This work was financially supported by the Scientific Research Fund of Flanders (FWO-Vlaanderen project n° 1.5.013.12N), the Special Research Fund of Ghent University (GOA project n° BOF12/GOA/022).
Chapter 6

General discussion and perspectives
Introduction

According to FAO, aquaculture is the fastest growing food sector globally and has established itself to fulfil the food demand since the natural resources are over exploited. Vibrios are very common in the aquatic environment, and most of them are the part of the normal flora of the water. In many cases, they are opportunists, only causing vibriosis disease when the host organism is immune-suppressed or otherwise physiologically stressed, i.e. poor environmental conditions, inadequate diet and poor husbandry techniques (Peddie and Wardle, 2005). Therefore, understanding the host and bacteria interactions is fundamental for determining more effective strategies for disease prevention and control. It is proposed that the outcome of disease is the imbalance of environment, host and microbial relationship. How the bacteria interact with each other and the host to cause disease is represented in Figure 6.1 in the V. anguillarum/fish model and will be discussed throughout the following paragraphs.

Over the past decade, vibriosis caused by V. anguillarum is one of the most prevalent fish diseases and results in large economic losses. In a previous study with a model system with gnotobiotic sea bass larvae (Rekecki et al., 2012), an increasing number of bacteria was localised in the gastrointestinal tract lumen post exposure to V. anguillarum strain HI610. No significant colonization of the skin was observed. Bacterial adhesion to the apical portion of gut enterocytes was already observed at 2 hours post exposure and adhesion to the oesophageal mucosa was demonstrated at 48 hours post exposure. The presence of V. anguillarum within the swim bladder and within both mid and hindgut enterocytes was related to the pathogenesis of this bacterium. However, the mechanisms of pathogenicity behind the disease caused by V. anguillarum are not yet completely understood. This study addresses the role of bacterial cell-to-cell communication and bacteria-host interactions on the virulence of V. anguillarum in this sea bass larvae model system.
The impact of cell-to-cell signaling on the virulence of *V. anguillarum*

The QS mechanisms of vibrios vary in complexity and cellular output even though the components are quite similar. This may reflect the plasticity of the Vibrio genomes to respond to a broad spectrum of activities in a highly variable aquatic environment, including associations with animal tissues (Milton, 2006). Several phenotypes and gene products associated with virulence in some pathogenic species of vibrios have been shown to be QS-regulated. In contrast to *V. harveyi*, *V. vulnificus*, *V. alginolyticus* and *V. salmonicida*, where QS plays an essential role in virulence towards hosts, no direct correlation between virulence and AHL/three-channel QS system has been described in *V. anguillarum* (Table 6.1). It is confirmed in this study that
inactivation of neither the AHL-based QS, nor the three-channel system resulted in decreased virulence of *V. anguillarum* towards sea bass larvae (Chapter 3).

The *rpoS* mutant of *V. anguillarum* was attenuated for virulence towards sea bass larvae and deficient in biofilm formation and EPS production, but increased in motility. The changes on mRNA levels of selected genes that are involved in these phenotypes further confirmed our findings. These data indicate that RpoS has a strong impact on not only the virulence-related phenotypes expression but also the virulence of *V. anguillarum*, which has already been documented before by Ma et al. (2009) in an injection model with zebrafish. RpoS also has been reported to be essential for virulence in pathogens *B. burgdorferi* and *S. enterica* (Dong and Schellhorn, 2010). In *V. cholerae*, RpoS is required for efficient colonization and mucosa escape response, but not critical for *in vivo* survival in infant mouse (Yildiz and Schoolnik, 1998). In *V. vulnificus*, RpoS positively regulates stress response, extracellular protease and motility; however the impact in virulence towards animals has not been tested (Hülsmann et al., 2003).
Table 6.1 *Vibrio* pathogens in aquaculture and the link with quorum sensing.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>QS signals</th>
<th>Regulatory proteins</th>
<th>Phenotype</th>
<th>Virulence in animal models</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vibrio</em> harveyi</td>
<td>3-hydroxy-C4-HSL, HAI-1 HSL, Al-2, CAI-1</td>
<td>LuxM/N, LuxS/PQ, CqsA/S, LuxO, LuxR&lt;sub&gt;v&lt;/sub&gt;</td>
<td>Siderophore, type III secretion, chitinase, exotoxin T1, polysaccharide, metalloprotease</td>
<td>AI-2 and CAI-1, but not HAI-1, were needed for full virulence of <em>V. harveyi</em> towards brine shrimp larvae. HAI-1 and Al-2, but not CAI-1, were needed for full virulence towards giant fresh water prawn larvae.</td>
<td>Henke and Bassler, 2004a, b; Defoirdt et al., 2008; Natrah et al., 2011; Pande et al., 2013</td>
</tr>
<tr>
<td><em>Vibrio</em> anguillarum</td>
<td>3-hydroxy-C4-HSL, 3-oxo-C6-HSL, Vanl/R, VanM/N, VanS/PQ, CqsA/S, VanO, VanT</td>
<td>LuxS/PQ, LuxO, LuxR&lt;sub&gt;v&lt;/sub&gt;</td>
<td>Biofilm, metalloprotease, serine and glycine protease, pigment, siderophore, EPS, haemolysin, lipase, neurotoxic acetylcholinesterase</td>
<td>Inactivation of neither the AHL-based QS, nor the three-channel system resulted in decreased virulence of <em>V. anguillarum</em> towards brine shrimp and fish. Addition of indole decreased its virulence towards sea bass larvae in this study.</td>
<td>Milton et al., 1997; Milton et al., 2001; Croxatto et al., 2002; Defoirdt et al., 2005; Buchholtz et al., 2006</td>
</tr>
<tr>
<td><em>Vibrio</em> vulnificus</td>
<td>C4-HSL, 3-oxo-C6-HSL, C10-HSL, C12-HSL, minor AHLs, AI-2</td>
<td>LuxS/PQ, LuxO, SmcR (LuxR&lt;sub&gt;v&lt;/sub&gt; homologue)</td>
<td>Metalloprotease, cytolysin, haemolysin, EPS, siderophore, toxin RTX</td>
<td>Mutation of LuxS or SmcR significantly decreased the virulence in mice.</td>
<td>Kim et al., 2003; Lee et al., 2007b; Valiente et al., 2009</td>
</tr>
<tr>
<td>Pathogens</td>
<td>QS signals</td>
<td>Regulatory proteins</td>
<td>Phenotype</td>
<td>Virulence in animal models</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------------------</td>
<td>---------------------</td>
<td>------------------------------------------</td>
<td>---------------------------------------------------</td>
<td>---------------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>AI-2, CAI-1</td>
<td>LuxI/R homologues</td>
<td>Type III secretion, type VI secretion systems,</td>
<td>Unknown</td>
<td>Henke and Bassler, 2004b; Gode-Potratz and McCarter, 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>motility, EPS, iron transport, opacity</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio alginolyticus</em></td>
<td>3-hydroxy-C₄-HSL,</td>
<td>LuxM/N, LuxS/PQ, LuxO, LuxRᵥh master</td>
<td>type VI secretion, motility, extracellular protease,</td>
<td>Mutation of LuxT led to a nearly two-fold</td>
<td>Liu et al., 2012; Sheng et al., 2012</td>
</tr>
<tr>
<td></td>
<td>HSL, AI-2, CAI-1</td>
<td>regulator, LuxT regulator</td>
<td>EPS, biofilm</td>
<td>attenuation in the virulence in zebrafish.</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio salmonicida</em></td>
<td>C₆-HSL, 3-oxo-C₆-HSL</td>
<td>LuxI/R homologues, LuxS/PQ, LitR master</td>
<td>Cryptic bioluminescence, motility, adhesion,</td>
<td>Reduced mortality was observed in salmon</td>
<td>Nelson et al., 2007; Bjelland et al., 2012</td>
</tr>
<tr>
<td></td>
<td>HSL, AI-2</td>
<td>regulator</td>
<td>biofilm</td>
<td>infected with LitR mutant.</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio scophthalmi</em></td>
<td>3-hydroxy-C₁₂-HSL,</td>
<td>LuxS/PQ, SmcR master regulator</td>
<td>Biofilm</td>
<td>Unknown</td>
<td>García-Aljaro et al., 2008; García-Aljaro et al., 2012</td>
</tr>
<tr>
<td></td>
<td>unknown AHLS, AI-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio mimicus</em></td>
<td>AI-2 homologue</td>
<td>LuxS/PQ, LuxO, LuxRᵥh homologues</td>
<td>Protease, haemolysin</td>
<td>Unknown</td>
<td>Sultan et al., 2006</td>
</tr>
<tr>
<td><em>Vibrio ichthyoenteri</em></td>
<td>Three unknown AHLs,</td>
<td>LuxS/PQ homologues</td>
<td>Biofilm</td>
<td>Inactivation of LuxS had no effect on</td>
<td>Li et al., 2010</td>
</tr>
<tr>
<td></td>
<td>AI-2</td>
<td></td>
<td></td>
<td>virulence towards turbot.</td>
<td></td>
</tr>
</tbody>
</table>
The novel findings in this work are the regulation mechanisms of rpoS and indole in *V. anguillarum*. In our study, the rpoS mutant showed higher indole production and higher expression of the indole synthase gene tnaA than the wild-type. The addition of exogenous indole decreased biofilm formation, EPS levels as well as the expression of the EPS synthesis gene *wbfD* in wild type *V. anguillarum*. Furthermore, addition of indole to either the sea bass larvae rearing water or pre-treatment of wild-type *V. anguillarum* with indole before challenge resulted in a significantly decreased virulence. The addition of indole inhibitor restored part of the virulence of the rpoS mutant. These results are opposite to what has been reported for *V. cholerae*, where indole activates the expression of polysaccharide synthesis genes and increases biofilm formation (Mueller et al., 2009). However, in other pathogenic bacteria such as *Edwardsiella tarda*, mutation of indole synthase gene tnaA reduces LPSs production, antibiotic resistance and virulence towards zebrafish (Han et al., 2011b). In *Salmonella enterica*, indole increases drug resistance, represses various genes related to virulence and decreases bacterial motility and invasive activity (Nikaido et al., 2008; Nikaido et al., 2012). Indole and 7-benzyloxyindole attenuate the virulence of *Staphylococcus aureus* (Lee et al., 2013). In *P. aeruginosa*, indole has been reported to reduce virulence factors by modulating the expression of virulence and regulatory genes, which are reportedly regulated by the QS system (Kim and Park, 2013). Our results suggest that indole is an important signaling molecule to regulate virulence of *V. anguillarum*. However, whether or not indole interferes with QS system in *V. anguillarum* still needs further investigation.

Since hundreds of indole derivatives are commercially available (Bunders et al., 2011), further screening on a larger indole library is likely to generate potent therapeutics for *V. anguillarum* and possibly for other important pathogens. However, we need to be aware that indole might have additional effects in multispecies communities. Indole e.g. increases drug resistance in *Salmonella enterica* (Nikaido et al., 2008) and activates genes involved in polysaccharide production, increases biofilm formation in *V. cholerae* (Mueller et al., 2009). Moreover, indole and indole derivatives may have some biological functions in the host body, which might be either beneficial or
detrimental. Recently, it was shown for instance, that indole significantly increases expression of genes involved in strengthening the mucosal barrier and mucin production and expression of cytoskeleton genes, suggesting increased resistance to pathogen colonization in human (Bansal et al., 2010). The impact of indole towards fish is currently not known. However, we noticed that indole affected the sea bass larvae since they were clearly more active and pigmented in the 50 μM and 100 μM indole treatments (especially the 100 μM treatment), while 200 μM indole was toxic to sea bass larvae. Indole at different concentrations can have different effects (beneficial effects or harmful effects) on the hosts. Before indole or indole derivatives can be used as antivirulence compounds, further research will be needed on the effect of indole on harmless and beneficial bacteria as well as on the host.

The impact of host factor sensing on the virulence of V. anguillarum

In the previous works in our lab, Dierckens et al. (2009) set up a challenge model system with gnotobiotic sea bass larvae. However, in Chapter 2 we found out that the variable mortality of sea bass larvae between stocking (DAH 0) and the day of V. anguillarum bath challenge (DAH 7) significantly affected the survival of sea bass larvae after challenge. In order to accommodate for this, an improved experimental set-up was developed in which the vibrios were added to the culture water at \(10^5\) CFU ml\(^{-1}\) on DAH 3 together with homogenised fish (added at 3 mg l\(^{-1}\), equivalent to 3 larvae per 10 ml). The timelines of these two challenge models are shown in Figure 6.2.
In European sea bass, complete yolk absorption takes place at DAH 4, and the complete utilization of the oil globule takes place at DAH 6 (Klaoudatos et al., 1990). In sea bass hatcheries, first feeding with live *Artemia* nauplii is normally given at DAH 7 (Klaoudatos et al., 1990). In this thesis, the sea bass larvae were not fed during the entire trial (lasting up to DAH 11), and the animals were kept under conditions that aimed at minimizing energy consumption (constant blue light, gentle agitation). Experience from previous tests taught us that under our experimental conditions, larvae start to die from starvation after 14 days, and no significant mortality was observed in non-challenged larvae until the end of the experiments in this study. However, we acknowledge that starvation might have attributed to the sensitivity of the larvae to *V. anguillarum*. Indeed, starvation as a kind of stress has been shown to depress the disease resistance of Chinook salmon (*Oncorhynchus tshawytscha*) (Maule et al., 1988).
By the 1930s, it was apparent that interfering host defense mechanisms could increase the virulence of certain microbes. The first report was that meningococcal infections in mice are promoted by mucin (Miller and Castles, 1936). In a more recent report, *V. cholerae* shed from the human gastrointestinal tract showed greatly enhanced infectivity, which led the authors to hypothesize that epidemics may be propagated by the human host (Merrell et al., 2002). In this study, both *in vitro* and *in vivo* observations have shown that host factors present in the homogenised hosts (homogenised sea bass larvae or brine shrimp) strongly increased the motility of *V. anguillarum* as well as the virulence towards sea bass larvae (Chapter 2). This host-induced increase in virulence of *V. anguillarum* was further investigated by specifically characterizing the factors which might exist in the homogenised hosts (Chapter 4). Host factors mucin, the bile salts glycocholate and taurocholate, and cholesterol increased the virulence of *V. anguillarum* towards sea bass larvae, several virulence-related phenotypes (protease activity, flagellar motility, biofilm formation and exopolysaccharide production) as well as the mRNA levels of selected genes that are involved in these phenotypes. Our results indicate that *V. anguillarum* uses host mucin, bile salts, and cholesterol as cues to promote the expression of several important virulence traits.

Moreover, the presence of these host factors significantly increased the mRNA levels of alternative sigma factor *rpoS* (2.3-4.4 fold increase) (Table 6.2). RpoS is involved in the response of many bacteria to various stresses. It has been shown before to regulate the expression of the EmpA metalloprotease in *V. anguillarum* (Weber et al., 2008). RpoS has also been shown to regulate the expression of the EPS production in this study (Chapter 3). Here, our results indicate that RpoS is not only required for environmental stress response, but also involved in the host-pathogen interaction. However, further confirmation is needed by testing the responses of *rpoS* mutant to the host factors.
Table 6.2 Impact of the host factors cholesterol, bile salts and mucin (10 mg l⁻¹) on the expression of the alternative sigma factor rpoS (average ± standard error of three replicate *V. anguillarum* cultures).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Relative transcription (fold)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NB10</td>
</tr>
<tr>
<td>rpoS</td>
<td>Alternative sigma factor</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

¹mRNAs were isolated from three independent *V. anguillarum* cultures (grown to stationary phase; OD₅₅₀ 1.5) for each treatment. mRNA levels were normalized to the rpoA mRNA levels. For each gene, the expression in the untreated control was set at 1 and the expression in all other treatments was normalized accordingly using the 2ΔΔCT method. Asterisks indicate significant differences between the control and the respective treatment (* P<0.05; ** P<0.01).

Mucins are large, filamentous glycoproteins that coat the surfaces of the cells lining the respiratory, digestive, and urogenital tracts and the skin. Similar type of mucins, the transmembrane MUC1-type mucin and gel-forming MUC2-type mucins, have been identified in human as well as in the puffer fish (*Fugu rubripes*) and gilthead sea bream (*Sparus aurata*) (Lang et al., 2004; McGuckin et al., 2011; Pérez-Sánchez et al., 2013). It has been reported that despite considerable similarity in the overall chemical composition and physical structure, intestinal mucins from different hosts (rabbit, human and rat) showed significant differences in bacterial binding by *Yersinia enterocolitica* (Mantle et al., 1989). Therefore, it would be interesting to investigate whether there is a difference between the porcine stomach mucin used in this study and fish mucin with respect to virulence factor production in *V. anguillarum*.

Biofilm formation is thought to be a multi-step process that requires the primary adhesion of bacteria to a substrate surface followed by the formation of multiple cell layers (Von Eiff et al., 1999). Surface properties strongly influence the biofilm formation.
Adhesion to hydrophobic substrata (acrylic) for *Staphylococcus epidermidis* occurred to a greater extent than that to hydrophilic surfaces (glass) (Cerca et al., 2005). Biofilms of *P. aeruginosa* formed on mucin-coated surfaces developed large cellular aggregates and had increased tolerance to the antibiotic tobramycin compared with biofilm grown on glass (Landry et al., 2006). In this thesis, biofilm formation of *V. anguillarum* was measured on polystyrene substrata (96-well polystyrene plates). However, the structure and function might be different when *V. anguillarum* forms biofilm in vivo. Hence, it would be interesting to investigate biofilm formation on fish mucus-covered surfaces or on fish cell cultures.

Virulence varies amongst different *V. anguillarum* strains. Frans et al. (2013) has demonstrated that no relation could be found among virulence towards sea bass larvae and serotype, genotypic or virulence factors in 15 *V. anguillarum* strains belonging to 3 different serotypes. However, the reason for this is not yet known. Recent studies reveal that phenotypic heterogeneity is common in bacteria. During invasion of the hosts, formation of bacterial pathogen subpopulations is often controlled by epigenetic mechanisms that generate inheritable phenotypic diversity without altering the DNA sequence (Casadesús and Low, 2013). The host-pathogen interaction is an inherently unstable relationship. Recent findings suggest that epigenetic regulation can play a key role in phenotypic diversification and co-evolution of hosts and pathogens (Kasuga and Gijzen, 2013). Epigenetic regulation can be one possible reason why no correlation is found between genetic make up of *V. anguillarum* strains and virulence.

In Chapter 5, we examined the phenotypic responses of 15 *V. anguillarum* strains to the host factors mucin, bile salts and cholesterol. However, not all the *V. anguillarum* strains respond to host factors. Only four strains responded in the increase of the virulence-related phenotypes to three host factors. Furthermore, no significant correlations were obtained between three phenotypes (motility, biofilm formation and EPS production) and virulence to the sea bass larvae. Our work illustrates the complexity of the virulence mechanisms in *V. anguillarum* and suggests that virulence in *V. anguillarum* is highly multi-factorial. The knowledge of the impact of bacterial signaling
systems not only informs us how *V. anguillarum* interacts with aquatic organisms, but also provides further targets that could be pursued for antibiotic drug development.

**Proposed virulence mechanism**

In this thesis, we demonstrate that RpoS is essential for the virulence regulation of *V. anguillarum*. RpoS also plays a role in the host-pathogen interaction. The virulence of *V. anguillarum* is highly affected by indole and host factors. The mechanism how RpoS, indole and host factors affect the virulence of *V. anguillarum* is proposed in Figure 6.3.

![Proposed mechanism by which RpoS, indole and host factors affect the virulence of *V. anguillarum* NB10 towards sea bass larvae.](image)

**Figure 6.3** Proposed mechanism by which RpoS, indole and host factors affect the virulence of *V. anguillarum* NB10 towards sea bass larvae. The solid circles stand for positive effect and the dashed circle stands for negative effect on the virulence of *V. anguillarum* towards sea bass larvae. Solid lines with arrows stand for positive regulation. Dashed lines and short bars stand for negative regulation.
Further research

This study raises the following questions which could be dealt with in future research. We have proposed the possible mechanisms by which RpoS, indole and host factors affect the virulence of *V. anguillarum* towards sea bass larvae. However, further research is needed in order to unravel the exact mode of action. It is necessary to further confirm the role of RpoS in the virulence regulation of *V. anguillarum*. The complementation of the *rpoS* mutant needs to be tested in the challenge test, which would be predicted to restore the virulence. To verify if the reduction of the virulence is due to the over-expression of indole in *rpoS* mutant, *rpoS tnaA* double mutant should be generated and used to challenge sea bass larvae. Further research is needed on the identification of more host factors that can affect the virulence of *V. anguillarum*, i.e. stress hormones (adrenaline, noradrenaline and dopamine). Finally, it might be very interesting to evaluate the impact of the host factors on the virulence of different *V. anguillarum* strains in the sea bass challenge test.

Further perspectives on disease control and prevention

As a result of the growing awareness that antibiotics should be used with more care, there is a trend to develop alternative ways to kill pathogenic bacteria with antibiotics (Defoirdt, 2013).

Vaccine can prevent or ameliorate morbidity from infection by stimulating an individual’s immune system to develop specific immunity to a pathogen. However, specific immunity is not yet developed in fish larvae (Vadstein et al. 2013) and therefore, vaccination of larvae is not possible. RpoS is essential for virulence of *V. anguillarum*. Due to the virulence deficiency of *rpoS* mutants, they become potential candidate vaccine agents in *Salmonella enterica* (Coynault et al., 1996; Curtiss et al., 2009). However, we need to be aware that using *rpoS* mutant as a vaccine is serotype-dependent. Protection from infection of wild-type *S. enterica* Serovar Dublin can be achieved with preinoculation of *rpoS* mutants of *S. enterica* Serovar Dublin, but not with
a heterologous preparation made from subsp. *S. enterica* Serovar Typhimurium (Coynault and Norel, 1999).

Another alternative to antibiotics is preventing pathogens from attacking the host, without the need to kill them, i.e. antivirulence therapy (Clatworthy et al., 2007). Unlike antibiotics, antivirulence compounds diminish bacterial virulence without affecting cell viability and thus, may not lead to drug resistance (Kim and Park, 2013). Indole is likely to generate potent therapeutics for *V. anguillarum* and possibly for other important pathogens. Further research will be needed on screening of a larger indole derivatives and the effect of indole on harmless and beneficial bacteria as well as on the host.
APPENDICES


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Summary

*Vibrio anguillarum* is the major cause of haemorrhagic septicaemia, vibriosis, which is a severe disease affecting a variety of fish species and other aquatic animals, and causes large economic losses in the aquaculture industry worldwide. However, the mechanisms of pathogenicity of this bacterium are not yet completely understood. In this work, we investigated the impact of different regulation systems (including QS, and host factor sensing) on the virulence of *V. anguillarum* in a model system with gnotobiotic sea bass larvae.

First, a literature research was done on the current knowledge of the virulence and regulation mechanisms of *V. anguillarum* (Chapter 1).

In Chapter 2, an improved challenge set-up of the gnotobiotic sea bass model was established and the effect of dead hosts on the virulence of *V. anguillarum* was investigated by using this improved model system. Addition of homogenised hosts led to significantly increased larval mortality of challenged larvae. Our results suggest that dead hosts increase infectivity of *V. anguillarum*, not merely by offering nutrients to the bacteria, but also by increasing virulence-associated activities such as motility.

In Chapter 3, the impact of QS and RpoS on the virulence of *V. anguillarum* towards gnotobiotic sea bass larvae was investigated. No direct correlation between virulence and quorum sensing has been found in *V. anguillarum*. RpoS is essential for the virulence of *V. anguillarum* and regulates several virulence-related phenotypes. Indole negatively affected exopolysaccharide production and decreased the virulence of *V. anguillarum* to sea bass larvae. The indole inhibitor acetyltryptophan restored part of the virulence of rpoS mutant.

In Chapter 4, how dead hosts modulate the virulence of *V. anguillarum* was further demonstrated. The effect of host factors (mucin, bile salts and cholesterol) on several virulence-related phenotypes in *V. anguillarum* and on the virulence of the bacterium towards gnotobiotic sea bass larvae was detected. Host factors increased the virulence of *V. anguillarum* towards sea bass larvae, although the effect was not
significant for cholesterol. All three host factors significantly increased several virulence-related phenotypes as well as the mRNA levels of selected genes that are involved in these phenotypes.

In Chapter 5, the response of 15 *V. anguillarum* strains to the host factors mucin, bile salts and cholesterol was examined and the correlations between this response and the virulence to sea bass larvae were calculated. Our results indicate that there is no significant correlation between phenotype responses and virulence to the sea bass larvae. However, there are strong linkages between each of the virulence-related phenotype response to three host factors.

Finally, in Chapter 6, the most important results are highlighted and discussed. Suggestions for further research are proposed, including perspectives on sustainable disease control and prevention, i.e. *rpoS* mutant as attenuated vaccine and indole as antivirulence compound.

In conclusion, the work presented in this thesis indicates the complexity of the virulence mechanisms in *V. anguillarum* and proposes a signaling mechanism by which RpoS, indole and host factors regulate the virulence of *V. anguillarum*.
Samenvatting

*Vibrio anguillarum* is één van de belangrijkste oorzaken van vibrios. Dit is een ernstige ziekte die verschillende vissoorten en andere aquatische dieren aantast en die wereldwijd grote economische verliezen in de aquacultuurindustrie veroorzaakt. Ondanks het grote belang van *V. anguillarum* voor de aquacultuur begrijpt men de pathogeniciteitsmechanismen van deze bacterie nog niet volledig. In dit werk hebben we de impact van verschillende regulatiesystemen, waaronder quorum sensing (bacteriële cel tot cel communicatie) en detectie van gastheerfactoren, op de virulentie van *V. anguillarum* onderzocht in een modelsysteem met gnotobiotische zeebaarslarven.

Eerst werd er een literatuurstudie uitgevoerd met betrekking tot de huidige kennis over virulentie en regulatiemechanismen in *V. anguillarum* (**Hoofdstuk 1**).

In **Hoofdstuk 2** werd een verbeterde experimentele set-up voor het gnotobiotische zeebaarsmodel ontwikkeld. Gebruikmakend van dit verbeterde modelsysteem werd het effect van dode gastheren (vislarven of pekelkreeftjes) op de virulentie van *V. anguillarum* onderzocht. Toediening van gehomogeniseerde larven van zeebaars of het pekelkreeftje leidde tot een significant verhoogde mortaliteit van geïnfecteerde zeebaarslarven. Onze resultaten laten vermoeden dat dode gastheren de infectiviteit van *V. anguillarum* verhogen, niet enkel door een sterkere vermeedering van de pathogene, maar ook door stimulatie van karakteristieken die een belangrijke rol spelen bij infectie, zoals “swimming motility”.

In **Hoofdstuk 3** werd de invloed van quorum sensing (bacteriële cel tot cel communicatie) en de alternatieve sigma factor RpoS op de virulentie van *V. anguillarum* tegenover gnotobiotische zeebaarslarven onderzocht. Er werd geen verband gevonden tussen virulentie en quorum sensing. RpoS bleek wel essentieel voor de virulentie van *V. anguillarum* en door de productie van verscheidene virulentie-gerelateerde fenotypes te regelen, waaronder de productie van de signaalmolecule indool. Indool verlaagde de virulentie van *V. anguillarum* tegenover zeebaarslarven door exopolysaccharide
productie te verlagen. De indool inhibitor acetyltryptofaan herstelde deels de virulentie van de avirulente rpoS mutant.

In Hoofdstuk 4 werd de manier waarop dode gastheren de virulentie van *V. anguillarum* beïnvloeden verder onderzocht. Er werd aangetoond dat de gastheerfactoren mucine, galzouten en cholesterol de virulentie van *V. anguillarum* tegenover gnotobiotische zeebaarslarven stimuleren. De drie gastheerfactoren verhoogden significant de expressie van verscheidene virulentie-gerelateerde fenotypes.

In Hoofdstuk 5 werden de reacties van 15 *V. anguillarum* stammen op de gastheerfactoren mucine, galzouten en cholesterol onderzocht en de correlaties tussen deze reacties en de virulentie tegenover zeebaarslarven werden berekend. Onze resultaten tonen aan dat er geen significante correlatie is tussen de respons op de gastheerfactoren en virulentie tegenover de zeebaarslarven. Darentegen zijn er sterke en significante correlaties wat betreft de fenotypische responsen van de stammen op de verschillende gastheerfactoren.

In Hoofdstuk 6 worden de belangrijkste resultaten benadrukt en bediscussieerd. Mogelijkheden voor verder onderzoek worden voorgesteld, waaronder voorstellen voor duurzame ziektebestrijding en preventie, zoals een rpoS mutant als vaccin en indool als antivirulentie product.

Ter conclusie, het werk voorgesteld in deze thesis toont de complexiteit van virulentiemechanismen in *V. anguillarum* aan en stelt een mechanisme voor waardoor RpoS, indool en gastheerfactoren de virulentie van *V. anguillarum* regelen.
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Publications


Li, X., Defoirdt, T., Bossier, P. (2014) Relation between virulence of *Vibrio anguillarum* strains and response to the host factors mucin, bile salts and cholesterol. (Manuscript in preparation)
Conference

Host-induced increase of larval sea bass mortality in a gnotobiotic challenge test with Vibrio anguillarum. (Poster presentation)

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