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MINIREVIEW

Vibrio vulnificus: Disease and Pathogenesis[▽]

Melissa K. Jones and James D. Oliver*

Department of Biology, University of North Carolina at Charlotte, Charlotte, North Carolina 28223

Vibrio vulnificus is an opportunistic human pathogen that is highly lethal and is responsible for the overwhelming majority of reported seafood-related deaths in the United States (30, 117). This bacterium is a part of the natural flora of coastal marine environments worldwide and has been isolated from water, sediments, and a variety of seafood, including shrimp, fish, oysters, and clams (4, 7, 25, 26, 43, 97, 109, 116, 118, 149, 165). Consumption of seafood (primarily raw oysters) containing *V. vulnificus* can result in a severe, fulminant systemic infection. Characteristics of this disease include fever, chills, nausea, hypotensive septic shock, and the formation of secondary lesions on the extremities of patients (11, 22, 41, 74, 115, 146). This primary septicemia is the most lethal infection caused by *V. vulnificus*, with an average mortality rate exceeding 50% (30, 41). A review of 459 U.S. cases reported by the FDA between 1992 and 2007 (J. D. Oliver, unpublished) revealed that 51.6% of the patients died. Interestingly, 85.6% of the cases were male; this aspect of the infection is discussed later in this review. Of 180 cases in 2002 to 2007 for which FDA data were available, 92.8% of patients had consumed raw oysters prior to the onset of symptoms and 95.3% had some preexisting disease(s). The latter are clearly associated with *V. vulnificus* infection, with liver diseases, such as cirrhosis or hepatitis, being the most common (116). In addition to septicemia, *V. vulnificus* can produce serious wound infections that typically result from exposure of open wounds to water harboring the bacterium (114). Wound infections are frequently contracted as a result of recreational swimming, fishing injuries, or seafood handling (7, 46). Like systemic disease, wound infections progress rapidly to cellulitis, ecchymoses, and bullae, which can progress to necrotizing fasciitis at the site of infection; however, the mortality rate for wound infections (ca. 25%) is lower than that for systemic disease (10, 13, 74). This organism possesses a wide array of virulence factors, including acid neutralization, capsular polysaccharide expression, iron acquisition, cytotoxicity, motility, and expression of proteins involved in attachment and adhesion. These factors likely require concerted expression for pathogenesis to take place and appear to be under the control of global regulators. Overall, *V. vulnificus* is a complex microorganism with physiological characteristics that contribute to its survival in the marine environment and in the human host.

* Corresponding author. Mailing address: University of North Carolina at Charlotte, Department of Biology, 9201 University City Blvd., Charlotte, NC 28223. Phone: (704) 687-8516. Fax: (206) 350-1385. E-mail: jdoliver@uncc.edu.

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BIOTYPES AND GENOTYPES

Biotypes. Strains of *V. vulnificus* are classified into biotypes based on their biochemical characteristics. Strains belonging to biotype 1 are responsible for the majority of human infections, while biotype 2 strains are primarily eel pathogens (2, 153). A recently identified third biotype (biotype 3) was shown to possess biochemical properties of both biotypes 1 and 2 and to cause human wound infection (7). Analyses comparing genomic similarities among the three biotypes indicated that biotype 3 is a hybrid of biotypes 1 and 2 (6). Furthermore, the use of multilocus sequence typing indicated similar results and divided strains of *V. vulnificus* into genetic lineages (24). This analysis also uncovered the presence of a 33-kb genomic island found only in lineage 1/biotype 1 strains (24). Several genes located within this genomic island are thought to play a role in pathogenesis of other pathogens; therefore, it was suggested that *V. vulnificus* strains possessing this island also possess an increased potential for virulence (24). While biotype 3 does cause human infections, these have to date been limited to Israel and to persons handling tilapia fish, and biotype 1 is the predominant cause of human infections. Thus, the remainder of this review will focus on the characteristics of biotype 1.

On average, 34 cases of *V. vulnificus* infection are reported annually by the U.S. Food and Drug Administration (115–117). However, in recent years this number has risen dramatically. The CDC reported *Vibrio* infections increased by 78% between 1996 and 2006, and in 2005, 121 cases of *V. vulnificus* disease were confirmed (14, 15). While reasons for these increases have yet to be determined, it has been noted that outbreaks of *V. vulnificus* disease in Israel are associated with record high temperatures (124). These data suggest that global climate change resulting in higher water temperatures may increase the frequency of *V. vulnificus* disease and influence the global distribution of this pathogen (124). Despite increases in the number of cases, the rate of infection remains relatively low. This contrasts with the ubiquity of this organism in marine habitats. At least part of the explanation for this difference is that *V. vulnificus* rarely causes severe disease in healthy individuals. However, persons with underlying disorders, such as chronic liver disease, diabetes, hemachromatosis, AIDS, malignancy, and immunocompromised states, are at high risk of infection (74, 115, 116). In fact, individuals with compromised immune systems or chronic liver disease are up to 80 times more likely than healthy individuals to develop primary septicemia (13). However, in that such underlying conditions put between 12 million and 30 million Americans at

risk for infection, the opportunistic nature of this pathogen alone cannot solely account for its low rate of infection (117).

Genotypes. The discrepancy between the large at-risk population and the low level of disease has spawned a flurry of studies investigating methods for subtyping *V. vulnificus* in hopes of identifying genetic indicators of virulence. One such method employed random amplified polymorphic DNA PCR to identify an amplicon present primarily in clinical isolates (156). Subsequent analysis of this virulence-correlated gene (*vcg*) revealed consistent sequence variations that correlate highly with the isolation source (131). Strains possessing the sequence common among clinical isolates were designated C type, while strains possessing the environmentally correlated sequence were designated E type. Clinical and environmental strains can also be differentiated by comparing 16S rRNA sequences, where the majority of environmental isolates possess the "A type" and clinical isolates generally have the "B type" (3, 67, 111). More recently, typing of the intergenic spacer region between 16S and 23S rRNA genes has further subdivided *V. vulnificus* based on PCR banding patterns. This analysis showed clinical strains were highly similar, grouping into one cluster that contained only B-type strains (32). Conversely, environmental isolates displayed much more variability, and though they grouped into a single cluster, the strains comprised a mixture of both A and B 16S rRNA. While 16S rRNA has been widely used for evaluating *V. vulnificus* strains, recent questions have been raised as to the effectiveness of this typing system since this RNA species appears to be highly polymorphic (113).

Typing methods have been employed to examine the environmental distribution of potentially virulent strains of *V. vulnificus*. We examined the presence of the C and E genotypes in oysters and surrounding seawater and found that while these genotypes were present in approximately equal proportions in water (46.9% E type versus 53.1% C type), there was a large preponderance (84.4%) of the potentially less virulent E types in oysters (155). Evaluation of market oysters using the A/B typing system reported similar results, with B-type strains comprising a minority among *V. vulnificus* isolates (78). Such results indicate that in addition to the opportunistic nature of *V. vulnificus*, the disparity of genotype distribution in oysters may also factor into the low incidence of disease (155). Examination of 75 cases reported by the FDA between 2002 and 2007 revealed that the onset of symptoms occurred within a range of 0 to 7 days (2.0 days average) whether a victim consumed a single oyster or 24 oysters, and symptoms typically occurred within a mean of 24 h (Oliver, unpublished). Similarly, of those consuming a single oyster, 33% died, whereas of those consuming >24 oysters, 25% died. These data indicate that the number of oysters consumed is not a factor either in the length of time before symptom development or in disease outcome, suggesting that the ingestion of a single oyster containing a sufficiently high number of *V. vulnificus* strains possessing a virulent genotype is likely adequate to initiate disease in predisposed individuals.

The variety of individual loci able to correlate *V. vulnificus* isolates with the isolation source points to chromosome-wide differences among strains of this bacterium. Thus, a subtyping method that simultaneously examines DNA polymorphisms at multiple loci has been used to further discriminate among *V. vulnificus* strains. Repetitive extragenic palindromic PCR (rep-PCR) targets conserved repetitive elements that are distributed throughout the bacterial genome, generating a "fingerprint" for each strain. In contrast to the case with intergenic spacer region typing (32), analysis of clinical and environmental isolates using this technique showed higher diversity among clinical isolates, while environmental strains were quite similar (18). rep-PCR analysis was also combined with single-locus typing methods to compare *V. vulnificus* strains isolated from various sources and worldwide locations. Results from this study found that the clinical profile was rarely seen among oyster isolates, supporting the findings noted above (18, 78, 155). Given the concerns regarding the highly polymorphic nature of rRNA and the congruence between rep-PCR results and those of other, single-locus typing methods (e.g., *vcgC* or *vcgE* and capsular polysaccharide (CPS) allele typing), this method of evaluating multiple loci seems to better characterize the genomic divergence of this bacterium. Additionally, these data support epidemiological findings and further link the environmental distribution and pathogenic potential of *V. vulnificus* to the incidence of disease.

HOST RESISTANCE

Acid resistance. The rapid onset of symptoms (as little as 7 h after raw oyster consumption [115]) and progression of infection indicate that *V. vulnificus* is able to quickly evade the immune response mounted by infected individuals. In order to better understand the pathogenic nature of this bacterium, researchers have investigated how *V. vulnificus* interacts with various aspects of host defenses. Since disease typically results from raw shellfish consumption, the highly acidic gastric environment is one of the first host defenses encountered by this bacterium. A common method employed by gram-negative bacteria to neutralize low-pH environments is through the breakdown of amino acids to yield amines and CO_2, and *V. vulnificus* appears to use a similar system when it encounters acidic environments in vitro. In this bacterium, lysine decarboxylase, which is encoded by the *cadBA* operon, breaks down lysine to form cadaverine (127). Expression of this operon, which is primarily regulated by *cadC*, increases upon acid exposure (126, 127). In addition to its function as an acid neutralizer, cadaverine also acts as a superoxide radical scavenger, suggesting a link between acid and oxidative stress tolerance (54, 65). In *V. vulnificus*, exposure to low pH results in increased cellular superoxide levels (66), and expression of both *cadBA* and activity of lysine decarboxlyase are increased upon exposure to superoxide stress (65). Under these conditions, *cadBA* expression is also regulated by *soxR* (65). This link between acid and oxidative stress tolerance is further indicated by the use of manganese superoxide dismutase (SOD) for acid neutralization in a variety of bacteria (23, 159, 160). In *V. vulnificus*, mutation of manganese SOD (encoded by *sodA*) resulted in an increased sensitivity to low pH (66). However, this gene is not directly induced by low pH but rather is induced by the oxidative stress that results from low pH exposure, suggesting that at acidic pH *sodA* is indirectly regulated through SoxR (66). Furthermore, mutation of *sodA* resulted in a 2-log increase in the 50% lethal dose (LD_{50}) after intraperitoneal (i.p.) injection, highlighting its need for bacterial sur-

vival within the host (53). Two other superoxide dismutase genes (*sodB* and *sodC*) were examined for their role in survival under low-pH conditions, and results showed that a loss of either gene also resulted in increases in the LD_{50} compared to that of the wild-type strain (53). Overall, these results demonstrate that loss of any SOD activity results in a decrease in virulence and suggest that *soxR* may serve as a link between acid neutralization and the oxidative stress pathways. The link between these two pathways may be what helps this bacterium survive the acidic environment of the human gut. While the genes discussed above are involved in acid survival, it should be noted that *V. vulnificus* is a poor survivor when placed directly in low pH under laboratory conditions (75). However, exposure to a slightly acidic pH prior to low-pH exposure dramatically increases survival of this organism, demonstrating the induction of a stress response(s) which provides a protective effect for the subsequent stresses (127). While there are limited data on protection against acid stress in this organism, nutrient starvation has been shown to induce cross-protective effects against oxidative stress (130). Furthermore, cross-protection following starvation has been shown to increase survival of this pathogen under both clinical and environmental conditions (9, 130). These cross-protective abilities are likely an important contributor to the survival of this organism and may work in concert with the mechanisms outlined above to allow its survival as it passes through the gastric environment.

Host immune response. Once *V. vulnificus* passes through the digestive tract and enters the bloodstream, one of the primary innate immune factors encountered by the bacterium is complement. Research examining the effects of *V. vulnificus* infection on complement activity demonstrated that blocking the classical complement cascade could eliminate the bactericidal effects of serum (108). Furthermore, opsonization as a result of complement activation was required for phagocytosis of *V. vulnificus* by polymorphonuclear leukocytes, demonstrating the need for this branch of the innate immune response in clearing *V. vulnificus* infection (108). While the precise role of polymorphonuclear leukocytes in *V. vulnificus* disease is not yet understood, these leukocytes are known to secrete cytokines that serve to recruit additional leukoctyes to the site of infection following complement-mediated phagocytosis. Several of these cytokines are also able to induce the inflammatory response, and it has been suggested that septic shock, which is a hallmark of *V. vulnificus* disease, may result from overactive cytokine induction (137). Analysis of cytokine activation during *V. vulnificus* disease has shown that several proinflammatory cytokines, namely interleukin 6 (IL-6), IL-8, and tumor necrosis factor alpha (TNF-α), are specifically induced and predominantly expressed during infection (81, 125, 137). These results are also supported by epidemiologic data which found increased levels of several cytokines, including TNF-α, IL-6, and IL-1, present in the blood of patients infected with the bacterium (137).

Other studies investigating activation of the innate immune response against *V. vulnificus* examined the role of Toll-like receptors. These receptors are commonly found on the surfaces of immune and epithelial cells, and some are specifically involved in recognizing antigens produced by gram-negative bacteria (28, 101). Studies examining the activation of Toll-like receptor 4 (TLR4) showed that it does not appear to participate in lymphocyte apoptosis upon exposure to *V. vulnificus* and is therefore unlikely to participate in the immune response during infection (56). However, TLR2 was shown to be activated by a surface lipoprotein (encoded by *ilpA*) of *V. vulnificus*, and its activation resulted in the production of TNF-α and IL-6, which have been established as associated with *V. vulnificus* infection (33). Examination of innate immune system activation by *V. vulnificus* surface structures revealed that flagellin, the flagellar structural subunit, was able to bind TLR5 and thus activate immune responses (89). These studies implicate TLR2 and TLR5 in activation of host immune responses to *V. vulnificus*; however, more research is needed to definitively establish their role in innate immune system activation. Additional investigations into TLR2 and TRL5 revealed that binding of these receptors by *V. vulnificus* "pathogen-associated molecular patterns" resulted in production of the immune regulatory element NF-κB and in turn led to secretion of the proinflammatory cytokine IL-8, which not only is involved in the inflammatory response but also is a potent activator of neutrophils (33, 81, 89).

Neutrophils are among the first responders to acute infection and readily phagocytize and degrade invading organisms. Blood taken from patients with chronic liver disease showed decreased neutrophil activity compared to the blood of healthy individuals, and survival of *V. vulnificus* in human blood is inversely correlated with phagocytosis by neutrophils (44). Based on these data and a study showing increased neutrophil concentrations in the peritoneal cavity upon infection with *V. vulnificus*, the response of neutrophils to *V. vulnificus* has been implicated as a disease determinant for this organism (44, 56, 144, 154). One of these studies, however, did use unencapsulated strains (the significance of which is discussed later in this review), thus diminishing the potential role neutrophils play in controlling *V. vulnificus* infection (44, 154). In addition to neutrophils, macrophages have also been suggested to play a part in defense against *V. vulnificus*. During infection, this bacterium reduces the number of lymphocytes through apoptotic activity, but neutrophil numbers do not decrease, indicating that other leukocytes are being targeted (56). Specific examination of macrophages revealed that clinical strains of *V. vulnificus* were able to induce macrophage apoptosis (57). Furthermore, in the absence of macrophages, neutrophils alone were not able to clear a *V. vulnificus* infection in mice (154). Together, these results suggest that the ability to kill leukocytes may be a virulence determinant of this organism, with macrophages playing the primary role in clearing *V. vulnificus* infection and neutrophils playing a secondary role (57, 154).

CPS. Along with the killing of immune cells, evasion of host defenses is another mechanism used by microorganisms to survive inside a host and cause disease. In *V. vulnificus*, evasion is achieved primarily through surface expression of CPS. The presence of capsule provides resistance to opsonization by complement and thus avoidance of phagocytosis by macrophages (57, 150, 151, 168). Expression of CPS also confers resistance to the bactericidal effects of serum, and it is hypothesized that CPS masks immunogenic structures that normally would activate these nonspecific host responses (57, 129, 168). Due to these effects, expression of CPS is one of the few known virulence factors of *V. vulnificus* that is recognized to be absolutely required for pathogenicity. Experiments comparing the

virulence of encapsulated versus unencapsulated strains have shown that CPS allowed cells to be cleared from the bloodstream more slowly and to be more invasive in subcutaneous tissue than nonencapsulated cells (168). Encapsulated strains also demonstrated increased lethality over that of unencapsulated strains upon i.p. injection into iron-loaded mice (163). Furthermore, insertional inactivation of a CPS transport gene (*V. vulnificus wza*) abolished surface expression of capsule, and this CPS-negative mutant showed dramatic increases in the LD_{50} (4.0×10^5 CFU) over that of the encapsulated parent (<10 CFU), confirming the role of CPS in *V. vulnificus* virulence (164).

Given the pathogenic benefits of CPS expression, it would seem disadvantageous for this bacterium to exist in a nonencapsulated state. However, *V. vulnificus* does undergo phase-variable expression of its capsule. This phenomenon is identifiable by reversion between opaque (Op) (encapsulated) and translucent (Tr) (reduced capsulation or nonencapsulated) colony morphologies (17, 40, 166). Switching between these phenotypes occurs at a base level of 10^{-3} to 10^{-4} (163, 168). However, increased levels of transition to the Tr state can be observed when Op cells are incubated at 37°C (17, 40). The role of phase-variable capsule expression in the virulence of this pathogen is unknown. However, one study reported that C-type strains switched to the Tr phenotype more slowly than E-type strains, indicating that the ability to undergo phase variation may possibly have evolved to increase environmental survival and not human pathogenicity (40).

The production and expression of capsular polysaccharide in *V. vulnificus* is dependent upon a variety of genetic loci. Primary among these is the group 1 CPS operon. This operon is comprised of highly conserved transport genes followed by more-variable biosynthetic genes (17). Reverse transcriptase (RT)-PCR analysis has demonstrated that downregulation of the *V. vulnificus* transport gene *wzb* resulted in an intermediate translucent phenotype which has been shown to possess only small amounts of capsule on the cell surface (17, 132). Additionally, sequence changes in the conserved region have been linked to changes in CPS expression, where site-specific deletion of *V. vulnificus wzb* resulted in an irreversible shift to the Tr phenotype (17). Multiple genes outside of the group 1 operon have also been shown to be essential for CPS expression (121, 170). Among these are two epimerase genes, *wcvA* and *wbpP*, which are likely involved in activating monosaccharides in the early stages of capsule development (121, 143, 148). Several glycosyltransferase enzymes (including *wecA*) and biosynthetic genes (e.g., *rml*) have also been identified and shown to be essential for capsule expression (143). Overall, the variety of genes involved in capsule production underscores the complexity and importance of this surface structure for *V. vulnificus*.

Recently a rugose phenotype, resulting from copious production of extracellular polysaccharide, was identified for *V. vulnificus* and demonstrated a variety of phenotypic differences from the encapsulated, nonrugose morphotype (35). This phenotype has been observed in the related species *Vibrio cholerae* and has been associated with human virulence (106). In *V. vulnificus*, rugose isolates exhibit decreased motility, increased resistance to human serum, and increased biofilm formation. However, it is possible that the increased biofilm formation is due to a loss of motility, since mutations in flagellar genes are known to result in this phenotype (107). Regardless, the role of the rugose phenotype in *V. vulnificus* pathogenicity is uncertain given that increased resistance to human serum was seen only in Op-rugose and not Tr-rugose isolates (35). The Tr-rugose phenotype was also avirulent in mouse models, suggesting that while the rugose phenotype may aid in environmental survival, it does not likely play a definitive role in human disease (36).

IRON ACQUISITION

Another feature highly associated with *V. vulnificus* disease is elevated serum iron levels in infected individuals. Early virulence assays conducted with mice demonstrated that the infectious dose of *V. vulnificus* was directly correlated with serum iron concentrations (162). Furthermore, injecting mice with iron prior to infection significantly lowered the LD_{50} and increased the mortality rate to 100% (145, 162). The importance of iron acquisition in *V. vulnificus* virulence is also seen in the differential survival of the C and E genotypes in human serum. The more-virulent C types are better able to survive than the E types, although the addition of exogenous iron allows both genotypes to survive equally well (9). Precisely how excess serum iron confers an advantage to *V. vulnificus* is still unclear; however, two theories have been put forth. One study evaluating growth of this bacterium in mice demonstrated that the presence of excess iron dramatically increased the growth rates of clinical strains, suggesting that excess iron enhances growth of this pathogen (144). Other researchers have found that excess iron resulted in decreased neutrophil activity and concluded that excess iron resulted in a compromised immune response (45). These differing conclusions indicate that elevated serum iron levels may play multiple roles in enhancing *V. vulnificus* infection, but they also highlight the need for further research in this area.

Iron acquisition systems. In human serum, most iron is bound to transferrin, making it unavailable to invading organisms (158). In order to scavenge iron from transferrin and other iron-binding compounds, *V. vulnificus* has developed multiple systems for iron acquisition (142). The primary system used by this organism involves siderophores, of which *V. vulnificus* produces two types: a catechol and a hydroxymate (141). The catechol siderophore (vulnibactin) is the chief means by which *V. vulnificus* acquires iron and is required for growth in iron-limited media (1, 61). Mutational analysis of genes involved in vulnibactin synthesis and transport has demonstrated that this siderophore is also required for scavenging iron from transferrin and holotransferrin (61, 157). The suspected role of vulnibactin in virulence was confirmed through mutation of several vulnibactin-associated genes (*vuuA*, *venB*, *vvsA*, and *vvsB*), which resulted in diminished virulence compared to wild-type strains (64, 94, 157). However, these mutants still retained a moderate level of virulence, signifying the ability of this bacterium to obtain iron by other means, possibly through the use of the hydroxymate siderophore. This second siderophore type has recently been shown to be involved in *V. vulnificus* infection, but its role in iron uptake is still unclear (1). Interestingly, *V. vulnificus* not only can utilize iron from its own hydroxymate but also can bind iron sequestered by exogenous hydroxymate molecules secreted by other micro-

organisms. The *desA* gene, which encodes a receptor for deferoxamine, a hydroxymate-type siderophore produced by *Streptomyces pilosus*, was recently found in *V. vulnificus* (42, 59). Examination of this gene showed it is expressed only when deferoxamine is present, confirming that *V. vulnificus* can take up iron independently of vulnibactin (59). In addition, *V. vulnificus* possesses a gene cluster involved in utilization of aerobactin that is produced by *Escherichia coli* (152). Similar to the case with deferoxamine, these genes are expressed only when aerobactin is present (152). Apart from siderophore-mediated iron uptake, *V. vulnificus* utilizes non-transferrin-bound iron through a heme receptor, HupA (92). It has been suggested that non-transferrin-bound iron is required for initiation of *V. vulnificus* growth; therefore, HupA may be necessary for initial growth once the organism enters the host (60, 92).

Regulation of iron acquisition. The expression of genes involved in iron acquisition is highly regulated in *V. vulnificus* and occurs primarily through the ferric uptake regulator, Fur. Expression of several genes involved in vulnibactin synthesis (*vvsA*, *vvsB*, *venB*, and *vis*) and uptake (*vuuA*) are under the control of Fur, which increases expression of these genes under iron-limiting conditions (1, 61, 64, 157). HupA is also regulated by Fur, as are the iron receptors encoded by *tonB* and *tolC* (1, 83, 93). While Fur is a common regulator in many organisms, in *V. vulnificus* Fur regulation is unique in that this protein likely activates its own expression under iron-depleted conditions (83, 84). Given the importance of iron in the disease process of *V. vulnificus*, further research into regulation of iron acquisition will be critical to understanding the pathogenesis of this organism.

CELLULAR DAMAGE AND CYTOTOXICITY

VvhA. Apart from direct uptake of iron, acquisition of this essential nutrient can also be facilitated through hemolytic factors that serve to release the iron from hemoglobin and provide an additional source of plasma iron (39). In *V. vulnificus*, an extracellular hemolysin encoded by *vvhA* contributes to iron release through its hemolytic activity and is also responsible, in part, for the bacterium's cytotoxic activity (161). Injection of the purified toxin into mice causes a variety of pathological effects, implicating its contribution in severe tissue necrosis. Additional symptoms resulting from hemolysin exposure include fluid accumulation, intestinal irregularities, partial paralysis, and lethality (37, 81). Direct examination of the effects of hemolysin on host cells revealed that toxin exposure increased vascular permeability, apoptosis of endothelial cells, induction of inducible nitric oxide synthase activity, increased nitric oxide production, and possibly increased neutrophil recruitment (55, 58, 80). Cell death caused by hemolysin occurs through pore formation in the cellular membrane, and it has been suggested that this activity ultimately leads to the vascular permeability and hypotension characteristic of *V. vulnificus* disease (62). Despite these various and significant virulence attributes, inactivation of hemolysin through *vvhA* mutation did not demonstrate a difference in the LD_{50} from that of the parent strain upon i.p. injection into iron-loaded mice (161). Moreover, mice injected with the mutant displayed tissue damage and necrosis similar to those with the wild type, suggesting that hemolysin is not responsible for the lethality of *V. vulnificus* and also is not solely responsible for the extensive tissue damage resulting from infection (161).

Because *vvhA* does not appear to be required for lethality of *V. vulnificus* and because loss of the toxin does not change the pathological results of i.p. infection, it was questioned whether this cytolysin is actively produced in vivo. To address this concern, enzyme-linked immunosorbent assay and RT-PCR studies were used to identify the toxin and its mRNA transcripts subsequent to infection. Results showed that the toxin is indeed produced in vivo (37, 88). Further investigation into *vvhA* expression revealed decreased levels of mRNA during swarming and upon loss of the AI-2 quorum sensing system, suggesting this toxin may play a greater role in environmental survival than in human infection (68).

Other studies investigating hemolysin activity within the host revealed that oligomerization of toxin monomers inhibits toxin activity and this oligomerization can be enhanced by the addition of exogenous cholesterol (20, 58, 122, 139, 140). Conversely, the presence of membrane-bound cholesterol increased toxin binding to the erythrocyte membrane and was shown to be required for activity of the toxin (167, 169). These results suggest that cholesterol possesses a hemolysin receptor and, depending on the state of cholesterol (i.e., bound versus free), allows for either increased or decreased toxin activity. In addition to bound cholesterol, hemolysin monomers can also be stabilized by human serum albumin, thereby delaying oligomerization and its associated toxin inactivation (20). Studies examining the effect of cholesterol on hemolysin led to research into the effects of low-density lipoprotein (LDL) cholesterol on *V. vulnificus* lethality in diseased mice. Results showed a decrease in lethality when LDL was administered prior to infection (122). However, lethality between a *vvhA* mutant and the wild-type strain after LDL exposure resulted in no observed differences (123). These results support previous data, which indicated that *vvhA* does not play a significant role in virulence in primary septicemia, and they further imply that the protective effect conveyed by LDL is not due to the loss of hemolytic activity (123).

VvpE. Another enzyme suggested to be involved in *V. vulnificus* virulence is an extracellular protease, designated VvpE, which is nonspecific and has broad substrate specificity. The purified enzyme has been shown to cause tissue necrosis and cutaneous lesions, as well as increased vascular permeability leading to edema, all of which are characteristic of the bullous lesions caused by systemic disease (16, 76, 104). The enhanced vascular permeability caused by VvpE occurs through the generation of bradykinin, a known vasodilator (96, 103). Bradykinin production was also shown to be important for invasion of this bacterium, since bradykinin inhibition reduced dissemination of *V. vulnificus* from the peritoneal cavity into the bloodstream (98). VvpE further contributes to local tissue damage through the degradation of type IV collagen (a component of the basement membrane) and activation of procaspase-3, an enzyme involved in cellular apoptosis (63, 103). However, production of VvpE by both virulent and avirulent strains of *V. vulnificus* indicated that this enzyme may not be involved in lethality (79, 105). Mutational analysis of *vvpE* confirmed this hypothesis, with the mutant not differing in LD_{50} from the parent strain in either local or systemic infections (50, 134). As a result of these findings, functions of VvpE other than in

tissue destruction have been investigated. It was reported that VvpE may contribute to the breakdown of heme-containing proteins, thereby releasing iron for use by siderophores, but further studies demonstrated that this is likely not the case (105, 112, 138). Interestingly, although the metalloprotease is apparently not needed for iron acquisition, the presence of iron is required for efficient transcription of *vvpE* (147). Overall, studies of the activity and action of VvpE indicate that it is not likely to be a major contributor to the virulence of *V. vulnificus*.

RtxA1. As noted above, single mutations in *vvpE* and *vvhA* did not result in changes in either cytotoxicity or lethality compared to their parent strains (50, 134, 161). More significantly, construction of a *vvhA vvpE* double mutant resulted in a strain which remained highly cytotoxic (29, 72). These results indicate that *V. vulnificus* must secrete another toxin responsible for this disease characteristic. Random chromosomal mutagenesis has been conducted by several laboratories to identify additional virulence factors in this bacterium, and such studies have identified a gene (*rtxA1*) homologous to the *rtxA* toxin gene of *V. cholerae* (38, 72, 85). RTX toxins are made of repeated structural subunits which form pores in cellular membranes and are found in a broad range of gram-negative bacteria. The amino acid sequence of *V. vulnificus* RtxA1 shows high homology to the RtxA sequence of *V. cholerae*, and organization of the Rtx gene cluster is also similar between the two species (72, 85). Two additional *rtxA* genes have also been identified and are designated *rtxA2* and *rtxA3*, but mutational analysis of these genes indicated they do not play a role in *V. vulnificus* cytotoxicity or virulence (51). In order to examine the cytotoxic and lethal effects of RtxA, isogenic mutants have been generated. These mutants showed decreased cellular damage compared to the parent strains and an inability to lyse cells or to disrupt either cell monolayers or tight junctions (72, 85, 95). Other observed cellular changes included rearrangement of cytoskeletal structure, bleb formation, and aggregation of actin resulting in cell rounding, which were all attributed to RtxA1 activity (72). It was concluded that such changes could lead to cellular necrosis and allow *V. vulnificus* to invade the bloodstream by crossing the intestinal epithelium (72). In support of this, an *rtxA* mutant demonstrated a significant reduction in spread to the liver, indicating the toxin plays a considerable role in the development of systemic disease (38). Overall, these results suggest an important function for RtxA1 in cell injury and possibly infection.

Investigation of RtxA1 activity revealed that incubation of *V. vulnificus* with host cells resulted in increased toxin expression, suggesting cell-to-cell contact was required for cytotoxicity (72). This physical contact requirement was further evidenced by the following: (i) a lack of cytotoxicity when *V. vulnificus* was coincubated with HeLa cells in a system that prevented their contact and (ii) the immediate induction of toxin expression when the bacterium encountered host cells (72, 82). This apparent cell contact requirement led to an investigation into the mechanism of toxin delivery. In *V. cholerae*, the *rtxBDE* operon comprises a type I secretion system responsible for secretion of RtxA (8, 31, 135). A similar operon-like structure is found in *V. vulnificus*, suggesting a similar method of RtxA1 delivery (82, 95). Mutation of *rtxE* showed the mutant strain was unable to secrete RtxA1, as evidenced by the absence of toxin in culture supernatants (82). Further, the *rtxE* mutant exhibited a reduction in epithelial cell death and decreased mouse lethality, similar to that observed for an *rtxA1* mutant, suggesting the changes in virulence were associated with an inability to secrete RtxA1 (82). The second portion of the Rtx gene cluster is the *rtxAC* region. In other *Vibrio* spp., RtxC is needed for activation of RtxA (90, 91). In *V. vulnificus*, mutation of *rtxC* results in full cytotoxicity with no differences in lethality from the parent, indicating an alternative function for this gene in RtxA1 expression (51). Although *rtxA1* is not regulated by *rtxC*, another potential regulator, HlyU, has been identified. HlyU has long been implicated in the virulence of *V. vulnificus* due to the presence of antibodies against this protein in the sera of infected patients (73). It has now been shown that this protein serves to regulate expression of *rtxA1* at the transcriptional level by binding to a region upstream of the *rtx* operon and initiating transcription (95). Furthermore, mutation in *hlyU* resulted in decreased *rtxA1* expression (95).

Given the involvement of RtxA1 in *V. vulnificus* disease, its role in relationship to *vvhA* and *vvpE* was investigated. These studies were facilitated by generation of double and triple mutations in *rtxA1*, *vvpE*, and *vvhA* and examination of their cytotoxicity to HeLa cells. As noted above, a *vvpE vvhA* double mutant remained highly cytotoxic; however, an *rtxA1 vvpE* double mutant showed a decrease in cytotoxicity similar to that of the *rtxA1* single mutant (72). Interestingly, a *vvhA rtxA*1 double mutant and a *vvpE vvhA rtxA*1 triple mutant were both devoid of cytotoxic effects. These results indicated that only mutation in *rtxA1* influences cell cytotoxicity (72). Moreover, these studies strongly indicate that RtxA1 plays the primary cytotoxic role in *V. vulnificus* disease, while the role of VvhA appears to be auxiliary and the role of VvpE is likely negligible (72). Mutation of *rtxA1* has also demonstrated this toxin is involved in cell death. These mutations have also shown this toxin plays a significant role in the lethal nature of *V. vulnificus*. Intragastric or i.p. injection of *rtxA1* mutants has resulted in higher LD_{50}s than those of wild-type strains (72, 82). Overall, it seems likely that RtxA1 is the primary toxin involved in both cytotoxicity and virulence of *V. vulnificus*.

LPS. Like tissue necrosis, endotoxic shock is also characteristic of *V. vulnificus* disease, and this symptom is thought to be due to the presence of lipopolysaccharide (LPS). In *V. vulnificus*, LPS is a known pyrogen that elicits a small cytokine response in mice and causes release of TNF-α (100, 125). Direct i.p. injection of LPS into rats and mice resulted in a dramatic drop in mean arterial pressure and rapid death of the animal, implicating its role in symptom development and lethality (100, 123). Injection of *N*-monomethyl-L-arginine, an inhibitor of nitric oxide synthase, was shown to mitigate the effects of LPS, indicating this endotoxin may stimulate the host immune response through nitric oxide synthase activity, which contributes to the pyrogenic nature of this toxin (27). Interestingly, LDL cholesterol and estrogen also appear to mitigate the effects of LPS. Injection of LDL prior to LPS exposure was shown to increase mouse survival and delay death in fatal cases (123). Likewise, estrogen has been implicated in the protection of females against the endotoxic activity of *V. vulnificus* LPS and is apparently the reason for the disproportionately small number of females developing this infection compared to males (102). So far, no link between LDL, estrogen, and their

effects on LPS has been reported, and while these results indicate that LPS plays a role in activation of host responses and host damage, the precise role of this cellular component in virulence of *V. vulnificus* has not been definitively elucidated.

ATTACHMENT AND MOTILITY

Pili. It is generally accepted that surface receptors are required for virulence since they are used by bacteria for attachment and invasion of a host. The role of attachment in the pathogenesis of *V. vulnificus* has been well documented with the finding that cell-cell contact is required for cytotoxicity (72). In fact, early studies investigating the pathogenesis of *V. vulnificus* found that preventing dissemination of the bacterium resulted in a lack of both localized disease and death of the infected animals (10). Because pili are used by many gram-negative bacteria for adherence to host cells, mutations in *pilA*, which encodes a pilin structural protein, and *pilD*, which encodes a prepilin peptidase, were generated in *V. vulnificus* (119, 120). Both mutants demonstrated a loss of attachment to epithelial cells and a slight increase in LD_{50} (1 and 2 logs for *pilA* and *pilD*, respectively) compared to the parent strains (119, 120). Furthermore, mutation in *pilD* reduced secretion of cytolysin, protease, and chitinase and was associated with an overall reduction in cytotoxicity (120).

Outer membrane proteins. Recently two additional proteins (OmpU and IlpA) suspected of contributing to adherence in *V. vulnificus* were identified (33, 34, 52). OmpU is an outer membrane protein able to bind fibronectin, and mutation of this gene resulted in decreased binding and adherence to Hep-2 cells (34). IlpA is a membrane-bound protein able to stimulate the immune response (33). Both mutants also showed reduced cytotoxicity but only a slight increase in the LD_{50} after i.p. injection into mice (33, 34). These results suggest these proteins may contribute to local damage but are not required for lethality. Interestingly, the loss of adherence in the *ompU* mutant was concomitant with a loss of cytotoxicity, again supporting the conclusion that cell contact is required for cytotoxicity of *V. vulnificus* (34).

Flagella. In addition to adhesion, flagellum-based motility is important for a variety of bacterial processes including biofilm formation and pathogenesis. In *V. vulnificus*, several flagellar genes have been mutated in order to investigate the role of flagella in the bacterium's pathogenesis. A loss of two flagellar structural components (encoded by *flgC* and *flgE*) each resulted in significant decreases in motility, cellular adhesion, and cytotoxicity compared to those of the parent strains (70, 86). Injection of *flgC* and *flgE* mutants into mice resulted in increased LD_{50}s (3-log increase after intragastric inoculation of *flgC* and 1 log after i.p. injection of *flgE*), indicating that the flagellum is necessary for virulence (70, 86). However, the use of a polar mutation and problems with complementation tempered the significance of these studies (70, 86). It has been suggested that decreases in motility, adhesion, and cytotoxicity may play a concerted role in reducing virulence, in that a loss of motility may lead to decreased adhesion and to an inhibition of cytotoxin delivery (70, 86). Overall, studies investigating the role of attachment in *V. vulnificus* virulence lend support to the hypothesis that host cell contact is required for *V. vulnificus* toxin secretion and pathogenicity.

VIRULENCE REGULATION

Quorum sensing. The experimental evidence presented thus far indicates that *V. vulnificus* requires coordinated expression of a variety of virulence factors in order for tissue destruction and lethality to occur. Therefore, the bacterium likely employs global regulators to control its pathogenesis. Quorum sensing (QS) through the LuxS/LuxR-type system was one of the first global regulatory mechanisms to be investigated in *V. vulnificus*, where LuxS is responsible for synthesis of autoinducer 2 and LuxR is the associated transcriptional regulator. Mutational analysis of both *luxS* and *luxR* (denoted *smcR* in *V. vulnificus* [99]) has demonstrated that a loss of either gene influenced the virulence of this pathogen by decreasing cytotoxicity and increasing the LD_{50} (69, 87). *luxS* was also shown to influence transcription of *vvhA* and *vvpE*, leading to increases and decreases of these toxins, respectively; however, their precise role in virulence is still unknown (69). Interestingly, the *smcR* mutant produced translucent colonies, which are associated with decreased lethality in mice (discussed above) and may partially contribute to the decreases in virulence seen with this strain (87). *luxS* and *smcR* mutants have also been evaluated for their ability to influence gene expression of mouse alveolar macrophages, and results indicated that the *V. vulnificus* QS system could influence gene expression of these macrophages and thus potentially contribute to survival of the bacterium during infection (136). Another signaling molecule suggested to regulate virulence of *V. vulnificus* is cyclic-di-GMP (c-di-GMP), which is a small molecule involved in signaling pathways and which allows bacteria to sense and adapt to their environmental surroundings (12). This messenger has been shown to play a role in virulence regulation in a variety of bacteria (133); however, in *V. vulnificus* its role in virulence is still not clear (110). Mutation of a gene purported to be involved in synthesis of c-di-GMP demonstrated that increased levels of intracellular c-di-GMP resulted in the production of an extracellular polysaccharide. However, this polysaccharide was not the CPS associated with virulence, and virulence was shown to be unaffected by changes in c-di-GMP levels (110).

Global virulence regulators. Another global regulator that has been extensively investigated for its role in both virulence and general metabolism is the cAMP-cAMP receptor protein (CRP) system. cAMP is generated through the cleavage of ATP by adenylate cyclase (encoded by *cya* in *V. vulnificus* [71]) and functions by binding the CRP, which then binds DNA to influence gene expression. Several putative virulence factors of *V. vulnificus* have been shown to be under the regulation of cAMP/CRP, including hemolysin, metalloprotease, and an iron acquisition system (5, 19, 21, 48, 49, 71). Mutation of *cya* dramatically reduced hemolysin production and cytotoxicity to HeLa cells, and further examination revealed cAMP to be a primary regulator of hemolysin activity, with CRP binding to the *vvhA* promoter region and activating its transcription (19, 71). This signaling cascade also appears to regulate iron acquisition through vulnibactin activity, since mutation of *crp* suppressed synthesis of vulnibactin and its receptor protein, leading to a loss of transferrin-bound iron utilization (21). While this system directly regulates several genes, it has also been show to indirectly regulate others, such as *vvpE*. In this case,

CRP works in concert with QS via SmcR and exerts its effects on *vvpE* transcription through the activity of RpoS (48, 49). The cAMP-CRP system is not only necessary for regulation of individual virulence factors but also contributes to lethality, since a *cya* mutant showed a 2-log increase in LD_{50} compared to the wild-type strain (71).

While the cAMP-CRP system appears to be a major regulator of *V. vulnificus* virulence, another global regulator has also been identified. AphB is a transcriptional regulator that is a member of the LysR family and controls activation of virulence factors in many bacteria, including *V. cholerae* (77). In *V. vulnificus*, AphB is involved in a wide variety of functions, including acid tolerance, cytotoxicity, adhesion, motility, and lethality (47, 128). Mutation of *aphB* results in decreased cell death compared to that of the wild type, and the mutant strain also demonstrated significantly less adhesion to epithelial cells than the parent strain (47). Examination of virulence revealed a 3-log increase in the LD_{50} of the *aphB* mutant over that of the wild type when i.p. injected into mice (47). In order to identify virulence genes potentially regulated by AphB, microarray analysis was used to compare gene expression between the wild type and the *aphB* mutant (47). Results showed that a gene encoding an outer membrane protein (belonging to the OmpC family) and a flagellar gene (*flaD*) both had reduced expression in the *aphB* mutant, while *hupA*, *venB*, and the gene encoding the Flp pilus assembly protein CpaC all showed increased expression. Interestingly, results also showed that several virulence factors are not regulated by AphB, particularly those involved in cytotoxicity, including *vvhA*, *vvpE*, and *rtxA1* (47). Overall, it appears that AphB primarily regulates genes involved in nutrient acquisition and metabolism and thus indirectly contributes to virulence by controlling growth and environmental adaptation.

HlyU. As mentioned above, the small regulatory protein HlyU is also a regulator of virulence in *V. vulnificus*. Antibodies against HlyU have been found in the serum of patients infected by *V. vulnificus*, which first highlighted the importance of this protein in the virulence of this pathogen (73). Mutation of *hlyU* resulted in a strain with reduced cytotoxicity and an increased LD_{50} compared to those of the wild type (73, 95). It was initially supposed that the change in cytotoxicity was due to *hlyU* regulation of *vvhA* expression; however, given that *vvhA* has been clearly shown to not be involved in virulence, *hlyU* was then suggested to regulate *rtxA1* (73, 95). Interestingly, microarray and real-time RT-PCR analysis of this mutant revealed a downregulation of both *vvhA* and *rtxA1* expression, and HlyU was further shown to regulate *rtxA1* expression at the transcriptional level by binding upstream of the *rtxA1* promoter region (95). This protein likely plays an important role in virulence of *V. vulnificus*, and knowledge of the way it works in concert with other mechanisms of virulence regulation is likely to be a significant advance in our understanding of virulence regulation in this pathogen.

CONCLUSION

V. vulnificus is a complex microorganism on many levels, from the disparity between its environmental ubiquity and the low number of infections it causes to the genomic heterogeneity seen among strains of this pathogen. While considerable progress has been made in deciphering the mechanisms responsible for virulence, recent studies indicate this bacterium, like most pathogens, requires a coordinated regulation of virulence factor expression in order for pathogenesis to occur. Furthermore, it appears many putative virulence factors (e.g., CPS, hemolysin, extracellular polysaccharide, etc.) serve primarily in environmental survival and pathogenesis in an auxiliary, but beneficial, function. Therefore, knowledge of what factors make this bacterium successful within its natural habitat may lead to a better understanding of the virulence of this significant human pathogen.

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