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Genetic Determinants of Virulence in the Marine Fish Pathogen *Vibrio anguillarum*

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Abstract

One of the most studied fish pathogens is *Vibrio anguillarum*. Development of the genetics and biochemistry of the mechanisms of virulence in this fish pathogen together with clinical and ecologic studies has permitted the intensive development of microbiology in fish diseases. It is the intention of this review to compile the exhaustive knowledge accumulated on this bacterium and its interaction with the host fish by reporting a complete analysis of the *V. anguillarum* virulence factors and the genetics of their complexity.

Keywords

Vibrio anguillarum; virulence factor; genetics

Vibriosis is one of the most ubiquitous fish diseases caused by bacteria belonging to the genus Vibrio. *Vibrio anguillarum* is a Gram-negative halophilic bacterial pathogen that causes Vibriosois with lethal hemorrhagic septicimia in cultured and natural fish and shellfish (Actis *et al*., 2011).

Canestrini (1893) reported epizootics in migrating eels (*Anguilla vulgaris*) that associated to a bacterium named *Bacillus anguillarum*. Bergman (1909) first described *V. anguillarum* as the etiological agent of 'Red Pest of eels' in the Baltic Sea. The pathology of the disease as well as the characteristics of the bacterium in these two reports indicated that the etiological agents were the same bacteria. Vibriosis caused by *V. anguillarum* has been recognized as a major problem for salmonid culture due to the significant economical loses it causes in the fish industry (Schiewe *et al*., 1977; Trust *et al*., 1981; Winton *et al*., 1983; Toranzo *et al*., 2005; Actis *et al*., 2011). It was demonstrated that isolates of *V. anguillarum* displayed an obvious heterogeneity that led to the division of these vibrios into two separate biotypes, 1 and 2 (Harrell *et al*., 1976; Schiewe *et al*., 1977). Later, Schiewe *et al*. (1981) proposed a new species for the *V. anguillarum* biotype 2 named *V. ordalii*, based on cultural and biochemical characteristics, as well as DNA homology with biotype 1. There are twenty-three O serotypes that have been reported in *V. anguillarum* (Sorensen and Larsen, 1986; Grisez and Ollevier, 1995; Pedersen *et al*., 1999), and serotypes O1, O2 and O3 are mainly causative agents of fish Vibriosis (Toranzo and Barja, 1990; Larsen *et al*., 1994; Tiainen *et al*., 1997).

To prevent vibriosis many research groups have attempted to understand the virulence mechanisms of this bacterium. Recently, using molecular genetic approaches have resulted

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in a large increase in the available information on the virulence of this bacterium. Here we review the main virulence factors identified by molecular genetics as well as biochemical techniques in *V. anguillarum*. The list of *V. anguillarum* virulence factors is shown in Table 1, and their action is exemplified in Fig. 1.

Iron transport systems

Iron is an essential metal for nearly all living organisms since it is indispensable for their metabolism. However the available free iron in the host environment is extremely limited because the majority of the iron inside the host is strongly bound to host factors such as transferrin and lactofrerrin thus pathogenic bacteria need to harbor high affinity systems to acquire iron from those complexes to survive and multiply inside the host and cause disease.

V. anguillarum virulence is associated with the presence of a plasmid-mediated iron uptake system (Crosa, 1980). The 65-kilobasepairs (kb) pJM1 plasmid that presents in the serotype O1 strain 775 encodes a very efficient iron uptake system mediated by the siderophore anguibactin. This is the most well studied and important virulence factor of *V. anguillarum*. The siderophore anguibactin is a small molecular weight peptide (348 Da) and it steals iron from the transferrin-iron complex. Many O1 strains of *V. anguillarum* carry the 65kb virulence plasmid pJM1 or pJM1-like plasmids. The pJM1 plasmid cured strain shows dramatically lower virulence than the wild type strain (the difference of LD_{50} is $> 10^4$) thus the pJM1 plasmid is an essential virulence factor for *V. anguillarum* to cause diseases. The pJM1 plasmid encodes the majority of the anguibactin biosynthesis as well as transport genes. In the pJM1 plasmid, *angB*, *angC*, *angD*, *angE*, *angG*, *angH*, *angM*, *angN*, *angR*, *angT* and *angU* are involved in anguibactin biosynthesis (Crosa, 1980; Tolmasky *et al*., 1988; Wertheimer *et al*., 1999; Welch *et al*., 2000; Crosa and Walsh, 2002; Di Lorenzo *et al*., 2003; Di Lorenzo *et al*., 2004; Liu *et al*., 2004; Alice *et al*., 2005; Liu *et al*., 2005; Di Lorenzo *et al*., 2008; Naka *et al*., 2008). The *fatA* gene encodes the cognate receptor for ferric anguibactin, and *fatB*, *fatC* and *fatD* encode an iron ABC transporter that is essential to lead the ferric siderophore from the periplasmic space to the cytosol (Actis *et al*., 1985; Actis *et al*., 1988; Koster *et al*., 1991; Actis *et al*., 1995; Lopez and Crosa, 2007; Lopez *et al*., 2007; Naka *et al*., 2010). In addition to the pJM1 plasmid, some of the anguibactin biosynthesis genes such as *vabA*, *vabB*, *vabC*, *vabD* and *vabE* are located in the chromosome, and *angC* and *vabC*, *angD* and *vabD*, and *angE* and *vabE* have been shown to be functional homologues (Alice *et al*., 2005; Naka *et al*., 2008). It has been shown that the plasmid pJM1-cured strain of *V anguillarum* can neither transport the ferric anguibactin complex nor does it colonize the skin (or colonized only 5–10% of the fish) although it colonizes the intestine, and shows a decrease in motility through the mucus as compared with the wild type strain (Weber *et al*., 2010). The authors also found that this plasmidless strain is more sensitive to lysozyme than the wild type strain.

pJM1-less O1 strains as well as O2 serotype strains produce a chromosome mediated siderophore vanchrobactin (Lemos *et al*., 1988; Soengas *et al*., 2008). The chromosomal locus encoding vanchrobactin biosynthesis (*vabA*, *vabB*, *vabC*, *vabD*, *vabE*, *vabF* and *vabG*), transport (*fvtA*), secreting (*vabS*) genes have been identified and characterized (Balado *et al*., 2006; Balado *et al*., 2008; Naka *et al*., 2008; Balado *et al*., 2009). The vanchrobactin system is important to grow in iron limiting conditions although it has not been tested whether vanchrobactin is important for virulence of *V. anguillarum* (Balado *et al*., 2006; Balado *et al*., 2008). Furthermore, this vanchrobactin system has also been found in the serotype O1 anguibactin producer harboring pJM1, but in this case one of the vanchrobactin biosyntheis gene *vabF* was interrupted by a transposon originated from pJM1 (Naka *et al*., 2008). It has been proposed that an anguibactin producer was generated

evolutionary because the anguibactin system is better fit to deal with the ability to scavenge iron as compared to the vanchrobactin system (Naka *et al*., 2008).

Furthermore, *tonB2* mediated energy transport systems consisting of *ttpC*, *tonB2*, *exbB2* and *exbD2* that are required for ferric-anguibactin and exogenous siderophore uptake are also essential virulence factors for *V. anguillarum* (Stork *et al*., 2004; Stork *et al*., 2007; Kuehl and Crosa, 2009; Kuehl and Crosa, 2010).

In addition to siderophore mediated iron transport system *V. anguillarum* also harbors another iron transport system, the heme system by which *V. anguillarum* can possibly acquire iron from heme-containing proteins that exist in host. The outer membrane heme receptor *huvA* gene has been identified, and an *huvA* mutant has growth defects under iron limiting condition in the presence of hemoglobin and shows lower virulence than the wild type strain in turbot pretreated with hemin (Mazoy *et al*., 2003). Furthermore, it was found that the *huvA* gene exists as linked to the *tonB1* cluster consisting of *huvA*, *huvZ*, *huvX*, *tonB1*, *exbB1*, *exbD1*, *huvB*, *huvC* and *huvD*, and the deletion mutant of *huvA*, *huvZ*, *huvX*, *huvB*, *huvC* and *huvD* showed hemin transport defect (Mourino *et al*., 2004). It has been demonstrated that *V. anguillarum* can use *tonB1* or *tonB2* systems for heme transport (Stork *et al*., 2004). In addition to *huvA* there is another heme receptor gene, *huvS*, identified in *V. anguillarum*, and it has been shown that the existence of *huvA* and/or *huvS* depend on the strains examined (Mourino *et al*., 2005).

Hemolysins

A hemolysin gene from *V. anguillarum*, *vah1*, a homologue of *V. cholerae* EI Tor hemolysin was first reported by Hirono *et al*. (1996). The product of this gene showed strong hemolytic activity to erythrocytes from fish such as carp and rainbow trout albeit lower activity to mammalian erythrocytes such as sheep, rabbit, bovine and horse. Rock and Nelson (2006) found that the mutation of *plp*, a phospholipase gene located downstream of *vah1* increased two- to threefold the hemolytic activity of *V. anguillarum* suggesting that the *plp* gene product must repress or destabilize the *vah1* transcripts. Furthermore, the upstream gene of *vah1* was annotated as a putative lactonizing lipase gene, *llpA*. Mutation of *vah1* or *llpA* in the *plp* mutant background decreased the hemolytic activity of the *plp* mutant to the wild type level. From those results they concluded that at least these three genes, *vah1*, *plp* and *llpA*, are involved in one of the hemolytic activities of *V. anguillarum*. Moreover, they also found that the highest hemolytic activity was detected in the exponential growth phase where the highest expression of *vah1* and *plp* also occurred. An insertion mutant obtained by integrating a suicide vector in *vah1*, attenuated *V. anguillarum* virulence for juvenile Atlantic salmon, even though the mutant still showed hemolytic activity similar to the wild type. Furthermore, insertion mutations in *llpA* or *plp* did not affect the virulence of *V. anguillarum*. Later on, the same group demonstrated that the *vah1* deletion mutant, that was constructed using the same parent strain and carrying an insert of the kanamycin resistance gene, did not show a reduction of the *V. anguillarum* virulence in the same animal model (Li *et al*., 2008). This could be due to the fact that the *vah1* insertion mutant that Rock and Nelson (2006) constructed caused a polar effect, or there was a secondary mutation. Since the deletion mutant would also affect downstream genes in the same operon albeit with a lower effect it seems that the *vah1* single mutation does not directly affect *V. anguillarum* virulence. Li *et al*. (2008) found that the residual hemolytic activity of the *vah1* mutant is due to the existence of another hemolysin gene, *rtxA* which is highly related to the repeat in toxin genes that are virulence factors of different Vibrio species such as *V. cholerae* and *V. vulnificus* (Lee *et al*., 2007; Liu *et al*., 2007; Olivier *et al*., 2009). The *rtxA* single insertion mutant still shows hemolytic activity whereas no hemolytic activity was observed in the double *vah1* and *rtxA* mutant. Furthermore, they discovered that both *vah1* and *rtxA*

participate in the cytotoxicity to Atlantic salmon kidney (ASK) cells. They determined that the RtxA toxin is necessary to cause cell rounding of the ASK cells, while Vah1 causes their vacuolation. Moreover, they demonstrated that RtxA is a major virulence factor of *V. anguillarum* as determined using intraperitoneal injection in juvenile Atlantic salmon. In addition to Vah1, four additional hemolysins (Vah 2–5) have been reported (Rodkhum *et al*., 2005). It was of interest that *E. coli* expressing each hemolysin gene showed hemolytic activity on rainbow trout blood agar indicating that those hemolysins can be exported outside of the *E. coli* cells. Furthermore, the purified hemolysin proteins showed hemolytic activities not only against fish erythrocytes, but also for sheep and rabbit red cells. Each deletion mutant of *vah 2–5* showed a reduction of virulence against rainbow trout as compared with the parent strain (low virulence strain cured of the pJM1 plasmid) indicating that each of the hemolysins contributes to the virulence of *V. anguillarum*. It is also clear from these studies that Vha4 is the most important for the virulence of *V. anguillarum*. However, all of the single mutants still show hemolytic activity, even though the activity is less than the wild type strain. Xu *et al*. (2010) identified the *mltD* gene, a homologue of *E. coli mltD* encoding a membrane-bound lytic murein transglycosylase D. The *mltD* mutant showed reduction in extracellular protease as well as gelatinase activity, and a total loss of hemolytic activity. They also found that the mutation in *mltD* resulted in better growth at higher concentration of sodium chloride and an increase antibiotic resistance i.e. to aminoglycosides, cephalosporines and penicillins as compared with the wild type strain. The purified MltD showed hemolytic, phospholipase, gelatinase and diastase activities. The *mltD* mutant showed higher virulence to zebrafish than the wild type strain indicating that *mltD* somehow affects the virulence of *V. anguillarum*.

Metalloproteases

Norqvist *et al*. (1990) found that a spontaneous rifampicin resistance mutant of *V. anguillarum* showed lower virulence, to rainbow trout as compared with the wild type. Comparison of the rifampicin mutant with the wild type revealed that the mutant also shows much lower protease activity than the wild type. The purified protease was 36 kDa and required Zn^{2+} for its activity and Ca^{2+} for its stability. Farrell and Crosa (1991) also identified and purified a 38 kDa protease from *V. anguillarum*. The protease was proposed to be a metalloprotease since the protease activity was inhibited by EDTA and 1,10-phenathroline, while classical inhibitors of serine, cysteine, and acid proteases did not inhibit the protease activity.

The *empA* gene encoding the metalloprotease was cloned and sequenced from *V. anguillarum* NB10 (Milton *et al*., 1992). EmpA consists of 611 amino acids with a molecular weight of approximately 66,700. This 611-amino-acid polypeptide may contain a putative signal sequence, a putative leader peptide and a mature protein. The signal peptide cleavage site was predicted in the Ala-25-Ala-26. The mature protein consists of 411 amino acids with a calculated molecular weight of 44,600. The purified protein was previously demonstrated to have a molecular mass of approximately 36 kDa, thus the mature protein is most likely processed a third time. The *V. anguillarum* metalloprotease EmpA was 69.3% and 47.3% identical to the *V. cholerae* HA/protease and the *Pseudomonas aeruginosa* elastase, respectively. Putative zinc-binding as well as other active sites were well conserved (greater than 30%) as compared with numerous other bacterial metalloproteases. The *empA* mutant constructed by integrating a suicide vector in the *empA* gene showed much lower proteolytic activity on 2% gelatin agar indicating that EmpA is responsible for the *V. anguillarum* protease activity. Varina *et al*. (2008) demonstrated that protease Epp processes a 46 kDa pro-EmpA to a 36 kDa mature EmpA by removing a peptide of about 10 kDa. The *epp* gene is located in the locus that includes the *vah1* gene coding for the Vah1 hemolysin and this cluster is considered to be a pathogenicity island of *V. anguillarum*. They also

showed that Epp can be secreted independently of EmpA, and secreted Epp and EmpA interact in the extracellular environment.

Yang *et al*. (2007a) performed mutational analysis of EmpA and found that mutation in the conserved residues, His^{346}, His^{350}, Glu^{347} and Glu^{370}, possibly involved in the zinc-binding sites and the active center of EmpA resulted in an almost complete loss of proteolytic activity as well as cytotoxicity against a flounder gill cell line. On the other hand the mutation in the predicted substrate-binding sites (Tyr^{361}, His^{429} and Asp^{417}) caused smaller reductions in both proteolytic activity and cytotoxicity indicating that mutations in the substrate-binding sites only affect the substrate specificity rather than the proteolytic activity. Zhang *et al*. (2006) demonstrated that EmpA is exported into the periplasm by using the Sec system (type II secretion system) when it is expressed in *E. coli*.

Milton *et al*. (1992) tested the effect of EmpA in *V. anguillarum* virulence using immersion and intraperitoneal injection. The immersion LD_{50}s were 2×10^2 bacteria/ml for the wild type and 1×10^4 bacteria/ml for the *empA* mutant, while the intraperitoneal LD_{50}s were one bacterium for the wild type and 20 bacteria for the *empA* mutant suggesting that *empA* is somewhat involved in the virulence of *V. anguillarum*. Denkin and Nelson (2004) performed virulence tests for *V. anguillarum* NB10 and M93Sm and their *empA* mutants using intraperitoneal and anal intubation in Atlantic salmon. They only found a reduction of the virulence when the anal intubation route was used for the *empA* mutant of M93Sm, while the reduction of virulence was observed in both intraperitoneal and anal intubation in case of the *empA* mutant of NB10. These data suggest that EmpA is a virulence factor during the infection of the gastrointestinal tract of Atlantic salmon by *V. anguillarum*. Yang *et al*. (2007b) purified from *E. coli* a recombinant *V. anguillarum* EmpA (rEmpA) with a His-tag. They showed that rEmpA displays cytotoxicity to flounder gill cells. Intraperitoneal injection of the purified rEmpA into turbot caused hemorrhage in the peritoneal cavity, necrotic signals at the site of injection in most injected fish, and death.

Another type of metalloprotease PrtV that is a homologue of *V. cholerae* PrtV has been identified and characterized (Mo *et al*., 2010). These authors demonstrated that a p*rtV* mutant shows lower enzymatic activities for gelatinase on gelatin agar, protease against azocasein, and glycosidases such as alkaline phosphatase, lucine arylamidase, trypsin and N-acetyl-β-glucosaminidase, while higher activities for esterase and esterase/lipase as compared to the parent strain. The *prtV* mutant also displayed a lower growth rate in turbot intestinal mucus, and showed lower virulence (at least one log difference) to turbot when the intraperitoneal route was used for inoculation

Motility

The correlation between flagellum and virulence in *V. anguillarum* has been proposed (Chart, 1983; Norqvist and Wolf-Watz, 1993). Four flagellin genes encoding FlaABCD and a potential flagellin gene encoding FlaE were identified in *V. anguillarum* NB10 (McGee *et al*., 1996; Milton *et al*., 1996). Those genes are located within two clusters, *flaEDB* and *flaAC*, and the organization of the two locus are very similar to the *V. parahaemolyticus* flagellin cluster. Mutations in each gene except for *flaE* showed a loss of the particular flagellin particle, but obvious structural loss in mutants was not observed. The full deletion of each gene showed that *flaA*-full gene deletion resulted in almost 50% decrease of motility, while each *flaBCDE* mutants showed only less than 15% decrease in motility. None of the full gene deletions affected virulence using an intraperitoneal route. However, when immersion challenge was used the *flaA*-full gene deletion mutant showed a decrease of approximately 10^3 in virulence, while each *flaBCDE* mutant showed only a slight decrease in virulence (approximately 10 to 10^2). Those results indicate that *V. anguillarum* requires

flagellae in the process of invasion but once they passed the integument flagella are no longer required.

O'Toole *et al*. (1996) identified non-motile mutants by using transposon mutagenesis. Genes interrupted by the transposon are homologues of *rpoN* encoding the sigma-54 subunit, *flgI* encoding the flagellar P-ring protein, *flgB* encoding the flagellar rod protein, *flgL* encoding the flagellar hook-associated protein HAP3, and *flhA* encoding one of the components of the flagellum export protein.

The mutations in *flgB, flgL* or *flhA* caused a reduction of virulence of 10^2 to 10^3 when using the immersion route, while there were no differences using the intraperitoneal route. The *flgI* mutant showed dramatically lower virulence in both immersion and intraperitoneal routes of infection.

The *flgI* or *flgB* mutants showed expression of at least one of the flagellin subunits FlaA and FlaB, whereas the *flgL* mutant shows wild-type production of flagellin subunits whereas the *flhA* mutant did not produce any flagellin. Electron microscopic observations revealed that the *flgI, flgB* or *flhA* mutants did not show any flagellum, while the *flgL* mutant showed a short cylindrical projection from one of the poles of the bacterium. Furthermore, they also identified a chemotaxis gene *cheR* located in upstream of *flgB*. Motility assays in broth showed that the *cheR* mutant swarmed rapidly but it did not exhibit the wild type tumbling motion associated with re-orientation of the direction of swimming. When soft agar was used for the assay, the *cheR* mutant showed similar phenotype with the aflagellate mutants although having wild-type expression of the flagellin subunits, and the presence of wild-type flagellae was confirmed by electron microscopy. Those results indicated that *the cheR* mutant possesses a flagellate non-chemotactic phenotype. Furthermore, they found that the *cheR* mutant exhibited a reduction of virulence of approximately 10^2 in immersion but not in intraperitoneal injection.

Ormonde *et al*. (2000) tested whether the mutation in *cheR* affects the adherence to CHSE cells, and found that the *cheR* mutant shows stronger adherence to the CHSE cells than the wild type strain. Thus the smooth swimming phenotype of the *cheR* mutant may increase the chance for *V. anguillarum* to find the attachment site on the cells.

O'Toole *et al*. (1999) isolated skin and intestinal mucus from rainbow trout, and showed that the wild type *V. anguillarum* displayed stronger chemotactic response to intestinal mucus than to skin mucus. The chemotactic response to both type of mucus was abolished when the *cheR* gene was mutated. By analyzing components of the intestinal mucus they demonstrated that free amino acids (glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, serine and threonine), carbohydrates (fucose, glucose, mannose and xylose) and lipid compounds (bile acids such as taurocholic acid and taurochenodeoxycholic acid) induce chemotactic responses. They also found that the concentration of those amino aids and carbohydrates identified as chemoatractants were much lower in skin mucus than in intestinal mucus suggesting an explanation for why intestinal mucus causes stronger chemotactis than the skin mucus. Furthermore, the mixture of all chemoattractants identified from intestinal mucus reconstituted a high level of chemotactic activity similar to that present in the intestinal mucus homogenate, while none of the single compounds showed similar level of chemotactic activity to the homogenate.

The importance of the *rpoN* sigma factor gene for *V. anguillarum* motility was also determined by screening of non-motile transposon mutants (O'Toole *et al*., 1996). The *rpoN* gene was cloned and the nucleotide sequence obtained (O'Toole *et al*., 1997). They found that the *V. anguillarum rpoN* gene consists of 1,458 bp and potentially encodes 486 amino acids. RpoN exhibited strong homology to the alternative sigma-54 factor of many bacteria.

The *rpoN* mutant did not express any flagellin subunits and it also lost the motility phenotype (O'Toole *et al*., 1996; O'Toole *et al*., 1997). The mutant showed an attenuation of virulence as compared with the wild type by immersion challenge but not by the intraperitoneal route suggesting that RpoN does not regulate virulence genes that are necessary after passing the integument (O'Toole *et al*., 1997).

Ormonde *et al*. (2000) identified the *motY* gene encoding a stator for the sodium-driven motor of the polar flagella. Mutations in the *motY* gene did not affect the structure of the flagellum, even that the mutant was non-motile. The *motY* mutant of *V. anguillarum* showed a 750-fold decrease in virulence in rainbow trout only by the immersion assay. Those results are very similar to the results observed with flagellum mutants. Therefore motility but not flagellin proteins are necessary for virulence of *V. anguillarum*. They also observed that the flagellum might not affect the adherence to the CHSE cells. The invasion analysis of the CHSE cells using either wild type or the mutants *motY* or *flaA*, demonstrated that motility might increase the frequency of invasion by increasing the chance of collision between the pathogen and the host cells. However, the *flhA* mutant missing the flagellum showed similar value to the wild type indicating that the flagellum might physically hinder the internalization of the nonmotile bacterium.

Lipopolysaccharides (LPS)

Aoki *et al*. (1985) demonstrated that *V. anguillarum* PT479 isolated from ayu (*Plecoglossus altivelis*) exhibits high virulence to ayu when it was passed seven times through ayu, while it exhibited low virulence when it was cultured 20 times in brain heart infusion medium. The difference of these high- and low-virulent strains was found in the LPS structure, the high-virulence strain possessing an extra high molecular weight band. Furthermore, they observed that the high-virulence strain transports iron more efficiently than the low-virulent strain.

A cluster consisting of four genes, *rmlBADC*, predicted to function in the biosynthesis of the O side chain precursor dTDP-rhamnose from its own precursor glucose 1-phosphate has been identified in the *V. anguillarum* O1 serotype strain 531A (pJHC1, a pJM1-like plasmid) (Welch and Crosa, 2005). A polar mutant in *rmlC* and a non polar mutant in *rmlD* resulted in the loss of the O1 side chain as well as loss of siderophore anguibactin mediated iron transport. Further studies using the *rmlD* mutant unveiled that the loss of ferric-anguibactin transport was due to instability of the ferric anguibactin outer membrane protein FatA in the *rmlD* mutant. The *rmlD* gene was also essential for the resistance of *V. anguillarum* to the bactericidal action of nonimmune serum from rainbow trout. From those results it was concluded that the lipopolysaccharide O1 side chain is indispensable for serum resistance as well as anguibactin-mediated iron transport (Welch and Crosa, 2005).

Norqvist and Wolf-Watz (1993) constructed a Tn5–132 transposon library *of V. anguillarum* 775.17B and they screened the mutants using an experimental infection of rainbow trout. They found that two strains, VAN20 and VAN70, showed lower virulence as compared with the wild type. VAN70 was identified as the Tn5–132 transposon inserted in the *virB* gene. They also identified another gene, *virA* in the same locus. Mutations in *virA* or *virB* caused an attenuation of the virulence of *V. anguillarum* by both intraperitoneal and immersion experimental infections. They also determined that both the *virA* and *virB* mutants did not react with a rabbit polyclonal antibody against whole cells of the wild type strain indicating that both the *virA* and *virB* products are major surface antigens, and found that the difference between wild type and mutants are in the LPS profiles. The *virA* and *virB* genes have been identified in different serotype O1 strains, while no expression was detected by Western blot analysis in O2 serotype strains. The mutation in each of the genes in an O1 clinical strain showed reduction of virulence as observed in 775.17B indicating that *virA* and *virB* are

serotype O1 specific genes. Jedani *et al*. (2000) reported that *virA* and *virB* are located in the *rfb* region of *V. anguillarum* O1, a homologue of the *V. cholerae rfb* region involved in O-antigen biosynthesis, thus they renamed *virA* and *virB* as *wbhS* and *wbhR*, respectively. Milton *et al*. (1995) analyzed VAN20 in which Tn5–132 transposed into two sites, and identified two genes. Each of the two genes thus identified were mutated, and virulence test were performed. One mutant in the *virC* gene showed reduction of virulence (a 10^4-fold for immersion and 10^6-fold for intraperitoneal) as compared with the wild type whereas the other mutant did not change the LD_{50} value indicating that only the *virC* gene is a virulence factor in *V. anguillarum*.

Exopolysaccharides (EPS)

Croxatto *et al*. (2007) identified two divergently transcribed operons, *orf1-wbfD-wbfC-wbfB* and *wza-wzb-wzc*. *wzabc* genes are conserved in bacteria that produce group-1 capsular polysaccharides. Wza is a surface-located outer membrane lipoprotein, Wzb is a protein tyrosine phosphatase and Wzc is a protein tyrosine kinase. The *wbfDCB* genes are similar to those of *V. cholerae* O139, which map in the *rfb*/capsule DNA locus. They demonstrated that the mutation in the *orf1*, *wbfD* and *wza* genes caused a decrease in the level of EPS but not LPS indicating that the *wza-wzb-wzc* locus is involved in EPS but not LPS biosyntheses in *V. anguillarum*. They also showed that the EPS may form a loose capsule or a slime layer that is not anchored to the cell and easily shed into the culture supernatant. The *orf1* and the *wza* mutants showed reduction of the virulence in both immersion and intraperitoneal inoculation routes, while the *wbfD* mutant exhibited attenuation of virulence only when challenging by immersion. These results indicated that the EPS biosynthesis gene may have different functions during infection. The authors also demonstrated that the wild type strain penetrates the skin mucosal layer within 5 hours after infection, proliferate and attach to the scales at the 12 hours time point forming a biofilm by 24–48 hours. On the contrary the *wza* mutant never proliferates in the fish mucus and was not detected after 24 hours post infection. Each *orf1*, *wbfD* and *wza* mutants showed less efficient penetration of the skin mucus as compared with the wild type. Biofilm formation on fish scales was compared between mutants and wild type. It is clear that *orf1* and *wza* are required to make biofilms, while the *wbfD* mutation did not affect biofilm formation. Furthermore, they found that *wza* and *orf1* are required for protease as well as mucinase activity, while the *wbfD* mutant showed again similar results to the wild type strain. Weber *et al*. (2010) demonstrated that *wzb* and *wzc* are essential for EPS transport as well as virulence to rainbow trout in both the immersion and intraperitoneal routes. The authors also showed that exopolysaccharide mutants such as Δ*wza*, Δ*wzb* and Δ*wzc* were impaired to colonize the skin (or colonized only 5–10% of the fish) and decreased in motility through the mucus as compared with the wild type strain, even that those mutants are still able to colonize the intestine. Moreover, they found that exopolysaccharide mutants are more sensitive not only to lysozyme but also to antimicrobial peptides such as polymixin B and parasin as compared with the wild type.

Conclusions

We have described that virulence factors in *V. anguillarum* can be classified into those that are required to gain access to the fish host and those that are necessary for bacterial proliferation. In the latter classification one of the most important factors concerns the ability of bacteria to use the otherwise unavailable iron that is bound to the specific iron binding proteins such as transferrin and lactoferrin and that is mostly encoded in the 65 kb plasmid pJM1. It is obvious that many factors such as proteases, hemolysins, LPS, and EPS are also important in allowing access to the fish. Demonstrating the power of the modern approaches 40 putative virulence factors have been identified by random genome sequencing of the *V. anguillarum* strain H775–3 (Rodkhum *et al*., 2006). The identified

putative virulence factors include a repeat toxin gene cluster *rtxABCD*, four hemolysin genes, an adheshin gene, 13 flagellum genes, four type IV pilus genes, three protease genes, seven LPS biosynthesis genes, a siderophore biosynthesis gene and *rpoN* in which some of them were originally identified by more genetic classical approaches.

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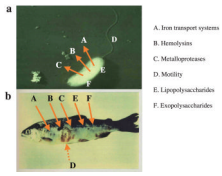

Fig. 1.

Production of *V. anguillarum* virulence factors and action on the host fish. (a) Virulence factors of *V. anguillarum*. Arrows indicate that virulence factors are secreted outside of bacterial cell. (b) Location where virulence factors affect the host fish during infection. Arrows indicate that virulence factors aid in the attachment or to go through the fish integument and/or inside the fish, while the dotted arrow indicates that the virulence factor only attaches to the fish integument.

Table 1

List of *V. anguillarum* genes associated with virulence

Category	Genes name	Functions or products
Iron transport systems	*angA–E, G, H, M, N, R, T* and *U*	Anguibactin biosynthesis
	vabA–E	Anguibactin biosynthesis
	fatA–D	Anguibactin transport
	ttpC, tonB2, exbB2 and *exbD2*	Energy transducer for anguibactin and heme transport
	huvA–D, S, X and Z	Heme transport
	tonB1, exbB1 and *exbD1*	Energy transducer for heme transport
Hemolysins	*rtxA*	Hemolytic and cytotoxic activity
	vah2–5	Hemolytic activity
	mltD	Hemolytic, phospholipase, gelatinase and diastase activity
Metalloproteases	*empA*	Hemolytic and cytotoxic activity
	prtV	Gelatinase, protease, and glycosidase activity
Motility	*flaA–E*	Flagellin
	flgI	Flagellar P-ring protein
	flgB	Flagellar rod protein
	flgL	Flagellar hook-associated protein HAP3
	flhA	Flagellum export protein
	rpoN	Sigma-54 subunit that affects expression of flagellin subunits
	cheR	chemotaxis
	motY	Stator for the sodium-driven motor of the polar flagella
Lipopolysaccharide	*rmlA–D*	O1 side chain biosynthesis
	virA (*wbhS*), *virB* (*wbhR*) *and virC*	O-antigen biosynthesis
Exopolysaccharide	*wza–c, wbfB–D, orf1*	Exopolysaccharide biosynthesis