Molecular Pathogenesis of *Vibrio vulnificus* and Use of Bacteriophage as Therapy for Disease in a Mouse Model of Infection

Paul A. Gulig, Keri L. Bourdage, Karen E. Cerveny, and Angela M. Starks

University of Florida College of Medicine, Department of Molecular Genetics and Microbiology
PO Box 100266, Gainesville, FL 32610-0266 USA

Vibrio vulnificus is a gram-negative bacterium found in estuarine waters and which frequently contaminates oysters. Consumption of raw contaminated oysters or contamination of wounds with *V. vulnificus* can lead to septicemia and wound infection, respectively. In fact, *V. vulnificus* is the leading cause of reported death from sea food in the United States (1-3). Both septicemia and wound infection are noted for the extremely rapid replication of bacteria in host tissues with extensive tissue damage to the skin. Mortality rates for septicemia can be as has high as 75%, even with treatment, and mortality rates for wound infection can be as high as 50% (2). Septicemia is associated with various predisposing conditions such as hemochromatosis, cirrhosis, diabetes, immune compromise, and kidney failure requiring dialysis (2,4-6). 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*) (2) and the high numbers of predisposed individuals who consume raw oysters, the numbers of cases of reported *V. vulnificus* disease are low (approximately 30 to 50 each year in the United States) (7). 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.

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 (7,8). *V. vulnificus* is an extracellular pathogen that relies on its capsular polysaccharide to avoid phagocytosis by host defense cells and complement (7,8). Unencapsulated mutants, either occurring naturally from phase variation or as the result of constructed mutations, are attenuated in mouse models of infection (9). Therefore the capsule is a critical virulence factor. Similarly, mutants in regulation of gene expression by iron or in the production of iron-acquiring siderophores are attenuated in mice (10,11). This result is interesting because iron overload of patients is a critical predisposing factor for disease. A mutation in a peptidase with pleiotropic effects involved in type 2 secretion was attenuated for virulence; however, because numerous secreted proteins were affected, it is impossible to discern the primary reason for attenuation (12). Most recently, Kim and Rhee (13) determined that flagella were necessary for virulence of *V. vulnificus* in both cell culture and mouse models. Much research has been conducted on two secreted proteins of *V. vulnificus*, 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 (14-17); however, mutants of *V. vulnificus* which do not produce either or both of these proteins are not significantly attenuated in animal models of infection (18-21). 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.

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 (9,12,14,15,17,17,22,22-27). As noted above, injection of culture supernatants or purified metalloprotease or

hemolysin can reproduce pathology of human infection or experimental infection. Although important information can be gained from such studies, results must be balanced with the caution that levels of expression of the putative virulence factors that were injected may not be attained during natural infection, or the putative virulence factor may not even be expressed during the natural disease process. The most widely accepted criteria for definitely identifying bacterial virulence factors are the molecular version of Koch's postulates, originally proposed by Stanley Falkow (28) and modified by Gulig (29). 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 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. 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 (23,26,27), which might enable bacteria to overwhelm host defenses or achieve levels of infection which they normally would not be able to achieve in a natural disease process. Injection of iron renders mice extremely susceptible, with LD50s as low as 1 CFU after intraperitoneal (i.p.) injection of bacteria (23,25,26). 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 (27) and others (19,30-32) have employed subcutaneous (s.c.) inoculation to place the bacteria into the host tissue in which they thrive during both septicemia and wound infection and to force 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 (27). 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. However, we were unable to develop a reproducible oral inoculation model. Fan et al. (18) successfully inoculated iron-treated, neutropenic mice with *V. vulnificus* to produce damage in the intestines. The combined predispositions of iron overload and neutropenia and possible intestinal epithelial damage by cyclophosphamide, although enabling oral inoculation, should be carefully considered in interpreting results, just as for injecting 10^7 CFU i.p. or in using parenteral inoculation of iron-treated mice.

All of this being said, we reported the development of a useful animal model with s.c. inoculation of outbred mice with *V. vulnificus* (27). Injection of iron dextran-treated mice with approximately 100 CFU of clinical strains yielded lethal infection within 24 hours, with extremely high numbers of bacteria in skin tissues (10^8 CFU/g), systemic infection (10^6 CFU/g liver), and histological damage that mimicked human disease. Specifically, we observed extensive edema in the dermis and subcutis, necrosis of all cell types in the region, especially neutrophils, and perivascular infection. We used this animal model to examine the virulence properties of three virulent clinical strains and three environmental strains that had been chosen from a collection based on their apparent attenuation in i.p. inoculated, iron dextran-treated mice. The naturally attenuated environmental strains had the potential to cause identical histological damage as the clinical strains; however, they required as high as 1000-fold increased inocula. This result suggested that these environmental strains did not lack damaging virulence factors, but rather virulence factors that enabled rapid growth or resistance to host defenses. As described below, this hypothesis was later shown to be true.

As opposed to the initial animal model studies described above, in which we examined *V. vulnificus* strains which had been previously characterized for virulence potential, we then used the mouse model to examine

virulence of a large collection of 25 clinical and 25 oyster isolates of *V. vulnificus* strains in an attempt to identify characteristics that might be used to predict virulence (33). The goal of the study was to determine if markers other than virulence itself could predict virulence. Expression of hemolysin and protease had already been shown not to correlate with virulence (18-21), and although capsule is necessary for virulence (9), not every encapsulated strain is virulent. Presence of plasmids and AFLP fragments among encapsulated *V. vulnificus* strains did not correlate with virulence in our mouse model. Most interesting was the fact that 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 (23,34), demonstrates that most environmental, encapsulated strains of *V. vulnificus* have the potential to cause disease in animal models, and by inference, humans. We also examined systemic manifestations of disease in terms of CFU/g liver and decrease in body temperature. The clinical strains were significantly greater in their abilities to cause systemic disease; however, this result must be balanced with the fact that the clinical strains had been preselected by having caused sepsis, systemic disease, in humans.

We then took this collection of clinical and oyster strains and examined their abilities to cause cytotoxicity in various cell culture models. First, we examined the abilities of the 50 strains to lyse INT407 human intestinal epithelial cells by measuring release of lactate dehydrogenase 24 hours after a 2 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. Again, 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 Transwell cultures. Again, there was no correlation with virulence. Finally, the ability of these strains to induce apoptosis in J774.1 macrophage-like cells did not correlate with mouse virulence, in contrast to the results of Kashimoto et al. (35) who reported that clinical but not environmental strains of *V. vulnificus* induced apoptosis. Therefore, none of these cell culture models could be used to predict virulence in animal models. Interestingly, mutants for hemolysin or metalloprotease were not significantly affected for cytotoxicity. Therefore, the major factor responsible for the observed cytotoxicity had not been identified at this step. As discussed below, for at least one strain, M06-24/0, the RtxA toxin appeared to be a major cytotoxic factor.

The differences in virulence between clinical strains and a set of preselected attenuated environmental strains was apparently in their ability to replicate in the host or resist host defenses, since inoculating enough CFU of these strains resulted in indistinguishable histological damage to virulent strains. A problem was that measuring the numbers of bacteria recovered from infected mice could not differentiate between the possibilities of differential growth versus death. In previous studies examining virulence of *Salmonella enterica* serovar Typhimurium, we had used a marker plasmid to enable the separate measurement of bacterial growth and killing in infected animals (36,37). The marker plasmid is an antibiotic resistance-encoding plasmid that does not replicate in the bacteria during infection of the animal. Therefore, each bacterial division in the animal host generates plasmid-cured derivatives with frequencies proportional to the number of divisions that have occurred. Similarly, as marker plasmid-containing bacterial cells are killed in the host, they cannot be replaced; hence, the total number of marker plasmid-containing bacteria recovered from infected animals is an indication of the amount of killing that has occurred. Unfortunately, the marker plasmid that was used in the salmonella studies did not work in *V. vulnificus* because it was not mobilizable and it depended on the body temperature of the mice to prevent temperature-sensitive plasmid replication. The decreased body temperature of mice infected with *V. vulnificus* therefore would have caused a problem. We therefore constructed a new marker plasmid system for use with *V. vulnificus*. The backbone of the plasmid is the suicide plasmid pUT-Km-2 originally constructed by de Lorenzo et al. (38) for use as a donor of the mini-Km-2 transposon. This plasmid has an R6K-based origin of replication that requires the B

protein encoded by the *pir* gene for replication. We therefore cloned the *pir* gene under the control of the p*BAD*/AraC system from pBAD-TOPO (Invitrogen) into the transposase gene of pUT-Km-2 yielding plasmid pGTR902. This disabled the transposase function, hence pGTR902 is no longer a transposon donor, and, more important, replication of pGTR902 is now under control of arabinose. If sufficient levels of arabinose are present in the growth environment, *pir* will be expressed, and the plasmid will replicate. However, in the absence of arabinose, the plasmid will not replicate and will segregate from the bacterial population with exponential kinetics with each generation. Mice apparently do not have enough arabinose available to enable replication of pGTR902 in *V. vulnificus* in tissues.

We conjugated pGTR902 into several *V. vulnificus* strains of either clinical or environmental origin which we had previously characterized for virulence in the iron dextran-treated mouse model to ask if differences in virulence were the result of differential growth or killing in the mouse host. In results to be reported in detail elsewhere 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. For one virulent strain infecting non-iron-dextran-treated mice resulted in slower growth while another clinical strain was both killed more effectively and grew more slowly in normal mice. These combined 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 min. Furthermore, even virulent clinical strains appear to be killed by greater than 90% shortly after inoculation into mice. This marker plasmid system should be amenable to use in almost any gram-negative bacteria.

Since some environmental strains exhibited higher susceptibility to host defenses, we examined the reason for different rates of bacterial killing in the mouse host. Since all of the strains discussed here were resistant to complement (27), we examined the role of neutrophils in differential killing by the host. Mice were rendered neutropenic by treatment with either cyclophosphamide or the anti-granulocyte monoclonal antibody RB6-8C5 (39). With either treatment virulent clinical strains were not increased in virulence in neutropenic mice compared with normal mice. However, some attenuated environmental strains were increased for virulence in neutropenic mice, suggesting that differential susceptibility to neutrophils was responsible, at least in part, for the decreased virulence of these strains. When the *V. vulnificus* strains were examined for susceptibility to neutrophils in an in vitro assay using peritoneally derived, proteose peptone-elicited neutrophils, the strains that were increased for virulence in neutropenic mice were also most susceptible to killing by neutrophils in vitro. These results suggest that neutrophils can have a suppressive effect against some *V. vulnificus* strains.

We have used a variety of genetics tools to examine virulence of *V. vulnificus* in our mouse model. For in vivo complementation for virulence, we cloned 5-kb fragments of genomic DNA from clinical strain LL728 (27) into the vector pCos5 (40) and conjugated the plasmids into attenuated environmental strain MLT367 (27). Pools of 200 recombinant bacteria were inoculated s.c. into iron dextran-treated mice at a dose too low for MLT367 to cause disease by itself. One mouse became ill with high numbers of bacteria being recovered from skin and liver. The recovered bacteria carried a single plasmid, pGTR600. When placed de novo into MLT367, pGTR600 significantly increased virulence of the attenuated *V. vulnificus* strain. A combination of deletion mutagenesis, subcloning, and DNA sequence analysis identified a single open reading frame that was both necessary and sufficient to confer the virulence-enhancing abilities of pGTR600 to MLT367. We named this gene *vvgA*, for *V. vulnificus* GGDEF protein A. DNA sequence analysis of *vvgA* revealed that it encoded a protein that possessed homology to a family of proteins named GGDEF. These proteins possess this amino acid sequence and are believed to be involved with signal transduction; however, specific roles in virulence or physiology have not been identified (41). We determined that the environmental strain MLT367

possessed a *vvgA* gene, and DNA sequence analysis revealed only three amino acid sequence differences between the *vvgA* genes of LL728 and MLT367. The MLT367 *vvgA* gene could increase the virulence of MLT367 when expressed from a recombinant plasmid. Therefore, the virulence-enhancing ability of *vvgA* appeared to be more related to levels of expression than complementing a missing or defective gene. Finally, when *vvgA* of virulent strain LL728 was mutated by insertional inactivation with a kanamycin resistance gene, there was no significant effect on local or systemic infection in our s.c. inoculated iron dextran-treated mouse model. Therefore, despite the ability of *vvgA* to increase the virulence of an attenuated environmental *V. vulnificus* strain, *vvgA* is not itself essential for virulence in our animal model.

We have begun using the signature-tagged mutagenesis (STM) system of Lehoux et al. (42), which is based on mini-kanamycin resistance gene insertional mutagenesis with 12 transposons each differentially labeled with a unique oligonucleotide sequence to enable specific amplification by PCR. Groups of 12 STM mutants were pooled and inoculated into two iron dextran-treated mice. Bacteria in skin lesion and liver from infected mice were grown, and genomic DNA was extracted. The presence of each of the inoculated 12 mini-Km mutations was examined by PCR. If a mutation was amplifiable from the inoculum but was absent or significantly decreased in skin and/or liver, the mutant was reexamined for virulence by itself, and if virulence was attenuated, the mutation was cloned and sequenced. One interesting mutation was located within the *fliP* gene, which is involved with flagellar biosynthesis. The mutant bacteria were non-motile and expressed aberrant flagella. The *fliP* mutant had wild-type ability to infect skin, but was significantly attenuated in its ability to cause systemic disease and infect to the liver. This result, combined with those published by Kim and Rhee (13), who also reported that flagella are essential for full virulence of *V. vulnificus*, will lead us into a more detailed analysis of the role of flagella in virulence in *V. vulnificus*.

To enable the focus of mutagenesis for identifying virulence genes, many groups have utilized TnPhoA mutagenesis, which creates alkaline phosphatase (PhoA) fusions and insertions into target genes (43). Productive PhoA activity indicates that a gene whose product is secreted beyond the cytoplasm has been mutated. Such genes are the most likely, but not exclusive, candidates for virulence genes. We made several attempts at using TnPhoA, but the numbers of mutants were prohibitively low. We therefore created our own PhoA fusion vector that is based on recombination, rather than transposition. The suicide vector pUT-Km-2 was digested with *Bgl*II, which deleted the transposase gene, and a *Bgl*II-'*phoA-Bam*HI fragment was cloned into the *Bgl*II site. This resulted in a unique *Bgl*II site upstream of the promoterless and leader peptide-deficient *E. coli phoA* gene. The goal was for sub-gene-length fragments of *V. vulnificus* genomic DNA to be cloned into the *Bgl*II site, sometimes creating gene fusions between the cloned open reading frame and the *phoA* gene. When this plasmid is conjugated into *V. vulnificus*, the suicide plasmid will integrate into the *V. vulnificus* genome via the cloned fragment, thereby creating a *phoA* gene fusion, insertional inactivation. $PhoA^+$ mutants could then be individually examined for virulence. To test this system, we cloned a fragment of the *V. vulnificus vvhA* gene encoding the secreted hemolysin into the PhoA fusion vector in frame with the *phoA* gene. As expected, the recombinant vibrios were $PhoA^+$ (produced blue colonies on plates containing the colorimetric substrate BCIP), and, as expected, these mutants were not attenuated for virulence. A problem with the PhoA mutagenesis system encountered by others was the endogenous PhoA activity of *V. vulnificus* which requires plating the bacteria on 2% glucose to suppress the PhoA activity (9,11). This procedure inhibits the growth of *V. vulnificus* and still enables background PhoA activity. We are therefore attempting to delete the *phoA* gene from *V. vulnificus* to dispense with the background activity. If successful, this new PhoA fusion/insertion mutagenesis system should be amenable for use by others who cannot use TnPhoA for this purpose.

The final ongoing genetic analysis in the laboratory involves the role of the RtxA toxin in virulence

and cytotoxicity of *V. vulnificus*. We had begun a preliminary genomic DNA sequencing project of strain MO6-24/0 and had identified a cloned with a 3.8-kb insert which contained a gene with homology to the *rtxA* gene of *V. cholerae* (44). We constructed a kanamycin resistance insertion mutation in the *V. vulnificus rtxA* gene by allelic exchange, and examined the resulting strain, FLA402, for cytotoxicity in cell culture and virulence in our mouse model. The RtxA$^-$ mutant was significantly inhibited in its ability to lyse INT407 cells in culture (lactate dehydrogenase release assay), destroy or detach INT407 monolayers (crystal violet staining assay), and to destroy polarized monolayer tight junctions in a Transwell assay (transepithelial electrical resistance for Caco-2 cells). Therefore, RtxA appeared to be a major, if not 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 at either one time or ten times the minimum lethal dose for the parent (300 CFU and 3,000 CFU, respectively), there were no significant differences in CFU recovered, and histological damage was identical to that of the parent strain. Therefore, as is the case for the hemolysin/cytolysin (19) and metalloprotease (20,21), the RtxA toxin does not appear to be essential for virulence in mammals. We were unable to examine the RtxA mutant strain for virulence in orally inoculated mice, because we cannot achieve consistent infection by this route.

A final aspect to our studies of virulence of *V. vulnificus* is the potential use of bacteriophage to prevent and/or treat disease. When bacteriophage were first discovered in the early 1900s, they were used as treatment for several bacterial diseases of humans (for a review, see (45)). However, an incomplete understanding of their usefulness and the development of antibiotics brought about an end to phage therapy, except in Eastern Europe. In the 1980s interest was renewed with basic studies in the West. To examine if phage can be used to treat disease caused by *V. vulnificus*, we obtained several *V. vulnificus*-specific phage and demonstrated that some of them had the potential to prevent disease and death in our mouse model (46). Phage that could lyse the infecting *V. vulnificus* strain in LB-sodium chloride could protect the mice, while phage that required the presence of seawater failed to protect. We are currently examining the ability of *V. vulnificus* phage to clear oysters of *V. vulnificus* contamination. We first had to develop an experimental infection system, since oysters would not readily take up vibrios simply added to their tanks. We pretreated oysters with rifampin to clear the abundant normal flora and observed that oysters could now take up exogenously provided *V. vulnificus*. We are currently examining the ability of *V. vulnificus*-specific phage to reduce or clear the experimental infection in this system. We believe that the experimental questions and parameters that we are investigating in both the mouse and oyster phage treatment systems will be useful to others interested in examining the potential usefulness of phage in fighting bacterial disease.

The broad array of studies briefly described in this paper demonstrates the multifaceted approach we are taking in examining the virulence of *V. vulnificus* and means to fight the disease process. Despite two decades of investigation, there is much more to be learned about how this opportunistic pathogen can cause such a devastating disease in predisposed people. This includes a more complete understanding of the host susceptibility, as well as identifying virulence factors and dissecting their mechanisms of action.

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