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Review

Vibrio anguillarum as a fish pathogen: virulence factors, diagnosis and prevention

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Abstract

Vibrio anguillarum, also known as *Listonella anguillarum*, is the causative agent of vibriosis, a deadly haemorrhagic septicaemic disease affecting various marine and fresh/brackish water fish, bivalves and crustaceans. In both aquaculture and larviculture, this disease is responsible for severe economic losses worldwide. Because of its high morbidity and mortality rates, substantial research has been carried out to elucidate the virulence mechanisms of this pathogen and to develop rapid detection techniques and effective disease-prevention strategies. This review summarizes the current state of knowledge pertaining to *V. anguillarum*, focusing on pathogenesis, known virulence factors, diagnosis, prevention and treatment.

Keywords: detection, pathogen profile, pathogenesis, treatment, vibriosis.

Introduction

In 1893, Canestrini reported an epidemic disease in migrating eels, *Anguilla anguilla* (L.), dating back to 1817 and referred to the causative bacterium as *Bacterium anguillarum* (Canestrini 1893). A few years later, Bergman proposed the name *Vibrio anguillarum* for the aetiological agent of the 'red pest of eels' in the Baltic Sea (Bergman 1909). Because of the high similarity of the disease signs and characteristics of the causal bacterium described in both reports, it was suggested that it concerned the same causative agent.

Currently, *V. anguillarum* is widely found in various cultured and wild fish as well as in bivalves and crustaceans in salt or brackish water causing a fatal haemorrhagic septicaemic disease, called vibriosis (Aguirre-Guzmán, Ruíz & Ascencio 2004; Paillard, Leroux & Borrego 2004; Toranzo, Magarinos & Romalde 2005). In this review, main characteristics of *V. anguillarum* and vibriosis are discussed with an emphasis on known virulence factors, pathogenesis, diagnosis, detection and prevention.

Vibrio anguillarum

Vibrio anguillarum is a Gram-negative, comma-shaped rod bacterium, belonging to the family *Vibrionaceae*. It is polarly flagellated, non-spore-forming, halophilic and facultatively anaerobic (Madigan, Martinko & Parker 2000; Buller 2004) (Fig. 1). The bacterium grows rapidly at temperatures between 25 and 30 °C on rich media containing 1.5–2% sodium chloride (NaCl), forming cream-coloured and round-shaped colonies.

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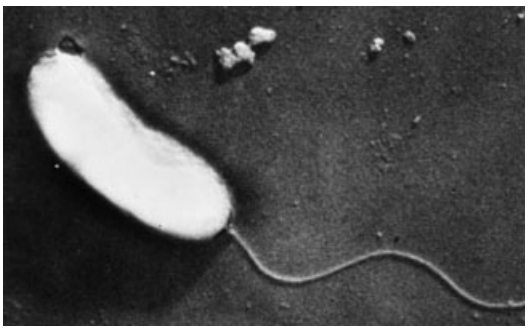

Figure 1 Electron micrograph of *Vibrio anguillarum* 775 showing the single polar flagellum. Shadow preparation, ×10 000 (Actis *et al.* 1999).
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Initially, *V. anguillarum* was divided into two separate biotypes (1 and 2) (Harrell, Novoty, Schiewe & Hodgins 1976). However, as a result of advances in DNA technology, *V. anguillarum* biotype 2 was reclassified as a new species, *Vibrio ordalii*, in honour of Erling J. Ordal (Schiewe, Trust & Crosa 1981). Based on 5S ribosomal RNA (rRNA) gene sequence analysis, *V. anguillarum* biotype 1 was reclassified as *Listonella anguillarum* (MacDonell & Colwell 1985). Nevertheless, it is generally still referred to as *V. anguillarum* because of its strong relatedness with other *Vibrio* species (Wiik, Stackebrandt, Valle, Daae, Rodseth & Andersen 1995; Thompson, Thompson, Vandemeulebroecke, Hoste, Dawyndt & Swings 2004; Thompson, Gevers, Thompson, Dawyndt, Naser, Hoste, Munn & Swings 2005; Thompson, Thompson, Dias, Naka, Dubay & Crosa 2011). For example, the 16S rRNA gene of *V. anguillarum* shows more than 95% similarity with corresponding genes of *V. alginolyticus*, *V. parahaemolyticus* and *V. fischeri*, a generally accepted guideline for defining a genus (Wiik *et al.* 1995; Clarridge 2004).

At present, 23 O serotypes (O1–O23) within *V. anguillarum* are distinguished, each displaying a different pathogenicity and host specificity. Of these, only serotypes O1 and O2, and to a lesser extent serotype O3, are associated with vibriosis in fish (Pedersen, Grisez, van Houdt, Tiainen, Ollevier & Larsen 1999). The other *V. anguillarum* serotypes represent environmental isolates from sediment, plankton or sea water that are mostly non-pathogenic. Within serogroup O2, three host-specific sero-subgroups, O2a, O2b and O2c, can be distinguished and a fourth sero-subgroup is suggested (Knappskog, Rødseth, Slinde & Endresen 1993; Larsen, Pedersen & Dalsgaard 1994; Tiainen, Pedersen & Larsen 1997; Mikkelsen, Lund, Martinsen, Gravningen & Schroder 2007; Mikkelsen, Lund, Larsen & Seppola 2011).

The total genome size of *V. anguillarum* is about 4.2 Mbp with a GC content of 43–46% (Naka, Dias, Thompson, Dubay, Thompson & Crosa 2011). It is composed of two circular chromosomes of 3.0 and 1.2 Mbp, respectively, which is a common feature among *Vibrio* species. Heidelberg, Eisen, Nelson, Clayton, Gwinn, Dodson, Haft, Hickey, Peterson & Umayam (2000) suggested that this might be a survival strategy allowing the pathogen to rapidly adapt to different environments (e.g. sea water, host). Random genome sequencing of *V. anguillarum* strain H775-3 resulted in a partial genome sequence, covering approximately 1.5 Mbp (Rodkhum, Hirono, Stork, Lorenzo, Crosa & Aoki 2006b). The assembly and annotation of the complete genome sequence of *V. anguillarum* strains 43 and HI-610 is currently ongoing. Strain 43 belongs to serotype O1 and was isolated from a diseased sea bass, *Dicentrarchus labrax* (L.), by Professor B. Austin (University of Stirling, UK). Dr O. Bergh (Institute of Marine Research, Bergen, Norway) isolated strain HI-610, belonging to serotype O2a, from cod, *Gadus morhua* L., suffering from vibriosis. Recently, strain 43 was reported to be non-pathogenic to sea bass larvae using a gnotobiotic sea bass larvae test system, in contrast to the highly pathogenic strain HI-610 (Dierckens, Rekecki, Laureau, Sorgeloos, Boon, Van den Broeck & Bossier 2008). In addition to the chromosomes, *V. anguillarum* serotype O1 possesses a virulence plasmid, designated pJM1 or pEIB1, encoding a siderophore-dependent iron sequestering system. The sequence of these plasmids has previously been reported and is around 65–67 kbp (Di Lorenzo, Stork, Tolmasky, Actis, Farrell, Welch, Crosa, Wertheimer, Chen, Salinas, Waldbeser & Crosa 2003; Wu, Ma, Zhang & Zhang 2004).

Vibriosis

Susceptible organisms

Vibrio anguillarum causes vibriosis in more than 50 fresh and salt-water fish species including various species of economic importance to the larviculture and aquaculture industry, such as salmon, *Salmo salar* L.; rainbow trout, *Oncorhynchus mykiss* (Walbaum); turbot, *Psetta maxima* (L.); sea bass, sea bream, *Sparus aurata* L.; cod, eel, and ayu, *Plecoglossus altivelis*

(Temminck & Schlegel) (Buller 2004; Toranzo *et al.* 2005). Bivalve molluscs and crustaceans are also occasionally affected by the bacterium (Aguirre-Guzmán *et al.* 2004; Paillard *et al.* 2004).

Clinical signs of the disease

As previously mentioned, *V. anguillarum* is the causative agent of a highly fatal haemorrhagic septicaemia known as vibriosis. Based on the clinical signs, vibriosis is also known as salt-water furunculosis (Rucker 1963), boil disease (Kubota & Takakuwa 1963) and ulcer disease (Bagge & Bagge 1956). Typical external clinical signs of vibriosis include weight loss, lethargy, red spots on the ventral and lateral areas of the fish and swollen and dark skin lesions that can ulcerate and bleed (Fig. 2). The eyes are also infected, resulting in opacity at first, and later in ulceration and exophthalmia. The pathogen is found in high concentrations in the blood and haematopoietic tissues. Internally, the intestines may be distended and filled with a clear, viscous liquid. Because of a pH gradient in the gastrointestinal tract, the pathology is more severe in the posterior gastrointestinal tract and rectum than in the anterior parts of the tract. Nevertheless, in acute epizootics, the infection spreads so rapidly that most of the infected fish die without showing any clinical signs (Actis, Tolmasky & Crosa 1999; Toranzo *et al.* 2005; Austin & Austin 2007).

Infection route of *Vibrio anguillarum*

The infection route of *V. anguillarum* is still a major point of discussion in the literature. Both infection through the skin as well as oral intake of the pathogen through contaminated water or food appears to cause vibriosis in fish (Grisez, Chair, Sorgeloos & Ollevier 1996; Svendsen & Bogwald 1997; O'Toole, von Hofsten, Rosqvist, Olsson & Wolf-Watz 2004; Weber, Chen & Milton 2010). In most cases, infection occurs through penetration of the fish skin. Although continuous renewal of the mucous layer prevents the adhesion of bacteria to the epithelial cells, injuries or a damaged mucous layer are typical points of entry for *V. anguillarum* (Svendsen & Bogwald 1997; Spanggaard, Huber, Nielsen, Nielsen & Gram 2000; O'Toole *et al.* 2004; Croxatto, Lauritz, Chen & Milton 2007; Weber *et al.* 2010). Occasionally, oral ingestion of *V. anguillarum* can also result in the development of vibriosis. Infection starts with the intake of the bacterium through contaminated food or water, after which *V. anguillarum* enters the gastrointestinal tract. Although its growth is greatly inhibited by low pH, research has shown that *V. anguillarum* is able to survive the acidic environment of the stomach (Larsen 1984). Moreover, the gastrointestinal tract of larvae is not fully developed having a pH which is not low enough to inactivate *V. anguillarum*. The pathogen is therefore often detected when a disease outbreak is reported in larvae (Spanggaard *et al.* 2000). After passage through the stomach, the gut becomes a site of adhesion, colonization and proliferation using the intestinal mucus as an important nutrient source (Olsson, Joborn, Westerdahl, Blomberg, Kjelleberg & Conway 1998; O'Toole, Lundberg, Fredriksson, Jansson, Nilsson & Wolf-Watz 1999; O'Toole *et al.* 2004). *V. anguillarum* then moves across the intestinal epithelium by endocytosis followed by the release of the bacteria in the lamina propria. Subsequently, the pathogen enters the blood stream, resulting in blood poisoning (septicaemia) or in the infection of various internal organs, such as liver, spleen and kidney (Grisez *et al.* 1996).

Figure 2 Haemorrhaging on the fins and around the operculum of a sea bass caused by *Vibrio anguillarum* (Austin & Austin 2007).
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Environmental factors influencing *V. anguillarum* infection

Important factors inducing vibriosis include chemical stress (e.g. water quality, pollution, diet composition), biological stress (e.g. population density, presence of other micro- or macro-organisms) and physical stress (e.g. temperature). Regarding the latter, an outbreak of vibriosis only occurs when water temperature exceeds 15 °C (Austin & Austin

2007). Larsen, Blackburn, Larsen & Olsen (2004) revealed that the chemotactic response of *V. anguillarum* towards fish mucus is influenced by both the temperature and the salinity of the water. Optimum growth rates require 1–2% NaCl. Nevertheless, *V. anguillarum* can also be found in freshwater environments such as Lake Biwa in Japan, where it is responsible for severe losses of ayu, one of the most important species in Japanese freshwater fisheries. Darkness, coldness, microaerobiosis and high cell density enhance survival of *V. anguillarum* in this environment, retaining its culturability and pathogenicity (Eguchi, Fujiwara & Miyamoto 2000; Eguchi, Fujiwara-Nagata & Miyamoto 2003). Fujiwara-Nagata & Eguchi (2003) suggested that the survival of this pathogen in the bottom sediments of Lake Biwa can be explained by the formation of a biofilm, a niche which is often associated with enhanced bacterial survival. The polymer matrix of a biofilm also protects *V. anguillarum* against environmental pollutants and provides a scavenging system for obtaining essential minerals and nutrients.

Fish immune system

Fish are constantly threatened by pathogenic micro-organisms, of which bacteria are an important group. However, under normal conditions, the fish retains its healthy state by defending itself against potential pathogens mainly by using a complex system of host defence mechanisms. First, physical and chemical barriers provide an innate defence mechanism that protects the fish against the attachment and invasion of micro-organisms. For instance, fish are protected by the continuous renewal of skin mucus containing various antibacterial substances such as antimicrobial peptides (pleurocidin), proteases (trypsin-like proteases and cathepsin L and B proteases), lectins and lysozymes. The gastrointestinal tract is a hostile environment of acids, bile salts and enzymes able to inactivate and digest various pathogenic bacteria. The blood serum also contains a range of factors that inhibit bacterial growth, such as iron-binding proteins (transferrin) and anti-proteases or factors such as lysozymes that are bactericidal (Ellis 1999, 2001; Magnadottir 2006).

Second, the complement system provides a pathogen-specific immune response. Under normal circumstances, the complement system is in an inactive state, but it can be activated by interaction of complement components with antibody–antigen complexes (classical complement activation), or by direct contact with Gram-negative bacteria (alternative antibody-dependent complement activation). Both activation routes result in the death of the susceptible pathogen either by creating pores in the bacterial cell membrane or by enhancing phagocytosis (Holland & Lambris 2002).

However, fish larvae rely on non-specific defence reactions as their adaptive immune system is still insufficiently developed. It takes, for example, about 2–3 months after hatching for cod larvae to develop a fully competent immune system (Engelsen, Sandlund, Fiksdal & Bergh 2008). Consequently, fish larvae are much more vulnerable to any pathogen present in the environment than adult fish resulting in high mortality rates (Bricknell & Dalmo 2005).

Virulence factors

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, an iron uptake system, lipopolysaccharides (LPSs) and extracellular products with proteolytic or haemolytic activity. The following presents an overview of some important virulence factors of this pathogen.

The importance of chemotactic motility and adhesion

Motility and chemotaxis are important virulence factors in several pathogenic bacteria (Ottemann & Miller 1997; Josenhans & Suerbaum 2002; Rediers, Rainay, Vanderleyden & De Mot 2005). In *V. anguillarum*, chemotaxis for components in the mucus layer on the skin or the gut is an important first step in infection (O'Toole, Milton & Wolf-Watz 1996; O'Toole *et al.* 1999). O'Toole *et al.* (1996) indeed showed that a mutation in the *cheR* gene, encoding a cytoplasmic methyl transferase essential for the response to chemotaxic signals, resulted in a decreased virulence in rainbow trout. However, no decreased virulence of the *cheR* mutant was observed when fish were infected intraperitoneally, suggesting that chemotaxis plays an important role in the movement of the pathogen towards the host but not in infection and persistence in the host.

The exact interaction between *V. anguillarum* and its host is not fully understood. It is, however, known that the flagellum of this mobile bacterium plays an essential role in the pathogenesis (O'Toole *et al.* 1996; Ormonde, Horstedt, O'Toole & Milton 2000). Once the bacterium has penetrated the fish skin, active motility seems no longer to be a requirement for the development of vibriosis. Nevertheless, it has been demonstrated that flagellin A, one of the five flagellin subunits, is required for persistence in systemic infections. Milton, O'Toole, Horstedt & Wolf-Watz (1996) showed that removing the conserved C terminus of flagellin A resulted in a decreased virulence when fish were infected intraperitoneally or through contaminated water. Similar phenomena were observed when other flagellin subunits such as *flaD* and *flaE* were mutated (McGee, Horstedt & Milton 1996). In *V. cholerae*, however, transcription of flagellin antisense RNA was upregulated during infection of its host (Camilli & Mekalanos 1995). A possible role of this antisense RNA is the downregulation of flagellin synthesis after the invasion of the host as motility is no longer required, and the flagellum is a possible target for the immune system.

Adhesion to the host is an important step in host colonization and subsequent infection. Bacterial adhesion to host epithelial cells is facilitated by the presence of so-called adhesins, such as pili and fimbriae, outer membrane proteins, LPS and extracellular polysaccharides (Pizarro-Cerda & Cossart 2006). Rodkhum *et al.* (2006b) identified several genes involved in biosynthesis (*pilC*) and assembly (*pilB*, *pilQ*) of type IV pili and suggested a possible role of the corresponding proteins in *V. anguillarum* virulence. Furthermore, Wang & Leung (2000) demonstrated the involvement of extracellular polysaccharides, LPSs, and glycoproteins in adhesion. However, to elucidate the adhesion processes and the importance of *V. anguillarum* adhesins in virulence, more research is required.

Invasion of host tissues

Protease activity. After adhesion of *V. anguillarum* to the skin or the intestinal mucus, it needs to penetrate the epithelium before it can cause a systemic infection in the host. The discovery of an extracellular metalloprotease (EmpA) with mucinase activity produced by *V. anguillarum* suggests that EmpA is possibly involved in mucus degradation and penetration (Norqvist, Norrman & Wolf-Watz 1990). As a result of the tissue damage, caused by the proteolytic activity of this protease, the pathogen can colonize and penetrate the fish and subsequently cause a systemic infection, affecting various organs such as liver and spleen (Milton, Norqvist & Wolf-Watz 1992; Denkin & Nelson 1999, 2004; Croxatto *et al.* 2007). Moreover, it was shown that the EmpA protease activity is strongly induced by elicitors within the mucus of the fish (Denkin & Nelson 1999). In other pathogenic *Vibrio* species such as *V. aestuarianus*, *V. cholerae*, *V. proteolyticus*, *V. splendidus*, *V. tubiashii* and *V. vulnificus*, it was also demonstrated that EmpAs contribute to their pathogenicity in fish and shellfish larvae (Hasegawa, Gharaibeh, Lind & Häse 2009). Despite these studies, it is not exactly known how *V. anguillarum* is able to persist in its host.

Haemolysin activity. Bacterial haemolysins are exotoxins that cause lysis of erythrocytes in the host and thus the release of the intracellular haem, and are therefore identified as important virulence factors of *Vibrionaceae* owing to their contribution to the haemorrhagic septicaemia (Zhang & Austin 2005). Moreover, haemolysins are known to also cause lysis in other cell types, including mast cells, neutrophils and polymorphonuclear cells. *V. anguillarum* has several extracellular haemolysins (VAH 1–5), each contributing to the haemolytic activity of the pathogen (Hirono, Masuda & Aoki 1996; Rodkhum, Hirono, Crosa & Aoki 2005). In addition, Li, Rock & Nelson (2008) identified a repeat in toxin (*rtx*) operon which also contributes to the haemolytic activity of *V. anguillarum*. This *rtx* gene cluster contains six genes (*rtxACHBDE*) where *rtxA* encodes the RTX toxin, *rtxC* encodes the RTX toxin activating protein (acylase), *rtxH* encodes a conserved hypothetical protein and *rtxBDE* encodes the RTX ABC transporter. The RTX toxins are a diverse group of pore-forming exotoxins, including cytolytic toxins, metalloproteases and lipases, synthesized by many Gram-negative bacteria (Rodkhum *et al.* 2006b). Fish infection studies by intraperitoneal injection demonstrated the importance of this RTX toxin as a major virulence factor of *V. anguillarum*. However,

further research on the exact role of this toxin during invasion of the intestinal epithelium is necessary to fully understand its role in *V. anguillarum* virulence (Li *et al.* 2008).

Iron sequestering system

Siderophore-dependent iron-acquisition system. Fish have a non-specific defence mechanism using iron-binding proteins such as transferrin, lactoferrin and ferritin, making iron unavailable for potential pathogenic micro-organisms. However, by producing siderophores and subsequent uptake of the ferri-siderophores into the cell, several bacterial fish pathogens, such as *Aeromonas salmonicida* subsp. *salmonicida*, *Tenacibaculum maritimum*, *Yersinia ruckeri*, and also *V. anguillarum*, can circumvent this defence mechanism (Avendano-Herrera, Toranzo, Romalde, Lemos & Magarinos 2005; Fernández, Méndez & Guijarro 2007; Najimi, Lemos & Osorio 2009; Lemos, Balado & Osorio 2010). Most of the *V. anguillarum* serotype O1 strains synthesize the siderophore anguibactin, encoded on the so-called virulence plasmid (Actis, Fish, Crosa, Kellerman, Ellenberger, Hauser & Sanders-Loehr 1986; Stork, Lorenzo, Welch, Crosa & Crosa 2002; Di Lorenzo *et al.* 2003; Wu *et al.* 2004). In contrast, strains belonging to serotype O2 usually express a chromosomally encoded siderophore, vanchrobactin (Conchas, Lemos, Barja & Toranzo 1991; Soengas, Anta, Espada, Paz, Ares, Balado, Rodríguez, Lemos & Jiménez 2006). Alice, Lopez & Crosa (2005) and Naka, Lopez & Crosa (2008), however, showed the coexistence of both the plasmid-encoded and the chromosomally encoded iron sequestering system in a *V. anguillarum* serotype O1 strain. Lemos *et al.* (2010) hypothesized that such *V. anguillarum* strains obtained the plasmid-encoded anguibactin by horizontal gene transfer. Furthermore, *V. anguillarum* serotype O3, also causing fish disease, possesses a third, chromosomally encoded, high-affinity iron uptake system, enabling growth in iron-limiting conditions. In addition to a catechol-like siderophore, serotype O3 strains also produce iron-regulated outer membrane proteins which may function as an iron acquisition system (Muino, Lemos & Santos 2001). The exact mechanism of iron uptake by siderophore-dependent iron-acquisition systems in *V. anguillarum* has been described in detail elsewhere (Stork *et al.* 2002; Stork, Di Lorenzo, Mourino, Osorio, Lemos & Crosa 2004; Balado, Osorio & Lemos 2006, 2008, 2009; Lopez & Crosa 2007; Stork, Otto & Crosa 2007; Lemos *et al.* 2010). Several genes involved in the anguibactin synthesis (*angB/G*, *angM*, *angN*, *angR* and *angT*) and iron-anguibactin uptake (*fatA*, *fatB*, *fatC* and *fatD*) have been isolated from the virulence plasmid, and several of these genes are clustered in the iron transport biosynthetic operon (ITBO) (Stork *et al.* 2002). A schematic overview of the *V. anguillarum* virulence plasmid pJM1 is presented in Fig. 3.

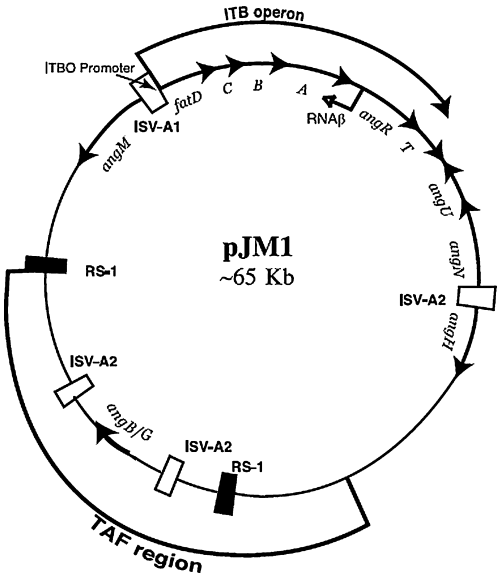

Figure 3 Schematic representation of the *Vibrio anguillarum* virulence plasmid pJM1 (Stork *et al.* 2002). Reprinted with permission from Elsevier.

Both the biosynthesis genes and the transporter genes are tightly regulated. Presently, five regulators have been identified which regulate the gene expression of this iron uptake system, i.e. the ferric uptake regulator protein (Fur), the plasmid-mediated antisense RNAs (RNAα and RNAβ), the so-called trans-acting factor, the anguibactin regulatory protein (AngR), and the siderophore itself. More information regarding the regulation of the siderophore-dependent iron acquisition system can be found in Crosa & Walsh (2002), Stork *et al.* (2002) and Lemos *et al.* (2010).

Siderophore-independent iron-acquisition system. In addition to siderophore-mediated iron uptake, *V. anguillarum* is also able to sequester iron from haem and haem-containing proteins such as

haemoglobin and haemoglobin-haptoglobin. This process is analogous to the iron-siderophore mechanism and subject to transcriptional regulation by the repressor protein Fur (Mazoy, Osorio, Toranzo & Lemos 2003; Mouriño *et al.* 2004; Mouriño, Rodriguez-Ares, Osorio & Lemos 2005; Mouriño, Osorio, Lemos & Crosa 2006; Lemos & Osorio 2007). Iron uptake from haem is facilitated by the production of bacterial haemolysins or cytotoxins resulting in the release of haem into the environment, as previously discussed.

The role of lipopolysacharides

As indicated earlier, the complement system of fish plays an important role in the host defence mechanism against pathogenic bacteria. However, some Gram-negative bacteria can circumvent the complement-mediated immune response by avoiding activation of the complement system or because the activated complement system is unable to attack the bacterium (Holland & Lambris 2002). It has been recognized that smooth strains of Gram-negative bacteria, containing a long polysaccharide side chain (the O antigen) in their LPS structure, are more resistant to the complement system than so-called rough strains, which do not contain the O-antigen (Muschel & Larsen 1970). Boesen, Pedersen, Larsen, Koch & Ellis (1999) demonstrated a correlation between the length of the O-antigen of the LPS structure of *V. anguillarum* and the susceptibility to complement-mediated killing of this pathogen. Recently, the same relationship was established in smooth virulent *A. hydrophila* strains and rough attenuated variants (Swain, Behera, Mohapatra, Nanda, Nayak, Meher & Das 2010). A possible explanation is that the O-antigen structure protects the bacterium from the complement system components because of steric hindrance (Boesen *et al.* 1999). In addition, the O-antigen of *V. anguillarum* appears to be involved in anguibactin-mediated iron uptake. A transposon insertion in the O-antigen biosynthesis operon resulted in ferri-siderophore uptake deficiency. It was shown that the mutant showed a drastic decrease in the outer membrane receptor for iron-anguibactin transport (FatA), suggesting that O-antigen-containing LPS are required for FatA to be properly translocated to or to be stably maintained in the outer cell membrane (Welch & Crosa 2005).

Outer membrane proteins

Because of the differences in the salinity of the host (0.85%) and sea water (3.5%), *V. anguillarum* requires an efficient osmolarity regulation to cope with changes in osmotic pressure. Five salt-responsive outer membrane proteins were identified that play a role in the adaptation to different salinities (Kao, Cheng, Kuo, Lin, Lin, Chow & Chen 2009). One of these proteins, Omp26La, a porine-like peptidoglycan-associated protein, is involved in the adaptation of *V. anguillarum* to low salinity, but the exact biological mechanism remains unknown. In contrast, OmpW and OmpU are porine-like proteins that are probably required for the efficient efflux of NaCl. Both proteins are upregulated in increased salt concentrations. Furthermore, the 38-kDa major outer membrane protein OmpU also plays an important role in bile resistance and therefore contributes to survival and colonization in the fish intestine. The synthesis of OmpU is regulated by the transcriptional activator ToxR in response to environmental changes such as osmolarity and nutrient limitation. In addition, changes in OmpU expression lead to alterations in the LPS and phospholipid profile in the outer membrane and hence in membrane permeability. Although OmpU is involved in bile resistance, the loss of this outer membrane protein does not result in a decrease in virulence for rainbow trout. It is therefore possible that *V. anguillarum* possesses two outer membrane proteins that are both involved in bile resistance (Wang, Lauritz, Jass & Milton 2002, 2003).

Regulation of virulence gene expression

Role of quorum sensing. The quorum sensing system, a bacterial cell–cell communication which regulates gene expression in response to population density, is often involved in the control of virulence gene expression in bacterial pathogens (de Kievit & Iglewski 2000; Whitehead, Barnard, Slater, Simpson & Salmond 2001; Winzer & Williams 2001). The production of quorum sensing signalling molecules was demonstrated in several Gram-negative fish pathogens, including *V. anguillarum*, suggesting an important role of quorum sensing during infection of the host (Bruhn, Dalsgaard, Nielsen, Buchholtz, Larsen & Gram 2005) which corroborates findings from other studies. For example, it was found that the use of specific

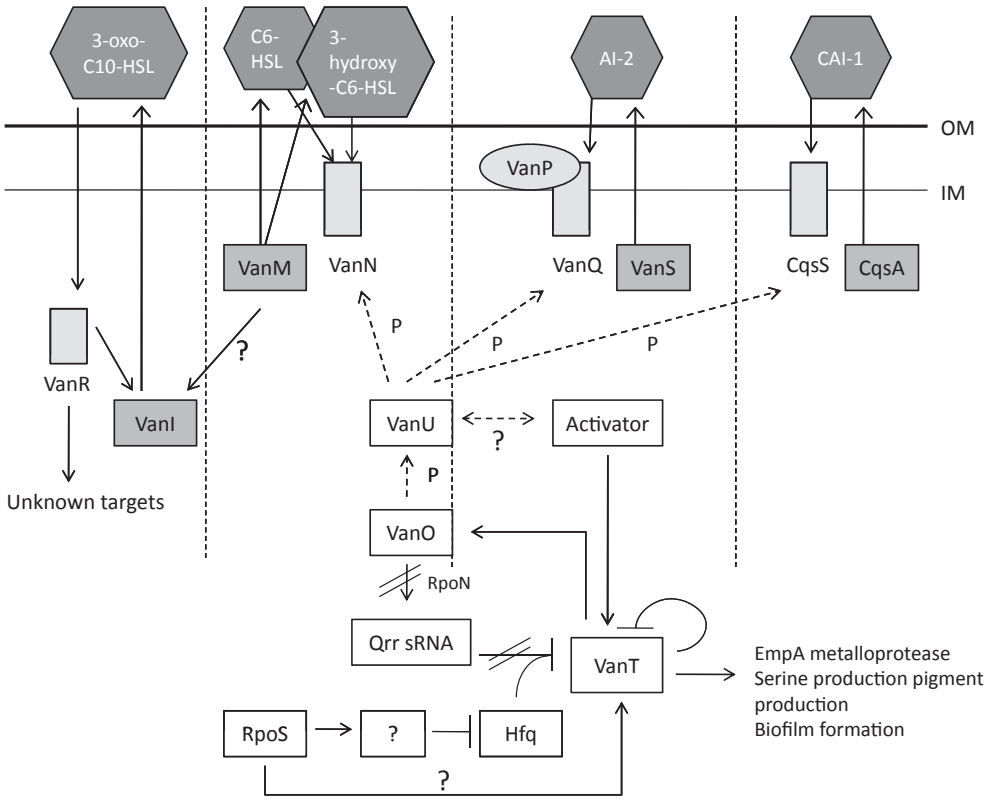

Figure 4 Quorum sensing systems in *Vibrio anguillarum*. A detailed description is given in the text. Dashed arrows indicate the relay of phosphate groups from one protein to another. Solid arrows indicate activation of gene expression while solid lines with a bar indicate repression of gene expression. IM, inner membrane; OM, outer membrane (adapted from Milton 2006; Weber *et al.* 2008).

quorum sensing inhibitors, such as furanone C-30, caused a reduced mortality in rainbow trout infected with *V. anguillarum* (Rasch, Buch, Austin, Slierendrecht, Ekmann, Larsen, Johansen, Riedel, Eberl, Givskov & Gram 2004). In addition, Brackman, Celen, Baruah, Bossier, Van Calenbergh, Nelis & Coenye (2009) illustrated the potential of quorum sensing inhibitors as promising antipathogenic agents.

Milton (2006) identified three quorum sensing systems in a *V. anguillarum* serotype O1 strain, including the VanM/N, VanS/PQ and VanI/R quorum sensing systems (Fig. 4). The VanM/N and Van-S/PQ systems are homologous to the respective LuxM/N and LuxS/PQ systems of the free-living marine bacterium *Vibrio harveyi*. The third quorum sensing system (VanI/R) is similar to the bioluminescent *Vibrio fisheri* LuxI/R system. Henke & Bassler (2004) suggested that quorum sensing in *V. anguillarum* is even more complicated to understand because of the presence of a fourth predicted quorum sensing system, i.e. the CqsA/S signal system synthesizing (S)-3-hydroxytridecan-4-one as a signal molecule. A schematic representation of the quorum sensing systems in *V. anguillarum* is given in Fig. 4. A more detailed description is given in Milton (2006) and Weber, Croxatto, Chen & Milton (2008). Briefly, in the VanM/N system, an *N*-acyl homoserine lactone (AHL) synthase (VanM) synthesizes two small diffusible signal molecules, *N*-hexanoyl-L-homoserine lacton (C6-HSL) and *N*-(3-hydroxyhexanoyl)-L-homoserine lacton (3-hydroxy-C6-HSL), which are detected by the sensor kinase VanN. In the VanS/PQ system, VanS synthesizes an AI-2 signal (3A-methyl-5,6-dihydro-furo(2,3-D)(1,3,2)dioxaborole-2,2,6,6A-tetraol), which binds the periplasmic protein VanP, which is detected by the sensor kinase VanQ. The binding of these signalling molecules results in a phosphorylation cascade which eventually leads to the activation of the quorum sensing-regulated transcriptional activator VanT (Croxatto, Pride, Hardman, Williams, Camara & Milton 2004). In the *V. anguillarum*

VanI/R system, VanI synthesizes *N*-(3-Oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL), which binds to VanR, a transcriptional activator of VanI, and probably other target genes that are as yet unknown (Milton, Hardman, Camara, Chhabra, Bycroft, Stewart & Williams 1997; Milton, Chalker, Kirke, Hardman, Camara & Williams 2001). As shown in *V. fisheri*, there is also a hierarchical relationship between the VanM/N and VanS/PQ signal systems and the VanI/R system of *V. anguillarum*. The deletion of VanM eliminates the production of all AHLs, suggesting that VanM also affects the VanI/R quorum sensing system (Milton *et al.* 2001).

Quorum sensing regulates a number of target genes, including a few virulence genes. For example, it was shown that a *V. anguillarum vanT* mutant is deficient in the EmpA, which is required for invasion of the pathogen (Croxatto, Chalker, Lauritz, Jass, Hardman, Williams, Camara & Milton 2002). Similarly, in *V. cholerae*, the VanT homologue (HapR) is required for expression of the haemagglutinin protease (Jobling & Holmes 1997). VanT also positively regulates pigment synthesis (*hpdA*) and biofilm formation (*vps73* and *sat*), which may play a role in the survival of *V. anguillarum* in its host (Croxatto *et al.* 2002). The *V. anguillarum hpdA* gene, encoding a 4-hydroxyphenylpyruvate dioxygenase (HPPD), is involved in the production of a red-brown pigment in response to stress conditions. The HPPD enzymes of *V. vulnificus* also have haemolytic activity, suggesting a possible role of the *V. anguillarum* homologue in virulence. Both *vps73* and *sat* genes are involved in the production of extracellular polysaccharides for biofilm formation (Croxatto *et al.* 2002). Thus, further research on the *hpdA* gene and the *vps73* and *sat* genes and their role in the pathogenesis of *V. anguillarum* is needed.

Sigma factors as virulence regulators. The σ^{54} sigma factor (RpoN) is involved in the transcription of a wide range of genes in response to environmental and metabolic changes, particularly in response to nitrogen limitation. The *V. anguillarum* σ^{54} factor also plays a role in the transcriptional regulation of flagellum biosynthesis. *rpoN* mutants are deficient in flagellin subunits and exhibit a non-motile aflagellate phenotype, resulting in a reduced virulence. To date, no additional virulence factors have been identified that are under transcriptional control of RpoN after the bacterium has penetrated the host. This suggests that RpoN plays an important role in movement towards the host, but probably not in persistence (O'Toole, Milton, Horstedt & Wolf-Watz 1997).

The σ^{38} sigma factor (RpoS), a key regulator during starvation and the stationary growth phase, also contributes to the pathogenesis of *V. anguillarum*. The importance of this sigma factor for virulence in zebra fish was demonstrated by the decreased pathogenesis of a *V. anguillarum rpoS* mutant (Ma, Chen, Liu, Zhang & Jiang 2009). In addition, it was shown that RpoS is involved in the transcriptional regulation of the EmpA (Denkin & Nelson 2004) and in the post-transcriptional regulation of VanT. RpoS possibly stabilizes *vanT* mRNA at high cell density by a mechanism involving the repression of the RNA chaperone Hfq, important in stabilizing the small quorum regulatory RNAs (Qrr sRNAs) (Weber *et al.* 2008). In addition, Ma *et al.* (2009) demonstrated that RpoS is also involved in the resistance of the pathogen to heat, UV irradiation and oxidative stress contributing to its survival in varying environmental conditions. Furthermore, the synthesis of extracellular enzymes, including phospholipases, diastases, lipases, caseinases, haemolysins, catalases and proteases is also under the control of RpoS.

Diagnosis and detection

Rapid, accurate and reliable detection is crucial in taking proper control measures to effectively minimize economic losses caused by *V. anguillarum*. Driven by the limitations of classical culture-dependent detection and identification techniques, these methods are increasingly being replaced or complemented by culture-independent techniques (Cunningham 2002; Altinok & Kurt 2003). In relation to *V. anguillarum* detection, the following discusses both methods.

Culture-dependent techniques

Traditional diagnostic methods include interpretation of clinical and histological signs, culturing of the pathogen in or on a selective medium and analysis of the morphological or biochemical characteristics of the presumptive pathogen. Different selective media, such as thiosulphate citrate bile salts sucrose agar medium (TCBS) and *V. anguillarum* medium (VAM), have been developed for the detection of *V. anguillarum* in water samples.

However, these media are not conclusive as other *Vibrio* spp. are also able to grow on TCBS and VAM (Bolinches, Romalde & Toranzo 1988; Alsina, Martinez-Picado, Jofre & Blanch 1994). Furthermore, *V. anguillarum* can also be present in a viable but non-culturable state during the winter months, resulting in false-negative results (Eguchi *et al.* 2003; Oliver 2009). The identity of putative *V. anguillarum* colonies can be confirmed by analysis of biochemical properties, such as nitrate reduction activity, oxidase and catalase activity, and indole production (Buller 2004). However, identification based on metabolic fingerprinting is not always conclusive, requiring additional confirmation assays (Grisez, Ceusters & Ollevier 1991; Santos, Romalde, Bandin, Magarinos, Nunez, Barja & Toranzo 1993; Austin, Alsina, Austin, Blanch, Grimont, Grimont, Jofre, Koblavi, Larsen, Pedersen, Tiainen, Verdonck & Swings 1995; Kühn, Austin, Austin, Blanch, Grimont, Jofre, Koblavi, Larsen, Möllby, Pedersen, Tiainen, Verdonck & Swings 1996; Vandenberghe, Thompson, Gomez-Gil & Swings 2003; Popovic, Coz-Rakovac & Strunjak-Perovic 2007).

Culture-independent techniques

Traditional identification methods suffer from a number of considerable drawbacks. Standard culture tests permit pathogen detection, but are time-consuming, labour-intensive and have a limited accuracy and reliability. Conventional techniques also do not lend themselves well to managing large numbers of samples. In addition, the tests that are routinely utilized for pathogen identification do not directly characterize virulence factors. Thus, these tests do not provide the needed information about the potential pathogenicity or virulence of the identified organism. These disadvantages have promoted the development of alternative, rapid and culture-independent detection and identification techniques. Essentially, these techniques can be divided into serological techniques, which make use of specific antibodies, and nucleic acid-based techniques.

A fluorescently labelled monoclonal antibody/DAPI (4′,6-diamidino-2-phenylondole) double staining technique was developed to detect *V. anguillarum* in water environments without the need for a cultivation step (Miyamoto & Eguchi 1997). In addition, a latex agglutination-based assay (BIONOR Mono-Va-kit) was developed for the rapid detection of *V. anguillarum* (Romalde, Magarinos, Fouz, Bandin, Núñez & Toranzo 1995). Although this kit contains latex particles coated with monoclonal antibodies against the three pathogenic serotypes O1, O2 and O3, some strains of the environmental serotypes O4, O5 and O7 were found to lead to false-positive results. Furthermore, cross-reactions with *Vibrio splendidus* and motile *Aeromonas* strains are frequently observed (Romalde *et al.* 1995; Gonzalez, Osorio & Santos 2004a). Recently, an ELISA-based (Bionor AQUA-RAPID-Va test) and a magnetic particle enzyme immunoassay (Bionor AQUAEIA-Va test) have been developed for *V. anguillarum* (Gonzalez *et al.* 2004a). These commercial kits suffer from the same limitations as more conventional techniques, i.e. cross-reaction with other serotypes or related bacteria. In addition, antibody-based identification techniques are known to have limited sensitivity, limiting their use in analysing environmental samples (Lievens, Grauwet, Cammue & Thomma 2005).

In contrast, nucleic acid-based techniques have the advantage of being both highly specific and sensitive, especially if they make use of polymerase chain reaction (PCR) amplification. A number of species-specific probes have been developed allowing identification of *V. anguillarum* through hybridization experiments (Martinez-Picado, Blanch & Jofre 1994; Ito, Ito, Uchida, Sekizaki & Terakado 1995). For instance, a single 17-base-pair oligonucleotide probe based on a 5S rRNA gene sequence was developed to detect *V. anguillarum* and its close relative *V. ordalii* (Ito *et al.* 1995). Increasingly, PCR-based techniques have been developed for *V. anguillarum* detection. Hirono *et al.* (1996) developed a PCR protocol for the amplification of a haemolysin gene (*vah1*). However, not all *V. anguillarum* isolates contain a *vah1* homologue, leading to false-negative results. For this reason, a multiplex PCR was developed for the detection of haemolysin-producing *V. anguillarum* using primers targeting the five known haemolysin genes (*vah1-vah5*) (Rodkhum, Hirono, Crosa & Aoki 2006a). Other PCR protocols, amplifying the *rpoN* gene, encoding the σ^{54} sigma factor, the *empA* gene, encoding an EmpA, the *amiB* gene, encoding a peptidoglycan hydrolase, or the *groEL* gene, encoding bacterial chaperonins, have been developed. In these protocols, special emphasis has been given to the capability to discriminate *V. anguillarum* from *V. ordalii*, which share around 98.8% 16S rDNA sequence identity, and to distinguish pathogenic and non-pathogenic

V. anguillarum strains (Wiik *et al.* 1995; Gonzalez, Osorio & Santos 2003; Demircan & Candan 2006; Hong, Kim, Bae, Ahn, Bai & Kong 2007; Xiao, Mo, Mao, Wang, Zou & Li 2009; Kim, Kim, Kim, Cho, Ahn & Kong 2010). To our knowledge, available PCR-based protocols for the detection and identification of *V. anguillarum* are currently based on conventional PCR, requiring time-consuming post-PCR handling steps for amplicon evaluation, such as agarose gel electrophoresis. However, among the various PCR strategies available, those based on monitoring the amplification reaction in real time are probably the most promising and are therefore expected to replace conventional end-point PCR strategies for rapid, sensitive and specific detection of microbes (Lievens *et al.* 2005; Zhou, Hou, Li & Qin 2007). Real-time quantitative reverse-transcriptase PCR has already been used to study the regulation of the metalloprotease gene and the transcription of haemolysin genes in *V. anguillarum* (Staroscik, Denkin & Nelson 2005; Rock & Nelson 2006). It is thus expected that real-time PCRs for detection and identification of *V. anguillarum* in water samples or fish tissues will be developed in the near future.

Another method that rapidly amplifies genomic DNA with high specificity and amplification efficiency is the loop-mediated isothermal amplification method (LAMP) (Notomi, Okayama, Masubuchi, Yonekawa, Watanabe, Amino & Hase 2000). This method is based on an auto-cycling strand displacement DNA synthesis, performed by a DNA polymerase with high strand displacement activity and a set of four or six specific primers. The entire procedure is performed under isothermal conditions and may be completed in 1 h producing a mixture of stem-loop DNAs with specific lengths (Notomi *et al.* 2000; Nagamine, Hase & Notomi 2002; Savan, Kono, Itami & Sakai 2005). Both Kulkarni, Caipang, Brinchmann, Korsnes and Kiron (2009) and Gao, Li, Zhang, Wang & Xiang (2010) successfully developed a LAMP assay to detect *V. anguillarum* based on the *amiB* gene and the *empA* gene, respectively. LAMP is a rapid, highly specific, sensitive and cost-effective alternative to PCR which can be used for the detection of *V. anguillarum* both in the laboratory and in the field. Although the methods described earlier are convenient for the detection of a single species, these methods are generally not suitable to simultaneously detect multiple pathogens. Nevertheless, multiplex detection, enabling detection of several pathogens simultaneously, may be advantageous to screen samples for more than one pathogen or to study interspecies relationships between different pathogens (Frans, Lievens, Heusdens & Willems 2008). Multiplex assays based on DNA array technology are increasingly developed for fish pathogen detection. A major advantage of this technology is that it combines powerful nucleic acid amplification with the massive screening capability of DNA array technology, resulting in a high level of sensitivity, specificity and throughput (Frans *et al.* 2008). Gonzalez, Krug, Nielsen, Santos & Call (2004b) as well as Warsen, Krug, LaFrentz, Stanek, Loge & Call (2004) described the development of a DNA array for the simultaneous detection of multiple important pathogenic bacteria including *V. anguillarum*. Recently, such an assay was extended to detect viral pathogens (Lievens, Frans, Heusdens, Justé, Jonstrup, Lieffrig & Willems in press) and also virulence or antibiotic resistance markers (Hamelin, Bruant, El-Shaarawi, Hill, Edge, Bekal, Fairbrother, Harel, Maynard, Masson & Brousseau 2006), making it even more useful for efficient pathogen diagnosis.

Prevention and treatment

For many years, antibiotics have been extensively used in the treatment of bacterial infections in cultured fish. For example, in cod fry production in Norway oxolinic acid and florfenicol are mainly used to treat vibriosis (Samuelsen & Bergh 2004; Seljestokken, Bergh, Melingen, Rudra, Olsen & Samuelsen 2006). However, the widespread use of antibiotics should be minimized because it causes antibiotic contamination of the environment and fish used for human consumption (Alderman & Hastings 1998), as well as leading to the development of antibiotic resistance. Colquhoun, Aarflot & Melvold (2007), for instance, observed oxolinic acid resistance among *V. anguillarum* strains isolated from diseased Atlantic cod. In spite of these drawbacks, antimicrobial therapy is still required for containment of vibriosis outbreaks.

To minimize the use of antibiotics and to obtain a more sustainable larviculture and aquaculture industry, a number of alternative measures, such as the use of antimicrobial peptides, application of probiotics, vaccination, immunostimulation and quorum sensing inhibitors have been investigated (Jia, Patrzykat, Devlin, Ackerman, Iwama & Hancock 2000; Brunt, Newaj-Fyzul & Austin 2007; Liu, Oshima & Kawai 2007; Brackman,

Defoirdt, Miyamoto, Bossier, Calenbergh, Nelis & Coenyie 2008; Lu, Lin & Liu 2008). Antimicrobial peptides, such as pleurocidin amide, an amidated form of a peptide found in winter flounder skin mucus, or oncorhyncin II, a histone H1-derived peptide from rainbow trout skin secretion, have been shown to protect fish against *V. anguillarum* infection (Jia *et al.* 2000; Fernandes, Kemp & Smith 2004). In this regard, it was postulated that the development of transgenic fish overexpressing such antimicrobial peptides may result in disease-resistant fish. Using probiotic bacterial strains as a biological control measure offers another promising alternative (Chabrillon, Arijo, Díaz-Rosales, Balebona & Moriñigo 2006; Brunt *et al.* 2007; Isnansetyo, Istiqomah, Muhtadi, Sinansari, Hernawan, Triyanto & Widada 2009). For instance, the use of a probiotic *Pseudomonas fluorescens* strain of aquatic origin (AH2) resulted in a decrease in vibriosis-caused mortality in rainbow trout (Gram, Melchiorsen, Spanggaard, Huber & Nielsen 1999). It was hypothesized that the antagonistic behaviour of *P. fluorescens* is caused by a more efficient siderophore-based iron uptake system, resulting in iron deprivation of the pathogen (Holmstrom & Gram 2003). Bacteria belonging to the genera *Phaeobacter, Roseobacter* and *Ruegeria* are other promising fish larvae probiotics. Bioencapsulation of these bacteria in rotifers is a preferable way of introducing them to fish larvae, after which they can colonize different niches in the production farms and produce tropodithietic acid (TDA), a sulphur-containing anti-bacterial compound (Planas, Pérez-Lorenzo, Hjelm, Gram, Fiksdal, Bergh & Pintado 2006; Porsby, Nielsen & Gram 2008). Multiple commercial vaccines have been developed to protect fish against outbreaks of vibriosis, for example MICROViB (Microtek International Inc.), ALPHA MARINE™ *Vibrio* (PHARMAQ AS), and Aqua-Vac® *Vibrio* and Norvax® Vibriose Marine (Schering-Plough Aquaculture). All these vaccines consist of inactivated strains of both *V. anguillarum* serotypes O1 and O2 and show good protection against *V. anguillarum* infections in several fish species (Angelidis, Karagiannis & Crump 2006; Corripio-Miyar, Mazorra de Quero, Treasurer, Ford, Smith & Secombes 2007; Mikkelsen *et al.* 2007). Besides the use of inactivated bacterial vaccines, DNA vaccines consisting of an antigen-encoding plasmid are being developed because they result in a strong and long-lasting immune response in fish. A DNA vaccine based on the major outer membrane protein OmpU or the zinc metalloprotease EmpA of *V. anguillarum* have already shown promising results (Kumar, Parameswaran, Ahmed, Musthaq & Hameed 2007; Yang, Chen, Yang, Zhang, Liu & Xue 2009). The administration route of the vaccine is important for efficient vaccination. Although intraperitoneal injection has proven to be the most effective vaccination method, this technique is very time-consuming, labour-intensive, causes handling stress and can induce the formation of granulomas, inflammation and pigmentation. Therefore, immersion vaccination of fish is often preferred even though booster vaccination is recommended because of the relatively short period of immunity against *V. anguillarum*. Vaccination through oral intubation is the least effective, probably due to the degeneration of the vaccine in the gastro-intestinal tract. Incorporation of the vaccine in the feed, coating of the feed, or bio-encapsulation in live feed, such as *Artemia* nauplii, can prevent degradation of the vaccine (Bowden, Menoyo-Luque, Bricknell & Wegiland 2002; Rajesh Kumar, Ishaq Ahmed, Parameswaran, Sudhakaran, Sarath Babu & Sahul Hameed 2008). Although most of the cultivated Atlantic cod in Norway are vaccinated against *V. anguillarum* serotypes O1 and O2, outbreaks of vibriosis caused by serotype O2 are not completely prevented (Mikkelsen *et al.* 2007). Another interesting strategy for fish disease management is the use of non-specific immunostimulants. Stimulation of the non-specific immune system can be achieved by the administration of high M alginate, an extract of algae containing poly-mannuronic acid, yeast DNA, containing unmethylated CpG motifs or pathogen-derived immunostimulants. However, more research is required to investigate the optimal administration route and the dose–response relationship (Skjermo & Bergh 2004; Huttenhuis, Ribeiro, Bowden, Van Bavel, Taverne-Thiele & Rombout 2006; Liu *et al.* 2007).

Conclusions

Vibrio anguillarum is an economically important pathogen in larviculture and aquaculture. It is, however, still a major challenge for the sector to avoid outbreaks of vibriosis, and it is clear that more knowledge regarding the interaction of *V. anguillarum* with its host is required. Insight into the pathogenesis mechanism could, for example, result in the development of new vaccines protecting fish

against *V. anguillarum* infections. Nonetheless, only some features of *V. anguillarum* virulence, such as quorum sensing or iron uptake, are well known. Recently, random genome sequencing, suppression subtractive hybridization (SSH) and *in vivo*-induced antigen technology (IVIAT) have been used to identify additional virulence-related genes of *V. anguillarum* (Rodkhum *et al.* 2006b; Wang, Xu, Jia, Chen, Mo & Zhang 2009; Zou, Mo, Hao, Ye, Guo & Zhang 2010). Although these techniques have produced interesting results, the complete mechanism of pathogenesis is still to be unravelled. For this reason, the development of complementary research strategies, such as *in vivo* expression technology (IVET) and whole genome sequencing, is ongoing. These combined research efforts should unravel the interaction of *V. anguillarum* with its host, which should ultimately result in the development of more effective disease prevention and remediation measures, and improved detection techniques. The latter could potentially be accomplished by implementing identified virulence markers on existing DNA array platforms for pathogen detection.

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