

Ocean acidification and disease: How will a changing climate impact
Vibrio tubiashii growth and pathogenicity to Pacific oyster larvae?

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Abstract

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Vibrio tubiashii (Vt) is a causative agent of vibriosis in molluscan bivalves. Recent re-emergence of vibriosis in economically valuable shellfish, such as the Pacific oyster (*Crassostrea gigas*) in Washington State, has increased the urgency to understand the ecology of this pathogen. It is currently unknown how predicted environmental changes associated with ocean acidification, such as elevated surface seawater temperature, increased partial pressure of CO₂ ($p\text{CO}_2$), and Vt abundance, will impact marine organismal health and disease susceptibility. This study investigates how environmental cues predicted with ocean acidification influence physiological changes and pathogenicity in Vt.

Using laboratory experiments to manipulate temperature and $p\text{CO}_2$, we examined how these environmental factors influenced pathogen growth. Larval susceptibility to vibriosis was determined by exposing *C. gigas* larvae to a combination of elevated $p\text{CO}_2$ and Vt concentrations. These experiments provide insight into the environmental parameters that may drive pathogenicity or influence proliferation of the bacterium. Investigation of single and multivariate parameters such as temperature, $p\text{CO}_2$, and pathogen levels will help assess how predicted shifts in ocean conditions can impact shellfish survival and disease resistance.

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Chapter I: Literature Review

Introduction

Commercial shellfish production in the United States occurs along all marine coasts with the most production occurring in Washington State (FAO 2011). In the Pacific Northwest, coastal and estuarine environments are used to propagate and cultivate economically significant commercial species of bivalve molluscs such as the Pacific oyster (*Crassostrea gigas*), kumamoto oyster (*C. sikamea*) and geoduck clam (*Panopea abrupta*). Successful, large-scale oyster production is highly dependent on the propagation of healthy oyster seed and reliance on hatcheries for distribution of settlement size larvae to growers (Elston et al. 1999; Barton et al. 2011; FAO 2011). Washington state is a large producer of molluscan shellfish larvae for export to growers both in the US and abroad. Production of shellfish in the US has increased dramatically in recent years to represent 35% of total aquaculture industry value in 2008, generating USD 323 million (FAO 2011). The economic contribution of the shellfish industry in Washington state is significant is estimated to be USD100 million (PCSGA 2010).

Within the last decade, marked declines in the abundance of marine invertebrate larvae and post-larval settlement from natural and hatchery populations have been observed in Washington state (White et al. 2009; Barton et al. 2011). These dramatic decreases in larval settlement correspond with production failures of hatchery produced oyster seed in Netarts Bay Oregon and Dabob Bay, Washington. Subsequent and re-occurring disease outbreaks of vibriosis caused by the marine bacterium, *Vibrio tubiashii* (Vt), has further exacerbated larval mortality in hatchery facilities, threatened production of seed, and led to severe economic losses for the industry within the last decade (Elston et al. 2008; Barton et al. 2011). One severe outbreak of vibriosis caused by Vt in early-stage shellfish was responsible for a dramatic loss of an estimated 59% in production at one Pacific Northwest hatchery (Elston et al. 2008). Total oyster larvae production in 2007 was only 51% of larvae produced in 2005 during the same period (Elston et al. 2008).

In concert with depressed oyster seed production and recurrent bacterial disease over the last decade, environmental shifts caused by an increase in anthropogenic CO₂ in ocean waters, known as ocean acidification (OA), has been identified in the Pacific

Northwest (Sabine et al. 2004; Feely et al. 2008). Seasonal upwelling events bring deep, CO₂-enriched seawater to regions of the eastern Pacific coast continental shelf along central North America exposing vulnerable calcareous marine larvae to corrosive waters with low in carbonate ion availability (Hales et al. 2005; Feely et al. 2008; Evans et al. 2011). Uncertainties in biological responses brought about by OA make it hard to anticipate the associated economic impacts on the shellfish industry (Cooley and Doney 2009). The combination of these stressors – seawater chemistry changes associated with OA and bacterial pathogen exposure – may have detrimental effects on normal molluscan larval physiological processes, energy allocation and survival.

It is unknown how predicted environmental changes, such as elevated surface seawater temperature, OA, and pathogen abundance, will impact marine organismal health and disease susceptibility (Elston et al. 2008). One of the most challenging aspects in understanding how OA influences life in the oceans is the lack of adequate baseline data with which to compare microbial physiology and marine ecosystem shifts. Continuing long-term research of molluscan species exposure to low CO₂ conditions is needed to investigate changes in molecular, cellular, and whole organism functions, including susceptibility to pathogens. Specifically, how OA may impact virulence and pathogenicity mechanisms in Vt and Pacific oyster disease susceptibility to vibriosis is of great interest. Using laboratory experiments manipulating temperature and pCO₂, we can examine how acidified seawater can influence pathogen growth and host susceptibility. Completion of this research will provide compelling data on the interactions between Vt, ocean acidification, and Pacific oyster larvae, which are fundamental to the success and preservation of Northwest shellfish aquaculture.

The pathogen: *Vibrio tubiashii*

The economic importance of Vt on the cultivation of bivalve molluscs has increased the urgency to understand the ecology of the pathogen. Vt is a causative agent for a toxigenic and invasive disease affecting early life stages of molluscan bivalves, called vibriosis (Brown and Losee 1978; Elston et al. 1981; Hasegawa & Hase 2009). Researchers have long speculated that epidemics of vibriosis, caused by members of marine *Vibrio* species, including Vt, might limit the recruitment and survivorship of valuable bivalve species. Disease outbreaks of vibriosis in bivalve larvae are characterized by bacterial swarming around the velum, loss of larval motility, extensive soft tissue necrosis, and rapid mortality (Elston and Leibovitz 1980; Nottage

and Birkbeck 1987). Vibriosis can cause dramatic larval mortality within intensive culture especially when optimal rearing conditions for larval shellfish are implemented, which include high population densities and elevated temperatures. In some cases, larval mortality can exceed 90% within 24 hours of initial exposure to the most pathogenic Vt strains (Estes et al. 2004).

Management of infectious disease, especially those caused by bacteria, has been problematic in shellfish aquaculture since its inception, often leading to severe economic losses in production (Tubiash et al. 1970; Elston et al. 1981; Elston 1990; Elston et al. 2008). Environmental conditions within shellfish hatcheries, such as temperature, salinity, pH, and algal culture, may exacerbate the spread of bacterial pathogens (Elston et al. 2008; Sainz-Hernandez and Maeda-Martinez 2005). Thus, opportunistic pathogens can easily multiply and produce larval mortalities within hatcheries. Significant research has focused on mitigation of pathogen proliferation including the use of routine bacterial sampling of algal cultures and larval tanks, water quality measurements of influent seawater, isolation and destruction of infected stocks, and identification of contaminant sources (Elston et al. 1981; Elston et al. 2008; Elston 1990; Sainz-Hernandez and Maeda-Martinez 2005; Hasegawa et al. 2009). In the natural environment, the factors that influence the presence of Vt and pathogenicity of vibriosis to bivalve species are still poorly defined; although abundance of the bacterium was correlated with warm, summer upwelled waters along the Pacific coast (Elston et al. 2008).

Bacterial characterization

Tubiash et al. (1965) first described strains of Vt as a causative agent for bacillary necrosis in larval and juvenile bivalve molluscs. Vt, a member of the family *Vibrionaceae*, inhabits a wide range of marine and estuarine environments and is a natural symbiont of many marine invertebrate species. The genus *Vibrio* contains more than 30 known species of bacteria and many are pathogenic to multiple taxa (Chakraborty et al. 1997). They are often free-living, but can form biofilm colonies on host tissue. Because of their ubiquitous presence in seawater, *Vibrio* species are commonly isolated from fish and shellfish with 100-fold higher concentration found in filter-feeding shellfish than the surrounding water (Wright et al. 1996).

Originally cultured from a moribund juvenile oyster, Vt is a Gram negative, curved, rod-shaped bacterium with a single polar flagellum for motility (Hada et al. 1984).

Early stage larvae and juvenile molluscan species, including crustaceans, are particularly susceptible to vibriosis. *Vibrio* infections may produce larval mortalities up to 90% within 24 hours of exposure to the most pathogenic strains (Tubiash et al. 1965; Hada et al. 1984; Nottage and Birkbeck 1987; Elston 1990; Elston and Leibovitz 1980; Estes et al. 2004; Elston et al. 2008), whereas adult shellfish experience minimal mortality even after weeks of bacterial exposure (Tubiash 1975).

Vt colonies are circular, smooth, opaque white, sometimes mucoid, and measure 1 – 4 mm in diameter when grown on marine agar 2216 plates (Tubiash et al. 1965; Hada et al. 1984). On Thiosulfate-Citrate-Bile-Sucrose agar, Vt produces yellow colonies characteristic of members of the *Vibrionaceae* that are able to ferment sucrose. The bacterium is oxidase and catalase positive, able to grow aerobically and possesses a fermentative metabolism for anaerobic conditions (Tubiash et al. 1965). Cells of Vt can grow at temperature ranging from 12 – 30°C; optimal growth temperature is 25°C (Tubiash et al. 1965). Vt is able to grow at a pH range of 6.5 – 8.0. Vt requires sodium and chloride ions for growth and cannot grow on media containing less than 0.5% NaCl (Hada et al 1984).

Specific ecology of Vt is not known, although most members of the family *Vibrionaceae* are distributed throughout seawater ecosystems including marine, brackish, or freshwater habitats (West and Colwell 1983). Vt is associated with healthy bivalve molluscan flora, but can also be isolated free within the water column. Some environmental factors contributing to the concentration of vibrios include organic and inorganic chemicals, pH, temperature, salinity, oxygen, and exposure to UV light (Chakraborty et al. 1997). Abundance of Vt strongly correlates with increased surface seawater temperature and coastal upwellings, which are high in CO₂ (Elston et al. 2008). High densities of Vt have been cultured in seasonal upwelled waters and, in some instances, with an absence of other culturable marine bacteria (Elston et al. 2008).

Hatchery isolates of Vt were tested for pathogenicity to oyster larvae in a study performed by Estes et al. (2004). Three Vt isolates (RE22, RE98, and RE101) were identified as pathogenic. Strain RE22 was determined to be most pathogenic with a lethal dose at 50% (LD₅₀) of 1.9×10^3 colony forming units per milliliter of seawater (CFU/ml) after 48 hours of exposure when tested in 4 mL of seawater (Estes et al. 2004). When tested using 1L of seawater, Vt LD₅₀ was 10-fold lower than that observed using 4 ml containers (Estes et al. 2004).

Virulence factors

Vibrios have various virulence factors that play a role in establishing infection and may contribute to the development of disease. Understanding the molecular mechanisms that drive virulence and pathogenesis are fundamental to predicting and controlling disease outbreaks. Extracellular products are postulated to play an important role in vibrio pathogenesis in fish and molluscan species (Rodriguez et al. 1992; Hasegawa et al. 2008; Hasegawa & Hase 2009a). These virulence factors include enterotoxins, hemolysins, cytotoxins, proteases, siderophores, and adhesive agents (Hasegawa and Hase 2009a; Hasegawa et al. 2008; Shinoda and Miyoshi 2011). Highly virulent Vt strains, such as RE22, release extracellular toxins, hemolysin and proteases, responsible for proteolytic and hemolytic functions (Hasegawa et al. 2008). Vt possesses several secreted proteins thought to influence virulence in larval shellfish vibriosis, including a zinc-containing metalloprotease (Kothary et al. 2001; Nottage and Birkbeck 1987; Delston et al. 2003; Hasegawa et al. 2008). Although these extracellular products are thought to contribute to Vt virulence, their specific roles in pathogenesis as well as the influence of environmental conditions on virulence are not known.

Nottage and Birkbeck (1987) demonstrated that seven *Vibrio* spp. pathogenic to fish and/or shellfish produce secreted antigenically similar protease(s) capable of producing toxicity. The study provides good evidence that Vt secreted protease acts as a virulence factor to shellfish. Protein fractionation peaks of *Vibrio* sp. culture supernatant gel filtration in spat toxicity assays revealed that protease activity and soft tissue necrosis followed by increased mortality was consistent with bacterial protease and hemolysin production. The quick disintegration of gill tissue seen with vibriosis infection suggested that protease(s) and/or cytolytic factors are involved in pathogenesis. Extracellular protease activity degrades host tissue, which can cause extensive tissue damage and enhance bacterial propagation (Maeda et al. 1996). Cytolytic toxins, such as hemolysin, cause lysis of red blood cells *in vitro* and are important factors in pathogenesis of disease in multiple pathogenic bacteria (Nomura et al. 1988; Rodriguez et al. 1992). Hemolysin and protease production in *Vibrio* spp. is reported to influence pathogenesis of disease in fish (Nomura et al. 1988; Rodriguez et al. 1992) and cytolytic toxicity was postulated to be a factor in *Vibrio* virulence to shellfish (Kothary et al. 2001).

Further characterization of the extracellular toxicity of Vt culture supernatants was performed by Hasegawa et al. (2008) and Hasegawa & Hase (2009a). These studies examined the role of extracellular protease and hemolysin production in vibriosis

infection of *C. gigas* larvae. Molecular analysis of Vt metalloprotease (VtpA) revealed high sequence similarity to several metalloproteases produced by multiple *Vibrio* species (*Vibrio* sp. strain MED222 (GenBank accession no. NZ_AAND01000005), *V. splendidus* strain 12B01 (accession no. ZP_00990032), *V. proteolyticus* (accession no. AAA27548), Vibrionales bacterium strain SWAT-3 (ZP_01816166), *V. anguillarum* strain M93Sm (accession no. AAR88093), *V. vulnificus* strain YJ016 (accession no. NP_937521), *V. cholerae* strain 623-39 (accession no. ZP_01980763), *V. aestuarianus* strain 01/32 (accession no. AAU04777), *V. angustum* strain S14 (accession no. ZP_01236251), *Photobacterium* sp. strain SKA34 (accession no. ZP_01158654), and *V. fluvialis* strain AQ0005 (accession no. BAB86344), suggesting that VtpA may function as a zinc metalloprotease (Hasegawa et al. 2008). Expression of Vt extracellular proteins, metalloprotease and hemolysin, is correlated to cell density of the culture. Hemolysin activity was shown to increase during early stage growth and decrease at stationary phase, while protease activity increased during all stages of bacterial growth, reaching the highest level of activity during stationary phase. Hasegawa and Hase (2009a) examined the function of metalloprotease and cytolysin/hemolysin on *C. gigas* larvae, using mutant strains of Vt and a series of protease inhibitor experiments. They concluded Vt metalloprotease (VtpA) was one of the most critical factors for toxicity to bivalve larvae, whereas Vt hemolysin did not influence larval mortality in supernatant toxicity experiments (Hasegawa et al. 2008; Hasegawa and Hase 2009a).

Vibrios utilize a complex cell-to-cell communication system called quorum sensing, which is a coordinated molecular response to cell density. Gram-negative bacteria use quorum sensing in a range of physiological activities such as horizontal gene transfer, antibiotic production, motility, and virulence (de Kievit and Iglewski 2000; Miller and Bassler 2001; Antonova and Hammer 2011). *V. cholerae*, a causative agent for the diarrheal disease cholera, and other *Vibrio* spp. utilize signal molecules, called autoinducer molecules, that control gene expression and coordinated group behaviors at high cell densities (Fugua et al. 1994). The concentration of autoinducers is proportional to the number of bacteria present in a colony. In *V. cholerae*, autoinducer signals trigger production of a transcription factor, HapR, which regulates biofilm formation and colonization in the host intestine (Zhu and Mekalanos 2003). HapR is significant in *V. cholerae* pathogenesis because it is required for production of the hemagglutinin protease, encoded by the *hapA* gene during colonization (Zhu et al. 2002). At low cell densities, the phosphorylated response regulator, LuxO, will initiate transcription of small

RNAs, ultimately repressing the translation of *hapR*, which encodes the quorum sensing master regulator (Hammer and Bassler 2007; Svennigsen et al. 2009; Rutherford et al. 2011). At high cell densities, autoinducers accumulate and bind to receptors that dephosphorylate and inactivate LuxO. LuxO no longer represses *hapR* expression. Subsequent production of HapR represses some genes and activates other genes, including activation of *hapA* and those involved in horizontal gene transfer (Zhu et al. 2002; Meibom et al. 2005; Antonova and Hammer 2011). Many species of *Vibrio*, such as *V. harveyi*, possess analogous pathways of *V. cholera*-like quorum sensing, suggesting that this pathway is genetically conserved within the genus (Zhu et al. 2002; Hammer and Bassler 2008).

Quorum sensing and biofilm formation may be important functions in pathogenic strains of Vt. In Vt, mechanisms of protease and hemolysin production have been investigated in response to cell density. Extracellular protein production in Vt is controlled by a TetR family of transcriptional regulators, VtpR, which shows high homology to HapR in *V. cholerae* (Hasegawa and Hase 2009b). TetR family transcriptional regulators are known to initiate secretion of metalloproteases in multiple *Vibrio* species including *V. anguillarum*, *V. vulnificus*, *V. cholerae*, and *V. harveyi* (Crozatto et al. 2002; Jeong et al. 2001; Jobling and Holmes 1997; Mok et al. 2003). VtpR actively controls production of metalloproteases *vtpA* and *vtpB*, hemolysins, and swimming motility, important for planktonic and biofilm phases of the Vt life cycle. Transcriptional regulators of *Vibrio* virulence (e.g., ToxR, TcpP, and ToxT in *V. cholerae*) are hypothesized to respond to external environmental cues such as temperature, pH, and osmolarity (Zhu et al. 2002). In experiments examining extracellular production of Vt in varying environmental conditions, VtpR activity was attenuated at higher salt concentrations (4 – 5%), causing a dramatic reduction in protease expression (Hasegawa and Hase 2009b). The relationship between environmental stressors and Vt virulence warrant further investigation. Exploration of both environmental and molecular factors will help to elucidate Vt pathogenesis of vibriosis. A list of *Vibrio* spp. with annotated metalloprotease, hemolysin, and/or Tet-R homologs within the National Center for Biotechnology Information (NCBI) database is provided in Table 2.

Vibriosis, associated pathology, and diagnostics

Vibrio species are commensal bacteria of many marine taxa, but are also considered opportunistic pathogens, associated with disease and mortality when host immune responses can be circumvented to establish infection. With the rise in early commercial hatcheries in the late 1950s, larval cultures were met with high incidences of epizootic mortalities (Tubiash et al. 1965). Poor hygienic procedures, incoming seawater, algal food supply, and high population densities contributed to persistent occurrence of vibriosis in hatchery environments (Elston et al. 1981; Sainz-Hernández and Maeda-Martínez 2005). Guillard (1959) first described disease of larval hard clams (*Venus mercenaria*) by exposing larvae to a pathogenic species of *Vibrio* originally cultured from a moribund animal. He described some larval pathology related to disease - detachment of the velum and abnormal ciliary movement - and recorded a 70% mortality of the original larval population.

A study performed by Tubiash et al. (1965) described bacillary necrosis of multiple bivalve species including: *C. virginica*, *Ostrea edulis*, *Mercenaria mercenaria*, *Argopecten irradians* and *Teredo navalis*. Bacillary necrosis in larval bivalves was associated with loss of motility, distended velum, and bacterial swarming. Researchers found that adult bivalves of the same species were not nearly as susceptible to *Vibrio* exposure as the larval stages, as they were able to survive exposure to high concentrations of the pathogenic serotypes used in this experiment. Brown (1973) confirmed this finding in exposure experiments, finding that susceptibility to vibriosis decreases as larvae age (10-d old < 2-d old < up to 48 hr old) (Brown 1973).

Oyster pathology associated with vibriosis has been examined in depth (Elston and Lebovitz 1980; Elston et al. 1999; Estes et al. 2004; Elston et al. 2008). Elston and Lebovitz (1980) described the observed pathogenesis of experimentally infected larval oysters. Three courses of infection were specified. The first phase, observed in all ages of larvae, was described as bacterial attachment to the external shell periostracum and proliferation of the bacteria inward along the internal shell surfaces. Invasion of visceral cavity soon followed, overwhelming the host immune response. The second phase of infection, observable in early veliger larvae, resulted in severe deformation of the velum, including retractor muscle detachment and extended vela, without bacterial invasion of tissues. Abnormal swimming patterns, reduced feeding, and further pathological symptoms such as erosion of digestive tract tissues and loss of ciliated velar cells were also observed at stage two. The third phase of disease progression, seen in late veliger

larvae, was characterized by inactivity of the larvae, the development of lesions within and atrophy of the viscera, cellular sloughing, bacterial proliferation of the epithelial surfaces of the digestive tract, and focal lesions in the digestive organs.

A summary of *Vibrio* spp. isolated from vibriosis outbreaks in multiple bivalve species worldwide is summarized in Table 1. Species of *Vibrio* associated with vibriosis have included *V. anguillarum*, *V. alginolyticus*, *V. tubiashii*, and *V. splendidus*, and *V. neptunium* (Brown and Losee 1978; Tubiash et al. 1970; Prado et al. 2005). *Vt* has recently been described as a re-emergent pathogen along the West Coast of North America causing mass mortalities in *C. gigas* larvae over the past decade (Elston et al. 2008). The hatchery industry in the Pacific Northwest has been severely impacted by vibriosis outbreaks caused by *Vt*. High concentrations of *Vt* associated with the decline of natural populations has not yet been examined, but is speculated to be a contributing factor (Elston et al. 2008).

Some larvae may survive an epizootic of vibriosis. The presence of surviving larvae suggests that selection for vibriosis-resistant stock may be possible if surviving larvae perform well as juveniles and adults in the field and traits associated with increased survival are heritable (Brown and Losee 1978). The ability to successfully spawn in natural or hatchery settings is crucial for the development of resistant lines. Genetic studies have begun using sequencing to identify genetic markers in disease resistant families of *C. gigas* associated with tolerance to extracellular toxin production of *Vt* and to high pCO₂ seawater (Camara and Chen, unpublished). This research will help identify robust stocks of oysters that can be reared commercially given the recent environmental conditions that have persisted over the past decade and the growing concern for the future of shellfish hatchery production.

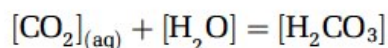
For a definitive diagnosis of vibriosis in a larval culture, appropriate biochemical, immunodiagnostic, or molecular method is needed (Bower 2009). Elston et al. (1981) developed a *Vibro*-specific fluorescent antibody detection method to help diagnose vibriosis in hatcheries by chemically illuminating *Vibrio* species on oyster larvae diagnosed with the disease. Elston's method was developed to validate vibriosis in a hatchery environment, but the methodology cannot be applied to environmental samples, nor is it species-specific. An azocasein protease test, able to detect proteolytic activity of pathogenic vibrios, has been used to detect possible virulent strains of *Vibrio*, but again, this method is not species-specific (Elston et al. 2008; Hasegawa et al. 2008). Traditional methods of diagnosis, such as histopathology and antigen-based tests, are

often time consuming and costly and fail to detect early stage infection. Highly specific genomic-based diagnostic tools have been increasingly common and widespread in detecting pathogenic agents in disease research. A sensitive molecular assay, quantitative PCR (qPCR) using the putative Vt virulence gene, metalloprotease (*vtpA*) was developed to aid in identification of the bacterium (Gharaibeh et al. 2009). Use of this assay in hatchery settings may limit losses of larval shellfish from vibriosis outbreaks caused by Vt with early and specific detection of the bacterium.

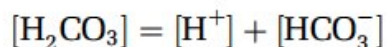
Ocean acidification: impact on larval shellfish and microorganisms

Global emissions of atmospheric CO₂ lead to the accumulation of CO₂ in marine surface waters and are thus contributing to chemical shifts in the pH and carbonate chemistry of our world's oceans. This phenomenon is referred to as OA. Within the past 250 years, seawater pH has decreased by 0.1 pH units and is expected to continue to decline an additional 0.3-0.4 units by the end of this century (Brewer 1997; Oee et al. 2005; Royal Society 2005). The Pacific Northwest coast of the United States has been one of the first areas to see major shifts in the marine environment due to OA. Seasonal oceanic upwellings bring CO₂-rich waters to the Northwestern coast and Puget Sound, exposing calcareous marine organisms to corrosive, low pH waters. Low total alkalinity (TA) values in the region (~2000-2100 μmol/kg) significantly reduce the buffering capacity of seawater and, in conjunction with the naturally occurring fluctuations in biological activity in coastal and inland marine waters, may exacerbate the impact of OA to biologically sensitive species (Lee et al. 2006; Fabry et al. 2008; Feely et al. 2008).

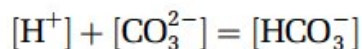
Seawater contains three major forms of dissolved inorganic carbon (DIC): bicarbonate ion (HCO₃⁻), carbonate ion (CO₃²⁻), and aqueous carbon dioxide (CO_{2(aq)}). When CO₂ dissolves in seawater, a series of chemical reactions occur. The pH scale is defined by the $-\log[H^+]$ and seawater pH decreases with the addition of CO_{2(aq)}. Carbonic acid (H₂CO₃) is formed when CO₂ initially dissolves in seawater:



H₂CO₃ quickly dissociates into a hydrogen ion (H⁺) and HCO₃⁻:



The H⁺ ion can react with CO₃²⁻ to form HCO₃⁻:



The overall reaction of the addition of CO_2 to seawater increases the amounts of H_2CO_3 , HCO_3^- , and H^+ and decreases the concentration of CO_3^{2-} , limiting the availability of carbonate ions marine biota utilize for calcification (Feely et al. 2004; Fabry et al. 2008; Miller et al. 2009; Gazeau et al. 2011).

The saturation state of carbonate ions (Ω), the building blocks of marine CaCO_3 structures, is dependent on seawater temperature, salinity, pressure, DIC, TA and mineral phase (calcite, magnesium calcite, or aragonite) (Feely et al. 2004). Formation of shells and skeletons of marine biota is favored when the saturation states of calcite (Ω_{cal}) and aragonite (Ω_{arag}) are >1.0 and dissolution is favored when saturation rates are <1.0 . Aragonite and magnesium calcite have lower saturation states at a given pCO_2 than the more stable calcite. With continued increases in anthropogenic CO_2 , undersaturation with respect to aragonite in seawater is projected to reach critical threshold levels by 2050 in high-latitude regions (Orr et al. 2005; Feely et al. 2008). Upwelled seawater along the coast of the North Pacific continental shelf with Ω_{arag} below 1.0 has been observed in recent years (Sabine et al. 2004; Feely et al. 2008), but little is known regarding how survivorship of marine calcifying organisms will be affected by chronic or acute exposure to these upwelled, corrosive waters.

Although the basic carbonate chemical reactions in seawater are well-studied (Millero et al. 2002), seawater carbonate chemistry and its parameters (pH, partial pressure CO_2 , carbonate ion availability, DIC, etc.) are complex and these parameters change in response to biological activity, temperature, and variable coastal and estuarine conditions (Boyd and Doney 2002; Doney et al. 2009). The combination of OA and nearshore biological and physical processes makes understanding OA effects on natural populations difficult to predict and differential responses hard to interpret. Furthermore, individual and ecological effects of reduced calcification in association with chronic exposure to OA are largely unknown.

Molluscan larval species that reside in marine surface waters are often sensitive to changes in pH and fluctuations in carbonate availability (Calderia and Wickett 2003; Gazeau et al. 2007; Portner et al. 2005; Guinotte and Fabry 2008). OA affects calcifying marine organisms in a variety of ways including: calcium deposition, acid-base balance, shifts in energy allocation and metabolic equilibria, embryonic development and growth, and reproductive success (Allen and Burnett 2008; Parker et al. 2009; Parker et al.

2010; Lannig et al. 2010). Numerous studies have been performed examining how OA can affect early biomineralization in molluscan shellfish and overall larval success through growth and development. Overall, research on larval oyster response to OA has been negative (Kurihara et al. 2007; Miller et al. 2009; Parker et al 2009; Watson et al. 2009; Parker et al. 2010; Talmage and Gobler 2010; Barton et al. 2011).

Biomineralization in bivalve larvae is a complex physiological and biochemical process that is influenced by environmental conditions and endogenous activity of the organism (Lee et al. 2006). Acidification and seawater undersaturated with respect to aragonite can have detrimental physiological effects on developing larvae following fertilization. Larval stages of calcifying marine organisms may be more acutely affected by high partial pressure of CO₂ (pCO₂), as the early stages of calcification rely on aragonite and amorphous calcium carbonate for shell formation (Weiss et al. 2002). Aragonite based shells of bivalve larvae begin to develop approximately 24 - 48 hours after fertilization at the trochophore larval stage and fully mineralize when larvae reach D-veliger stage of growth (Weiss et al. 2002; Lee et al. 2006). Most calcifying organisms investigated to date have shown reduced calcification in response to increases pCO₂, decreased CO₃²⁻, CaCO₃ depletion, and lowered pH (Gazeau et al. 2007; Kurihara et al. 2007; Talmage and Gobler 2009). Experimental parameters in these experiments are not uniform, but most of the current research performed utilizes dissolved CO₂ (µatm) to adjust pH and alter levels of carbonate ion availability.

Kurihara et al. (2007) exposed gametes of *C. gigas* to high concentrations of µatm CO₂ (pH = 7.4), with low aragonite saturation ($\Omega_{\text{arag}} = 0.68$), measured rates of fertilization, and examined development of larvae. More than 80% of D-veliger larvae exposed to high CO₂ conditions displayed malformed shells or remained unmineralized compared to the control group 24-hours post fertilization. Similar results were shown by Talmage and Gobler (2009) in experiment exposing two commercially valuable bivalve species (*M. mercenaria* and *Argopecten irradians*) to elevated pCO₂. They noted that at preindustrial levels of CO₂ (250 ppm) growing larvae displayed thicker, more robust shells when compared to larvae reared at present day and predicted levels of CO₂ concentrations (390 and 1500 ppm). According to Gazeau et al. (2007) mussel (*Mytilus edulis*) and oyster (*C. gigas*) calcification may decrease by 25 and 10%, respectively, in juvenile and adult bivalves when exposed to pCO₂ of 740 ppm.

Larvae are generally less robust than adults to environmental stressors and shifts in carbonate ion availability may lead to reduced performance or death (Miller et al.

2009). Multiple studies to date have examined bivalve larval performance, rates of metamorphosis, and survival under OA conditions. Talmage and Gobler (2009 and 2010) found slow growth and delayed time to metamorphosis in three species of bivalve larvae (*C. virginica*, *M. mercenaria*, and *Argopecten irradians*) exposed to 650 ppm CO₂. Watson et al. (2009) exposed Sydney rock oyster larvae (*Saccostrea glomerata*) to seawater pH levels of 7.6 – 8.1 and found a reduction in larval survival and development at the lowest pH. Gazeau et al. (2011) examined larval *C. gigas* growth and developmental success at three days post-fertilization under various acidified and carbonate ion concentrations. Researchers discovered that developmental rates and growth were not significantly impacted by low pH seawater directly, but low carbonate ion availability ($\Omega_{\text{arag}} < 1.0$) was in fact responsible for delayed growth and developmental abnormalities.

Larval growth and performance is of great interest to the commercial hatchery industry. Continuous years of depressed production of larval stocks in the industry have threatened availability of larval seed to growers. Some Pacific Northwest hatcheries have started to evaluate seawater chemistry of incoming seawater and monitor larval performance. Barton et al. (2011) investigated the impact of influent seawater to larval survival and growth rates in the summer of 2009. The hatchery, located in Netarts Bay, Oregon, found that the reduced growth rates and depressed larval production season corresponded with periodic influxes of low pH seawater, undersaturated in aragonite.

Bivalve transition from larvae into juveniles is energetically costly, resulting in naturally high mortality in coastal populations (Green et al. 2004). Further increases in mortality due to OA and resulting physiological shifts could have detrimental effects on molluscan populations (Guinotte and Fabry 2008) and the commercial hatchery industry as a whole. The combined energetic costs of biomineralization and development through early life stages suggest that early developmental stage oysters (zygotes, embryos, larvae, and post larvae) are most sensitive to changes in environmental conditions associated with OA. Reduced survival and fitness of calcareous marine organisms is likely due to the physiological compensation of maintaining normal processes (growth, shell formation, metamorphosis) in a low pH marine environment (Wood et al. 2008). The biological cost of OA in conjunction with additional environmental stressors, including pathogen abundance or disease, as seen in hatchery environments over the past decade, may have deleterious effects on the animal populations and associated commercial industries. Continued research of OA impacts on molluscan species is

necessary to fully examine the synergistic effects of oceanic chemistry, disease, and larval growth and survival.

Ocean acidification, microbiology, and Vibrio tubiashii

Bacteria provide essential ecological functions that maintain healthy marine ecosystems by mediating biogeochemical cycling (nitrogen, carbon and phosphorous). Predicting the changes OA may have on marine microorganisms, ecosystems, and these major biogeochemical processes, however, represents a major challenge to the scientific community (Doney et al. 2009; Joint et al. 2011). Most of the current research on microbes and OA has focused on how biogeochemical processes respond to acidification including examination of the effects on coccolithophore calcification and productivity (Riebesell et al. 2000; 2007), photosynthesis (Tortell et al. 1997), and primary productivity (Tortell et al. 2002). One recent study indicates if OA continues, pH could be a significant factor affecting the equilibrium between ammonia and ammonium in the world's oceans (Beman et al. 2010). This study revealed that OA could reduce nitrification rates by 3-44%, impacting nitrous oxide production, limiting supplies of oxidized nitrogen in surface waters, and may ultimately alter nitrogen cycling in the ocean. As a result of rising CO₂ concentrations, increased stratification in the upper layers of the ocean will decrease dissolved oxygen (O₂) concentrations, change O₂/CO₂ ratios, and possibly impact aerobic microbial communities (Keeling et al. 2010; Brewer and Peltzer 2009).

Variability in climate can affect both bacterial and host physiology in the environment. Numerous studies have correlated bacterial proliferation with increased water temperatures in a number of pathogenic *Vibrio* species, such as with *V. parahaemolyticus*, *V. vulnificus* and *Vt* (Kaneko and Cowell 1973; Huq et al. 1984; Pfeiffer et al. 2003; Elston et al. 2008). In Peru, temperature increases during the warmest months of the year coincide with *V. cholerae* presence and associated disease in humans (Colwell and Huq 1999; Lipp et al. 2003). In shellfish, 1°C increase in temperature produced a significant increase in mortalities of the European abalone (*Haliotis tuberculata*) caused by outbreaks of vibriosis from the pathogen *V. harveyi* (Travers et al. 2008). Meta-analyses done by Harvell et al. (2002) predict that just a 1.5°C rise in average global temperature may dramatically increase duration of vector-borne human and animal pathogen proliferation annually, modifying seasonal patterns of pathogens and subsequent disease occurrence. Furthermore, it is unknown how

elevated temperatures associated with climate change will alter the physiology and disease susceptibility of the host. It is possible that the interactions between elevated CO₂ concentrations and rising temperature could shift bacterial assemblages, influence bacterial virulence, and limit immune response of the host.

Mobile genetic elements of bacterial pathogens may be influenced by climate change and OA. In prokaryotes, a large proportion of genes are acquired laterally from different microbial species or viruses (Koonin et al. 2001; Rohwer and Thurber 2009). It is thought that since members of the genus *Vibrio* occupy similar ecological niches in the marine environment, the utilization of mobile genetic elements, within and between species, may assist populations of bacteria to maintain and adapt to a changing environment (Hazen et al. 2010).

Genetic transfer of putative virulence genes between *Vibrio* spp. has been observed. Gonzalez-Escalona (2006) identified a thermostable direct hemolysin-related (*trh*) gene in *V. alginolyticus* sharing a 98% homology to the *trh* gene found in *V. parahaemolyticus*, suggesting horizontal genetic transfer between the two species. Environmental cues such as nutrient limitations and presence of chitin have been identified as signals for horizontal gene transfer in *Vibrio* spp., e.g. conversion from O139 to O1 El Tor serotype in *V. cholerae* (Blokesch and Schoolnik 2007) and a classical type cholera toxin prophage movement between strains of *V. cholerae* (Udden et al. 2008). There are many examples where environment stressors, such as temperature, nutrient availability, and pH, can produce phenotypic changes, such as polysaccharide production and biofilm structure (McCarter 1998; Enos-Berlage et al. 2005; Hilton et al. 2006).

Multidrug resistance, an integrative conjugative element, in a human pathogen, *V. cholerae*, has been shown to increase the expression of horizontal gene transfers under stress (Beaber and Waldor 2004). Environmental cues have been linked to putative virulence expression as well. Flagellar expression, important for swarming behavior and thought to aid in bacterial pathogenesis for some species, has been linked to environmental signals such as iron limitation (Jacques and McCarter 2006). To further understanding of bacterial ecology, metatranscriptomic and/or proteomic research can give much insight into the physiological activities of microorganisms under varying oceanic conditions, as well as identifying candidate genes that can give insight into key regulatory, metabolic, or virulent pathways (Bowler et al. 2009).

The integration of cell biology and genomics to facilitate oceanographic research has yet to be fully explored (Bowler et al. 2009). Lack of genetic sequence data poses an impediment to understanding the differences in Vt virulence. Whether major differences in Vt virulence among strains exist due to differential expression of existing virulence genes, insertion or deletion of virulence factors, or due to genetic transfer among strains remains unknown. Pathogenesis of vibriosis and how OA may influence disease expression in Vt is of great interest. Advances in high throughput sequencing technology can be utilized to identify genomic variation and key genes that may encode Vt virulence factors. We can utilize molecular approaches with the application of new sequencing technologies to gain a better understanding of the fundamental genetic differences of strain variation as performed with other pathogenic vibrio species, such as *V. vulnificus* (Gulig et al. 2010). Table 2 provides a list of species within Vibrionaceae that have genome data publically available in the NCBI database. Although many vibrios have been sequenced, more genome data is still needed to provide sufficient data for comparative genomic analyses. Genomic data generated by the present research may offer important baseline knowledge in future work with Vt and may offer insight into vibriosis pathogenesis. Genomic analyses can complement our current knowledge of Vt virulence and can serve as an anchor for interpreting complex pathogen/host relationships and physiological changes that occur at the gene level. Since OA will drive both large-scale and individual, small-scale physiological microbial processes in the ocean, species-specific physiological responses to OA need to be examined further.

Oyster immune response and environmental influences

The coastal environment is dynamic in nature; physical and chemical properties such as temperature, salinity, and gas saturation can change drastically temporally and spatially, influencing the ecology and abundance of bacterial communities. Environmental stressors in the intertidal can also limit the bivalve health. Normal physiological processes, such as nutrient uptake, elimination of bacteria, respiration, or digestive functions may be altered. Under intensive culture conditions, environmental stressors (e.g., elevated temperatures, high population densities) can directly or indirectly affect interactions between microbiota and host physiology, resulting in disease. Because larval stage oysters are more susceptible to certain disease agents than adults, it is critical to understand how varying environmental stressors predicted with OA will impact larval response to pathogen presence and disease susceptibility.

Bivalve immune mechanisms include both cellular and humoral components. Bivalve molluscs have an open circulatory system where hemolymph is pumped into arteries, sinuses, and interstitial spaces by the heart (Kennedy 1996). Hemolymph cells, or hemocytes, operate to provide protection from invading microorganisms and foreign material (Pruzzo et al. 2005). Cellular response in bivalves, carried out by circulating hemocytes, consists of recognition and response to non-self particles, damaged host cells, and debris. Aside from playing roles in nutrition and cation transport, hemocytes are involved in inflammation, wound repair, shell repair, excretion, and internal defense (Kennedy 1996). Hemocytes are primary defense cells in bivalve species that act by recognition, adhesion, phagocytosis and encapsulation of foreign or unwanted material and can show chemotactic activity towards pathogens or their products (Rinkevich and Miller 1996; Prieur et al. 1990).

Hemocytes also have the capability to secrete immune response factors including, antimicrobial peptides (Gueguen et al. 2006; Stensvåg et al. 2008), proteases, protease inhibitors (Zhu et al. 2006; Xue et al. 2006), lysozymes (Bachali et al. 2002; Matsumoto et al. 2006) and lectins (Gourdine et al. 2007; de Lorgeril et al. 2011) to enable binding and ingestion of foreign particles. Two groups of receptors aid in bivalve hemocyte phagocytosis: receptors binding to integral components of bacteria and receptors that recognize hemolymph serum components and coat foreign particles to act as opsonins (Pruzzo et al. 2005). Additionally, humoral system chemical reactive molecules in bivalves, such as reactive oxygen species (ROS), act as cytotoxins and are important in anti-microbial activity (Carballal et al. 1997).

Environmental changes, such as fluctuations in salinity and increases in CO₂, have been associated with increased oyster cell mortality, reduction of hemocyte enzymatic activities, and slower responses of oyster hemocytes when presented with foreign material, inhibiting the ability to kill bacteria (Fisher and Newell 1986; Gagnaire et al. 2006). Reduction in ROS activity in the Eastern oyster (*Crassostrea virginica*) hemocytes has been observed with fluctuations in pH, CO₂, and O₂ in dynamic intertidal environments (Boyd and Burnett 1999). Metabolic depression from hypercapnia (acidosis of tissues due to elevated CO₂ in the marine environment) can lead to decreased larval development, impact adult oyster immune responses, and influence parasite-disease interactions in molluscan larvae (Portner et al. 2005). Overall, the ability to cope with and compensate for environmental stressors can vary between species, although the most fundamental physiological functions of these organisms are almost

always modified, making them more susceptible to disease (Harvell et al. 1999; Portner et al. 2005).

Summary

Given the large-scale environmental shifts predicted with OA, this research assesses how environmental stressors influence disease susceptibility in the economically important Pacific oyster. The goal of this research is to characterize the factors that threaten commercial aquaculture industry and wild shellfish populations. It is critical to examine the factors that influence larval survival by systematically assessing how elevated CO₂ concentrations predicted with OA may (1) impact the physiology of the pathogen, *Vt*, and (2) influence disease susceptibility of oyster larvae when exposed to a combination of pathogen and environmental stressors. The information gathered from this research will provide a basis for assessing mortality risk in commercial hatcheries and evaluate how environmental perturbations will impact valuable and ecologically significant bivalve species.

Chapter 2 : The influence of ocean acidification on *Vibrio tubiashii* growth and impact on *Crassostrea gigas* disease susceptibility.

Abstract

A complete understanding of the relationships among environment, host, and pathogen is important in order to mitigate disease outbreaks in natural and aquaculture settings. Recurrent bacterial disease caused by *Vibrio tubiashii* (Vt) has caused detrimental production losses in Pacific Northwest molluscan bivalve hatcheries. Seawater chemistry changes associated with ocean acidification (OA) in combination with bacterial pathogen exposure may have adverse effects on bivalve larval survival. Examination of larval survival under environmental stressors, such as elevated $p\text{CO}_2$, may identify driving variables that contribute to disease presence in bivalve larvae. To examine disease susceptibility of early stage Pacific oyster (*Crassostrea gigas*) larvae, laboratory trials were used to expose D-veliger and prodissoconch I veliger larvae to a combination of Vt and three levels of CO_2 gas for 72 h in 16°C seawater: current CO_2 levels (approx. 390 ppm), approx. 600 ppm $p\text{CO}_2$, and an extreme $p\text{CO}_2$ level similar to those recently observed in Hood Canal, WA (approx. 1600 ppm $p\text{CO}_2$). Independent Vt cultures were grown at these same $p\text{CO}_2$ levels to investigate Vt abundance and differences in growth. Vt grew faster and in greater abundance at elevated $p\text{CO}_2$ levels at 16°C, but not at 25°C. No difference in larval mortality was detected at elevated $p\text{CO}_2$ compared to ambient $p\text{CO}_2$ conditions at either larval stage. These data indicate $p\text{CO}_2$ may not influence bacterial virulence, but acceleration of growth at higher $p\text{CO}_2$ may account for natural blooms of the pathogen when exposed to higher temperatures within larval rearing environments. Although the present study does not provide a definitive answer to how elevated $p\text{CO}_2$ levels predicted with OA may affect *C. gigas* larvae when exposed to Vt, it does offer important insight into how natural fluctuations of $p\text{CO}_2$ may contribute to episodes of vibriosis in Pacific Northwest hatcheries.

Introduction

Global emissions of atmospheric carbon dioxide (CO_2) are contributing to chemical shifts in the world's oceans resulting in the accumulation of aqueous CO_2 in marine surface waters. This phenomenon is described as "ocean acidification" (OA). Within the past 250 years, seawater pH has decreased by 0.1 pH units and is expected

to continue to decline an additional 0.3-0.4 units by the end of this century (Brewer 1997; Orr et al. 2005; Royal Society 2005). The Pacific Northwest coast of the United States has been one of the first areas to see major shifts in the marine environment due to OA. Seasonal oceanic upwelling events bring CO₂-rich waters to the coast of the Northwest Pacific and enters coastal embayments and inland marine waters (e.g. Puget Sound, WA), exposing coastal marine organisms to corrosive, low pH waters (Hales et al. 2005; Feely et al. 2008; Evans et al. 2011; Barton et al. 2012). Uncertainties in biological responses brought about by OA make it hard to anticipate the associated economic impacts on the shellfish industry (Cooley and Doney 2009).

Any diversion from an evolutionary optimal environment can exceed physiological tolerances of developing invertebrates. Marine larval species that reside in surface waters are sensitive to changes in pH and fluctuations in carbonate availability (Caldeira and Wickett 2003; Gazeau et al. 2007; Portner et al. 2005). OA affects calcifying marine organisms in variety of ways including: calcium deposition, acid-base balance, shifts in energy allocation and metabolic equilibrium, embryonic development and growth, and reproductive success (Allen and Burnett 2008; Parker et al. 2009; Parker et al. 2010; Lannig et al. 2010). Environmental changes occurring from OA have been shown to reduce immune function and respiration as well as lower the physiological tolerances of oysters to additional stressors such as acute thermal stress (Willson and Burnett 2000; Portner et al. 2005; Lannig et al. 2010). Metabolic depression from hypercapnia (acidosis of tissues due to elevated CO₂ in the marine environment) can ultimately lead to decreased development and influence parasite-disease interactions in molluscan larvae (Portner et al. 2005).

Environmental stressors can alter tolerances or susceptibility of bivalve larvae to pathogens. Shifts in naturally occurring low pH, high CO₂, and low O₂ in dynamic intertidal environments have been shown to reduce reactive oxygen species of hemocytes of the Eastern oyster, *Crassostrea virginica* (Boyd and Burnett 1999). Metabolic depression from hypercapnia can lead to decreased larval development, impact adult oyster immune responses, and influence parasite-disease interactions in molluscan larvae (Portner et al. 2005). Overall, the ability to cope with and compensate for environmental stressors can vary between species, although the most fundamental physiological functions of these organisms are almost always modified, making them more susceptible to disease (Harvell et al. 1999; Portner et al. 2005).

The shellfish industry in Washington state is important to the state's economy; US production of shellfish has increased dramatically in recent years to represent 35% of total aquaculture industry value in 2008, generating USD 323 million (FAO 2011). The Pacific oyster, *Crassostrea gigas*, is a major cultivated species and a valuable economic resource for the state (FAO 2011). Successful, large-scale oyster production is highly dependent on the propagation of healthy oyster seed and reliance on hatcheries for distribution of settlement size larvae to growers (Elston et al. 1999; Barton et al. 2012; FAO 2011).

Opportunistic diseases, specifically bacterial diseases, have been the leading cause of larval and juvenile mortality in the hatchery industry (Tubiash et al. 1970; Estes et al. 2004; Elston et al. 2008). Management of infectious disease is problematic in shellfish aquaculture, often leading to severe economic losses in production (Elston et al. 2008). In recent years, re-emergence of a bacterial pathogen, *Vibrio tubiashii* (Vt), was a source of detrimental production losses in Pacific Northwest bivalve hatcheries (Elston et al. 2008). The disease, vibriosis, can cause dramatic larval mortality within high population densities and elevated temperatures common in intensive aquaculture. In some cases, larval mortality exceeds 90% within 24 h of initial exposure to the most pathogenic Vt strains (Estes et al. 2004). Disease outbreaks of vibriosis in bivalve larvae are characterized by bacterial swarming around the velum, loss of larval motility, extensive soft tissue necrosis, and rapid mortality (Elston and Leibovitz 1980; Nottage and Birkbeck 1987). Losses in Oregon and Washington State bivalve hatcheries have increased the urgency to understand the ecology of Vt (Elston et al. 2008).

It is unknown how predicted environmental changes associated with OA, such as elevated surface seawater temperature, increased partial pressure of CO₂ ($p\text{CO}_2$), and pathogen abundance, will impact marine organism health and disease susceptibility (Elston et al. 2008). This study investigated how two environmental stressors, temperature and elevated $p\text{CO}_2$, affected the physiology of Vt and assesses its impact on larval bivalve disease. We used laboratory experiments to manipulate $p\text{CO}_2$ to examine how environmental factors predicted with OA influence Vt growth and Pacific oyster disease susceptibility to vibriosis. Three levels of $p\text{CO}_2$ were targeted in disease trials: current-day ambient level (approx. 390 microatmospheres (μatm)), a $p\text{CO}_2$ level predicted by the Intergovernmental Panel for Climate Change for the end of this century (IPCC 2007) (750 μatm), and an elevated $p\text{CO}_2$ level representative of low pH conditions similar to those recently observed in Hood Canal, WA (2000 μatm). The aim of this

research was to establish Vt growth and *C. gigas* larval susceptibility to Vt at these three $p\text{CO}_2$ levels. This research facilitates understanding the interrelationships between Vt, OA, and Pacific oyster larvae, fundamental to the success and preservation of Pacific Northwest shellfish aquaculture.

Materials and Methods

Seawater chemistry

Seawater was collected from Elliott Bay outside of the Seattle Aquarium (Seattle, Washington) (N 47°36.440' W 122°20.523') in May – June 2011. All seawater was autoclaved before each experiment. Seawater pH of discrete seawater samples were measured using the spectrophotometric technique outlined in SOP 6b by Dickson et al. (2007) using *m*-cresol purple as an indicator dye (Sigma-Aldrich). Salinity (ppt) measurements were performed with a refractometer. For each laboratory trial, pH measurements were taken prior to bacterial inoculation, every 24 h after inoculation, and at experiment completion.

After each trial was performed, seawater from each treatment were submitted to NOAA's Pacific Marine Environmental Laboratory (PMEL) for seawater chemistry analysis to measure total alkalinity. Sample collection was performed under PMEL sampling guidelines of SOP 1 (Dickson et al. 2007) with an added siphon step to collect seawater from each treatment. Total alkalinity (TA), temperature, salinity, and spectrophotometric pH measurements were used to calculate resulting $p\text{CO}_2$ of treatments using the program CO_2Calc (Robbins et al. 2010). Parameters of calculations were based upon Lueker et al. (2000) CO_2 equilibration constants, Dickson (1990) KHSO_4 constants, and total pH scale (mol kg^{-1}).

Vibrio tubiashii growth at elevated $p\text{CO}_2$

Growth curves were determined using batch cultures of Vt strain RE22 (Estes et al. 2004). Strain RE22 was grown overnight (17 h) in sterile seawater augmented with 0.25% tryptone at room temperature (RT, approx. 22°C). Cultures were agitated on a serological rocker (Thermolyne Speci-Mix). The spread plate technique using 1% tryptone, 2% NaCl (T1N2) agar was used to estimate bacterial growth of original culture used to inoculate the seawater treatment described below.

Temperatures used in this experiment represent a mean summer temperature associated with upwelling and observation of Vt in the Northeast Pacific, 16°C (Elston et

al. 2008), and an elevated temperature, 25°C, that is typically used to rear *C. gigas* larvae in bivalve hatcheries. Vt growth curves were determined at three $p\text{CO}_2$ levels (approx. 390 (ambient), 750 and 2000 ppm CO_2) at 16°C and two $p\text{CO}_2$ levels (approx. 390 (ambient) and 750 ppm CO_2) at 25°C. Each trial was performed in triplicate 1L Erlenmeyer flasks and included a fourth flask that served as a sterile control to check for contamination. Specialty gas mixes of CO_2 with 21% O_2 balance (2000 and 750 ppm $p\text{CO}_2$) (Praxair, Inc.) were bubbled into culture vessels to elevate $p\text{CO}_2$ in the treatment cultures. Ambient air was bubbled into control treatments. All Erlenmeyer flasks were covered with a rubber stopper through which the appropriate gas mixture passed into the container. Filters (0.22 μm) were placed on each incoming air supply to prevent aerosol contamination. Seawater media was pre-equilibrated with the appropriate gas mixture for approximately 40 h before inoculating with Vt. Water baths maintained cultures at 16°C or 25°C.

Vt was added to each sterile replicate (beginning concentration of 2.58×10^2 CFU/ml was used in the 16°C trial and 1.6×10^3 CFU/ml used in the 25°C trial). Cultures at 16°C were grown for 122 h and cultures at 25°C were grown for 72 h. Aliquots of each replicate were taken every 4-18 h to estimate bacterial abundance by plate count. T1N2 plates were incubated at 30°C for 24 h prior to being counted. Control flasks were plated directly onto T1N2 agar to check for contamination at each time point. Vt growth rate and generation time was calculated at each $p\text{CO}_2$ level using the formula:

$$\text{Growth rate } (k) = (\log_{\text{cells at end of incubation}}) - (\log_{\text{cells at beginning of incubation}}) / (0.301 * \text{time interval between two points})$$

$$\text{Generation time } (t_{\text{gen}}) = 1/k$$

Data analysis

Predicted growth curves of viable cell counts were calculated using the Gompertz equation (Gompertz 1825):

$$y = ae^{(-b2*b3)^x}$$

The parameters of the Gompertz equation represent: the asymptote (*a*); the parameter value when $x = 0$ (*b2*); and the parameter value related to the scale of the x -axis (*b3*).

Goodness of fit for each growth curve was evaluated using residual plots of the predicted y values graphed relative to hours of growth. Parameters of the equation were estimated with the nonlinear self-start Gompertz function *stats* package in R statistical software, version 2.12.0 (R Development Core Team 2011). This equation was used to fit a regression line of predicted Vt abundance at each time point.

Pairwise two-sample T-tests were used to test differences in mean Vt abundance during stationary phase. ANCOVA was used to test for differences in exponential phase of Vt growth at each treatment. CFU/ml at hours 8 to 56 were used to test differences in exponential growth and hours 72 to 122 were used to test differences in total Vt abundance at stationary phase in the 16°C growth trial. CFU/ml at hours 2 to 14 were used to test differences in exponential growth and hours 38 to 62 were used to test differences in total Vt abundance at stationary phase in the 25°C growth trial. Welch's T-test was used to test differences in Vt abundance at stationary phase between 16°C and 25°C. Vt growth at the 72 hour time point during the 25°C trial was excluded from analysis because cultures had entered death phase.

C. gigas larval susceptibility to vibriosis at elevated $p\text{CO}_2$

Disease challenges were performed exposing *C. gigas* larvae to a combination of elevated $p\text{CO}_2$ and Vt dosages (Table 3). These experiments examined *C. gigas* larval susceptibility to vibriosis caused by Vt at three target $p\text{CO}_2$ levels (ambient (approx. 390), 750 and 2000 ppm $p\text{CO}_2$). Specialty gas mixtures of CO_2 (2000 and 750 ppm $p\text{CO}_2$) (Praxair, Inc.) were used to produce elevated $p\text{CO}_2$ conditions. Autoclaved seawater was placed into 1000 ml Erlenmeyer flasks and bubbled with premixed, elevated $p\text{CO}_2$ gas as described above for each treatment. Seawater was held at 16°C until use.

Two developmental stages of *C. gigas* larvae were used in each treatment: early stage D-veliger larvae (approx. 72 h old) and prodissoconch I veliger larvae (approx. 10 days old). Experiments were used to determine the lethal Vt dose needed to produce 50% larval mortality (LD_{50}) for each larval age. Disease trials for each stage larvae were conducted in the same manner. All larvae used for this experiment were transported on ice from the Taylor Shellfish Farms hatchery (Quilcene, Washington) directly to University of Washington (Seattle, Washington). *C. gigas* broodstock were spawned and resulting larvae were reared under routine hatchery procedures prior to transportation. Once larvae arrived at the lab, they were distributed into 3 groups and gently rinsed with

sterile seawater equilibrated to either ambient (approx. 390 ppm) or elevated (750 or 2000 ppm) $p\text{CO}_2$. Aliquots containing 40 larvae were placed into each well of a 12-well tissue culture plate in a final volume of 4ml seawater equilibrated to the appropriate $p\text{CO}_2$ level.

Vt cultures of strain RE22 were prepared as described above. Trials A-C exposed larvae to 2000 ppm and ambient CO_2 ; trials D-E exposed larvae to 750 ppm and ambient CO_2 (Table 3). Five concentrations of Vt were used to estimate larval LD_{50} : 10^2 , 10^3 , 10^4 , 10^5 , and 10^6 CFU/ml. Six replicates of each dose and six sterile control wells, prepared on a separate culture plate, were used for each $p\text{CO}_2$ treatment level (two trials of each $p\text{CO}_2$ treatment level, $n=12$ per dose, per treatment). Once plates were prepared with larvae, filled with seawater, and inoculated with Vt, they were placed into airtight plastic chambers into which either ambient (approx. 390 ppm) or elevated $p\text{CO}_2$ (750 or 2000 ppm) flowed; temperature was maintained at 16°C . A reservoir of each seawater treatment used to fill larval wells (approx. 1500 ml) was kept in each airtight container to monitor pH levels throughout the experiment. LD_{50} experiments were held static for the 72-hour experiment. Every 24 h, larval mortality was counted in each well by light microscopy. Larvae were considered dead when no ciliary movement was visible at 400x magnification. Disease trials were replicated at least two times per $p\text{CO}_2$ level.

Seawater pH was measured at the start of each trial, every 24 h after Vt inoculation and upon termination of the experiments using the spectrophotometric technique described above. The seawater reservoirs from each treatment were sampled for TA after experiment completion. Half of the samples were analyzed at PMEL using potentiometric titration method (Dickson et al. 2003) and half were analyzed at Friday Harbor Labs (University of Washington), using the open cell titration of SOP 3b (Dickson et al. 2007). All seawater chemistry samples were collected as described above.

LD_{50} determination and analysis of larval survival

LD_{50} estimations by day were analyzed using R statistical software MASS library with the logistic equation:

$$P_i = 1 / (1 + e^{-(a + b * x)})$$

To compare treatments, a generalized linear model was used to calculate LD₅₀. A binomial logit model was used to test proportional survival as distributed by $p\text{CO}_2$ treatment and dose Vt each day. Calculations were based upon unweighted proportions of larval survival due to the over dispersion of the weighted non-linear regression model. Two-way ANOVA tests were used to compare larval survival at elevated $p\text{CO}_2$ levels and Vt dosages by day. One-way ANOVA tests were used to test differences in larval mortality among replicate trials.

Results

Seawater Chemistry

Measurements of $p\text{CO}_2$, pH, salinity, and TA for each experiment are summarized in Table 4. Calculated $p\text{CO}_2$ values generally yielded lower values than the premixed CO_2/O_2 gas bubbled into seawater (Table 4). Calculated $p\text{CO}_2$ values through the duration of each experiment were plotted (Figures 1a, 1b, 2).

The 16°C Vt growth trial resulted in an average of 489 (± 4.34 SE) (ambient control), 735 (± 17.43 SE) (750 ppm treatment), and 2106 (± 21.88 SE) (2000 ppm treatment) $\mu\text{atm CO}_2$ throughout the 122 h of growth. The 25°C Vt growth trial produced an average elevated $p\text{CO}_2$ treatment of 325 (± 27.37 SE) μatm of ambient control cultures and treatment $\mu\text{atm CO}_2$ measurements of 620 (± 18.06 SE) at 24 h and 695 (± 3.22 SE) at 72 h (750 ppm treatment) (Table 4).

Disease susceptibility trials average treatment $p\text{CO}_2$ levels were 388 (ambient control) (all trials), 605 (750 ppm treatment) (Trial D and E), 1627 (2000 ppm treatment) (Trials A and B) μatm of CO_2 . Trial C (2000 ppm) yielded an average elevated treatment of 964 $\mu\text{atm CO}_2$. Trial C was eliminated due to the lower than expected μatm of CO_2 values (Table 4).

Vibrio tubiashii growth at elevated $p\text{CO}_2$

Vt grew significantly faster and reached higher abundance at 16°C in the cultures maintained at elevated $p\text{CO}_2$ (2000 ppm) relative to those held under ambient conditions ($p < 0.001$) (Fig. 3). Differences in exponential Vt growth at 16°C was not detected at 750 ppm when compared to ambient $p\text{CO}_2$ ($p = 0.101$), but significant differences in relative abundance were detected at stationary phase ($p < 0.001$) (Fig. 4). At higher temperature, the 25°C cultures showed no significant differences in Vt growth or relative abundance at 750 ppm $p\text{CO}_2$ compared to ambient cultures ($p = 0.184$ stationary growth, $p = 0.099$

exponential growth) (Fig. 5). Comparing Vt growth at different temperatures (16°C vs. 25°C), Vt growth at ambient $p\text{CO}_2$ was significantly greater at 25°C ($p < 0.001$), but no differences in Vt abundance was detected at 750 ppm $p\text{CO}_2$ ($p = 0.23$).

At 16°C, Vt grew at a rate of 0.14 generations/hour with a generation time of 404 min at ambient $p\text{CO}_2$, 0.16 generations/hour with a generation time of 367 min at 750 ppm $p\text{CO}_2$, and 0.19 generations/hour with a generation time of 316 min at 2000 ppm $p\text{CO}_2$. At 25°C, Vt grew at a rate of 0.49 generations/hour with a generation time of 124 min ambient $p\text{CO}_2$ and 0.53 generations/hour with a generation time of 114 min at 750 ppm CO_2 .

C. gigas larval susceptibility to vibriosis at elevated $p\text{CO}_2$

No significant differences in larval survival were detected from two-way ANOVA tests comparing elevated $p\text{CO}_2$ (approx. 600, approx. 1600 $\mu\text{atm CO}_2$) to ambient levels (p -values $\gg 0.05$). Figure 6 illustrates the proportions surviving at both larval stages over the 72 h of Vt exposure. No significant differences were seen in larval mortality among replicate trials ($p > 0.05$). Although calculated LD_{50} values were lower in the 2000 ppm treatment with the prodissoconch I larval stage when compared to LD_{50} values calculated for the ambient and 750 ppm treatments, differences were not significant (Table 5, $p > 0.05$).

Discussion

Increasing seawater acidification due to rising levels of atmospheric CO_2 coupled with near-shore biological and physical processes can negatively impact calcifying organisms, including oysters (Portner et al. 2005; Kurihara et al. 2007; Miller et al. 2009; Feely et al. 2010; Dickinson et al. 2011; Gazeau et al. 2011; Barton et al. 2012). Predicting the changes OA may have on economically significant marine species represents a major challenge to the scientific community (Cooley and Doney 2009). Strategies to mitigate disease in bivalve hatchery populations depend on a detailed understanding of the interactions among climate change, pathogen physiology, and host susceptibility. Examining larval survival of under single stressors, such as high $p\text{CO}_2$, is a starting point that may help identify driving variables that contribute to pathogenesis of vibriosis.

Vibrio tubiashii growth at elevated $p\text{CO}_2$

This research is the first to investigate the effects of elevated levels of $p\text{CO}_2$ on Vt abundance and disease susceptibility to vibriosis in larval bivalve molluscs. Through laboratory trials, we were unable to detect differences in susceptibility to vibriosis caused by Vt exposure at elevated $p\text{CO}_2$ although Vt growth alone, under the same environmental conditions, exhibited significantly greater growth at elevated $p\text{CO}_2$. Vt abundance was shown to significantly increase with elevated $p\text{CO}_2$ (750 and 2000 ppm) when grown at 16°C but exhibited no significant differences in growth at 25°C. Even though pathogen virulence was unaffected by CO_2 level, enhanced Vt growth under acidified conditions may lead to outbreaks of vibriosis by enabling the bacterium to reach lethal levels as was observed in bivalve shellfish hatcheries during periods of upwelling in 2005 and 2007 (Elston et al. 2008). Thus, enhanced growth may help explain one mechanism driving these episodes of vibriosis in Pacific Northwest hatcheries (Elston et al. 2008, Barton et al. 2012).

The economic importance of Vt on the cultivation of bivalve molluscs has led to continued investigation of factors contributing to the virulence of the bacterium (Tubiash et al. 1965; Nottage and Birbeck 1987; Estes et al. 2004; Hasegawa et al. 2008; Hasegawa and Hase 2009a; Hasegawa and Hase 2009b). The highly pathogenic Vt strain used in this study, RE22, isolated from vibriosis epidemics in Pacific Northwest bivalve hatcheries, is extremely toxic to *C. gigas* larvae, producing massive mortality within 24 - 48 h of exposure with LD_{50} dosages as low as 3.6×10^4 CFU/ml (Estes et al. 2004). The strategies used by bacterial pathogens, such as Vt, to circumvent host immune responses and produce infection is still unclear, although current research has focused on the role of excreted extracellular toxins produced by the bacterium (Hasegawa et al. 2008; Hasegawa and Hase 2009a; Hasegawa and Hase 2009b).

Extracellular protease and hemolysin production in vibriosis infection of *C. gigas* larvae, specifically the expression of Vt extracellular proteins, metalloprotease and hemolysin, is correlated to cell density (Hasegawa and Hase 2009a; Hasegawa and Hase 2009b). While hemolysin activity was shown to increase during early stage growth (8 to 16 h growth at 30°C) and decrease at stationary phase, protease activity increased during all stages of bacterial growth, reaching the highest level of activity during stationary phase (>20 h growth at 30°C) (Hasegawa and Hase 2009a). An excreted Vt metalloprotease (VtpA) was determined to be responsible for toxicity to bivalve larvae, whereas Vt hemolysin did not influence larval mortality in experimental trials (Hasegawa et al. 2008; Hasegawa and Hase 2009a). It is speculated that although hemolysin is not

a major virulence factor of Vt, hemolysin and other secreted extracellular proteases may contribute to the overall pathogenicity of Vt (Hasegawa et al. 2008; Hasegawa and Hase 2009a).

While it is interesting that levels of Vt virulence-associated extracellular proteins differ with growth phases of the bacterium, potential complex pathogen-host-environment interactions are still likely to exist. While numerous studies have correlated elevated temperature with increased vibrio abundance (Kaneko and Cowell 1973; Huq et al. 1984; Pfeffer et al. 2003), research investigating species-specific responses of vibrios to elevated $p\text{CO}_2$ levels predicted with OA is lacking. It is possible that the interactions between elevated CO_2 concentrations and rising temperatures could influence bacterial virulence and may also impact immune response of host species. The present study demonstrates that Vt abundance increases with elevated $p\text{CO}_2$, but the influence of $p\text{CO}_2$ on Vt virulence to bivalve larvae is still unknown.

C. gigas larval susceptibility to vibriosis at elevated $p\text{CO}_2$

Based on our current knowledge of the physiological changes that occur in bivalve larvae when exposed to OA conditions, we hypothesized that elevated $p\text{CO}_2$ would influence mortality when exposed to an additional stressor (infectious disease) in laboratory trials. This study illustrated that while Vt grew faster and reached higher abundance under elevated $p\text{CO}_2$ levels at a temperature reflective of the conditions in natural settings (i.e. Washington state estuaries), high $p\text{CO}_2$ did not influence its virulence or pathogenicity to larval *C. gigas*. While this study provides interesting results on larval disease susceptibility when exposed to elevated $p\text{CO}_2$, effects of the multivariate environmental conditions associated with climate change - salinity fluctuations, elevated temperature and possible ecosystem shifts - should not be downplayed. Reduced growth rates and depressed larval production have been correlated with periodic influxes of low pH seawater, undersaturated in aragonite at one Pacific Northwest bivalve hatchery (Barton et al. 2012). Aragonite saturation states (Ω_{arg}) observed in our study were above 1.0 in the 750 ppm $p\text{CO}_2$ treatment (mean $\Omega_{\text{arg}} = 1.5$), but well below 1.0 in the 2000 ppm $p\text{CO}_2$ treatment (mean $\Omega_{\text{a}} = 0.64$). The short duration of our study may have precluded examination of the synergistic effects of aragonite undersaturation and associated energetically expensive physiological compensation due to seawater chemistry stressors (low pH and Ω_{a} ; Timmins-Schiffman et al. in review) combined with disease. A recent study observed a synergistic effect of combined salinity and CO_2

stressors on juvenile oysters as evidenced by reduced tissue energy stores (glycogen and lipid), negative soft tissue growth, and significant increases in mortality, which also indicates energy deficiency in juvenile oysters (*C. virginica*) (Dickinson et al. 2011).

Larval mortality or survival upon pathogen exposure can be influenced by multiple factors including host nutritional status, environmental conditions at spawning, and population tolerances to pathogen exposure. Disease trials were performed over a number of weeks and, each week, different cohorts of larvae were exposed to Vt at our target $p\text{CO}_2$ levels. It is possible population tolerances to the pathogen and/or vibriosis may fluctuate, contributing to the amount of variability seen in larval survival among treatments even though differences in larval mortality between replicate trials was not detected. Recently, genetic studies have begun using molecular sequencing to identify genetic markers in disease resistant families of *C. gigas* associated with tolerance to extracellular toxin production of Vt and high $p\text{CO}_2$ seawater (Camara and Chen, unpublished). The potential use of vibriosis-resistant stocks may provide hatcheries immediate and substantial benefit to larval survival and growth in hatchery environments where bacterial mortalities are persistent.

The experimental design of the disease trials can be modified to produce consistent $p\text{CO}_2$ levels. Elevated $p\text{CO}_2$ conditions were maintained with airtight containers plumbed with premixed CO_2/O_2 gas to maintain $p\text{CO}_2$ levels. This procedure relied on atmospheric pressure of CO_2 to maintain treatment conditions. In the present study, fluctuations in $p\text{CO}_2$ levels of treatment seawater was observed in most laboratory trials and may have contributed to our results in the disease trials. Carbonate ion availability and saturation states are an essential part of larval biomineralization, development, and ultimately influence larval survivorship (Dickinson et al. 2011; Gazeau et al. 2011; Barton et al. 2012). Synergistic effects of carbonate ion saturation states and the energetic costs hypercapnia may have on developing larvae might be better understood in experiments of longer duration. However, in natural nearshore and inland marine waters CO_2 levels typically fluctuate over the course of a single day or days (Barton et al. 2012, Feely et al. 2008; 2010). While current research has found OA to have negative effects on molluscan species, bivalves may possess the physiological capability to cope with stress conditions by modifying cellular, biochemical, and metabolic processes. For future studies, modification of experimental procedures to include flowing seawater conditions, manipulation of temperature and carbonate ion

saturation states and longer trials are needed to further investigate the relationship between Vt and its host: *C. gigas* larvae.

Conclusions

These research results indicate elevated $p\text{CO}_2$ increases pathogen growth and altering hatchery practices to reduce pathogen proliferation may help control disease epidemics. It is likely that increased temperature is a stronger environmental driver in Vt growth rather than elevated $p\text{CO}_2$. If Vt is consistently in the environment, and elevated $p\text{CO}_2$ causes growth to reach levels associated with mortality, an immediate remedy could be altering hatchery conditions to mitigate vibriosis outbreaks. Specifically, decreasing seawater temperature during larval rearing may facilitate management of vibriosis by decreasing Vt growth. Although it is important to note, this environmental modification comes at a cost because development, growth, and settlement success of *C. gigas* larvae is optimal at higher temperatures (Kheder et al. 2010).

These trials indicate elevated $p\text{CO}_2$ does not directly impact oyster larvae susceptibility to a lethal exposure of Vt. If bacterial cultures were grown initially under elevated $p\text{CO}_2$ conditions and subsequently exposed to *C. gigas* larvae, pathogenesis could also vary. Examination of Vt virulence factors under predicted OA conditions – a combination of salinity gradients, elevated temperature, and low pH – may reveal important physiological changes within the pathogen itself that contribute to disease in bivalve populations.

In conclusion, the present study does not provide a definitive answer to how elevated $p\text{CO}_2$ levels predicted with OA may affect *C. gigas* larvae when exposed to Vt, but provides compelling data on an important environmental factor influencing Vt growth: elevated $p\text{CO}_2$. Further long-term studies are needed to better assess the potential consequences that OA may have on *C. gigas* larval disease susceptibility and the environmental variables contributing to Vt virulence. Immediate mitigation of vibriosis outbreaks caused by Vt in bivalve molluscan hatcheries should decrease rearing temperatures to reduce bacterial abundance, especially when elevated $p\text{CO}_2$ conditions are present.

Chapter 3 : Exploratory genomic analysis of two *Vibrio tubiashii* strains: RE22 and ATCC 19106.

Abstract

Vibrio tubiashii (Vt) is a causative agent of vibriosis, a disease that affects early life stages of many aquatic species and is a re-emerging problem for molluscan bivalves. Management of infectious disease, including that caused by Vt, has been problematic in shellfish aquaculture since its inception, often leading to severe economic losses in production. Understanding the genetic factors responsible for pathogenicity and physiology of the bacterium is hampered by lack of genomic resources. In this study, we used high throughput sequencing technology to provide genomic resources for two Vt strains: the highly pathogenic strain, RE22, and a nonpathogenic type strain, ATCC 19106. This approach was taken to 1) provide novel sequence information and 2) evaluate factors associated with *Vibrio* virulence. Analysis of the newly sequenced genomes revealed 1,690 putative genes, including 931 genes associated with bacterial virulence. Genomic libraries from both strains contained proteins of interest including those involved in pilin production, a cholera toxin transcriptional activator, and quorum sensing proteins associated with pathogenesis in related species. Three putative virulence proteins were examined in detail including metalloprotease M6, extracellular zinc metalloprotease, and ToxR transcriptional activator. *In silico* analysis indicated specific regions of high sequence dissimilarity likely associated with disparate physiological function. Genomic data generated by this research offered important baseline genomic knowledge and the development of genomic tools that can be utilized in future functional analysis experiments.

Introduction

Vibrio tubiashii (Vt), a toxigenic and invasive bacterium, is a causative agent of vibriosis, a disease affecting early life stages of a variety of aquatic species, including molluscan bivalves (Brown and Losee 1978; Elston et al. 1981; Hasegawa and Hase 2009a). Management of infectious disease, especially those caused by members of the genus *Vibrio* have been problematic in shellfish aquaculture since its inception, often leading to severe economic losses in production. Vt has been a recurring problem since its initial observation in the 1960s (Tubiash et al. 1965; 1970; Elston et al. 1981; Elston

1990; Elston et al. 2008). Vibriosis in bivalve larvae is characterized by bacterial swarming around the velum, loss of larval motility, extensive soft tissue necrosis, and swift mortality (Brown 1973; Elston and Leibovitz 1980; Nottage and Birkbeck 1987). In intensive molluscan culture, vibriosis causes dramatic larval mortality especially under rearing conditions commonly used for larval shellfish, including high population densities and elevated temperatures. Recent re-emergence of vibriosis by Vt has been a source of substantial economic losses, particularly in Pacific oyster (*Crassostrea gigas*) rearing facilities (Elston et al. 2008).

Vt is a member of the family *Vibrionaceae* and is a Gram-negative, curved rod-shaped bacterium that inhabits a wide range of marine and estuarine environments. The genus *Vibrio* contains more than 30 known species of bacteria (Chakraborty et al. 1997). Vibrios are free-living or may live commensally with a variety of marine taxa, but are also considered opportunistic pathogens associated with disease and mortality when host immune responses are circumvented and infection is established (Elston and Leibovitz 1980; Elston et al. 1999; Estes et al. 2004; Elston et al. 2008). *Vibrio* spp. produce various pathogenic factors that play a role in establishing infection and causing disease: extracellular products are postulated to play an important role in its pathogenesis in fish and molluscan species (Rodriguez et al. 1992; Hasegawa et al. 2008; Hasegawa & Hase 2009a). Extracellular factors, lipases, exopolysaccharides, and metalloproteases found in marine *Vibrio* species are similar to those seen in Vt (Park et al. 2004; Hasegawa and Hase 2008; Hasegawa et al. 2009a; Shinoda and Miyoshi 2011), but few potential virulence factors in Vt have been examined in detail using experimental or genomic studies (Hasegawa and Hase 2008; Hasegawa and Hase 2009a; 2009b).

Lack of genomic information limits our understanding of Vt virulence. Advances in high-throughput sequencing technology can be utilized to identify genomic variation and key genes that may encode for virulence factors in pathogenic bacteria. These molecular approaches can be used to gain a better understanding of the genetic diversity and virulence among bacterial strains as demonstrated in other genomic analyses of *Vibrio* species (e.g., *V. vulnificus*, Gulig et al. 2010). Comparative genomics offers us the opportunity to understand the complex systems that are responsible for differences among strains well beyond what has been possible with gene centric studies (Laing et al. 2011).

In this study, genome sequence information was obtained from two strains of Vt: the highly pathogenic strain, RE22, and a nonpathogenic type strain, ATCC 19106.

Previous research demonstrated differential pathogenicity to oyster larvae among Vt strains (Estes et al. 2004). Strain RE22 is highly pathogenic, with a lethal dose at 50% (LD_{50}) of 3.6×10^4 colony forming units per milliliter of seawater (CFU/ml) after 48 hours of exposure to oyster larvae at 20°C (Estes et al. 2004). Larval mortality exceeded 90% within 24 hours of initial exposure to RE22 (Estes et al. 2004). Under the same experimental conditions, strain ATCC 19106 demonstrated a lack of pathogenicity to oyster larvae (Estes et al. 2004). The aim of this research was to utilize high-throughput sequencing technology to 1) provide novel sequence information from two Vt strains and 2) evaluate factors associated with *Vibrio* virulence. The information provided as part of this study provides essential genomic information for future environmental and molecular research efforts.

Materials and Methods

DNA isolation and sample preparation

Isolates of Vt strains RE22 and ATCC 19106 were individually streaked onto 10% tryptone 20% NaCl (T1N2) agar plates and grown overnight at 30°C. Individual colonies were grown in natural seawater media supplemented with 1% tryptone for 24 hours at room temperature. Bacterial cultures (1ml) were concentrated by centrifugation (12,000 rpm for 10 minutes) and DNA was extracted from the resultant pellet using DNeasy Blood & Tissue kit (Qiagen) following manufacturer's instructions specific to Gram-negative bacteria. Library preparation and sequencing was conducted by the University of Washington High Throughput Genomics Unit (UWHTGU) on the SOLiD 4 System (Applied Biosystems) using standard protocols.

Library preparation and sequence assembly

All sequence analysis was performed with CLC Genomics Workbench version 4.0 (CLC Bio). Sequence reads from each library were quality trimmed using a minimum quality score of 0.05 with a minimum number of 20 nucleotides. *De novo* assemblies were performed on each Vt strain library using the following parameters: mismatch cost 2, limit of 8, color space error cost of 3, a nucleotide vote was used for conflict resolution, and non-specific matches were selected randomly. Minimum contiguous sequence (contig) length was set to 200 base pairs (bp). Open reading frames (ORFs) were discovered using the Bacterial and Plant Plasmid translation table defined by the

National Center for Biotechnology Information (NCBI) taxonomy group (Wheeler et al. 2000) with a minimum length of 100bp.

Gene annotation

Contig sequences with greater than 20x coverage were compared to the UniProtKB/Swiss-Prot database (<http://uniprot.org>) using the BLASTx algorithm (Altschul et al 1997). BLASTx results were limited to e-values <1e-05. SwissProt identifiers were associated with respective Gene Ontology terms (GO) (<http://www.geneontology.org/>). GO definitions were then used to classify sequences into parent categories using the GO slim database. Comparisons based on top BLAST hits were made across strains to identify shared gene sets MS Access and Galaxy (<https://main.g2.bx.psu.edu/>). Sequence based comparisons were made using BLASTn among two genomes with an e-value limit <1e-05.

Protein alignments

Select protein sequences (3) associated with *Vibrio* virulence, including extracellular zinc metalloprotease, metalloprotease M6, and ToxR transcriptional activator were identified within each Vt strain. Changes in amino acid sequences were quantified relative to corresponding sequences in NCBI Genbank from *V. tubiashii* NCIMB 1337 (accession number AHHF00000000) (Temperton et al. 2011). Identification of conserved domains for each protein sequence was performed using NCBI Conserved Domains search engine using default parameters. Percentage of identical sequence alignment positions was used to calculate percentage sequence similarity within the alignment range.

Results

Sequencing summary

Sequencing two genomic libraries (strain RE22 and ATCC 19106) generated 83,732,779 and 70,883,333 reads, respectively, with an average read length of 42 bp for both libraries. Average depth of coverage was 189x per contig for both strains. After quality trimming, 56,024,538 (RE22) and 49,217,456 (ATCC 19106) reads remained from each library.

De novo assembly

De novo assemblies resulted in a total of 6,558 contigs with an average 384x depth of coverage for RE22 and 6,098 contigs with an average 350x depth of coverage for strain ATCC 19106 (Dorfmeier 2012a). RE22 contig length ranged from 198 – 4,657 bp with a mean contig length of 713 (Table 6). ATCC 19106 contig lengths ranged from 196 – 6,350 bp with a mean contig length of 737 (Table 6) (Dorfmeier 2012b). Sequence information from the RE22 library included 4.7 Mbp in combined contigs and ATCC 19106 contained a total of 4.5 Mbp in combined contigs. DNA G+C content was 46% in strain RE22 and 45% for ATCC 19106. ORF discovery revealed 3,821 ORFs in strain RE22 and 3,402 in ATCC 19106.

Gene annotation

Using the Swiss-Prot database 2,465 genes in the RE22 library and 2,375 genes in the ATCC 19106 library were putatively identified (Dorfmeier 2012c; 2012d). Of the genes identified, 1,690 were identified in both libraries. A total of 775 genes were identified in the RE22 library, but not found in ATCC 19106. Likewise, 685 genes were identified in the ATCC 19106 library, but not seen in the RE22 library (Fig. 7). Table 7 summarizes gene annotations for each based on Gene Ontology.

Genes associated with bacterial virulence (1,699) were identified based on previously reported GO terms (Tsai et al. 2009). Of these virulence genes, 55% were identified in both libraries. Comparisons between the two libraries revealed 1,339 virulence-associated genes in the RE22 library (Dorfmeier 2012e) and 1,291 in the ATCC 19106 library (Dorfmeier 2012f). A summary of genes associated with virulence based on Gene Ontology is provided in Table 8.

Protein comparison

Deduced amino acid of M6 metalloprotease, extracellular zinc metalloprotease, and ToxR transcriptional activator from both libraries were compared to respective proteins from *V. tubiashii* NCIMB1337 (accession number AHHF00000000) (Temperton et al. 2011) (Fig. 8, 9, 10). ToxR transcriptional activator did not contain a conserved domain within the range of amino acid sequence examined. Conserved protein domains included peptidase M6 super family, immune inhibitor A peptidase M6 within the M6 metalloprotease deduced acid sequences from respective strains (Fig. 8). The deduced protein sequences from RE22 and ATCC1906 contained two conserved domains including LasB zinc metalloprotease and peptidase M4 family neutral protease (Fig. 9).

Compared to NCIMB 1337, Vt strain RE22 contained 77 amino acid changes within the ToxR transcriptional activator protein (reference position 152 – 395) and shared 70% sequence similarity. The metalloprotease M6 amino acid sequence from RE22 contained 9 amino acid changes (reference position 226 – 390, positions 391-512 not counted due to low sequence similarity) compared with NCIMB 1337 and shared 81% sequence similarity. A total of 72 amino acid changes within the extracellular zinc metalloprotease protein sequence (reference position 265 – 520) were found in RE22 with 68% shared sequence similarity to NCIMB 1337.

ATCC 19106 contained 5 amino acid changes compared to NCIMB 1337 (reference position 137-425) within ToxR transcriptional activator protein with 98% shared sequence similarity. In the metalloprotease M6 protein of ATCC 19106, 5 variations were found (reference position 281 – 591) compared to NCIMB 1337 with 99% shared sequence similarity. The ATCC 19106 extracellular zinc metalloprotease protein contained 3 amino acid changes compared to NCIMB 1337 (reference position 353–520) with 98% shared sequence similarity.

Discussion

High-throughput sequencing was used to generate novel genomic resources and compare two strains of Vt, a causative agent of vibriosis in early stage molluscan bivalve larvae. These data provide important genomic tools to aid in future experimental and genetic research with the bacterium. Sequencing two Vt strains - the highly pathogenic RE22 and the non-pathogenic type strain ATCC 19106 - allowed identification of candidate genes of key metabolic or virulent pathways and contributes to the identification of central genomic characteristics among strains.

V. tubiashii genomics

One objective of this study was to characterize genomic information between two strains of Vt. Sequencing and assembly revealed approximately 87% total genomic sequencing from RE22 and 83% from ATCC 19106. Prior sequencing efforts revealed that the full genome Vt size is approximately 5.4 Mbp in size with two circular chromosomes containing 4,868 genes, of which, approximately 3,600 genes have known functional annotation (Temperton et al. 2011).

Since coverage gaps between the two genomes exist, we are unable to make comprehensive conclusions of genomic differences between the strains. However,

gleaning significant information about the genomic content of each strain is still possible, including, quorum sensing systems in the genome, select virulence mechanisms, and quantification of genomic sequence changes within selected virulence proteins.

Quorum sensing in Vt

Vt utilizes a coordinated cell-to-cell communication system in response to cell density, called quorum sensing. Previous sequencing of the Vt genome, NCIMB 1337 (ATCC 19106), describes quorum sensing communication systems in the luxM/N system (Temperton et al. 2011). Gram-negative bacteria use quorum sensing in a range of physiological activities such as horizontal gene transfer, antibiotic production, motility, and virulence (de Kievit and Iglewski 2000; Miller and Bassler 2001; Antonova and Hammer 2011). Genomic sequencing performed here reveals multiple genes homologous to other members of the genus involved in quorum sensing. Regulatory proteins discovered include an autoinducer 2-binding periplasmic protein (LuxP), LuxR, LuxM, LuxN, LuxQ, LuxO, LuxU, CAI-1 autoinducer sensor kinase/phosphatase CqsS, and an RNA polymerase sigma factor, RpoS found in one or both libraries (RE22 and ATCC 19106) (Dorfmeier 2012c; 2012d). These proteins are directly involved in quorum sensing in a related species, *V. cholerae*. Many members of *Vibrio*, such as *V. harveyi*, possess homologous pathways of *V. cholerae*-like quorum sensing, suggesting that this pathway is genetically conserved within the genus (Zhu et al. 2002; Hammer and Bassler 2008). Quorum sensing and biofilm formation may be important functions in pathogenic strains of Vt.

Pilin production, Type II and Type III secretion systems

Sequencing revealed key virulence factors within the Vt genome, including pilin assemblies, type II and type III secretion systems (Dorfmeier 2012e; 2012f). Type IV pili homologous to *Aeromonas hydrophila* pilin (*tapB*, *tapC*), toxin coregulated pilus biosynthesis protein I, and a *V. cholerae* type IV pilin assembly (*pilC*) were seen in one or both genomes (Dorfmeier 2012e; 2012f). Type IV pili are involved in cell adhesion to host tissue, a necessary step involved in most bacterial pathogenesis. The type IV pilus assembly shares homology to the Type II secretion system, a significant metabolic pathway involved in vibrio pathogenesis, although different pilin sequences may form different adherence structures and different invasion capabilities into host epithelial cells (Finlay and Falkow 1997). Similarly, the toxin coregulated pilus biosynthesis protein I

homolog seen in both sequenced strains, is directly associated with *V. cholerae* colonization of gut epithelia (Harkey et al. 1994).

Homologous Type III secretion system genes were discovered in RE22 including: a probable ATP synthase, *yscN* and *yscR* (Dorfmeier 2012e). Gram-negative bacteria secrete a number of proteins for a variety of functions including generation of adhesion and motility, nutrient uptake, and virulence (Hueck 1998). Six secretion systems have been identified that mediate protein transport through inner and outer membranes. In particular, the Type III secretion apparatus in Gram-negative bacteria is used to transfer virulence proteins from the bacterium into the cytosol of eukaryotic cells (Hueck 1998). Translocated proteins facilitate pathogenesis by interfering with host cell signal transduction and cellular immune responses.

Toxins: RTX and hemolysin

Pathogens, such as Vt, encounter host tissue barriers that inhibit bacterial colonization, such as extracellular matrices, epidermal layers, and viscera. Bacterial proteases may target these protein structures and proteolysis may assist in soft tissue necrosis to aid in bacterial colonization. Homologous genes of putative virulence discovered within the Vt genome include an RTX-I toxin translocation ATP-binding protein, cholera toxin transcriptional activator, and hemolysins (Dorfmeier 2012e; 2012f). The RTX-I toxin translocation ATP-binding protein found in RE22 (Dorfmeier 2012e) is homologous to the protein in *Actinobacillus pleuropneumoniae* (*Haemophilus pleuropneumoniae*). RTX (repeat in toxin), a large multifunctional bacterial toxin that induces depolymerization of actin stress fibers through actin cross-linking, weakening host epithelial cells, may aid in bacterial colonization of the host gut (Sheahan et al. 2004). RTX is an important virulence factor for other bacterial pathogens including *V. vulnificus*, *V. cholera* and *Salmonella enterica* SpvB (Lui et al. 2009; Aktories et al. 2011).

Other homologous *Vibrio* hemolysins were also discovered including a hemolysin secretion protein, hemolysin VIIY, and hemolysin secretion protein (Dorfmeier 2012f). Extracellular products, including cytolytic toxins such as hemolysin, cause lysis of red blood cells *in vitro* and are an important factor in pathogenesis of disease caused by multiple pathogenic bacteria (Nomura et al. 1988; Rodriguez et al. 1992). Hemolysin production in *Vibrio* spp. is reported to influence pathogenesis of disease in fish (Nomura et al. 1988; Rodriguez et al. 1992) and cytolytic toxicity was postulated to be a factor in

Vibrio virulence to shellfish (Kothary et al. 2001). Toxicity of hemolysin to bivalve larvae was relatively unknown until recent examination of putative virulence factors in Vt determined that hemolysin production did not influence larval mortality in supernatant toxicity experiments with Pacific oyster (*Crassostrea gigas*) larvae (Hasegawa et al. 2008; Hasegawa and Hase 2009), although hemolysin is speculated to contribute to the overall pathogenicity of the bacterium (Hasegawa et al. 2008; Hasegawa and Hase 2009a).

Protein sequence analysis: metalloprotease and ToxR transcriptional activator

Of the known putative Vt virulence factors, the metalloprotease gene, *vtpA*, and its transcriptional regulator, VtpA, has been the subject of recent research (Hasegawa et al. 2008; Hasegawa and Hase 2009a; Hasegawa and Hase 2009b). Extracellular protease activity of this toxin degrades host tissue and enhances bacterial colonization (Maeda et al. 1996). Two metalloprotease proteins, an extracellular zinc metalloprotease and M6 metalloprotease, found in both Vt strains (Dorfmeier 2012e; 2012f) were examined for amino acid variations to investigate differences between the two strains.

The M6 protein family found in the Vt genome has been found in various species of environmental bacteria including *Vibrio*, *Shewanella*, *Clostridium*, *Geobacillus* and *Bacillus*, suggesting that there might be a role for this type of protease in bacterial environmental persistence, survival, and virulence (Rawlings et al. 2006; Vaitkevicius et al 2008). One domain within the amino acid sequence of the M6 metalloprotease, peptidase M6 super family contains a homolog *V. cholerae* immune inhibitor A, PrtV. This immune inhibitor can degrade antimicrobial peptides from host hemolymph and plays an important in *V. cholera* pathogenesis (Vaitkevicius et al 2008). Variations between the newly sequenced strains and the reference strains were similar although slightly more variations were observed in RE22 (RE22 n=9; ATCC 19106 n=5) (Fig. 8).

Examination of the extracellular zinc metalloprotease protein showed high dissimilarity in amino acid sequence of Vt strain RE22 (n=72) in contrast to that of ATCC 19106 (n=2). The active sites of the peptidase M4 family domain were especially dissimilar. A number of the enzymes included in this domain - thermolysin, protealysin, aureolysin, and neutral protease endopeptidases - are linked to virulence of several pathogenic bacterial species, including *V. cholerae*, *Helicobacter pylori*, and *Clostridium perfringens* (Booth et al. 1983; Smith et al. 1994; Jin et al. 1996). The enzymes in the M4 family have a two-domain structure containing an active site and zinc binding site.

The N-terminal contains the HEXXH zinc-binding motif and the helical C-terminal domain carries a third zinc ligand (Adekoys and Sylte 2009). RE22 shows 3 amino acid changes within the zinc binding sites of the domain, while ATCC 19106 has one (Fig. 9).

The virulence factor cholera toxin transcriptional activator homolog of the human pathogen *V. cholerae* (Provenzano et al. 2001) was also seen in both Vt genomes (Dorfmeier 2012e; 2012f). Contigs containing the cholera toxin transcriptional activator homolog from both libraries shared sequence similarity to the ToxR transcriptional activator amino acid sequence found in *V. tubiashii* NCIMB 1337. ToxR, a major regulator of pathogenicity in *Vibrio* spp. (Beauburn et al. 2009), was first discovered as the positive transcriptional regulator of the cholera toxin, CTX (Miller and Mekalanos 1984) and an important virulence factor in pathogenic strains of *V. cholerae* (Bhadra et al. 1995). Protein sequences revealed large variation in RE22 (77 variations) within the 241 bp protein sequence compared to the type strain ATCC 19106 (5 variations) (Fig. 10). Amino acid sequence comparisons did not capture sequence coding for the response regulator effector domain (NCBI accession cd00383) found in *V. cholerae* ToxR transcriptional activator protein sequence. Thus, it is unknown if any changes in RE22 sequence could be indicative of functional differences within this conserved domain.

The high amount of genotypic variation in RE22 the extracellular zinc metalloprotease and ToxR transcriptional activator proteins may contribute to differential virulence among Vt strains, but further characterization is needed to determine if the sequence variation seen in RE22 when compared to ATCC 19106 can be linked to functional differences between strains. Future studies should investigate proteolytic and cytolytic activity differences. Furthermore, comparative genomic examination of multiple Vt strains of varying pathogenicity (Estes et al. 2004) would elucidate the genetic factors that contribute to virulence and possibly identify patterns of genetic variation in the major bacterial virulence factors discussed here.

Conclusions

In summary, genomic analyses reveal novel information on Vt biology and provide critical resources for future research efforts. Both libraries sequenced here share multiple genes including proteases, pilin production, cholera toxin transcriptional activator, and quorum-sensing proteins associated with pathogenesis of other vibrio pathogens such as *V. cholerae* and *V. vulnificus*. RE22, the highly pathogenic strain,

contains multiple homologous proteins of putative virulence associated with other bacterial pathogens including *Salmonella*, *Shigella* and *E. coli*. These proteins include RTX-I translocation ATP-binding protein, and type III secretion system genes, *yscN* and *yscR*, that may play roles in pathogenesis to invertebrate hosts by helping to establish bacterial colonies, aid in bacterial proliferation, and produce toxins. Both strains possess homologous metalloprotease and hemolysin proteins, quorum sensing systems, and antibiotic resistance proteins homologous to other *Vibrio* spp. *In silico* protein analysis of major virulence factors indicate specific regions of significant sequence dissimilarity that are likely associated with physiological differences. Further genetic and biochemical studies are needed to elucidate how these variations may impact functional and metabolic pathways of the strain, including response to changing environmental conditions.

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Figures

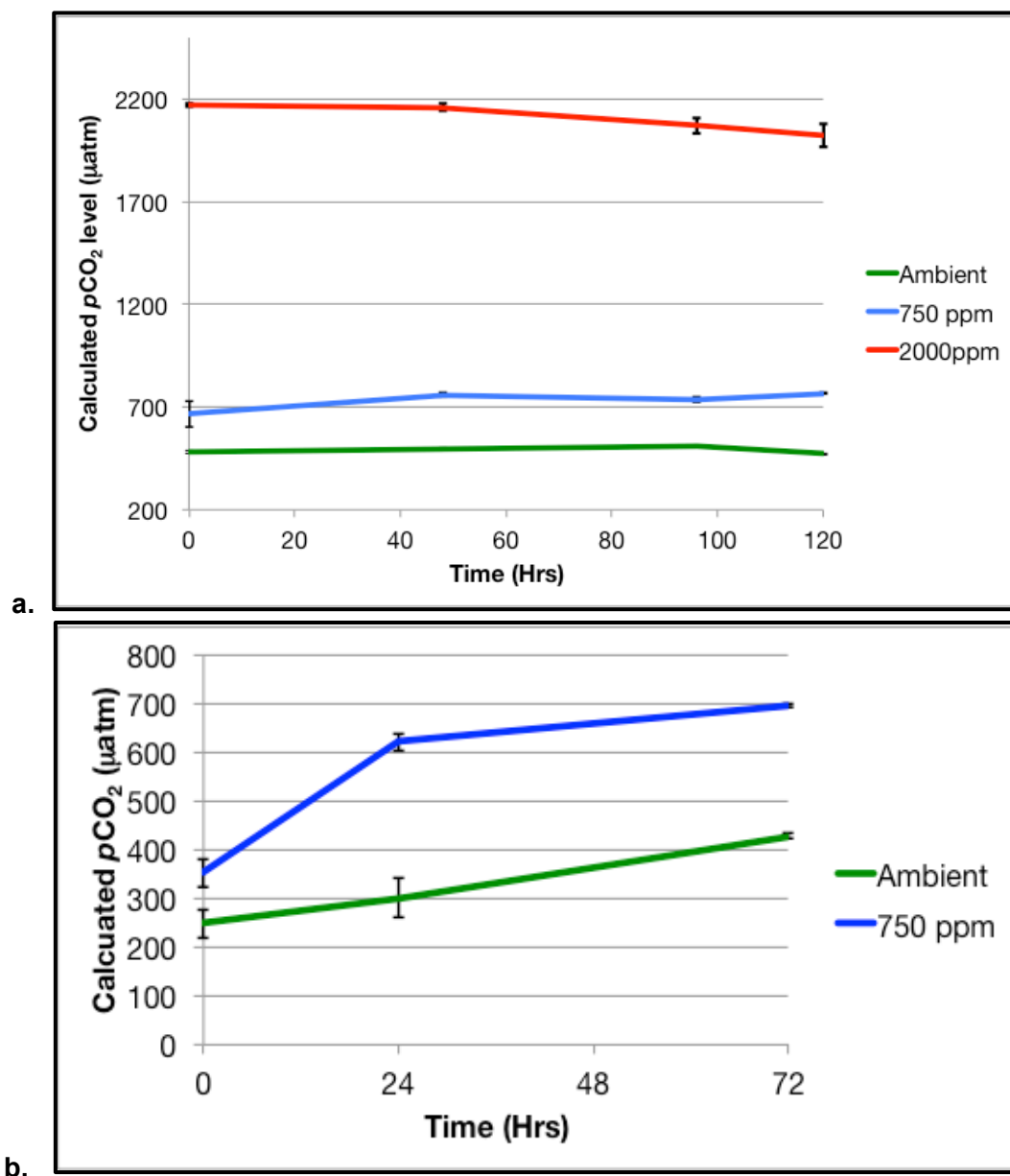


Figure 1a and 1b. Calculated $p\text{CO}_2$ concentrations during *V. tubiashii* growth trials at 16°C (a) and 25°C (b). Error bars represent ± 1 SE.

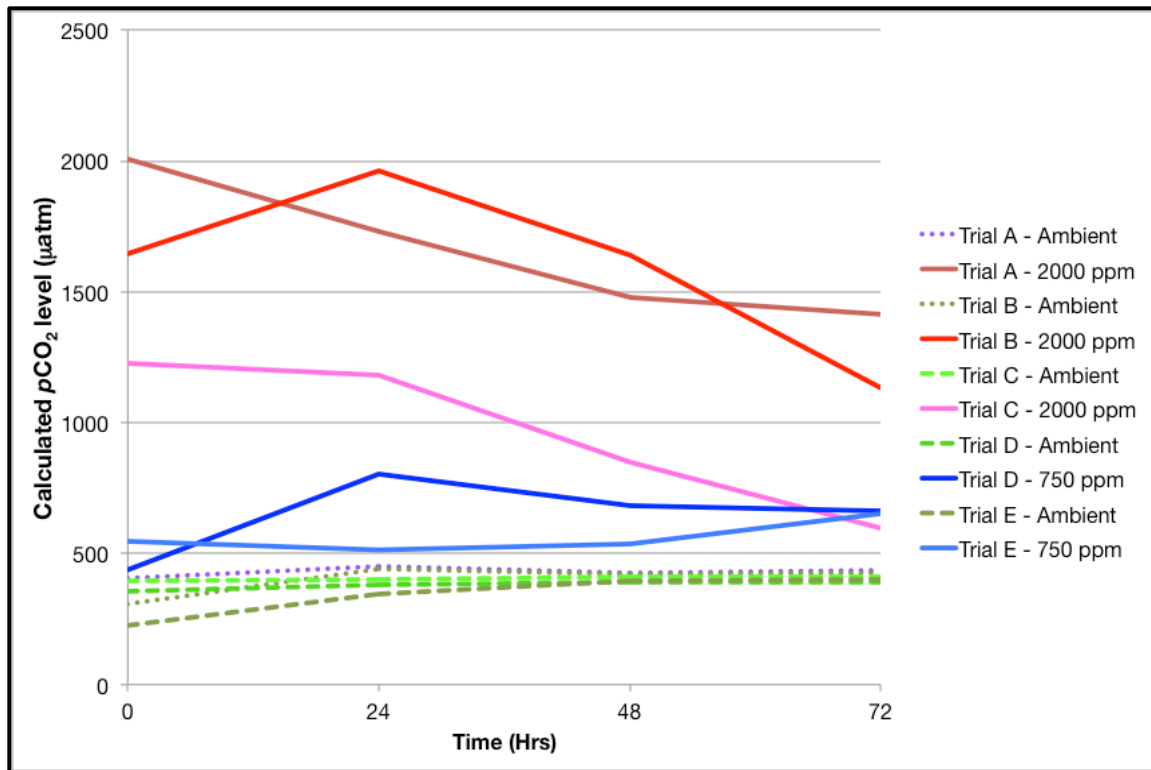


Figure 2. Calculated $p\text{CO}_2$ concentrations of *C. gigas* disease trials.

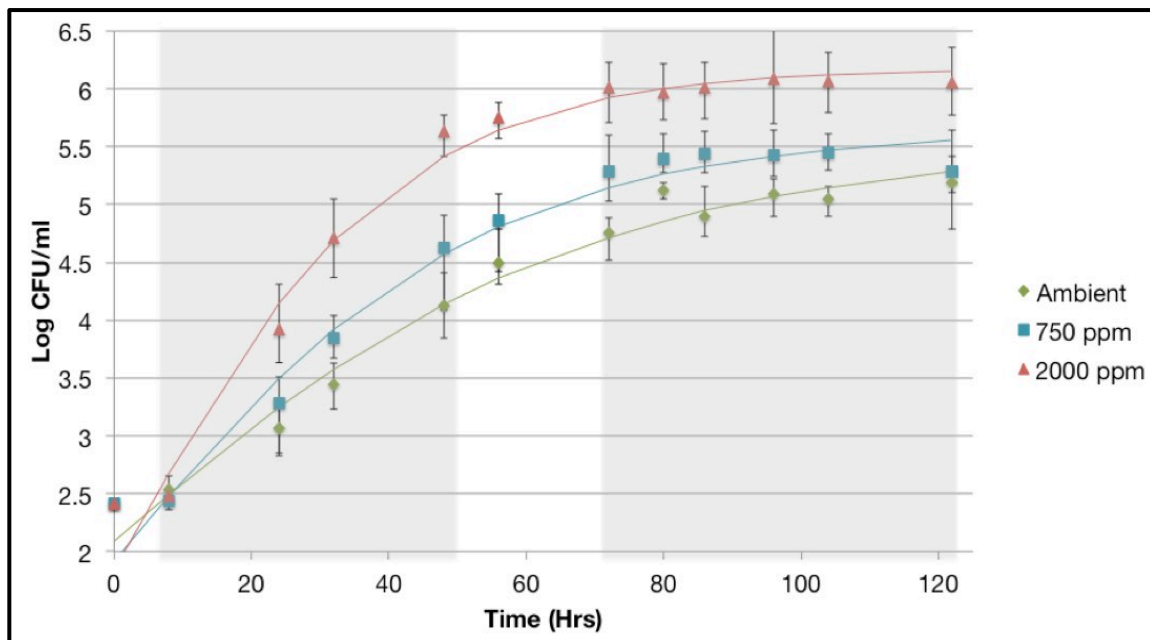


Figure 3. Growth of *Vt* at 16°C. *Vt* cultures were grown at three $p\text{CO}_2$ concentrations: ambient (approx. 390), 750 and 2000 ppm. Error bars represent \pm 95% CI. Gompertz growth curve was used for predicted values in regression line. Shaded areas represent time points used to test for differences in exponential and stationary phase growth.

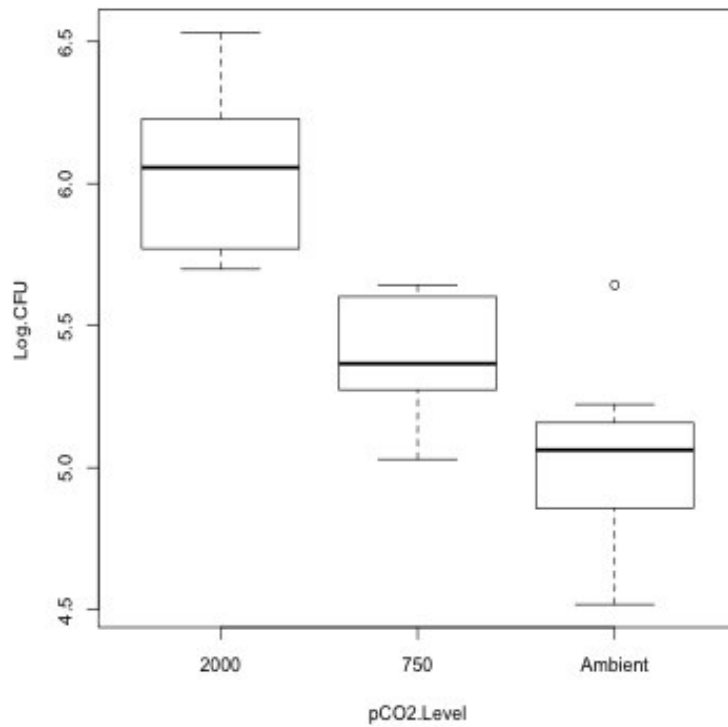


Figure 4. Box plot of stationary phase Vt growth during 72 – 122 hrs of growth at 16°C under three $p\text{CO}_2$ levels. X-axis represents $p\text{CO}_2$ level (ambient (approx. 390), 750, and 2000 $p\text{CO}_2$); Y-axis represents log CFU/ml of Vt.

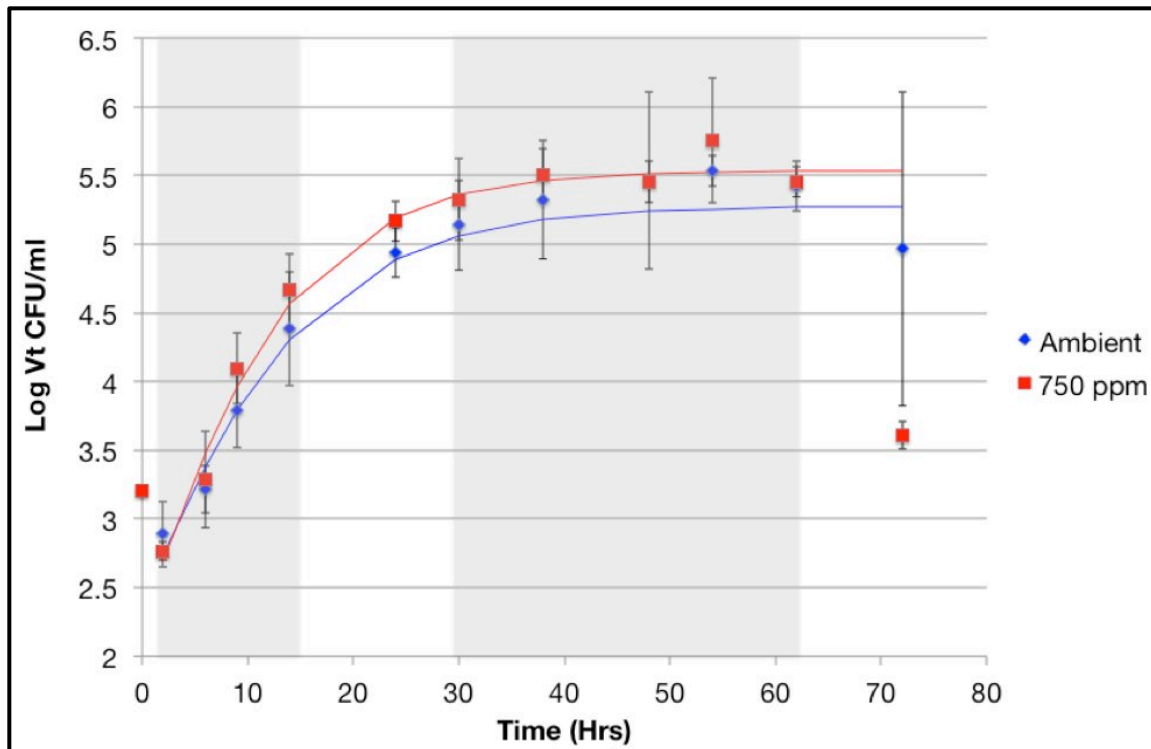


Figure 5. Growth of *Vt* at 25°C. *Vt* cultures were grown at two $p\text{CO}_2$ concentrations: ambient (approx. 390) and 750 ppm. Error bars represent \pm 95% CI. Gompertz growth curve was used for predicted values in regression line. Shaded areas represent time points used to test for differences in exponential and stationary phase growth.

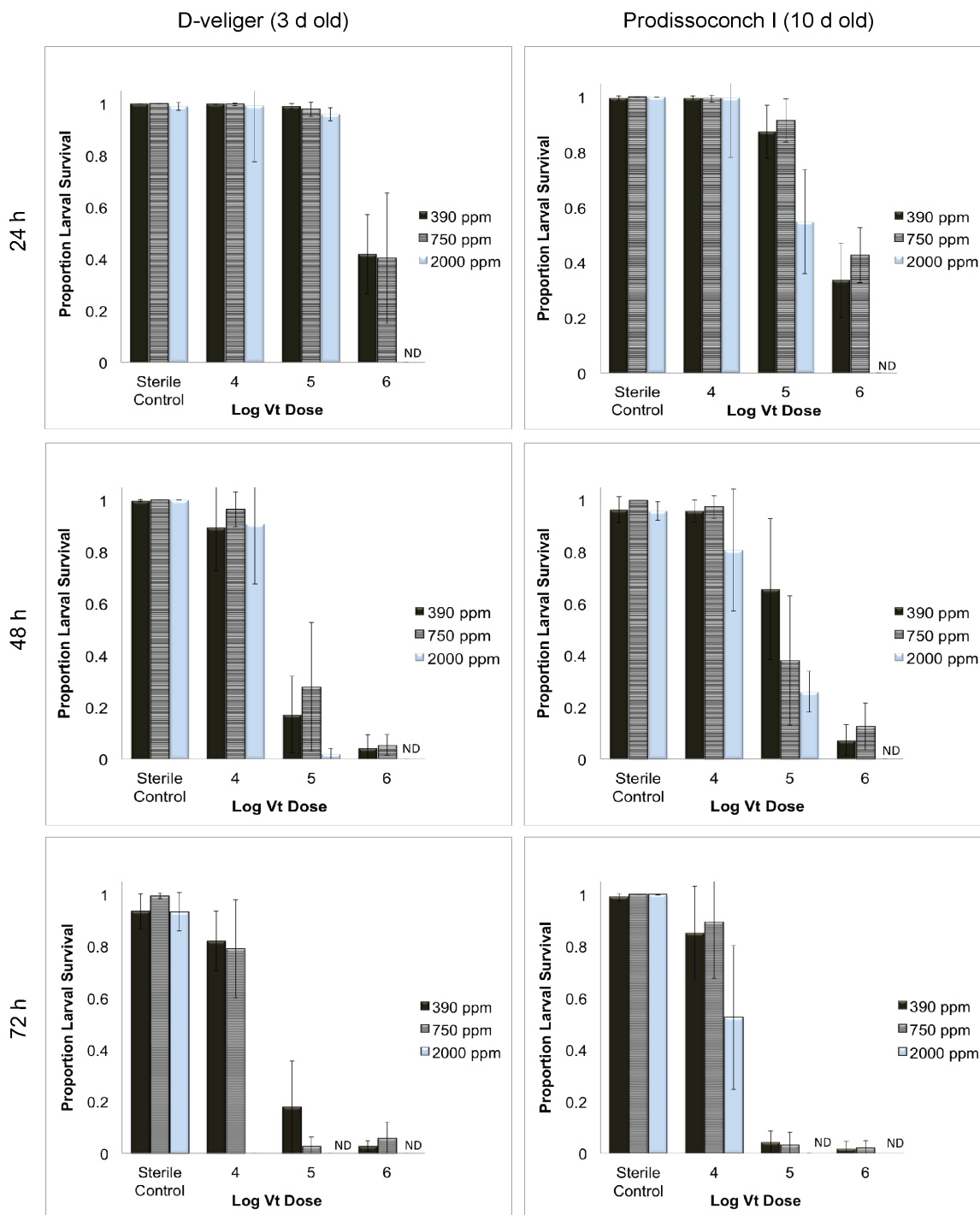


Figure 6. Survival of early D-veliger stage and prodissococonch I stage *C. gigas* larvae when exposed to three $p\text{CO}_2$ levels over 72 h ($p >> 0.05$). X-axis represents log *V. tubiashii* abundance and Y-axis represents proportion of larval survival. Error bars represent 95% CI. ND = not done

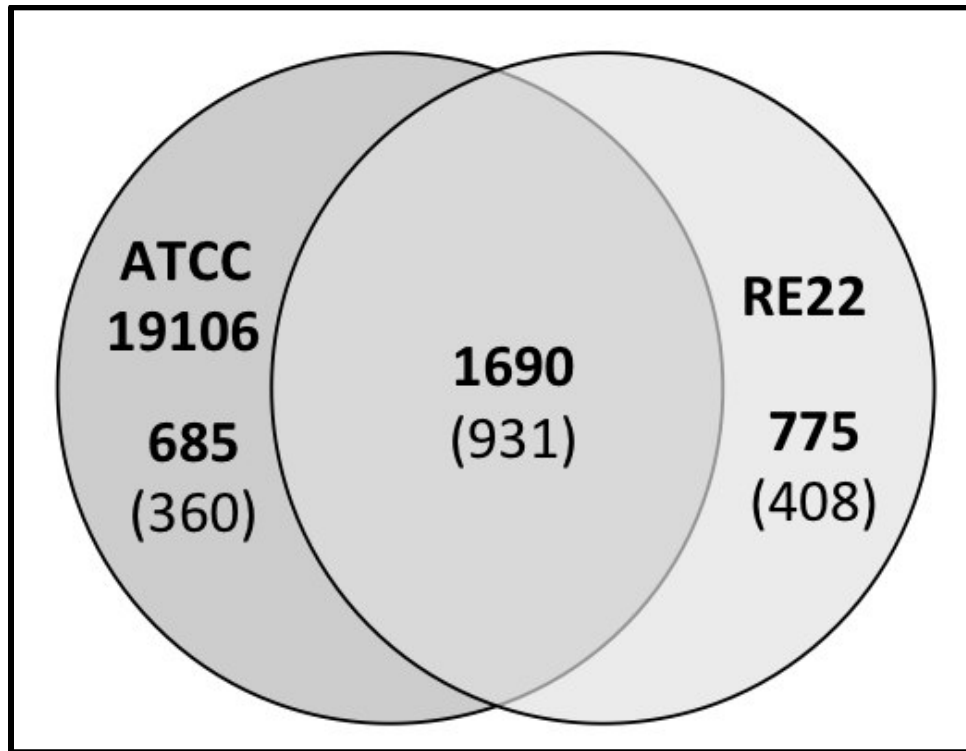


Figure 7. Venn diagram of unique and shared annotated genes between *V. tubiashii* strains ATCC 19106 and RE22 with e-values $\leq 1e-05$. The diagram represents all genes annotated from respective *de novo* assemblies. Bold numbers represent the total numbers of annotated genes either unique or shared between both libraries. Annotated genes with putative virulence are denoted in parentheses.

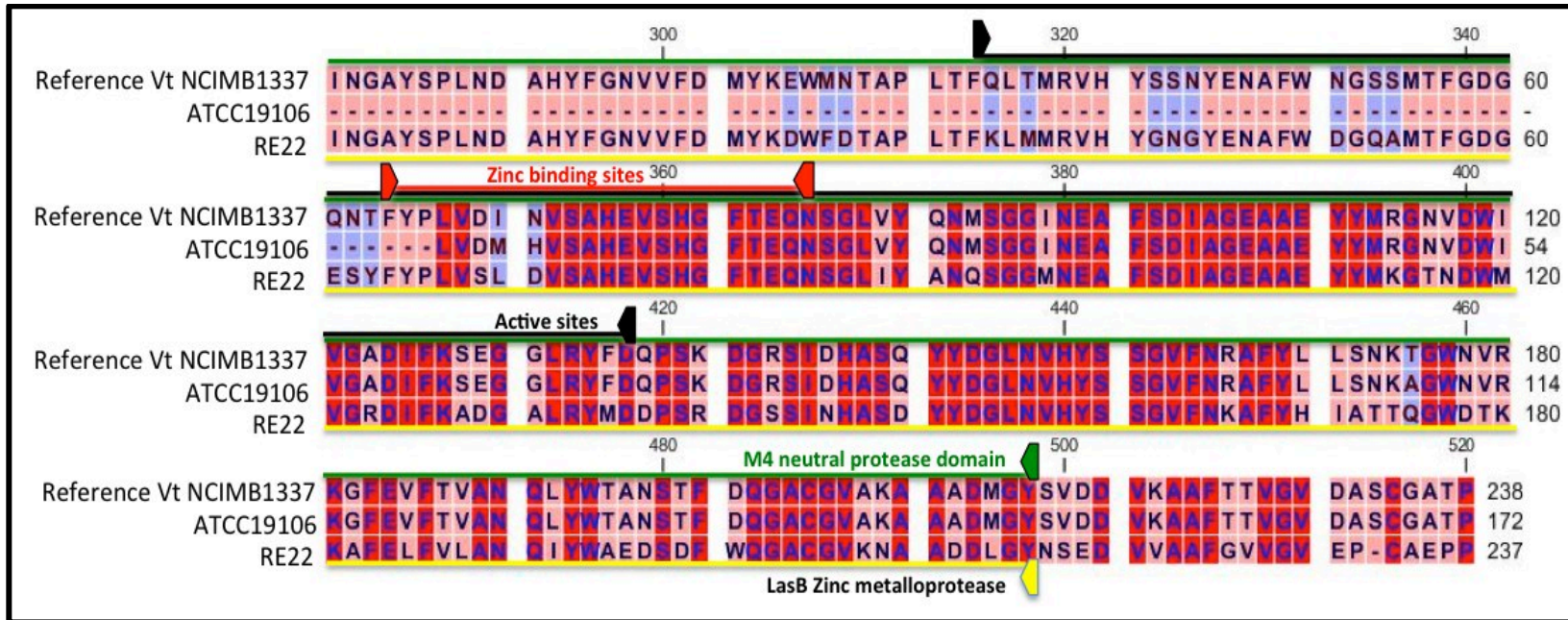


Figure 9. Extracellular zinc metalloprotease protein alignment. Yellow arrows note regions of the conserved domain LasB (Zinc metalloprotease (elastase) (COG3227) ($1.55e-121$ e-value). Green arrows note conserved domain for peptidase M4 family neutral protease (cd09597) ($3.57e-92$ e-value). Black and red arrows mark regions of the active sites and zinc binding sites of the M4 family neutral protease domain. Dashes in sequence indicate areas of the strain that do not contain sequence information.



Figure 10. ToxR transcriptional activator protein alignment. Dashes in sequence indicate areas of the strain that do not contain sequence information.

Tables

Table 1. List of vibrio pathogens associated with recent disease outbreaks of molluscan larvae.

Pathogenic species	Origin / Strain	Host*	Life stage	Reference
<i>V. alginolyticus</i>	Culture collection	<i>M. galloprovincialis</i>	Larvae	Beltrán et al. 2004
<i>Vibrio sp.</i>	Mortality outbreak	<i>C. gigas</i> ; <i>O. edulis</i>	Larvae	Estes et al. 2004
<i>V. splendidus</i> biovar II	Mortality outbreaks	<i>R. decussatus</i>	Larvae	Gómez-León et al. 2005
<i>V. alginolyticus</i>	Mortality outbreaks	<i>R. decussatus</i>	Larvae	Gómez-León et al. 2005
<i>V. neptunius</i> ; <i>Vibrio sp.</i>	Mortality outbreaks	<i>O. edulis</i>	Larvae	Prado et al. 2005
<i>V. pectenecida</i> ; <i>V. splendidus</i> -like	Mortality outbreaks	<i>P. maximus</i>	Larvae	Sandlund et al. 2006
<i>Vibrio sp.</i>	Pacific oyster	<i>C. virginica</i>	Larvae	Gómez- León et al. 2008
<i>V. tubiashii</i>	Pacific oyster	<i>C. gigas</i>	Larvae	Elston et al. 2008
<i>V. tapetis</i>	Symptomatic clams	<i>R. philippinarum</i> ; <i>R. decussatus</i>	Adults	Allam et al. 2001; 2002; 2006a and 2006b
<i>V. splendidus</i>	Moribund oysters	<i>C. gigas</i>	Juveniles	Lacoste et al. 2001
<i>V. splendidus</i> biovar II (<i>V. chagasii</i>)	Mortality outbreak	<i>C. gigas</i>	Juveniles	Waechter et al. 2002
<i>V. splendidus</i> -like	Moribund/ healthy oysters	<i>C. gigas</i> , <i>R. philippinarum</i>	Adults, seed	Gay et al. 2004
<i>V. aestuarianus</i>	Mortality outbreak, Diseased/ healthy oysters	<i>C. gigas</i>	Adults	Labreuche et al. 2006a; 2006b
<i>V. aestuarianus</i> subsp. <i>francensis</i>	Mortality outbreak	<i>C. gigas</i>	Adults	Garnier et al. 2008
<i>Vibrio sp.</i>	Moribund/ healthy oysters	<i>C. gigas</i> , <i>C. virginica</i>	Juveniles	Gay et al. 2004; Gómez- León et al. 2008

*Abbreviations: C = Crassostrea, M = Mytilus, O = Ostrea, P= Pecten, R = Ruditapes
Table modified from Romalde and Barja 2010

Table 2. List of species in the *Vibrionaceae* family that have been sequenced to date and/or contain homologs to TetR transcriptional regulators, metalloprotease, and/or hemolysin proteins available in the National Center for Biotechnology Information database.

Species	Genome Sequenced	Metalloprotease	TetR	Hemolysin
<i>Vibrio aestuarianus</i>		X		
<i>Vibrio agarivorans</i>				
<i>Vibrio albensis</i>				
<i>Vibrio alginolyticus</i>	X	X	X	
<i>Vibrio alginolyticus</i> subsp. <i>iophagus</i>				
<i>Vibrio anguillarum</i>		X		
<i>Vibrio areninigrae</i>				
<i>Vibrio artabrorum</i>				
<i>Vibrio atlanticus</i>				
<i>Vibrio atypicus</i>				
<i>Vibrio azureus</i>				
<i>Vibrio brasiliensis</i>	X			
<i>Vibrio breoganii</i>				
<i>Vibrio calviensis</i>				
<i>Vibrio campbellii</i>	X		X	
<i>Vibrio caribbenthicus</i>	X	X		
<i>Vibrio carchariae</i>				
<i>Vibrio casei</i>				
<i>Vibrio celticus</i>				
<i>Vibrio chagasii</i>				
<i>Vibrio cholerae</i>	X	X	X	X
<i>Vibrio cincinnatiensis</i>				
<i>Vibrio comitans</i>				
<i>Vibrio communis</i>				
<i>Vibrio coralliilyticus</i>	X	X		
<i>Vibrio costicola</i>				
<i>Vibrio crassostreae</i>				
<i>Vibrio cyclitrophicus</i>	X			
<i>Vibrio damsela</i>				
<i>Vibrio diabolicus</i>				
<i>Vibrio diazotrophicus</i>				
<i>Vibrio ezurae</i>				
<i>Vibrio fischeri</i>		X		
<i>Vibrio fluvialis</i>		X		
<i>Vibrio fortis</i>				
<i>Vibrio furnissii</i>	X	X		
<i>Vibrio gallaecicus</i>				
<i>Vibrio gallicus</i>				

Table 2. Continued.

Species	Genome Sequenced	Metalloprotease	TetR	Hemolysin
<i>Vibrio gazogenes</i>				
<i>Vibrio gigantis</i>				
<i>Vibrio haliotocoli</i>				
<i>Vibrio hangzhouensis</i>				
<i>Vibrio harveyi</i>	X	X	X	X
<i>Vibrio hepatarius</i>				
<i>Vibrio hippocampi</i>				
<i>Vibrio hispanicus</i>				
<i>Vibrio hollisae</i>				
<i>Vibrio ichthyenteri</i>	X	X		
<i>Vibrio iliopiscarius</i>				
<i>Vibrio inusitatus</i>				
<i>Vibrio kanaloae</i>				
<i>Vibrio lentus</i>				
<i>Vibrio littoralis</i>				
<i>Vibrio logei</i>				
<i>Vibrio mangrovi</i>				
<i>Vibrio marinus</i>				
<i>Vibrio marisflavi</i>				
<i>Vibrio maritimus</i>				
<i>Vibrio mediterranei</i>				
<i>Vibrio metschnikovii</i>	X			
<i>Vibrio mimicus</i>	X	X		
<i>Vibrio mytili</i>				
<i>Vibrio natriegens</i>				
<i>Vibrio navarrensis</i>	X			
<i>Vibrio neonatus</i>				
<i>Vibrio neptunius</i>				
<i>Vibrio nereis</i>				
<i>Vibrio nigripulchritudo</i>	X			
<i>Vibrio ordalii</i>	X			
<i>Vibrio orientalis</i>	X			
<i>Vibrio owensii</i>				
<i>Vibrio pacinii</i>				
<i>Vibrio parahaemolyticus</i>	X	X	X	X
<i>Vibrio pectenicida</i>				
<i>Vibrio pelagius</i>				
<i>Vibrio penaeicida</i>				

Table 2. Continued.

Species	Genome Sequenced	Metalloprotease	TetR	Hemolysin
<i>Vibrio plantisponsor</i>				
<i>Vibrio pomeroyi</i>				
<i>Vibrio ponticus</i>				
<i>Vibrio porteresiae</i>				
<i>Vibrio proteolyticus</i>		X		
<i>Vibrio rarus</i>				
<i>Vibrio rhizosphaerae</i>				
<i>Vibrio rotiferianus</i>	X			
<i>Vibrio ruber</i>				
<i>Vibrio rumoiensis</i>				
<i>Vibrio sagamiensis</i>				
<i>Vibrio salmonicida</i>				
<i>Vibrio scophthalmi</i>	X			
<i>Vibrio shilonii</i>	X			
<i>Vibrio sinaloensis</i>	X			
<i>Vibrio splendidus</i>	X	X	X	
<i>Vibrio stylophorae</i>				
<i>Vibrio succinogenes</i>				
<i>Vibrio superstes</i>				
<i>Vibrio tapetis</i>				
<i>Vibrio tasmaniensis</i>				
<i>Vibrio trachuri</i>				
<i>Vibrio tubiashii</i>	X	X	X	X
<i>Vibrio variabilis</i>				
<i>Vibrio viscosus</i>				
<i>Vibrio vulnificus</i>	X	X	X	X
<i>Vibrio wodanis</i>				
<i>Vibrio xuii</i>				
<i>Vibrio sp. RC341</i>	X			
<i>Vibrio sp. Ex25</i>	X		X	X
<i>Vibrio sp. RC586</i>	X			
<i>Vibrio sp. MV-1</i>	X			
<i>Vibrio sp. AND4</i>	X			
<i>Vibrio sp. HV10</i>	X			
<i>Vibrio sp. MED222</i>	X	X		
<i>Vibrio sp. EJV3</i>	X			
<i>Vibrio sp. N418</i>	X			

Table 3. Trial data summary of larval *C. gigas* disease experiments.

Trial	pCO₂ treatment (ppm)	D-Veliger	Prodissoconch I veliger
A	2000	X	X
B	2000	X	X
C	2000	X	X
D	750	X	X
E	750	X	X

Table 4. Seawater chemistry summaries for each trial performed: larval disease trials (top) and *V. tubaishii* growth trials (bottom). Dissolved inorganic carbon, $p\text{CO}_2$, and saturation states were calculated from spectrophotometric pH values, salinity, and total alkalinity. Confidence interval values represent the lower (5%) and upper limits (95%). Spect pH = spectrophotometric pH measurement; TA = total alkalinity; DIC = dissolved inorganic carbon; Ω_{arg} = aragonite saturation state; Ω_{cal} = calcite saturation state

Disease Trials									
Trial	Treatment (ppm)	Average Spect pH	5% CI Spect pH	95% CI Spect pH	Average $p\text{CO}_2$	5% CI $p\text{CO}_2$	95% CI $p\text{CO}_2$	Salinity	TA
A	2000	7.34	7.29	7.39	1658.94	1448.21	1938.43	30.50	1966.85
A	Ambient	7.86	7.85	7.88	430.99	415.84	449.76	32.20	2080.42
B	2000	7.40	7.35	7.49	1595.66	1262.43	1881.86	30.38	2137.42
B	Ambient	7.91	7.88	7.98	400.17	365.56	437.75	32.11	2195.19
C	2000	7.58	7.48	7.68	963.82	722.74	1204.90	32.00	2104.05
C	Ambient	7.89	7.89	7.90	403.47	398.29	409.79	32.00	2086.51
D	750	7.73	7.67	7.83	647.81	555.58	776.16	30.83	2096.62
D	Ambient	7.87	7.86	7.89	380.42	365.03	391.89	29.49	1913.76
E	750	7.76	7.72	7.79	562.71	525.05	626.99	32.00	2034.17
E	Ambient	7.96	7.90	8.06	342.02	284.86	400.53	32.00	2069.07

Trial	Treatment (ppm)	Average DIC	5% CI DIC	95% CI DIC	Average Ω_{arg}	5% CI Ω_{arg}	95% CI Ω_{arg}	Average Ω_{cal}	5% CI Ω_{cal}	95% CI Ω_{cal}
A	2000	1974.12	1960.09	1992.33	0.56	0.50	0.62	0.88	0.78	0.99
A	Ambient	1908.53	1902.32	1914.07	1.94	1.88	2.00	3.02	2.94	3.13
B	2000	2127.31	2100.50	2148.75	0.71	0.62	0.87	1.12	0.98	1.37
B	Ambient	1994.06	1965.85	2011.22	2.27	2.11	2.56	3.55	3.30	4.00
C	2000	2033.66	1999.58	2067.32	1.10	0.92	1.44	1.72	1.43	2.25
C	Ambient	1904.47	1902.42	1906.95	2.04	2.01	2.06	3.18	3.16	3.23
D	750	1955.86	1925.93	1981.72	1.45	1.26	1.78	2.28	1.98	2.79
D	Ambient	1761.74	1755.10	1765.99	1.72	1.69	1.78	2.71	2.65	2.80
E	750	1906.66	1898.58	1921.42	1.52	1.41	1.61	2.38	2.19	2.51
E	Ambient	1857.14	1814.25	1888.30	2.32	2.07	2.79	3.62	3.17	4.36

<i>V. tubaishii</i> Growth Trials											
Growth Trial (ppm)	Average Spect pH	5% CI Spect pH	95% CI Spect pH	Average $p\text{CO}_2$	5% CI $p\text{CO}_2$	95% CI $p\text{CO}_2$	Salinity	TA	Average DIC	5% CI DIC	95% CI DIC
Ambient	7.82	7.81	7.83	488.68	477.28	503.81	30.75	2052.85	1908.73	1905.38	1913.10
750	7.66	7.64	7.68	733.26	691.20	764.14	30.87	2008.30	1921.37	1914.11	1926.58
2000	7.26	7.24	7.27	2106.10	2057.22	2168.81	32.02	2067.75	2096.93	2094.07	2100.57

Table 5. Calculated *V. tubiashii* LD₅₀ values for two developmental stages of *C. gigas* larvae at 24, 48, and 72 h.

		D-veliger larvae (3 days old)					
		Day 1		Day 2		Day 3	
pCO₂		LD₅₀	SE	LD₅₀	SE	LD₅₀	SE
~390		8.49E+05	1.35	1.58E+05	1.70	2.28E+04	1.66
750		8.05E+05	1.37	9.69E+04	1.51	2.09E+04	1.53
2000		5.39E+05	1.84	3.52E+05	3.51	7.20E+03	2.06
		Prodissoconch I larvae (10 days old)					
		Day 1		Day 2		Day 3	
pCO₂		LD₅₀	SE	LD₅₀	SE	LD₅₀	SE
~390		6.16E+05	1.55	6.16E+05	1.55	2.35E+04	1.31
750		7.79E+05	1.59	6.26E+04	1.46	1.98E+04	1.53
2000		3.77E+05	1.76	5.83E+04	1.43	1.86E+04	1.48

Table 6. *De novo* assembly properties of *V. tubiashii* libraries RE22 and ATCC 19106.
bp = basepairs

<i>V. tubiashii</i> Strain	RE22
Assembly	<i>de novo</i>
Reads	56,024,538
Matched	44,869,510
% Mapped Reads	80%
Contigs	6,588
Average bp/contig	713
Average coverage/contig	384x

<i>V. tubiashii</i> Strain	ATCC 19106
Assembly	<i>de novo</i>
Reads	49,217,456
Matched	39,480,379
% Mapped Reads	80%
Contigs	6,098
Average bp/contig	737
Average coverage/contig	350x

Table 7. Summary of genes identified in *V. tubiashii* strain ATCC 19106 and strain RE22 genomic libraries based on Gene Ontology terms.

GO Category		ATCC 19106	RE22
		(Annotated gene count)	
Cellular Component	cytoskeleton	6	7
	cytosol	1	0
	extracellular matrix	1	0
	non-structural extracellular	82	102
	plasma membrane	1133	1141
	translational apparatus	34	38
Molecular Function	enzyme regulator activity	0	1
	kinase activity	186	219
	nucleic acid binding activity	560	615
	signal transduction activity	98	108
	transcription regulatory activity	10	14
	transporter activity	75	83
Biological Process	cell adhesion	4	0
	cell cycle and proliferation	62	71
	cell organization and biogenesis	182	191
	cell-cell signaling	1	0
	death	41	54
	developmental processes	2	0
	DNA metabolism	226	222
	protein metabolism	139	137
	RNA metabolism	793	875
	signal transduction	238	273
	stress response	235	233
	transport	1491	1491

Table 8. Summary table of genes with putative bacterial virulence by gene ontology descriptions in *V. tubiashii* strains ATCC 19106 and RE22.

GO Category		ATCC19106	RE22
		(Annotated gene count)	
Cellular Component	cell outer membrane	106	118
	cell wall	6	5
	cyanelle	1	0
	extracellular region	75	87
	membrane	1194	1198
	periplasmic space	110	127
Molecular Function	ATP binding	718	755
	protein binding	5	1
	protein tyrosine kinase activity	1	2
	zinc ion binding	206	206
Biological Process	alginate biosynthetic process	3	0
	cell adhesion	4	0
	cell wall organization	66	68
	coenzyme A biosynthetic process	1	3
	cytolysis	41	53
	diaminopimelate biosynthetic process	9	13
	DNA repair	87	88
	lipid A biosynthetic process	10	12
	lipopolysaccharide biosynthetic process	20	29
	pathogenesis	48	48
	peptidoglycan biosynthetic process	44	47
	phosphate transport	13	17
	polysaccharide transport	1	3
	response to toxin	3	3
	rhamnose metabolic	1	2
	teichoic acid biosynthetic process	0	1
	transport	860	849