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Molecular Pathogenesis of *Vibrio vulnificus*

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***Vibrio vulnificus* is an opportunistic pathogen of humans that has the capability of causing rare, yet devastating disease. The bacteria are naturally present in estuarine environments and frequently contaminate seafoods. Within days of consuming uncooked, contaminated seafood, predisposed individuals can succumb to sepsis. Additionally, in otherwise healthy people, *V. vulnificus* causes wound infection that can require amputation or lead to sepsis. These diseases share the characteristics that the bacteria multiply extremely rapidly in host tissues and cause extensive damage. Despite the analysis of virulence for over 20 years using a combination of animal and cell culture models, surprisingly little is known about the mechanisms by which *V. vulnificus* causes disease. This is in part because of differences observed using animal models that involve infection with bacteria versus injection of toxins. However, the increasing use of genetic analysis coupled with detailed animal models is revealing new insight into the pathogenesis of *V. vulnificus* disease.**

Key words: *Vibrio vulnificus*, virulence, molecular pathogenesis, genetics

Vibrio vulnificus and disease

V. vulnificus is a gram-negative bacterium that is found in estuarine waters and frequently contaminates oysters and other seafood. Consumption of raw contaminated seafood or contamination of wounds with *V. vulnificus* can lead to septicemia and wound infection, respectively. Septicemia can also be a complication of wound infection. Additionally, *V. vulnificus* has been reported to cause gastroenteritis. *V. vulnificus* is the leading cause of reported death from seafood in the United States with approximately 40 cases reported per year (Food and Nutrition Board - Institute of Medicine, 1991; Hlady *et al.*, 1993; Hlady and Klontz, 1996). In Taiwan, between 1996 and 2000, the number of cases of *V. vulnificus* infection ranged from 13 to 26 per year (Hsueh *et al.*, 2004). A recent survey of emergency medicine physicians in Japan estimated that 425 cases of *V. vulnificus* sepsis occurred per year (vulnificus infection is not reportable in Japan, so there are no official numbers) (Osaka *et al.*, 2004). Japan could have the highest rate of vulnificus infection because of its warmer coastal waters, which are conducive to *V. vulnificus* growth, and because of higher levels of raw seafood consumption. A study of 422 infections reported that wound infection accounted for 45% of cases, and primary septicemia after consumption of contaminated seafood accounted for 43% of cases of *V. vulnificus* disease (Shapiro *et al.*, 1998). The remaining 12% of cases were thought to be associated with gastroenteritis or unknown exposure. The role that *V. vulnificus* may play in causing a gastroenteritis-type illness is not clear. *V. vulnificus* can be cultured from stool samples, but examination for other potential pathogens, which could also be responsible for the illness, is often not conducted (Strom and Paranjpye, 2000).

Both septicemia and wound infection are noted for the extremely rapid replication of bacteria in host tissues with extensive tissue damage to the skin. Even with treatment, mortality rates for septicemia can be as high as 75%, and mortality rates for wound infection can be as high as 50% (Hlady and Klontz, 1996). In fact, death can occur within 24 hours after contact with the bacteria (Hlady and Klontz, 1996; Vollberg and Herrera, 1997; Strom and Paranjpye, 2000). Typically, individuals infected by *V. vulnificus* exhibit fever, chills, hypotension, and characteristic secondary bullous skin lesions (Halow *et al.*, 1996; Vollberg and Herrera, 1997; Kumamoto and Vukich, 1998). These large bullous lesions are filled with a hemorrhagic fluid, but they can form necrotic ulcers or even become gangrenous (Musher, 1989). The skin manifestations are rapidly progressive and typically confined to the subcutaneous (s.c.) regions (Musher, 1989; Halow *et al.*, 1996). The extremities exhibiting these lesions are usually swollen and painful as a result of vascular leakage. The severe tissue destruction that occurs often requires extensive tissue debridement or amputation of the affected

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limbs (Blake *et al.*, 1979; Linkous and Oliver, 1999; Strom and Paranjpye, 2000). *V. vulnificus* is susceptible to many antibiotics, but the fatality rate increases as the time between initial infection and initiation of antibiotic treatment increases (Hlady *et al.*, 1993).

Septicemia is associated with various predisposing conditions such as hemochromatosis, cirrhosis, diabetes, immune compromise, and kidney failure requiring dialysis (Bullen *et al.*, 1991; Kraffert and Hogan, 1992; Hlady and Klontz, 1996; Kumamoto and Vukich, 1998). *V. vulnificus* sepsis in Taiwan is associated with liver disease such as Hepatitis B and C, which are less prevalent in the United States (Hsueh *et al.*, 2004). Despite the extremely high rates of contamination of oysters with *V. vulnificus* (it has been estimated that nearly all oysters harvested from the Gulf of Mexico during the summer months are contaminated with *V. vulnificus*) (Hlady and Klontz, 1996) and the high numbers of predisposed individuals who consume raw oysters, the numbers of cases of reported cases of *V. vulnificus* disease are low (approximately 30 to 50 each year in the United States) (Strom and Paranjpye, 2000). Clearly, much more needs to be learned about the nature of predisposing conditions and relevant epidemiological considerations in determining the frequency of *V. vulnificus* disease. For example, Powell *et al.* (2003) observed that elevated levels of reduced glutathione in sera of alcoholic patients were associated with elevated cytokine release from mononuclear cells upon infection with *V. vulnificus*. This result suggests that serum reduced glutathione could be used as a marker for susceptibility to vulnificus disease.

Classification of *V. vulnificus* strains

V. vulnificus has been classified based on biotypes, lipopolysaccharide (LPS) antigens, capsule, and most recently genetic sequences. There are three biotypes of *V. vulnificus*. Biotype 1 is the predominant human pathogen, biotype 2 is primarily associated with eels, and biotype 3 was recently identified in humans handling fish in Israel (Bisharat *et al.*, 1999). Biotype 2 strains possess a single type of LPS, which resulted in its designation as serogroup E (Biosca *et al.*, 1996b). In contrast, a limited number of strains of Biotype 1 was initially divided into five LPS groups based on reaction with monoclonal antibodies (Martin and Siebeling, 1991). When this panel of monoclonal antibodies was used to examine a larger set of strains from clinical, environmental, and retail seafood sources, a significant number of strains was not typable (39% to 49%), indicating that LPS is more complex in its antigenic heterogeneity. However, there was an interesting correlation of one particular LPS type, 1/5, that was significantly more prevalent among clinical strains, 33%, than environmental and retail strains. This result suggests that either the presence of this LPS type itself causes increased virulence or that the LPS type is a marker for related strains that possess higher potential for causing disease (similar to ribotype profiles - see below).

Although there has not been a comprehensive analysis of the antigenic heterogeneity of the *V. vulnificus* capsule, there have been some studies of the biochemical composition. Bush *et al.* (1997) examined the biochemical composition of 120 different *V. vulnificus* strains by acid hydrolysis and high-performance anion-exchange chromatography at high pH. This analysis yielded 94 distinguishable patterns, demonstrating extreme variability among the strains.

Most recently, the use of genetic techniques such as ribotyping and pulse field gel electrophoresis (PFGE) has revealed relatedness strains and possibly clues to virulence. Buchrieser *et al.* (1995) used clamped homogenous electric field (CHEF) gel electrophoresis to examine genetic heterogeneity of *V. vulnificus* isolates from three oysters. From analysis of the heterogeneity they observed among 118 typable strains, they concluded that a single oyster could harbor approximately 1,000 different strains of *V. vulnificus* and that the genetic heterogeneity among strains was immense. Hor *et al.* (1995) examined 77 *V. vulnificus* from environmental sources around Taiwan using ribotyping and observed great heterogeneity. More than half of the environmental strains were virulent in a mouse model. Tamplin *et al.* (1996) used PFGE and ribotyping to examine genetic relatedness of 141 strains of clinical and environmental origin and found PFGE to be too discriminatory, i.e., it yielded too many individual patterns. However, ribotyping yielded four clusters of strains (A-D). The fact that few clinical strains were present in cluster C suggested some relationship to virulence. Most recently, Nilsson *et al.* (2003) examined 16S rRNA of 67 clinical and environmental strains by PCR and restriction digestion and observed that 26 of 31 clinical strains were in group B, while 31 of 33 environmental strains were in group A. This significant relationship suggests that the group B strains have greater potential for virulence. Although the rRNA sequences are likely not directly involved with virulence, these results suggest that there are sets of related strains, some of which possess traits that enable them to be more adept at causing serious human disease. As detailed below, there is considerable effort being directed at determining what these virulence determinants are.

Pathogenesis of disease

Despite a considerable volume of published research attempting to elucidate virulence factors of *V. vulnificus* that are responsible for the remarkable disease process, very little definitive information has been gained (Linkous and Oliver, 1999; Strom and Paranjpye, 2000). For example, the two hallmarks of *V. vulnificus* disease are the

extreme destruction of host tissues and the rapid proliferation of the bacteria in the host. However, other than factors that are directly tied to the survivability of the bacteria in the host, e.g., capsule, and one that aids in growth, viz., acquisition of iron, our understanding of these major elements of disease is lacking.

Capsular polysaccharide

It is clear that the most important virulence factor for *V. vulnificus* is its capsular polysaccharide (CPS). *V. vulnificus* is an extracellular pathogen that relies on its CPS to avoid phagocytosis by host defense cells and complement (Linkous and Oliver, 1999; Strom and Paranjpye, 2000). Unencapsulated mutants, either occurring naturally from phase variation or as the result of constructed mutations, are attenuated in mouse models of infection (Yoshida *et al.*, 1985; Simpson *et al.*, 1987; Wright *et al.*, 1990) and are susceptible to bactericidal activity in human serum (Shinoda *et al.*, 1987). Presence of capsule is related to the colony morphology, with encapsulated strains being opaque and unencapsulated strains being translucent (Yoshida *et al.*, 1985; Wright *et al.*, 1999). As is the case for other bacteria that rely on their capsule to resist host defenses during systemic disease, antibodies to the *V. vulnificus* capsule are protective in animal models (Devi *et al.*, 1995; Devi *et al.*, 1996). Unfortunately, the heterogeneity, at least at the biochemical level (Bush *et al.*, 1997), makes the development of a capsule-based vaccine with broad coverage of *V. vulnificus* strains nearly impossible.

Before the elucidation of the genomic sequences of two *V. vulnificus* strains, identification of genes involved with capsular production was accomplished primarily through transposon insertion mutagenesis. Zuppardo and Siebeling (1998) first identified a gene encoding a sugar epimerase enzyme as being essential for capsule production and virulence. Wright *et al.* (2001) later identified a group 1-like capsular biosynthetic system and suggested genetic mechanisms for the opaque to translucent phase variation known to occur with *V. vulnificus*. Smith and Siebeling (2003) subsequently performed extensive transposon insertion mutagenesis and identified four genes essential for capsule biosynthesis and virulence, in addition to other genes with supporting roles in capsule biosynthesis, e.g, rhamnose biosynthesis and transferases. The identification of other genes involved in sugar metabolism suggested that capsular biosynthesis could be related to LPS biosynthesis. The elucidation of the genomic sequence of *V. vulnificus* confirmed the presence of an intact group 1 capsular biosynthesis system. Most recently, Chatzidaki and Wright (unpublished) determined that phase variation is related to various forms of genetic changes. Interestingly, deletion of sequences in *wzb*, a gene whose product is involved with subunit transport and which is flanked by direct repeat DNA sequences, irreversibly causes loss of encapsulation. In addition to the on-off phase variation associated with opaque to translucent colony type, Wright *et al.* (1999) determined that capsule biosynthesis was not constitutive, and that capsule production was decreased in stationary phase and elevated at 30°C relative to 37°C. Production of the capsule is inversely related to the ability of *V. vulnificus* to form biofilms *in vitro* (Joseph and Wright, 2004). The significance of these relationships to either the stability of *V. vulnificus* in the environment or the host-pathogen interaction in diseases is not known.

Acquisition of iron

It has been known since the earliest studies of *V. vulnificus* pathogenesis that increased iron in the host resulted in vastly increased susceptibility to infection. As noted above and reviewed by Weinberg (2000), *V. vulnificus* is one of only a small subset of opportunistic pathogens which rely on iron imbalance of the host and other host susceptibilities that can lead to iron imbalance. Wright *et al.* (1981) showed that iron treatment of mice reduced the intraperitoneal (i.p.) LD_{50} from 10^6 CFU to 1 CFU. Liver damage by carbon tetrachloride increased susceptibility of mice in a manner proportional to the levels of iron in serum. As with other invasive bacterial pathogens, iron-scavenging siderophores and proteins that bind host iron-containing proteins were identified in *V. vulnificus*. Simpson and Oliver (1983) identified both phenolate and hydroxamate siderophore production by *V. vulnificus* grown under iron-limiting conditions. Helms *et al.* (1984) determined that hemoglobin, methemoglobin, and hematin could serve as iron donors during growth of *V. vulnificus* in i.p. inoculated mice. Bullen *et al.* (1991) showed that *V. vulnificus* grows better in the blood of people with hemochromatosis, if the saturation of transferrin with iron is increased, or if hematin is added. A couple of studies indicated that the protease produced by *V. vulnificus* could be involved in acquisition of iron from heme proteins (Nishina *et al.*, 1992; Okujo *et al.*, 1996). As might be expected with an organism whose pathogenesis is centered on iron imbalanced hosts and acquisition of iron, this element of *V. vulnificus* pathogenesis is complex.

Litwin and Calderwood (1993) cloned the *V. vulnificus fur* gene, which encodes the central regulator in iron metabolism in many bacteria. Okujo *et al.* (1994) determined the structure of one of the siderophores, vulnibactin, to be a dihydroxybenzoic acid-containing compound. The essential role for vulnibactin in virulence was confirmed by Litwin *et al.* (1996) using a *venB*::Tn*PhoA* mutant that was attenuated in intragastrically inoculated suckling mice. This mutant could not obtain iron from transferrin. Similarly, a *V. vulnificus* mutant for VuuA, the ferric vulnibactin receptor, could not use vulnibactin and was decreased for virulence in mice. Biosca *et al.* (1996a) subsequently showed that the hydroxamate siderophore was used for obtaining iron from transferrin. Outer mem-

brane proteins that bind heme and hemoglobin were initially identified in Biotype 2 *V. vulnificus* (Fouz *et al.*, 1997), and Litwin and Byrne (1998) later identified HupA as the heme receptor on a Biotype 1 *V. vulnificus* strain. The HupA mutant could not use heme or hemoglobin. The role of HupA in virulence has not been examined.

A few studies have examined the mechanism(s) by which iron imbalance either increases the susceptibility of hosts to infection or increases the virulence of *V. vulnificus* in animal models. It should be noted that these are different concepts. For example, excess iron could inhibit phagocyte function (van Asbeck *et al.*, 1984; Patruta *et al.*, 1998) or could increase the growth rate of pathogens in the host or upregulate virulence factors. Interestingly, Ashrafian (2003) recently reviewed the role of iron in host susceptibility and microbial pathogenesis and proposed that hepcidin, an antimicrobial protein expressed from the liver that is involved in regulation of iron by the host, could be an important factor determining increased *V. vulnificus* disease in iron-compromised humans. Examining infection of iron-dextran-treated mice, Hor *et al.* (2000) proposed that increased susceptibility of iron-treated mice was primarily the result of increased replication of the bacteria, but also noted that neutrophil function was inhibited by high iron. We used a genetic marker plasmid system that enabled us to measure the growth rate and death rate of bacteria in infected animal hosts, independently of total recovery of bacteria (Starks, A.M. *et al.*, submitted to Molecular Microbiol.). Our results showed that the greatest effect of treating s.c. inoculated mice with iron dextran was significantly increasing the replication rate of *V. vulnificus* in skin tissues. However, one strain also was killed less effectively in iron dextran-treated mice. Clearly, there is much more to be learned in the *V. vulnificus*-host interaction as well as for infectious diseases related to iron.

Flagella and motility

Kim and Rhee (2003) used a mariner-based transposition mutagenesis system to look for *V. vulnificus* mutants that had lost cytotoxicity against HeLa cells. They used a strain that already possessed *vvhA* and *vvpE* mutations, knocking out hemolysin and metalloprotease, respectively (see below). They noted that mutations in either of these genes did not significantly affect cytotoxicity, hence they were searching for other cytotoxins. One of the cytotoxic-defective mutants had the Tn*Himar1* insertion in the *flgC* gene, which encodes the flagellar basal body. In addition to the expected defect in motility, the *flgC* mutant was also decreased for adherence to HeLa cells. Most important, the *flgC* mutant was attenuated for virulence in orally inoculated suckling mice with an increase in LD_{50} by 10,000-fold over the *vvhA/vvpE* double mutant parent. These authors noted that the decreased adherence could have prevented delivery of toxic factors to host cells or that the flagella could act as a type III secretion system for toxins, as has been noted for *Yersinia enterocolitica* (Young *et al.*, 1999). Lee *et al.* (2004a) subsequently constructed a knockout mutation in the *flgE* gene of *V. vulnificus*, which encodes a flagellar hook protein. As expected, the mutant lacked flagella and was nonmotile. The LD_{50} of the *flgE* mutant was increased 10-fold over the wild-type parent in i.p. injected, non-iron-treated mice. In s.c. inoculated, iron dextran-treated mice, the LD_{50} of the *flgE* mutant was increased 1,000-fold. In addition to attenuation of mouse-virulence, the *flgE* mutation decreased the ability of *V. vulnificus* to form biofilms *in vitro*. Upon screening a signature-tagged insertional mutation library of *V. vulnificus*, our laboratory identified an insertion in the *fliP* gene, which encodes a protein involved with flagellar biosynthesis (Lang, S-S, and P.A. Gulig, unpublished data). This mutant was nonmotile and was attenuated for virulence in s.c. inoculated iron dextran-treated mice. Interestingly, the *fliP* mutant was capable of causing severe skin infection, but was defective at causing systemic infection, as measured by liver CFU.

Hemolysin/cytolysin and metalloprotease

The subjects of perhaps the greatest amounts of published research on virulence of *V. vulnificus* are two secreted proteins, a hemolysin/cytolysin and metalloprotease; however, their roles in virulence are enigmatic. When the purified proteins are injected into animals, some of the pathology of *V. vulnificus* infection is reproduced (Kreger and Lockwood, 1981; Gray and Kreger, 1985; Kothary and Kreger, 1987; Kook *et al.*, 1996); however, mutants of *V. vulnificus* which do not produce one or both of these proteins are not significantly attenuated in animal models of infection (Wright and Morris 1991; Jeong *et al.*, 2000; Shao and Hor, 2000; Fan *et al.*, 2001). This paradox either demonstrates problems inherent in studying virulence by injecting putative virulence factors into animals or it demonstrates problems with the animal models used to study disease processes of humans.

Hemolysin/cytolysin

Kreger and Lockwood (1981) and Johnson and Calia (1981) first described hemolytic and cytolytic activity of *V. vulnificus* in 1981. Activity of the toxin is inhibited by cholesterol, suggesting that this is part of the receptor (Gray and Kreger, 1985; Shinoda *et al.*, 1985). The gene encoding the hemolysin was cloned by Wright *et al.* (1985). Injection of the hemolysin induced skin damage in mice that resembled damage caused by infection (Gray and Kreger, 1987). This result suggested that the cytotoxin could be responsible for most of the damage caused during infection. Several biochemical activities have been ascribed to the hemolysin/cytolysin. The hemolysin acts by forming pores in host cell membranes (Kim *et al.*, 1993) and stimulates guanylate cyclase (Kook *et al.*,

1996). Such activity in endothelial cells of blood vessels could lead to vasodilation and edema, which are characteristic of infection. Treatment of murine peritoneal macrophages with hemolysin induced iNOS activity and increased nitric oxide production (Kang *et al.*, 2002). Kwon *et al.* (2001) reported that treatment of endothelial cells with cytolysin induced apoptosis. Treatment of pulmonary endothelial cells with cytolysin stimulated increase in P selectin expression, which would cause increased neutrophil adherence to capillaries (Kim and Kim, 2002). However, as noted above, all of these results obtained by injection of purified toxin are clouded by the lack of attenuation of hemolysin/cytolysin mutants in animal models (Wright and Morris, Jr., 1991). This caused some to question if the toxin was even expressed during infection. Proof of *in vivo* expression was obtained by detecting cytolysin in infected mice by ELISA and immunofluorescence (Gray and Kreger, 1989) and indirectly by antibody responses to the toxin in a patient who survived *V. vulnificus* infection and also in experimentally infected mice (Gray and Kreger, 1986). Most recently, Lee *et al.* (2004b) demonstrated by both ELISA and RT-PCR that cytolysin was produced by *V. vulnificus* during infection of mice, supporting a possible role for the toxin in virulence. Lee *et al.* (2004c) showed that administration of trifluoperazine, a calcium-calmodulin antagonist, inhibited the increased permeability of polarized endothelial cell membranes *in vitro* after treatment with cytolysin. Administration of trifluoperazine with antibiotics increased the survival of mice infected with *V. vulnificus* over administration of antibiotic alone. This result suggests that cytolysin contributes to pathogenesis.

Two other *V. vulnificus* hemolysins have been identified. Chang *et al.* (1997) cloned the *vllY* gene by its ability to induce hemolytic activity to *E. coli*. The DNA sequence of the gene revealed similarity to another hemolysin, legiolysin, produced by *Legionella pneumophila*. A role for VllY in virulence was not examined. Chen *et al.* (2004a) serendipitously identified a gene, *hlyIII*, encoding a third hemolysin because of its presence near another gene that they had identified, *trkA*. HlyIII is similar to hemolysin III of *Bacillus cereus*, and mutagenesis of the *V. vulnificus* *hlyIII* gene resulted in attenuation in i.p. inoculated mice.

Metalloprotease

The *V. vulnificus* metalloprotease was originally discovered because of its collagenase activity (Smith and Merkel, 1982). The protease was subsequently purified, and additional activities of elastase, caseinase, metalloprotease (Zn^{2+}) were identified (Kothary and Kreger, 1985; Miyoshi *et al.*, 1987). As was the case for the hemolysin/cytolysin, injection of the purified protease into mice caused dermal necrosis (Kothary and Kreger, 1987) and increased vascular permeability and edema (Molla *et al.*, 1989). The ability of the protease to cause edema was explained by its ability to cause histamine release from mast cells and increased vascular permeability from activation of hagemann factor and prekallikrein and generation of kinins (Miyoshi and Shinoda, 1988; Molla *et al.*, 1989; Miyoshi and Shinoda, 1992). Consistent with a role for the protease stimulating vascular permeability by activating the kinin pathways, administration of bradykinin potentiated the spread of *V. vulnificus* after i.p. infection of mice, and administration of a bradykinin antagonist inhibited spread (Maruo *et al.*, 1998). As noted above, the metalloprotease appears to have a role in acquisition of iron from heme-binding proteins (Nishina *et al.*, 1992; Okujo *et al.*, 1996).

The gene for the metalloprotease was cloned and sequenced by three different groups, each of which gave it a different name: *vvp* (Cheng *et al.*, 1996), *empV* (Chuang *et al.*, 1997), *vvpE* (Jeong *et al.*, 2000). To examine the role of the metalloprotease in virulence during infection of animals, two groups constructed knockout mutations. Shao and Hor (2000) observed no attenuation of the *V. vulnificus* mutant in i.p. inoculated mice and even increased virulence in orally inoculated mice. This latter result supports a role for the hemolysin in gut pathogenesis, since elimination of the protease could cause a net increase in hemolytic activity. Jeong *et al.* (2000) reported that a protease mutant was not attenuated for cytotoxicity in cell culture models and was not attenuated for virulence in s.c. inoculated mice, either in terms of damage to the skin or invasion to the liver, with or without administration of iron. These results cast doubt on the role of the metalloprotease in virulence during infection, despite the effects of injecting purified protein. One possibility is that the hemolysin/cytolysin and metalloprotease may be redundant virulence factors, so that mutation of one would be compensated by the other. Fan *et al.* (2001) therefore created a double mutant for protease and hemolysin/cytolysin. Infection of mice with the double mutant revealed no attenuation by the i.p. route. Interestingly, the double mutant lost the increased virulence caused by mutation of the protease alone. This latter result is consistent with the protease degrading the hemolysin in the gut. The double mutant strain retained some cytotoxic activity in cell culture, suggesting the presence of another cytotoxin.

Other putative/possible virulence factors

Early studies of *V. vulnificus* identified the expression of numerous extracellular enzymes: protease, mucinase, lipase, chondroitinase, hyaluronidase, DNase, esterase, and sulfatase (Desmond *et al.*, 1984; Oliver *et al.*, 1986). A periplasmic nuclease, Vvn, was identified and mutated (Wu *et al.*, 2001). Loss of the nuclease enabled an increased uptake of DNA by 10-fold, but had no effect on virulence in i.p. inoculated mice.

RtxA toxin

The most recently identified and studied virulence factor for *V. vulnificus* is a member of the RTX family of toxins. These toxins share a common motif of a repeated nine amino acid sequence and are expressed by a limited set of gram-negative bacteria (reviewed in (Lally *et al.*, 1999)). Four genes comprise the *rtx* operon: *rtxA* encodes the toxin, *rtxC* encodes an essential acylase of RtxA, *rtxB* encodes an ATP binding cassette transporter for RtxA, and *rtxD* encodes a gene with unknown function *in trans*-port. Genomic DNA sequence analysis of *V. vulnificus* ultimately revealed the presence of a complete four gene set on chromosome 2, an incomplete set that includes *rtxA* on chromosome 2, and a third partial *rtxA* gene on chromosome 1. The Rhee laboratory initially identified the *V. vulnificus* RtxA toxin by screening mutants that were inhibited for cytotoxicity in cell culture (Rhee *et al.*, 2001). The RtxA mutant exhibited a 100-fold increase in LD_{50} in suckling mice, suggesting a critical role in virulence. They subsequently determined that RtxA causes depolymerization of actin in HeLa cells in a contact-mediated, de novo protein synthesis mediated manner (Rhee *et al.*, 2002). Lee *et al.* (2003c) then showed that RtxA causes pore formation in red blood cells and necrotic cell death in Hep2 cells. Finally, Kim *et al.* (2004) examined structure-function relationships for RtxA and found cellular targets of RtxA by a yeast two-hybrid system. Their results suggested a very complex interaction of multiple domains of the toxin with numerous host cell targets.

We have also examined a role for the *V. vulnificus* RtxA toxin in cytotoxicity and mouse virulence and arrived at somewhat different results (Bourdage *et al.*, 2003). During a preliminary genomic DNA sequencing project of strain MO6-24/0, we identified a clone exhibiting homology to the *rtxA* gene of *V. cholerae*. We constructed a kanamycin resistance insertion mutation in the *V. vulnificus rtxA* gene and examined the RtxA mutant for cytotoxicity in cell culture and virulence in mice. The $RtxA^-$ mutant was significantly inhibited in its ability to lyse INT407 cells in culture, destroy or detach INT407 monolayers, and to destroy polarized monolayer tight junctions in a Transwell assay. Therefore, RtxA appeared to be the major cytotoxic factor for *V. vulnificus* MO6-24/0. However, when the $RtxA^-$ mutant was examined for virulence in s.c. inoculated iron dextran-treated mice, effects were not as dramatic as would have been expected if cytotoxicity predicted animal virulence. At ten times the minimum lethal dose for the parent, 3,000 CFU, the *rtxA* mutant was not reduced for either skin or liver infection. At one minimum lethal dose, 300 CFU, there were no significant differences in s.c. lesion CFU, and histological damage was identical between the wild-type parent and mutant strains. However, systemic infection to the liver was significantly reduced for the *rtxA* mutant. The differences in results for the role of RtxA in *V. vulnificus* virulence between the Rhee laboratory and ours need to be resolved.

Pili

Gander and LaRocco (1989) first identified pili on *V. vulnificus* cells by electron microscopy and related presence of the pili to adherence to human epithelial cell lines. Paranjpye *et al.* (1998) constructed a mutation in a gene encoding a PilD peptidase, *vvpD*, involved in type 2 secretion that exhibited pleiotropic effects. For example, not only were pili no longer produced, but secretion of hemolysin/cytolysin, protease, and chitinase was also inhibited. The *vvpD* mutant was attenuated for virulence in mice; however, because pili and numerous secreted proteins were affected, it is impossible to discern the primary reason for attenuation.

Models for studying pathogenesis

Animal models

Several animal models have been used to examine the disease process and identify virulence factors for *V. vulnificus*. Most of these involve rodents and injection of purified vulnificus proteins or viable bacteria (Wright *et al.*, 1981; Gray and Kreger, 1985, 1987; Kothary and Kreger, 1987; Miyoshi and Shinoda, 1988; Wright *et al.*, 1990; Stelma *et al.*, 1992; Kook *et al.*, 1996; Jackson *et al.*, 1997; Paranjpye *et al.*, 1998; Starks *et al.*, 2000). The most commonly used animal models for *V. vulnificus* infection are injection of mice or rats with bacteria, with or without pretreatment of the animals with some form of iron to mimic the common human predisposing condition. Without iron treatment, extremely high numbers of bacteria must be injected, on the order of 10^7 CFU (Wright *et al.*, 1981; Stelma *et al.*, 1992; Starks *et al.*, 2000). Injection of iron renders mice extremely susceptible, with LD_{50}s as low as 1 CFU after i.p. injection of bacteria (Wright *et al.*, 1981; Stelma *et al.*, 1992; Jackson *et al.*, 1997). Treatment with carbon tetrachloride to induce liver damage also increases susceptibility of mice to *V. vulnificus* infection (Brennaman *et al.*, 1987).

The route of infection is also an important consideration. Most investigators have injected bacteria i.p. with or without iron treatment. However, by this route, the bacteria are administered to the deepest sites without having to invade through any host tissues. We (Starks *et al.*, 2000) and others (Bowdre *et al.*, 1981; Yoshida *et al.*, 1985; Gray and Kreger, 1986; Wright and Morris, 1991) have employed s.c. inoculation to place the bacteria into the skin tissue in which they thrive during both septicemia and wound infection, thereby forcing the bacteria to invade both tissues and vasculature to cause systemic disease. Histological damage observed after s.c. inoculation of mice reproduces that reported for human disease, although the bullous skin lesions do not occur (Starks *et*

al., 2000). Although s.c. inoculation reproduces the encounter of humans with *V. vulnificus* for wound infection, oral inoculation would be ideal for reproducing the encounter for postingestion septicemia. Fan *et al.* (2001) successfully inoculated iron-treated, neutropenic adult mice with *V. vulnificus* to produce damage in the intestines.

We recently used our iron dextran-treated mouse model of *V. vulnificus* infection to examine the ability of *V. vulnificus*-specific bacteriophages to prevent disease and death (Cerveny *et al.*, 2002). When they were first discovered in the early 1900's, bacteriophage were used to treat human infectious diseases; however, their use was discontinued with the development of antibiotics (reviewed by Duckworth and Gulig (2001). The continuing practice of phage therapy in eastern Europe and recent promising results in animal models have caused a emergence of interest in phage therapy. We found that intravenous injection of *V. vulnificus*-specific phages could prevent both infection and death of mice s.c. inoculated with *V. vulnificus* (Cerveny *et al.*, 2002). However, because of the rapidly fulminating nature of infection in the animal model, there was only a brief window of a few hours in which effective phage treatment could be administered.

Clinical versus environmental V. vulnificus strains

A major question in understanding virulence of *V. vulnificus* is the proportion of virulent strains contaminating seawater, oysters, and other seafood. Kaysner *et al.* (1987) reported that over half of 40 environmental strains were virulent in non-iron-treated mice and that all of the strains were lethal to iron-treated mice. Moreno and Landgraf (1998) found that nearly half of opaque *V. vulnificus* strains isolated from seafood could kill non-iron-treated mice when injected at 10^8 CFU. DePaola *et al.* (2003) used the s.c. iron dextran-treated mouse model to examine virulence of 25 clinical and 25 oyster isolates of *V. vulnificus* strains. Nearly all strains of either clinical or oyster origin were virulent in s.c. inoculated iron dextran-treated mice, as determined by CFU/g in skin tissues. This result, in agreement with those of others (Tison and Kelly, 1986), demonstrated that most environmental, encapsulated strains of *V. vulnificus* have the potential to cause disease in animal models, and by inference, humans. The clinical strains were significantly greater in their abilities to cause systemic disease, as measured by liver CFU; however, this result must be balanced with the fact that the clinical strains had been preselected by having caused sepsis, a systemic disease, in humans.

This latter result could also explain two interesting facts about *V. vulnificus* disease of humans. First, wound infection does not require predisposing conditions, as is seen with postingestion sepsis. Second, not every case of wound infection progresses to sepsis. It is as if there exist two populations of *V. vulnificus* strains - nearly all have the potential to cause destructive, localized infection of the skin, but a subset lacks the ability to cause systemic disease in humans. In fact, we characterized an environmental strain of *V. vulnificus* that causes severe skin infection in s.c., iron dextran-treated mice, but is incapable of causing liver infection (Bourdage, K.L. *et al.*, manuscript in preparation). As opposed to most of the other virulent strains in our collection, this strain may represent the non-systemic infecting population. Identifying the systemic virulence factors that this strain and others like it lack will be important in understanding *V. vulnificus* wound infection and sepsis.

As discussed above relative to the role of iron in *V. vulnificus* infection, we used our marker plasmid to examine differential growth rate and killing rate among several *V. vulnificus* strains of either clinical or environmental origin which we had previously characterized for virulence in the iron dextran-treated mouse model (Starks, A.M. *et al.*, submitted to Mol. Microbiol.). We determined that one environmental strain grew more slowly than did the clinical strains, while another environmental strain was killed more effectively by the mouse host. These results demonstrate the heterogeneity among *V. vulnificus* strains in their interactions with the mouse host and the usefulness of the marker plasmid system to dissect these differences. Additionally, we determined that most of the bacterial replications that occur in s.c. inoculated, iron dextran-treated mice take place within the first four hours after inoculation with doubling times as short as 15 minutes. Furthermore, even virulent clinical strains appear to be killed by greater than 90% shortly after inoculation into mice.

Cell culture models

Surprisingly, other than treatment of cell cultures with purified toxins (discussed above), very little has been published in terms of infection of host cells by *V. vulnificus*. As noted above, cell culture has been used to examine adherence to host cells by *V. vulnificus* in relation to pili (Gander and LaRocco, 1989; Paranjpye *et al.*, 1998). Adherence to epithelial cells was also inhibited by mutations affecting flagella (Kim and Rhee, 2003). We determined that *V. vulnificus* does not invade epithelial cells in culture (Gallman *et al.*, 1999) in agreement with a lack of type III secretion systems or other cellular invasion-associated genes in the genomic DNA sequences (Chen *et al.*, 2003).

Apoptosis

After the initial reports that treatment of cell culture with cytolysin could induce apoptosis (Kwon *et al.*, 2001; Rho *et al.*, 2002), Kashimoto *et al.* (2003) examined a set of five clinical and four environmental strains of *V. vulnificus* for the ability to induce apoptosis in murine J774 macrophage-like cells after infection. Using a variety of mea-

surements for apoptosis, they found that the five clinical isolates, but none of the four environmental isolates, could induce apoptosis in these cells. By using an *ex vivo* assay with murine macrophages harvested from the peritoneal cavity of s.c. inoculated mice, they showed that apoptosis in macrophages apparently also occurred during infection of mice. By using the same J774 infection assay, we examined the apoptotic abilities of the set of over 50 clinical and environmental *V. vulnificus* strains examined by DePaola *et al.* (2003). We confirmed that most strains of *V. vulnificus* have the ability to induce apoptosis in macrophages; however, we found that the apoptotic ability of any *V. vulnificus* strain was unrelated to either its origin or its level of virulence in s.c. inoculated mice (Bourdage and Gulig, manuscript in preparation). These discrepancies must be addressed and could be related to the relatively small sample of strains examined by Kashimoto *et al.* (2003).

We also used this collection of clinical and oyster strains to examine their abilities to cause cytotoxicity in various cell culture models (Bourdage *et al.*, 2002). First, we examined the abilities of the 50 strains to lyse INT407 human intestinal epithelial cells by measuring release of lactate dehydrogenase 24 h after a 1 hour infection period. We observed a wide range of lysis (7% to 81%), but lysis did not correlate with mouse virulence. We measured the ability of the *V. vulnificus* strains to cause the detachment of the INT407 cells using a crystal violet staining assay. A range of values was obtained (23% to 100%), but these values did not correlate with virulence. We examined a subset of these strains for their ability to destroy the transepithelial electrical resistance using Caco-2 polarized monolayers *in Trans* well cultures. Again, there was no correlation with virulence. Therefore, no cell culture models examined could be used to predict virulence in animal models. Interestingly, as also reported by Kim and Rhee (2003), mutants for hemolysin or metalloprotease were not significantly affected for cytotoxicity. As discussed above, the RtxA toxin appears to be a major cytotoxic factor, but its role in virulence is not agreed upon.

Studies of molecular pathogenesis

The most widely accepted criteria for definitively identifying bacterial virulence factors are the molecular version of Koch's postulates, originally proposed by Falkow (1988) and modified by Gulig (1993). In brief, these rules state that there must be an association between a putative virulence factor and disease, a mutation be made that knocks out expression of the factor, virulence must be diminished as a result of the mutation, and that the mutation and virulence be restored by complementation with the cloned wild-type gene expressed *in trans*. There are several potential problems in implementing the molecular version of Koch's postulates with *V. vulnificus*, including suitability of different animal models and the possibility of redundancy of function for specific attributes of virulence. As discussed above, these rules have been applied to identify several confirmed virulence factors of *V. vulnificus* including capsule, flagella, iron acquisition systems, and prepilin peptidase. Application of the molecular version of Koch's postulates to hemolysin/cytolysin and metalloprotease has been problematic.

Kim *et al.* (2003b) recently used a novel method to identify *V. vulnificus* genes that are expressed exclusively during infection of animal hosts - the IVIAT method (Handfield *et al.*, 2000). The method is based on creating expression libraries of genomic sequences from a pathogen in *E. coli* and then probing the expression library using patient convalescent sera that have been exhaustively absorbed with *in vitro*-grown organisms. Twelve clones were identified, including three potentially involved in signaling, most interestingly a GGDEF family member, three involved in biosynthesis, two involved with secretion, two transcriptional activators, including the *hlyU* gene involved with virulence gene regulation, and two hypothetical proteins. Mutations were then constructed in these *in vivo*-expressed genes. The *hlyU* and two biosynthesis mutants (*purH*, *pyrH*) were attenuated for cytotoxic activity against HeLa cells. These three mutants were also attenuated in i.p. inoculated, non-iron-treated mice, as determined by LD_{50}. Clearly, this method shows promise in identifying virulence genes based on capitalizing on unique aspects of the host-pathogen interaction.

Chen *et al.* (2004b) looked for *V. vulnificus* genes encoding serum resistance factors. They screened 3,000 Tn*5* mutants for sensitivity to human serum. Of the fifteen sensitive mutants, three were opaque and grew normally. One insertion was in the *trkA* gene, encoding TrkA, a NAD^+ binding protein involved with K^+ uptake in *E. coli*. The *trkA* mutant was sensitive to serum, protamine, and polymyxin B. Most interestingly, the mutant was attenuated for virulence in both iron-treated and non-treated mice by i.p. and s.c. routes in terms of LD_{50}. The exact mechanism of TrkA for serum resistance and virulence is not known; however, since the opaque mutant was rendered serum-sensitive, it indicates that the capsule is an essential, but not sufficient, virulence factor for serum resistance.

Regulation of virulence genes

To fully understand the host-pathogen interaction during disease, one must identify the virulence genes and then determine how and when these genes are expressed during infection. Several studies have been conducted to dissect the regulation of virulence gene expression. These studies have focused on regulatory systems well characterized in other gram-negative bacteria as well as the closely related pathogen, *V. cholerae*.

Lee *et al.* (2000) identified the *toxRS* genes of *V. vulnifi-*

cus. A mutant was constructed and resulted in decreased expression of hemolysin. Interestingly, *V. vulnificus* does not encode a *toxT* homologue, which is known to interact with the ToxRS system of *V. cholerae* in expression of cholera toxin and several other virulence genes (Krukonis and DiRita, 2003). The *toxRS* mutant was not examined for virulence.

RpoS is a well characterized alternative sigma factor for RNA polymerase that is involved with gene expression under stationary phase, starvation, and stress conditions. Jeong *et al.* (2001) identified two promoters expressing the *vvpE* protease - a constitutive log phase (PL) and induced stationary (PS) promoter. The PS promoter was decreased in *crp* and *rpoS* mutants during stationary phase (when *vvpE* is normally expressed). Lee *et al.* (2003a) examined the proteome of a *V. vulnificus* RpoS mutant and identified Fur as being down-regulated, i.e., RpoS is necessary for full expression of Fur. Consistent with a role for RpoS in resistance to stress, Hulsmann *et al.* (2003) reported that an RpoS mutant was more sensitive to oxidative, osmotic, and acid stress. The mutant also demonstrated decreased protease production and motility. Park *et al.* (2004) reported that an RpoS mutant was susceptible to peroxide, starvation, UV light, and acid during exponential phase growth. None of these studies reported the effects of the *rpoS* mutations on virulence.

Catabolite repression mediated by cAMP and the CAP protein has been linked to expression of hemolysin (Bang *et al.*, 1999; Choi *et al.*, 2002). The effects of either an adenylate cyclase (*cya*) mutation or *cap* mutation on virulence have not been reported.

Quorum sensing is the cell density-dependent regulation of gene expression by bacteria using the accumulation of small molecules (autoinducers) to affect gene expression. Quorum sensing was first identified in relatives of *V. vulnificus*, *Vibrio fischeri* and *Vibrio harveyi* (Miller and Bassler, 2001). *V. vulnificus* possesses a *luxS* gene, involved in autoinducer-2 production, as well as the gene encoding the regulator that senses the autoinducer, *luxR* (in *V. vulnificus* the gene was named *smcR*) (McDougald *et al.*, 2001). Shao and Hor (2001) constructed a LuxR (SmcR) mutant and found that metalloprotease production decreased while cytolysin increased, suggesting inverse regulatory networks for these extracellular proteins. The *luxR/smcR* mutation had no effect on virulence in mice. Jeong *et al.* (2003) subsequently showed that SmcR (LuxR) was required for full expression of metalloprotease by working synergistically with CRP and RpoS. Consistent with these results, Kim *et al.* (2003a) showed that LuxS, the autoinducer producer, was required for full expression of protease and appeared to inhibit expression of hemolysin. In contrast to the results of Shao and Hor (2001) who reported that a *luxR* mutant was not affected for virulence, Kim *et al.* (2003a) found a 10 to 750-fold increase in LD_{50} for the *luxS* in mutant in iron-treated and non-treated mice, respectively. Most recently, Kawase *et al.* (2004) reported that the protease gene, *vvp*, was regulated by LuxS during growth of *V. vulnificus* in LB, but in serum, protease expression was related to amounts of iron, not expression of LuxS. These results indicate the need for caution in interpreting gene expression in *in vitro* conditions. The ultimate environment for examining virulence gene expression is in the infected animal host, itself.

The genomic DNA sequences of V. vulnificus CMCP6 and YJ016

The genomic DNA sequences of two different *V. vulnificus* strains, CMCP6 and YJ016, have been deposited in GenBank (accession numbers NC. 005139, NC. 005140, NC. 004459, NC. 004460). Chen *et al.* (2003) analyzed the genomic sequence of strain YJ016. As for other *Vibrio* spp., *V. vulnificus* has two chromosomes. The larger chromosome 1 consists of 3.35 MB, and the smaller chromosome 2 consists of 1.86 MB. Unlike the other sequenced strain, YJ016 possesses a 48.5-kb plasmid. There are 4,959 chromosomal coding sequences. In supplemental information to their publication (genome.nhri.org.tw/vv/), Chen *et al.* (2003) delineate putative and confirmed virulence genes: carbohydrate metabolism and transport involved in capsule and LPS biosynthesis, 13 cytotoxin homologues including hemolysins and 3 RTX toxins, genes involved in type IV pilin biogenesis, and iron and heme uptake. Many of these putative virulence genes are encoded on the small chromosome. As noted above, there are no homologues to cellular invasion genes. It appears that most rearrangement and diversity has occurred in chromosome 2 of *V. vulnificus* relative to the other two major pathogens in the genus, *V. cholerae* and *V. parahaemolyticus*.

The presence of these two genomic DNA sequences will be invaluable in ongoing studies of molecular pathogenesis. The most important question now becomes how the sequence data will be utilized to better understand the organism and its disease. It is tempting to identify genes that are homologues of other known or putative virulence gene from other organisms, mutate those homologues, and examine their effects on virulence. We believe that one of the most important uses of the genomic DNA sequences will be in the construction of whole genome microarrays for the analysis of genomic-wide gene expression under various conditions *in vitro*, in cell culture, and during growth in animal models. The extremely high levels of recovery of *V. vulnificus* from infected skin tissues of mice should enable the recovery of sufficient amounts of RNA to perform such microarray analysis.

Conclusion

Despite the immense progress that has been obtained over the past 20 years in understanding the cellular and molecular bases for the devastating but rare diseases

caused by *V. vulnificus*, we still lack answers to several critical questions. How does the organism replicate so rapidly in the host? How do the bacteria evade the host defenses they encounter in tissues? How do the bacteria invade so quickly and efficiently though host tissues? How do the *V. vulnificus* bacteria that accumulate in tissues as a result of rapid growth and evasion of host defenses create such massive and destructive damage to host cells and tissues? We believe that the development of animal and cell culture models coupled with the existence of two sequenced genomes and tools for creating mutations and cloned genes will enable the answers to these questions to be obtained at an accelerated rate. Perhaps the answers to these questions will enable the development of tools to prevent or intervene in *V. vulnificus* disease.

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