Potential Mechanisms in the Pathogenesis of Virulent *Aeromonas hydrophila* in Channel Catfish, *Ictalurus punctatus*

by

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Abstract

Aeromonas hydrophila are Gram-negative bacteria ubiquitous in freshwater ecosystems. *A. hydrophila* are opportunistic pathogens with a broad host range that includes mammals, fish, reptiles, amphibians, and invertebrates. In Southeastern catfish production systems, *A. hydrophila* are common opportunistic pathogens causing muscle necrosis and, less often, septicemia, with outbreaks occurring following primary immune insult. In 2009, outbreaks of motile aeromonad septicemia (MAS) of epidemic proportions occurred in west Alabama catfish production systems resulting in significant mortalities, with some ponds exceeding 60% mortality of market-size catfish. The etiologic agent, a hypervirulent *A. hydrophila*, referred to herein as vAh, is capable of causing disease as a primary pathogen. Since 2009, vAh-induced MAS (vMAS) outbreaks in west Alabama and east Mississippi catfish productions systems have resulted in $60 - $70 million dollar in losses. Because no prerequisite environmental conditions have been identified, management practices have failed to limit or prevent vMAS outbreaks. In an attempt to elucidate mechanisms of vAh pathogenesis, a comparison of vAh proteins secreted under two primary ecological niches – biofilm and planktonic growth - was undertaken. To further understand how growth niche could influence pathogenicity, gene expression of putative virulence factors was compared. Lastly, to determine the importance of these secreted proteins in vAh virulence, a secretion-deficient mutant was created by recombineering, and functional screening of mutant and wild-type secretomes were performed *in vitro*, and *in vivo* challenges were performed in channel catfish. The results of this work show that vAh niche occupation significantly influences secretion of degradative and toxigenic proteins. Furthermore, gene expression is intimately tied to niche occupation, with 35% of all genes differentially expressed

based on growth condition. Biofilm transcriptomes revealed upregulation of multiple putative virulence factor classes that, while required for biofilm production and maintenance, could act secondarily to increase host colonization and invasiveness. Toxin genes were upregulated in planktonic transcriptomes, suggesting that planktonic growth may more closely mimic bacterial growth *in vivo* and may prime vAh to cause disease within a host. Finally, functional screening of a T2SS mutant revealed complete loss of degradative and hemolytic ability in a secretion-deficient mutant *in vitro* as well as complete attenuation of virulence *in vivo*. Whole-pathway T2SS complementation completely restored proteolytic and hemolytic functionality *in vitro* and restored *in vivo* virulence, demonstrating that T2SS is the primary secretory pathway for degradative and hemolytic proteins in vAh, and that a functional T2SS is required for vAh virulence. These results established that T2SS and the T2S effectors play a vital role in the pathogenicity of vAh, and provide convincing evidence for the role of toxins in the development of vMAS. The research presented herein underscores the importance of considering pond ecology and natural bacterial niche ecology in the study of bacterial pathogenicity and virulence.

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This dissertation is dedicated to the memory of my parents, Jimmy and Martha Jiles, who taught me the value of hard work and, above all, that cultivating kindness, love, and compassion are lifelong endeavors.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

vAh	Virulent *Aeromonas hydrophila*
MAS	Motile aeromonad septicemia
vMAS	vAh-induced MAS
T2SS	Type II secretion system
ECP	Extracellular protein
PVF	Putative virulence factor
DEG	Differentially expressed gene
FC	Fold change
LFC	Log_2 fold change

Chapter I

Introduction and Review of Literature

1. Introduction

Aeromonas hydrophila is a ubiquitous Gram-negative bacterium capable of inhabiting a wide range of environments and acting as an opportunistic pathogen in fish, reptiles, amphibians, and mammals. In channel catfish production systems, *A. hydrophila* outbreaks in fish are common in spring and summer, particularly in Southeastern pond systems. Traditionally, *A. hydrophila* has been considered a secondary pathogen with low morbidity and mortality. However, in 2009 a novel, highly-virulent strain of *A. hydrophila* was responsible for epidemic outbreaks of peracute motile aeromonad septicemia (MAS) in Alabama catfish production ponds. The bacterium continues to cause significant losses in the Southeastern catfish industry. This epidemic strain, referred to as virulent *Aeromonas hydrophila* (vAh), shows significant genetic variation from the traditional *A. hydrophila* and is capable of producing disease as the primary pathogen, resulting in rapid and devastating mortality in catfish production ponds. Whole genome sequence comparisons of virulent and traditional *A. hydrophila* revealed significant genetic variation and *in vivo* challenges in a channel catfish (*Ictalurus punctatus*) model have confirmed the increased virulence of the epidemic strain. However, little is known about the mechanisms of pathogenicity that are responsible for the isolate's hypervirulence. The research proposed herein will attempt to 1) determine how ecological niche adaptation in the bacterial environment influence the production and secretion of potentially toxigenic extracellular proteins, 2) compare gene expression of putative virulence factors produced by vAh when cultured planktonically and within a biofilm, and 3) determine the importance of the type II

secretion system in the secretion of potential toxins and other virulence factors. The goal of the proposed research is to develop an understanding of the environmental stimuli that precede host invasion, the mechanisms of pathogenicity that lead to increased virulence, and the factors responsible for peracute mortality in virulent *A. hydrophila* infections.

A. hydrophila are capable of surviving in a variety of environments and have the metabolic capacity to invade a wide range of hosts (Beaz-Hidalgo and Figueras 2013). They also contain a broad range of virulence factors that could be related to pathogenicity. The primary step of infection is host colonization. *Aeromonas* spp. produce a number of adhesins, both filamentous and non-filamentous, that could promote initial attachment to fish skin or gill tissue. Flagella, both polar and lateral, are commonly used in host adhesion and biofilm formation, and quorum sensing which likely occurs in *Aeromonas* spp biofilms has been found to be responsible for increased production of extracellular proteases and hemolysins. These extracellular proteins, or ECPs, are another potential source of virulence of *A. hydrophila*. Pridgeon et al (2013) reported 228 ECPs in the supernatant of vAh isolates, 23 of which were believed to function as virulence proteins. vAh also contain a Type II secretion system (T2SS), which has been reported to secrete common toxins such as aerolysins, amylases, DNases, and proteases from Aeromonads. In *A. hydrophila*, T2SS is constitutively expressed. Constitutive expression of the T2SS genes is also seen in *Vibrio cholerae* and is likely due to the need to secrete substrates in multiple environments (Sandkvist, 2001). Constitutive expression of secretion genes (*exe* genes in *A. hydrophila*) requires tightly regulated expression of ECP-associated genes. Therefore, extracellular secretion via T2SS will generally occur when bacteria have reached a density threshold and the secreted proteins will be those that increase survival in the specific location of the bacteria.

Likely, ECP production is regulated by environmental conditions (temperature, nutrient availability, etc) and is specific to the ecological niche occupied at a specific time. However, only vAh ECPs produced in broth culture have been studied. Because most bacteria in natural environments are generally maintained in biofilms, studying the secreted proteome (secretome) of vAh growing in a biofilm, as well as planktonic cells, may shed light on the role that ecological niche adaptations play in vAh pathogenicity, and may pinpoint proteins that play vital roles in attachment, adhesion, and colonization. Environmental regulation of bacterial genes means genes responsible for the epidemic nature of vAh infections may only be transcribed under specific growth conditions. To further investigate how ecological niche could influence vAh virulence, gene expression of planktonic and biofilm-associated vAh, focusing on previously described putative virulence genes, will provide a snapshot into the role putative virulence factors (PVFs) play in bacterial niche partitioning and host virulence. Rapid mortality in fish challenge models (3-5 hours IP, 6-8 hours immersion) with minimal lesions present in tissues suggests toxin-mediated killing. If secreted proteins are vital for host invasion and mortality, secretion-null mutants would be avirulent. To determine the role secreted proteins play in the pathogenicity and virulence of vAh and to establish the mode of protein secretion, T2SS-deficient mutants will be constructed by homologous recombination. Secreted protein profiles will be examined for protein content and functional proteolytic and toxin screenings will be performed. Whole-pathway complementation will be performed and complemented mutants will be screened for restored function. Channel catfish will then be challenged by wild-type vAh and secretion-deficient and complemented mutants to determine influence of T2SS and its effectors on *in vivo* virulence.

This dissertation describes the influence of environmental niche adaptation on the secretion of degradative and toxigenic proteins of vAh, and on the gene expression of putative virulence factors that likely contribute to strain hypervirulence. Furthermore, this research identified a pivotal role of Type II secretion in the pathogenicity of vAh, and provides further support for the hypothesis that vMAS is a toxin-mediated disease.

2. Review of Literature

2.1 Life history of *Aeromonas hydrophila*

The genus *Aeromonas* is currently comprised of 32 species of rod-shaped, Gram-negative, oxidase-positive, facultative anaerobes capable of glucose fermentation, moderately halotolerant, and capable of growth from 0°C to 42°C (Abbott, Cheung, & Janda, 2003). In the mid-1980s, based on molecular characterization, the genus was recharacterized from the family *Vibrionaceae* to *Aeromonadaceae*. The genus *Aeromonas* is divided into two major groups, motile, mesophilic species and non-motile, psychrotrophic species. Psychrotrophic species, such as the economically important *A. salmonicida*, generally cause disease only in fish, while mesophilic species are opportunistic pathogens with a broad host range (Austin & Adams, 1996; Gosling, 1996a; Janda & Abbott, 2010).

Mesophilic aeromonads have a global distribution and are almost ubiquitous in aquatic environments. Mesophilic aeromonads have been isolated from fresh water, estuarine waters, surface waters, including recreational waters, drinking water, including chlorinated, well, and bottled water. Aeromonads are also pollution tolerant and have been isolated from polluted waters and wastewater effluent sludge. While not considered marine organisms, aeromonads can be isolated from brackish waters. Mesophilic *Aeromonas* spp. are habitat generalists and *r*-strategists and can grow to large numbers in nutrient rich waters with peak growth generally

occurring in the warm temperatures accompanying summer months in freshwater lakes (Holmes, 1996). Mesophilic *Aeromonas* spp. can cause mild to severe cutaneous wound infections in humans, typically occurring in extremities following traumatic aquatic injuries. These infections can range in seriousness from mild cellulitis to severe, with some cases of near-fatal necrotizing fasciitis resulting in severe disfiguration and limb amputations. The four species most commonly associated with wound infections are *A. hydrophila*, *A. veronii*, *A. schubertii*, and *A. dhakensis.* Human diarrheal diseases are the most common manifestation of *Aeromonas* contracted through environmental exposure, with Traveler's diarrhea likely the most common presentation in the U.S. (Igbinosa, Igumbor, Aghdasi, Tom, & Okoh, 2012). The use of medicinal leeches has also been linked to cases of serious *Aeromonas* spp. wound infections and sepsis. *Aeromonas* spp. reside in the leech gut as mutualistic symbionts, assisting in the hemolysis of blood ingested by the leech, and can be transmitted to humans through medicinal leech therapy (Sartor et al., 2002; Verriere et al., 2016).

Aeromonas hydrophila is a wide-spread and diverse species of mesophilic *Aeromonas* ubiquitous in freshwater aquatic ecosystems. *A. hydrophila* can be isolated from almost every environmental niche, with the exception of the most extreme environments (Holmes, 1996; Janda & Abbott, 2010). *A. hydrophila* are routinely isolated from fresh and estuarine systems, municipal and bottled drinking water, raw seafood, meat, poultry, milk, and fresh fruits and vegetables (Grim et al., 2013; Igbinosa et al., 2012; Janda & Abbott, 2010) and can tolerate wide ranges in saline concentrations and temperature (Jahid, Mizan, Ha, & Ha, 2015; Rahman, Suzuki, & Kawai, 2001b). They are resistant to chlorine treatment and some produce β-lactamases, which break down penicillin and cephalosporin antibiotics (Janda & Abbott, 2010). *A. hydrophila* is an extremely metabolically diverse species, with different strains displaying a

wide variety of carbohydrate utilization profiles, the capacity for dissimilatory sulfate reduction, and possible bioremediation of contaminants and wastewater (Ogugbue & Sawidis, 2011; Sandkvist, 2001b). *Aeromonas hydrophila* are generally considered opportunistic pathogens with a broad host range, causing disease in frogs, snakes, alligators, and mammals, and has been proposed as an emerging pathogen due to the increase of human-related cases of necrotizing fasciitis and isolation of antibiotic-resistant strains (Gosling, 1996a; Grim et al., 2014; Grim et al., 2013; Igbinosa et al., 2012; Janda & Abbott, 2010).

2.2 *Aeromonas* infections in catfish production systems

In aquaculture, *A. hydrophila* is an important cause of disease in most freshwater production systems. In catfish production systems, *A. hydrophila* is an important secondary pathogen, commonly responsible for cutaneous ulceration and muscle necrosis and, occasionally, motile aeromonad septicemia (MAS), which can result in high mortalities (Austin & Adams, 1996; Beaz-Hidalgo & Figueras, 2013; Camus, Durborow, Hemstreet, Thune, & Hawke, 1998; Griffin et al., 2013). These *A. hydrophila* infections, referred to herein as traditional *A. hydrophila*, are generally considered stress-induced, with outbreaks common following extensive handling, cutaneous injury, high water temperature, low dissolved oxygen, and other poor water quality conditions common in intensive fish culture ponds (Beaz-Hidalgo & Figueras, 2013; Camus et al., 1998; Cipriano, 2001). *A. hydrophila* are also considered part of the normal intestinal microbiome of healthy fish, cattle, and other livestock (Cipriano, 2001; Grim et al., 2013; Janda & Abbott, 2010), emphasizing the diverse heterogeneity that exists within the species.

Aeromonas hydrophila, along with *A. sobria* and *A. caviae*, make up a triad of motile aeromomads that commonly cause a complex of diseases referred to as motile aeromonas

infection (Austin & Adams, 1996; Camus et al., 1998; Gosling, 1996a). Aeromonad infections, specifically those where *A. hydrophila* is the etiologic agent, commonly present in one of three ways. The most common manifestation in traditional strains of *A. hydrophila* is acute onset of ulcerative lesions that progress to severe muscular necrosis. With this cutaneous form of disease, mortality rates are low, and fish may survive for extended periods of time despite the presence of severe ulcerative lesions (Camus et al., 1998; Cipriano, 2001; Gosling, 1996a). A second, more severe form of infection is an acute form of motile aeromonad septicemia, or MAS. MAS infections occur when bacteria enter the bloodstream and are rapidly disseminated. In traditional *A. hydrophila* strains, fish with this disease manifestation present with a grossly distended abdomen and exopthalmia, and will swim weakly near the water's surface. Internally, significant liver and kidney necrosis is present (Camus et al., 1998; Cipriano, 2001). Acute MAS can result in significant mortalities in stocked ponds. The third and most severe presentation is a peracute form of septicemia. In this form, fatal septicemia occurs so rapidly that few or no clinical symptoms are seen internally or externally on gross evaluation and minimal histologic lesions are present in liver, kidney, and spleen on necropsy (Camus et al., 1998; Cipriano, 2001). Extremely high mortalities are often reported with peracute MAS. Despite the three distinct presentations of motile aeromonad infections, it is not uncommon for all manifestations of *A. hydrophila* infections to be referred to motile aeromonad septicemia (MAS). This reference is misleading. It is important to distinguish between the types of *A. hydrophila* infections, as each presentation is likely influenced by the mechanism(s) of pathogenicity which may be attributed to the strain's virulence (Gosling, 1996b; Tomas, 2012).

2.3 Virulent *Aeromonas hydrophila*

In 2009, a new, highly virulent strain of *A. hydrophila* was isolated from a diseased channel catfish, *Ictalurus punctatus*, within a production pond in West Alabama. This strain, referred to as virulent *Aeromonas hydrophila*, or vAh, was responsible for outbreaks of peracute motile aeromonad septicemia of epidemic proportions (Griffin et al., 2013; Hossain et al., 2014; Hossain et al., 2013; Pridgeon & Klesius, 2011; Pridgeon et al., 2013; Tekedar et al., 2013). vAh appears to act as a primary pathogen, and may not be preceded by immune insult (Griffin et al., 2013). vAh-induced MAS (vMAS) is responsible for the loss of over 3 million pounds of market-size catfish each year (Abdelhamed, Ibrahim, Nho, et al., 2017), resulting in the loss of 30 million pounds of marketable channel catfish from production farms in West Alabama since its appearance in 2009. Since the initial isolation, vAh has been isolated from ponds in Alabama, Mississippi, and Arkansas (Griffin et al., 2013), three of the four leading producers of catfish in the US. Further spread of vAh among these states could result in devastating losses in US catfish production. Furthermore, potential spread of vAh to tilapia or other cage-reared fish could have far reaching impacts on aquaculture in the Southeast.

2.4 Phenotypic and genotypic variation in virulent *Aeromonas hydrophila*

To date, several differences have been identified between vAh and traditional *A. hydrophila* (tAh). Unlike tAh, vAh causes disease as the primary pathogen, with no notable stress factors preceding vAh outbreaks (Abdelhamed, Ibrahim, Baumgartner, Lawrence, & Karsi, 2017; Baumgartner, Ford, & Hanson, 2017; Griffin et al., 2013). Another important difference between vAh and tAh is the predominant disease seen with each strain. While tAh generally presents as cutaneous muscle necrosis in a few fish, vAh outbreaks are peracutely septicemic in nature and result in mass mortalities throughout ponds (Griffin et al., 2013). In aquarium

challenges using a channel catfish model, vAh was found to be more virulent than tAh, with a substantial reduction in the infective dose causing significant mortality when injected intraperitoneally (Hossain et al., 2014; Pridgeon & Klesius, 2011). Pridgeon and Klesius (2011) reported an infective dose of $2.8x10^7$ cfu/fish for tAh, but only $1.3x10^5$ cfu/fish for vAh when challenged by IP injection.

At necropsy, differences also exist between tAh and vAh-induced MAS mortalities. Baumgartner et al. (2017) studied 22 fish from 15 natural pond infections of vMAS, with pond mortalities ranging from 10% to 90%. Grossly, external signs of disease, including generalized petechiation, dermal necrosis, and exopthalmia are present in both MAS and vMAS fish, to varying degrees. However, signs of endomeningitis with necrotizing cellulitis, similar to the hole-in-the-head presentation of *Edwardsiella ictaluri* infections, was present in some vMAS cases. Internally, few vAh bacteria were present in spleens of vMAS fish, but the presence of vascular necrosis and hemorrhage suggests a toxin-mediated necrosis may be occurring. When bacteria were present in decomposing splenic tissue, they were present in high numbers, leading the researchers to deduce that vAh thrive in anaerobic tissues. Significantly, severe gastric hemorrhage and edema, which are not typical in MAS, were present in vMAS cases. Furthermore, intestines, liver and trunk kidney, which are common targets for *A. hydrophila* were not inflamed in vMAS. In catfish challenged with vAh by intraperitoneal injection (IP), morbidity is extremely rapid, with death occurring in three to five hours. In these infections, very few, if any, lesions are present grossly, and minimal histologic changes are seen in tissues (Newton, personal communication). These findings further support the hypothesis that vMAS is a toxin-mediated disease.

Whole genome comparison between vAh and tAh isolates also show significant genetic variability (Hossain et al., 2013; Tekedar et al., 2013), including the presence of multiple transposons, unique prophage elements, unique O-antigen structure, and an inositol utilization pathway in the genome of vAh. While tAh strains are genetically variable, vAh are clonal in nature, sharing 99% homology and a close relation to a Chinese isolate, ZC1, which was isolated from a diseased grass carp (Hossain et al., 2014). This suggests that vAh may have been introduced to the US from fish imported from China. Interestingly, U.S. vAh isolates are more virulent to catfish and carp when compared to the Chinese isolate. Hossain et al. (2013) identified 313 predicted genes present in vAh strains that were absent from tAh strains. Of these genes, 35% were present on genomic islands likely acquired through lateral genetic transfer. The presence of an inositol utilization pathway allows vAh catabolism of myo-inositol, which is present in high amounts within catfish tissue and fish feed, as a sole carbon source. This could offer vAh a competitive advantage over tAh, which is unable to utilize myo-inositol, and enable vAh to exploit a previously unavailable environmental niche. Pilus biogenesis clusters were present on two of the genomic islands. Pili are common colonization factors and may contribute to vAh invasiveness. The presence of a unique O-antigen structure may protect vAh from complement-mediated lysis within the host and help to evade host immune cells, thus increasing the likelihood of vAh colonization within catfish. Recent research found that LPS O-antigen was not required for vAh virulence, but modification of the O-antigen capsule resulted in attenuation of virulence in a catfish model. Furthermore, challenge with the capsular mutant resulted in a strong adaptive immune response in catfish, perhaps due to the exposure of strongly-antigenic outer membrane proteins (Thurlow, Hossain, et al., 2019). However, the virulence mechanisms of *A. hydrophila* are not well understood and it is unclear what genetic variation contributes to

the pathogenicity of vAh. The increased virulence of vAh is likely multifactorial, and may include increased production of potential virulence factors. *A. hydrophila* possess multiple virulence factors, including toxigenic extracellular proteins (ECPs), adhesins, iron acquisition mechanisms, quorum sensing, and secretion systems (Bales, Renke, May, Shen, & Nelson, 2013; Beaz-Hidalgo & Figueras, 2013; Carvalho-Castro et al., 2010; Castro et al., 2014; Gavin et al., 2003; Janda & Abbott, 2010; Khajanchi et al., 2009; Tomas, 2012). In a catfish challenge model, intraperitoneal and intramuscular injection of vAh causes rapid death, usually within 3-5 hours, with minimal significant histological lesions present in tissues (personal communication, Joseph Newton), suggesting a toxin-mediated killing may occur during vAh infections. Histology of naturally-infected vMAS fish also revealed histologic lesions with toxic signature (Baumgartner et al., 2017). This hypothesis is supported by research by Pridgeon (2011, 2013), who reported the presence of multiple potentially toxigenic extracellular proteins in the supernatant of vAh cultured *in vitro* and that injection of vAh ECP fractions into channel catfish fingerlings resulted in death. To understand the increased virulence and the role of toxins in vAh infections, study of the modes of pathogenicity are necessary.

The hypervirulence of vAh is an enigma. Though vastly more pathogenic than traditional vAh in catfish models, virulence factors often considered in direct correlation with pathogenicity, such as Type III and Type VI secretion systems, are absent from U.S. isolates (Tekedar et al., 2019). Lateral flagella, which have been reported as of paramount importance for *A. hydrophila* adhesion and invasion into multiple cell lines, are also absent from vAh (Gavin et al., 2003; Gavin et al., 2002; Lowry, Balboa, Parker, & Shaw, 2014). Furthermore, intraperitoneal laboratory challenges and natural pond infections result in severe and, often, peracute vMAS with extremely high mortality. However, aquarium challenge models by immersion require

scarification of the catfish by removal of the adipose fin followed by exposure to unnaturally high CFUs in order to cause disease (Zhang et al., 2017) and, even using this technique, reproducibility has been elusive (Abdelhamed, Ibrahim, Nho, et al., 2017; Peatman et al., 2018). In this way, vAh behaves more likes its opportunistic relatives than the devastating primary pathogen that wreaks havoc *in natura*. The suggestion that vAh infections must be preceded by scarification to create a route of entry for vAh, as has been suggested (Zhang, Moreira, Shoemaker, Newton, & Xu, 2016; Zhang, Xu, & Shoemaker, 2016), is questionable. While scarification likely serves as a mode of entry for traditional *A. hydrophila*, as outbreaks often accompany handling and seining, tAh infections typically occur in small numbers of fish with much lower morbidity. It seems unlikely that 60% - 100% of all fish in a production pond would acquire an entry wound and would subsequently become infected with vAh and perish in a 48 hour span. When fish are scarified or challenged with vAh by IP injection, major, and arguably the most important, hurdles of natural infection - adhesion and colonization - are bypassed (Martínez, 2013).

A. hydrophila-induced MAS in catfish has long been considered a multivariate disease, and *A. hydrophila* do not lack for virulence determinants (Canals et al., 2006; Gavin et al., 2002; Romero et al., 2016; Tomas, 2012; Vilches et al., 2004; Yu et al., 2004; Yu et al., 2005). Yet, MAS outbreaks are sporadic and short-lived, suggesting that *A. hydrophila* exists successfully in non-host aquatic environs. Virulence factors are defined as genetic attributes that increase the possibility of causing disease in a host and their presence is often used to categorize an isolates' potential for pathogenicity. However, presence of these putative virulence genes in the genome do not guarantee that an organism will cause disease in any or all potential hosts (Diard & Hardt, 2017). It is all too easy, when focusing on the obvious presence of virulence determinants in an

organism to overlook the life history of that organism in nature, and how evolutionary adaptations and ecological response effect pathogenicity. For example, to date, all challenge experiments using vAh have used cells cultured planktonically in nutrient rich media, typically Tryptic Soy Broth (TSB). This culture method is simple and reproducible, and data generated in these studies have contributed greatly to our understanding of vAh pathogenicity. However, it is imperative to understand bacterial life history, and how the organism's role as a resident in the natural environment may contribute to its overall pathogenicity and virulence.

2.5 Bacterial Generalism, Plasticity, and the Evolution of Virulence

Stanley Falkow, the father of molecular microbial pathogenesis, said, "the goal of a bacterium is to become bacteria". For bacterial generalists, this idiom is particularly true. Bacterial generalists are organisms with the ability to metabolize multiple sources of organic carbon and utilize a broad ecological niche (Brown, Cornforth, & Mideo, 2012; Kassen, 2002; Mou, Sun, Edwards, Hodson, & Moran, 2008) and are generally *r*-strategists; organisms that undergo rapid growth in response to favorable ecological inputs (Andrews & Harris, 1986). Understanding generalism and microbial phenotypic plasticity can increase understanding of how opportunists become pathogens, when the shift from opportunism to pathogenesis occurs, and how to impede plastic responses to regulate virulence (Brown et al., 2012).

Bacterial gene expression is intimately linked to environmental conditions and there is relevance in considering the role of niche-dependent adaptations in virulence. For example, a change in temperature triggers the transcription of a host of genes that allow the organism to adapt to this new condition (Herbst et al., 2009; Hurme & Rhen, 1998; Konkel & Tilly, 2000). This is particularly important for pathogens who are subject to significant temperature increase when invading a mammalian host but may be of lesser importance for pathogens of

poikilotherms. However, there are a host of environmental stimuli that may alter gene expression that leads to bacterial adaption. This ability of an organism to rapidly alter gene expression to maximize fitness is referred to as adaptive phenotypic plasticity (Brown et al., 2012; Leff & Lemke, 1998) and it is a hallmark of *r*-strategists and opportunistic pathogens (Weinbauer & Hofle, 1998). Plasticity is vital for survival in chaotic environments, allowing bacteria to adapt rapidly to take advantage of nutrient pulses or colonize a new niche, as well as increasing bacterial survival in hostile environments through mechanisms such as biofilm formation and sporulation (Brown et al., 2012; Maughan, Birky, & Nicholson, 2009; Prosser et al., 2007; Weinbauer & Hofle, 1998).

In the environment, bacterial survival relies on the ability to express genes whose products will maximize fitness regardless of environmental condition. A universal theme in ecology, and pond ecology in particular, is the concept of spatial and temporal environmental heterogeneity (Strayer, Ewing, & Bigelow, 2003). Variations in temperature, light, nutrient availability, dissolved oxygen, wave action, turbidity, and substrate vary horizontally as well as vertically – not seasonally or daily, but hourly. Larger aquatic fauna – fish, crustaceans, and macroinvertebrates – are less sensitive to microenvironmental fluctuations, as their environment is The Pond (Pinel-Alloul & Ghadouani, 2007). Bacterioplankton, on the other hand, must quickly adapt to each microenvironmental upheaval in order to maximize fitness, and rely on instantaneous genomic response to adapt. Thus, plasticity is often seen as expression of metabolic pathways, secretion of adhesins, flagella, extracellular enzymes and their secretion pathways, iron acquisition systems, and quorum sensing, all of which are proposed virulence factors for pathogenic bacteria (Beaz-Hidalgo & Figueras, 2013; Gosling, 1996b; Grim et al.,

2013; Howard, Macintyre, & Buckley, 1996; Lowry et al., 2014; Singh, Chaudhary, Mani, Jain, & Mishra, 2013).

While phenotypic plasticity allows bacterial survival in an ever-changing landscape, there are also trade-offs to maintaining this flexibility. Maintenance of the regulatory machinery required to control expression of genes that are responsive to both physical and social factors is energetically expensive. For example, *Pseudomonas aeruginosa* maintains at least 25 sigma factors that regulate expression of large numbers of genes in response to environmental stimuli (Schulz et al., 2015). However, the challenge of surviving in a heterogeneous environment selects for phenotypic plasticity, as the ability to adapt to environmental variation or to partition a new niche outweighs the cost of maintaining the machinery that regulates plastic response (Auld, Agrawal, & Relyea, 2010; Brown et al., 2012; Callahan, Maughan, & Steiner, 2008). Maintenance of these sigma factors allow *P. aeruginosa* to be exceptionally adaptable to environmental conditions and makes them opportunistic pathogens with a vast host range (Brown et al., 2012). The prevailing theoretical paradigm for the evolution of bacterial virulence states that virulence factors yield a net benefit during host exploitation because of positive effects on within-host growth and/or transmission (Ben-Ami & Routtu, 2013; Brown et al., 2012; Leggett, Buckling, Long, & Boots, 2013). In the case of *A. hydrophila*, a primarily environmental bacterial species, we must assume that the pond environment is the primary site of bacterial adaptation, and that selection from environmental pressures subsequently generate pre-adaptations that increase pathogencity to the secondary environment i.e., the host. So, although virulence factors confer selective advantage within the host, the evolution of the virulence factor was driven by selective pressures outside the host. This is sometimes referred to a coincidental virulence (Adiba, Nizak, van Baalen, Denamur, & Depaulis, 2010).

Ecology and evolution are intimately entwined, and any environmental change can influence the fitness of an organism and the evolution of its virulence. Too often, anthropogenic perturbations are responsible for the evolution of bacterial virulence. Misuse of antibiotics and intensive farming are two common causes of anthropogenic virulence evolution (Diard & Hardt, 2017). In the case of *A. hydrophila*, intensive farming practices may well play a role in the evolution of hypervirulent strains. Catfish aquaculture practices involve extremely high stocking rates, and high host density selects for virulence by facilitating host-to-host transfer. Furthermore, when hosts are abundant, host survival becomes irrelevant, and mortality as the endpoint of infection increases. Furthermore, the heavy feed load introduced to grow-out ponds contributes to pond eutrophication and supports vAh growth. *A. hydrophila* do not rely on a living host for replication and can gain nutrients from decaying fish and wasted fish feed, thus, host mortality is inconsequential. Peatman et al. (2018) reported anecdotal evidence that farmers consistently reported vAh outbreaks corresponded to heavy feeding, and found higher vAh mortality in catfish challenged post-prandially as compared to their fasted counterparts. This supports findings of severe gastric hemorrhage and stomach involvement seen in vMAS, which are not common lesions of tAh-induced MAS (Baumgartner et al., 2017). The link between feeding and vAh infection have yet to be elucidated. Perhaps the vast input of nitrate and phosphorus could result in a vAh growth boom. With bacterial counts exploding, feeding fish may take in large numbers of bacteria. Post-prandial increases in cortisol and stress-hormones may also decrease host immune response, and increases in digestion-related mucins could further trigger expression of vAh toxins in the gut. While amending feed with probiotics has shown some promise for reducing eutrophication and improving fish health (Kuebutornye, Abarike, & Lu, 2019; Thurlow, Williams, et al., 2019), these methods have largely not been adopted by

farmers. *A. hydrophila* exhibit extraordinary phenotypic plasticity and genetic diversity that allows them to occupy broad ecological roles, playing the roles of non-harmful environmental bacteria, opportunistic pathogens, secondary pathogens, commensal symbionts, and hypervirulent primary pathogens. Unfortunately, this makes understanding bacterial interaction in the environment difficult. Adaptation to a benign environment can result in reduced virulence when a virulence factor is not energetically favorable. This may explain the loss of T3SS and T6SS from US vAh isolates. It is curious, then, that a primary environmental bacterium which generally lives in peaceful coexistence, such as vAh, would retain a host of PVFs that appear to be of little relevance in a pond. However, plasticity allows virulence factors to be decoupled from growth in a benign niche, allowing host-specific virulence factors to be expressed only within the infective environment.

2.6 Biofilms

A method commonly employed by phytoplankton to mitigate niche disruption is the formation of a biofilm. Generalist bacteria within aquatic ecosystems, such as *A. hydrophila*, are capable of occupying three niches: 1) suspended in the water column as individual planktonic cells 2) within a biofilm attached to submerged plant, detritus, or sediment, and 3) within a host (De Schryver & Vadstein, 2014; Weinbauer & Hofle, 1998). Although fish and macroinvertebrates have been hypothesized as reservoirs for *A. hydrophila* (Leff & Lemke, 1998), it is unlikely that large numbers of vAh are maintained within a host, as vMAS outbreaks are sporadic and short-lived. Therefore, it stands to reason that vAh largely occupy one of the other two niches. What is unclear is the niche occupied at the time of host invasion and how the microenvironment of each particular niche impacts regulation of genes whose products allow for host invasion. As a general rule, bacteria that remain suspended in the water column are slow

growing K-strategists as the water column is nutrient poor (Weinbauer & Hofle, 1998). However, catfish production ponds are eutrophic, with high amounts of dissolved and suspended organic matter. In these ponds, artificially high nutrient loading occurs due to high feed input, excrement from heavily stocked fish, and nutrients from decaying fish (Camus et al., 1998; Cipriano, 2001; Pandey, Bharti, & Kumar, 2014). In these systems, *r* -selected bacteria may be capable of thriving as planktonic cells, even if planktonic residence is short-lived in response to nutrient pulses. The potential for vAh to be resident as planktonic cells has been supported by Zhang et al (Zhang, Xu, Shoemaker, & Beck, 2020), who reported commercial catfish feed supported vigorous planktonic growth *in vitro*. In addition, bacteria disrupted from their niche or translocating to a new niche will remain suspended in the water column for a limited time and host invasion at this point could be beneficial. While some bacteria may remain as individual planktonic cells in the water column, most free-living *r*-strategists in aquatic systems reside primarily in biofilms (Balcázar, Subirats, & Borrego, 2015; Bales et al., 2013; Hall-Stoodley, Costerton, & Stoodley, 2004; Johnson et al., 2014; Weinbauer & Hofle, 1998). While biofilms are often considered imperative for survival in harsh and hostile environments (Hall-Stoodley et al., 2004), biofilm production is also important for colonization in nutrient rich environments (Bjarnsholt et al., 2013; Flemming & Wingender, 2010; Jefferson, 2004; Ye et al., 2019). In a recent study, water, sediment, and biofilm samples were collected from catfish production ponds during the growing season (July - October) and analyzed for the presence of vAh by qPCR (Cai et al., 2018). In all samples across all months, vAh was absent from the water samples in all three ponds. In sediment samples, vAh was detected in 62.5 % and 6.2% of samples tested from two ponds and was absent from a third. However, biofilm samples tested positive for vAh in all three ponds, with 56.2%, 31.2%, and 18.7% of samples containing vAh. While the number of ponds

and samples included in this study are small, these data suggest that vAh may primarily be resident in biofilms, and biofilms and sediments may act as a vAh reservoirs. vAh forms a tenacious biofilm when cultured on special high-nutrient media *in vitro*. However, although growth occurs in nutrient-poor minimal broth, biofilms will not form on minimal biofilm media (personal observations). This further supports the hypothesis that vAh may reside primarily in biofilm along the sediment in the presence of nutrient rich detritus.

To understand the pathogenesis of vAh, the study of the organisms in biofilm is imperative. Bacteria within biofilms exhibit emergent properties – properties present in a group that do not exist in the individual (Flemming & Wingender, 2010; Konopka, Lindemann, & Fredrickson, 2015). These emergent properties cannot be predicted by studying only free-living cells. Biofilm formation is a dynamic process and there is likely stage-specific gene expression. Aquatic copiotrophic biofilms, those composed of *r*-strategists in nutrient rich environments, are stratified along an oxygen gradient, and would be comprised of aerobic, microaerophilc, and anaerobic organisms (Flemming, Neu, & Wozniak, 2007; Flemming & Wingender, 2010). All three of these metabolic states would likely change expression of genes involved in nutrient acquisition, adhesion, and biogeochemical processing. Abiotic factors within each niche including nutrient availability and form, culture temperature, viscosity, pH, salinity, and oxygen availability have all been reported to impact bacterial phenotype (Holmes, 1996; Jahid et al., 2015; Maurelli & Sansonetti, 1988; Rahman, Suzuki, & Kawai, 2001a; Shapiro & Cowen, 2012).

Biofilm-associated bacteria generally have increased adhesive properties (Bales et al., 2013; Bjarnsholt et al., 2013; Costerton et al., 1987; Gavin et al., 2003; Lowry et al., 2014) and may also increase production of proteolytic enzymes, both of which could increase their virulence (Bi, Liu, & Lu, 2007; De Schryver & Vadstein, 2014; Khajanchi et al., 2009; Redfield,

2002). Extracellular proteases are expressed when diffusion and/or mixing is reduced because degraded proteins would be sequestered near bacteria and could be assimilated by the bacteria, thus increasing growth (Redfield, 2002). This phenomenon has been described as 'external digestion' and is a collaborative biofilm mechanism (Flemming & Wingender, 2010). Gosling (1996a) suggests that *Aeromonas* spp. produce extracellular enzymes to allow the organisms to feed in aquatic environs and that bacterial adhesins aid in the attachment and colonization of benthic surfaces. In aquatic environments, these enzymes provide nutrients by degrading the multitude of environmental organic compounds including detritus, benthos, and animal tissues, and are not host specific (Bales et al., 2013; Brown et al., 2012; Holmes, 1996). If this is the case, vAh in biofilm may have an advantage to host invasion due to the increased expression of proteolytic enzymes and potential adhesins which may facilitate attachment to fish tissue. Bi et al (2007) reported protease, nuclease, and hemolytic activity were decreased in an *A. hydrophila* quorum-sensing-regulator deletion mutant. Khajanchi et al. (2009) also reported quorum sensing regulators were responsible for type VI secretion and protease production by a clinical *A. hydrophila* isolate. These findings suggest that quorum sensing, which is vital for biofilm formation, is also imperative for the expression of some putative virulence factors by vAh. A study by Pridgeon et al. (2013) reported the presence of 228 ECPs in the culture supernatant of vAh, of which at least 23 were putative virulence factors. However, only ECPs of vAh produced in broth cultures have been studied to date, and this may not reflect ECP production *in natura*. Likely, ECP production is regulated by the niche the organism is occupying (planktonic, biofilm, or within host) as well as environmental influences, such as temperature and nutrient availability (Bi et al., 2007; Hall-Stoodley et al., 2004; Holmes, 1996; Khajanchi, Kozlova, Sha, Popov, & Chopra, 2012; Khajanchi et al., 2009). Because many bacteria in natural environments are

largely maintained in a biofilm and not as sustained planktonic populations, study of phenotypic and genotypic expression of PVFs by vAh cultured under different conditions, including within biofilm, could shed light on the proteins that allow attachment and invasion in to the fish host. Biofilms in a pond setting are likely multispecies biofilms, which may also alter gene expression and phenotype. In this way, the study of a single-species vAh biofilm *in vitro* likely will vary somewhat from biofilms *in natura*. However, growth on rich laboratory media likely supports growth, at least planktonically, in a similar fashion to ponds with heavy nutrient loading from fish feed (Zhang et al., 2020). Comparisons of vAh proteolytic secretion and regulation of putative virulence genes under planktonic and biofilm growth is vital to understand mechanisms of vAh pathogenicity.

2.7 Bacterial Secretion Systems

Assuming a role of secreted virulence factors in the pathogenicity, it is important to evaluate the pathways of secretion. The secretion of proteins from within the bacterial cytoplasm through the outer membrane is vital for bacterial survival and to this end multiple secretory pathways have evolved. Protein secretion is essential for bacterial cell growth and survival, and secretion systems perform numerous physiological functions required for niche-specific propagation and fitness. Some bacteria secrete proteins that help them adapt to or alter an environmental niche. Some have evolved systems that can inject proteins, often toxins, directly into a target cell, be it eukaryotic host or bacterial competitor. Some secretion systems are capable of secreting a vast array of proteins, while some are more specific, dedicated to secreting as few as one protein. Most bacterial species harbor multiple secretion pathways that play important roles in different aspects of survival, niche occupation, nutrient acquisition, and

pathogenicity (Abby et al., 2016), with *Proteobacteria* having the broadest range, as *Gammaproteobacteria* have representative species containing Type 1 – Type V1 secretions systems.

Secreted substances play key roles in the bacterial environmental response. Physiological responses including adhesion, adaptation and survival, and pathogenicity rely on efficient and timely secretion. Secreted substances have three fates: they may remain attached to the bacterium, anchored in the outer membrane, they may be released indiscriminately into the external cellular milieu, or they may be injected directly into a target cell (Costa et al., 2015).

Gram-negative bacteria have evolved at least eight secretion pathways. Two, the general secretion (Sec) and twin-arginine translocation (Tat) pathways, span the inner membrane and act to transport folded or nascent proteins into the periplasm. Five secretion systems, Type I, II, III, IV and VI, span both the inner and outer membranes, and Type V, which spans only the outer membrane. Apart from these, the chaperone-usher pathway and the curli biogenesis pathway, sometimes referred to as Type VIII secretion system, which span only the outer membrane, are used for type 1 pilus and curli assembly and integration into the outer membrane (Barnhart & Chapman, 2006; Bhoite, van Gerven, Chapman, & Remaut, 2019; Busch & Waksman, 2012). Recently, a novel secretion system, Type IX, was identified that is unique to *Bacteriodetes*, including the commensal environmental bacterium, *Flavobacterium johnsoniae,* and the human oral pathogen, *Porphyromonas gingivalis*. In the former, Type IX secretion systems export substrates used in gliding motility and chitin and cellulose degradation, and, in the latter, secreted substrates, including adhesins and multiple proteolytic enzymes, which are required for virulence (Lasica, Ksiazek, Madej, & Potempa, 2017; McBride, 2019).

Extracellular protein secretion via one of the six secretion systems involves a one-step or two-step secretion mechanism. Secretion systems utilizing a one-step mechanism translocate proteins from within the cytoplasm directly through the inner and outer membrane into the external cell environment, while a two-step mechanism requires initial transport into the periplasm via the Sec or Tat pathway, followed by secretion through the outer membrane (Rêgo, Chandran, & Waksman, 2010).

Many excellent reviews are available that detail genetic, mechanistic, and evolutionary processes of Gram-negative bacterial secretion systems and associated pathways (Bhoite et al., 2019; Burkinshaw & Strynadka, 2014; Busch & Waksman, 2012; Campos, Cisneros, Nivaskumar, & Francetic, 2013; Cianciotto, 2005; Costa et al., 2015; Delepelaire, 2004; Galán, Lara-Tejero, Marlovits, & Wagner, 2014; Gerlach & Hensel, 2007; Green & Mecsas, 2016; Nivaskumar & Francetic, 2014). Provided below are concise overviews of the main secretory systems in Gram-negative bacteria.

Type I Secretion System

The Type I Secretion System (T1SS), also referred to as the ABC-transporter dependent pathway, is highly conserved across bacterial species and is known to secrete biologically diverse proteins, ranging from < 10 KDa bacteriocins to massive adhesins > 1MDa (Delepelaire, 2004; Thomas, Holland, & Schmitt, 2014). In the genomic study performed by Abby et al. (2016), T1SS was the most common secretion system, appearing in 68% of all genomes compared. Traditionally, the T1SS has been characterized as a one-step secretion mechanism, where substrates are exported from cytoplasm to the external cell environment with no periplasmic intermediate (Delepelaire, 2004). While T1SS largely function in this manner, it was recently discovered that some adhesins are transported via T1SS in a two-step manner (Smith,

Sondermann, & Toole, 2018). T1SS are relatively simple tripartite systems, consisting of only three independent parts; an ABC transporter, a membrane fusion protein (MFP), and an outer membrane pore, whose genes are generally coded in two loci (Abby et al., 2016). While T1SS are capable of secreting a broad array of proteins, the ABC transporter and MFP form a complex which is typically dedicated to sensing a single Type 1 substrate. The OM pore is less specific, and is recruited by the ABC transporter-MFP complex following substrate-transporter interaction (Delepelaire, 2004; Smith et al., 2018).

Type II Secretion System

The Type II Secretion System (T2SS) secretes folded, fully functional protein from the periplasm to the extracellular environment in two-step process. First, either the Sec or Tat pathways translocate the protein from the cytoplasm into the periplasm, then the protein is secreted in an indiscriminate manner through the outer membrane. The T2SS is the only known exporter of Tat proteins, which fold in the cytoplasm prior to export (Nivaskumar & Francetic, 2014). T2SS are present in hundreds of bacterial species, both pathogenic and nonpathogenic, but is largely restricted to the *Proteobacteria* (Abby et al., 2016). Where the T1SS is simplistic in construction, the T2SS is very complex, containing 12 – 16 different proteins, 12 of which are conserved across bacterial genomes, often encoded on a single operon (Abby et al., 2016; Korotkov & Sandkvist, 2019; Korotkov, Sandkvist, & Hol, 2012). The T2SS is considered a **g**eneral **s**ecretion **p**athway, and in many species, a Gsp-based nomenclature is used to denote T2 genes and proteins. While this is a common practice, it is not universally accepted (Douzi, Filloux, & Voulhoux, 2012) and many important opportunistic pathogens, including *Vibrio*, *Aeromomas*, and *Pseudomonas* have genus-specific nomenclature. The T2SS membrane-spanning machinery is referred to as the secreton and is comprised of three sub-complexes; the

inner membrane platform and traffic ATPase, the transperiplasmic protein, and the outer pore. The inner membrane platform is comprised of three structural proteins plus the ATPase and is connected by the transperiplamic protein to the outer membrane pore. The pore, called secretin, is a dodecameric protein structure that inserts into the outer membrane, creating the secretion channel. The last component is a periplasmic pseudopilus. The pseudopilus is comprised of six of the 12 conserved *gsp* genes, five of which code for pseudopilins and one that codes for the prepilin peptidase required for maturation of the pseudopilus. The pseudopilus is believed to act like a piston - pushing substrates out of the periplasm through the secretin pore either into the extracellular milieu, or to be displayed on the cell surface (Douzi et al., 2012; Korotkov et al., 2012; Peter Howard, 2013; Rondelet & Condemine, 2013; Sandkvist, 2001a). T2SS are highly similar to the Type 4 pilus assembly mechanism, and the pseudopilus is closely related to Type IV pilin. Unlike Type IV pilins, which are anchored in the outer membrane as surface exposed proteins, the pseudopili are localized in the periplasm (Douzi et al., 2012; Nivaskumar & Francetic, 2014). Unlike one-step transport systems that recognize substrates by a secretion system-specific motif, the recognition process of T2SS substrates is a mystery. Recognition may even be species or strain specific (Douzi et al., 2012).

T2SS is the only known system capable of transporting both Sec and Tat-associated periplasmic proteins. Most T2SS substrates are highly stable and can be found in extremophiles adapted to extremes of temperature, salt, and pressure (Nivaskumar & Francetic, 2014). T2SS is also a common secretion pathway in environmental generalists and opportunistic pathogens, especially those resident in aquatic environs, such as *Vibrio* and *Aeromonas*. Here, a multitude of hydrolytic enzymes capable of degrading carbohydrates, proteins, and lipids, as well as toxins and adhesins, function primarily in nutrient acquisition, niche partitioning, and niche

colonization. From a bacterial perspective, the primary function of Type 2 secretion is nutrient acquisition. T2SS substrates also facilitate adhesion to biotic and abiotic surfaces and colonization of host species. Many of these substrates, while directed toward environmental nutrient sources, also act as virulence factors, and proteins used for adhesion can promote host invasion (Cianciotto & White, 2017; Korotkov et al., 2012; Nivaskumar & Francetic, 2014). In this way, the host is inadvertently caught in the crossfire - collateral damage in the environmental war for survival. Other important plant and animal pathogens secrete virulence factors required for pathogenicity through T2SS. *Legionella pneumophila* secretes more than 20 putative virulence enzymes via T2SS. *Chlamydia*, an obligate intracellular pathogen, requires T2 substrates to utilize host glycogen reserves, and many plant pathogens including *Dickeya dadanfii*, *Erwinia* spp., and *Xanthamonas campestris* secrete a variety of plant cell wall-degrading enzymes via T2SS (Cianciotto, 2013; Cianciotto & White, 2017; Korotkov & Sandkvist, 2019).

Despite all the research, multitudes of secreted substrates, highly conserved nature, and its role in adaptation and survival in both environmental and pathogenic bacteria, much of the function and substrate recognition of T2SS remains a mystery (Nivaskumar & Francetic, 2014).

Type III Secretion System

The Type III Secretion System (T3SS) is likely the best studied secretion system due to its involvement in host-pathogen interaction and its role in human disease modulation (Coburn, Sekirov, & Finlay, 2007; Hu et al., 2017). Phylogenetic analyses support the hypothesis that T3SS is an evolutionary exaptation of bacterial flagella (Abby & Rocha, 2012; Hu et al., 2017; Troisfontaines & Cornelis, 2005). T3SS is genetically complex, with the structure consisting of

20 or more proteins. Only nine T3SS core genes are highly conserved and eight of those genes are shared with flagellar apparatus in other bacteria. Ten to 20 accessory proteins, which play critical roles in T3SS function, are present in different bacterial species (Burkinshaw & Strynadka, 2014; Troisfontaines & Cornelis, 2005). T3SS use a one-step mechanism to translocate effector proteins from bacterial cytoplasm directly into the host target cell using a needle-like apparatus called an injectisome. The T3SS is inducible in nature, with expression believed to be regulated by external cues from the target host. T3SS effectors are delivered from the cytoplasm to the T3 assembly by chaperones. There, these chaperones also aid in the assembly of the injectisome. The specialized tip of the injectisome contains multiple copies of an adapter protein and performs multiple functions, including sensing contact with the host cell, regulating secretion of effector proteins, and assisting with injectisome insertion into the host cell plasma membrane (Blocker et al., 2008; Burkinshaw & Strynadka, 2014; Costa et al., 2015)

T3SS are found in a host of pathogenic bacteria including *Salmonella, Shigella, Yersinia*, *Pseudomonas*, and enteropathogenic *E. coli*, to name a few. T3 effectors promote bacterial invasion, colonization, and establishment of disease in so many pathogens that the mere presence of T3SS within a bacterial genome is often used as a predictor of bacterial virulence. T3SS are typically acquired by horizontal gene transfer, and may be located in the chromosome as well as on plasmids. T3SS genes are often encoded in pathogenicity islands and some bacteria, such as *Salmonella typhimurium*, contain two T3SS, each encoded by a separate pathogenicity island (Mecsas & Strauss, 1996). So much horizontal acquisition of T3SS has occurred that highly homologous species may have very dissimilar systems, while evolutionarily distinct species may share very similar systems (Troisfontaines & Cornelis, 2005).

Type IV Secretion System

Type IV Secretion Systems (T4SS) are unique in that they are capable of transporting DNA as well as proteins. There are three functional types of T4SS. The first is a conjugative T4SS, where DNA is transferred from one cell to another in a contact-dependent manner. Conjugative T4SS are often encoded along with other fitness-increasing genes of self-transmissible plasmids. These plasmids often provide selective advantage to the cell by promoting metabolic flexibility and antibiotic resistance.

A second type of T4SS also mediates DNA uptake from and export to the extracellular environment. This functional type is uncommon, currently only characterized in *Helicobacter pylori, Neisseria gonorrhoeae,* and *Campylobacter jejuni* (Cascales & Christie, 2003; Green & Mecsas, 2016). *N. gonorrhoeae* use T4SS to promote the acquisition of virulence genes by importing environmental DNA.

The third type of T4SS is a protein secretion system, sometimes referred to as the T4 effector translocation. T4 effector translocation uses a T3SS-like injectisome to introduce proteins, mostly toxic in nature, to the host cell. Many important human pathogens including *Legionella pneumophila*, *H. pylori*, and *Brucella* spp., as well as the plant pathogen, *Agrobacterium tumefaciens*, require T4 effector translocation for disease initiation and progression. *Bordetella pertussis*, the etiologic agent of whooping cough, also uses T4 effector translocation to deliver its toxin. However, instead of using the injectisome to target a specific host cell, pertussis toxin is released into the extracellular environment indiscriminately (Cascales & Christie, 2003; Shrivastava & Miller, 2009). In contrast to *L. pneumophila*, which secretes over 200 effector proteins into host cells by T4 secretions, the T4 effector translocon of *B. pertussis* secretes only pertussis toxin.

Type V Secretion System

Type V Secretion Systems (T5SS) are autotransporters, and are the most simplistic secretions systems. T5SS is highly conserved, previously discovered in 62% of studied genomes (Abby et al., 2016). T5SS is divided into three sub-families, but all function very similarly (Henderson, Navarro-Garcia, Desvaux, Fernandez, & Ala'Aldeen, 2004). T5SS substrates are secreted in a two-step manner and rely on the Sec machinery for translocation through the inner membrane into the periplasm, where T5SS exoproteins may remain fused to the outer membrane or may be released extracellularly after proteolytic cleavage (Depluverez, Devos, & Devreese, 2016). Many T5 substrates, including extracellular proteases, lipases, and adhesins, are important virulence factors (Leo, Grin, & Linke, 2012).

Type VI Secretion System

Type VI Secretion Systems (T6SS) have only recently been characterized. Like T3 and T4SS, T6SS also employ an injectisome to deliver effector proteins directly into the host cell. The T6SS is required for virulence in many pathogens of humans and animals, including *Y. pestis*, *S. typhimurium V. cholereae*, *Francisella tularensis*, pathogenic *E. coli* and *Burkholderia mallei*, as well as the plant pathogens *A. tumefaciens*, *Pectobacterium atrosepticum*, and *Xanthamonas oryzae* (Basler & Mekalanos, 2012; Navarro-Garcia, Ruiz-Perez, Cataldi, & Larzábal, 2019). T6SS are also prevalent among emerging and opportunistic pathogens such as *P. aeruginosa, Edwardsiella tarda,* and *A. hydrophila* and, like T3SS, are often considered harbingers of virulence.

T6SS likely evolved as a weapon of bacterial competition. Bacterial cells possessing a T6SS gained a competitive advantage by injecting bacterial rivals with antibacterial proteins, initiated by cell-to-cell contact. Thus, many T6 effectors are likely antibacterial rather than, or in

addition to, anti-eukaryotic. Such a system could provide bacteria with a significant competitive advantage against other bacteria, both in the natural environment and within a host. *Serratia marcescans* and *P. aeruginosa* specifically target toxins to bacterial competitors (Basler & Mekalanos, 2012).

The proteins delivery system in T6SS appears to differ from those of T3 and T4SS in that multiple effector proteins are injected into the host cell all at once as part of the injectisome complex, followed by delivery effectors. Within the host cell, the different proteins are chaperoned to different subcellular locations by signals carried within the effectors (Ho, Dong, & Mekalanos, 2014)

2.8 Secretion in virulent *Aeromonas hydrophila*

The breadth of niche adaption and host range suggests that *A. hydrophila* have an arsenal of ECPs at their disposal. The vast range of virulence proteins almost assuredly play a vital role in vAh pathogenicity and virulence. Assuming a role of ECPs in vAh virulence, the secretion of these enzymes and potential toxins must occur through a bacterial secretion system. Many *A. hydrophila* possess a T3SS and T6SS, and these systems have been reported as a major virulence factor in other Aeromonads and *A. hydrophila* strains (Burr, Pugovkin, Wahli, Segner, & Frey, 2005; Carvalho-Castro et al., 2010; Chacon, Soler, Groisman, Guarro, & Figueras, 2004; Tomas, 2012; Vilches et al., 2004; Vilches et al., 2008). However, U.S. vAh isolates possess neither a T3SS nor a complete T6SS, instead possessing genes encoding T1SS and T2SS. Interestingly, all U.S. isolates also possess two T6SS genes which, surprisingly, have been found to play some role in virulence (Tekedar et al., 2019).

The T2SS spans the inner and outer membrane and functions to export folded, fully functional proteins from the periplasm through a pore, called secretin, in the outer membrane

(Campos et al., 2013; Cianciotto, 2005; Cianciotto & White, 2017; Douzi et al., 2012; Peter Howard, 2013; Sandkvist, 2001a, 2001b; Vanderlinde et al., 2014). In general, T2SS are associated with organisms that colonize surfaces and generally do not invade cells (Cianciotto, 2005; Sandkvist, 2001b). Other *A. hydrophila* that possess a T2SS secrete toxins via this pathway (Howard et al., 1996; Strozen et al., 2011; Vanderlinde et al., 2014). The secreted proteases, lipases, phospolipases, and other proteins that are associated with type II secretion are important for providing nutrients to bacteria outside of a host, but may also aid in colonization or invasion of the host by tissue destruction (Costa et al., 2015; Douzi et al., 2012; Gosling, 1996b; Howard et al., 1996; Peter Howard, 2013).

Unlike many bacterial species that use *gsp* nomenclature for identifying T2SS genes and proteins, the T2SS genes of *Aeromonas* are denoted *exe* genes and, uncharacteristically, are encoded on two disjunct operons. The main *exe* operon contains genes *exeC-exeN*, which are contiguous and transcribed in the same direction (Douzi et al., 2012; Peter Howard, 2013; Strozen et al., 2011). Two genes present on another operon, *exeA* and *exeB*, are believed to be involved in the assembly of *exeD*, the secretin pore through which substrates exit the bacterial cell (Ast et al., 2002; Cianciotto, 2005; Costa et al., 2015; Douzi et al., 2012; Filloux, 2004; Strozen et al., 2011; Vanderlinde et al., 2014). While T2SS genes are inducible in some organisms (Putker et al., 2013; Sandkvist, 2001b), constitutive expression is believed to occur in generalist bacteria that secrete proteins in multiple environments (Cianciotto, 2005; Filloux, 2004). Both *V. cholerae* and *A. hydrophila* are thought to have constitutive expression of T2SS genes (Sandkvist, 2001b). When secretion genes are constitutively expressed, as hypothesized with vAh, ECP expression is under tight regulation (Sandkvist, 2001b). Regulation of genes encoding ECPs could be regulated by quorum sensing *asp*A or other sigma factors. This would

mean that extracellular secretion of specific proteins would only occur when vAh had reached some critical mass or some specific location (Bi et al., 2007; Khajanchi et al., 2012; Khajanchi et al., 2009). This would further support the hypothesis that biofilm-associated vAh could have a greater ability to colonize the host because they would be secreting potential virulence factors. Transposon mutagenesis of *exe* genes in *A. hydrophila* have resulted in secretion-null mutants (Ast et al., 2002; Peter Howard, 2013) and have helped verify the role of T2SS for some ECP secretion. However, targeted gene deletions of specific *exe* genes could help clarify the role of type II secretion in vAh pathogenicity. The recent improvements in genetic engineering technologies now allows for a more methodical approach to studying the secretion systems**.**

The recent advent of homologous recombination in bacteria through the use of the lambda red recombination system, also called recombineering, has given scientists the potential to create specific, accurate, targeted gene deletions within the genome of bacteria (Hossain, Thurlow, Sun, Nasrin, & Liles, 2015; Lesic & Rahme, 2008; Sharan, Thomason, Kuznetsov, & Court, 2009; Thomason, Sawitzke, Li, Costantino, & Court, 2014). Genetic modification by recombineering allows for the precise deletion of genes by homologous recombination of a selectable marker into the target genomic region. Hossain et al. (2015) developed a recombinant vAh mutant, ML09-119+pMJH65. This recombinogenic mutant will allow precise genetic modification in regions of interest. Recombineering works by utilizing three homologous recombination enzymes from the lambda Red (λ Red) bacteriophage expressed from within vAh. The three enzymes, Exo, Beta, and Gam, work in conjunction to allow incorporation of foreign DNA into the bacterial host genome by homologous recombination (Lesic & Rahme, 2008; Sharan et al., 2009; Thomason et al., 2014). When a selectable marker cassette flanked by sequence homologous to the gene of interest is introduced as linear DNA by electroporation,

Gam prevents the host restriction modification systems from degrading the insert. Next, Exo, which has 5′-3′ exonuclease activity, creates single-stranded DNA overhangs of the homologous flanking regions which are then bound by Beta. Beta promotes the binding of the overhang with its complementary sequence within the genome thus, effectively replacing the gene of interest with a selectable marker (Sharan et al., 2009). These recombinant mutants are then selected using growth media supplemented with the antibiotic from the selectable marker cassette. The selectable marker can be removed to make a markerless mutant by taking advantage of Flp-*FRT* recombination (Hoang, Karkhoff-Schweizer, Kutchma, & Schweizer, 1998). This system requires the incorporation of a flippase (Flp) recognition site, a specific palindromic sequence, flanking the selectable maker cassette. Following recombinogenic knock-out of the target gene, a plasmid containing the Flp recombinase can be introduced into the mutant by conjugative transfer. Flp- *FRT* excision machinery is temperature inducible, and growth of the mutant containing the Flp plasmid at the inducible temperature will initiate Flp-*FRT* excision. A plasmid containing Flp recombinase has previously been introduced into vAh and successful removal of marker cassettes has been demonstrated (Hossain et al., 2015). Using the recombinogenic vAh strain, ML09-119+pMJH65, it may be possible to delete specific T2SS genes to determine their role in extracellular secretion and to elucidate the role of type II secretion in the virulence of vAh.

The goal of this dissertation is to increase our understanding of the mechanisms of vAh pathogenicity and how niche adaptions may increase vAh virulence in natural systems. This research will attempt to elucidate how vAh plastic response to biofilm and planktonic niche occupation - through protein secretion and gene expression - may affect vAh virulence. Bacterial econiche adaptation regularly involves secretion of adhesins and enzymes that increase fitness,

and *A. hydrophila* are capable of producing a host of degradative and toxigenic proteins. The rapid mortality and minimal postmortem histologic tissue changes observed in vMAS infections suggest that vMAS may be a toxin-mediated disease. To elucidate the roll of secreted proteins and toxins in vMAS, and to determine the primary vAh secretory pathway, T2 secretion-deficient mutants will be constructed and secreted protein profiles will be examined for protein presence and functionality.

Chapter II

Differential Production and Secretion of Potentially Toxigenic Extracellular Proteins from an Epidemic Strain of *Aeromonas hydrophila*

1. Abstract

Virulent *Aeromonas hydrophila* is an emerging disease in freshwater aquaculture that results in the loss of 3 million pounds of marketable channel catfish, *Ictalurus punctatus*, and channel catfish hybrids each year from freshwater catfish production systems in Alabama. Virulent *Aeromonas hydrophila* isolates are clonal in nature and are genetically unique from, and significantly more virulent than, traditional *A. hydrophila*. Even with the increased virulence, natural infections cannot be reproduced in aquaria challenges making it difficult to determine modes of infection and the pathophysiology behind the devastating mortalities that are commonly observed. Despite the intimate connection between environmental adaptation and plastic response, epidemiological vAh research has largely ignored the role of bacterial econiche adaptive response and how niche partitioning and growth affects vAh pathogenicity and virulence. In this study, secreted proteins of vAh cultured as free-living planktonic cells and within a biofilm were compared to elucidate the role of biofilm growth on virulence. Functional proteolytic assays found significantly increased degradative potential in biofilm secretomes and increased toxigenic potential, measured by hemolysis assays, in planktonic secretomes. Intramuscular injection challenges in a channel catfish model proved that *in vitro* degradative potential translated to *in vivo* tissue destruction. Identification of secreted proteins by HPLC-MS/MS revealed the presence of many putative virulence proteins under both conditions, regardless of significant difference. Biofilm growth resulted in differential secretion of

proteolytic enzymes and adhesins, while planktonic secretomes had significant increases in toxins, porins, and fimbrial proteins, further supporting the usefulness of functional assays in determining the potential activity of secreted proteins under differing culture conditions. The results of this research underscore the need for alternative culture methods that more closely mimic natural ecological habitat growth to increase understanding of pathogenesis *in natura*.

2. Introduction

Aeromonas hydrophila is a wide-spread and diverse species of Gram-negative bacteria ubiquitous in freshwater aquatic ecosystems. *Aeromonas hydrophila*, a rapidly growing and metabolically diverse generalist (Holmes, 1996; Jaccard et al., 2014; Janda & Abbott, 2010; Peatman et al., 2018; Seshadri et al., 2006), is capable of exploiting a variety of ecological habitats and a broad range of hosts. *A. hydrophila* has been isolated from almost every aquatic environment and from diseased mammals, reptiles, amphibians, insects, and fish. *A. hydrophila* has been found in association with processed poultry, meats, fish, and even bottled water. It is capable of withstanding chlorination and is resistant to multiple antibiotics. In short, *A. hydrophila* is the ultimate survivor.

In aquaculture, *A. hydrophila* is an important cause of disease in most freshwater production systems. Historically, *A. hydrophila* has been an important secondary pathogen in catfish production systems, commonly responsible for cutaneous ulceration and muscle necrosis and, occasionally, motile aeromonad septicemia (MAS), which can result in high mortalities (Austin & Adams, 1996; Beaz-Hidalgo & Figueras, 2013; Camus et al., 1998; Cipriano, 2001; Griffin et al., 2013). In 2009, a new, highly virulent strain of *A. hydrophila* was isolated from a diseased channel catfish, *Ictalurus punctatus*, within a production pond in West Alabama. This

strain, referred to as virulent *Aeromonas hydrophila*, or vAh, was responsible for outbreaks of peracute motile aeromonad septicemia of epidemic proportions (Griffin et al., 2013; Hossain et al., 2014; Hossain et al., 2013; Pridgeon & Klesius, 2011; Pridgeon et al., 2013; Tekedar et al., 2013). Virulent *Aeromonas hydrophila* appears to act as a primary pathogen, and may not be preceded by immune insult (Griffin et al., 2013). To date, vAh has been responsible for the loss of 30 million pounds of marketable channel catfish from production farms in West Alabama. In 2017, *A. hydrophila* infections were responsible for the loss of 3.4 million pounds of farm-raised catfish in Alabama alone, more than twice as much than the second leading cause of loss, *Flavobacterium columnare*. vAh has been the primary or secondary cause of loss in Alabama since the primary outbreak in 2009 (Hemstreet, AL Fish Farming Center).

While much current research is focused on disease prevention, and rightly so, the focus on prevention of clinical disease should not distract from research toward understanding the mechanisms of pathogenesis and the bacterial-host interaction. Furthermore, current studies of pathogenesis and virulence are performed almost exclusively from planktonically-cultured bacteria. One study reported the presence of 228 extracellular proteins in the supernatant of vAh broth cultures, at least 23 of which were putative virulence factors (Pridgeon et al., 2013). However, only proteins secreted by planktonically-cultured bacteria have been studied, despite the fact that most free-living generalist bacteria in aquatic systems reside primarily within biofilm (Bales et al., 2013; Costerton et al., 1987; Hall-Stoodley et al., 2004; Jefferson, 2004; Johnson et al., 2014; Weinbauer & Hofle, 1998). A recent study by Cai et al. (2018) found no vAh present in the water column through the survey period, (July- October), while vAh resident in biofilm and pond sediment was detected at an increasing rate in the same sampling period, suggesting that biofilms offer a stable niche that allow vAh survival when planktonic conditions

are less favorable. Biofilm-associated bacteria generally have increased adhesive properties (Bales et al., 2013; Bjarnsholt et al., 2013; Costerton et al., 1987; Gavin et al., 2002; Lowry et al., 2014) and may have increased production of proteolytic enzymes, both of which could increase virulence (Austin & Adams, 1996; De Schryver & Vadstein, 2014; Khajanchi et al., 2009; Redfield, 2002). Redfield (2002) suggests that extracellular proteases are expressed when diffusion and/or mixing is reduced. Degraded proteins would then be sequestered near the bacteria and could be rapidly assimilated, thus increasing bacterial growth. If this is the case, vAh within a biofilm may have an advantage to host invasion due to increased secretion of proteolytic enzymes and adhesins which may facilitate attachment and invasion to fish tissue. If vAh are residing primarily in biofilm, a study of virulence factors secreted during biofilm growth could shed light on the process of host attachment and invasion *in natura*. In this study, we compared the secreted protein profiles (secretomes) of biofilm-associated and planktonically cultured vAh strain ML09-119 to determine how niche habitation effects protein production and secretion, and to determine if niche occupation could influence vAh pathogenicity in natural environments.

3. Methods and Materials

Bacterial Strain. Virulent *Aeromonas hydrophila* strain ML09-119 was isolated from a diseased channel catfish from a MAS outbreak in a West Alabama aquaculture facility in 2009. Molecular characterization and genome sequencing of vAh ML09-119 were performed (Tekedar et al., 2013) and gene sequence deposited into GenBank (Accession CP005966). Aliquots of vAh ML09-119 were cryogenically stored in 10% glycerol freeze medium at -80°C.

Culture Media and Culture Conditions. Tryptic soy broth (TSB) (Bacto TSB, BD) prepared according to manufacturer's directions was used as the culture medium for planktonic growth.

Biofilm media was prepared by adding 0.2% agar powder (AlfaAesar) to TSB media prior to sterilization. Approximately 70ml of molten biofilm agar was poured into deep well petri dishes (Fisher) and allowed to solidify.

Bacterial strain vAh ML09-119 was removed from cryogenic storage and inoculated into 25ml TSB media and grown overnight at 30°C with shaking. A 1ml aliquot of overnight culture was transferred to 70ml of TSB and grown at 30°C on an orbital shaker to mid-log phase, approximately 16 hours. Biofilm agar plates were inoculated from overnight culture by stab inoculation. Plates were sealed with parafilm and incubated at 30°C for 72 hours, until an adherent bacterial film covered the agar surface.

Planktonic and biofilm cultures were performed in triplicate.

Secretome Preparation.

Planktonic Secretome: vAh ML09-119 was cultured as described above. Cells were pelleted by centrifugation at 20,000 *x* g for 15 minutes at 4°C; supernatant was decanted and retained. Cells were washed twice with cold, sterile PBS, pelleted as above, and the wash was added to supernatant. Remaining cells were removed by passage through a low-binding 0.22µ vacuum filter (VWR). Cell-free supernatants were used as the starting point for secreted protein purification.

Biofilm Secretome. vAh ML09-119 cells were gently removed from biofilm media surface with sterile cell scraper, transferred to 50ml conical tube, and washed twice with cold, sterile PBS as described above. Cell wash was decanted and retained. To collect secreted proteins within biofilm media, the plates were disrupted using a sterile disposable probe until the soft agar had formed a slurry. The agar slurry was transferred to a sterile 50ml conical tube, centrifuged at 20,000 *x* g for 15 minutes at 4°C to pellet the agar. Following centrifugation, the liquid media was decanted from the agar plug and retained. The agar plug was then resuspended in 20ml cold sterile PBS, centrifuged as above, and the wash solution decanted and retained. All wash solutions and liquid media were combined and filtered, first through a low-binding 0.45µm vacuum filter (VWR), then through a low-binding 0.22µ vacuum filter to remove any residual agar and bacterial cells. This cell-free supernatant was used at the starting point for biofilm secretome purification.

Ammonium Sulfate Precipitation: Secreted proteins were precipitated from cell-free supernatants by the addition of ammonium sulfate crystals (Fisher Scientific) to achieve 65% saturation, followed by incubation at 4°C on a rotary platform shaker with gently mixing for 24 hours. Precipitated proteins were collected by centrifugation at 30,000 *x* g for 45 minutes at 4°C, then dissolved in 10 ml cold Tris buffer (20mM Tris-Hcl, pH 7.6) + protease inhibitor (Complete tablets, mini, EDTA-free (Roche)). Resuspended proteins were dialyzed twice, for 18 hours and 12 hours, respectively, against the same buffer in 10Kda dialysis cassettes (Slide-A-Lyzer (Thermo Fisher)). After dialysis, the total volume was adjusted to 20 ml by the addition of cold Tris buffer. Protein concentration of each sample was determined by Bradford assay (Pierce Coomassie Plus Protein Assay, Thermo Fisher). These concentrated proteins were used for all assays.

Enzymatic Activity. The *in vitro* activity of secreted proteins was measured using multiple substrates to determine degradative and toxigenic potential of planktonic and biofilm secretomes.

Hemolysis: Hemolytic potential was measured using the method of Peatman et al. (2018) with some modifications. In brief, heparinized blood from three channel catfish was pooled and diluted 1:10 in sterile phosphate buffered saline (PBS). A suitable dilution of protein in 150µl PBS buffer was added to 25µl diluted blood in sterile microcentrifuge tubes. Tubes were incubated at 30°C in an orbital shaker for 2 h. Positive control tubes representing 100% hemolysis contained 150µl sterile distilled water in place of protein samples. Negative control tubes contained 150µl sterile PBS in place of protein samples. Controls were incubated with 25µl diluted blood as above. Following incubation, tubes were centrifuged at 1,000 *x g* to pellet un-lysed cells and 150µl of supernatant was transferred to 96-well flat bottom plates. Erythrocyte lysis was quantitated by measuring absorbance of released hemoglobin at 415nm in multi-mode plate reader (Synergy HTX, Bio-Tek) and hemolysis was reported as percent of positive control.

Universal Protease Activity: Non-specific proteolytic activity was measured using HiLyteFluor 488-labeled casein as the substrate, following manufacturer's protocol with minor modifications (Sensolyte Green Fluorimetric Protease Assay Kit, AnaSpec, Inc.). Briefly, a suitable concentration of protein in 50µl deionized water was added to triplicate wells of black, flat-bottom 96-well plates with non-binding surfaces (Greiner Bio-One). Trypsin, diluted 50-fold in deionized water, acted as a positive control and sterile deionized water was a substrate control. Following the addition of 50 µl labeled casein substrate to each well, plates were mixed briefly and fluorescent intensity was measured at Ex/Em = 490nm/520nm every five minutes for one hour in a multi-mode plate reader (Synergy HTX, Bio-Tek) with 30° incubation temperature. Data was plotted as relative fluorescence units versus time for each sample.

Elastase Activity: Elastase-specific activity was measured using 5-FAM/QXL^{TM} 520 labelled elastin as the substrate, following manufacturer's protocol with minor modifications (Sensolyte Green Fluorimetric Elastase Assay Kit, AnaSpec, Inc.). Briefly, a suitable concentration of protein in 50µl deionized water was added to triplicate wells of black, non-binding, flat-bottom 96-well plates. Elastase, diluted 50-fold in assay buffer, acted as a positive control and sterile, deionized water was a substrate control. Following the addition of 50µl labeled elastase substrate, plates were mixed briefly and fluorescent intensity was measured continuously at Ex/Em = 490 nm/520 nm, and data recorded every five minutes for one hour in a multi-mode plate reader with 30° incubation temperature. Data was plotted as relative fluorescence units versus time for each sample.

***In vivo* Proteolysis.** Extracellular protein activity was measured *in vivo* using channel catfish fingerlings to determine potential proteolytic and cytotoxic tissue effects.

Catfish: Specific-pathogen free channel catfish fingerlings maintained under Auburn University IACUC-approved protocol 2018-3251 (Catfish Production and Maintenance) were used for challenges. All challenges were performed adhering to the guidelines of AU-IACUC-approved protocol 2016-2900 (Identification of toxigenic proteins of virulent *Aeromonas hydrophila* and evaluation of host response).

Protein Preparation: Ten microgram aliquots of secreted planktonic and biofilm-associated proteins, prepared as above, diluted in 100µl sterile PBS were used for injection challenges.

Challenge Model: Channel catfish fingerlings were transferred to 15-gallon glass aquaria and acclimated at 30°C for two days prior to challenge. Triplicate tanks containing five

fish each represented planktonic ECP, biofilm-associated ECP, and injection control groups. Prior to injection, fingerlings were transferred to sedation aquaria containing 70mg/ L tricaine methanosulfate (MS-222) buffered to neutrality with sodium bicarbonate. Following sedation, characterized by decreased opercular movement and loss of equilibrium, 100µl of sterile PBS containing 10µg of total protein was injected intramuscularly just below the dorsal fin using tuberculin syringes fitted with 26 gauge needles. Control fish were injected with 100µl sterile PBS. Fish were then returned to the appropriate aquarium and monitored until fully recovered. Fish were maintained in aquaria at 30°C for 7 days. Moribund fish or fish developing external lesions were euthanized by prolonged exposure to buffered MS-222, the tissues were collected and fixed in 10% neutral-buffered formalin. After 7 days, remaining fish were humanely euthanized and samples were collected and prepared as above.

Histology: Formalin-fixed tissues were paraffin-embedded and 4 micron sections were prepared and stained with hematoxylin and eosin according to standard methods (Feldman & Wolfe, 2014). Slides were evaluated and photographed using an Olympus BX53 microscope fitted with an Olympus DP26 digital camera.

Secretome Analysis. To determine how vAh niche occupancy might influence protein production, secreted protein profiles of vAh cultured within a biofilm and in broth were compared by liquid chromatography with tandem mass spectrometry (LC MS/MS) analysis at the UAB Mass Spectrometry/Proteomics shared facility to identify and quantify proteins present in each sample.

Ion-exchange chromatography: Dialyzed biofilm and planktonic proteins, prepared as above, were filtered through low-binding 0.45µ syringe filters, and the filtered solution was

loaded at a flow rate of 1ml/minute onto a 5ml HiTrap QHP prepacked column (GE Healthcare) previously equilibrated with 10 column volumes of loading buffer (20Mm Tris-HCl, pH 7.6) connected to a Biologic LP Chromatography system (Bio-Rad). Samples were eluted at a flow rate of 1ml/minute using a linear gradient (0-100%) of elution buffer (1M NaCl in 20Mm Tris-HCl, pH 7.6). Eluate was monitored at 280nm and visualized on LP Data View software (v1.03, Bio-Rad). Two ml fractions were automatically collected with Model 2110 fraction collector (Bio-Rad) and fractions containing proteins were pooled and de-salted by passage through a 5ml HiTrap desalting prepacked column (GE Healthcare). Protein concentrations were determined using a Bradford assay kit (Coomassie Plus (Thermo Scientific). Fractions were then submitted for protein identification and quantitation via liquid chromatography- tandem mass spectrometry (HPLC MS/MS).

Proteomics analysis: Samples were prepared for analysis as follows: 20µg of protein per samples was diluted to 35µl in NuPAGE LDS sample buffer (Invitrogen), reduced with dithiothreitol, and denatured at 70°C for 10 minutes prior to loading onto Novex NuPage 10% Bis-Tris protein gel (Invitrogen). The gel was separated as a short stack (10 min, 200V constant) and stained overnight with Novex Colloidal Blue Staining kit (Invitrogen). Gels were destained and each lane was cut into single molecular weight fractions and equilibrated in 100mM ammonium bicarbonate. Each plug was then digested overnight with Trypsin Gold (Mass Spectrometry grade (Promega)) following manufacturer's instructions and peptide extracts were reconstituted to 0.1µg/µl in 0.1% formic acid.

Mass Spectrometry: Prepared peptide digests (8µl) were injected onto a 1260 Infinity nHPLC stack (Agilent Technologies) and separated using a 71µ I.D. X 15cm pulled-tip C-18 column (Jupiter C-18 300 Å, 5 micron (Phenomenex)) running in-line with a Thermo Orbitrap

Velos Pro hybrid mass spectrometer, equipped with a nano-electrospray source (Thermo Fisher). All data were collected in CID mode. nHPLC was configured with binary mobile phases comprised of 0.1% formic acid (solvent A) and 0.1% formic acid in 85% acetonitrile (solvent B). Following each parent scan (300-1200 m/z at 60K resolution), fragmentation data (MS2) were collected on the top most intense 15 ions. For data-dependent scans, charge-state screening and dynamic exclusion were enabled with a repeat count of 2, repeat duration of 30s, and exclusion duration of 90s.

Mass spectrometry data conversion and searches: Xcalibur RAW files were collected in profile mode, centroided, and converted to mzXML using ReAdW v3.5.1 (IonSource). The mgf files were then created using MzXML2Search (included in Trans-Proteomics Pipeline v3.5) for all scans. The data were searched using SEQUEST (Thermo Fisher), which was set for two maximum missed cleavages, a precursor mass window of 20ppm, trypsin digestion, variable modification C at 57.0293, and M at 15.9949. Searches were performed with a species-specific subset of the UniRef 100 database.

Peptide filtering, grouping, quantification and statistical analyses: The list of peptide IDs generated based on SEQUEST search results were filtered using Scaffold (Protein Sciences, Portland, Oregon), which filters and groups all peptides to generate and retain only high confidence IDs while also generating normalized spectral counts across all samples to allow for relative quantification. Filter cut-off values were set with minimum peptide length of >5 amino acids, with no MH+ charge states, peptide probabilities of >80% C.I., and with the number of peptides per protein ≥2. Proteins probabilities were then set to a >99% C.I. with false discovery rate <1.0. Scaffold incorporates the two most common methods for statistical validation of large proteome data sets, the false discovery rate (FDR) and protein probability. Relative

quantification across samples were then performed via spectral counting and, when relevant, spectral count abundances were normalized between samples. Proteins present in at least two experimental replicates were included in analyses. To determine statistical significance, two non-parametric statistical analyses were performed between each pair-wise comparison, including reproducibility-optimized test statistic (ROTS) (bootstrapping value = 1000) combined with single-tail t-test ($p < 0.05$) (Pursiheimo et al., 2015; Suomi & Elo, 2017). These were then sorted according to the highest statistical relevance in each comparison. For protein abundance ratios determined by normalized spectral counts, a fold change threshold ≥1.5 was set for significance. Protein abundance of proteins present in only one experimental group was set as the average of the normalized quantitative value.

Protein Function: To define the potential function of secreted proteins, major biological processes of significant proteins were determined from gene ontology annotation in UniProt (Consortium, T.U. 2018) and QuickGO (Binns et al., 2009).

4. Results

Proteolytic Assays. Universal protease activity was measured using HiLyteFluor488-labelled casein as substrate. Protease activity in biofilm samples was >2 times higher than in planktonic samples, and 1.2 times higher than the trypsin positive control (Figure 1). Elastase-specific activity showed similar results, with biofilm sample activity 2.7 times higher than planktonic samples, but not significantly higher than the elastase positive control (Figure 2). Hemolytic activity, which represents toxigenic potential, was greatly increased in planktonic samples, more than 6 times higher than biofilm samples (Figure 3).

***In vivo* Protein Activity.** To determine if *in vitro* activity would correspond to *in vivo* activity, 10μg of protein from each growth condition was injected intra-muscularly into channel catfish. Two hours post-injection, loss of dermal pigment was noted at the injection site in biofilm-injected fish. After 24 hours, substantial tissue necrosis was observed grossly in all biofilm-injected fish (Figure 4). Moribund fish were humanely euthanized and tissues were collected for histopathology. Fish injected with planktonic-associated ECPs developed no gross lesions after 7 days. No control fish developed any gross lesion at the injection site after 7 days.

Histopathology. Histopathology was performed on skin and subcutaneous tissues collected from injection sites of channel catfish. Biofilm-injected fish tissue was edematous, hemorrhagic, and there was extensive tissue necrosis at the site of injection (Figure 5). Despite substantial tissue damage, few inflammatory cells were present. Fish injected with planktonic ECPs were identical to the control fish, with no perceptible damage to skin, subcutaneous adipose tissue, or muscle.

Secretome Analysis. The observed variability in the *in vitro* assays and *in vivo* challenges when using secreted proteins from biofilm versus planktonically-cultured vAh supports the hypothesis that niche occupancy plays a role in production and secretion of vAh exoproteins. To further test this hypothesis, a secretome analysis was performed to identify specific differentially secreted proteins present under the two culture conditions. A total of 272 proteins were identified in the secretomes of biofilm and planktonically-cultured vAh. Eighty-two proteins were identified in the secretomes under both parameters, while 98 were identified only in biofilm-associated secretomes and 92 were unique to planktonic secretomes. Reproducibility-optimized test statistic (ROTS) and T-test analyses identified 53 proteins that significantly (FDR < 0.05, $p = 0.01$) varied in abundance. Protein abundance ratios of 52 ROTS-identified proteins were above the significant fold change threshold of ≥1.5 (Table 1). A single protein, maltoporin (*AHML_06215*)

which was found in higher abundance in biofilm secretomes, had a fold change of 1.46 and, thus, was excluded from analyses. Thirty-five proteins were significantly increased in the biofilm secretomes, 20 of which were unique in biofilms, and 15 proteins were significantly increased in planktonic secretomes, including nine unique proteins. Of the proteins that varied significantly in their abundance, at least 15 from planktonic secretomes and 30 from biofilm secretomes have been indicated in virulence. Not all secreted putative virulence proteins were differentially secreted, however. A multitude of PVFs were identified in secretomes under both conditions.

Predicted protein function was assessed by determining major biological processes through gene ontology. Using these data, eight functional groups were established, and proteins were sorted into these groups based on their primary biological function (Figure 6). A further comparison was made by compiling all proteins in each functional group from both biofilm and planktonic secretomes and expressing as parts of a whole, with side-by-side comparisons for each secretome type (Figure 7). Functional group comparisons revealed extensive secretion of degradative enzymes and toxins in both biofilm and planktonic secretomes, with degradative enzymes, such as elastase, metalloprotease, chitinase, and endochitinase, dominating biofilm secretomes and cytotoxic and cytotonic toxins, such as *ahh1*-type hemolysin and extracellular lipase enriched in planktonic secretomes. In both planktonic and biofilm secretomes, degradative enzymes and toxins made up the majority of significant proteins, representing 79.8% of planktonic proteins and 55.7% of biofilm proteins. Proteins involved in transport (16.5%), carbohydrate metabolism (8.5%), and pilus and flagellin (3.6%) contributed significantly to the biofilm secretome, while pilus and flagellin proteins (5.8%), outer membrane proteins (4.0%), and proteins involved in transcriptional regulation and electron transport (3.5%) were other significant contributors to planktonic secretomes (Figure 7). Of particular interest were the

presence of polar flagellar proteins (*AHML_09345* and *_09350*) present in higher quantities in the biofilm secretome and type I pili proteins (*AHML_2665* and *_2690*) that were present in planktonic secretomes, but absent from biofilm secretomes. Polar flagella, typically considered motility flagella, are important in adhesion and invasion in *A. hydrophila* that lack lateral flagella, such as vAh (Canals et al., 2006), while type I pili are thought to contribute to host colonization, but not host invasion (Dacanay, Boyd, Fast, Knickle, & Reith, 2010).

5. Discussion

While vAh has established itself as a primary pathogen in natural settings (Griffin et al., 2013), laboratory-cultured vAh appears to mimic its opportunistic relatives during immersion challenges. Planktonically-cultured vAh has been proven to be extremely virulent, causing death in a matter of hours in intraperitoneal injection challenges. However, models meant to mimic more natural infections including submersion and gavage have been unreliable, even when challenged with artificially high CFUs. Zhang et al. reported consistent MAS mortality was attainable in channel catfish immersion trials only following scarification and challenge with $2x10^7$ cfu/ml of planktonically-cultured vAh (Zhang, Xu, et al., 2016). This suggests that some environmental stimuli are not present in artificial broth culture, which, in pond systems, could be responsible for turning this mild-mannered opportunist into the havoc-wreaking pathogen responsible for MAS outbreaks of epidemic proportions. Since most environmental bacteria spend much of their time in biofilm, either attached to a substrate or floating as bacterial flocs (Bales et al., 2013; Davey & O'toole, 2000; Hall-Stoodley et al., 2004; Jefferson, 2004; Weinbauer & Hofle, 1998), biofilm-associated vAh may produce proteins that could increase invasiveness and allow initial colonization *in vivo* (Stewart & Franklin, 2008). The ability to form a biofilm is commonly considered a virulence factor, particularly in human disease

conditions (Reid, 1999). However, for environmental opportunistic pathogens, such as *A. hydrophila*, biofilm formation is likely an adaptive trait – part of *A. hydrophila* life history that may inadvertently increase pathogenicity or invasiveness. *Aeromonas* spp. produce extracellular enzymes to allow the organisms to feed in aquatic environs and produce adhesins that aid in the attachment and colonization of benthic surfaces (Holmes, 1996). In aquatic environments, these enzymes provide nutrients by degrading the multitude of environmental organic compounds including detritus, benthos, and animal tissues, and these enzymes are not host specific (Bales et al., 2013; Brown et al., 2012; Holmes, 1996).

Previous research reported the presence of 23 potentially toxigenic extracellular proteins in the supernatant of planktonically-cultured vAh (Pridgeon et al., 2013). Because many bacterial generalists in a natural environment are sustained largely in biofilms and not as sustained planktonic populations, a comparison of vAh proteins secreted under different conditions, including within a biofilm, could shed light on the proteins that allow attachment and invasion into the fish host. A recent survey of commercial catfish ponds found total *Aeromonas* sp. counts were significantly higher in biofilm and sediment samples as compared to water samples throughout the sampling period, and reported vAh, specifically, was present in >35% of all biofilm samples collected, leading the researchers to hypothesize that sediment and biofilms may act as vAh reservoirs (Cai et al., 2018). The work presented here found degradative activity was significantly increased in the supernatant of biofilm-associated vAh (Figures 1 and 2). Furthermore, when proteins were injected into the muscle of channel catfish, significant necrosis and cytolysis occurred within 24 hours, while secreted proteins of planktonically-cultured vAh failed to produce necrotic lesions after seven days.

To further examine how niche residency may influence virulence, we compared secreted protein profiles of vAh cultured within a TSB biofilm and planktonically in TSB media. These data revealed variability in the secretomes of the two cultures, both in complexity and quantity. The biofilm secretome contained 248 proteins, including 183 unique proteins, while planktonic secretomes contained 183 total proteins, including 101 unique proteins. Of the 82 proteins secreted under both culture conditions, at least 36 had previously been identified as putative virulence factors (Pang et al., 2015; Pridgeon et al., 2013; Rasmussen-Ivey, Figueras, McGarey, & Liles, 2016). Under both growth conditions, vAh secreted an abundance of potential virulence proteins, the majority of which were not statistically significant in differential secretion analyses. However, secretomes of vAh cultured in biofilm were significantly more varied and, in general, the proteins were secreted in higher amounts.

Assays to measure general and specific proteolytic potential of the secreted proteins revealed significant increases in both caseinolytic and elastinolytic activity in biofilm secretomes when compared to planktonic ECPs (Figures 1 and 2). A significant difference in proteolytic potential was also seen upon inspection of the secretome analysis. ROTS analysis revealed at least seven degradative proteins present in the biofilm secretomes at significantly higher observed abundance relative to planktonic secretomes. There was a 5-fold increase in elastase abundance in biofilm secretomes, with an average quantitative protein value (QPV) of 122, compared to an average QPV of 23 in planktonic secretomes. There was a 3-fold increase in the M66 - family metalloprotease *AHML_05230* in biofilm secretomes, with average QPVs of 103 and 30 in biofilm and planktonic secretomes, respectively. Both elastase and the M66 zinc metalloprotease are considered significant virulence factors of *A. hydrophila* as well as other pathogens, such as *Vibrio choleraee*, and enterohemorrhagic *Escherichia coli* (Russell &

Herwald, 2005; Yu, Worrall, & Strynadka, 2012). Five other proteolytic enzymes were significantly secreted in biofilm secretomes but were not detected in the planktonic secretomes and likely increase the overall proteolytic potential of biofilm ECPs (Figure 6, Table 1).

While the majority of the differentially secreted degradative enzymes present in the biofilm secretome were proteolytic, two important glycolytic proteins, chitinase and chitin binding protein (CBP) were found in significantly higher amounts in biofilm secretomes. While chitinase and CBP are integral in the breakdown of environmental chitin, they may also play integral roles in virulence. Though vAh has been proven capable of using chitin as a sole carbon source (Zhang et al., 2017), it seems unlikely that either laboratory growth condition would make chitinase and CBP production energetically feasible as no chitin was present in either medium. Thus, it could be hypothesized that these chitin-associated proteins play another important role in vAh fitness. In other pathogens, chitinases and CBPs are considered virulence factors not because they target chitin, per se, but because of their interactions with target substrates other than chitin. In some virulent *E. coli* and *V. cholerae*, chitinases and CBPs target host glycoproteins and glycolipids that contain N-acetylglucosamine (GlcNAc), the monomer that composes mucus (Low et al., 2013; Tran, Barnich, & Mizoguchi, 2011). Outer membrane-expressed chitinases and CBPs have also been indicated as accessory molecules responsible for initiating host cell adhesion and invasion (Bhowmick et al., 2008; Low et al., 2013; Tran et al., 2011). In a murine model, *E. coli* chitin-binding domain interacts with intestinal epithelial cells, increasing invasiveness and pathogenicity (Low et al., 2013). In *V. cholerae*, Bhowmick et al. (2008) found chitinases function to break down the GlcNAc of mucin and reported upregulation of chitinases resulted from exposure to exogenous mucin. Furthermore, the *V. cholerae* chitin binding protein, GbpA, binds specifically to the protective mucus layer of mammalian intestinal

epithelium, resulting in bacterial colonization and disease initiation. Likewise, chitinases and CBPs produced by clinical *Pseudomonas aeruginosa* strains isolated from patients with cystic fibrosis (CF) were also upregulated in response to mucin-containing sputum and likely play an integral role in primary adhesion to lung epithelium in the initiation of CF (Tran et al., 2011). In fish, the mucosal barrier covering the gills, skin, and intestinal surfaces are considered the first line of defense against invading pathogens (Dash, Das, Samal, & Thatoi, 2018; Peatman, Lange, Zhao, & Beck, 2015). The presence of chitinases and CBP may act to degrade not only the catfish slime coat, but also to bind to and degrade the epithelial mucins in the digestive tract, increasing vAh invasiveness. Peatman et al (2018) reported a direct link between feed consumption and vAh-induced MAS, with survival in vAh-challenged catfish decreasing significantly when fish were fed to satiation 4 hours prior to challenge. The mucus coating of the intestinal epithelium may decrease after eating, as ingesta moves through the digestive tract and takes mucus with it. Chitinases and CBPs may then be capable of breaking down the remaining mucus, gaining access to the underlying epithelium and, eventually, the bloodstream (Newton, personal communication, (Johansson, Sjovall, & Hansson, 2013). The presence of chitinase and CBP could help explain the intestinal epithelial damage found on necropsy in fish naturally infected with vMAS (Baumgartner et al., 2017). Although significantly higher in biofilm secretomes, chitinase and CBP was prominent in both planktonic and biofilm secretomes, suggesting they play an important role in bacterial fitness regardless of growth condition.

Whereas biofilm secretomes were flush with degradative exoenzymes, such as elastase, chitinases, and multiple Zn-dependent and metalloproteases, planktonic secretomes consistently produced more hemolytic and cytotoxic ECPs. Notably, both aerolysin-type and *ahh1*-type hemolysins were detected in much higher quantities in planktonic secretomes, as were two

extracellular serine proteases - neither of which were identified in any biofilm fraction - and extracellular lipases, all of which exert hemolytic activity against erythrocytes, and have been shown to be cytotoxic to cells (Tomas, 2012; Vences, Rivas, Lemos, Husmann, & Osorio, 2017). Interestingly, the alpha-hemolysin, phospholipid-cholesterol acyltransferase, which was present in planktonic secretomes but absent in biofilm, has been reported to produce significant lysis of salmon erythrocytes following activation by serine protease (Eggset et al., 1994). The presence of substantial amounts of both proteins in the planktonic secretomes suggests that the production of these proteins could allow a multi-pronged approach to cell death, with each toxin acting independently, but increasing in virulence upon interaction with other proteins. Aerolysin-type hemolysin has been implicated as the main virulence factor of *A. hydrophila* (Tomas, 2012), and was significantly higher in planktonic secretomes, with a three-fold increase compared to biofilm. However, ahh1-type hemolysin was present in planktonic secretomes at greater than three times the amount of aerolysin-type hemolysin. Ahh1 hemolysins are homologous to hlyA hemolysins of *V. cholerae* (Heuzenroeder, Wong, & Flower, 1999).The activity of this pore-forming hemolysin is not erythrocyte-specific, but targets erythrocytes, leukocytes, lymphocytes, and epithelial and endothelial cells in a multitude of eukaryotes (Chenal, Sotomayor-Perez, & Ladant, 2015) and, as such, are considered cytotoxins. This supports the *in vitro* hemolysis assay results that found 80% hemolysis of channel catfish erythrocytes in one hour when exposed to planktonic supernatants, versus less than 15% average hemolysis of erythrocytes incubated with biofilm supernatants (Figure 3). The presence of these hemolysins and other cytotoxins in planktonically-cultured vAh may also help explain the rapid mortality seen in catfish when challenged by intraperitoneal injection, as these bacteria may be primed to produce vast amounts of toxins *in vivo*.

Biological functions of secreted proteins as analyzed by GO found carbohydrate utilization to be the dominant function of secreted proteins under both conditions. Proteins involved in hemolysis, lipid and nucleotide catabolism, arginine biosynthesis, protein folding and transport were dominant biological functions of planktonic secretomes. Significant biofilm proteins were largely involved in transmembrane transport, amino acid processing, and transport of ions, amino acids, and carbohydrates. Interestingly, flagellar motility was also important in biofilms. This is likely due to *A. hydrophila*'s use of flagella in biofilm construction and not actually in bacterial motility (Canals et al., 2006). This increase in polar flagella may also contribute to an increased host colonization in biofilm-associated vAh. While lateral flagella are often considered imperative for biofilm production and adhesion (Gavin et al., 2003; Gavin et al., 2002), Aeromonads that lack lateral flagella are capable of using polar flagella for biofilm formation as well as cellular adhesion (Canals et al., 2006; Kirov, Castrisios, & Shaw, 2004; Lowry et al., 2014; Rabaan, Gryllos, Tomás, & Shaw, 2001). The increased polar flagella required for biofilm formation could act secondarily as adhesins when biofilm bacteria come into contact with catfish and could act in concert with other secreted invasins to colonize and destroy host mucosal barriers.

The research presented here represents the first comparison of the secreted proteomes of vAh produced in two distinct ecological niches. These data give insight into the adaptive physiological response of vAh based solely on growth condition and increase understanding of how environmental niche partitioning could affect vAh pathogenicity and virulence. Increased secretion of colonization factors and degradative enzymes in biofilm growth may increase bacterial attachment and host invasiveness, while increased secretion of hemolysins, porins, and other potential toxins under planktonic growth could result in increased host mortality.

Functional protein screening using *in vitro* methods appear to function well as a screening tool to measure virulence, as degradative proteolytic screening were supported by both *in vivo* challenge results as well as the presence of significantly increased degradative proteins, particularly elastase, in biofilm secretomes. Furthermore, the *in vitro* screening for toxigenic potential was supported by significantly increased hemolysins and other potentially cytolytic proteins in the planktonic secretomes.

While the secretome analyses conducted in the current work highlighted the variability in the secretion of potential virulence proteins, the degree in which central niche occupation holds over gene expression is currently unknown. Future research will focus on transcription analyses of vAh grown under these same conditions. Taken together, these data may help unmask the role of vAh growth in vAh-induced MAS.

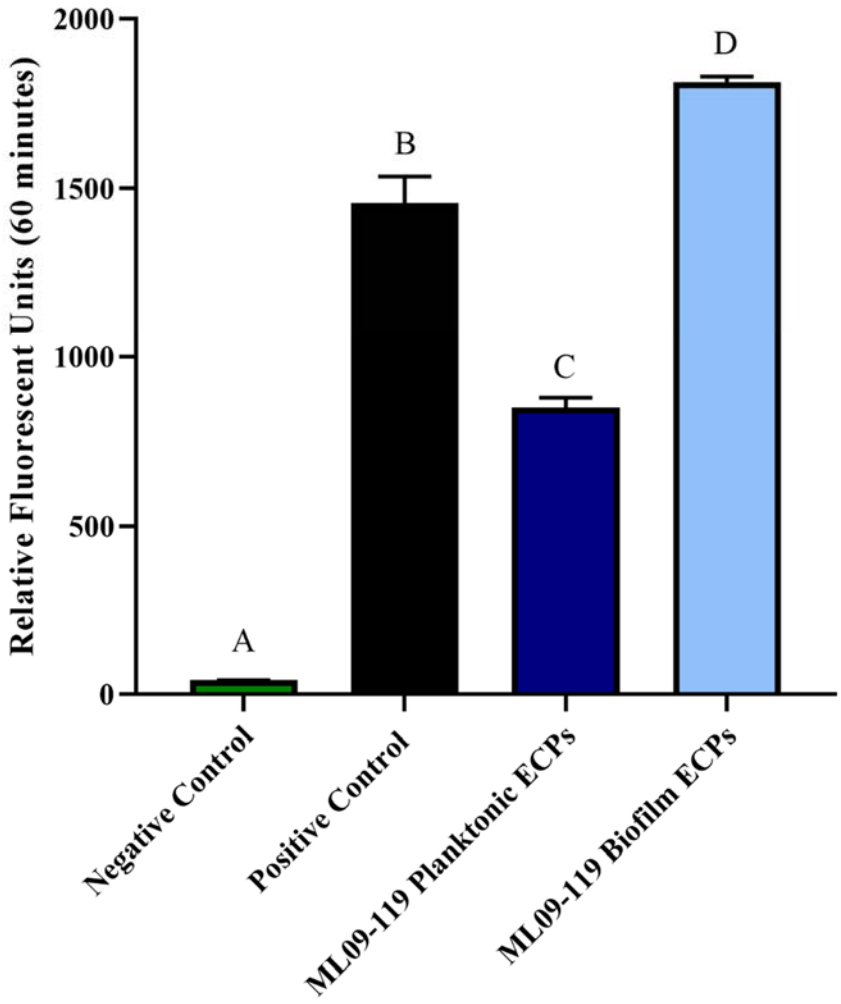

Figure 1. General proteolytic potential of vAh proteins secreted under biofilm and planktonic growth. The general proteolytic potential of biofilm and plankonic secretomes was measure using HiLyteFluor 488-labeled casein as a substrate. Secreted protein from each condition was incubated at 30°C with labeled casein and fluorescent intensity was measured at Ex/Em = 490nm/520nm every five minutes for one hour. Data were plotted as relative fluorescence units versus time for each sample. Three individual experiments were performed, and all samples were performed in triplicate. Statistical analysis consisted of one-way ANOVA followed by Tukey's multiple comparisons post-test with significance set at $p < 0.05$.

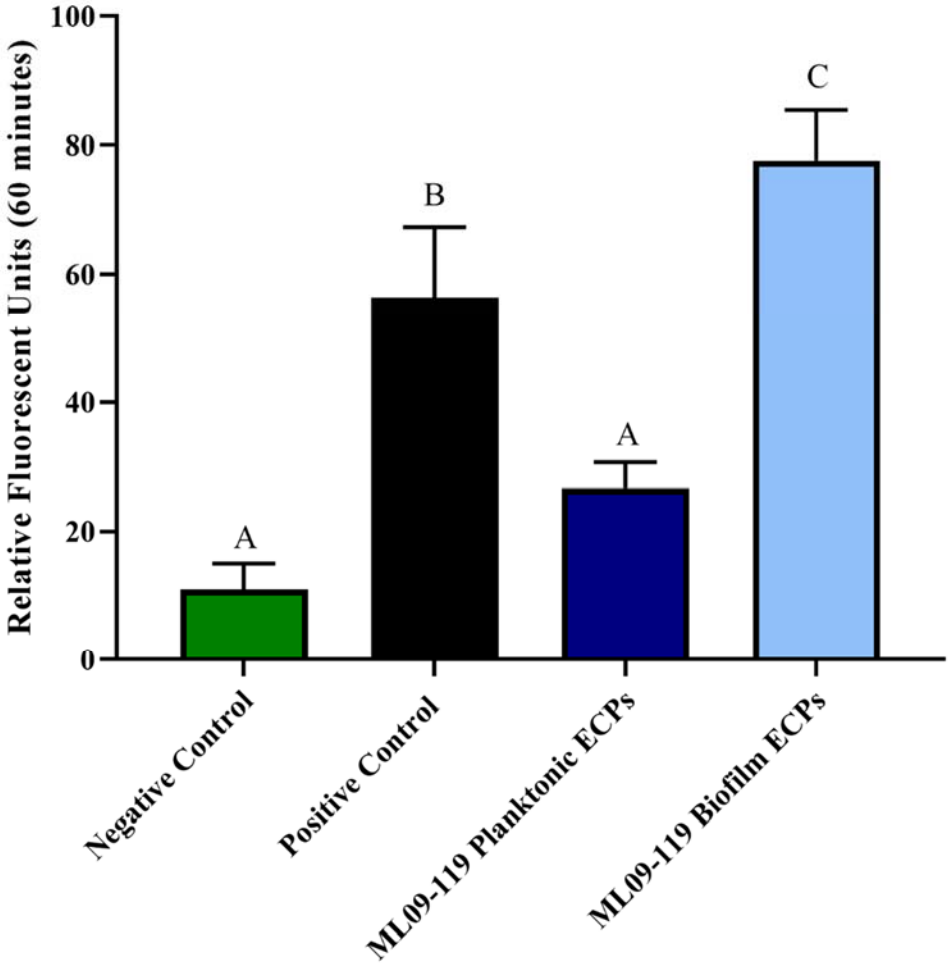

Figure 2. Elastase-specific degradative potential of vAh proteins secreted under biofilm and planktonic growth. The elastase activity of biofilm and plankonic secretomes was measure using 5-FAM/QXL™ 520-labeled elastin as a substrate. Secreted protein from each condition was incubated at 30°C with labeled elastin and fluorescent intensity was measured at Ex/Em = 490nm/520nm every five minutes for one hour. Data were plotted as relative fluorescence units versus time for each sample. Three individual experiments were performed, and all samples were performed in triplicate. Statistical analysis consisted of one-way ANOVA followed by Tukey's multiple comparisons post-test with significance set at $p < 0.05$.

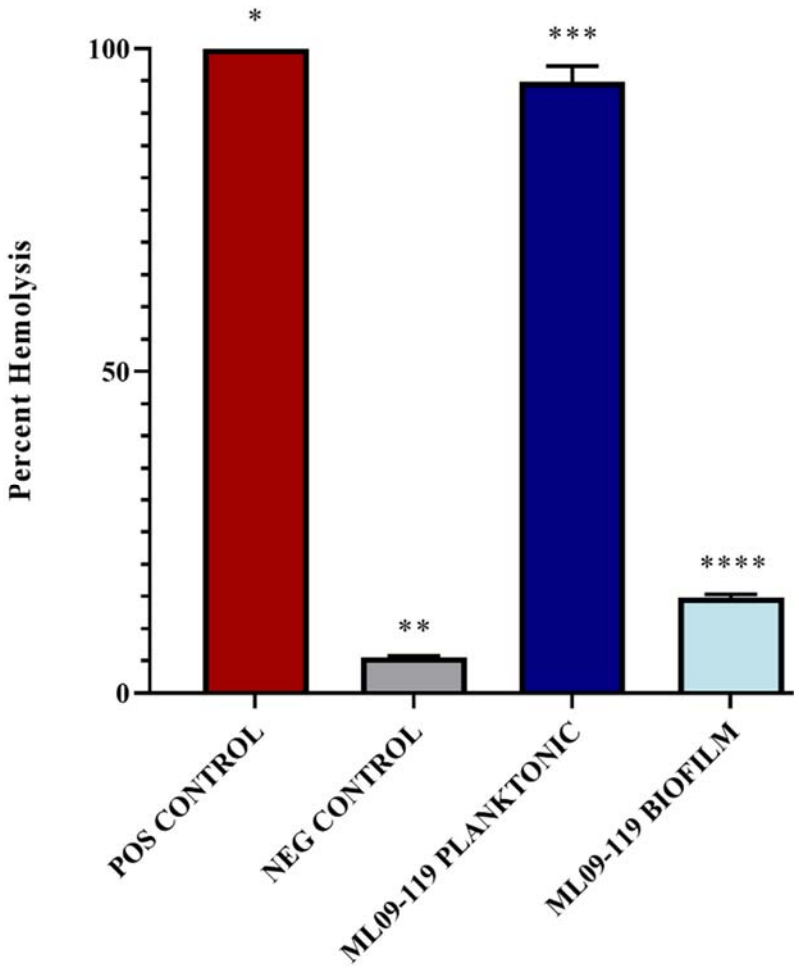

Figure 3. Hemolytic potential of vAh proteins secreted under biofilm and planktonic growth. The hemolytic ability of vAh secreted proteins was measured using channel catfish erythrocytes as the substrate. 2.5µg secreted proteins from each culture condition was incubated with 25µl catfish blood diluted 1:10 in sterile PBS. at 30°C with shaking. Sterile, deionized water served as positive control and sterile PBS served as a negative control. Lysis was calculated by measuring sample absorbance at 415nm, and reported as percent positive control. All samples were asayed in triplicate. Statistical analysis consisted of one-way ANOVA followed by Tukey's multiple comparisons post-test with significance set at $p < 0.05$.

Figure 4. Gross lesions of channel catfish injected with biofilm-cultured vAh secreted proteins 24 hours post-injection. To determine *in vivo* degradative potential of vAh secreted proteins, 10µg of total protein from each condition was injected intramuscularly into channel catfish fingerling. Sterile PBS was used as injection control. After 24 hours, all fish injected with biofilm-associated ECPs had developed large, necrotic lesions at the injection site. No gross lesions developed after 7 days in any control fish or in any fish injected with planktonic EPCs. TOP – Representative Control, BOTTOM – 3 Representative Biofilm-ECP injected fish

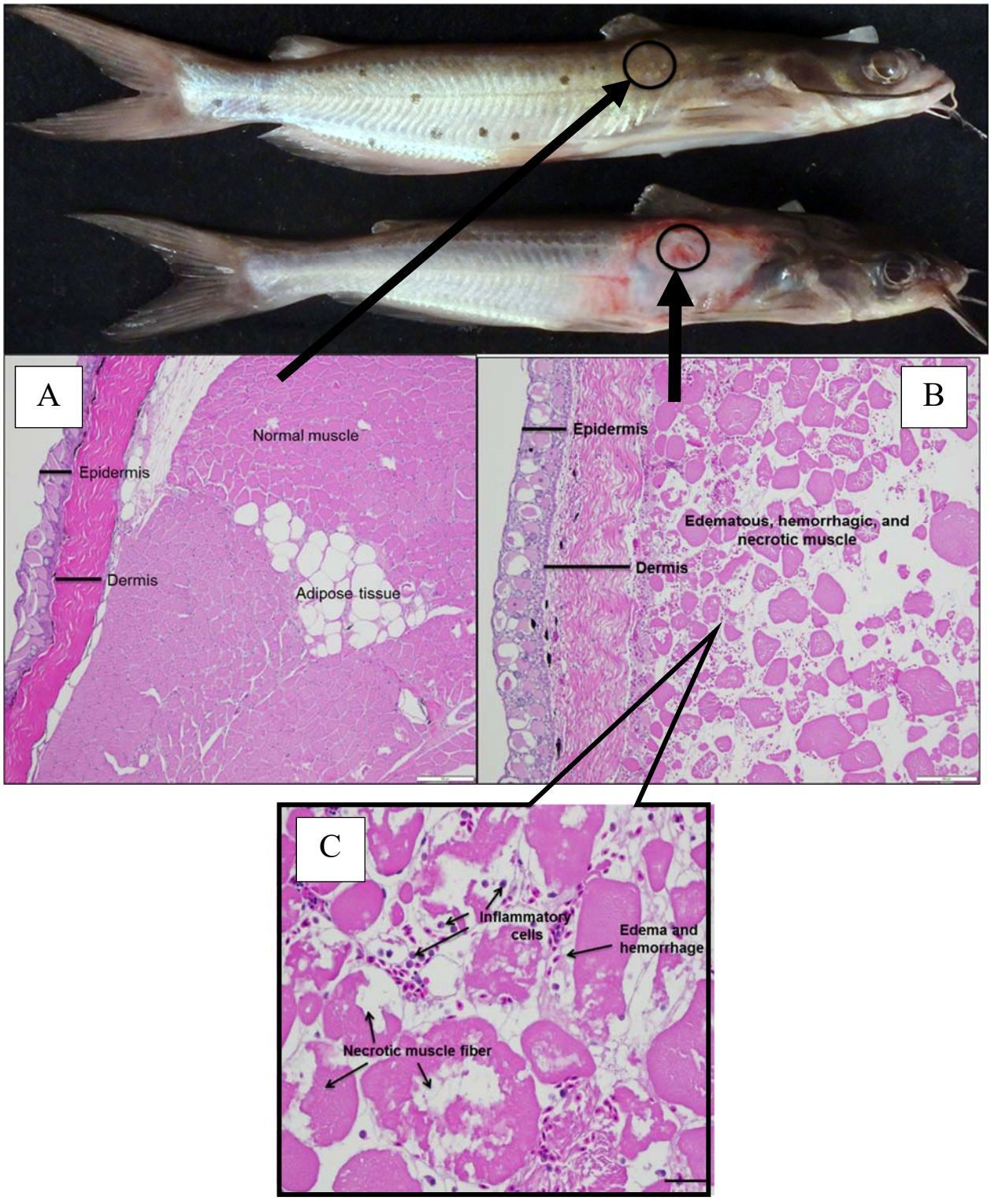

Figure 5. Histopathology of channel catfish muscle following intramuscular injection with vAh secreted proteins. Histologic sections prepared from paraffin-embedded tissues were stained with

hematotoxylin and eosin. (A) Control – No perceptible damage to skin, subcutaneous adipose, or muscle. Fish injected with planktonic ECPs were indistinguishable from controls. (B) Biofilm-injected fish tissue (10x) 24 hours post-injection. Tissue was edematous, hemorrhagic, and necrotic at the injection site. Despite substantial tissue damage, few inflammatory cells were present. (C) Biofilm-injected muscle (40x) showing detailed myonecrosis, edema, and hemorrhage.

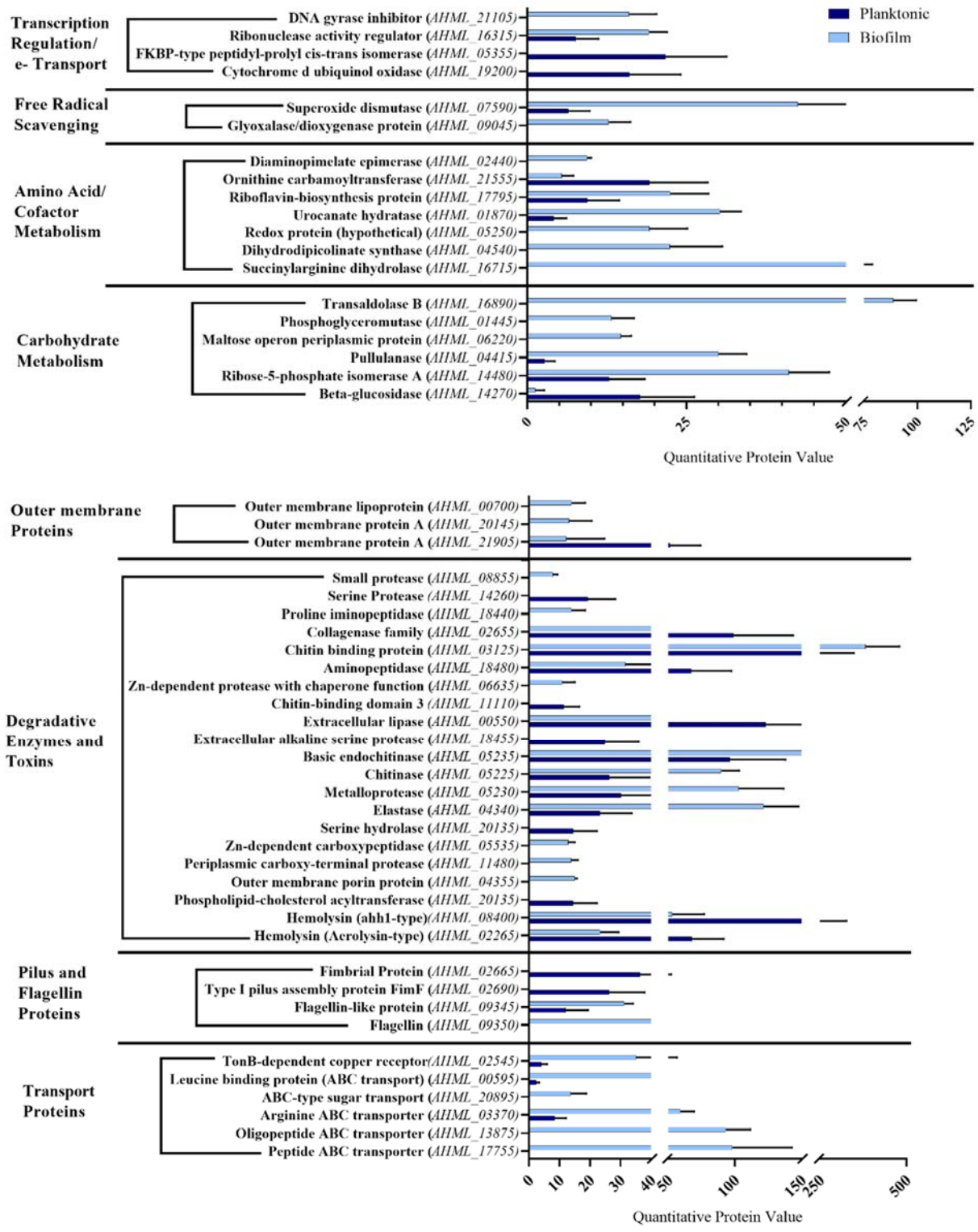

Figure 6. Primary biological function of differentially secreted vAh proteins. Protein function was assessed by gene ontology analyses and grouped into eight functional categories.

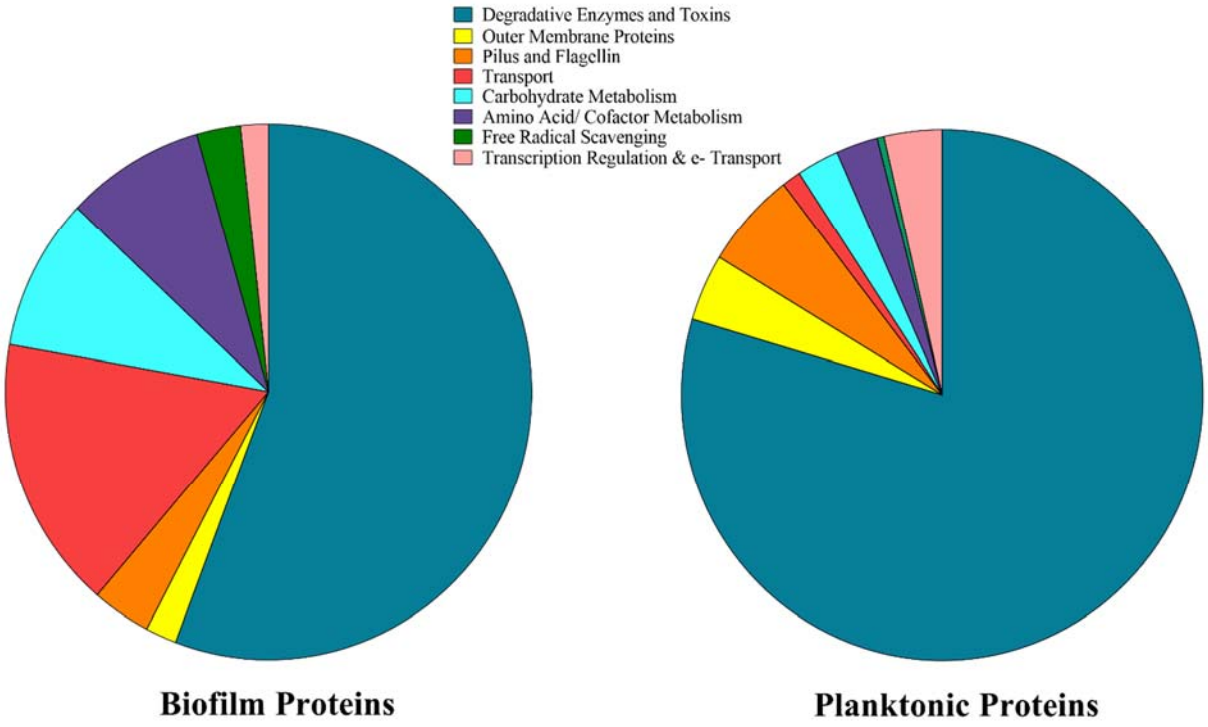

Figure 7. Functional categories of differentially secreted proteins from vAh cultured planktonically and within a biofilm. Proteins were grouped into eight functional categories based on their gene ontology annotation and plotted as part-of-a-whole. In both biofilm and planktonic secretomes, differentially secreted proteins were dominated by degradative enzymes and toxins.

Table 1. Differentially secreted proteins of ML09-119 cultured planktonically (TSB) and within a biofilm (BIO). Protein abundance fold change marked with * denotes protein identified in only one condition and is reported as the average Quantitative Protein Value. FDR = False Discovery rate

Secreted Protein	Locus Tag	ROTS-Statistic	*p* value	FDR	Protein Abundance Fold Change	Significant Experimental Group	Quantitative Protein Value TSB	Quantitative Protein Value BIO
Peptide ABC transporter	AHML_17755	3.2	0.00	0	98*	BIO	0	98
Oligopeptide ABC transporter	AHML_13875	6.05	0.00	0	93*	BIO	0	93
Transaldolase B	AHML_16890	8.08	0.00	0	89*	BIO	0	89
Succinylarginine dihydrolase	AHML_16715	5.49	0.00	0	66*	BIO	0	66
Flagellin	AHML_09350	5.42	0.00	0	43*	BIO	0	43
Dihydrodipicolinate synthase	AHML_04540	2.35	0.01	0	22*	BIO	0	22
Redox protein (hypothetical)	AHML_05250	2.33	0.01	0	19*	BIO	0	19
Leucine binding protein (ABC transport)	AHML_00595	3.74	0.00	0	18	BIO	2	41
DNA gyrase inhibitor	AHML_21105	2.2	0.01	0	16*	BIO	0	16
Outer membrane porin protein	AHML_04355	2.91	0.01	0	15*	BIO	0	15
Maltose operon periplasmic protein	AHML_06220	2.57	0.01	0	15*	BIO	0	15
Periplasmic carboxy-terminal protease	AHML_11480	2.28	0.01	0	14*	BIO	0	14
Phosphoglyceromutase	AHML_01445	1.94	0.01	0	13*	BIO	0	13
Zn-dependent carboxypeptidase	AHML_05535	2.15	0.01	0	13*	BIO	0	13
Pullulanase	AHML_04415	3.62	0.00	0	10	BIO	3	30
Urocanate hydratase	AHML_01870	3.71	0.00	0	8	BIO	4	30
Arginine ABC transporter	AHML_03370	4.58	0.00	0	7	BIO	8	59
Superoxide dismutase	AHML_07590	2.95	0.01	0	6	BIO	7	43
Elastase	AHML_04340	4.68	0.00	0	5	BIO	23	122
Chitinase	AHML_05225	4.07	0.00	0	3	BIO	26	90
Metalloprotease	AHML_05230	2.73	0.01	0	3	BIO	30	103
Basic endochitinase	AHML_05235	3.15	0.01	0	3	BIO	25	73
Ribose-5-phosphate isomerase A	AHML_14480	2.92	0.01	0	3	BIO	13	41
Flagellin-like protein	AHML_09345	2.05	0.01	0	2.6	BIO	12	32
Outer membrane protein A	AHML_21905	-1.99	0.01	0	4	TSB	52	12
Hemolysin (Aerolysin-type)	AHML_02265	-2.37	0.01	0	3	TSB	68	23
Hemolysin (ahh1-type)	AHML_08400	-2.69	0.01	0	4	TSB	227	53

Secreted Protein	Locus Tag	ROTS-Statistic	*p* value	FDR	Protein Abundance Fold Change	Significant Experimental Group	Quantitative Protein Value TSB	Quantitative Protein Value BIO
FKBP-type peptidyl-prolyl cis-trans isomerase	AHML_05355	-2.10	0.01	0	22*	TSB	22	0
Extracellular alkaline serine protease	AHML_18455	-2.22	0.01	0	25*	TSB	25	0
Type I pilus assembly protein FimF	AHML_02690	-2.29	0.01	0	26*	TSB	26	0
Fimbrial Protein	AHML_02665	-2.61	0.01	0	36*	TSB	36	0
Extracellular lipase	AHML_00550	-4.16	0.01	0	3	TSB	123	41
Chitin-binding domain 3	AHML_11110	-1.50	0.02	0.02	12	TSB	12	0
Zn-dependent protease with chaperone function	AHML_06635	1.51	0.02	0.02	11*	BIO	0	11
Ribonuclease activity regulator	AHML_16315	1.55	0.02	0.02	2.5	BIO	8	19
Phospholipid-cholesterol acyltransferase	AHML_20135	-1.55	0.02	0.02	14*	TSB	14	0
Aminopeptidase	AHML_18480	-1.55	0.02	0.02	2	TSB	67	32
Outer membrane lipoprotein	AHML_00700	-1.58	0.02	0.02	13*	TSB	13	0
Chitin binding protein	AHML_03125	1.59	0.02	0.02	1.6	BIO	243	382
Beta-glucosidase	AHML_14270	-1.68	0.02	0.0	14	TSB	18	1.25
Cytochrome d ubiquinol oxidase	AHML_19200	-1.71	0.02	0.02	16	TSB	16	0
ABC-type sugar transport	AHML_20895	1.77	0.02	0.02	14*	BIO	0	14
TonB-dependent copper receptor	AHML_02545	1.79	0.02	0.02	9	BIO	4	35
Diaminopimelate epimerase	AHML_02440	1.82	0.02	0.03	9*	BIO	0	9
Proline iminopeptidase	AHML_18440	1.88	0.01	0.03	14*	BIO	0	14
Glyoxalase/dioxygenase protein	AHML_09045	1.89	0.01	0.03	13*	BIO	0	13
Collagenase family	AHML_02655	-1.90	0.01	0.03	2.5	TSB	99	40
Serine Protease	AHML_14260	-1.91	0.01	0.03	19	TSB	19	0
Outer membrane protein A	AHML_20145	1.46	0.02	0.04	13*	BIO	0	13
Ornithine carbamoyltransferase	AHML_21555	-1.35	0.03	0.05	3.5	TSB	19	5
Small protease	AHML_08855	1.37	0.03	0.05	8*	BIO	0	8
Riboflavin-biosynthesis protein	AHML_17795	1.4	0.02	0.05	2.4	BIO	9	22

Chapter III

Comparative gene expression of putative virulence factors in planktonic and biofilm-associated cultures of virulent *Aeromonas hydrophila*

1. Abstract

Virulent *Aeromonas hydrophila* (vAh) is an emerging bacterial pathogen in farmed catfish production systems in the Southeastern US. vAh-induced outbreaks of motile aeromonad septicemia (vMAS) have reached epidemic proportions, with pond mortalities often in excess of 60%. *A. hydrophila* exploits a variety of ecological habitats and hosts; however, it is unclear how niche dynamics influence vAh virulence. Previous research revealed significant variability in the extracellular proteins of vAh cultured planktonically and within a biofilm. In order to explore how these changes in gene expression relate to virulence, the transcription of putative virulence factors was assessed under each growth condition. Differential expression analysis of the RNA-Seq results supported the hypothesis that niche occupancy plays a vital role in gene expression, with 35% of all genes differentially expressed. Upregulation of putative virulence genes related to motility, adhesion, iron binding, antibiotic resistance, and proteolysis was observed in biofilm-cultured vAh, while genes related to porins, toxins, O-antigen, and enzymes related to LPS production were upregulated in planktonically-cultured vAh. These data suggest that vAh residing in biofilms *in natura* may be capable of survival, adhesion, and destruction of physical barriers, facilitating host invasion and increasing infectivity, while planktonic growth may prime vAh to evade host defense mechanisms and cause septicemia *in vivo*.

2. Introduction

Bacterial gene expression is intimately tied to the environment, particularly for bacterial generalists. While specialists have evolved specific adaptations required to survive in a defined niche, generalists are metabolically diverse and capable of inhabiting multiple environmental niches thank to phenotypic plasticity (Chen et al., 2020). Plastic response relies upon sensing and responding to ever-present fluctuations in biotic and abiotic environmental stimuli, and relies upon a host of sensor kinase receptors on the cell surface that trigger response regulators, which respond by initiation or repression of transcription. Many times, regulons controlling multiple genes or operons are involved in environmental adaptation, switching on and off clusters of genes. Bacteria are not only capable of sensing physicochemical fluctuations; they are also capable of tactile sensing (Harapanahalli, Younes, Allan, van der Mei, & Busscher, 2015; Hug, Deshpande, Sprecher, Pfohl, & Jenal, 2017). Tactile sensing allows substrate recognition of biotic and abiotic surfaces and triggers the production of products necessary for survival on, or avoidance of, the surface. Biofilm formation relies on both chemosensory and mechanosensory signals, from the environment and through cell-to-cell contact communication.

Bacterial generalists in aquatic environments are often resident in biofilms, either attached to some submerged structure or floating in the water column as flocs (Balcázar et al., 2015; Davey & O'toole, 2000). If this is the case, it is likely that many bacterial-host interactions occur when bacteria are in biofilms. In biofilm growth, increased production of adhesins and degradative enzymes could inadvertently increase pathogenicity in opportunistic pathogens by increasing potential for host colonization and barrier destruction. The emergent properties inherent in biofilms make their study invaluable in determining mechanisms of pathogenesis.

Since 2009, virulent *Aeromonas hydrophila* (vAh) has emerged as a major threat to catfish aquaculture in the Southeastern US. Unlike traditional *A. hydrophila*, which causes disease with limited mortality as a secondary pathogen, vAh can act as the primary pathogen, inducing motile aeromonad septicemia (vMAS) with significant mortality, often greater than 60%. vAh have the genetic capacity to produce a multitude of virulence determinants, many of which have been previously defined (Pang et al., 2015; Pridgeon et al., 2013; Rasmussen-Ivey, Figueras, et al., 2016). Previous work in this lab found significant differences in the secreted protein profiles of vAh when cultured within a biofilm versus growing as free-living, planktonic cells. However, not all virulence factors are secreted proteins. In a continued effort to determine the role of niche dynamics in the pathogenicity of vAh, comparative transcriptomics, focusing on previously identified putative virulence factors, was performed on biofilm and planktonically-cultured vAh.

3. Methods and Materials

Bacterial Strain. vAh strain ML09-119 was isolated from a diseased channel catfish, *Ictalurus punctatus*, from a MAS outbreak in a West Alabama aquaculture facility in 2009. Molecular characterization and genome sequencing of vAh ML09-119 were performed (Tekedar et al., 2013) and the gene sequence deposited into GenBank (Accession CP005966). Aliquots of vAh ML09-119 were cryogenically stored in 10% glycerol freeze medium at -80°C.

Culture Media and Culture Conditions. Tryptic soy broth (TSB) (Bacto TSB, BD) prepared according to manufacturer's directions was used as the culture medium for planktonic growth.

Biofilm media was prepared by adding 0.2% agar powder (AlfaAesar) to TSB media prior to sterilization. Approximately 70ml of molten biofilm agar was poured into deep well petri dishes (Fisher) and allowed to solidify.

Bacterial strain vAh ML09-119 was removed from cryogenic storage and inoculated into 25ml TSB media and grown overnight at 30°C with shaking. An aliquot of overnight culture was transferred to 70ml of TSB and grown at 30°C on an orbital shaker to mid-log phase, approximately 16 hours. Biofilm agar plates were inoculated from overnight culture by stab inoculation. Plates were sealed with parafilm and incubated at 30°C for 72 hours, until bacterial film covered and adhered to the agar surface.

Planktonic and biofilm cultures were performed in triplicate.

RNA Isolation. RNA was isolated from planktonic cells and biofilm cells using MOBio UltraClean Microbial RNA Isolation kit and MOBio PowerBiofilm RNA Extraction kit (MO Bio Laboratories, Inc.), respectively.

RNA was isolated from 1.8 ml planktonic culture following manufacturer's protocol and was eluted in 50µl RNase-free water. RNA was then treated with DNase I using RNA Clean & Concentrator -5 (Zymo Research) following manufacturer's protocol, and DNA-free RNA was eluted in 15µl RNase-free water.

RNA was extracted from 0.07 – 0.1g biofilm-cultured cells. Biofilm cells were peeled from the surface of the plate with a sterile cell scraper, and RNA was extracted following manufacturer's instructions. RNA was eluted with 100µl RNase-free water.

rRNA depletion was performed on each RNA sample using Illumina Ribo-Zero Bacterial rRNA Removal Kit for Gram-Negative bacteria (Illumina) following manufacturer's protocol. Depleted

RNA was recovered in ~90µl supernatant. Depleted RNA was then purified and concentrated using RNA Clean & Concentrator -5, as above, and large and small RNAs were eluted.

Sequencing. Purified rRNA-depleted RNA samples were submitted to the Auburn University Genomic and Sequencing Laboratory for library preparation and pair-end sequencing. Sequencing was performed on Illumina HiSeq System.

Read Processing. Reads were mate-paired using Set Paired Reads in Geneious R10.2.2 software (www.geneious.com). Adapter removal and quality trimming were performed in Geneious R10.2.2 using the BBDUK plug-in with an error probability set at 0.01 and a minimum read length of 50bp. Reads were then aligned to the vAh ML09-119 reference genome (NCBI Accession: CP005966.1) and mapped with five iterations using Geneious R10 assembler.

Read Counting. Raw read counts were determined for each sample using the Annotate and Predict function in Geneious R10. Raw read counts were exported as .csv file for processing in R (R Core Team (2017)) and RStudio (RStudio Team (2017)).

Raw reads were normalized using a relative log expression normalization in the DESeq2 package (v 1.17). Dispersion estimates were performed on normalized reads (Figure 1). Blind regularized log (rlog) transformation was applied to normalized count matrix and a PCA plot was generated to check sample variance within and between experimental groups (Figure 2). Sample-to-sample distances were calculated using the rlog-transformed data and a distance matrix heatmap was generated using RColorBrewer package (version 1.1.2) (Figure 3).

Differential Expression Analysis. Based on dispersion estimates, differential gene expression was calculated from normalized reads using a local fit type. Resulting log fold changes were shrunk to further minimize the impact of large fold changes due to low gene counts. MA and

volcano plots were generated to visualize gene expression log fold change and significance between the two experimental groups (Figure 4 and 5).

Comparison of gene expression of putative vAh virulence factors. Differential expression results were exported to Excel and putative virulence genes were selected using vAh locus tags for genes previously identified as potential vAh virulence factors (Pang et al., 2015; Pridgeon et al., 2013; Rasmussen-Ivey, Figueras, et al., 2016; Zhang et al., 2017) (Table 2). These data were imported into R and an MA plot was generated to compare expression of putative virulence genes between experimental groups (Figure 6). Virulence genes were then separated into one of 15 classes, and MA plots were generated to compare gene expression of each virulence class between experimental groups (Figure 7).

4. Results

Sequencing and Read Processing. The number of mapped reads for all planktonic replicates exceeded 16 million, with quality values at ≥ Q20 of 99%, and ≥ Q30 of 97%. Other quality scores, such as pairwise % identity and confidence means were also high (Table 1). Mapped sequences of biofilm replicates were highly variable, with mapped read counts ranging from 6.7 million to 39 million reads. Ref-Seq % scores were lower, as well, with values ranging from 71% - 93%. Quality values, though slightly lower than planktonic samples, were still high, with ≥ Q20 of 98% and ≥ Q30 of 97% (Table 1). While sequencing depth of 10 million mapped reads was the target, 5 – 10 million reads from rRNA-depleted libraries are sufficient to profile approximately 90% of transcriptional activity in *V. cholerae.* Furthermore, differentially expressed virulence genes were identifiable with high statistical significance with as few as 2 – 3 million mapped reads, and with relatively low between-replicate correlation (Haas, Chin, Nusbaum, Birren, & Livny, 2012).

Read Counting. Raw read counting was performed in Geneious R10, and reads were processed in R and RStudio. Because of the between-replicate variability, raw reads were normalized using the DESeq2 method. The DESeq2 program uses relative log expression normalization, which adjusts for differences in sequencing depth and for differences in library composition, taking into account genes that are only expressed under one condition. The use of log averages and median values minimize the impact of extreme gene values that result from high or low outliers (Love, Huber, & Anders, 2014). After normalization, rlog transformation was applied to count matrix to further minimize differences between genes with low counts and to further normalize counts with respect to library size. Rlog transformation acts to further stabilize variance within and between experimental groups. From rlog-transformed counts, PCA (Figure 2) and sample-to-sample distances (Figure 3) were estimated. PCA analysis revealed a total variance of 79%, with 54% of variance between planktonic and biofilm counts attributable to culture condition and 25% of variance attributed to between-replicate variation. Sample-to-sample distance confirmed clustering of planktonic replicates, with increased distance between biofilm replicates. The increased variability between biofilm replicates is likely due to the inherent variability in biofilm bacteria. Biofilm formation is a dynamic process and mature biofilms consist of bacterial cells in multiple stages of growth, and cell growth phase is an important variable in gene expression. Gene expression and cell growth are interconnected in multiple ways, both passive and growth-rate dependent (Klumpp & Hwa, 2014) and phase variation, a hallmark of biofilm bacteria, further adds to the within-sample variability (Beitelshees, Hill, Jones, & Pfeifer, 2018). While these phenomena increase transcriptional complexity and, thus, sample to sample variation, this transcriptional complexity also increases biofilm bacterial persistence, fitness, and, for

pathogenic organisms, virulence (Martínez & Vadyvaloo, 2014). This variation underscores the need for comparative transcriptomics between sessile and free-living bacteria.

Differential Expression Analysis. Dispersion estimates were performed in order to select the best fit for calculation of gene expression from normalized reads (Figure 1), and a local fit type was selected. DESeq2 categorizes expression values > 0 and < 0 with $p < 0.05$ to be significant. Based on this methodology, differential expression analysis of 4357 genes revealed upregulation of 1060 genes (24%) in biofilm transcriptomes and upregulation of 1145 genes (26%) in planktonic transcriptomes, with 225 genes (5.2%) discarded as outliers. However, fold changes ≥ 2, which corresponds to Log_2 fold change (L_2FC) of 1 and -1 with $p < 0.05$, are generally accepted as statistically significantly for transcriptome analysis. At the level of $L_2FC \geq 1, \leq -1$ corresponding to up and down regulation, 739 genes (17%) were upregulated in biofilm transcriptomes and 774 genes (18%) were downregulated in biofilm transcriptomes. These results indicate 35% of all genes were differentially expressed at fold change ≥ 2 based on culture condition. Differentially expressed genes (DEG) between experimental groups were visualized by MA and volcano plots (Figures 4 and 5).

Comparative Gene Expression of Putative Virulence Factors. To explore the role of niche occupation on vAh virulence, gene expression of 165 previously-identified putative vAh virulence factors (PVF) was compared (Table 2, Figure 6). In biofilm trancriptomes, 30 PVF genes (18%) were upregulated, while 17 PVF genes were upregulated in planktonic transcriptomes. The majority of PVF genes, 118 (72%), were not differentially expressed. However, lack of differential expression did not correlate to low expression values and many PVF genes were highly expressed under both conditions. Overall, biofilm transcriptomes were upregulated in seven virulence classes, including amonabactin, antibiotic resistance, enzymes,

Flp pilus, heme uptake, polar flagella, and siderophores. Planktonic PVF upregulation primarily occurred in three virulence classes, including porins, toxins, and type IV pilus. The class designated vAh Virulence included vAh strain-specific virulence factors previously defined by Pridgeon et al. (2013). In this virulence class, differential expression of multiple PVFs was seen under both conditions. Even when not differentially expressed, there was substantial expression of many these genes found under both growth conditions (Figure 7).

To determine if secretome values accurately represented gene expression, gene expression values were compared to previously identified differentially secreted biofilm and planktonic proteins. Of 53 significant secretome proteins, 38 (72%) were differentially expressed in the corresponding culture condition. Gene expression of 15 proteins (28%) did not correlate to secretome analysis. However, of those 15 proteins, six corresponding genes did not show differential expression between the two culture classes. Furthermore, four proteins identified only in biofilm secretome but differentially expressed in planktonic genomes (outer membrane porins, outer membrane proteins, flagellins, and ABC transporters) were membrane-bound or periplasmic proteins that were likely released into biofilm secretomes by mechanical stress placed on biofilm organisms during secretome preparation.

5. Discussion

Variability in gene expression profiles between biofilm and planktonic bacteria has been documented in multiple bacterial species (Beenken et al., 2004; Dötsch et al., 2012; Levipan & Avendano-Herrera, 2017; Levipan, Quezada, & Avendaño-Herrera, 2018; Safadi et al., 2012; Sánchez et al., 2019; Schembri, Kjaergaard, & Klemm, 2003; Stanley, Britton, Grossman, & Lazazzera, 2003). However, the degree of transcriptome variation between sessile and planktonic growth modes appears inconsistent between, and sometimes within, species.

Comparative transcriptomic analysis of sessile and free-living *Bacillus subtilus* found differential expression of approximately 6% of genes in mature biofilms (Stanley et al., 2003). An early report comparing *Pseudomonas aeruginosa* growing planktonically and within a biofilm (Whiteley et al., 2001) reported only 73 genes, approximately 1%, were differentially expressed in biofilm culture. More recent transcriptomics of *P. aeruginosa* reported that despite substantial similarities in gene expression under both growth classes, there were significant differences in many genes, suggesting that certain gene expression profiles are likely a distinct adaptive response to growth niche occupation (Bielecki et al., 2011; Dötsch et al., 2012). Safadi et al. (2012) reported that when compared to planktonic cultures, biofilm growth of a particularly virulent strain of *E. coli,* 0104:H4, resulted in enhanced virulence gene expression and increased the likelihood for renal damage in a murine model. The authors suggested that while the primary objective of biofilm growth is environmental survival and persistence, overexpression of biofilm-related genes inadvertently increase virulence. This hypothesis was supported by comparative genomics in *Staphylococcus aureus*, which found that the group of biofilm-associated DEGs were involved in bacterial persistence, which inadvertently increased pathogenicity by decreasing antibiotic sensitivity and by increasing acid resistance (Beenken et al., 2004). Likewise, two recent studies involving transcriptome comparisons of the fish pathogen *Flavobacterium psychrophilum*, reported that between 15% and 18% of genes were differentially expressed during biofilm growth, and that biofilm growth transcriptionally enhanced virulence in *F. psychrophilum* (Levipan & Avendano-Herrera, 2017; Levipan et al., 2018).

The data presented herein indicate that overall vAh gene expression is heavily influenced by niche occupation, and that transcription response to biofilm cells differs substantially from their planktonic counterpart, with 35% of all genes displaying differential expression with FC ≥

2. This amount of transcriptome variation is much higher than reported from other bacterial species. However, the majority of transcriptome comparisons between biofilm and planktonic cultures have been performed on clinical isolates, which likely have more host-specific adaptations than environmental isolates. On the other hand, *A. hydrophila* is a master of genetic manipulation, and this phenotypic plasticity is likely responsible for the aquatic ubiquity of the organism. *A. hydrophila* is capable of causing disease in a host of vertebrates, crustaceans, mealworms, and even amoebae, as well as inhabiting intestinal tracts of leeches in a mutualistic association (Romero et al., 2016). It stands to reason, then, that the extent of phenotypic variability would be much higher in an organism equipped to survive in multitudinous environments.

The aim of the current study was to characterize vAh gene expression under two common niche lifestyles, within a mature biofilm and as free-living cells, and to determine the potential virulence of each vAh phenotype by evaluating differential expression of putative virulence genes. Putative virulence genes were selected from previously published data (Pang et al., 2015; Pridgeon et al., 2013; Rasmussen-Ivey, Figueras, et al., 2016; Zhang et al., 2017) but, in no way should be considered an exhaustive list. These PVF genes were separated into 15 virulence families (Figure 7, Table 2) in an attempt to determine how biofilm or planktonic niche occupation could affect vAh virulence. No significant differential expression was found in four groups: MSH pilus, O-antigen, TAP pilus, and quorum sensing. Increased expression in planktonic samples was found in Type IV pilus and porins. Upregulation of genes in biofilm occurred in six groups, including amonabactin, antibiotic resistance, FLP pilus, heme uptake, polar flagella, and siderophores. Upregulation of certain PVF genes was seen under both growth conditions in enzymes, toxins, and vAh virulence groups. With few exceptions, even when

expression was not statistically significant, PVF gene expression was high in both biofilm and planktonic samples.

Based on these results, it appears that iron sequestration and uptake are of paramount importance to biofilm-associated vAh. Along with a heme uptake system, vAh possess a catechol siderophore, amonabactin, and a pyoverdine – like siderophore, as well as multiple siderophore receptors. The role of iron acquisition in bacterial pathogenicity has been well studied, and the consensus that iron scavenging and heme uptake systems function as important, and sometimes obligate, virulence factors has long been established (Cassat & Skaar, 2013; Parrow, Fleming, & Minnick, 2013; Richard, Kelley, & Johnson, 2019; Russell & Herwald, 2005; Skaar, 2010; Zughaier & Cornelis, 2018). While no less important for bacterial niche adaptation, less consideration has been given to the necessity of iron in biofilm development and maintenance in environmental isolates. While the biofilm-iron interactions are not well understood, research has consistently shown the crucial role of iron in biofilm. This interplay has been studied in several ESKAPE species, particularly *P. aeruginosa* (Post, Shapiro, & Wuest, 2019). In *P. aeruginosa* iron transport by the siderophore, pyoverdine, is crucial for biofilm development and maintenance, and chelation or iron replacement substantially reduces biofilm formation and destroys established biofilm (Banin, Brady, & Greenberg, 2006; Kang & Kirienko, 2018; Singh, Parsek, Greenberg, & Welsh, 2002; Smith, Lamont, Anderson, & Reid, 2013). In *E. coli*, iron appears to regulate type I fimbria expression and, thus, decreased attachment and biofilm dispersal occurs under iron-limiting conditions (Wu & Outten, 2009). Iron limitation has also been reported to prevent initial adhesion and biofilm formation in *Staphylococcus aureus* by reducing adhesin production, and that biofilm formation is restored after the addition of Fe_2SO_4 (Lin, Shu, Huang, & Cheng, 2012). The abundance of siderophores in biofilm vAh likely

contribute to increased virulence. Peatman et al (2018) reported that the addition of the xenosiderophore, deferoxaminemesylate (DFO), to planktonic culture media significantly increased the virulence of vAh strain ML10-51K in catfish scarification-submersion challenges and increased hemolytic potential. Interestingly, DFO had no impact on vAh growth in TSB media, which is replete with iron, nor were iron acquisition genes significantly upregulated. This suggests that, even in the presence of plentiful iron, siderophores play a role in increasing virulence.

Polar flagella are required for biofilm formation (Merino, Wilhelms, & Tomás, 2014) and have also been indicated in cell adhesion in the absence of lateral flagella. vAh lack lateral flagella and, though likely functioning in biofilm formation and stability, polar flagella would add another host-cell adhesin to its virulence repertoire.

Like other species, vAh biofilm likely functions as a key mechanism for environmental survival and persistence that may inadvertently increase virulence by increasing initial host adhesion and invasion, and by evading initial host immune defense. This could help explain the difficulty in recreating natural infection using planktonically-cultured cells. Likely, planktonically-cultured cells more closely mimic cells in active infection *in vivo*. Vast increases in toxins, such as 7-fold increase in aerolysin and 10 fold increase *ahh1*-type hemolysin, could explain the toxic signature seen in vMAS tissue (Baumgartner et al., 2017) and the rapid host mortality following vAh intraperitoneal challenges. Furthermore, increases in porins, and some degradative enzymes could increase invasion and nutrient acquisition *in vivo*.

The scope of this study focused on expression of vAh PVF genes present in the literature, but should not be considered a complete enumeration of all possible virulence determinants. A cursory study of the top 150 differentially expressed genes (DEGs) finds only two of the six most

highly upregulated genes were previously identified PVFs (Table 3). However, all six of those genes had previously been indicated in virulence of other bacterial species. Of particular interest, three genes, *AHML 13715, AHML 13720,* and *AHML 13725* with LFC of 7.6, 8.2, and 9.0, respectively, are hypothetical proteins with significant homology to a tripartite pore-forming toxin in the *Bacillus* hemolytic enterotoxin family. In *Bacillus cereus*, this toxin contributes to diarrheal disease by damaging the plasma membrane of the small intestine epithelium (Senesi & Ghelardi, 2010). Recently, this toxin was formally identified and structurally analyzed in *A. hydrophila* and was found to require all three subunits, denoted AhlA, AhlB, and AhlC, for maximal lytic activity (Wilson et al., 2019). This significant upregulation suggests an important role in biofilm niche maintenance *in vitro*. *In vivo*, this toxin may act as another hemolysin, or could function to destroy skin, gill, or intestinal epithelium, thereby inadvertently increasing the biofilm cells' invasion potential.

The second most highly upregulated DEG, *matB*, *AHML_00355*, has previously been reported as a highly conserved gene in *E. coli* which is expressed in a highly virulent, clonal group responsible for meningeal infections and septicemia in newborns. Presence of the *mat* operon, which is comprised of *matABCDEF*, is considered a fitness determinant of *E. coli* (Lehti, Heikkinen, Korhonen, & Westerlund-Wikström, 2012) and promotes biofilm formation in newborn meningitis (Lehti et al., 2010; Lehti et al., 2012; Pouttu et al., 2001). In the vAh biofilm, *matB*, *AHML_00355*, along with three hypothetical proteins with homology to pilus chaperone and assembly proteins, *AHML_00335*, *AHML_00345*, and *AHML_00350*, are upregulated with LFC = 8.5, 4.3, 4.2, and 6.2, respectively. In *E.coli*, *matA* functions as the positive regulator of the *mat* operon (Lehti et al., 2012). In vAh ML09-119 genome, the regulator appears to be disjunct from the *mat* operon, and the regulatory agent is not readily obvious. VAh

gene *AHML_00360*, positive restriction regulator EvgA, is upregulated 2.5 fold and shares 38% homology with *matA*. However, the vAh *mat* genes are transcribed from the negative strand, while EvgA is on the positive strand and likely regulates virulence sensor protein BvgS, *AHML_00365*, which is upregulated 3-fold in biofilms. As in *E. coli*, vAh *matB* likely promotes, and may be required for, biofilm formation. Increased fimbrial adhesion to host surfaces by this extraordinary expression would likely be increased, again lending to the possibility of increased host colonization and invasion in biofilm vAh.

The research presented herein represents the first transcriptomic comparison of biofilm and planktonic vAh and provides the first insights into the biofilm lifestyle of *A. hydrophila*. The goal of this research was to compare how biofilm growth affected overall gene expression in vAh and to specifically compare expression of genes previously implicated in vAh virulence. Upregulation of PVF in biofilm seem to show the primary functions of iron scavenging and uptake, adhesion, and nutrient acquisition by production of proteolytic enzymes. Each of those functions are imperative for environmental biofilm niche maintenance, while also contributing significantly to the virulence and pathogenicity of the organism (Jefferson, 2004; Post et al., 2019; Zughaier & Cornelis, 2018). Repressed biofilm genes, which can be considered upregulated planktonic genes, included toxins, specifically hemolysins, multiple porins, and type IV pilus. Increases in these PVFs suggest that planktonic culture may more closely mimic *in vivo* growth. Type IV pili may help colonize the intestinal tract, while the porins and toxins target cells. This may also help explain the difficulty creating a natural infection model using planktonically-cultured vAh, while challenge by IP injection results in peracute mortality. Planktonically cultured cells appear to be less equipped for invasion, but well equipped for initiating septicemia. The results of PVF gene expression comparisons supports the hypothesis

presented for other bacterial species, that gene expression in biofilm growth primarily functions to support niche persistence, but these gene products may be used 'off-label' to increase pathogenicity and virulence. The results of this work underscore the importance and necessity of considering bacterial behavior in the natural habitat when trying to unravel the modes of pathogenicity of 'accidental' pathogens, such as vAh. Broth-based batch laboratory culture of bacteria is simple and reproducible and countless significant scientific breakthroughs have been made investigating bacterial species under such conditions. However, we must not forget to consider the vast ecological variation in natural systems whose pressures have shaped bacterial evolution and life history. In our aims to unravel the mystery of vAh-induced MAS, we must attempt to examine bacterial behavior under multiple modes of survival if we are to understand and prevent these deadly outbreaks. RNA sequencing technology generates vast amounts of data, and, while outside the scope of the current work, further investigation into potential roles of DEGs in vAh life history and pathogenicity is needed and will continue.

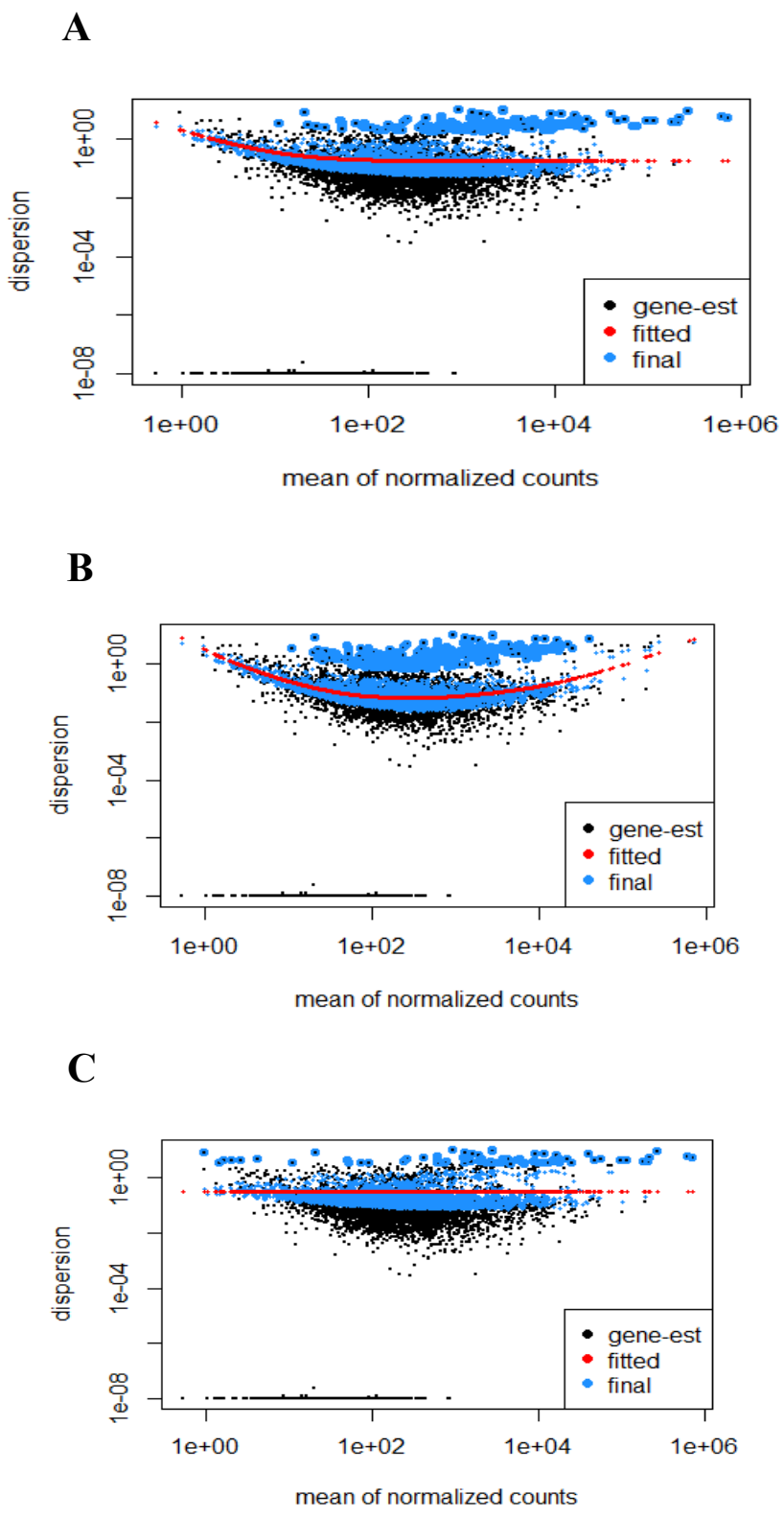

Figure 1. Dispersion estimates were performed on normalized raw reads to determine best fit. (A) Mean fit, (B) Local fit, (C) Parametric fit. A local fit was selected for further analyses.

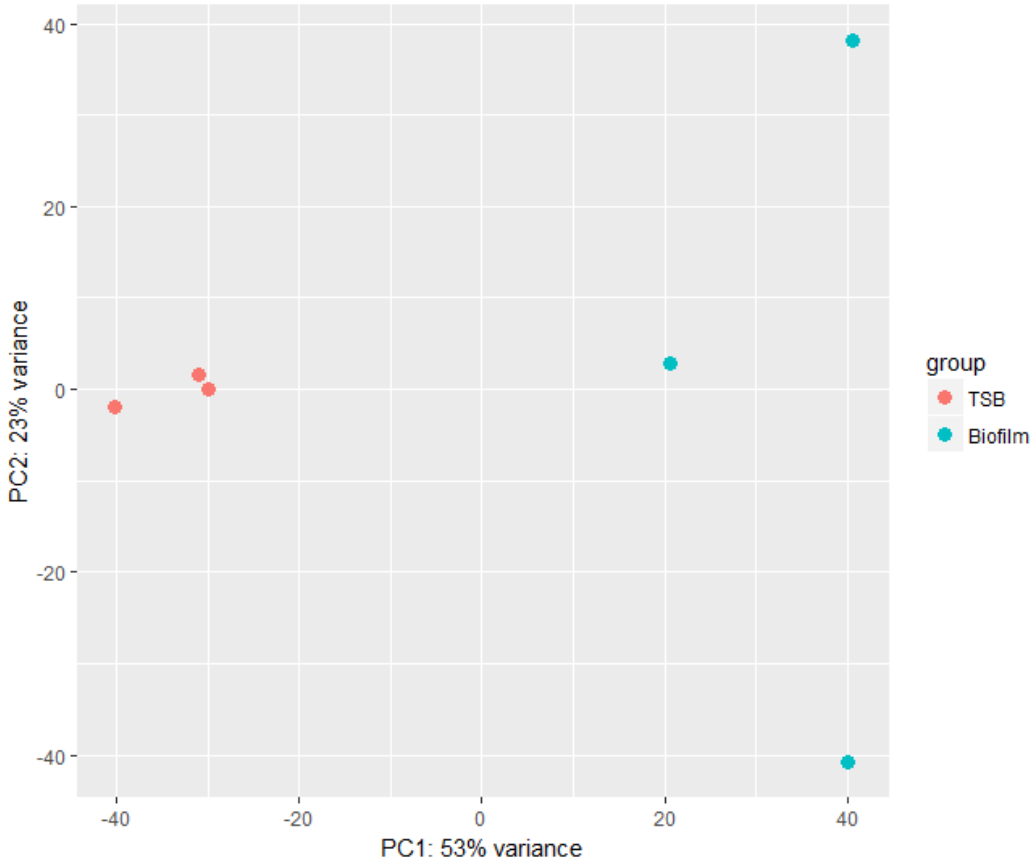

Figure 2. Principle component analysis (PCA) was performed on rlog-transformed normalized count matrix to check sample variation within and between experimental groups. Analysis revealed tight clustering of planktonic (TSB) samples, with significant variance between experimental groups. Biofilm samples had significantly greater within-group variance.

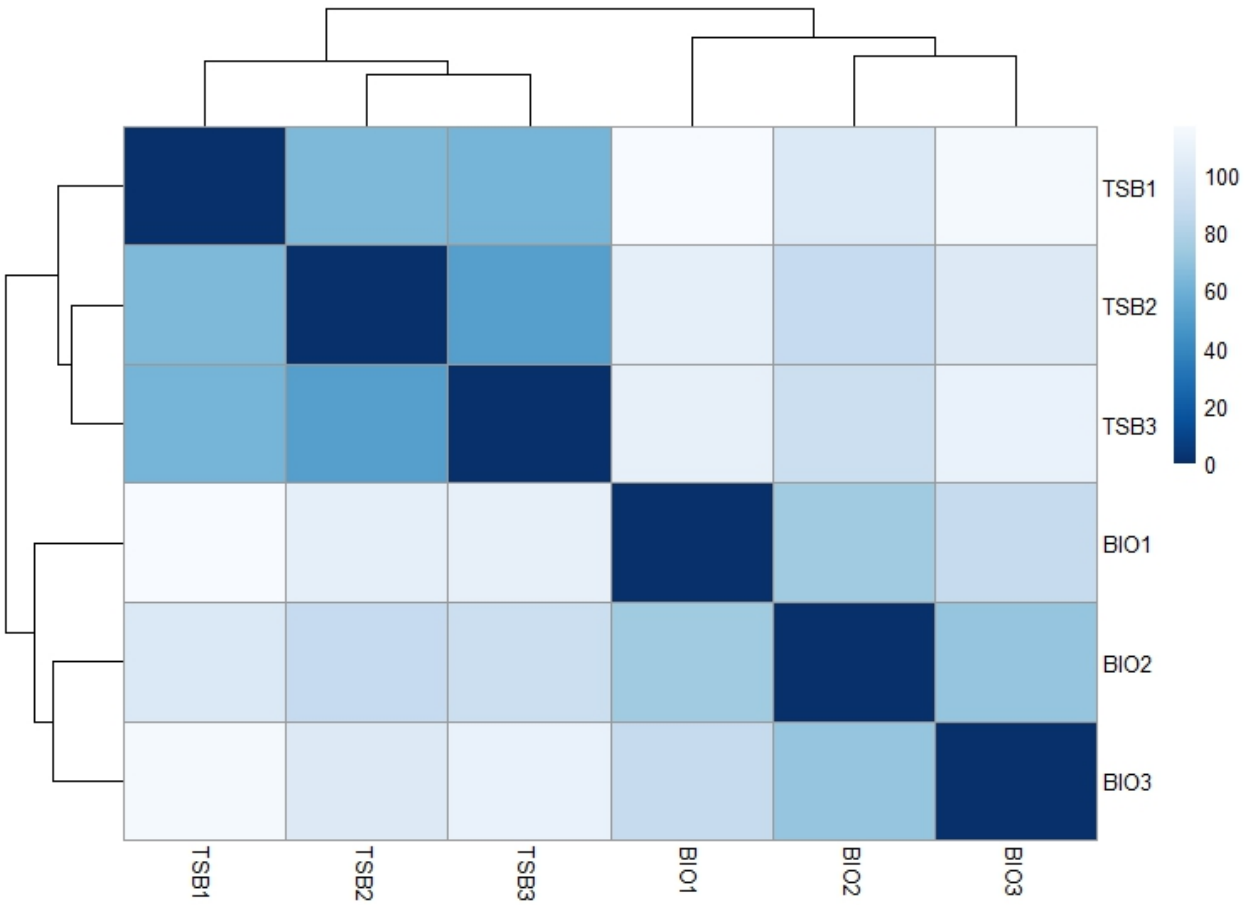

Figure 3. Distance matrix heatmap showing sample-to-sample distances calculated using rlog-transformed normalized reads. TSB = Planktonic sample relicates, BIO = Biofilm sample replicates.

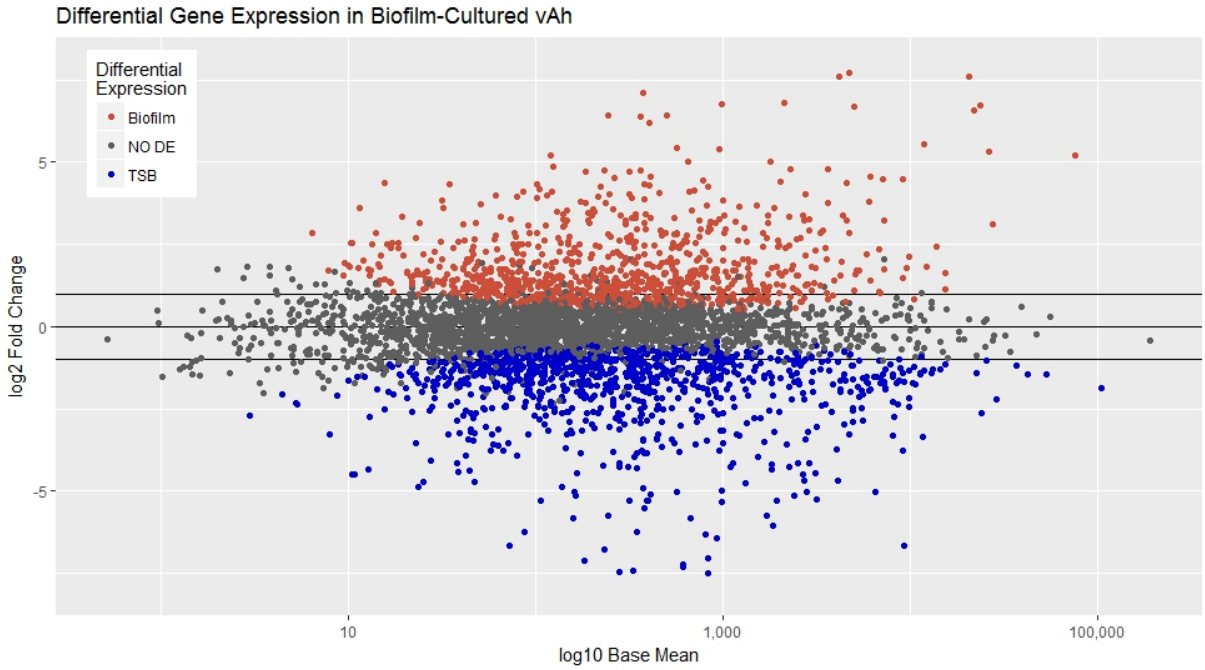

Figure 4. MA plot generated from differential gene expression analysis of normalized reads. Log fold changes were shrunk to minimize the impact of large fold changes due to low gene counts.

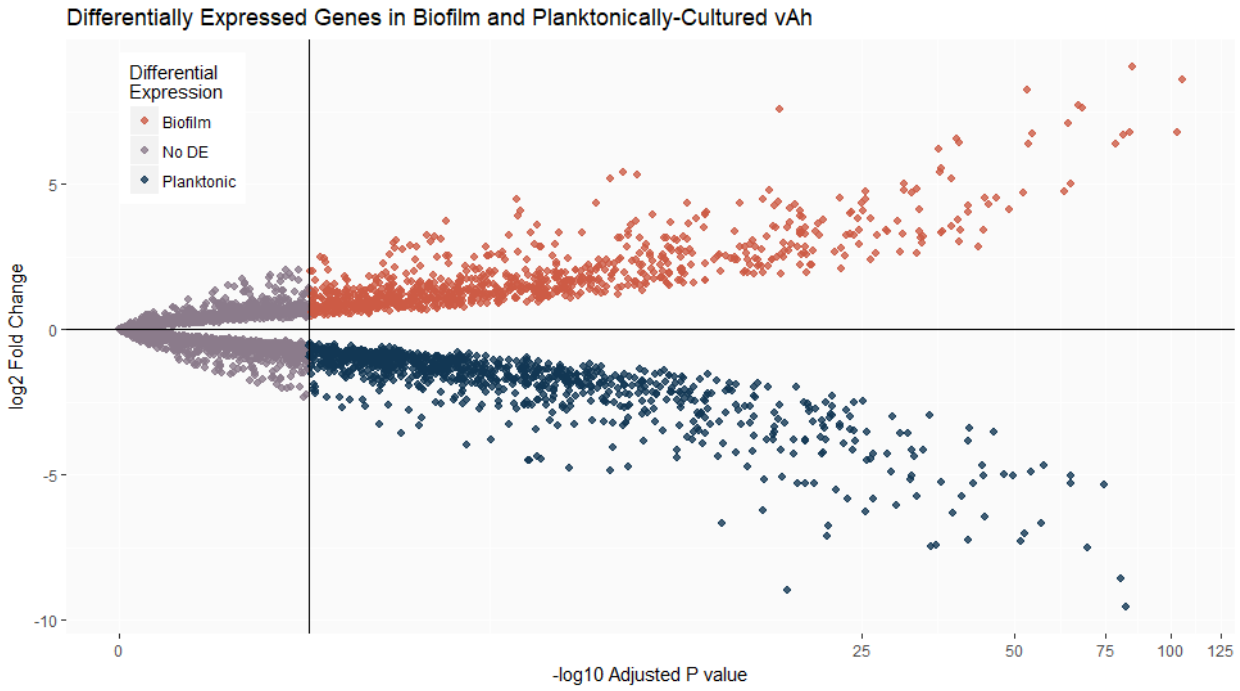

Figure 5. Volcano plot of differentially expressed gene in biofilm and planktonically-cultured vAh. Significant differential expression set at $L_2FC > 1$, $p < 0.01$.

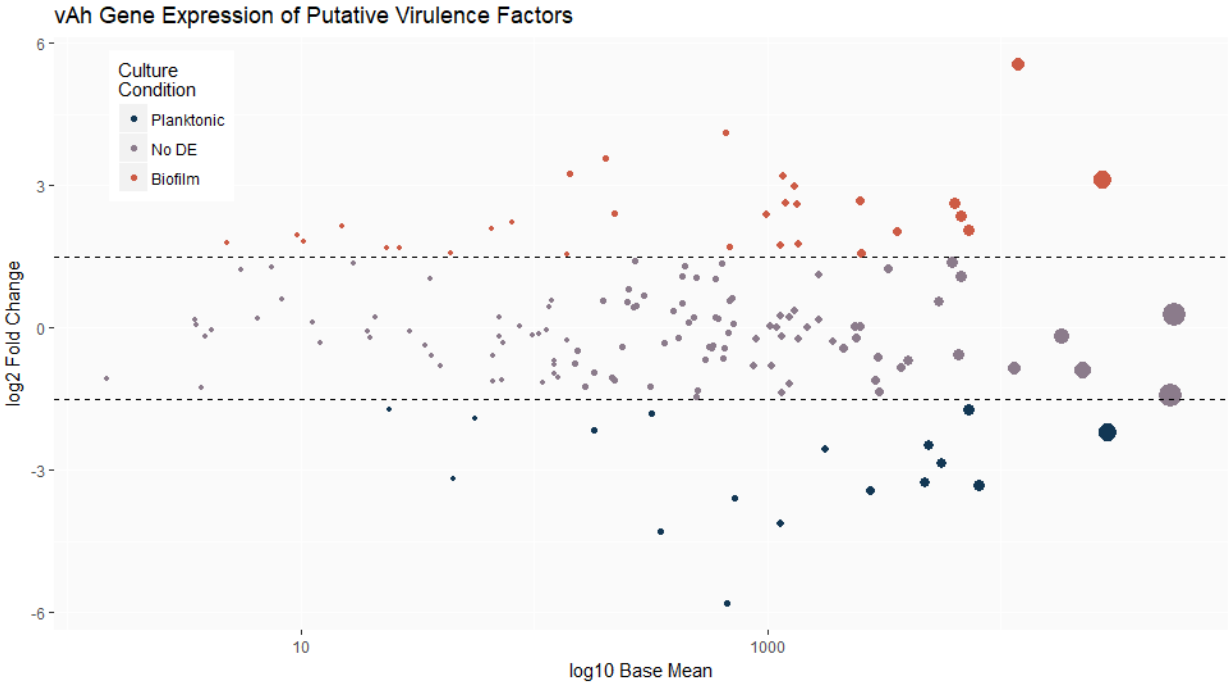

Figure 6. Differential expression of putative virulence gene when vAh is cultured planktonically and within a biofilm. Significant differential expression set at $L_2FC > 1$, $p < 0.01$.

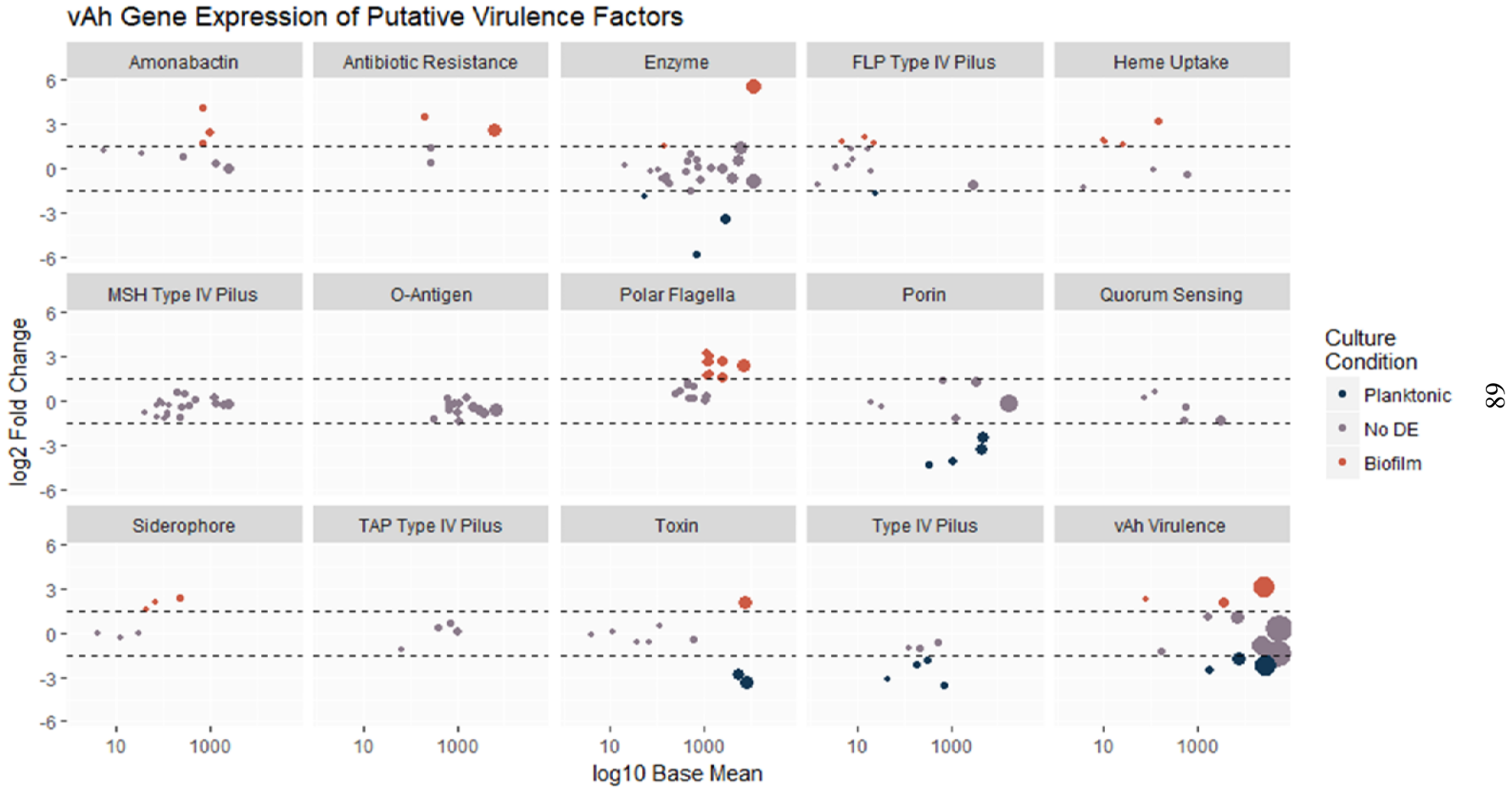
vAh Gene Expression of Putative Virulence Factors
Amonabactin
Antibiotic Resistance
Enzyme
FLP Type IV Pilus
Heme Uptake
MSH Type IV Pilus
O-Antigen
Polar Flagella
Porin
Quorum Sensing
Siderophore
TAP Type IV Pilus
Toxin
Type IV Pilus
vAh Virulence
log2 Fold Change
6
3
0
-3
-6
10
1000
log10 Base Mean
Culture Condition
Planktonic
No DE
Biofilm

Figure 7. MA plots of putative virulence gene expression by class. Putative virulence genes were separated into 15 classes based on ontology and gene expression was plotted to compare expression of each virulence class between experimental groups. $L_2FC > 1$, $p > 0.01$

Table 1. Transcriptome quality analysis.

Sample	# Mapped Sequences	Pairwise % Identity	Ref-Seq %	Confidence Mean	Quality Value		
					≥ Q20	≥ Q30	≥ Q40
Planktonic 1	16,992,934	97.3	91.6	38.5	99%	97.9%	58.4%
Planktonic 2	18,931,067	97.6	97.6	38.6	99.2%	98.2%	60.1%
Planktonic 3	22,080,825	96.6	96.6	38.5	99.1%	97.9%	58.4%
Biofilm 1	12,519,788	99	71.3	38.2	98.7%	96.8%	53.5%
Biofilm 2	6,799,829	96.8	78.9	38.5	99.1%	97.9%	58%
Biofilm 3	39,003,009	98.9	93.9	38.2	98.7	97%	54.6%

Table 2. Putative virulence factor gene by virulence class

*p value adjusted for multiple testing using Benjamini-Hochberg method in DESeq2

Negative Log_2 Fold Change corresponds to a downregulation in biofilm secretomes, or upregulation in planktonic secretomes.

Virulence Class	Product	Locus Tag	baseMean	p Value *Adjusted	log_2 Fold Change
Polar Flagella	Flagellar hook-associated protein 3	*AHML_15255*	1354.211	1.66E-10	1.771997
	Flagellar hook protein FlgK	*AHML_15260*	2473.424	1.67E-30	2.670439
	Flagellar rod assembly protein/muramidase FlgJ	*AHML_15265*	1197.018	7.63E-33	2.619285
	Flagellar P-ring protein	*AHML_15270*	1299.383	9.52E-34	2.984102
	Flagellar basal body L-ring protein	*AHML_15275*	1162.339	6.99E-34	3.19739
	Flagellar basal body rod protein FlgG	*AHML_15280*	1344.401	8.88E-20	2.591948
	Flagellar basal body rod protein FlgF	*AHML_15285*	441.32	5.45E-07	1.291838
	Flagellar hook protein FlgE	*AHML_15290*	6751.353	3.78E-10	2.36193
	Flagellar hook capping protein	*AHML_15295*	1136.75	4.04E-06	1.725497
	Flagellar basal body rod protein FlgC	*AHML_15300*	601.4134	0.005354	1.034531
	Flagellar basal body rod protein FlgB	*AHML_15305*	483.8363	0.682623	0.20849
	Chemotaxis protein CheR	*AHML_15310*	1096.11	0.995355	0.003069
	Chemotaxis protein CheW	*AHML_15315*	1135.192	0.498741	0.250917
	Flagella basal body P-ring formation protein FlgA	*AHML_15320*	249.7012	0.165508	0.543331
	Flagellar biosynthesis anti-sigma factor FlgM	*AHML_15325*	2520.742	0.001249	1.567961
	Flagellar protein FlgN	*AHML_15330*	596.2659	0.43904	0.216704
	FlaA/ DUF2750 domain-containing protein	*AHML_15395*	293.8713	0.449773	0.679851
	FlaB/ hypothetical protein	*AHML_15400*	430.443	0.397061	1.094512
FLP Type IV pilus	Pilin	*AHML_08075*	23.45072	0.007856	1.693998
	Peptidase	*AHML_08080*	16.71887	0.030072	1.35987
	Flp pilus assembly protein CpaB	*AHML_08085*	6.485065	0.852289	0.19606
	secretin	*AHML_08090*	19.88078	0.745822	-0.22179
	Hypothetical protein_08095	*AHML_08095*	1.480022	0.449364	-1.07938
	Flp pilus assembly protein FlpE	*AHML_08100*	3.494514	0.898393	0.173035
	Flp pilus assembly protein FlpF	*AHML_08105*	7.462278	0.102292	1.285934
	Flp pilus assembly protein TadB	*AHML_08110*	3.568557	0.966285	0.054842
	Pilus assembly protein TadC	*AHML_08115*	2879.395	1	-1.09984
	Flp pilus assembly protein TadD	*AHML_08120*	14.98848	0.004461	2.153709
	Hypothetical protein_08125	*AHML_08125*	4.831127	0.073514	1.792273
	Hypothetical protein_08130	*AHML_08130*	8.248418	0.455572	0.593929
	Hypothetical protein_08135	*AHML_08135*	23.82367	0.000387	-1.71845

MSH Type IV Pilus	RNase E specificity factor CsrD	*AHML_01890*	458.966	0.857032	0.115614
	ACC deaminase/D-cysteine desulfhydrase	*AHML_01895*	98.24224	0.664142	-0.16441
	Hypothetical protein_01900	*AHML_01900*	237.9616	0.154073	-0.41222
	Hypothetical protein_01905	*AHML_01905*	197.7198	0.185736	0.575497
	MSHA biogenesis protein MshJ	*AHML_01910*	274.3845	0.251798	0.473566
	MSHA biogenesis protein MshK	*AHML_01915*	86.24064	0.957588	0.022698
	Pilus (MSHA type) biogenesis protein MshL	*AHML_01920*	1237.166	0.43588	0.20909
	General secretion pathway protein GspA	*AHML_01925*	122.1791	0.121384	-0.77342
	Hypothetical protein_01930	*AHML_01930*	39.5657	0.183573	-0.81008
	MSHA biogenesis protein MshE	*AHML_01935*	362.7493	0.391957	-0.32601
	GspF family protein	*AHML_01940*	219.3312	0.000608	-1.10434
	Hypothetical protein_01945	*AHML_01945*	137.9625	0.603737	-0.27672
	MSHA biogenesis protein MshB	*AHML_01950*	1363.059	0.549875	-0.23692
	MSHA pilin protein MshA	*AHML_01955*	2387.398	0.616949	-0.20185
	Msha pilin protein mshc	*AHML_01960*	72.89909	0.001356	-1.10706
	Msha pilin protein mshd	*AHML_01965*	108.8037	0.003654	-1.16377
	MSHA biogenesis protein MshO	*AHML_01970*	122.3584	0.008485	-0.97495
	Hypothetical protein_01975	*AHML_01975*	73.96113	0.37368	-0.32636
	MshQ protein	*AHML_01980*	1912.221	0.347925	-0.29854
TAP Type IV Pilus	Hypothetical protein_20495	*AHML_20495*	699.9772	0.132492	0.62112
	Ype IV-A pilus assembly ATPase PilB	*AHML_20505*	1023.663	0.936118	0.041274
	Type 4 fimbrial assembly protein PilC	*AHML_20510*	393.6049	0.387279	0.365497
	Prepilin peptidase	*AHML_20515*	66.35606	0.001066	-1.11915
Type IV Pilus	Fimbrial protein_20665	*AHML_2665*	719.2718	1.59E-30	-3.58471
	Hypothetical protein_02670	*AHML_02670*	45.15967	6.00E-09	-3.19521
	Fimbrial assembly protein	*AHML_02675*	181.3471	5.16E-11	-2.16275
	Fimbrial chaperone protein	*AHML_02680*	214.8308	4.68E-05	-1.04023
	Type 1 fimbrial protein	*AHML_02685*	317.9826	3.54E-07	-1.81485
	Type I pilus assembly protein FimF	*AHML_02690*	125.8151	0.000435	-1.04267
	Transcriptional regulator	*AHML_02695*	542.5522	0.114537	-0.67715
O-Antigen Cluster	Polysaccharide export protein	*AHML_15490*	6549.659	0.160977	-0.55732
	O-antigen chain length determinant protein	*AHML_15495*	1153.915	0.000319	-1.37879
	dTDP-4-dehydrorhamnose 3,5-epimerase	*AHML_15500*	676.4227	0.790743	-0.10892
	Glucose-1-phosphate_15505	*AHML_15505*	1157.056	0.681411	-0.17402
	NAD(P)-dependent Oxidoreductase	*AHML_15510*	897.9077	0.377967	-0.22753
	Undecaprenyl-phosphate alpha-N-Acetylglucosaminyl 1-phosphate transferase	*AHML_15515*	1043.867	0.060701	-0.81682

	Phosphomannomutase	*AHML_15520*	315.6024	0.000715	-1.24374
	Mannose-1-phosphate Guanylyltransferase/mannose-6-phosphate isomerase	*AHML_15530*	2123.696	0.290915	-0.41635
	GDP-fucose synthetase	*AHML_15540*	2953.666	0.174526	-0.60633
	GDP-mannose 4,6-dehydratase	*AHML_15545*	3744.191	0.039513	-0.83668
	Group 1 glycosyl transferase	*AHML_15560*	640.8112	0.251952	-0.63213
	Acetyltransferase (isoleucine patch superfamily)-like protein	*AHML_15565*	614.9088	0.72221	0.198961
	WzxB protein	*AHML_15570*	650.7361	0.220995	-0.42069
	Glutamine--scyllo-inositol Aminotransferase	*AHML_15575*	1665.019	0.730891	0.163906
	Glucose-1-phosphate Thymidylyltransferase	*AHML_15590*	614.9088	0.72221	0.198961
Toxins	Enterotoxin	*AHML_04100*	36.39608	0.349655	-0.59581
	Hemolysin (Ahh1)	*AHML_08400*	8063.568	2.09E-11	-3.31914
	Hemolysin (HlyA)/HlyC/CorC family transporter	*AHML_15145*	576.553	0.224937	-0.42022
	Hemolysin III family protein	*AHML_18530*	66.46756	0.21953	-0.59005
	Thermostable hemolysin	*AHML_17235*	3.905473	0.885852	-0.17645
	ATPase RavA stimulator ViaA	*AHML_03730*	116.5128	0.370122	0.440805
	Aerolysin	*AHML_02265*	5578	4.22E-14	-2.87205
	3-phosphoshikimate 1-carboxyvinyltransferase	*AHML_13050*	416.7831	0.475351	-0.21946
	Toxin-activating lysine-acyltransferase	*AHML_07365*	11.28466	1	0.107432
	Structural toxin protein RtxA	*AHML_07370*	7267.663	0.083154	2.066895
Enzymes	DNA adenine methylase	*AHML_17080*	121.6029	0.093407	-0.69973
	Elastase	*AHML_04340*	11841.42	1.08E-36	5.559866
	Enolase	*AHML_04185*	11402.97	0.174046	-0.85373
	Peptidase M35	*AHML_14405*	2352.727	0.956526	0.030219
	tRNA uridine-5-Carboxymethylaminomethyl(34) synthesis enzyme MnmG	*AHML_22245*	713.3476	0.807033	0.07879
	Lipase_00550	*AHML_00550*	5449.086	0.303577	0.541539
	Lipase chaperone	*AHML_02630*	181.0289	1	-0.95455
	Lipase_02635	*AHML_02635*	493.6545	0.005874	1.071272
	Phospholipase C precursor	*AHML_03265*	137.6534	0.000486	1.533643
	Ribonuclease R	*AHML_03605*	6146.869	0.000164	1.391816
	Peptidase S8	*AHML_14260*	2747.276	1.58E-24	-3.41895
	Chitinase	*AHML_05225*	4037.457	0.060145	-0.70441
	Metalloprotease	*AHML_05230*	492.1347	1.19E-05	-1.4566
	Basic endochitinase	*AHML_05235*	875.7167	0.150685	-0.81892
	UDP-glucose 4-epimerase GalE	*AHML_21615*	671.031	4.05E-27	-5.81753
	UTP--glucose-1-phosphate uridylyltransferase	*AHML_18560*	1475.165	0.986706	-0.00742
	U32 family peptidase_09610	*AHML_09610*	56.07383	2.35E-07	-1.9104
	STM-protease	*AHML_09615*	70.74153	0.721294	-0.19633
	Collagenase-like protease	*AHML_05510*	104.4715	0.729027	-0.1206
	U32 family peptidase_05420	*AHML_05420*	432.2382	0.142804	0.52666
	Prolyl endopeptidase	*AHML_03915*	685.5121	0.121532	0.580903
	Periplasmic serine Protease/membrane protein protease SohB	*AHML_05015*	20.93168	0.712372	0.209773
		AHML_16720	152.7369	0.10044	-0.49004

	GlyGly-CTERM sorting domain-containing protein	*AHML_17785*	148.4383	0.005888	-0.76423
Quorum Systems	Acyl-homoserine-lactone synthase	*AHML_02845*	502.2785	0.000105	-1.31917
	Transcriptional activator protein AhyR/AsaR	*AHML_02850*	3005.402	0.001227	-1.35572
	S-ribosylhomocysteine lyase	*AHML_03595*	561.3239	0.083789	-0.40449
	DNA-binding response regulator	*AHML_17260*	71.2174	0.652569	0.222393
	Two-component sensor histidine kinase	*AHML_17265*	118.3207	0.079076	0.560631
Amonobactin	Phenylalanine--tRNA ligase subunit beta	*AHML_11325*	2491.311	0.898003	0.043315
	Integration host factor subunit alpha	*AHML_11330*	1314.712	0.401575	0.345157
	Histidine kinase	*AHML_11335*	995.3304	1.22E-14	2.395492
	Response regulator	*AHML_11340*	690.9056	2.16E-05	1.721128
	Acetyltransferase	*AHML_11345*	35.9802	0.025186	1.026272
	NAD-dependent deacylase	*AHML_11350*	665.1842	2.39E-33	4.122762
	Isochorismate synthase_02725	*AHML_02725*	253.0802	0.091227	0.810465
	Isochorismate synthase_13155	*AHML_13155*	5.508389	0.167676	1.22099
Heme Uptake	Heme ABC transporter ATP-binding protein	*AHML_05160*	3.735193	0.225219	-1.27915
	Iron ABC transporter permease	*AHML_05165*	9.6213	0.005982	1.953779
	Hemin ABC transporter substrate-binding protein	*AHML_05170*	10.26073	0.010153	1.812593
	Heme utilization cystosolic carrier protein HutX	*AHML_05175*	26.39275	0.000551	1.668692
	Heme utilization protein HutZ	*AHML_05180*	112.9299	1	-0.0392
	Outer Membrane Heme Receptor TonB-dependent hemoglobin/transferrin/lactoferrin family receptor	*AHML_05200*	141.4805	6.30E-25	3.254786
Siderophores	Pyoverdine chromophore biosynthetic protein PvcB	*AHML_17545*	12.17201	0.667799	-0.31369
	Pyoverdine chromophore biosynthetic protein PvcA	*AHML_17550*	4.123654	0.972272	-0.03882
	TonB-dependent siderophore receptor_02375	*AHML_02375*	29.34478	0.943337	-0.07366
	TonB-dependent siderophore receptor_10545	*AHML_10545*	66.05957	1.14E-07	2.075218
	Siderophore-interacting protein	*AHML_16150*	43.63784	5.94E-06	1.581637
	TonB-dependent siderophore receptor_22255	*AHML_22255*	219.2392	2.28E-13	2.417797
	Ferric iron uptake transcriptional regulator	*AHML_8495*	580.2996	0.355158	-0.36933
Antibiotic Resistance	CMY/LAT/MOX/ACT/MIR/FOX family class C beta-lactamase	*AHML_16865*	6297.46	1	2.630895
	Zn-dependent hydrolase	*AHML_17145*	202.7053	1.88E-38	3.5737
	MBL fold metallo-hydrolase	*AHML_11820*	270.2805	1.01E-05	1.423794
	Chorismate synthase	*AHML_11150*	265.07	0.108217	0.441424

vAh-Specific Putative Virulence Factors	Chitin Binding Protein/N-acetylglucosamine-binding protein GbpA	*AHML_03125*	27304.14	3.53E-11	3.11859
	Bifunctional metallophosphatase/5'-nucleotidase	*AHML_05380*	7240.271	2.25E-05	-1.7194
	Flagellin-like protein	*AHML_09345*	6775.755	0.000624	1.081511
	Long-chain fatty acid transporter	*AHML_12310*	3589.65	1.53E-08	2.033673
	Maltoporin	*AHML_06215*	55751.28	0.704943	0.286953
	Nuclease	*AHML_12120*	1666.312	0.007803	1.10942
	Porin_15025	*AHML_15025*	53339.96	0.026969	-1.42916
	Outer membrane receptor-mediated transport energizer protein TonB	*AHML_22120*	80.00953	4.46E-12	2.232063
	Arginine deiminase	*AHML_21565*	28930.61	7.15E-07	-2.22461
	Type I glyceraldehyde-3-phosphate dehydrogenase	*AHML_19130*	22484.47	0.278137	-0.88579
	PLP-dependent transferase	*AHML_10515*	1762.19	1.16E-05	-2.56522
	Ornithine carbamoyltransferase_03050	*AHML_03050*	164.4429	0.006939	-1.22791
Porins	Outer membrane porin, OprD family	*AHML_05565*	637.8244	5.04E-06	1.3641
	Porin OmpA_06750	*AHML_06750*	18214.1	0.685765	-0.17323
	Major outer membrane protein OmpAI	*AHML_06755*	4916.218	1.10E-07	-2.48244
	Outer membrane protein_02040	*AHML_02040*	19.4186	0.909911	-0.07619
	Porin_02385	*AHML_02385*	34.032	0.492467	-0.36414
	Porin_04355	*AHML_04355*	4708.969	4.38E-15	-3.27004
	Aquaporin family protein	*AHML_09065*	1132.456	7.58E-34	-4.14063
	Sucrose porin_16090	*AHML_16090*	345.6693	1.42E-21	-4.28441
	Porin OmpA_20145	*AHML_20145*	3289.963	0.000179	1.257551
	Porin OmpA_21905	*AHML_21905*	1243.764	0.000297	-1.1981

Table 3. Top differentially expressed genes, grouped sequentially by locus tag

*p value adjusted for multiple testing using Benjamini-Hochberg method in DESeq2

Negative Log_2 Fold Change corresponds to a downregulation in biofilm secretomes, or upregulation in planktonic secretomes.

Product	Locus tag	Base Mean	p Value *Adjusted	log_2 Fold Change
Hypothetical protein FimC pilus assembly protein	*AHML_00335*	405.0220926	2.50E-45	4.300309849
Hypothetical protein FimD OM usher protein	*AHML_00345*	833.798625	2.21E-41	4.262146844
Hypothetical protein PapD pili and Flagella assembly chaperone	*AHML_00350*	404.1086726	2.67E-36	6.204158838
Fimbrillin MatB	*AHML_00355*	12557.13166	4.31E-106	8.590599998
ABC transporter ATP-binding protein	*AHML_00575*	199.2205662	7.45E-37	3.383289247
ABC transporter ATP-binding protein	*AHML_00580*	324.1587622	1.08E-36	3.32320648
High-affinity branched-chain amino acid ABC transporter permease LivM	*AHML_00585*	388.7741469	3.01E-44	3.414563125
Branched chain amino acid ABC transporter substrate-binding protein	*AHML_00595*	6118.328611	8.79E-47	4.551698359
Hybrid sensor histidine kinase/response regulator	*AHML_02080*	382.5486301	7.22E-12	4.333673614
TorS-like protein	*AHML_02085*	31.82595468	2.49E-12	3.833991983
Sigma-B regulator RsbS	*AHML_02090*	15.66242172	7.34E-08	4.360289529
Chemotaxis protein	*AHML_02095*	140.7629508	1.74E-25	3.414774214
dipeptidase	*AHML_02580*	2700.932986	4.34E-44	-4.689477728
Nucleoside permease NupC	*AHML_02760*	981.1567891	2.72E-48	-4.980482246
Hypothetical protein	*AHML_02795*	173.32531	2.82E-25	3.81124845
2',3'-cyclic-nucleotide 2'-phosphodiesterase	*AHML_02860*	2185.69549	1.38E-31	-3.579749357
Protein CsaA	*AHML_03000*	101.3324402	3.38E-19	4.329968446
Hypothetical protein	*AHML_03100*	43.97527124	5.81E-06	-3.437900074
Glycine radical enzyme, YjjI family	*AHML_03180*	1096.549921	9.64E-15	-4.251162133
Hypothetical protein	*AHML_03220*	547.0824744	2.13E-26	-3.533625243
Hypothetical protein	*AHML_03245*	65.42980269	7.75E-06	3.334429834
DUF4432 domain-containing protein	*AHML_03700*	11807.49753	3.29E-81	-8.55020997
Glutamine ABC transporter substrate-binding protein	*AHML_03890*	13107.20222	4.59E-83	-9.556692191
Dipeptidase	*AHML_02580*	2700.932986	4.34E-44	-4.689477728
Fimbrial protein	*AHML_02665*	719.2718387	1.59E-34	-3.584706257
ABC transporter	*AHML_03895*	1314.498524	6.30E-07	-4.770025116
Glutamine ABC transporter permease	*AHML_03900*	609.9006012	2.47E-41	-7.234797688

Glutamine/glutamate ABC transporter permease	*AHML_03905*	344.9474143	3.62E-26	-6.257982467
Threonine synthase	*AHML_03910*	232.083307	3.01E-22	-6.764825156
Transposase	*AHML_04260*	233.442479	3.08E-26	4.736397423
Methyl-accepting chemotaxis protein	*AHML_05460*	1023.130886	6.92E-29	3.398528756
Methyl-accepting chemotaxis protein	*AHML_05470*	1214.818107	3.09E-21	3.645919344
Anti-sigma B factor antagonist	*AHML_05480*	205.1330088	1.56E-18	3.496805775
Response regulator	*AHML_05485*	705.8272629	1.37E-49	4.140360106
Methyl-accepting chemotaxis protein	*AHML_05490*	683.8465111	4.83E-20	3.52740831
Hypothetical protein	*AHML_05880*	5038.654498	5.20E-82	6.694150254
Deoxyribodipyrimidine photo-lyase	*AHML_05890*	984.1145109	1.27E-103	6.774801164
Transcriptional regulator	*AHML_05895*	377.4537624	3.41E-64	7.104097825
DUF1365 domain-containing protein	*AHML_05910*	121.0666153	1.95E-38	5.205655448
Cyclopropane-fatty-acyl-phospholipid synthase	*AHML_05915*	184.3110707	1.26E-52	4.724778236
Pyridoxal kinase	*AHML_06350*	265.2476979	2.20E-44	4.511041805
MATE family efflux transporter	*AHML_06355*	2299.384922	4.11E-31	4.801698781
Protease LasA	*AHML_06855*	*4723.943072*	2.97E-67	7.71479333
Aldolase	*AHML_07115*	*391.4986364*	5.07E-25	-3.830755382
Microcompartments family protein	*AHML_07255*	*11.51824507*	5.48E-06	3.599616821
Sulfurtransferase FdhD	*AHML_07285*	*345.9763424*	2.36E-41	-3.839491989
Hypothetical protein	*AHML_07360*	*22.90675649*	0.00361520	-3.556643412
Hypothetical protein	*AHML_07985*	*257.7817674*	2.10E-17	3.558188275
Glycerol kinase	*AHML_09060*	4032.738647	1.88E-21	-3.722206783
Aquaporin	*AHML_09065*	1132.456293	7.58E-34	-4.140625892
MFS transporter	*AHML_09070*	9309.170613	4.46E-57	-6.645025345
Glycerol-3-phosphate dehydrogenase	*AHML_09080*	1852.243753	5.35E-30	-6.046892383
Glycerophosphoryl diester phosphodiesterase	*AHML_09085*	1709.574632	5.36E-33	-5.747069825
Alpha-2-macroglobulin	*AHML_09315*	26299.45217	1.94E-09	5.320653162
Hypothetical protein	*AHML_09550*	105.4927858	1.04E-18	4.176172499
MFS transporter	*AHML_09920*	236.1687038	3.16E-30	3.752477402
Hypothetical protein	*AHML_09480*	2735.920875	2.08E-41	4.042702253
4a-hydroxytetrahydrobiopterin dehydratase	*AHML_10195*	1002.378369	8.55E-27	3.834764058
Pterin-4-alpha-carbinolamine dehydratase	*AHML_10200*	190.7490367	1.21E-19	3.441234605
Hypothetical protein	*AHML_10705*	114.6984559	1.51E-24	3.993614583
Tricarboxylic transport TctC	*AHML_10710*	318.9871648	6.80E-13	4.034312913
bifunctional PTS fructose transporter subunit IIA/HPr protein	*AHML_11190*	2493.356682	3.73E-32	-4.149464473
1-phosphofructokinase	*AHML_11195*	2782.808079	2.51E-44	-5.041344191
PTS fructose, IIBC component	*AHML_11200*	6501.330443	1.64E-50	-5.039584811
2-methylcitrate dehydratase	*AHML_11600*	1803.541271	3.74E-31	5.012365465

Citrate synthase/methylcitrate synthase	*AHML_11605*	778.3159565	3.52E-26	4.466189775
Methylisocitrate lyase	*AHML_11610*	819.9047794	5.34E-11	3.701587687
Reductase	*AHML_12530*	361.6520115	2.57E-79	6.384352976
Phosphodiesterase	*AHML_12535*	197.8496719	9.95E-29	3.433356449
Cyclic nucleotide-binding protein	*AHML_12805*	505.4555667	6.79E-54	6.412681079
Membrane protein	*AHML_12815*	948.7120684	6.98E-09	5.410899394
Cation acetate symporter	*AHML_12820*	3645.259274	4.47E-17	4.789035544
TonB-dependent receptor	*AHML_13095*	565.8669277	2.21E-36	5.425884162
Periplasmic-binding protein	*AHML_13120*	242.314201	1.33E-39	6.417207862
Non-ribosomal peptide synthase	*AHML_13125*	19.41986912	1.29E-06	3.35376343
Non-ribosomal peptide synthetase	*AHML_13140*	101.2901822	1.28E-19	3.906739348
Isochorismatase	*AHML_13145*	50.7496966	0.00061891	3.728134828
Formate dehydrogenase subunit alpha	*AHML_13240*	2393.923895	1.02E-16	-5.154049075
Formate dehydrogenase subunit alpha	*AHML_13245*	826.5575115	6.68E-53	-7.031556208
Electron transporter HydN	*AHML_13250*	828.2202153	8.23E-70	-7.493970851
Electron transporter	*AHML_13265*	183.2695628	4.05E-22	-7.128060799
Hydrogenase 4 subunit B	*AHML_13270*	806.8515579	1.34E-38	-6.304683218
Hydrogenase 3 membrane subunit	*AHML_13275*	372.9400592	1.20E-54	-4.893190185
Hydrogenase 3 large subunit	*AHML_13280*	986.0395808	4.34E-75	-5.319400903
Hydrogenase-4 component H	*AHML_13285*	242.1450095	1.49E-18	-8.967776463
Hydrogenase	*AHML_13290*	615.9299844	3.75E-52	-7.290514378
Formate hydrogenlyase maturation protein HycH	*AHML_13295*	282.2169641	4.87E-35	-7.464937738
Hydrogenase 3 maturation endopeptidase HyCI	*AHML_13300*	331.1548348	7.52E-36	-7.436862663
L-selenocysteinyl-tRNA(Sec) synthase	*AHML_13305*	59.56572729	7.35E-13	-3.590231989
(Fe-S)-binding protein	*AHML_13585*	1824.848227	6.48E-16	-4.173949207
Hypothetical protein	*AHML_13590*	230.9782595	1.11E-21	-4.236675055
Oxidoreductase	*AHML_13600*	473.527059	5.37E-16	-3.654982254
Lactate permease (LctP) family protein	*AHML_13605*	379.9080641	4.71E-20	-3.855241595
Hypothetical protein (Bacillus haemolytic enterotoxin Family protein)	*AHML_13715*	4147.892613	2.04E-68	7.616667833
Hypothetical protein (Non-Hemolytic Enterotoxin Lytic Component L1)	*AHML_13720*	6368.689384	1.63E-53	8.26221557
Hypothetical protein (Subunit P of AhlC tripartate pore forming toxin)	*AHML_13725*	4077.764953	5.21E-85	9.06518307
Peptidase S8	*AHML_14260*	2747.276213	1.58E-24	-3.418946879
Hypothetical protein	*AHML_14265*	375.551677	1.78E-12	-3.45767443
Cytochrome c nitrite reductase subunit NrfD	*AHML_14630*	335.8825876	9.10E-24	-3.940018839
4Fe-4S ferredoxin	*AHML_14635*	281.4329346	3.57E-27	-4.255273188
Formate-dependent nitrite reductase	*AHML_14640*	144.5224722	8.15E-12	-3.688088523

Hypothetical protein	*AHML_14935*	79.29636621	6.67E-14	-3.906804073
Toll-interleukin receptor	*AHML_14995*	3165.063854	1.38E-36	-5.262133093
Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide Acyltransferase component	*AHML_15420*	7175.507307	1.33E-16	4.480597737
Pyruvate dehydrogenase E1 component subunit beta	*AHML_15425*	3644.721799	2.11E-09	3.747935393
Pyruvate dehydrogenase (acetyl-transferring) E1 component subunit alpha	*AHML_15430*	4356.733884	4.71E-09	3.374817799
Sucrose-6-phosphate hydrolase	*AHML_16075*	367.7336303	5.68E-22	-3.767534048
PTS sucrose transporter subunit IIBC	*AHML_16080*	587.3589479	5.12E-19	-3.796211944
Aminoimidazole riboside kinase	*AHML_16085*	282.0998857	1.03E-24	-4.147172867
Sucrose porin	*AHML_16090*	345.669275	1.42E-21	-4.284407479
4-aminobutyrate transaminase	*AHML_16190*	408.0718117	1.09E-19	3.320337737
Purine-nucleoside phosphorylase	*AHML_16245*	32.45321759	6.56E-12	3.629386033
Leucyl aminopeptidase	*AHML_16450*	548.3055313	2.41E-07	3.323151303
Hint domain-containing protein	*AHML_16485*	216.0397326	4.08E-10	3.961264012
Two-component sensor histidine kinase	*AHML_16560*	1548.832825	0.000249941	-3.969382814
Membrane protein	*AHML_16640*	175.0520854	9.95E-10	-3.824790242
Dipeptide and tripeptide permease B	*AHML_16860*	1933.07172	7.54E-65	-5.27370819
Zn-dependent hydrolase	*AHML_17145*	202.7052595	1.88E-38	3.573699943
Acetyl-coenzyme A synthetase	*AHML_17870*	4544.033515	1.04E-25	4.362451208
Alkaline serine protease	*AHML_18455*	20678.68407	6.88E-18	7.607946054
Competence protein ComEA	*AHML_18555*	284.6793315	5.81E-21	3.366944589
PAP2 family protein	*AHML_18655*	9080.050096	1.83E-05	4.493740972
SMR family multidrug efflux pump	*AHML_18675*	184.7271765	1.68E-39	3.778462554
SMR family multidrug efflux pump	*AHML_18680*	85.99647832	1.51E-05	4.11108953
Hypothetical protein	*AHML_16750*	67.74298544	4.16E-12	-3.77685538
Hypothetical protein	*AHML_16760*	2663.240488	1.91E-26	-4.487347239
EAL family, Rtn protein	*AHML_16805*	165.0414193	5.92E-32	-5.139841464
Hypothetical protein	*AHML_19475*	243.0111944	3.86E-40	-5.73944864
Phage protein	*AHML_19480*	160.1036286	3.17E-32	-5.018343493
Hypothetical protein	*AHML_19485*	106.4722279	5.04E-20	-5.275655443
Transcriptional regulator	*AHML_19490*	44.63623689	1.45E-13	-4.382569867
Hypothetical protein	*AHML_19495*	10.59645527	8.63E-06	-4.486830642
Hypothetical protein	*AHML_19500*	25.17796327	4.58E-09	-4.707808058
Hypothetical protein	*AHML_19505*	47.47327706	1.64E-15	-4.69965572
DNA methyltransferase	*AHML_19510*	57.65764456	1.97E-17	-3.775286556
Phage capsid protein	*AHML_19565*	382.3415792	4.83E-23	-5.517572564
Phage major capsid protein, P2 family	*AHML_19570*	399.9741961	2.43E-42	-5.296232201
Terminase	*AHML_19575*	73.17740108	7.44E-14	-6.648952352

Phage head completion protein	*AHML_19580*	23.75121629	2.17E-08	-4.868911088
Phage protein	*AHML_19585*	39.01205654	2.75E-11	-4.415698111
Phage protein	*AHML_19590*	38.18513726	2.75E-11	-4.160113582
Phage protein	*AHML_19595*	137.4534168	2.22E-29	-4.889579934
Phage protein	*AHML_19600*	63.74388177	2.32E-17	-3.629227489
Phage protein	*AHML_19605*	10.77476916	9.49E-06	-4.495443472
Phage holin, lambda family	*AHML_19610*	27.80788682	1.82E-08	-4.073529989
Phage lysin	*AHML_19615*	42.24008976	1.18E-12	-3.902187024
Phage protein	*AHML_19630*	87.8891126	1.36E-16	-6.240363979
Phage tail tape measure protein	*AHML_19635*	71.05385712	3.22E-12	-3.52441976
Phage protein	*AHML_19640*	12.77503839	5.28E-06	-4.34474161
PspC domain-containing protein	*AHML_19875*	61.33851194	7.53E-13	3.994867994
Phage shock protein PspA	*AHML_19880*	409.4447204	2.33E-23	4.551320237
Transposase	*AHML_21250*	314.6718603	3.18E-32	4.724597094
Galactoside ABC transporter permease MglC	*AHML_21590*	957.8789184	4.32E-20	-3.774145694
Galactose/methyl galactoside ABC transporter ATP-binding protein MglA	*AHML_21595*	2267.686196	6.66E-29	-4.280539099
UDP-glucose 4-epimerase GalE	*AHML_21615*	671.0310245	4.05E-27	-5.81753135
Galactose-1-phosphate uridylyltransferase	*AHML_21620*	925.8141258	1.37E-44	-6.432065837
Galactokinase	*AHML_21625*	410.3776064	3.98E-18	-5.083023684
Galactose-1-epimerase	*AHML_21630*	394.4442325	1.96E-19	-5.299181868
Aldo/keto reductase	*AHML_21635*	165.8625802	4.10E-06	-4.469968724

Chapter IV

The Importance of the Type II Secretion System in the Pathogenicity of Hypervirulent *Aeromonas hydrophila* Responsible for Epidemics in Channel Catfish, *Icatlurus punctatus*

1. Abstract

The rapid mortality that occurs when catfish are challenged by intraperitoneal injection with vAh suggests that vAh-induced MAS may be a toxin-mediated disease. *Aeromonas hydrophila* are known to produce a multitude of proteolytic and potentially toxigenic proteins. While T3SS and T6SS have generally been considered indicators of the virulence of *A. hydrophila* strains, no U.S. isolates of vAh possess complete Type 3 or Type 6 secretion systems. Instead, US vAh isolates possess Type 1 and Type 2 secretions systems. In order to determine the role of secreted protein in vAh-induced disease, and to determine the extent of protein secretion by the Type 2 secretion pathway, a T2 secretion-deficient mutant was created by homologous recombination. Secretomes were analyzed for presence or absence of proteins and secretome functional analyses were performed to measure degradative and toxigenic potential. T2 secretion-deficient mutants had a near complete loss of secreted proteins and complete loss of functionality. T2SS complementation by whole-pathway cloning restored protein secretion and restored degradative and toxigenic potential of vAh. *In vivo* challenges in channel catfish found complete attenuation of virulence in T2 secretion-deficient mutant, while T2SS complementation restored virulence. These results indicate a vital role of secreted proteins in vAh virulence, and confirm that T2SS is the primary secretory pathway utilized for secretion of virulence determinant in US isolates of vAh.

2. Introduction

Type II secretion systems (T2SS) are found in myriad human, animal, and plant pathogens and Type II secretion (T2S) mediates a host of pathogenic processes, including host adhesion and invasion, host cell destruction, and tissue necrosis (Korotkov & Sandkvist, 2019). While the role of T2SS in bacterial pathogenicity has been the primary focus of research, T2S is also crucial for survival in environmental species, as well as for opportunistic pathogens whose primary niche is outside the host.

Environmental niche manipulation is a hallmark strategy to increase fitness in generalist bacteria, and secretion of proteolytic, glycolytic, and metal-reducing molecules assist in bacterial habitat remodeling (Chen et al., 2020; San Roman & Wagner, 2018). Bacterial influence over available habitat provides a competitive advantage; thus, bacteria possessing the capacity to release 'remodeling' substances directly into the environment may be capable of rapid colonization of a self-created niche. T2S provides such a capability. By secretion of substrates that facilitate adhesion to biotic and abiotic surfaces, as well as proteins involved in biofilm formation, T2SS may support the shift between free-living and sessile lifestyles common in environmental bacteria and may increase niche exploitation capacity. Furthermore, T2SS substrates are generally highly stable in the environment, as evidenced by their presence in many extremophiles (Rondelet & Condemine, 2013). While vAh is capable of causing disease as a primary pathogen it is, by and large, an environmental opportunist. While T2SS are widespread in *A. hydrophila* (Tekedar et al., 2019), presence of a T3SS or T6SS is generally considered more important for virulence (Tomas, 2012) and, in some cases, essential to the infectious process (Vilches, Jimenez, Tomás, & Merino, 2009; Yu et al., 2004). Both T3SS and T6SS rely on

needle-like ‘injectisomes’ to mediate effector molecules directly from the bacterial cytoplasm into the target host cell, while T2SS substrates are released from the periplasm through the outer membrane, in a seemingly indiscriminate manner, into the extracellular milieu. T3SS and, likely, T6SS are inducible systems (Vilches et al., 2009), while T2SS is believed to be constitutively expressed in *A. hydrophila* as well as its marine counterpart, *Vibrio cholerae* (Sandkvist, 2001b). Interestingly, genomic comparisons of secretion systems in vAh found all 27 isolates lacked a T3SS, and 18 of the 27 isolates, including all US isolated strains, lacked a complete T6SS, though all vAh genomes contained at least three genes from the T6SS cluster (Tekedar et al., 2019). Lack of T6SS in US strains does not appear to reduce virulence in these strains, as mortalities in channel catfish immersion challenges reached 90% in one study (Rasmussen-Ivey, Hossain, et al., 2016). However, no vAh isolate included in the aforementioned study possessed a complete T6SS. Perhaps the presence of a T6SS in these isolates would have a cumulative effect on mortality. Nevertheless, neither a T3SS nor T6SS appear to be prerequisite for hypervirulence in US pathovars and the continued ability for US vAh isolates to cause disease suggests that T3SS and T6SS are redundant.

Lack of T3SS and T6SS support the hypothesis that these virulence factors provide no benefit for US vAh strains, and the energistic expense of maintenance outweighs the benefit in the non-host habitat (Brown et al., 2012). If vAh-induced MAS (vMAS) mortality results from one or more secreted toxins, an alternate secretion pathway must be utilized. To determine the extent of protein secretion via T2SS, and to determine the importance of vAh ECPs in vAh virulence, a T2 secretion-deficient mutant was created by homologous recombination and this deficiency was complemented using T2SS whole-pathway cloning. Secretion mutants, both secretion-deficient and whole-pathway complemented, were screened for ability to secrete

proteins into the growth media, and the growth media secretomes were analyzed for proteolytic and toxigenic potential. Intraperitoneal challenges using wild type and secretion mutants were then performed in a channel catfish model to determine the role of T2SS and its substrates *in vivo*.

3. Methods and materials

Bacterial Strains and Plasmids. Bacterial strains and plasmids used in this study are presented in Table 1. Virulent *Aeromonas hydrophila* strain ML09-119 containing the recombinogenic plasmid, pMJH65 (Hossain et al., 2015), was used as the wild-type vAh in which deletion mutations were generated.

Culture Media and Culture Conditions. Tryptic Soy Broth (TSB) (Bacto TSB, BD) and Luria Broth (LB) prepared according to manufacturer's directions was routinely used as the culture medium for growth of vAh and *Escherichia coli*, respectively, with the addition of 1.5% agar powder (Alfa Aesar) for solid culture. When necessary for selection, media were supplemented with chloramphenicol (Cam) (25µg/ml), tetracycline (Tet) (10µg/ml), colistin (Col) (10µg/ml), kanamycin (Kan) (30µg/ml), or combinations of those antibiotics. Super Optimal Broth (SOB) (Difco SOB, BD) was used for culture of electrocompetent cells and Super Optimal Broth with Catabolite Repression (SOC) was used as a recovery medium following electroporation.

Biofilm media was prepared by adding 0.2% agar powder (AlfaAesar) to TSB media prior to sterilization. Approximately 70ml of molten biofilm agar was poured into deep well petri dishes (Fisher) and allowed to solidify. When necessary, media was supplemented with chloramphenicol (25µg/ml).

Bacterial strain vAh ML09-119 was removed from cryogenic storage and inoculated into 25ml TSB media and grown overnight at 30°C with shaking. An aliquot of overnight culture was transferred to 70ml of TSB and grown at 30°C on an orbital shaker to mid-log phase, approximately 16 hours. Biofilm agar plates were inoculated from overnight culture by stab inoculation. Plates were sealed with parafilm and incubated at 30°C for 72 hours, until bacterial film covered and adhered to agar surface.

Preparation of Electrocompetent Cells. To make electrocompetent cell for recombineering experiments, an isolated colony of vAh ML09-119 containing the recombinogenic plasmid pMJH65 (ML09-119 + pMJH65) was inoculated into 5ml TSB + 10µg/ml TET and incubated overnight at 30°C with shaking at 200 rpm. 0.5 ml of overnight culture was transferred to a 250 ml baffled flask containing 50ml SOB supplemented with 10µg/ml TET and 10mM arabinose, and was incubated at 30°C with shaking until OD_{600}=0.6. Cells were then transferred to pre-chilled 50ml conical tubes and centrifuged at 6,000 rpm for 8 minutes at 4°C. Supernatant was decanted and the cell pellet was carefully resuspended in 30ml ice-cold 10% glycerol. Cells were then pelleted, as above, and this wash process was repeated three more times, for a total of four washes. After the final wash step, cells were carefully resuspended in 150µl of cold 10% glycerol and placed on ice until use. Electrocompetent vAh cells were freshly prepared for each electroporation experiment, and were used immediately.

Electrocompetent *E. coli* SM10 λpir were prepared as above with minor modifications. An isolated *E. coli* SM10 λpir colony was inoculated into 5ml LB and incubated overnight at 37°C with shaking, then a 0.5ml aliquot was transferred to 50ml SOB without antibiotic or arabinose supplementation and was cultured at 37°C with shaking until OD_{600}=0.6. Cells were then pelleted, washed, and prepared as above.

T2SS *exeD* Gene Deletion by Recombineering

Preparation of antibiotic cassette for gene replacement: Chloramphenicol was chosen as the selectable marker for gene deletion by recombineering. The pKD3 plasmid (Datsenko & Wanner, 2000) was used as the donor for the chloramphenicol resistance gene, *cat*. The *cat* cassette, along with flanking FRT sequences, were extracted using appropriate primers (Table 2). To add homologous sequences required for recombineering, PCR was performed on the FRT*cat* cassette using FRT*cat*-specific primers with 60 and 65 base pair homology to upstream *exeC* and downstream *exeE* sequences, respectively. PCR amplicon was purified using MicroElute Cycle-Pure kit (OMEGA Bio-Tek) following manufacturer protocol. This purified DNA was used as the template for preparatory PCR using phosphorothioate primers. Following PCR, ten, 50µl reactions were pooled, purified using ReliaPrep DNA Clean-up and Concentration system (Promega), and eluted in molecular-grade water to yield 590ng/µl dsDNA. This *exeC*-FRT-*cat*-*exeE* linear dsDNA was then used as the selectable marker for *exeD* gene deletion.

***exeD* gene deletion by recombineering**: 50µl of freshly-prepared electrocompetent vAh Ml09-119 + pMJH65 was transferred to pre-chilled 1.5ml tubes. 1500ng *exeC*-FRT*cat*-*exeE* linear dsDNA (2.5µl) was added to electrocompetent cells, gently mixed, and transferred to chilled, 0.1cm electroporation cuvettes. Cells were subjected to a single exponential decay pulse of 1.2kV, 25µF, and 200 Ω using an Eppendorf Eporator (Hamburg, Germany), followed by the immediate addition of 1ml SOC recovery media, and incubated overnight with shaking at 30°C. Cells were then spread onto 2xYT agar plates supplemented with 25µg/ml CAM and incubated at 30°C for up to 72 hours to select for successful transformants.

Confirmation of FRT*cat* insertion and *exeD* deletion: To verify the deletion of *exeD* and the integration of the FRT*cat* cassette, PCR was performed on colonies that appeared on CAM-selective media within 72 hours using primers with homology to *exeC* and *exeE*. Isolated colonies were carefully removed from the agar and resuspended in 20µl sterile water, and 5µl of this mixture was used as colony template. Wild-type ML09-119+pMJH65 was used as the *exeD*+ control, and FRT*cat* cassette served as *Cat*+/*exeD*- control. PCR was performed using EconoTaq Plus Green 2X Master Mix (Lucigen) in a Biorad T100 Thermal Cycler. PCR conditions included an initial denaturation step at 95°C for 3 minutes, followed by 34 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. The final (35^{th}) cycle included extension at 72°C for 5 minutes. Upon completion of PCR, 10µl aliquots of each reaction were loaded onto a 0.8% agarose TAE gel containing 10ng/µl ethidium bromide, and electrophoresed at 100V for 1.5 hours. Gel visualization and documentation was performed using the Gel Doc EZ Imager (BioRad) platform.

Removal of pMJH65 plasmid from *exeD*:FRT*cat* mutant: The pMJH65 plasmid, which confers Tet resistance, was cured from *exeD*:FRT*cat* mutants by heat induction as follows. *exeD*:FRT*cat* mutants were grown in TSB at 30°C with shaking at 200 rpm to an OD_{600} = 1.0. Culture temperature was then increased to 43°C for one hour, followed by overnight culture at 37°C to induce plasmid loss. Following heat induction, cells were streaked for isolation on non-selective TSA plates, then isolated colonies were streaked onto TSA+Tet selective agar. pMJH65 plasmid loss was confirmed in isolated colonies by the inability to grow in the presence of Tet.

Generation of T2SS Δ*exeD* markerless mutant by *flp*-mediated recombination. Following successful generation of *exeD*:FRT*cat* mutants via recombineering, a markerless mutant, Δ*exeD*, was generated by *flp*-mediated recombination using the *flp*-recombinase plasmid, pCMT-flp

(Hossain et al., 2015). The donor *E. coli* strain, SM10 λpir, was selected to mobilize pCMT-flp into vAh *exeD*:FRT*cat* via conjugation, as follows.

Isolation of pCMT-flp and electroporation into mobilizable *E. coli* SM10 λpir: The pCMT-flp plasmid, which confers Tet resistance, was purified from a common cloning strain of *E. coli*, E.cloni 10G (Lucigen), using E.Z.N.A. Plasmid DNA Mini Kit I (Omega Bio-Tek) following manufacturer's protocol, and was eluted at a final concentration of 11ng/µl. Electrocompetent *E.coli* SM10 λpir were prepared as described above. pCMT-flp plasmid DNA (11ng) was introduced via electroporation following the protocol above, but with a single pulse of 1.6kV, 25µF, and 200Ω, and incubated at 37°C with shaking at 200 rpm for 4 hours. Cells were then plated onto 2xYT+10µg/ml Tet. Presence of the pCMT-flp plasmid was verified by the ability of colonies to grow on Tet-selective media.

Conjugal transfer of pCMT-flp from *E.coli* SM10 λpir to *exeD*:FRT*cat*: The *exeD*:FRT*cat* mutant was cultured in TSB+CAM at 30°C and *E.coli* SM10 λpir + pCMT-flp was cultured in LB+TET at 37°C with shaking to an OD_{600}=1.0. 1ml aliquots of donor and recipient cultures were transferred to separate sterile 1.5ml microcentrifuge tubes, cells were pelleted by centrifugation at 10,000*x g* for 5 minutes, and supernatants were removed from cell pellets. To remove residual antibiotics, cells were washed three times by resuspension in 1ml antibiotic-free LB, followed by centrifugation and removal of supernatants. After the final wash, cells were resuspended in 500µl LB. To prepare the conjugation mixture, 125µl of *E.coli* SM10 λpir + pCMT-flp was combined with 500µl *exeD*:FRT*cat*, to a 1:4 donor: recipient ratio. Conjugation was performed using a spread-plate method, as follows. A 75µl aliquot of the conjugation mixture was spread onto a blood agar plate, sealed with parafilm, and incubated overnight at 30°C. Plates were then divided into 8 sections, and 1/8 of the bacterial cells were removed from

the agar surface using a disposable sterile inoculating loop and resuspended in 500µl LB. 75µl aliquots were spread onto selective LB plates containing 10µg/ml Tet + 10 µg/ml Col, and plates were incubated at 30°C for up to 72 hours. *exeD*:FRT*cat* transconjugants containing pCMT-flp were capable of growth in the presence of Tet.

Removal of *cat* gene from *exeD*:FRT*cat* by *flp*-mediated recombination: *flp*-mediated excision of the *cat* gene was initiated by heat induction as follows. Transconjugant *exeD*:FRT*cat* + pCMT-flp colonies were removed from selective media and streaked for isolation onto TSA + Tet plates, and incubated at 30°C for 24 hours. An isolated colony was then inoculated into TSB broth containing no antibiotic and grown at 30°C with shaking at 200 rpm to an OD_{600}=1. Culture temperature was then increased to 37°C, and culture continued for 1 hour to allow for flp-frt mediated gene deletion. The induced culture was then streaked for isolation on non-selective TSA and incubated at 30°C for 24 hours. Isolated colonies were resuspended in 50µl sterile water, and 5µl of this template was used for colony PCR as described above. Colony template was also transferred to selective media containing Cam or Tet to confirm presence of pCMT+flp plasmid and the excision of *cat* gene. Isolated colonies that failed to grow in the presence of Cam and that revealed flp-deletion 'scar' by PCR were confirmed as Δ*exeD* mutants. Prior to complementation, pCMT-flp plasmid was cured from Δ*exeD* mutants following the heat induction protocol outlined above. Successful loss of plasmid was confirmed by loss of Tet resistance in isolated colonies.

Restoring T2SS by whole-pathway complementation. To restore T2SS functionality in Δ*exeD*, a whole-pathway complementation procedure was implemented.

Construction and cloning of T2SS vector: From an overnight culture of wild-type vAh ML09-119, high molecular weight genomic DNA (HMW gDNA) was isolated using MagAttract HMW DNA kit (Qiagen) following manufacturer's protocol. HMW gDNA was sent to Varigen Biosciences, and CRISPR-Cas9 restriction was performed using guide RNAs specific to the T2SS genomic region. T2SS DNA was assembled into linearized pBAC-S vector containing overlap sequence specific to the T2SS fragment and conferring resistance to Cam and apramycin. The T2SS vector (pBAC + T2SS) assembly was transformed into *E. coli* BAC-Optimized Replicator v2.0 electrocompetent cells and colonies recovered on selective media. Successful cloning was confirmed by colony PCR, NotI restriction digestion, and sequencing.

Complementation of T2SS by tri-parental mating: pBAC + T2SS was conjugally transferred from the donor strain (*E. coli* BAC-Optimized Replicator v2.0) to Δ*exeD* by triparental mating, using the helper strain, *E. coli* HB101 pRK2013, to mobilize pBAC + T2SS plasmid. An isolated colony of Δ*exeD* was inoculated into TSB and grown at 30°C, with shaking at 200 rpm to an OD_{600}=1.0. Donor and helper strains were inoculated into LB containing Cam and kanamycin, respectively, and grown at 37°C, with shaking at 200 rpm to and OD_{600} = 0.6. A 1ml aliquot of Δ*exeD* was pelleted by centrifugation at13,000 rpm for 2 minutes at room temperature. Supernatant was removed and cells were resuspended in 1ml LB and centrifuged as above. This wash step was repeated twice more. Following the final wash, Δ*exeD* was resuspended in 500µl LB. 1.5ml aliquots of donor and helper strains were removed, pelleted, and washed as above. Following the final wash, donor and helper strains were resuspended in 250µl LB. To prepare the conjugation mix, 62.5µl donor and 62.5µl helper were added to 500µl Δ*exeD* for a 1:1:4 donor:helper:recipient ratio. 100µl aliquots were spread onto blood agar plates and incubated at 30°C for 24 hours. Following incubation, cells were removed from 1/8 of the

plate, as described above, and resuspended in 300µl LB. 75µl aliquots were spread onto selective LB plates supplemented with 10µg/ml colistin and 25µg/ml Cam. Plates were incubated at 30°C for up to 72 hours. Colonies present on selective media within 72 hours were transferred to fresh LB + Cam + Col plates and streaked for isolation. An isolated colony was then selected and used as template for colony PCR. Transconjugant colonies that produced both *exeD* gene and *flp* scar amplicons were confirmed to have successful pBAC-S + T2SS vector integration, and were denoted *exeD*::T2SS.

Determining functional role of T2SS. To determine the role of T2SS in secretion of putative virulence products, functional screening of wild-type and vAh T2SS mutants were performed. The necessity of T2SS for *in vivo* pathogenicity was measured in channel catfish host.

Growth curves: To ensure cell viability in T2SS mutant, growth curves were performed. Cells were diluted in TSB to an OD_{600} = 0.01, and 200µl of cell suspension was placed in sterile, non-binding, 96-well polystyrene plates in quadruplicate. Plates were incubated at 30°C, with shaking, for 24 hours in Synergy HTX multi-mode reader, and readings were taken at OD_{600} every 30 minutes.

Secretome Preparation: To determine the role of T2SS in the secretion of putative virulence factors by vAh, secreted proteins of wild-type and T2SS mutants were isolated under planktonic and biofilm culture, as follows.

Planktonic Secretome: vAh ML09-119 wild-type and all secretion mutants were cultured as described above. Cells were pelleted by centrifugation at 20,000 *x* g for 15 minutes at 4°C; supernatant was decanted and retained. Cells were washed twice with cold, sterile PBS, pelleted as above, and the wash was added to supernatant. Remaining cells were removed by

passage through a low-binding 0.22µ vacuum filter (VWR). Cell-free supernatants were used as the starting point for secreted protein purification.

Biofilm Secretome: vAh ML09-119 wild-type and all secretion mutants, cultured as described above, were gently removed from biofilm media surface with sterile cell scraper, transferred to 50ml conical tube, and washed twice with cold, sterile PBS as described above. Cell wash was decanted and retained. To collect secreted proteins within biofilm media, the plates were disrupted using a sterile disposable probe until the soft agar had formed a slurry. The agar slurry was transferred to a sterile 50ml conical tube, centrifuged at 20,000 *x* g for 15 minutes at 4°C to pellet the agar. Following centrifugation, the liquid media was decanted from the agar plug and retained. The agar plug was then resuspended in 20ml cold sterile PBS, centrifuged as above, and wash solution decanted and retained. All wash solutions and liquid media were combined and filtered, first through a low-binding 0.45µm vacuum filter (VWR), then through a low-binding 0.22µ vacuum filter to remove any residual agar or bacterial cells. This cell-free supernatant was used at the starting point for biofilm secretome purification.

Ammonium Sulfate Precipitation: Secreted proteins were precipitated from cell-free supernatants by the addition of ammonium sulfate crystals (Fisher Scientific) to achieve 65% saturation, followed by incubation at 4°C on a rotary platform shaker with gentle mixing for 24 hours. Precipitated proteins were collected by centrifugation at 30,000 *x* g for 45 minutes at 4°C, then dissolved in 10ml cold Tris buffer (20mM Tris-Hcl, pH 7.6). Resuspended proteins were dialyzed twice, for 18 hours and 12 hours, respectively, against the same buffer in 10Kda dialysis cassettes (Slide-A-Lyzer (Thermo Fisher). After dialysis, the total volume was adjusted to 20ml by the addition of cold Tris buffer. Protein concentration of each sample was determined by

Bradford assay (Pierce Coomassie Plus Protein Assay, Thermo Fisher). These concentrated proteins were used for all assays.

PAGE Analysis. Secreted protein profiles were examined by polyacrylamide gel electrophoresis (PAGE). Aliquots of secreted proteins reduced with β-mercaptoethanol were loaded onto a pre-cast 12% Tri-glycine stain-free gel (NuSep) and electrophoresed at 200v for 45 minutes. Gels were developed and documented using Gel Doc EZ imaging system (Bio-Rad).

Enzymatic Activity. *In vitro* activity of secreted proteins was measured using multiple substrates to determine degradative and toxigenic potential of secretomes.

Hemolysis: Hemolytic potential was measured using the method of Peatman et al. (2018) with some modifications. In brief, heparinized blood from three channel catfish was pooled and diluted 1:10 in sterile phosphate buffered saline (PBS). A suitable dilution of protein in 150µl PBS buffer was added to 25µl diluted blood in sterile microcentrifuge tubes. Tubes were incubated at 30°C in an orbital shaker for 2 hours. Positive control tubes representing 100% hemolysis contained 150µl sterile distilled water and negative control tubes contained 150µl sterile PBS in place of protein samples and were incubated with 25µl diluted blood as above. Following incubation, tubes were centrifuged at 1,000*x g* to pellet unlysed cells and 150µl of supernatant was transferred to 96-well flat bottom plates. Erythrocyte lysis was measured by measuring hemoglobin absorbance at 415nm in multi-mode plate reader (Synergy HTX, Bio-Tek) and hemolysis was reported as percent of positive control.

Universal Protease Activity: Non-specific proteolytic activity was measured using HiLyteFluor 488-labeled casein as the substrate, following manufacturer's protocol with minor modifications (Sensolyte Green Fluorimetric Protease Assay Kit, AnaSpec, Inc.). Briefly, a

suitable concentration of protein in 50µl deionized water was added to triplicate wells of black, flat-bottom 96-well plate with non-binding surface (Greiner Bio-One). Trypsin, diluted 50-fold in deionized water, acted as a positive control and sterile deionized water was a substrate control. Following the addition of 50 µl labeled casein substrate, plates were mixed briefly and fluorescent intensity was measured at Ex/Em = 490nm/520 nm every five minutes for one hour in a multi-mode plate reader (Synergy HTX, Bio-Tek) with 30°C incubation temperature. Data was plotted as relative fluorescence units versus time for each sample.

Elastase Activity: Elastase-specific activity was measured using 5-FAM/QXL^{TM} 520 labelled elastin as the substrate, following manufacturer's protocol with minor modifications (Sensolyte Green Fluorimetric Elastase Assay Kit, AnaSpec, Inc.). Briefly, a suitable concentration of protein in 50µl deionized water was added to triplicate wells of black, flat-bottom 96-well plate with non-binding surface. Elastase, diluted 50-fold in assay buffer, acted as a positive control and sterile, deionized water was a substrate control. Following the addition of 50µl labeled elastase substrate, plates were mixed briefly and fluorescent intensity was measured continuously at Ex/Em = 490nm/520nm, and data recorded every five minutes for one hour in a multi-mode plate reader with 30°C incubation temperature. Data was plotted as relative fluorescence units versus time for each sample.

***In vivo* virulence**. Channel catfish were challenged with wild-type and T2SS mutants to determine role in *in vivo* virulence.

vAh cell preparation: Isolated colonies of wild-type vAh, ML09-119, and T2SS mutant, Δ*exeD* were inoculated into 20ml of TSB. An isolated colony of T2SS complemented mutant, *exeD*::T2SS, was inoculated into 20ml TSB + 25µg/ml Cam. All isolates were incubated at 30°C

with shaking at 200 rpm to an OD_{600}= 1.0. Ten ml of each culture was pelleted by centrifugation at 6,000 rpm for 15 minutes, and supernatant was decanted. Cells were washed by resuspension in 10 ml sterile phosphate buffered saline (PBS), centrifuged as above, and cell washed decanted. Cells were then resuspended in 16 ml sterile PBS.

Catfish challenge: Specific – pathogen free channel catfish, reared under IACUC maintenance protocol #2018-3251, were transferred to 30 gallon aquaria and allowed to acclimate for 1 week under flow-through conditions, with water temperature of 30°C, controlled by in-tank heaters. Airstones present in each tank provided aeration. Challenges were performed under the IACUC-approved guidelines described in IACUC #2020-3671 (Studies into the pathogenesis of virulent *Aeromonas hydrophila* in channel catfish). Each challenge group was comprised of ten fish per tank, with three experimental replicate tanks, for a total of 30 fish per challenge group. Challenge groups consisted of PBS injection control, wild-type vAh, ML09-119, T2SS-null mutant, Δ*exeD*, and T2SS-complemented mutant, *exeD*::T2SS. Prior to challenge, fish were sedated by immersion in an anesthesia tank containing 80mg/L tricaine methanosulfate (MS-222) buffered to neutrality with sodium bicarbonate. Sedation was characterized by slowing opercular movement and total loss of equilibrium. Sedated fish were challenged with $5x10^7$ CFU total cells in 200µl PBS introduced by intraperitoneal injection. Control fish were injected with 200µl sterile PBS. Fish were then returned to appropriate experimental replicate tank and monitored until fully recovered, as indicated by normal swimming and response to visual stimuli. Fish were monitored hourly for 8 hours, then twice daily for five days, and moribund fish were euthanized by prolonged immersion in 300mg/L neutral-buffered MS-222.

Statistical Analyses. Statistical analyses were performed in Prism 8.2.0 (Graphpad). One-way ANOVA followed by Tukey's multiple comparisons post test were performed on triplicate data with significance set at $p < 0.05$. Graphical representations of data were produced in Prism 8.2.0.

4. Results

Deletion of *exeD* gene by recombineering and creation of marker-less mutant. To determine the role of the T2SS in the virulence of vAh, the *exeD* gene, which codes for secretin, was targeted for deletion by recombineering. The three genes required for gene deletion via recomineering, *exo, bet,* and *gam*, were provided by the pMJH65 plasmid which had previously been introduced into vAh ML09-119 (Hossain et al., 2015). As the pMJH65 plasmid confers tetracycline resistance, chloramphenicol was chosen as the selectable marker for *exeD* gene deletion. The *cat* cassette, along with the flanking FRT sequences, was extracted from pKD3 primer. Overlap sequences with homology to the upstream, *exeC*, and downstream, *exeE*, nucleotide sequences were added by overhang PCR, and this recombineering product was amplified with phosphorothioated primers at 5' and 3' ends to minimize exonuclease degradation. This recombineering cassette was then introduced into ML09-119 + pMJH65 by electroporation, and six colonies were recovered on selective media. Colony PCR verified the deletion of *exeD* and the incorporation of FRT*cat* (Figure 1). In-frame deletion of *exeD* was confirmed by RT-PCR with *exeE* primers (Figure 2) and growth curves were performed to verify cell viability following *exeD* deletion (Figure 3). Positive *exeD* deletion colonies were denoted *exeD*:FRT*cat.* The pMJH65 plasmid was effectively cured from *exeD*:FRT*cat* by heat induction. To create the markerless mutant, Δ*exeD*, pCMT-flp plasmid containing flippase machinery and conferring tetracycline resistance (Hossain et al., 2015), was electroporated into the mobilizing *E. coli* SM10 λ*pir* and dozens of successful transformants were recovered on selective media.

Conjugal transfer of pCMT-flp from *E. coli* SM10 λ*pir* + pCMT-flp to *exeD*:FRTcat was performed using a 1:4 donor : recipient ratio incubated at 30°C for 24 hours on blood agar plates, with *exeD*:FRT*cat* + PCMT-flp transconjugants recovered on selective media containing tetracycline and colistin. Flp-frt-mediated gene deletion was initiated in *exeD*:FRT*cat* + PCMT-flp by heat induction, then cells were streaked for isolation. Colony PCR was performed and amplicons from colonies with successful gene deletion revealed the FRT 'scar' (Figure 4). Colonies that failed to grow in the presence of chloramphenicol and produced an FRT 'scar' signature on PCR were denoted Δ*exeD*. The pCMT-flp plasmid was cured by heat induction, resulting in the loss of tetracycline resistance in the marker-less Δ*exeD* mutant.

Restoring T2SS by whole-pathway complementation. HMW gDNA isolated from vAh ML09-119 was sent to Varigen Biosciences for pathway cloning using CRISPR-Cas9 restriction system. Using guide RNAs specific to the T2SS genomic region, Cas9 restriction was performed, and the T2SS fragment extracted. The T2SS fragment was assembled into pBAC-S vector and transformed to *E. coli* BacOpt v.2.0. Hundreds of transformants were recovered and successful cloning was confirmed by colony PCR and NotI restriction digest (Figure 5).

Conjugal transfer of pBAC + T2SS into Δ*exeD* mutant by triparental mating required a helper strain of *E. coli*, HB101 pRK2013, to mobilize the plasmid from the donor. A cell ratio of 1:1:4 donor:helper:recipient was used for conjugal transfer using a spread-plate method, and transconjugants were recovered on selective media containing Cam and Col. Transconjugants were screened by PCR for the presence of *exeD* gene and *flp*-scar amplicons. Three colonies were selected for screening, and all three contained both *exeD* gene and *flp*-scar amplicons (Figure 6). These colonies were denoted *exeD*::T2SS.

Determining the functional role of T2SS. Cell growth curves were performed on WT and Δ*exeD* to ensure cell viability in secretion-null mutants. While growth was slower, Δ*exeD* was capable of reaching cell densities comparable to WT, but revealed a reduced stationary phase, with decline beginning after approximately 3 hours (Figure 3).

To confirm that deletion of *exeD* resulted in a secretion-null mutant, and that T2SS complementation restored secretory function, secretomes of WT and mutants were prepared and visualized by PAGE (Figure 7). The Δ*exeD* mutant secretome was nearly devoid of protein, confirming the loss of T2 secretion, while complementation with whole-pathway T2SS largely restored secretion. To determine if hemolytic and proteolytic proteins were secreted via T2SS, secretomes of WT and mutants were screened against multiple substrates. Hemolytic potential of planktonically-secreted proteins was measured using catfish erythrocytes as the target substrate. Δ*exeD* secretomes showed no hemolytic activity, while complementation fully restored, and perhaps increased, hemolytic activity (Figure 8). Universal proteolytic potential of planktonic and biofilm-secreted proteins was measured against a casein substrate. No casein proteolysis was present in either the planktonic or the biofilm secretomes of Δ*exeD*. Complementation restored the caseinolytic potential in the *exeD*::T2SS mutant (Figure 9). Elastase-specific activity of planktonic and biofilm secretomes was also measured. No elastinolytic activity was observed in planktonic or biofilm secretomes of Δ*exeD*, while complementation of TS22 restored elastinolytic capacity in both *exeD*::T2SS secretomes (Figure 10).

To determine the importance of T2SS and, thus, T2S proteins in the pathogenicity of vAh, *in vivo* challenges were performed on channel catfish fingerlings. Catfish were challenged with $5x10^7$ CFU vAh WT and mutants by IP injection. Fish challenged with vAh WT were

dead or moribund after 4 hours, with average mortality of 96%. *exeD*::T2SS-challenged fish reached 73% mortality by 6 hours, while Δ*exeD*-challenge produced 3% mortality after 5 days (Figure 11). These results provide convincing evidence of the prerequisite presence of T2S substrates in vMAS outbreaks.

5. **Discussion**

Aeromonas hydrophila are well-documented producers of putative virulence proteins. Historically, these proteins have been documented to target host cells by secretion through T3 and/or T6SS (Rosenzweig & Chopra, 2013; Tomas, 2012; Vilches et al., 2004; Yu et al., 2004), while T2SS is considered a general secretion pathway, playing important roles in environmental organisms (Cianciotto, 2005) and opportunistic pathogens (Cianciotto & White, 2017).

No vAh isolated from the US possess a T3SS or a complete T6SS, though all isolates have at least three remnant T6SS genes (Tekedar et al., 2019). While vAh do possess T1SS, as well as a tight adherence (Tad) secreton, primarily responsible for exporting Flp pili (Tomich, Planet, & Figurski, 2007), these secretion pathways transport specific proteins and are likely not capable of the exportation of a vast array of proteins, particularly from within the periplasmic space. Therefore, the aim of this research was to study the role of T2SS and its effectors in the pathogenicity and virulence of vAh. To this end, a T2 secretion-deficient mutant was generated using a recombineering method to create an in-frame mutation by replacing T2SS gene coding for secretin, *exeD*, with the selective marker, *cam*. The flp-mediated excision of *cam* created the markerless mutant, Δ*exeD*. PAGE analysis verified the inhibition of secretion by Δ*exeD*, while growth curves demonstrated cell viability in this secretion-deficient mutant. A third technique, made possible by the use of cas-9 restriction and whole-pathway cloning, was used to

complement the T2SS by tri-parental mating. PAGE analysis verified the restoration of secretion in this complemented mutant, *exeD*::T2SS.

In vitro functional assays assessing the degradative and hemolytic potential of WT and mutant vAh found a complete loss of degradative capacity against casein and elastase in Δ*exeD*. While vAh virulence is almost assuredly multi-factorial, the role of elastase in disease initiation and progression has been well documented. One study reported that the presence of elastase was required for *A. hydrophila* virulence in a rainbow trout (*Oncorhynchus mykiss*) model, with LD_{50} values two orders of magnitude higher in elastase-deficient mutant (Cascón et al., 2000). In *Pseudomonas aeruginosa*, elastase contributes to the invasiveness of the organism, likely by working in concert with other secreted proteins to weaken epithelial barrier function (Kamath, Kapatral, & Chakrabarty, 1998; Li et al., 2019). In *P. aeruginosa*, elastase is also responsible for significant tissue damage, especially in cystic fibrosis patients and those with other chronic respiratory infections. Elastin is critical for elasticity in lung tissue, and degradation by elastase has been implicated in lung fibrosis (Bleves et al., 2010; Li et al., 2019). Elastase may also be capable of degradation of other structural proteins, such as collagen types III and IV, which may lead to the destruction of the basement membrane, granting bacterial ingress to the dermal tissue (Cascón et al., 2000; Kamath et al., 1998). Elastase works in concert with other secreted proteins to increase tissue destruction and previous work in this lab identified a plethora of secreted proteins with degradative potential. The loss of general and elastase-specific degradation in Δ*exeD* supports the hypothesis that T2SS is the main secretory network used by vAh ML09-119, and is likely responsible for secretion of a vast majority, if not all, degradative virulence proteins. In previous work, we demonstrated the significant increase in degradative proteins when vAh was cultured as a biofilm; identifying many proteins in the secretome as well as the significant

upregulation of these genes in the transcriptome. The increased presence of degradative proteins in biofilm-cultured vAh suggests that, *in natura*, these proteins act on natural substrate as a means of nutrient acquisition. Thus, while secreted proteins are likely of paramount importance for initial host colonization, the peracute nature of vAh-induced MAS mortality is most likely attributable to the host of hemolysins, enterotoxins, and cytotoxins produced by vAh. In order to test the degree of vAh toxin secretion through T2SS, secretomes of wild type and mutants were screened for hemolytic capacity. *A. hydrophila* hemolysins have been implicated as major virulence factors in fish and human *A. hydrophila* isolates and are often considered the main virulence factor of pathogenic *A. hydrophila* (Bi et al., 2007; Tomas, 2012; Wu & Guo, 2010). Both the secretomes and transcriptomes reveal vast amounts of hemolysins are secreted by planktonically-cultured vAh, and *in vitro* hemolysin assays verify the hemolytic potential in these secretomes. In contrast, Δ*exeD* secretomes had a complete loss of lytic potential (Figure 8), again, suggesting the vast majority of vAh hemolysins are secreted via T2SS. This is somewhat surprising, as *E. coli* and closely-related *V. cholerae* transport hemolysin via T1SS (Thomas et al., 2014), and an increase in ABC-transport proteins, often associated with T1 secretion, was found in the secretomes and transcriptomes of biofilm-associated vAh. It would not have been unexpected for a certain degree of hemolytic activity to be retained by Δ*exeD*. The restoration of proteolytic and hemolytic potential by whole-pathway T2SS complementation further supports the hypothesis that vAh requires T2SS for virulence.

In vivo challenges in catfish provide convincing evidence of the role of T2SS substrates in the development of vMAS and for vAh virulence. Complete attenuation of virulence in Δ*exeD* and restoration of virulence in *exeD*::T2SS provide convincing evidence that vMAS is, in large part, a toxin-mediated disease, and that T2SS and its substrates are required for vAh virulence.

In conclusion, the experiments performed herein have demonstrated that T2SS and the T2S effectors play a vital role in the pathogenicity of vAh, and in the development of vAh-induced MAS in channel catfish. Most importantly, these data have shown that T2S is essential for the secretion of proteolytic and hemolytic proteins, and that a functional T2SS is prerequisite for the development of vMAS.

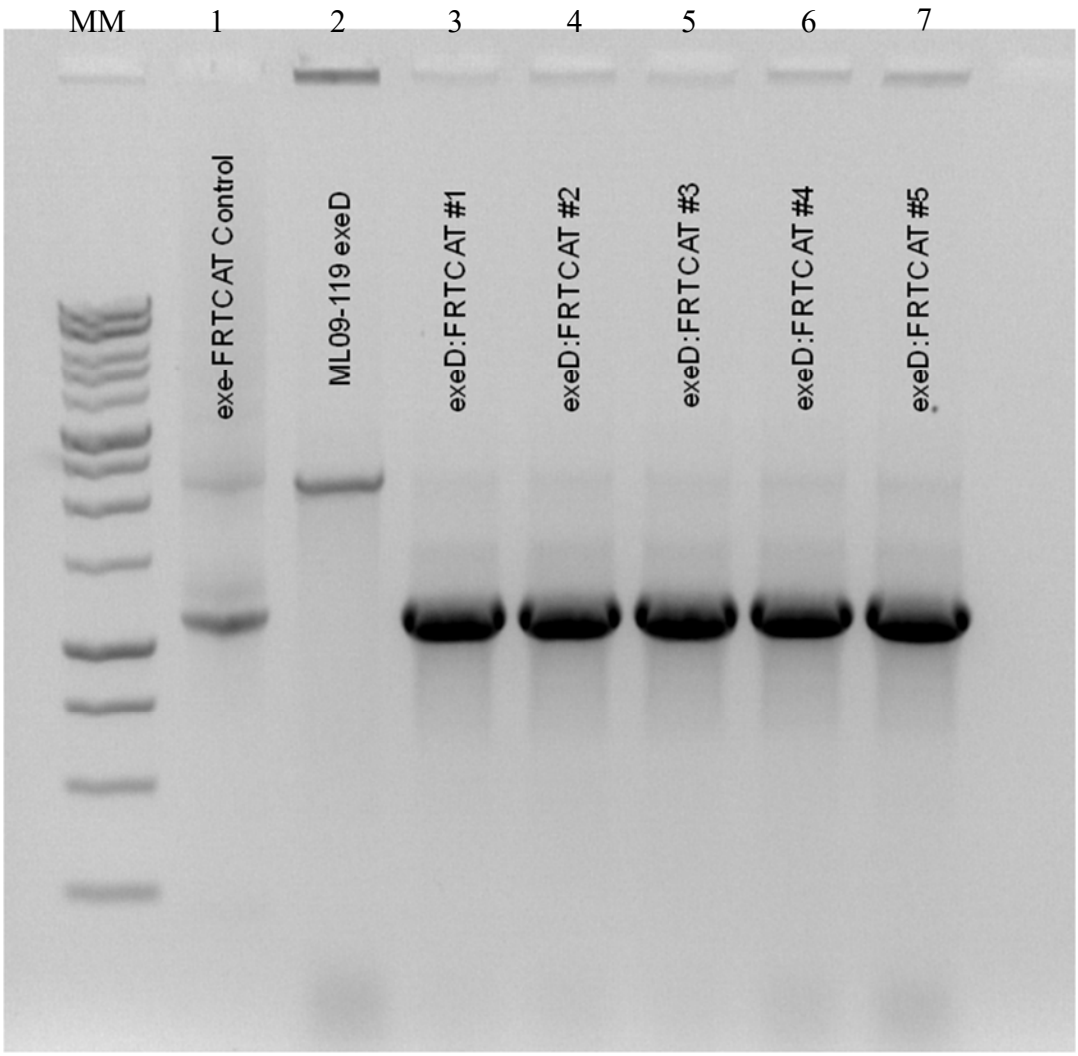

Figure 1. Confirmation of *exeD* deletion by homologous recombination in *exeD*:FRT*cat* mutants. Following electroporation of FRT*cat* cassette into vAH ML09-119 + pMJH65, colonies growing on selective media supplemented with CAM were selected and deletion of *exeD* gene was verified by colony PCR using primers that flanked *exeD*. MM. Molecular Marker Lane 1. Positive Control, Lane 2. Negative Control, Lanes 3 – 7. Recombinant *exeD*:FRT*cat* mutants showing successful deletion of *exeD* and incorporation of FRT*cat* cassette.

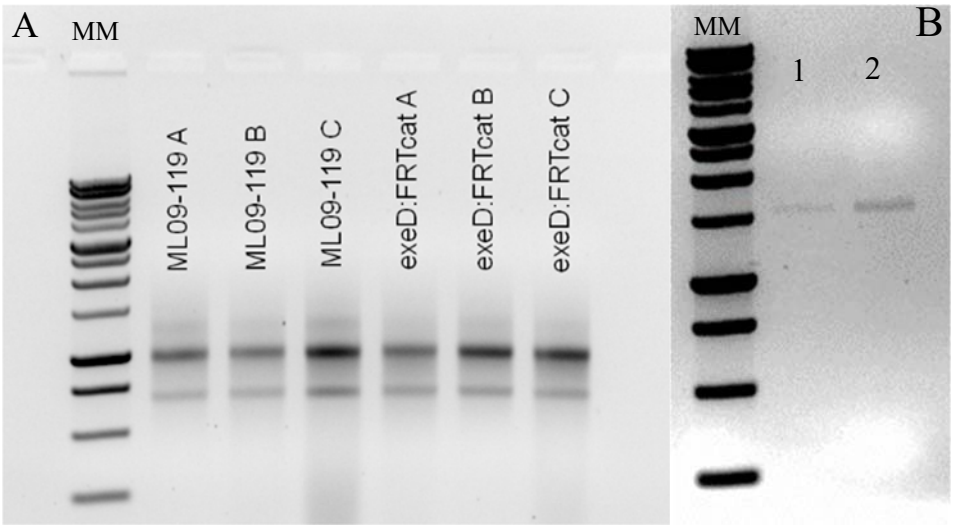

Figure 2. Verification of in-frame deletion of *exeD* in *exeD*:FRT*cat* mutants. RNA extraction was performed on wild type vAh ML09-119 and *exeD*:FRT*cat* mutants, followed by RT-PCR using *exeE*-specific primers. Following RNA isolation, RNA integrity was verified by electrophoresis through a 0.5% bleach gel. **A.** Two distinct bands, representing 23S and 16S RNA confirm RNA integrity prior to RT-PCR. **B.** Agarose gel of RT-PCR products using *exeE* -specific primers. *exeE* amplicon is visible at 1500 bp. MM = Molecular Marker. Lane 1. WT ML09-119 Lane 2. *exeD*:FRT*cat* RT-PCR.

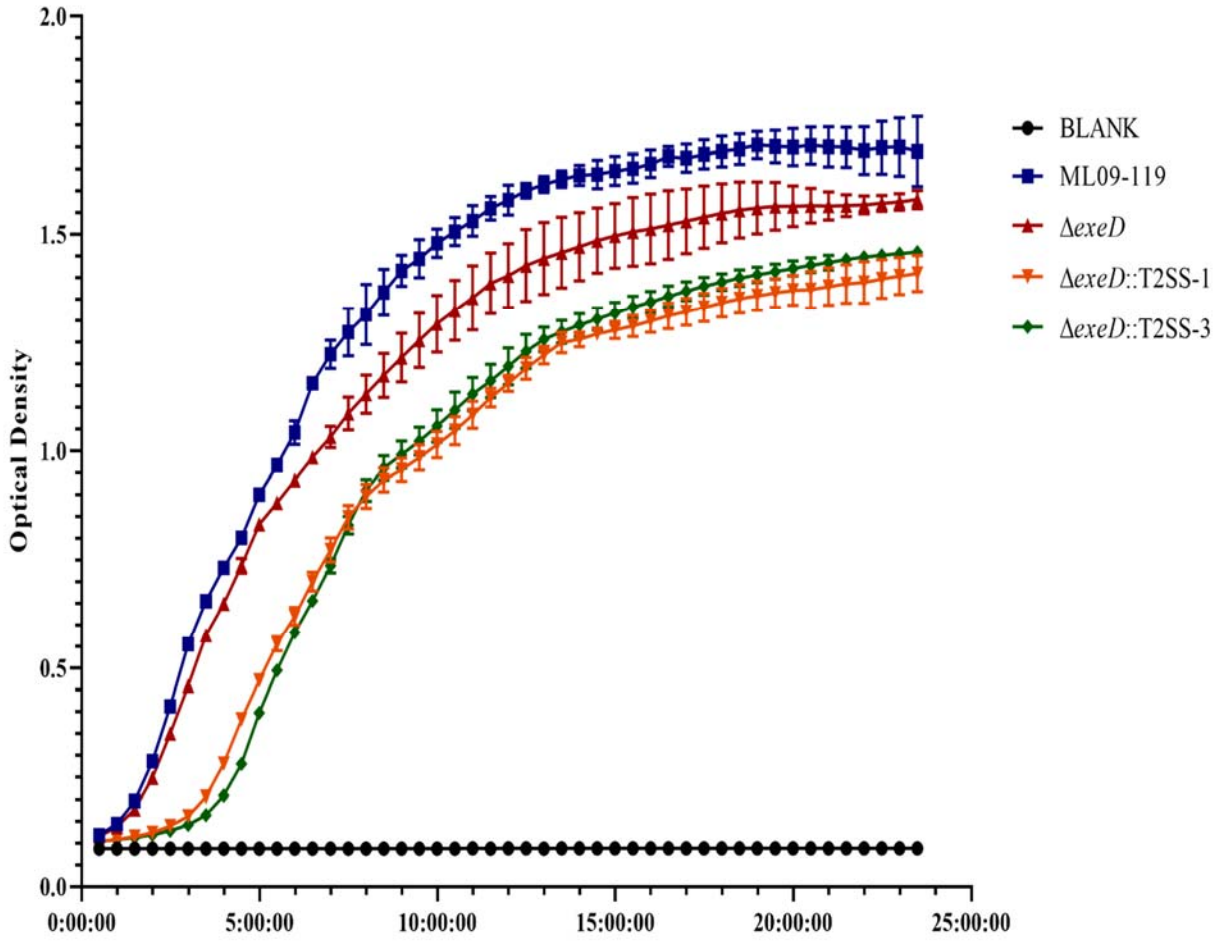

Figure 3. 24-hour growth curve of WT ML09-119, Δ*exeD*, and *exeD*::T2SS mutants. WT growth is more rapid, with a longer stationary phase. Δ*exeD* growth is slower and cell density is slightly decreased. *exeD*::T2SS mutant growth rate is decreased, however, substantial growth occurs, confirming cell viability in all mutants.

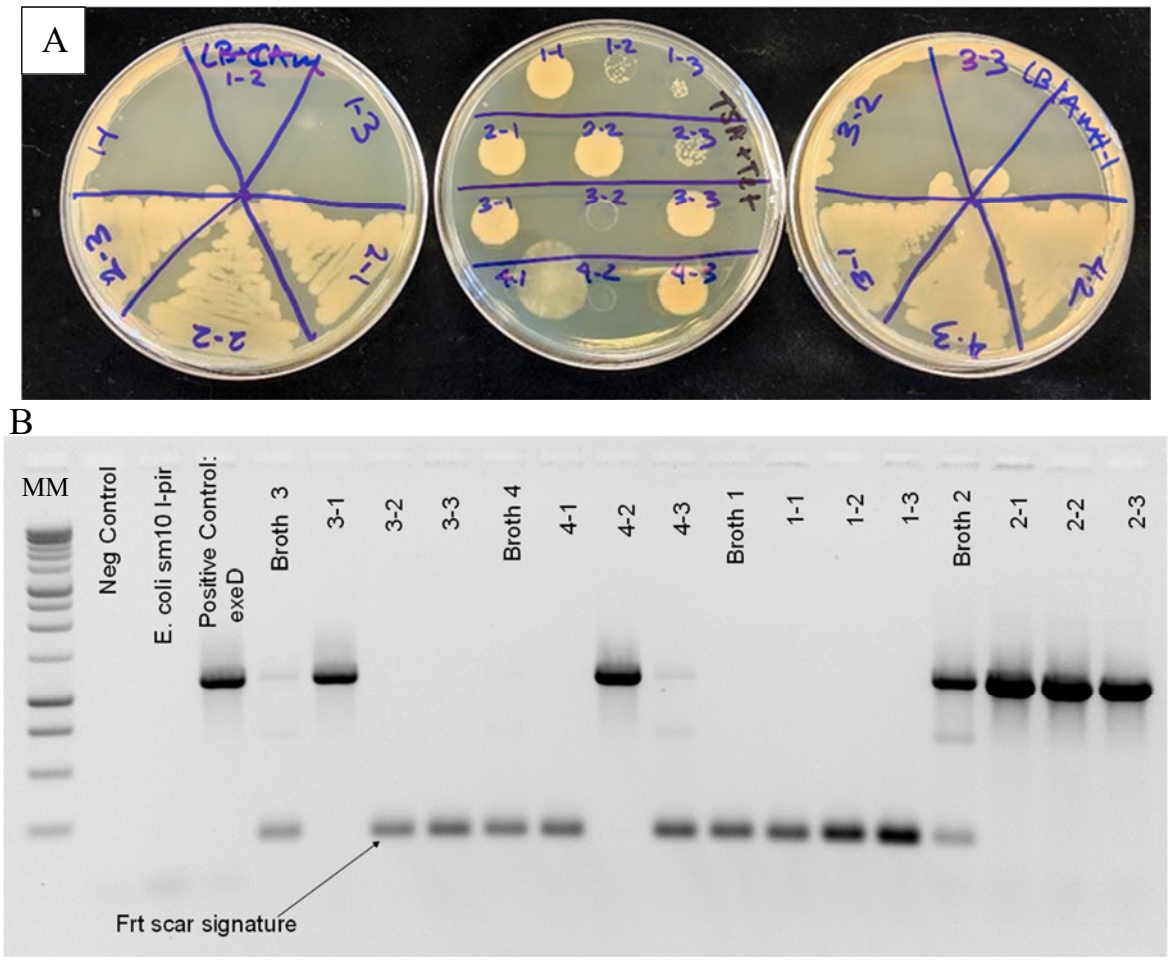

Figure 4. Creation of Δ*exeD* mutant by FLP - mediated recombination. Following conjugal transfer of plasmid pCMT-flp into *exeD*:FRT*cat* mutant, flp-recombinase was heat induced for 1 hour to allow removal of *cat* cassette. pCMT-flp plasmid, which carried Tet resistance, was cured by continued growth at 37°C for 4 hours. Cultures were streaked for isolation on non-selective media, then isolated colonies were plated on media containing Tet and Cam (**A**). Colony PCR was performed on colonies that failed to grow on Cam, Tet, and Cam and Tet using primers flanking *exeD* (**B**). Colonies with amplicons showing the frt 'scar' signature, and that were unable to grow in the presence of Cam or Tet had successful FLP-mediated excision of *cat* gene and had successfully been cured of pCMT-flp plasmid. Lane 1. Molecular Marker, Lane 2.

Negative Reagent Control, Lane 3. Negative Control, *E. coli* SM10 λpir, Lane 4. Positive Control, *exeD*:FRT*cat* mutant, Lanes 5 – 20. *exeD*:FRT*cat*:*flp* colonies

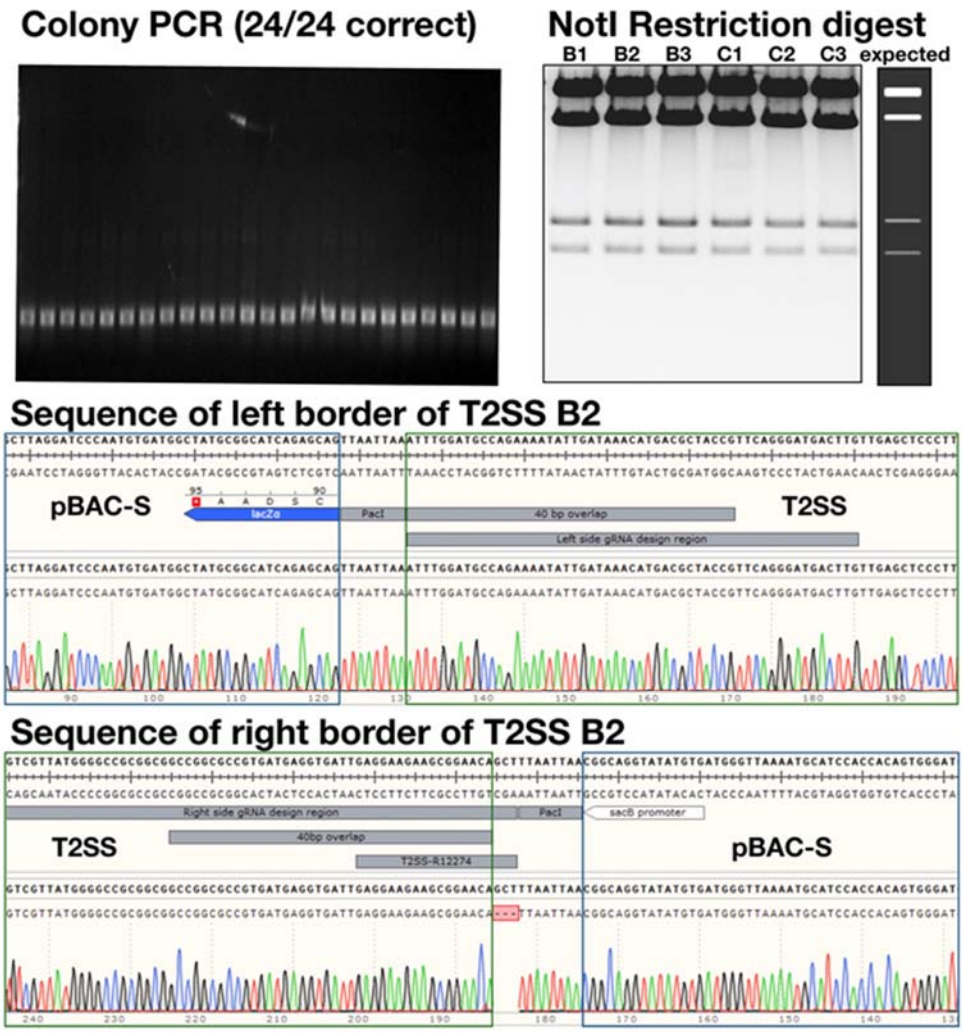

Figure 5. T2SS whole-pathway cloning validation. Pathway cloning was performed and validated by Varigen BioSciences. Using a proprietary CRISPR-Cas-9 restriction method with guide RNAs specific to the T2SS genomic region, the pathway was assembled into pBAC-S vector and transformed into BAC-optimized electrocompetent cells. Successful cloning was confirmed by colony PCR, *Not*I restriction digestion, and sequencing.

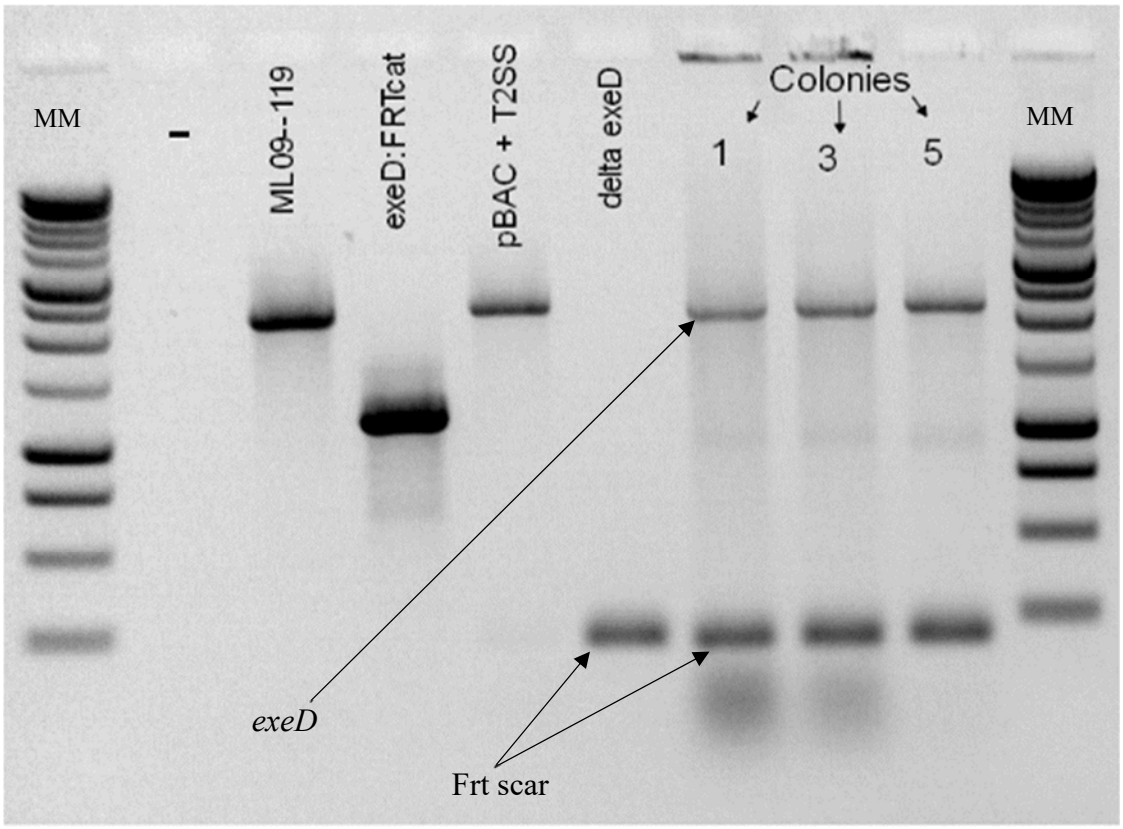

Figure 6. T2SS pathway complementation. Following tri-parental mating, transconjugants were recovered on selective media. Colony PCR was performed using *exeD*-specific primers to confirm the presence of the T2SS pathway. Successful complementation was verified by the presence of *exeD* gene and frt 'scar' amplicons. Lane 1. Molecular Marker, Lane 2. Negative Reagent Control, Lane 3. Positive Control (*exeD* gene amplified from wild type), Lane 4. Negative Gene Control (FRT*cat* insert amplified from *exeD*:FRT*cat* mutant), Lane 5. Positive pBAC Control (*exeD* gene amplified from the T2SS within pBAC), Lane 6. Frt scar Positive Control (amplification of 'scar' signature from Δ*exeD*), Lanes 7-9. *exeD*::T2SS transconjugants, Lane 10. Molecular Marker

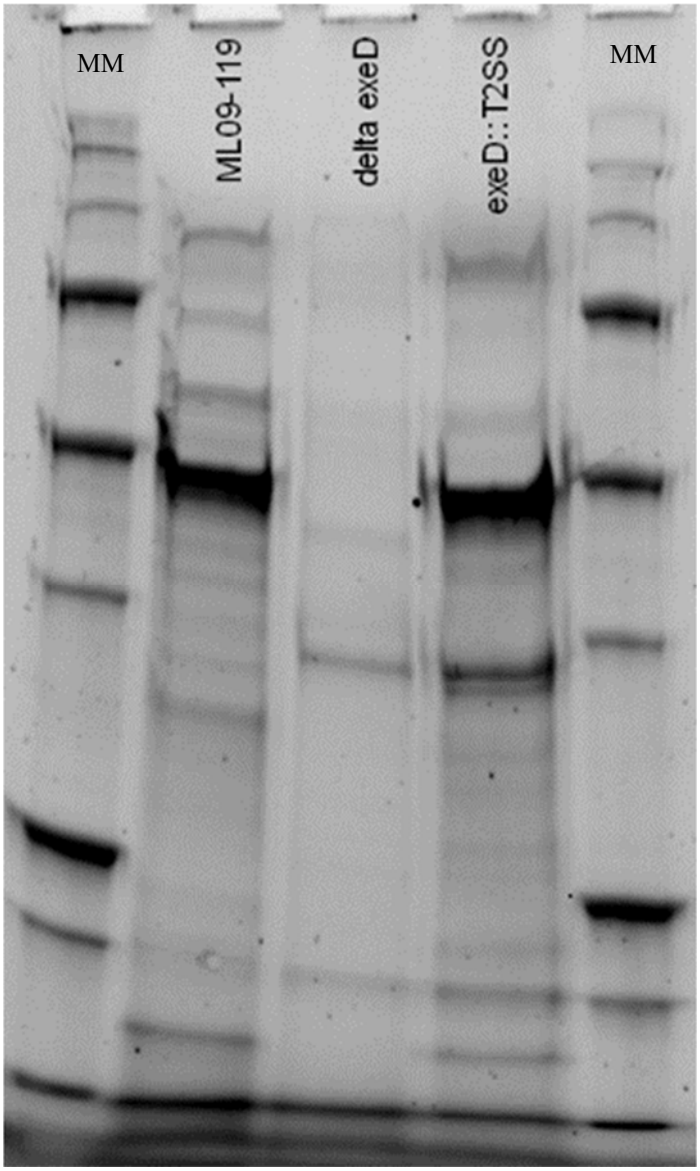

Figure 7. SDS-PAGE of proteins present in growth media of wild type ML09-119, Δ*exeD* mutant, and T2SS-complemented mutant, *exeD*::T2SS. Minimal protein in the growth media of Δ*exeD* is restored in the *exeD*::T2SS mutant, confirming the restoration of secretory function. MM = Molecular Marker.

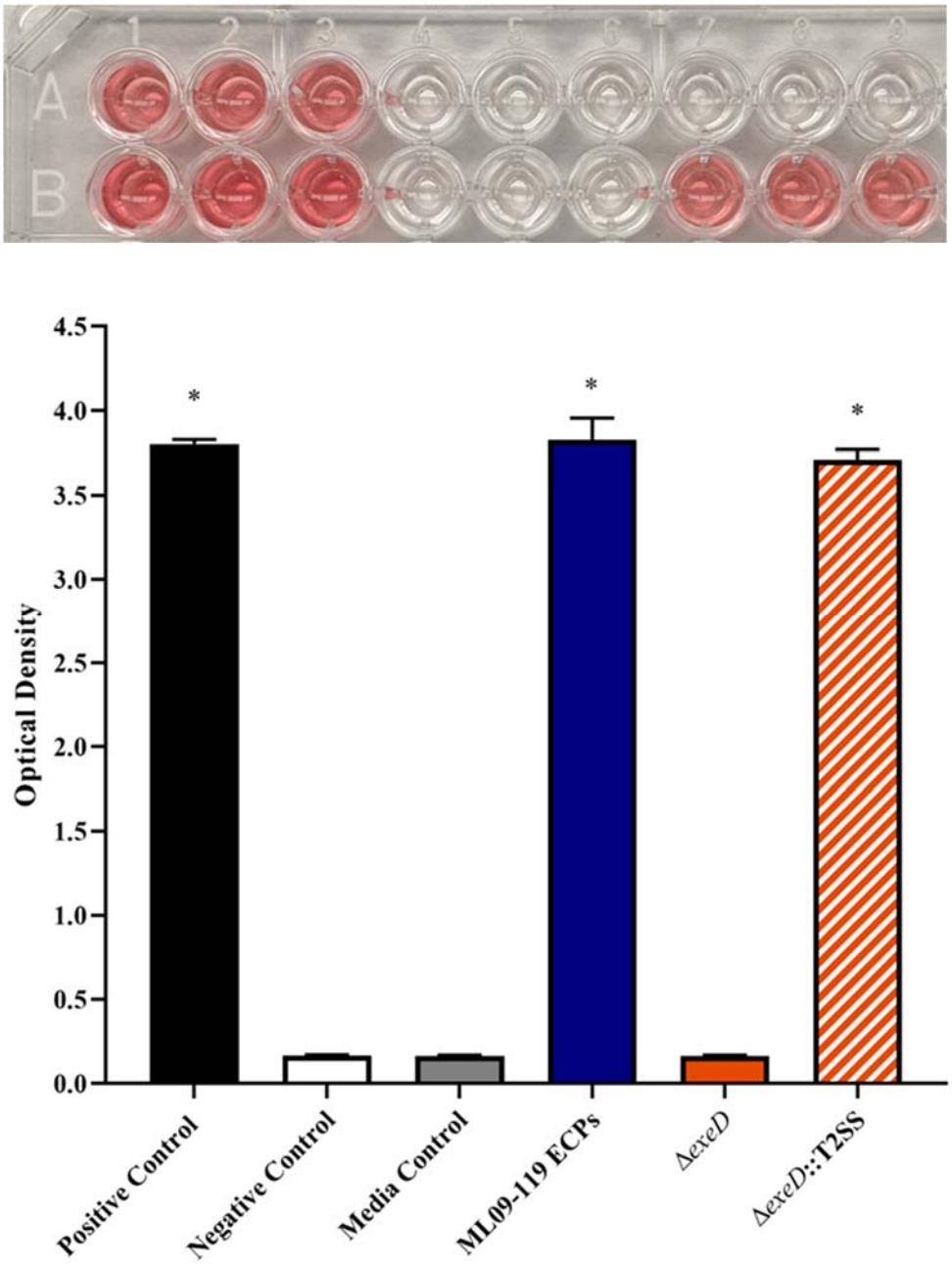

Figure 8. Hemolytic potential of wild type and mutant secretomes. Top: Plate containing triplicate results of hemolysis assay (top), with corresponding optical density measurements (bottom). Row A: 1-3, Positive Control. Row A: 4-6, Negative Control. Row A: 7-9, Media Control. Row B: 1-3, Wild type ML09-119. Row B: 4-6 Δ*exeD*. Row B: 7-9 Δ*exeD*::T2SS. All

samples were assayed in triplicate. Statistical analysis consisted of one-way ANOVA followed by Tukey's multiple comparisons post-test with significance set at $p < 0.05$.

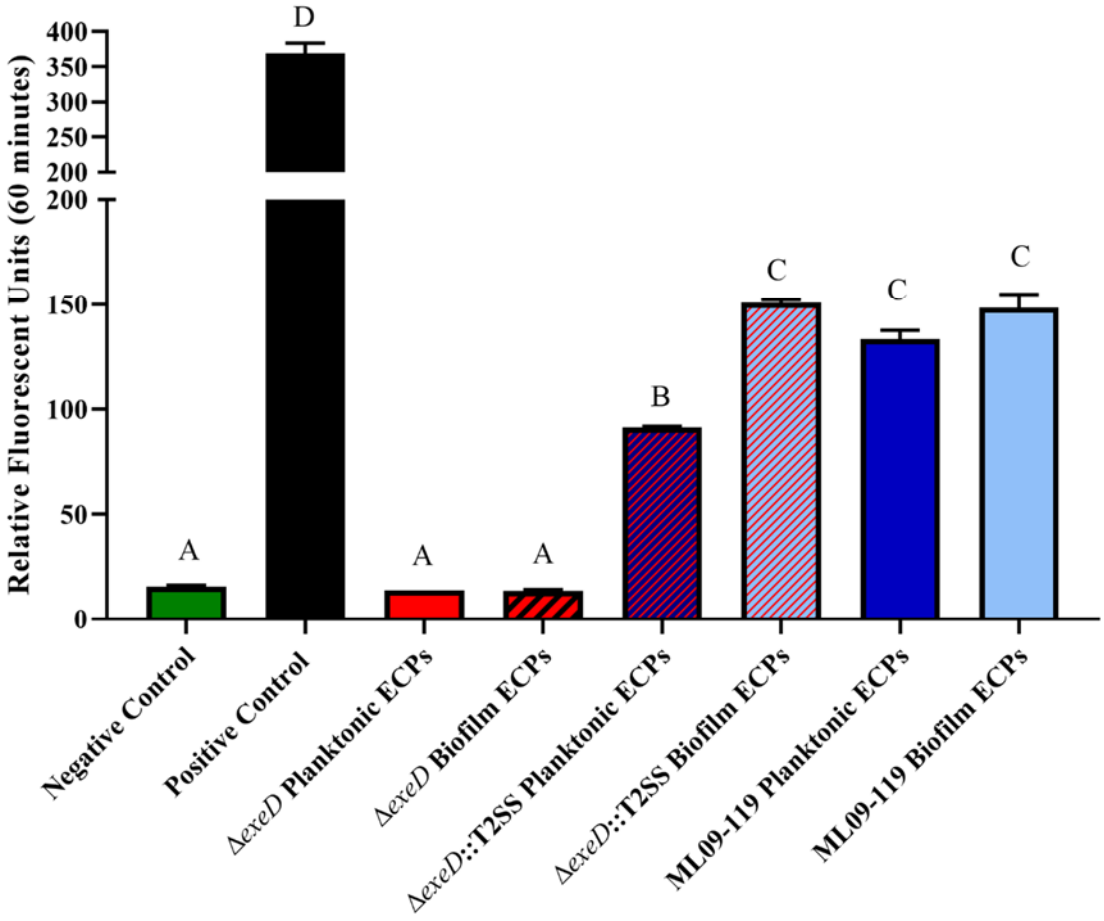

Figure 9. General proteolytic potential of vAh wild type and T2SS mutant proteins secreted under biofilm and planktonic growth. The general proteolytic potential of biofilm and plankonic secretomes was measure using HiLyteFluor 488-labeled casein as a substrate. Secreted protein from each condition was incubated at 30°C with labeled casein and fluorescent intensity was measured at Ex/Em = 490nm/520nm every five minutes for one hour. Data were plotted as relative fluorescence units versus time for each sample. Degradative potential was lost completely in Δ*exeD* mutants, but was restored in Δ*exeD*::T2SS complemented mutants. All samples were assayed in triplicate. Statistical analysis consisted of one-way ANOVA followed by Tukey's multiple comparisons post-test with significance set at $p < 0.05$.

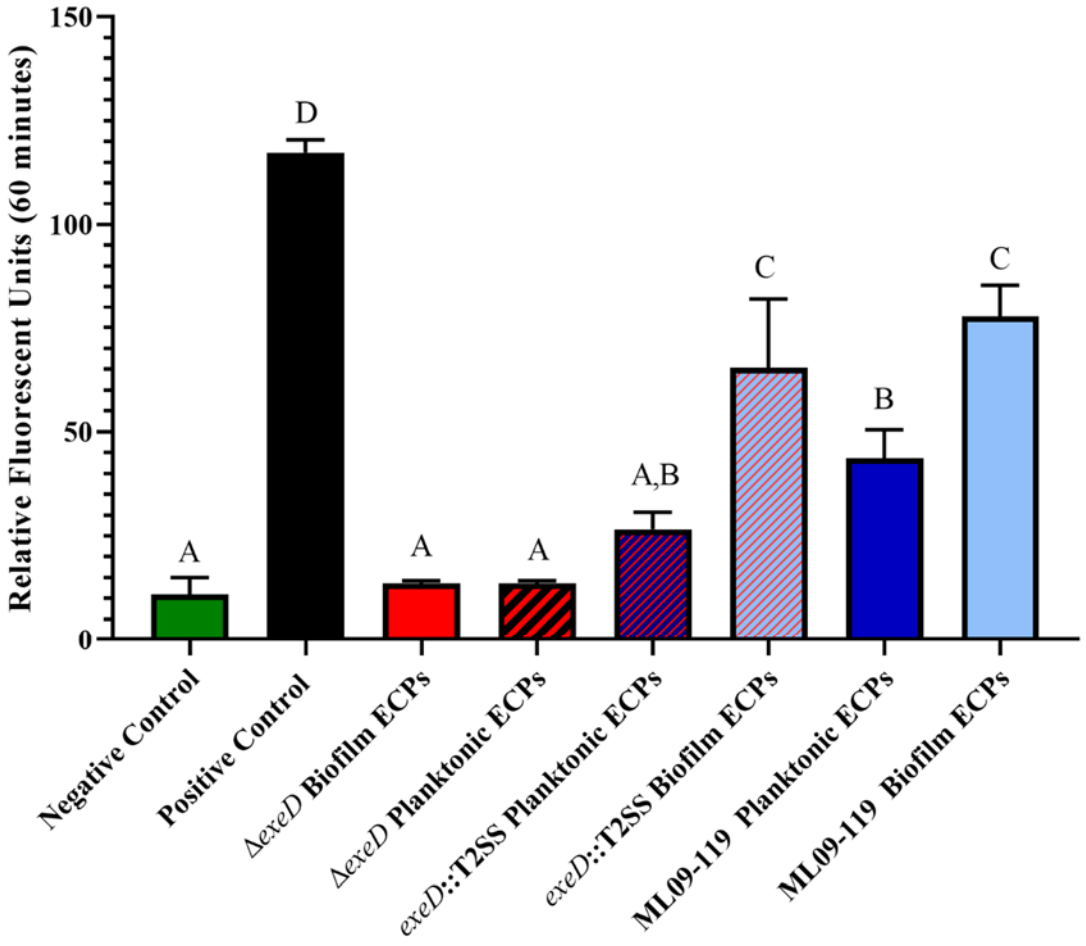

Figure 10. Elastase-specific degradative potential of vAh wild-type and T2SS mutant proteins secreted under biofilm and planktonic growth. The elastase activity of biofilm and plankonic secretomes was measure using 5-FAM/QXL™ 520-labeled elastin as a substrate. Secreted protein from each condition was incubated at 30°C with labeled elastin and fluorescent intensity was measured at Ex/Em = 490nm/520nm every five minutes for one hour. Data were plotted as relative fluorescence units versus time for each sample. Degradative potential was lost completely in Δ*exeD* mutants, but was restored in Δ*exeD*::T2SS complemented mutants. All samples were assayed in triplicate. Statistical analysis consisted of one-way ANOVA followed by Tukey's multiple comparisons post-test with significance set at $p < 0.05$.

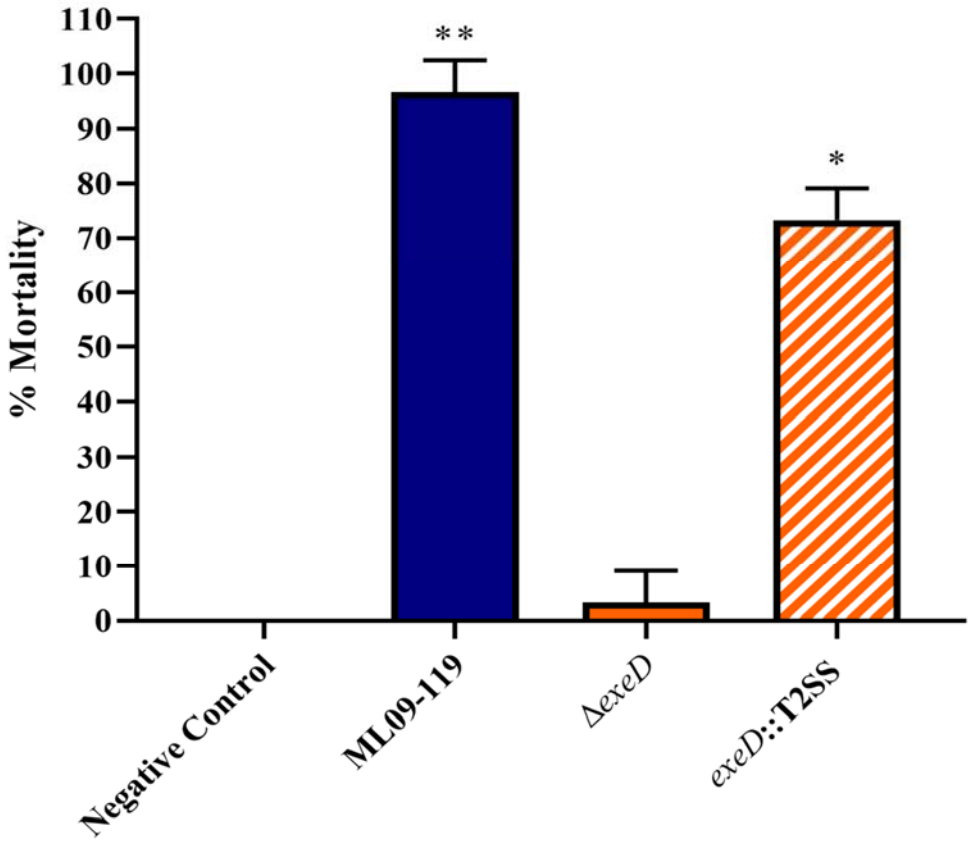

Figure 11. Percent channel catfish mortality when challenged with wild type and T2SS mutants. Fish were challenged with 200µl PBS containing 5 x 10^7 CFU of bacteria by intraperitoneal injection. Negative control fish were injected with 200µl sterile PBS. Fish were incubated in aquaria at 30°C. Wild type mortality was 96%, with death occurring in under 5 hours. T2SS-complemented mutant, *exeD*::T2SS, mortality was 67%. Survival was 100% in both Negative Control and T2SS deletion mutant, Δ*exeD*. The complete loss of virulence seen in Δ*exeD* mutant was largely restored by T2SS complementation, supporting the hypothesis that T2SS and T2SS substrates play a significant role in the pathophysiology of vMAS. Three individual experiments were performed. Statistical analysis consisted of one-way ANOVA followed by Tukey's multiple comparisons post-test with significance set at $p < 0.05$.

Table 1. Primers and plasmids used in the construction and complementation of Δ*exeD.*

Bacterial Strains and Plasmids	Description	Source
Aeromonas hydrophila strains		
ML09-119	Wild-type, vAh, Isolated from diseased channel catfish in 2009	Hossain et al., 2013
ML09-119 +pMJH65	Wild-type vAh carrying recombineering plasmid, pMJH65, Tet^r	Hossain et al., 2015
E. coli strains		
SM10λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Kan^r *λpir*	Szewczyk et al., 2006
HB101	F^- *mcrB mrr* hsdS20(r_B^- m_B^-) *recA*13 *leuB6 ara*-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20(Sm^R) glnV44 λ^-	Liles Lab
E.cloni®10G	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) *end*A1 *rec*A1 Φ80*dlac*ZΔM15 Δ*lac*X74 *ara*D139 Δ(*ara,leu*)7697 *gal*U *gal*K *rps*L *nup*G λ- *ton*A	Lucigen
BAC-Optimized Replicator v2.0®	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *end*A1 *rec*A1 Φ80d*lac*ZΔM15 Δ*lac*X74 *ara*D139 Δ(*ara,leu*)7697 *gal*U*gal*K *rps*L *nup*G (*att*L *ara*C-PBAD-*trf*A250 *bla att*R) λ-	Lucigen
Plasmids		
pKD3	Template plasmid for frt-flanked Cat cassette, Cat^r	Datsenko and Wanner, 2000
pMJH65	Conjugally transferable recombinogenic plasmid, Tet^r	Hossain et al., 2015
pCMT-flp	Temperature sensitive flp-recombinase, Tet^r	Hossain et al., 2015
pBAC-S	Non-self-transmissible expression vector, $Apra^r$, Cam^r	Varigen Biosciences
pRK2013	Helper plasmid for mobilization of non-self-transmissible plasmids, Kan^r	Liles Lab

Table 2. Oligonucleotides used in the construction and complementation of Δ*exeD.*

Oligonucleotide ID	Sequence	Applications
exeC-FRTCAT F	G*G*A *C*AG CTC TAC GAC GTT TAT G TC GGC TTG TCA GAA TAA TGA TTT GG A GTA GCA CCA AGA GTG TAG GCT GG A GCT GCT TC	Amplification of FRT-CAT sequence from pKD3 plasmid and addition of homologous sequence for recombineering
exeE-FRTCAT R	C*A*G *C*TC GGG CAG GGC TGC CGG C AT GTC AAC GCC ATC CAG CTG GTA TG C CGC CAT TAC TTG CAT ATG AAT ATC CTC CTT A	Amplification of FRT-CAT sequence from pKD3 plasmid and addition of homologous sequence for recombineering
exeC Homology F	G*G*A *C*AG CTC TAC GAC GTT TAT G TC GG	*exeD* amplification to check gene presence/deletions/insertions
exeE Homology R	C*A*G *C*TC GGG CAG GGC TGC C	*exeD* amplification to check gene presence/deletions/insertions
exeE Gene Forward	ATG GCG GCA TAC CAG CTG G	RT-PCR
exeE Gene Reverse	TCA GTC TTC CCG GGT CAC GC	RT-PCR

Chapter V

Conclusions

The research herein was performed with the goal of increasing our understanding of the mechanisms of pathogenicity of vAh. In natural infections, vAh acts as a primary pathogen, and causes devastating pond mortality in rapid, seemingly random, outbreaks of vMAS. However, natural infection models have been largely unsuccessful, with vAh mimicking traditional *A. hydrophila* in aquarium challenges. Most aquatic bacterial generalists, such as *A, hydrophila*, spend the majority of time resident in biofilms and host-microbe interactions are likely influenced by niche-specific microbial phenotype. Because biofilm-associated bacteria have emergent properties that cannot be elucidated by the study of free-living cells, it is imperative to study organisms within biofilms to understand how niche adaptations may influence overall pathogenicity and virulence. To this end, phenotypic and transcriptomic comparisons of vAh growing within a biofilm and as planktonic cells was undertaken in an attempt to determine how these two bacterial states could contribute to vAh virulence. Furthermore, vMAS causes rapid mortality with minimal histologic lesions in tissues, suggesting the vMAS is a peracute toxin-mediated disease. *A. hydrophila* have the capability to produce a vast array of degradative and potentially toxigenic secreted substrates. In order to determine if secreted proteins were instrumental in vAh pathogenicity and if the T2SS was the primary secretory pathway for these toxins, a T2SS deletion-deficient mutant was created by homologous recombination, and the gene deletion was complemented by whole-pathway complementation, restoring protein secretion and secretome function.

To determine how niche adaptation influences protein secretion, the secreted protein profiles (secretomes) of planktonic and biofilm-associated vAh were compared, both

functionally and quantitatively. *In vitro* functional analyses included toxin screening by measuring hemolytic activity against channel catfish erythrocytes and degradative capacity was measured using casein and elastin as substrates. Hemolytic activity in planktonic ECPs was more than 6 times higher than hemolytic activity in biofilm ECPs. However, degradative potential against casein and elastin was more than twice as high in biofilm ECPs than in planktonic ECPs. These increased proteolytic activity in the ECPs from biofilm-associated vAh may play an important role in the pathophysiology of vAh by increasing invasiveness by compromising skin, gill, and intestinal epithelial barriers. To determine if *in vitro* activity corresponded to *in vivo* activity, ECPs were injected intramuscularly in to channel catfish fingerlings. After 24 hours, all biofilm-ECP injected fish had developed large, necrotic lesions at the injection site. Histopathology of the biofilm-ECP tissues revealed edema, hemorrhage, and necrosis of skeletal muscle and adipose tissue at the injection site. In contrast, fish injected with planktonic ECPs were identical to the control fish, with no perceptible damage to skin, adipose, or muscle after seven days.

Analysis of biofilm and planktonic vAh secretomes was performed by HPLC MS-MS to identify and quantify proteins in each secretome. A total of 272 proteins were identified in the secretomes of biofilm and planktonically-cultured vAh. Eighty-two proteins were present in both secretomes, while biofilm-associated secretomes had 98 unique proteins and planktonic secretomes had 92 unique proteins. ROTS analysis was performed to determine statistically significant differences in secreted proteins, and those proteins above the significant fold-change threshold of ≥ 1.5 were considered differentially secreted. Thirty-five proteins were significantly increased in biofilm secretomes, 20 of which were unique to biofilm, and 15 proteins were significantly increased in planktonic secretomes, including nine unique proteins. Of the

significantly increased proteins, at least 30 have previously been implicated in virulence. Not all secreted putative virulence factors (PVFs) were differentially expressed, and many PVFs were present in both secretomes. Many significant biofilm proteins, including elastase, metalloprotease, and chitinase, were degradative in nature, while significant planktonic proteins, including hemolysins, lipase, and serine peptidase, were cytolytic and cytotonic in nature. Other significant biofilm proteins were associated with transport, carbohydrate metabolism, and polar flagella structure. Other significant planktonic proteins were outer membrane proteins and type 1 pili. The result of this research supports the hypothesis that niche occupancy plays a vital role in the production and secretion of vAh exoproteins, and provides insight into the adaptive physiological response of vAh based solely on growth condition. Increased secretion of colonization factors and degradative enzymes in biofilm growth may increase host invasiveness, while increased secretion of hemolysins, porins, and other potential toxins under planktonic growth could result in increased host mortality. Functional protein screening of ECPs using *in vitro* methods appear to function well as a screening tool to measure virulence, as degradative proteolytic screening were supported by both *in vivo* challenge results as well as the presence of significantly increased degradative proteins, particularly elastase, in biofilm secretomes. Furthermore, the *in vitro* screening for toxigenic potential was supported by significantly increased hemolysins and other potentially cytolytic proteins in the planktonic secretomes.

While many PVFs were present in the secretomes of vAh, sample preparation and assay conditions used in secretome analysis were not exhaustive, which likely led to other secreted proteins being either missed or lost during sample prep. Additionally, many proteins were present in one or both culture condition, but were excluded because they did not meet statistical significance. Furthermore, not all PVFs are secreted proteins. In order to determine niche

influence over gene expression, and how that influence could impact vAh pathogenicity, comparative transcriptomics were performed on planktonic and biofilm-associated vAh.

Differential expression analysis of the RNA-Seq results supported the hypothesis that niche occupancy plays a vital role in gene expression, with 50% of all genes differentially expressed. In order to explore how these changes in gene expression relate to virulence, the transcription of putative virulence factors was assessed under each growth condition. Upregulation of putative virulence genes related to motility, adhesion, iron binding, antibiotic resistance, and proteolysis was observed in biofilm-cultured vAh, while genes related to porins, toxins, O-antigen, and enzymes related to LPS production were upregulated in planktonically-cultured vAh. Upregulation of PVFs in biofilm seem to show the primary functions of iron scavenging and uptake, adhesion, and nutrient acquisition by production of proteolytic enzymes. Each of those functions are imperative for environmental biofilm niche maintenance, while also contributing significantly to the virulence and pathogenicity of the organism. These data suggest that vAh residing in biofilms *in natura* may be capable of survival, adhesion, and destruction of physical barriers, facilitating host invasion and increasing infectivity, while planktonic growth may prime vAh to evade host defense mechanisms and cause septicemia *in vivo*. Genes upregulated under planktonic conditions included toxins, specifically hemolysins, multiple porins, and type IV pilus. Increases in these PVFs suggest that planktonic culture may more closely mimic *in vivo* growth. Type IV pili may help colonize the intestinal tract, while the porins and toxins target cells. This may also help explain the difficulty creating a natural infection model using planktonically-cultured vAh, while challenge by IP injection results in peracute mortality. Planktonically-cultured cells appear to be less equipped for invasion, but well equipped for initiating septicemia. Along with previously published PVFs, which were the focus

of this research, many other differentially expressed genes have been indicated in virulence in other organisms. For example, a tripartite toxin previously identified in *Bacillus* and recently described in *A. hydrophila* was upregulated more than 300 times in biofilm, and a fimbrillin, essential for biofilm formation and adhesion in an extremely virulent clinical *E. coli* isolate, was upregulated more than 300 times in biofilm. While outside the scope of this research, further investigation into the potential roles of DEGs in vAh pathogenicity and virulence is needed and will continue.

The results of PVF gene expression comparisons supports the hypothesis presented for other bacterial species, that gene expression in biofilm growth primarily functions to support niche persistence, but these gene products may be used indiscriminately to increase pathogenicity and virulence. The results of this work underscore the importance and necessity of considering bacterial behavior in the natural habitat when trying to unravel the modes of pathogenicity of 'accidental' pathogens, such as vAh. In our aims to unravel the mystery of vAh-induced MAS, we must attempt to examine bacterial behavior under multiple modes of survival if we are to understand and prevent these deadly outbreaks. RNA sequencing technology generates vast amounts of data, and, while outside the scope of the current work, further investigation into potential roles of DEGs in vAh pathogenicity and virulence is needed and will continue.

The rapid mortality that occurs when catfish are challenged by intraperitoneal injection with vAh suggests that vMAS may be, in part, a toxin-mediated disease. *Aeromonas hydrophila* are known to produce a multitude of proteolytic and potentially toxigenic proteins. While T3SS and T6SS have generally been considered indicators of virulence in *A. hydrophila* strains, no US isolates of vAh possess complete T3SS or T6SS. Instead, US vAh isolates possess T1 and Type 2

secretions systems. In order to determine the role of secreted protein in vAh-induced disease, and to determine the extent of proteins secretion by the T2SS, a T2 secretion-deficient mutant, Δ*exeD*, was created by homologous recombination. Secretomes were analyzed for presence or absence of proteins and secretome functional analyses were performed to measure degradative and toxigenic potential. Δ*exeD* had a near complete loss of secreted protein on PAGE analysis. Hemolytic potential was completely lost in Δ*exeD* mutant, as were degradative potential in both planktonic and biofilm-associated Δ*exeD*. T2SS complementation by whole-pathway cloning restored protein secretion and completely restored degradative and toxigenic potential of vAh. The restoration of proteolytic and hemolytic potential by whole-pathway T2SS complementation further supports the hypothesis that vAh requires T2SS for virulence. To further explore the role of T2SS and its substrates in virulence, *in vivo* challenges were performed in a catfish model using wild-type, Δ*exeD*, and T2SS complemented mutant, *exeD*::T2SS. These challenges revealed total loss of virulence in Δ*exeD*. Whole T2SS pathway complementation restored virulence (73% mortality versus 96% mortality in wild-type), offering convincing evidence of the role of T2SS substrates and the prerequisite presence of a functional T2SS in vMAS. The experiments performed herein have demonstrated that T2SS and the T2S effectors play a vital role in the pathogenicity of vAh, and in the development of vMAS in channel catfish. Most importantly, these data have shown that T2SS is essential for the secretion of proteolytic and hemolytic proteins, and that a functional T2SS is prerequisite for the development of vMAS. These results indicate a vital role of secreted proteins in vAh virulence, and confirm that T2SS is the primary secretory pathway utilized for secretion of virulence determinant in U.S. isolates of vAh.

In conclusion, the research presented in this dissertation represents the first secretome and transcriptomic comparison of biofilm- and planktonic-grown vAh, and provides the first insights into the biofilm lifestyle of virulent *A. hydrophila*. The goal of this research was to compare how biofilm growth affected secretion of degradative and toxigenic proteins, the overall gene expression in vAh, and to specifically compare expression of genes previously implicated in vAh virulence. This research also provides convincing evidence of the role of T2SS in the secretion of multiple degradative and toxigenic proteins implicated in the pathogenicity of vAh and demonstrated that these T2SS substrates and a functional T2SS are essential in the development of vMAS.

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