THE ROLE OF TOXINS AND SECRETED FACTORS IN THE PATHOGENESIS OF *Vibrio vulnificus*

By

JENNIFER LEE JOSEPH

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2009
To my parents, William and Susan Joseph
ACKNOWLEDGMENTS

I thank my mentor, Paul Gulig, who has guided me with patience throughout my graduate career. With his wisdom and advice, he has served as a mentor not only in the laboratory, but in life as well. I acknowledge my committee members, Henry Baker, Shouguang Jin, and Anita Wright. They have brought new perspectives to my research and have kept me constantly thinking and questioning everything. My mentor and committee members have provided for me the foundation to have a successful career.

I would also like to express my appreciation to all of my colleagues in the Gulig lab (past and present) for making my time in lab enjoyable. I am especially grateful for all of the assistance provided by Roslyn Brown, Julio Martin, Rupam Sharma, and Jessica Ascencio. Of all of my colleagues, I am most grateful to Patrick Thiaville. With his constant encouragement, assistance, and friendship, he has gone above and beyond his role as a coworker, and has become a cherished friend.

Above all, I could not have done this without my friends and family, especially my parents. They have pushed me harder than anyone else and have never lost faith in me or my abilities. They express unconditional love and encouragement, and I realize everyday how blessed I am to have them by my side.
TABLE OF CONTENTS

ACKNOWLEDGMENTS............................................................................................................ 4
LIST OF TABLES.......................................................................................................................... 9
LIST OF FIGURES.......................................................................................................................... 10
ABSTRACT.................................................................................................................................. 12
CHAPTER

1 INTRODUCTION..................................................................................................................... 14

\textit{Vibrio vulnificus}.................................................................................................................. 14
Disease Caused by \textit{V. vulnificus}................................................................................................. 14
Classification of \textit{V. vulnificus}................................................................................................. 15
Virulence Factors of \textit{V. vulnificus}......................................................................................... 18
\hspace{0.5cm} The Polysaccharide Capsule.................................................................................... 18
\hspace{0.5cm} Acquisition of Iron from the Host ........................................................................... 19
\hspace{0.5cm} Flagella....................................................................................................................... 21
\hspace{0.5cm} Pili and Attachment .................................................................................................... 21
\hspace{0.5cm} Extracellular Toxins.................................................................................................. 22
\hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} Hemolysin/cytolysin ............................................................... 23
\hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} Other hemolysins ........................................................................ 23
\hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} Metalloprotease .................................................................................. 24
\hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} Phospholipases .................................................................................. 25
Focus of Investigation.................................................................................................................. 26
\hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} Specific Aim 1: Examine Role of the RtxA1 Toxin in Pathogenesis of \textit{Vibrio vulnificus} .................................................................................. 26
\hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} Specific Aim 2: Examine the Other RTX Loci in Virulence of \textit{V. vulnificus} and the Importance of Activation of the RtxA Toxins ............................................ 27
\hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} Specific Aim 3: Examine the Type VI Secretion System of \textit{V. vulnificus} ....... 28

2 MATERIALS AND METHODS.................................................................................................. 30

Standard Microbiological and Animal Infection Protocols ......................................................... 30
\hspace{0.5cm} Bacterial Cultures, Chemicals, and Media....................................................................... 30
\hspace{0.5cm} Infection of Mice ........................................................................................................... 31
\hspace{0.5cm} Histological Analysis...................................................................................................... 32
\hspace{0.5cm} Tissue Culture ............................................................................................................... 32
\hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} Infection of monolayers ............................................................................... 32
\hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} Crystal violet assay for detachment and destruction of monolayers .............. 34
\hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} Lactate dehydrogenase assay for measuring lysis of INT-407 Cells ...... 34
\hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} Apoptosis assay ................................................................................................. 36
\hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} \hspace{0.5cm} Statistical Analysis ........................................................................................... 37
Molecular Genetics and Mutagenesis

Southern Blots

Extraction and digestion of genomic DNA

Preparation of digoxigenin labeled probes for Southern blot analysis

Transfer of DNA

Hybridization and detection of the labeled DNA probe

Mutagenesis of V. vulnificus

USER-friendly cloning

Three-way USER-friendly cloning

Construction of mutations by conjugation of plasmid DNA into V. vulnificus

Chitin induced natural transformation

3 ROLE OF THE RtxA1 TOXIN IN PATHOGENESIS OF Vibrio vulnificus

Rationale for Study

Introduction

Results

Disruption of rtxA1 by aph Insertion

Cytotoxicity of rtxA1::aph mutant

Virulence of rtxA1::aph mutant in mice

Deletion of rtxA1

Cytotoxicity of ΔrtxA1::aph mutants

Virulence of ΔrtxA1::aph mutants in mice

Reconstruction of the ΔrtxA1::aph Mutation by Chitin-Induced Natural Transformation

Virulence of reconstructed ΔrtxA1::aph mutants

Attempted complementation of ΔrtxA1::aph with the wild-type rtxA1 allele

Histopathology of s.c. lesions of ΔrtxA1::aph infected mice

Insertion of Ωaph at 5’ End of rtxA1

In vitro characterization of rtxA1::Ωaph

Virulence of rtxA1::Ωaph in mice

Verification of the virulence defect of the rtxA1::Ωaph mutant

RtxA1 Causes Apoptosis

A Combination of RtxA1 and VvhA Contributes to Cytotoxicity

Construction of a double mutation of rtxA1::Ωaph and ΔvvhA

Cytotoxicity of rtxA1::Ωaph/ΔvvhA

Virulence of rtxA1::Ωaph, ΔvvhA

Prevalence of rtxA1 in V. vulnificus

Discussion

RtxA1 is the Major Cytotoxic Factor of V. vulnificus

RtxA1 has a Role in Virulence of V. vulnificus

Are Other Factors Involved in Cytotoxicity and Tissue Damage?

Presence of the rtxA1 Gene is Widespread Among V. vulnificus Strains

What is the Function of RtxA1 in Virulence?
4 THE ROLE OF THE OTHER RTX LOCI IN VIRULENCE OF V. vulnificus AND
THE IMPORTANCE OF ACTIVATION OF THE RtxA TOXINS .............................. 96

Rationale for Study ......................................................................................................... 96
Introduction ...................................................................................................................... 96
Results ............................................................................................................................... 98
   Identification and Examination of RtxA2 ................................................................. 98
   Mutation of rtxA2 to Examine a Role in Virulence ............................................. 99
      Construction of rtxA2::Ω .................................................................................. 99
      Cytotoxicity of the rtxa2::Ω mutant ................................................................. 100
      Virulence of the rtxA2::Ω mutant in iron-treated mice ..................................... 100
   Double Mutation of rtxA1 and rtxA2 ............................................................... 101
      Virulence of double rtxA1/rtxA2 mutant in mice ........................................... 102
      Construction of ΔrtxA1::aph, rtxA2::Ω ......................................................... 103
      Cytotoxicity of ΔrtxA1::aph, rtxA2::Ω ............................................................ 104
      Virulence of ΔrtxA1::aph, rtxA2::Ω ............................................................... 104
   Identification and Examination of RtxA3 ............................................................... 105
   Deletion of the rtxA3 Gene to Examine a Role in Virulence ............................. 106
      Construction of the ΔrtxA3 mutant ............................................................... 106
      Cytotoxicity of the ΔrtxA3 mutant .................................................................. 106
      Virulence of the ΔrtxA3 mutant ..................................................................... 106
   Future Direction: Construction of a Triple rtxA Mutant ................................... 107
      Examination of RtxC1 in Virulence ............................................................... 107
      Deletion of VV2_0480 encoding RtxC1 ......................................................... 108
      In vitro analysis of the ΔrtxC1 mutant ............................................................ 109
      Virulence of ΔrtxC1 in iron-treated mice ....................................................... 109
   Discussion .................................................................................................................... 110
      RtxA2 is Not Essential for Virulence ............................................................... 111
      RtxA3 is Not Essential for Virulence ............................................................... 113
      RtxC is Not Required for Virulence Caused by RtxA1 .................................... 114

5 EXAMINING THE ROLE OF THE TYPE VI SECRETION SYSTEM IN
PATHOGENESIS OF Vibrio vulnificus ......................................................................... 125

Rationale for Study .......................................................................................................... 125
Introduction ..................................................................................................................... 125
Results .............................................................................................................................. 129
   Identification and Deletion of V. vulnificus vgrG ............................................. 129
      Cytotoxicity of ΔvgrG::aph mutant ................................................................. 130
      Virulence of ΔvgrG::aph in mice ................................................................. 132
   Identification and Deletion of V. vulnificus hcp ............................................. 132
      Cytotoxicity of Δhcp::cat ............................................................................... 134
      Virulence of Δhcp::cat ................................................................................. 134
   Deletion of the T6SS Factors in an rtxA1 Background ................................ 135
      Cytotoxicity of T6SS, rtxA1 double mutants ................................................ 136
      Virulence of T6SS, rtxA1 double mutants ..................................................... 137
   Discussion .................................................................................................................... 138
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Bacterial strains used in this study</td>
<td>44</td>
</tr>
<tr>
<td>2-2</td>
<td>Plasmids used in this study</td>
<td>46</td>
</tr>
<tr>
<td>2-2</td>
<td>Oligonucleotides used in this study</td>
<td>48</td>
</tr>
<tr>
<td>3-1</td>
<td>Southern blot to detect <em>rtxA1</em> in <em>V. vulnificus</em> isolates</td>
<td>95</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>Schematic of rtx gene clusters of <em>Vibrio vulnificus</em> strain CMCP6.</td>
<td>78</td>
</tr>
<tr>
<td>3-2</td>
<td>Detachment/destruction of INT-407 monolayers by <em>rtxA1::aph</em> mutant.</td>
<td>79</td>
</tr>
<tr>
<td>3-3</td>
<td>Virulence of <em>rtxA1::aph</em> mutant in iron dextran-treated mice.</td>
<td>80</td>
</tr>
<tr>
<td>3-4</td>
<td>Skin lesions of mice infected with <em>rtxA::aph</em>.</td>
<td>81</td>
</tr>
<tr>
<td>3-5</td>
<td>Detachment/destruction of INT-407 monolayers by the Δ<em>rtxA1::aph</em> mutant.</td>
<td>82</td>
</tr>
<tr>
<td>3-6</td>
<td>Virulence of Δ<em>rtxA1::aph</em> mutants in mice.</td>
<td>83</td>
</tr>
<tr>
<td>3-7</td>
<td>Detachment/destruction of INT-407 cell monolayers by chitin-recreated Δ<em>rtxA1::aph</em> mutants.</td>
<td>84</td>
</tr>
<tr>
<td>3-8</td>
<td>Virulence of Δ<em>rtxA1::aph</em> mutants recreated by chitin transformation.</td>
<td>85</td>
</tr>
<tr>
<td>3-9</td>
<td>Cytotoxicity to INT-407 cells by Δ<em>rtxA1::aph</em> complemented with wild-type <em>rtxA1</em> in trans.</td>
<td>86</td>
</tr>
<tr>
<td>3-10</td>
<td>Complementation of virulence of Δ<em>rtxA1::aph</em> by expressing wild-type <em>rtxA1</em> in trans on pGTR1227.</td>
<td>87</td>
</tr>
<tr>
<td>3-11</td>
<td>Histopathology of s.c. lesions of Δ<em>rtxA1</em>-infected mice.</td>
<td>88</td>
</tr>
<tr>
<td>3-12</td>
<td>Detachment/destruction of INT-407 monolayers by <em>rtxA1::Ωaph</em> mutant.</td>
<td>89</td>
</tr>
<tr>
<td>3-13</td>
<td>Virulence of the <em>rtxA1::Ωaph</em> mutant at increasing inocula.</td>
<td>90</td>
</tr>
<tr>
<td>3-14</td>
<td>Apoptosis of J774 cells infected with <em>rtxA1</em> mutants.</td>
<td>91</td>
</tr>
<tr>
<td>3-15</td>
<td>Detachment/destruction of INT-407 monolayers infected with *rtxA1, vvhA, or rtxA1/vvhA mutants.</td>
<td>92</td>
</tr>
<tr>
<td>3-16</td>
<td>Virulence of double <em>rtxA1::aph, ΔvvhA::tet</em> mutant.</td>
<td>93</td>
</tr>
<tr>
<td>3-17</td>
<td>Histopathology of s.c. lesions of mice infected with ΔvvhA and <em>rtxA1::Ωaph</em> mutants.</td>
<td>94</td>
</tr>
<tr>
<td>4-1</td>
<td>Detachment/destruction of INT-407 monolayers infected with <em>rtxA2::Ω</em>.</td>
<td>116</td>
</tr>
<tr>
<td>4-2</td>
<td>Virulence of <em>rtxA2::Ω</em> in mice.</td>
<td>117</td>
</tr>
<tr>
<td>4-3</td>
<td>Cytotoxicity to INT-407 cells caused by the <em>rtxA1::aph, rtxa2::Ω</em> double mutant.</td>
<td>118</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>4-4</td>
<td>Virulence of $rtxA1::aph$, $rtxA2::\Omega$ mutant in mice.</td>
<td>119</td>
</tr>
<tr>
<td>4-5</td>
<td>Lysis of INT-407 cells infected with $\Delta rtxA1::aph$ or $\Delta rtxA1::aph$, $rtxA2::\Omega$ mutants</td>
<td>120</td>
</tr>
<tr>
<td>4-6</td>
<td>Virulence of $\Delta rtxA1::aph$, $rtxA2::\Omega$ mutant in mice.</td>
<td>121</td>
</tr>
<tr>
<td>4-7</td>
<td>Virulence of the $\Delta rtxA3$ mutant in mice.</td>
<td>122</td>
</tr>
<tr>
<td>4-8</td>
<td>Cytotoxicity to INT-407 cells caused by the $\Delta rtxC$ mutant.</td>
<td>123</td>
</tr>
<tr>
<td>4-9</td>
<td>Virulence of $\Delta rtxC1$ mutant in mice.</td>
<td>124</td>
</tr>
<tr>
<td>5-1</td>
<td>Detachment/destruction of cell monolayers by $\Delta vgrG::aph$.</td>
<td>143</td>
</tr>
<tr>
<td>5-2</td>
<td>Apoptosis induced by $\Delta vgrG::aph$.</td>
<td>144</td>
</tr>
<tr>
<td>5-3</td>
<td>Virulence of $\Delta vgrG::aph$ in s.c. inoculated mice.</td>
<td>145</td>
</tr>
<tr>
<td>5-4</td>
<td>Detachment/destruction of cell monolayers by $\Delta hcp::cat$.</td>
<td>146</td>
</tr>
<tr>
<td>5-5</td>
<td>Apoptosis of J774 cells infected with $\Delta hcp::cat$.</td>
<td>147</td>
</tr>
<tr>
<td>5-6</td>
<td>Virulence of $\Delta hcp::cat$ in s.c. inoculated mice.</td>
<td>148</td>
</tr>
<tr>
<td>5-7</td>
<td>Cytotoxicity of $\Delta rtxA1::tetA$, $\Delta vgrG::aph$ double mutant.</td>
<td>149</td>
</tr>
<tr>
<td>5-8</td>
<td>Cytotoxicity of $rtxA1$ and T6SS double mutants to J774 cells.</td>
<td>150</td>
</tr>
<tr>
<td>5-9</td>
<td>Virulence of $rtxA1$, T6SS double mutants in mice.</td>
<td>151</td>
</tr>
<tr>
<td>5-10</td>
<td>Subcutaneous lesions of mice infected with T6SS, $rtxA1$ mutants.</td>
<td>152</td>
</tr>
</tbody>
</table>
Vibrio vulnificus is a gram-negative bacterium capable of causing serious infections after ingestion of contaminated seafood or contact of wounds with contaminated water or objects. The bacteria are highly cytotoxic to host cells and cause extensive tissue damage during infection. The factors involved in this damage remain unknown. The focus of this investigation was to examine the role of toxins and other secreted factors of V. vulnificus in virulence and cytotoxicity. We examined the Repeats in Toxin (RTX) toxins and proteins secreted via the type VI secretion system (T6SS) by constructing mutations in V. vulnificus and evaluating their effects on virulence in mice and cytotoxicity in cell culture models.

V. vulnificus encodes three RTX toxins, RtxA1, RtxA2, and RtxA3. We examined all three toxins and their activation by RtxC. rtxA1 mutants were defective in cytotoxicity to intestinal epithelial INT-407 cells and the ability to induce apoptosis in J774 murine macrophage-like cells. rtxA1 mutants were also attenuated for virulence in subcutaneously inoculated, iron dextran-treated mice; however, they were still able to cause subcutaneous lesions similar to the wild type, suggesting that other virulence...
factors cause these lesions. Additionally, RtxA1 does not require activation by RtxC for virulence or cytotoxicity. Deletion of either \textit{rtxA2} or \textit{rtxA3} had no significant effect on cytotoxicity in cell culture or virulence in mice.

The recently discovered T6SS was examined for its role in cytotoxicity and virulence. The hemolysin coregulated protein (HCP) and Valine-glycine repeat protein (VgrG) are suggested to be secreted effectors and components of the T6SS apparatus. Deletion of each of these genes in \textit{V. vulnificus} had no effect on cytotoxicity in cell culture or virulence in mice, suggesting that the T6SS is not essential in virulence and tissue damage caused by \textit{V. vulnificus}.

These studies evaluated several toxins and secreted factors of \textit{V. vulnificus} for cytotoxicity and virulence. We identified RtxA1 as the major cytotoxic factor; however, other accessory toxins contribute to cytotoxicity. Despite examining several toxins and identifying the major cytotoxic factor, the key factor(s) involved in tissue damage remains elusive.
**Vibrio vulnificus**

*Vibrio vulnificus* is a gram-negative, motile, curved rod-shaped bacterium found commonly in estuarine waters, such as the coastal regions of the Gulf of Mexico. *V. vulnificus* can be free living in the water, as well as in association with filter feeding shellfish, such as oysters and clams (1). It resides in areas with temperate climates and thrives when water temperatures are above 18°C (1, 2). During the warm summer months, nearly all of the oysters harvested from Gulf of Mexico coastal waters are contaminated with *V. vulnificus* (1).

**Disease Caused by *V. vulnificus***

*V. vulnificus* is the leading cause of reported seafood-related deaths in the United States (1). Infection occurs by two routes: ingestion of raw, contaminated seafood and contact of open wounds with contaminated water or objects. Ingestion of contaminated seafood, such as raw oysters, results in primary septicemia, characterized by fever, chills, and the formation of secondary bullous lesions on the lower extremities (3-5). The disease progresses rapidly, with the onset of symptoms as soon as 24 hours after ingestion, and death can occur within 48 hours (1, 2). Contamination of open wounds after contact with *V. vulnificus* results in wound infection. In severe cases, wound infection may progress to necrotizing fasciitis, requiring debridement of the tissue or amputation of the infected limb, and in extreme cases will lead to secondary sepsis and death (2, 6). Individuals with elevated serum iron levels due to conditions such as hemochromatosis and those with other predisposing conditions, such as hepatic disease, are at highest risk for systemic infection due to either ingestion or wound
infection (1, 5, 7). Both septicemia and wound infection are characterized by extremely rapid replication of the bacteria in the host and extensive tissue damage to the skin. The rapid nature of the disease makes treatment difficult, resulting in mortality rates as high as 50% for septicemia and 30% for wound infection (1).

**Classification of *V. vulnificus***

*V. vulnificus* can be classified based on biotypes, lipopolysaccharide (LPS) antigens, and genetic sequences. There are three biotypes of *V. vulnificus*. Biotype 1 is associated with oysters and predominantly causes disease in humans, biotype 2 primarily affects eels and fish, and biotype 3 is an emerging biotype affecting people handling fish in Israel (8). Strains can also be classified by serotyping the LPS O antigen. Biotype 2 strains possess a single serotype; however, there is much more heterogeneity among the biotype 1 strains (9, 10).

More recently, various forms of molecular typing have been used to differentiate strains of *V. vulnificus*. Not all strains of *V. vulnificus* have the same virulence potential, and many researchers are interested in identifying genetic markers or patterns that are able to distinguish those which are more adept at causing disease from those which are less adept. Many of these studies involve molecular typing of clinical isolates from human patients and environmental isolates from water and oysters.

Nilsson, *et al.* (11) examined the small subunit (16S) rRNA of 67 clinical and nonclinical isolates, and determined there are 17-nucleotide differences throughout the sequence. These differences in the 16S rRNA divide *V. vulnificus* strains into two major groups, designated types A and B. The majority of nonclinical isolates (31 of 33) were of the A type, while the majority of the clinical isolates (26 of 34) were type B (11).
Development of a real-time PCR-based assay to determine the 16S rRNA type revealed a third type, designated AB, which correlated with nonclinical isolates (12).

Warner and Oliver (13) used random amplification of polymorphic DNA (RAPD) to differentiate *V. vulnificus* isolates. Using this method, they found extensive heterogeneity among the samples. However, they identified a band that correlated with the clinical *V. vulnificus* isolates and was occasionally present in the environmental isolates. This group subsequently developed a PCR-based assay to identify the presence of this band, designated as “virulence correlated gene” (*vcgC* or *vcgE*), in clinical (C) and environmental (E) type strains, respectively. Using this PCR method, they could distinguish between the C-type and E-type strains, and their classification matched the A and B ribotyping of Nilsson, et al. for strains that overlapped in the two studies (14).

Chatzidaki-Livanis, et al. (15) used an alternative typing method called Repetitive Extragenic Palindromic DNA PCR (rep-PCR) to classify isolates of *V. vulnificus*. Rep-PCR is a genetic typing method that targets conserved repetitive elements at multiple loci throughout the genome, also distinguished between strains of clinical versus environmental origin (15). As opposed to the other typing methods, which classified *V. vulnificus* into two major groups, this method showed greater genetic diversity among the strains of clinical origin. 68 strains were divided into seven groups based on patterns of PCR amplicons. Groups I, IV, and VII corresponded to clinical isolates, and group III corresponded to environmental strains. These rep-PCR groups matched the A and B ribotyping of Nilsson, *et al.* and the E and C RAPD results of Warner and Oliver. Chatzidaki-Livanis, *et al.* also examined polymorphisms in group 1.
capsule polysaccharide (CPS) gene sequences by PCR and identified 2 allele groups: CPS allele 1 corresponded to clinical isolates that classified as mostly rep-PCR groups I, IV, and VII and CPS allele 2 corresponded to environmental isolates in rep-PCR group III (15).

Multi Locus Sequence Typing (MLST) examining DNA sequence polymorphisms of housekeeping genes has also been used to examine genetic relationships of V. vulnificus strains. Bisharat, et al. (16,17) used MLST of a set of housekeeping genes to type 159 V. vulnificus strains and determined that V. vulnificus strains could be placed into two clusters. Cluster 1 was comprised mainly of environmental isolates, and cluster 2 was comprised primarily of clinical isolates. Cohen, et al. (18) subsequently examined 6 genes to type 67 V. vulnificus strains by MLST. This study developed different designations opposite that of Bisharat and coworkers. Almost all lineage 1 strains were of clinical origin, while lineage 2 comprised most environmental isolates. Additionally, Cohen, et al. identified a genomic island that was present in most lineage 1 strains, but was absent from most lineage 2 strains examined.

The different typing methods have successfully related genotype to source of isolation of a strain, implying that strains associated with clinical isolation would be of increased virulence. V. vulnificus strains fall primarily into two clades based on the different typing methods. Clade 1 is comprised of strains primarily isolated from the environment that are ribotypes A and AB, vcgE, MLST group 1, and lineage 2. Clade 2 consists of strains primarily isolated from clinical samples that are ribotype B, vcgC, MLST group 2, and lineage 1. Recently, our laboratory performed a detailed analysis of the virulence of 71 strains from clinical and environmental sources in the
subcutaneously (s.c.) inoculated, iron dextran-treated mouse model of infection to
determine the relationship of genotype to virulence (Thiaville, et al., in preparation).
Almost all of the strains caused severe skin infection. However, only a subset of strains
had potential to cause systemic infection and death. This higher virulence potential
significantly correlated with the genotypes classified into clade 2 strains (vcgC; B type
16S rRNA; MLST type 2; lineage 1), although there were several highly virulent clade 1
strains, as well as attenuated clade 2 strains. From these studies, it is evident that the
clade 2 V. vulnificus strains are more adept at causing systemic infection and death in
our mouse model than the clade 1 strains, although there are exceptions for in both
directions.

**Virulence Factors of V. vulnificus**

The major hallmarks of V. vulnificus disease are the rapid growth of the organism,
extensive tissue damage, and resistance to host innate defenses. Although studies in
pathogenesis of V. vulnificus have revealed factors necessary for virulence, the means
by which V. vulnificus causes such a rapid and destructive infection remain uncertain (2,
6, 19). Several virulence factors have been proposed, including a polysaccharide
capsule, mechanisms for iron acquisition, LPS, flagella, pili, hemolysin/cytolysin,
metalloprotease and several exoenzymes (19).

**The Polysaccharide Capsule**

The polysaccharide capsule is the most important virulence factor identified to
date. The presence of the capsule relates to colony morphology, as encapsulated
bacteria form opaque colonies and unencapsulated bacteria form translucent colonies
(20, 21). All virulent strains of V. vulnificus are encapsulated, and unencapsulated
isolates occurring naturally in the environment are attenuated in mouse models of
infection (20, 22, 23). Wright, et al. (22) demonstrated the importance of the capsule by examining translucent, acapsular transposon mutants of a virulent, encapsulated strain of *V. vulnificus*. These acapsular mutants were more sensitive to normal human sera than the encapsulated parent strain, and they were highly attenuated in intraperitoneal (i.p.) inoculated mice (22). *V. vulnificus* is an extracellular pathogen, so to cause a successful infection, it must have the ability to resist phagocytic activities of host defense cells and complement-mediated lysis. The capsule appears to be the main mechanism for the resistance to these host defenses, demonstrated by the serum sensitivity and the sensitivity to phagocytosis by translucent, acapsular isolates (22, 24, 25).

**Acquisition of Iron from the Host**

The importance of iron for growth of microorganisms has long been recognized (26). Free iron is limited in the host environment due to sequestration by iron-binding proteins, such as transferrin, lactoferrin, and hemoglobin. Pathogens have developed various mechanisms for sensing low iron levels and acquiring iron from the host during infection, including the production of low molecular weight siderophores for binding iron and receptors for host iron-containing proteins.

Iron overload in the host is one of the most important susceptibility factors for *V. vulnificus* disease, suggesting that the ability to sequester iron from the host is critical for virulence of the organism. Wright, et al. (27) experimentally demonstrated the importance of excess iron for pathogenesis of *V. vulnificus* infection. Iron treatment of mice reduced the i.p. LD$_{50}$ from $10^6$ CFU to 1 CFU. Starks, et al. (28) emphasized the importance of iron in the host by demonstrating that during subcutaneous (s.c.) inoculation of virulent strains, the inocula must be increased by $10^5$-fold in non iron
dextran-treated mice to achieve similar levels of bacteria in tissues as in iron-treated mice. Additionally, the mechanisms of iron sensing and iron acquisition have proven to be important virulence factors of *V. vulnificus*. *V. vulnificus* produces a catechol-type siderophore, vulnibactin, which enables *V. vulnificus* to utilize iron from transferrin, a host iron-binding protein (29). Litwin, *et al.* (29) characterized a mutant that was unable to utilize transferrin-bound iron and found it was defective in production of the catechol-type siderophore. This mutant was attenuated for virulence in an infant mouse model of infection.

Different mechanisms for the enhanced virulence of *V. vulnificus* in iron-overloaded hosts have been proposed. Elevated iron levels are speculated to increase host susceptibility by inhibiting certain innate immune defenses. For example, excess iron in the host has been demonstrated to have an inhibitory effect on phagocyte function (30). Hor, *et al.* (31) demonstrated that the activity of neutrophils during infection with *V. vulnificus* was decreased in iron-treated mice compared to non-iron-treated mice. Excess iron also contributes to increased virulence of *V. vulnificus* by increasing the growth rate of the bacteria. In the same study as above, Hor, *et al.* (31) also demonstrated that iron-treatment results in increased growth of *V. vulnificus* during infection. Using a marker plasmid system, Starks, *et al.* (32) demonstrated that treating s.c. inoculated mice with iron dextran significantly decreased the replication time of the *V. vulnificus* during infection. While iron contributes to both host susceptibility and virulence of *V. vulnificus*, it appears that the main role of iron scavenging by *V. vulnificus* is to facilitate growth of the bacteria in the host.
Flagella

*V. vulnificus* possesses a single polar flagellum and is motile. Kim and Rhee (33) investigated an insertion mutation of the *flgC* gene, encoding a flagellar basal body rod protein, of a *vvhA/vvpE* mutant strain. Their results revealed a defect in motility, a decrease in adherence and cytotoxicity to HeLa cells, and attenuation in mice (33). Lee, *et al.* (34) examined a flagellum-deficient mutant by a knockout of the *flgE* gene. This mutant had an increase in the LD$_{50}$ over the wild type in s.c. inoculated, iron dextran-treated mice. Similar to the *flgC* mutants, *flgE* mutants also showed a severe defect in adherence to epithelial cells (34).

Our laboratory has investigated the role of flagella in virulence in mice. Signature-tagged mutagenesis screens identified an attenuated mutant containing an insertion in the *fliP* gene involved in flagellar biosynthesis. This mutant was nonmotile and unable to cause systemic infection in the mice; however, it maintained the ability to cause severe skin infection. Further investigation of the flagellin gene clusters, *flaCDE* and *flaFBA*, revealed that the *flaCDE* flagellin genes are necessary for motility and for systemic disease in our s.c. inoculated, iron dextran-treated mouse model of infection, but that the *flaFBA* genes are dispensable for motility and virulence (Tucker, *et al.*, in preparation).

Pili and Attachment

To initiate infection at host surfaces, bacteria must be able to attach and colonize these surfaces. Attachment is mediated by production of adhesions, including pili. Gander and LaRocco (35) identified the presence of pili on the surface of *V. vulnificus* strains, particularly on clinical isolates. The authors determined the number of adherent
bacteria on HEp-2 cells and concluded that clinical strains were more adherent than were environmental strains (35).

Paranjpye, et al. (36) examined a pilD mutation that abolished expression of surface pili and resulted in a 100-fold increase in LD50 in an iron-treated, i.p. inoculated mouse model of infection. This study revealed a potential role of pili in virulence. The pilD gene encodes the type IV pilus leader peptidase/N-methyltransferase that is involved in pilus formation, as well as processing of other type 2 secretion system-secreted proteins. As expected, the pilD mutation had other pleiotropic effects, including defective secretion of the hemolysin/cytolysin, protease, and chitinase, so the defect in virulence could not be definitively attributed to the absence of pili. To examine pili further, Paranjpye and Strom (37) examined a pilA mutant to assess the effect on adherence and virulence. Although pili were still present on the surface, the pilA mutant was defective in adhesion to HEp-2 cells and had a 10-fold increase LD50 in i.p. inoculated, iron-treated mice (37).

**Extracellular Toxins**

*V. vulnificus* produces and secretes many extracellular proteins and toxins. It is hypothesized that some of these secreted proteins contribute to the significant tissue damage observed during infection. The most well studied extracellular proteins have been the metalloprotease and the hemolysin/cytolysin; however, their roles in pathogenesis have remained questionable. Other putative hemolysins have been suggested, such as the Hemolysin III (HlyIII) (38) and a homolog of Legiolysin (VIIY) (39). Phospholipase activity has also been implicated in virulence, although no isogenic mutant has been studied to date (40,41). Finally, the three RTX toxins, examined during this investigation, have been speculated to play a role in virulence. As discussed
later, only the RtxA1 toxin has been confirmed to be a cytotoxic factor with a role in virulence.

**Hemolysin/cytolysin**

Kreger and Lockwood (42) first identified the hemolytic and cytotoxic ability of *V. vulnificus*. The hemolysin/cytolysin protein, VvhA, was later purified and shown to have hemolytic activity (43). Injection of purified VvhA into animals produced skin damage very similar to that seen after infection with bacteria (44), suggesting that VvhA could be responsible for the damage caused by the bacteria during infection. Subsequent studies using purified toxin preparations have dissected various mechanisms of cytotoxicity, such as apoptosis and pore-formation (45, 46). Despite the evidence that the purified VvhA hemolysin causes tissue damage, Wright and Morris (47) demonstrated that an isogenic mutant strain remained virulent in mouse models of infection. These results cast doubt on the role of VvhA in virulence and tissue damage during infection.

**Other hemolysins**

In addition to VvhA, *V. vulnificus* produces other proteins with putative hemolytic activities. Chen, *et al.* (38) identified a gene with similarity to the hemolysin III (*hlyIII*) of *Bacillus cereus*, and it is highly similar to other putative hemolysin, including ones in *V. cholerae*, *Yersinia pestis*, and *Salmonella enterica*. *E. coli* expressing the *V. vulnificus* HlyIII from a plasmid was hemolytic towards human erythrocytes. However, a *V. vulnificus hlyIII* mutant was still hemolytic on sheep blood agar plates, indicating that the presence of other hemolysins compensate for the loss of *hlyIII* or that *V. vulnificus* HlyIII is not hemolytic to sheep erythrocytes. While it is not certain if HlyIII is a true hemolysin of *V. vulnificus*, it does have a role in virulence. The *hlyIII* mutant was
attenuated by 16-fold in i.p. inoculated, non iron-treated mice (38). We have also examined a *V. vulnificus hlyIII* mutant and observed approximately a 3-fold attenuation in s.c. inoculated, iron dextran-treated mice (unpublished data).

Another, more ambiguous hemolysin is the VllY protein. Chang, *et al.* (39) identified a clone from a *V. vulnificus* genomic library that confers hemolysis and pigment production to transformed *E. coli*. The gene identified had similarity to the *lly* gene encoding the legiolysin of *Legionella pneumophila*. The VllY and Lly are also related to the family of 4-hydroxyphenylpyruvate dioxygenase (HppD) proteins involved in the catabolism of tyrosine. The exact function of HppD in bacteria is uncertain, although it is involved in the production of pyomelanin, which has been implicated in stress survival and colonization for other bacteria (48). During our studies into the role of toxins in *V. vulnificus*, we deleted *vllY* and determined that it was not essential for hemolysis on blood agar plates or cytotoxicity in cell culture. The Δ*vllY* mutant did have a slight attenuation in virulence in s.c. inoculated, iron dextran-treated mice; however, the exact function during infection remains unknown (unpublished data).

**Metalloprotease**

The zinc-dependent metalloprotease produced by *V. vulnificus*, VvpE, degrades elastin and collagen(49,50). As with VvhA hemolysin, injection of purified VvpE into mice caused dermal necrosis, similar to what is seen during infection (49). Two groups constructed *vvpE* mutations and observed no attenuation of the mutants in i.p. or s.c. inoculated mice (51,52). Our laboratory showed that the *vvpE* mutation had no effect on skin damage or liver (systemic) infection in s.c. inoculated mice (52), indicating that VvpE is not likely involved in virulence or tissue damage. These results were similar to
what was seen with VvhA, emphasizing the importance of studying isogenic mutants to evaluate virulence, as opposed to studying the effects of a purified protein.

It remained a possibility that the hemolysin/cytolysin and metalloprotease may be redundant virulence factors, so that mutation of one of them could be compensated for by the other. Fan, et al. (53) reported that a strain with constructed mutations in both of these genes retains some cytotoxicity in cell culture and is virulent in mice. This suggests that there are other cytotoxins being produced that are contributing to tissue damage.

**Phospholipases**

The phospholipase activity of *V. vulnificus* also has been proposed to have a role in virulence. Nearly 25 years ago, Testa, et al. (40) demonstrated that *V. vulnificus* possesses phospholipase A1/A2 and lysophospholipase activities, but not phospholipase C activity. In 2007, Koo, et al. (41) proposed that this phospholipase A (PLA) activity was important for virulence in a mouse model. The researchers inhibited phospholipase activity during infection of mice by treatment with tetracycline and determined that the attenuation that they observed was due to the inhibition of PLA activity (41). There were many gaps in the study by Koo, et al. (41), including the lack of construction and analysis of a mutant deficient in PLA activity.

We followed up on the report by Koo, et al., to determine if PLA activity of *V. vulnificus* was important for virulence. We deleted two genes encoding phospholipase/lecithinase/hemolysin (*tlh*) and an outer membrane phospholipase A (*ompla*). Both mutants were hemolytic on rabbit or sheep blood agar plates and had phospholipase activity on egg yolk agar plates. Both mutants were also as virulent as the wild type in s.c. inoculated, iron-treated mice (unpublished data). Without a PLA
deficient mutant, the claims made by Koo, et al. cannot be completely disregarded. Phospholipase activity may still contribute the virulence of *V. vulnificus*.

**Focus of Investigation**

The goal of this investigation was to examine the role of toxins and extracellular proteins of *V. vulnificus* in pathogenesis. Despite years of research, the factors causing the extensive tissue damage during infection remain unknown. We speculated that secreted toxins are causing destruction of host cells, leading to the tissue damage observed during infection. We followed the molecular version of Koch's postulates (54, 55) to evaluate a role in virulence for the three RTX toxins (RtxA1, RtxA2, and RtxA3) and the type VI secretion system (T6SS). We analyzed these mutants for cytotoxicity in cell culture and virulence in the s.c. inoculated, iron dextran treated mouse model (28). This model allows us to examine both the ability of *V. vulnificus* to cause a local, skin infection and the ability to cause systemic infection and death.

**Specific Aim 1: Examine Role of the RtxA1 Toxin in Pathogenesis of *Vibrio vulnificus***

RtxA1 of *V. vulnificus* belongs to the Multifunctional Autoprocessing RTX (MARTX) family of RTX toxins (56). It is a very large protein (>500-kDa), consisting of conserved amino acid repeat regions N-terminus and C-terminus, flanking less conserved central domains with enzymatic activities. RTX toxins are cytotoxic in cell culture by a variety of mechanisms, and many of them contribute to the virulence. Previous studies in our laboratory identified the RtxA1 as a major cytotoxic factor of *V. vulnificus* strain MO6-24/O. These studies also showed it had a minor role in virulence. During this investigation, we analyzed the role of the RtxA1 in *V. vulnificus* strain CMCP6. We constructed mutations in the *rtxA1* gene and analyzed the mutant strains for cytotoxicity...
in cell culture and virulence in our mouse model of infection. We concluded that RtxA1 is the major cytotoxic factor of *V. vulnificus* CMCP6 and that it has a role in virulence in the mouse model.

It is interesting that, although RtxA1 is the major cytotoxic factor of *V. vulnificus*, *rtxA1* mutants are still able to cause significant amounts of gross tissue damage, as well as systemic infection and death in the mouse model. RtxA1 may contribute to tissue damage, but it is clear that there are other factors involved, possibly VvhA hemolysin/cytolysin. We examined strain with mutations in the *rtxA1* gene and the *vvhA* gene. Deletion of *vvhA* in the *rtxA1* mutant background eliminated residual cytotoxicity; however, the double mutant was as virulent as the *rtxA1* mutant. The factor causing tissue damage remains unknown.

**Specific Aim 2: Examine the Other RTX Loci in Virulence of *V. vulnificus* and the Importance of Activation of the RtxA Toxins**

In addition to RtxA1, CMCP6 encodes two other large, putative RTX toxins, RtxA2 and RtxA3. The residual cytotoxicity and virulence phenotypes of *rtxA1* mutants hinted that there could be an interesting story with the RTX toxins and pathogenesis. We speculated the RTX toxins could act in concert and that mutation of any one of them could affect cytotoxicity or virulence. To determine if these other RTX proteins contributed to tissue damage and virulence, we constructed mutations in the *rtxA2* and *rtxA3* genes. Interestingly, the *rtxA2* and *rtxA3* genes proved to be nonessential for cytotoxicity in cell culture and for virulence in mice. A double *rtxA1/rtxA2* mutant was examined for virulence in mice, and, despite a reduction in systemic infection, it was still able to cause skin infection and tissue damage in mice.
RTX toxins typically require activation by an acyltransferase, RtxC. CMCP6 only encodes one RtxC in its genome. The gene rtxC1 is located directly upstream of rtxA1. We investigated the requirement of RtxC1 for activation of the RtxA toxins by deleting the rtxC1 gene and examining the mutant strain for cytotoxicity and virulence. We expected the phenotype of an rtxC1 mutant to be similar to the rtxA1 mutant examined in Specific Aim 1. Interestingly, the rtxC1 mutant was cytotoxic in cell culture, and virulent in the mouse model. We concluded that activation by RtxC1 is not required for RtxA activity. While this differs from what is known about typical RTX toxins, it must be remembered that RtxA1 belongs to the MARTX family of toxins, and the requirement for activation of this subfamily of RTX toxins has not yet been shown.

Specific Aim 3: Examine the Type VI Secretion System of V. vulnificus

We identified the presence of a putative type VI secretion system (T6SS) in V. vulnificus by BLAST searches with known T6SS genes. The T6SS is newly discovered, but it has already been implicated to be involved in virulence of several gram-negative pathogens, including Vibrio cholerae. We examined T6SS by deleting the genes encoding Hcp and VgrG. These two proteins are suggested to be necessary for secretion, as well as being secreted substrates themselves (57). Deletion of these two genes had no effect on cytotoxicity in cell culture and no effect on virulence in mice, indicating that T6SS is not important for virulence.

Because all of the mutants that we constructed and tested, including combinations of mutations, retained significant virulence and pathology in infected skin tissues, we speculate that an unidentified toxin or combination of toxins and secreted factors, including those studied here, contribute to damage. If the latter is true, then it is
possible that deletion of just one or two toxins at time will not have an effect on virulence, because any of them could be sufficient for virulence.
CHAPTER 2
MATERIALS AND METHODS

Standard Microbiological and Animal Infection Protocols

Bacterial Cultures, Chemicals, and Media

Bacterial strains used in this study are listed in Table 2-1. The virulent clinical strain of V. vulnificus, CMCP6, was used as the background strain for all mutations discussed. Some mutations were originally constructed in FLA399, a spontaneous rifampicin-resistant mutant of CMCP6; however, we halted the use of mutants in this background due to concerns over the virulence of FLA399. E. coli Top10 (Invitrogen) and E. coli EC100D (Epicentre) were used for routine cloning. E. coli S17-1λpir (58) was used as the donor strain for plasmid conjugations with V. vulnificus.

V. vulnificus and E. coli strains were grown in Luria-Bertani broth containing 0.85% (w/v) NaCl (LB-N) or LB-N plates containing 1.5% (w/v) agar. Strains were stored at -80°C in LB-N with 35% (v/v) glycerol. Strains were grown on LB-N plates containing 6% (w/v) sucrose for counterselection for loss of suicide plasmids. When required, antibiotics were included in the media at the following concentrations for E. coli: ampicillin (100 µg/mL), tetracycline (12.5 µg/mL), chloramphenicol (30 µg/mL), kanamycin (40 µg/mL). Antibiotics were used at the following concentrations for V. vulnificus: ampicillin (10 µg/mL), tetracycline (6.25 µg/mL), chloramphenicol (5 µg/mL), kanamycin (100 µg/mL), rifampicin (50 µg/ml), and colistin (10² U/mL). To select for V. vulnificus and against donor E. coli during filter-mating conjugations, VVM agar (59) or LB-N agar containing 10² U/mL colistin and appropriate antibiotics was used.
Unless noted otherwise, components for media were from Difco (Franklin Lakes, NJ), chemicals were from Sigma (St. Louis, MO), DNA extraction and purification kits were from Qiagen (Valencia, CA), molecular genetics enzymes were from New England Biolabs (Ipswich, MA), and oligonucleotides were from IDT (Coralville, IA).

**Infection of Mice**

For mouse infections, a static overnight culture of the bacteria was grown in LB-N at room temperature. Prior to infection, the starter cultures were diluted 1:20 into LB-N and shaken at 37°C until the bacteria reached exponential growth phase, when the optical density at 600 nm (OD$_{600}$) reached 0.4 to 0.6. The cultures were diluted in phosphate-buffered saline (PBS) to an appropriate concentration for infection. CFU/ml was determined by diluting and plating.

All mouse infections used the s.c. inoculation model described by Starks, *et al.* (28). Seven to ten-week-old female ICR mice (Harlan Sprague-Dawley, Indianapolis, Ind.) housed under specific pathogen-free conditions were used for all experiments. At least 1 hr prior to inoculation, mice were injected i.p. with 250 μg iron dextran (Sigma, St. Louis, MO) per gram body weight. Mice were injected s.c. into the right lower flank with bacteria suspended in 0.1 mL PBS. When the mice became moribund, as indicated by a rectal temperature below 33°C, they were euthanized by carbon dioxide asphyxiation. If the mice did not become moribund, they were euthanized at 20 hr postinoculation.

After the mice were euthanized, their skin was peeled back to reveal the s.c. lesion at the injection site. For quantitative analysis of the bacteria in the tissues, samples of the s.c. lesion and the liver were aseptically removed from mice, homogenized in 5 mL of PBS using glass tissue homogenizers, diluted, and plated on LB N agar. Strains
carrying plasmids were plated both nonselectively (LB-N agar) and selectively (LB-N agar containing appropriate antibiotics). Samples were not taken from mice with no visible lesion. When no CFU were recovered from a skin lesion or liver sample, a minimum detectable CFU/g was used for these mice for statistical analysis.

**Histological Analysis**

Samples of the subcutaneous lesions resulting at inoculation sites were collected immediately after sacrifice of mice and fixed by immersion in 10% (v/v) buffered formalin (60). Formalin-fixed tissues were embedded in paraffin and cut into 5 μm sections at the University of Florida Department of Pathology, Immunology, and Laboratory Medicine Diagnostic Referral Laboratory. Histological sections were stained with hematoxylin-eosin.

**Tissue Culture**

Two cell lines were used in this study, the human intestinal epithelial cell line, INT-407, and the murine macrophage-like cell line, J774, both obtained from American Type Culture Collection (ATCC, Manassas, Virginia). All tissue culture was maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% (w/v) fetal bovine serum (FBS) and an antibiotic-antimycotic mix with a final concentration of 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 ng/ml Amphotericin B. All tissue culture media components were supplied by Invitrogen (Carlsbad, California). All tissue cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂.

**Infection of monolayers**

For measurement of monolayer destruction/detachment and cell lysis, INT-407 cells were washed with Hank’s balanced salt solution (HBSS), suspended in DMEM with FBS and antibiotics at a concentration of 1x10⁵ cells/mL, and seeded in 24-well
tissue culture plates (Corning, Cambridge, Massachusetts). The plates were incubated for 2 days until the monolayers reached 80-90% confluency. Two hours prior to infection, antibiotic-containing medium was removed from each well and replaced with 1 ml antibiotic-free DMEM with FBS. All *V. vulnificus* strains, grown to logarithmic phase as detailed above, were pelleted, suspended in 3 ml antibiotic-free DMEM with FBS and diluted to $2 \times 10^7$ CFU/ml. 0.5 ml of appropriate bacterial suspension was added to each well, in triplicate, for a multiplicity of infection (MOI) of 10. After 1 hour of incubation at 37°C, 100 μg/ml of gentamicin was added to each well to kill the bacteria. Following antibiotic treatment, plates were incubated at 37°C up to 24 hours. It should be noted that cell cultures were not washed after addition of gentamicin, so that any extracellular products and toxins produced by the bacteria during the initial 1 hour infection period would remain in the cell culture. 24 h later, infected cell cultures were assayed for destruction or detachment of INT-407 monolayers or lysis of INT-407 cells.

For measurement of apoptosis, J774 cells were washed as above, diluted to a concentration of $1 \times 10^5$ cells/ml, and 0.1 ml of the J774 cells were added to each well of a black Nunc F96 MicroWell™ plate (Nunc). The plates were incubated for 2 days, to allow the monolayers to reach 80-90% confluency. Two hours prior to infection, antibiotic-containing medium was removed from each well and replaced with 0.1 ml antibiotic-free medium. All *V. vulnificus* strains, grown to logarithmic phase as detailed above, were pelleted, suspended in 5 ml antibiotic-free DMEM with FBS, and diluted to $2 \times 10^7$ CFU/ml. 0.1 ml ($2 \times 10^6$ CFU) of appropriate bacterial suspension was added in triplicate to the 96-well plates. After 1 hour of incubation at 37°C, extracellular bacteria were killed by addition of gentamicin to each well at a final concentration of 100 μg/ml.
Following antibiotic treatment, plates were incubated at 37°C for 3 hours, at which time infected cell cultures were assayed for apoptosis.

**Crystal violet assay for detachment and destruction of monolayers**

A crystal violet staining assay, adapted from Ruff and Gifford (61), was used to assess destruction and detachment of infected INT-407 monolayers. 24 hr after infection, supernatants were aspirated, and wells were washed two times with 1 ml PBS to remove dead or damaged cells. The cells remaining attached to the culture well were stained with 1 ml of 0.05% (w/v) crystal violet diluted in PBS and incubated at room temperature for 10 min. The wells were washed four times with 1 ml PBS, and 1 ml 95% ethanol was added to solubilize the remaining crystal violet. Uninfected INT-407 cells and blank wells containing media only were used as positive and negative controls, respectively, for presence of epithelial cells. 150 µl of the crystal violet/ethanol solution from each well was transferred to a 96-well plate, and absorbance was measured at 490 nm (A_{490}) using an ELx800uv microplate reader (Bio-Tek Instruments, Inc.). Percent monolayer detachment was calculated as follows:

1. Raw A_{490} (from each infected and uninfected well) - A_{490} media only (average of all three wells) = Normalized A_{490}

2. Normalized A_{490} (for each infected well) / Normalized A_{490} of uninfected cells (average of all three wells) = ratio of attached infected cells to attached uninfected cells

3. 100 - (ratio x 100) = % detachment/destruction (cells detached from monolayer)

**Lactate dehydrogenase assay for measuring lysis of INT-407 Cells**

The Cytotoxicity Detection Kit (Roche Applied Science, Indianapolis, IN) was used to detect lysis of the infected INT-407 cells. This is a colorimetric assay that measures lactate dehydrogenase (LDH), a cytoplasmic protein released by eukaryotic cells as a
result of lysis. LDH interacts with tetrazolium salt (yellow) in the LDH assay reagent supplied in the kit, resulting in the formation of formazen salt (red). The color formed, measured by absorbance at 490 nm, is proportional to the number of lysed cells. 24 hr after infection of INT-407 monolayers, 150 μl of supernatant from each well was removed and transferred to a microcentrifuge tube. The supernatant was centrifuged at 200 x g at 4°C for 10 minutes to remove cells and debris, and 25 μl was transferred to a 96-well plate. This represented the released LDH. 75 μl PBS was added to each well, bringing the volume to 100 μl. To measure total LDH activity of either infected or uninfected cells, TritonX-100 (Tx) was added to each well for a final concentration of 1% and was mixed vigorously to induce lysis of the remaining monolayer. 150 μl was removed from each well, centrifuged, and 25 μl was transferred to a 96-well plate and diluted with PBS. 100 μl LDH assay reagent was added to each well of the 96-well plate, according to the manufacturer’s instructions, and the plate was incubated in the dark at room temperature for 8 minutes. The reaction was stopped with 50 μl 1M HCl, and the amount of LDH released was measured using an ELx800uv microplate reader (Bio-Tek Instruments, Inc.). Percent lysis was calculated as follows:

1. Raw A_{490} (from each infected and uninfected well, both TritonX treated and untreated) – A_{490} media only (average of all three wells) = Normalized A_{490}

2. \[ \frac{100 \times (\text{Normalized A}_{490} \text{ of infected well (no Tx treatment)} \times 0.525 \text{ mL})}{((\text{Normalized A}_{490} \text{ (no Tx treatment)} \times 0.15 \text{ mL}) + (\text{Normalized A}_{490} \text{ of maximum release of infected well (with Tx treatment)} \times 0.425 \text{ mL})} = \% \text{lisis} \]

For each well, we computed the LDH activity in the supernatant divided by the total LDH activity in the well. The percent LDH release by uninfected cells, approximately 15%, was subtracted from the percent LDH release in V. vulnificus-
infected wells. Triplicate wells were run for each sample, and each experiment was performed at least twice.

**Apoptosis assay**

The Apo-One Homogeneous Caspase-3/7 Assay (Promega, Madison, Wisconsin) was used to measure the ability of *V. vulnificus* strains to cause apoptosis to J774 cells. This assay measures the activities of caspase-3 and caspase-7, members of the cysteine aspartic acid-specific protease family which are key effectors in the process of apoptosis in mammalian cells. Measurements of these caspases are used as indicators of apoptosis in cell culture models. The Apo-One caspase-3/7 substrate rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R110), exists as a profluorescent substrate prior to the assay. Upon sequential cleavage and removal of the DEVD peptides by caspase-3/7 activity, the rhodamine 110 leaving group becomes intensely fluorescent allowing apoptosis to be quantified.

J774 cells were infected with *V. vulnificus* as described above and incubated for three hours at 37°C post addition of gentamicin. Following incubation, 100 µl of the Homogeneous Caspase-3/7 Reagent was added to each well and then incubated shaking gently at room temperature in the dark for 30 minutes. Fluorescence of each well was measured in the FLx800 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc., Winooski, Vermont) at an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm. The apoptotic agent gliotoxin was used as a positive apoptotic control, wells containing media only were used as negative controls, and uninfected J774 cells were used to establish a background level of apoptosis. The following formula was used to calculate apoptosis:

\[
\text{Apoptosis} = \text{F} - \text{B}
\]
1. Raw wavelength reading (from each infected and uninfected well) - Wavelength reading of media only (average of all three wells) = Normalized wavelength reading

2. For % apoptosis: \[100 \times \frac{\text{Normalized wavelength reading (from each infected well)}}{\text{Normalized wavelength reading (from gliotoxin treated wells, average of three wells)}}\] = % apoptosis

3. For apoptosis over background: \[
\frac{\text{Normalized wavelength reading (from each infected well)}}{\text{Normalized wavelength reading (from uninfected wells, average of three wells)}}\] = ratio of apoptosis over cell background

Means and standard deviations of the percent apoptosis or wavelength ratios obtained for each set of triplicate wells were calculated for each strain.

**Statistical Analysis**

The Student’s t-test was used to examine for significant differences between means of two sample groups. For experiments with more than two sample groups, an ANOVA was performed to determine if a significant difference was present in the group \(P \leq 0.05\). If a significant difference was detected, a Fisher’s Least Significant Difference (LSD) was used for pairwise comparisons. \(\chi^2\) tests were used in mouse experiments to determine if the number of mice with detectable CFU was significantly changed in mutant versus wild-type infections. Statistical analyses were performed using Excel and XLSTAT. The statistical test used is delineated in the text for each experiment. Values were considered statistically significant for \(P \leq 0.05\)

**Molecular Genetics and Mutagenesis**

**Southern Blots**

**Extraction and digestion of genomic DNA**

For Southern blot analysis, genomic DNA was extracted from a selection of clinical and environmental strains representing different genotypes with varying cytotoxicities and virulence (Thiaville, et al., in preparation). Genomic DNA was isolated from each
strain using the Qiagen DNeasy Blood and Tissue Kit. The genomic DNA was digested with the restriction endonuclease *Eco*RI for 3 hr at 37°C. 3 μg of the digested DNA was resolved in a 0.8% (w/v) agarose gel. The digoxigenin-labeled DNA Molecular Weight Marker II (Roche Applied Science, Indianapolis, IN) was used as a size standard. The gel was stained for 10 min with 0.5 μg/mL of ethidium bromide and photographed.

**Preparation of digoxigenin labeled probes for Southern blot analysis**

Four probes (rtxA1-A through rtxA1–D) spanning the *rtxA*1 gene of CMCP6 were used for detection of *rtxA* genes in multiple strains of *V. vulnificus*. Each probe (approximately 1.5 kb in length) was PCR amplified. The PCR amplicons were labeled with digoxigenin by random primed labeling using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Mannheim, Germany). Each labeled probe was purified using the QIAquick PCR Purification Kit (Qiagen) to remove excess contaminating proteins prior to use. Efficiency of probe labeling was detected by spotting serial dilutions of labeled probe onto a positively-charged nylon membrane and proceeding with the detection protocol described in the kit technical manual.

**Transfer of DNA**

After the agarose gel was stained with ethidium bromide and photographed, the DNA was depurinated by incubating the gel in 250 mM HCl with shaking using an orbital shaker at room temperature for 15 minutes. The gel was rinsed briefly two times with filtered ddH₂O. The DNA in the gel was then denatured in denaturing solution (0.5 M NaOH; 1.5 M NaCl) for 15 min shaking at room temperature. After incubation in the denaturing solution, the gel was briefly rinsed with ddH₂O two times. Next, the gel was incubated in a neutralization solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 15 min shaking at room temperature. The gel was briefly washed two times with ddH₂O.
gel was equilibrated for 10 min in 20X SSC (3M NaCl; 0.3M sodium citrate; pH 7.0) prior to transfer of the DNA to a positively charged nylon membrane (Roche). The transfer of the DNA from the gel to the positively-charged nylon membrane was accomplished by overnight capillary transfer. After overnight transfer, the DNA was fixed to the membrane by UV crosslinking using a GS Gene Linker UV Chamber (Bio-Rad, Hercules, CA).

**Hybridization and detection of the labeled DNA probe**

The membrane was prehybridized with 10 ml prewarmed DIG Easy Hyb (Roche) in a hybridization bag at 42°C for 30 min with gentle rocking. 350 ng of DIG-labeled probe was denatured by boiling and added to 5 ml prewarmed DIG Easy Hyb. The prehybridization solution was discarded, the hybridization solution containing the probe was added to the membrane, and the bag was sealed. The hybridization proceeded overnight at 42°C with gentle rocking. Hybridization solutions containing DIG-labeled probes were saved, stored at -20°C, and reused. To denature probes stored in DIG Easy Hyb, the hybridization solution was heated at 68°C for 10 min.

Chemiluminescent detection with CSPD was used to visualize the hybridized probe. The DIG Wash and Block Buffer Set (Roche) was used in the detection process. Detection of the hybridized probe was carried out as described in the Roche kit technical manual.

**Mutagenesis of *V. vulnificus***

**USER-friendly cloning**

USER (Uracil-Specific-Excision Reagent)-Friendly cloning (New England Biolabs, Ipswich, MA) is a method for easily capturing PCR products into a USER-compatible vector. Target DNA was PCR-amplified using oligonucleotide primers designed with
eight additional nucleotides, either GGAGACAU or GGGAAAGU, complementary to overhangs on the linearized USER vector. PCR products were treated with the USER enzyme, a mixture of uracil DNA glycosylase, which excises the uracil residue leaving an abasic site, and the DNA glycosylase-lyase Endo VIII, which breaks the phosphodiester backbone flanking the abasic site, releasing the terminal seven nucleotides. The PCR product, flanked by the 8 nucleotides, was ligated with the USER vector which had been digested with the nicking enzyme Nt.BbvCI and the restriction enzyme XbaI, to create 8-nucleotide overhangs complementary to the 8-nucleotide 3’ extensions on each end of the PCR product, allowing for easy capture of the product.

Our laboratory has constructed USER-friendly versions of cloning and allelic exchange vectors commonly used in V. vulnificus, described in detail in Gulig, et al. (62). An allelic exchange vector, pCVD442 (63), was modified by insertion of lacZ with a USER cloning site to yield pGTR1113. The cat gene, conferring chloramphenicol resistance, was inserted into pGTR1113, to yield pGTR1129. Both of these vectors were subsequently used to construct plasmids for mutation of selected genes. 10 µl of PCR product was incubated with 1 unit USER enzyme and 20 ng USER vector at 37°C for 15 minutes. T4 DNA ligase and ligase buffer were added to the mixture and incubated for 15 minutes at room temperature. The reaction was cleaned using the DNA Clean and Concentrator™ Kit (Zymo Research Corporation, Orange, CA), electroporated into E. coli EC100Dpir⁺ and plated LB-N agar plates containing 40 µg/ml X-gal and either 30µg/ml chloramphenicol or 100 µg/ml ampicillin.

Three-way USER-friendly cloning

We modified the original USER cloning method to clone upstream and downstream flanking DNA together in a single step. 500- to 1000-bp upstream and
downstream DNA sequences flanking a target gene were amplified using oligonucleotides designed for capture into a USER vector. The oligonucleotides used to amplify the outside ends of the upstream and downstream DNA included the USER sequences complementary to the vector. The inside ends of the two fragments to be joined were amplified with oligonucleotides designed with compatible USER ends (ACCCGGGU), containing a SmaI site. Equal amounts of the upstream and downstream PCR products were mixed together, cleaned using the DNA Clean and Concentrator™ Kit, and eluted in 10 µl water. The cleaned PCR products were mixed with 20 ng USER vector and incubated at 37°C for 15 min with 1 unit USER enzyme. The remainder of the USER cloning procedure was followed as described above.

To allow for selection of the deletion, an antibiotic resistance cassette was inserted between the upstream and downstream cloned sequence. The deletion plasmid was digested with SmaI at the site engineered between the upstream and downstream sequences. A blunt-end antibiotic resistance cassette (aph, encoding kanamycin resistance; cat, encoding chloramphenicol resistance; or tet, encoding tetracycline resistance) was ligated into the SmaI site with T4 DNA ligase and incubated overnight at room temperature. The insertion of the antibiotic resistance cassette was selected on the appropriate antibiotic, the correct size of the plasmid was confirmed on an agarose gel, and the presence of the insert was confirmed by PCR amplification across the upstream and downstream sequences.

**Construction of mutations by conjugation of plasmid DNA into V. vulnificus**

Allelic exchange plasmids were electroporated into E. coli S17-1λpir for conjugation into V. vulnificus via filter mating. Static overnight cultures of V. vulnificus and E. coli were grown in LB-N at room temperature and 37°C, respectively. The
cultures were diluted in fresh LB-N and grown shaking at 37°C until they reached exponential phase. The two cultures were mixed in a ratio of 3 to 1, donor *E. coli* cells to recipient *V. vulnificus* cells, filter-mated, and transconjugants were selected on either VVM or LB-N containing 10^2 U/mL colistin and appropriate antibiotics.

Once the correct insertion of the allelic exchange vector into the chromosome was confirmed by PCR, the single crossover isolates were grown on LB-N agar containing 6% (w/v) sucrose at room temperature to enrich for cells in which a second crossover event occurred, resulting in loss of the plasmid. Sucrose-resistant colonies were passaged onto LB-N containing appropriate antibiotics to select for the mutation and screened for loss of antibiotic resistance encoded by the vector. Allelic exchange of the mutant DNA for the wild type DNA was confirmed by PCR.

**Chitin induced natural transformation**

Chitin-induced natural transformation of *V. vulnificus* was performed as described in Gulig, *et al.* (62). Sterile pieces of crab shell from blue crabs were used as a source of chitin. Static cultures of *V. vulnificus* were grown overnight in LB-N at room temperature. The overnight cultures were diluted 1:20 into fresh LB-N and grown shaking at 37°C until the cultures reached exponential phase. The bacteria were centrifuged, washed with filter-sterilized seawater (University of Florida Whitney Laboratory for Marine Bioscience) diluted to a salinity of 25 ppt, centrifuged again, and suspended in a volume of 25-ppt seawater twice the starting culture volume. 2 mL of the bacteria in seawater were placed in a well of a 12 well tissue culture plate (Costar, Corning, NY) with a piece of sterile crab shell, and the plate was incubated overnight at 30°C. The following day the supernatant was removed and replaced with 2 mL of fresh 25-ppt seawater, and 1 μg of linearized plasmid DNA or 2 μg of genomic DNA was
Plasmid DNA, prepared using the Qiagen QIAprep Spin Miniprep Kit, was linearized by digestion with an enzyme that cut the vector opposite the insert sequences. Genomic DNA was prepared using Qiagen DNeasy Blood and Tissue Kit. The plates were incubated overnight at 30°C. The following day, the supernatants were removed and plated on LB-N with appropriate antibiotics. The crab shells were placed in a 50-mL conical tube containing 2 mL of PBS, vortexed to release bacteria, and the supernatant was plated on LB-N with appropriate antibiotics. Mutations were confirmed by PCR to verify correct allelic exchange of the mutant DNA for the wild type DNA.
<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant Characteristics</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOP10</td>
<td>F− mcrA Δ (mrr-hsdRMS-mcrBC) Φ80lacΔM15 ΔlacX74 recA1 araD139Δ(ara-leu)7697 galU galK rpsL (Strr) endA1 nupG Invitrogen</td>
<td></td>
</tr>
<tr>
<td>S17-1λpir</td>
<td>λ-pir lysogen; thi pro hsdR hsdM⁺ recA RP4-2 Tc::Mu-Km::Tn7(Tpr Sm⁻)</td>
<td>(58)</td>
</tr>
<tr>
<td>EC100D pir+</td>
<td>F− mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacΔM15 ΔlacX74 recA1 endA1 araD139Δ(ara, leu)7697 galU galK λ⁻ rpsL nupG pir+(DHFR) Epicentre</td>
<td></td>
</tr>
<tr>
<td><strong>V. vulnificus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMCP6</td>
<td>Clinical Isolate</td>
<td>(64,65)</td>
</tr>
<tr>
<td>FLA399</td>
<td>Spontaneous Rif derivative of CMCP6</td>
<td>This study</td>
</tr>
<tr>
<td>FLA413</td>
<td>FLA399rtxA1::aph; Km⁻</td>
<td>This study</td>
</tr>
<tr>
<td>FLA439</td>
<td>FLA399rtxA1::aph, rtxA2::Ω; Km⁻</td>
<td>This study</td>
</tr>
<tr>
<td>FLA441</td>
<td>FLA399rtxA2::Ω</td>
<td>This study</td>
</tr>
<tr>
<td>FLA554</td>
<td>CMCP6rtxA1::aph; Km⁻</td>
<td>This study</td>
</tr>
<tr>
<td>FLA558</td>
<td>CMCP6rtxA1::aph, rtxA2::Ω; Km⁻</td>
<td>This study</td>
</tr>
<tr>
<td>FLA590</td>
<td>CMCP6rtxA1::aph reversion</td>
<td>This study</td>
</tr>
<tr>
<td>FLA591</td>
<td>CMCP6ΔrtxC</td>
<td>This study</td>
</tr>
<tr>
<td>FLA899</td>
<td>CMCP6ΔrtxA3</td>
<td>This study</td>
</tr>
<tr>
<td>FLA900</td>
<td>CMCP6ΔrtxA1::aph isolate #1; Km⁻</td>
<td>This study</td>
</tr>
<tr>
<td>FLA901</td>
<td>CMCP6ΔrtxA1::aph isolate #2; Km⁻</td>
<td>This study</td>
</tr>
<tr>
<td>FLA904</td>
<td>CMCP6rtxA1::Ωaph; Km⁻</td>
<td>This study</td>
</tr>
<tr>
<td>FLA912</td>
<td>CMCP6ΔvvmA::tetAR; Tc⁻</td>
<td>This study</td>
</tr>
<tr>
<td>FLA917</td>
<td>CMCP6ΔvvmA::tetAR,rtxA1::Ωaph; Tc⁻ Km⁻</td>
<td>This study</td>
</tr>
<tr>
<td>FLA923</td>
<td>CMCP6ΔrtxA1::aph (via chitin transformation with FLA901 genomic DNA); Km⁻</td>
<td>This study</td>
</tr>
<tr>
<td>FLA943</td>
<td>CMCP6ΔrtxA1::aph (via chitin transformation with FLA900 genomic DNA); Km⁻</td>
<td>This study</td>
</tr>
<tr>
<td>FLA943(pGTR1228)</td>
<td>CMCP6ΔrtxA1::aph (rtxA1⁺); Km⁻, Cm⁻</td>
<td>This study</td>
</tr>
<tr>
<td>FLA943(pGTR1204)</td>
<td>CMCP6ΔrtxA1::aph (vector); Km⁻, Cm⁻</td>
<td>This study</td>
</tr>
<tr>
<td>FLA947</td>
<td>CMCP6ΔrtxA1::aph, rtxA2::Ω; Km⁻</td>
<td>This study</td>
</tr>
<tr>
<td>FLA954</td>
<td>CMCP6ΔrtxA1::tetA; Tc⁻</td>
<td>This study</td>
</tr>
<tr>
<td>Strains</td>
<td>Relevant Characteristics</td>
<td>Reference or Source</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>FLA960</td>
<td>CMCP6ΔrtxA1::tetA, ΔvgrG::aph; Tc(^r), Km(^r)</td>
<td>This study</td>
</tr>
<tr>
<td>FLA965</td>
<td>CMCP6ΔrtxA1::tetA, Δhcp::cat; Tc(^r), Cm(^r)</td>
<td>This study</td>
</tr>
<tr>
<td>FLA1030</td>
<td>CMCP6Δhcp::cat; Cm(^r)</td>
<td>This study</td>
</tr>
<tr>
<td>FLA1035</td>
<td>CMCP6ΔvgrG::aph; Km(^r)</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Source or Reference</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>pBRΩaph</td>
<td>pBR322 with Ω-aph cloned at EcoRI site; Source for Ω-aph element containing transcription and translation termination signals; Ap⁺, Km⁺, Tc⁺</td>
<td>(66,67)</td>
</tr>
<tr>
<td>pCOS5</td>
<td>Cloning vector containing cat, oriT, ColE1, and oriV; Ap⁺ Cm⁺</td>
<td>(68)</td>
</tr>
<tr>
<td>pCR2.1 TOPO</td>
<td>TOPO cloning vector for capturing PCR products using TA cloning, Ap⁺</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pCVD442</td>
<td>R6K ori mob sacB bla; suicide vector for allelic exchange; Ap⁺</td>
<td>(63)</td>
</tr>
<tr>
<td>pNEB206A</td>
<td>USER friendly cloning vector; Ap⁺</td>
<td>NEB</td>
</tr>
<tr>
<td>pUC19</td>
<td>Cloning vector; lacZα; Ap⁺</td>
<td>(69)</td>
</tr>
<tr>
<td>pGTR256</td>
<td>1-kb region of rtxA2 amplified from CMCP6 with oligos vv-rtxA2-A and vv-rtxA2-B captured into pCR2.1-TOPO</td>
<td>This study</td>
</tr>
<tr>
<td>pGTR257</td>
<td>pGTR256 digested with HindIII, filled in with Klenow fragment, and religated, for loss of HindIII site</td>
<td>This study</td>
</tr>
<tr>
<td>pGTR259</td>
<td>pGTR257digested with NarI and regligated; deletion of portion of aph gene</td>
<td>This study</td>
</tr>
<tr>
<td>pGTR260</td>
<td>Insertion of Ωaph cassette at SspI site in rtxA2 fragment of pGTR259</td>
<td>This study</td>
</tr>
<tr>
<td>pGTR261</td>
<td>rtxA2::Ωaph from pGTR260 fragment subcloned into pUC19 with HindIII site deleted</td>
<td>This study</td>
</tr>
<tr>
<td>pGTR262</td>
<td>Digestion of pGTR261 with HindIII and religation to delete aph of Ωaph</td>
<td>This study</td>
</tr>
<tr>
<td>pGTR263</td>
<td>rtxA2::Ω; rtxA2::Ω excised from pGTR262 and subcloned at SspI and XbaI of pGTR1122</td>
<td>This study</td>
</tr>
<tr>
<td>pGTR265</td>
<td>ΔrtxC; rtxC upstream and downstream PCR amplicons digested with NotI and USER-cloned into pGTR1113; Ap⁺</td>
<td>This study</td>
</tr>
<tr>
<td>pGTR267</td>
<td>ΔrtxA3; rtxA3 upstream and downstream amplicons captured into pGTR1113 by three-way USER cloning; Ap⁺</td>
<td>This study</td>
</tr>
<tr>
<td>pGTR268</td>
<td>ΔrtxA3; rtxA3 upstream and downstream amplicons captured into pGTR1129 by three-way USER cloning; Ap⁺, Cm⁺</td>
<td>This study</td>
</tr>
<tr>
<td>pGTR272</td>
<td>rtxA1 upstream and downstream PCR amplicons captured into pGRT1129 by three-way USER cloning (isolate 1); Ap⁺, Cm⁺</td>
<td>This study</td>
</tr>
<tr>
<td>pGTR273</td>
<td>rtxA1 upstream and downstream PCR amplicons captured into pGRT1129 by three-way USER cloning (isolate 2); Ap⁺, Cm⁺</td>
<td>This study</td>
</tr>
<tr>
<td>pGTR274</td>
<td>ΔrtxA1::aph; aph inserted at Smal site between the rtxA1 upstream and downstream sequences of pGTR272; Ap⁺, Cm⁺ 500-bp upstream and downstream flanking site in 5' end of rtxA1 gene captured into pGTR1129 by three-way USER cloning; Ap⁺, Cm⁺, Km⁺</td>
<td>This study</td>
</tr>
<tr>
<td>pGTR276</td>
<td>rtxA1::Ωaph; Ωaph cassette inserted at Smal site between cloned rtxA1 sequences of pGTR276 for insertion into 5' end of rtxA1; Ap⁺, Cm⁺, Km⁺</td>
<td>This study</td>
</tr>
</tbody>
</table>

Table 2-2. Plasmids used in this study.
<p>| pGTR285 | (\Delta rtxA1::\text{tet}; \text{tetA gene subcloned into the Smal wite between rtxA1 upstream and downstream sequences of pGTR273; Ap', Tc'}) | This study |
| pGTR288 | (\Delta rtxA3::\text{cat}; \text{cat gene subcloned into Smal site between rtxA3 upstream and downstream sequences in pGTR267; Apr, Cm'}) | This study |
| pGTR652 | 3.8-kb internal rtxA1 fragment from MO6-24/O sequencing clone inserted into Smal site of pCVD442; Ap' | This study |
| pGTR653 | rtxA1::aph; aph cassette from pUC4K inserted in rtxA1 fragment in pGTR652; Ap', Km' | This study |
| pGTR1113 | pCVD442::lacZa; pCVD442 with lacZa from pUC19 with USER Friendly cloning oligonucleotide linker incorporated (62) | |
| pGTR1119 | rtxA1::aph reversion; tetAR gene inserted at blunt-ended XbaI site of pGTR652; Ap', Tc' | This study |
| pGTR1122 | pCVD442::lacZa, cat; cat cloned at blunt-ended Ndel of pCVD442::lacZa; Ap', Cm' (62) | |
| pGTR1129 | pCVD442::lacZa::USER, catcloned into EcoRV site of pGTR1113; Ap', Cm' (62) | |
| pGTR1204 | pCOS5::lacZa::USER; USER-friendly cloning site from pGTR1113 incorporated into pCOS5::lacZa; Ap', Cm' (62) | |
| pGTR1208 | 500-bp vvhA upstream and downstream PCR amplicons captured into pGTR1129 by three-way USER cloning; Ap', Cm' | This study |
| pGTR1221 | (\Delta vvhA::\text{tetAR}; \text{tetAR cassette subcloned into Smal site between vvhA upstream and downstream sequences in pGTR1208; Ap', Cm', Tc'}) | This study |
| pGTR1227 | (\Delta rtxA1) complementation; 15.6-kb rtxA1 gene PCR amplified from CMCP6 and cloned into pGTR1204 at SacI and XbaI sites; Ap', Cm' | This study |
| pGTR1303 | 500-bp hcp upstream and downstream PCR amplicons captured into pGTR1113 by three-way USER cloning; Ap' | This study |
| pGTR1304 | 500-bp vgrG upstream and downstream PCR amplicons captured into pGTR1113 by three-way USER cloning; Ap' | This study |
| pGTR1305 | (\Delta hcp::\text{cat}; \text{cat cassette subcloned into Smal site between hcp upstream and downstream sequences of pGTR1303; Ap', Cm'}) | This study |
| pGTR1307 | (\Delta vgrG::\text{aph}; \text{aph cassette subcloned into Smal site between vgrG upstream and downstream sequences of pGTR1304; Ap', Cm', Km'}) | This study |</p>
<table>
<thead>
<tr>
<th>Oligonucleotide primer</th>
<th>Sequence 5’ – 3’ a</th>
</tr>
</thead>
<tbody>
<tr>
<td>rtxA1 test down</td>
<td>ATCGGTGTAGCGTCAAAACACAGG</td>
</tr>
<tr>
<td>rtxA1 test up</td>
<td>GGAACCTTGATGCTCCGGGGC</td>
</tr>
<tr>
<td>rtxA1 up 5’</td>
<td>ACCCCGGGU TAAAGCCAACA TCTTCTTCTTAGGAG</td>
</tr>
<tr>
<td>rtxA1 up 3’</td>
<td>GGAGACAU UGAGCTTGCAGCAGCGAGAGTGA</td>
</tr>
<tr>
<td>rtxA1 down 3’</td>
<td>GGGAAAAGU CGCTTATGGCAACGGAATTCCG</td>
</tr>
<tr>
<td>rtxA1 down 5’</td>
<td>ACCCCGGGU CAACGAGGCACGAGTATAGAG</td>
</tr>
<tr>
<td>rtxA1 insert up 5’</td>
<td>ACCCCGGGU UACAGTGA CATGATCATCAACCAC</td>
</tr>
<tr>
<td>rtxA1 insert up 3’</td>
<td>GGAGACAU TGGCAGAATGGCAGCAGCAGG</td>
</tr>
<tr>
<td>rtxA1 insert down 5’</td>
<td>ACCCCGGGU CGGATCG ATTTGGTGCAACCGG</td>
</tr>
<tr>
<td>rtxA1 insert down 3’</td>
<td>GGGAAAAGU GACCAAATGGCTTGTCAACGAC</td>
</tr>
<tr>
<td>rtxA1 omega down 5’</td>
<td>GGTAATATCGCCCACTTTGCC</td>
</tr>
<tr>
<td>rtxA1 omega</td>
<td>CGGTGGATGACCTTTTGAATG</td>
</tr>
<tr>
<td>rtxA1 omega up 3’</td>
<td>CCCCTTCATCGTGCTGTCAG</td>
</tr>
<tr>
<td>rtxA1 clone 3’</td>
<td>GCATGAGCTCGTGAATATTACACCCTTATACCTT</td>
</tr>
<tr>
<td>rtxA1 clone 5’</td>
<td>TCGATCTAGACTCTCAAAGAAGATTTGGCTTATG</td>
</tr>
<tr>
<td>rtxA1 A 5’</td>
<td>CACCGTTTACCCCTTCTTATGGA</td>
</tr>
<tr>
<td>rtxA1 A 3’</td>
<td>GAGATGTTGGAGGTCA TGGTG</td>
</tr>
<tr>
<td>rtxA1 B 5’</td>
<td>CCATGTTTACGATCAGATTGCC</td>
</tr>
<tr>
<td>rtxA1 B 3’</td>
<td>CACGTTTCTGGTTCTTCTGCTG</td>
</tr>
<tr>
<td>rtxA1 C 5’</td>
<td>GTCTGGGCAATTGTCAGAACG</td>
</tr>
<tr>
<td>rtxA1 C 3’</td>
<td>GTACAAAGTCCAGTTGGAGTCGAC</td>
</tr>
<tr>
<td>rtxA1 D 5’</td>
<td>CGGTCAGGTCTCTTCTCGACAG</td>
</tr>
<tr>
<td>rtxA1 D 3’</td>
<td>CGCCGACGATGGGAACACAG</td>
</tr>
<tr>
<td>rtxA2 a</td>
<td>GTCTAGAGGTGGCCAATGGTTGAAGATGG</td>
</tr>
<tr>
<td>rtxA2 b</td>
<td>CGAGCTCGTGATCAGCAGCGCGTGCTGCAACC</td>
</tr>
<tr>
<td>rtxA3 down 3’</td>
<td>GGGAAAAGU GCTCAAATGGCGCATGTGTCG</td>
</tr>
<tr>
<td>rtxA3 down 5’ Smal USER</td>
<td>ACCCCGGGU TGCTGTCTGCAATTGGGTCTAAG</td>
</tr>
<tr>
<td>rtxA3 up 3’ Smal USER</td>
<td>ACCCCGGGU AGATGACTGTAGTACTTTTCGTC</td>
</tr>
<tr>
<td>rtxA3 up 5’</td>
<td>GGAGACAU GTAACCAATACCACGCTGCTAAG</td>
</tr>
<tr>
<td>rtxC down 3’</td>
<td>GGGAAAAGU AGTACGGTCTAG TTATTGCCC</td>
</tr>
<tr>
<td>rtxC down 5’</td>
<td>GATATAAGGGCGAGCGGCGGCCTAAAGAAGAGTTTGCGCTTATG</td>
</tr>
<tr>
<td>Oligonucleotide primer</td>
<td>Sequence 5’ – 3’ a</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>rtxC up 3’</td>
<td>CTCTTCTTTAGGGCGGCCGCTCTGCCCTATATACCAACCATATG</td>
</tr>
<tr>
<td>rtxC up 5’</td>
<td>GGAGACAUTTGAGCTTGCGAGCCGAGAG</td>
</tr>
<tr>
<td>vvha up 5’ USER</td>
<td>GGAGACAUCGCCTTACCGTACTCTGCTG</td>
</tr>
<tr>
<td>vvhA up 3’ USER</td>
<td>ACCGGGUATCTTTTTTCTCCTAGATTGGG</td>
</tr>
<tr>
<td>vvha down 5’ USER</td>
<td>ACCGGGUUGCTCTGTTGCGCTAGCCCG</td>
</tr>
<tr>
<td>vvha down 3’ USER</td>
<td>GGGAAAGUCAATTGCGAGCGTAGGTAGGAG</td>
</tr>
<tr>
<td>vgrG down 5’</td>
<td>ACCGGGUAGAAATGCGAATATGCCGATCTTTT</td>
</tr>
<tr>
<td>vgrG up 3’</td>
<td>GGGAAAGUAGAAGCGGTGATCAACGATCG</td>
</tr>
<tr>
<td>hcp up 5’</td>
<td>GGAGACAUCACCATGACCTACTGCTTGG</td>
</tr>
<tr>
<td>hcp up 3’</td>
<td>ACCGGGUAGAAATGCGAATATGCCGATCTTTT</td>
</tr>
<tr>
<td>T6SS up 5’</td>
<td>GGAGACAUCACAGGCAGAAAACCGGTGTAG</td>
</tr>
<tr>
<td>T6SS up 3’</td>
<td>ACCGGGUUGTAAACCTGCATCTGCTTGG</td>
</tr>
<tr>
<td>T6SS down 5’</td>
<td>ACCGGGUUTCTTATACAATATGCTCAGAG</td>
</tr>
<tr>
<td>T6SS down 3’</td>
<td>GGGAAAGUGACTTATTATCTCTGTCGAG</td>
</tr>
<tr>
<td>vgrG hcp down 5’</td>
<td>ACCGGGUAGGAGATGCGATTTCATATTTCATTC</td>
</tr>
<tr>
<td>vgrG hcp down 3’</td>
<td>GGGAAAGUAGGAGATGCGATTTCATATTTCATTC</td>
</tr>
<tr>
<td>T6SS int hcp 5’</td>
<td>GCACCTTTCGACACCTCAGGTAAG</td>
</tr>
<tr>
<td>T6SS int hcp 3’</td>
<td>CTACAAAAATAGCGAGCATCTCAG</td>
</tr>
<tr>
<td>vgrG outside 5’</td>
<td>GTTCCAGCACCAGCTGGCC</td>
</tr>
<tr>
<td>vgrG outside 3’</td>
<td>CACCGCGCTGAAACTCCTTTCA</td>
</tr>
<tr>
<td>hcp outside 5’</td>
<td>CACCAAGGTTAACGCCGAGGAG</td>
</tr>
<tr>
<td>hcp outside 3’</td>
<td>GAAAGCAGCTACGCTGCAATCC</td>
</tr>
<tr>
<td>lacZ test 5’</td>
<td>CAAGGCGATTAGTTGGTGTAAC</td>
</tr>
<tr>
<td>lacZ test 3’</td>
<td>GACCAGTGATTACGCAAAGCTC</td>
</tr>
<tr>
<td>sacB5’-2</td>
<td>AAGTTTCTGAATTCGATTCGTC</td>
</tr>
<tr>
<td>sacB3’-2</td>
<td>CCTTTCGCTTGAGGTACAGCG</td>
</tr>
</tbody>
</table>

a Restriction sites are underlined and USER cloning sequences are bolded.
CHAPTER 3
ROLE OF THE RtxA1 TOXIN IN PATHOGENESIS OF *Vibrio vulnificus*

Rationale for Study

During a genomic sequencing project of the *V. vulnificus* clinical isolate MO6-24/0 in our laboratory, a clone was identified which contained a 3.8-kb insert with homology to the *V. cholerae* rtxA structural gene. Initial studies in the laboratory, performed by Angela Starks, Ph.D., indicated that RtxA1 had a major role in the cytotoxicity of *V. vulnificus* MO6-24/O in vitro and that it played a detectable, yet minor role in virulence. We were interested in conducting a more in depth analysis of the RtxA1 toxin in the virulent, clinical strain CMCP6, whose genome has been completely sequenced. There was some discrepancy in the virulence the original rtxA1::aph mutation in strain MO6-24/O, studied previously, and other mutations constructed in strain CMCP6, discussed here. These results demonstrated that RtxA1 is not only a major cytotoxic factor, but also plays a role in the virulence of *V. vulnificus* CMCP6. However, the residual virulence of rtxA1 mutants demonstrated that other, as of yet unidentified, virulence factors contribute to the damage to host tissues caused by infection with *V. vulnificus*.

Introduction

The RTX (repeats-in-toxin) toxins are produced by many gram-negative bacteria, and include such toxins as *E. coli* HlyA (70), *Bordetella pertussis* CyaA (71), *Neisseria meningitidis* FrpA (72), and *V. cholerae* RtxA (73). Each member of the family is characterized by a glycine-rich repeated motif at the C-terminal end of the protein. Generally, the RTX toxins, encoded by rtxA, are secreted by a type I secretion system (T1SS), encoded by *rtxBD* in the same operon and *tolC* encoded elsewhere (74,75). An
acyltransferase, RtxC, is encoded upstream of rtxA and has been shown in several of RTX-encoding bacteria to be essential for toxin activity (76-79).

The amino acid sequence identified during the MO6-24/O genomic sequencing project shared homology with the V. cholerae RtxA, the prototypical member multifunctional autoprocessing RTX toxins (MARTX) (56). The genomic sequences of two other clinical isolates, CMCP6 and YJ016 (64), revealed that each strain encodes two rtx gene clusters on chromosome 2 and one rtx gene on chromosome 1. The first gene cluster contains five genes in a similar order to the rtx locus of V. cholerae (73) (Figure 3-1 shows gene organization). The genes are grouped into two divergent operons. The first operon encodes rtxA1, a 15.62-kb gene encoding the toxin structural protein; rtxC1, encoding an acyltransferase putatively essential for activity of the RTX toxin; and VV2_0481, encoding an uncharacterized hypothetical protein. The second operon encodes the T1SS proteins, RtxB1, RtxD1, and RtxE1. RtxB1 and RtxE1 are ABC-transport proteins, and RtxD is a membrane fusion protein (80,81). Together along with tolC, located elsewhere on the chromosome, these genes encode a T1SS for export of RtxA.

The second rtx gene cluster located on chromosome 2 includes a 13.96-kb rtxA2 gene and the genes rtxB2 and rtxD2 putatively encoding parts of the secretion system for RtxA2. This cluster lacks rtxC that would normally encode the toxin activator, suggesting that the product of rtxA2 may not become an active RTX toxin. The third locus on chromosome 1 contains only an 8.8-kb gene encoding an RtxA toxin, rtxA3, and lacks the accessory secretion and activation genes.
MARTX toxin proteins have four important components (56): N-terminal repeats, internal enzymatic domains, a cysteine protease domain, and the C-terminal RTX repeats. The N-terminal and C-terminal repeats are highly conserved among the MARTX toxins; however, the internal regions are variable and are predicted to encode the cytotoxic functions of the toxins.

Typical RTX toxins are pore-forming toxins and not multifunctional, with the exception of B. pertussis CyaA, which has both pore-forming ability and adenylate cyclase activity (71). The RtxA of V. cholerae, the best characterized MARTX toxin, does not form pores or cause cell lysis (73,82). Instead, the RtxAvc causes rounding of cells and loss of the integrity of tight junctions of polarized cells by covalently crosslinking actin-monomers into multimers (73,82,83). The actin-crosslinking domain (ACD) is one of the central enzymatic domains of RtxAvc. While ACD is essential for actin crosslinking, deletion of the domain did not ablate the ability to cause cell rounding (84). This cell rounding is mediated through inactivation of small Rho GTPases leading to the downstream effect of disassembly of the actin cytoskeleton (85). The domain important for this activity is the Rho GTPase inactivation domain (RID) (85). These enzymatic domains carry out their function inside the host cell. It is predicted that the repeats at the N-terminus and C-terminus play a role in translocation of the internal domains into the host cell, and the domains are released by autocleavage of itself at a cysteine protease domain (CPD) located just upstream of the C-terminal repeats (56,86).

Bioinformatics analyses of RtxA1 of V. vulnificus revealed that it does not have the ACD domain, and functional studies have shown that V. vulnificus does not induce actin
crosslinking (84,87). RtxA1 of *V. vulnificus* does encode the RID domain, essential for the inactivation of Rho GTPases, which may account for observed cell rounding that occurs prior lysis of cells (87,88). *V. vulnificus* RtxA1 has three additional domains of putative enzymatic activity; however, the function of these remains unknown (56).

The RtxA1 toxin of *V. vulnificus* has become a topic of interest in recent years. Liu, *et al.* (89) searched for CMCP6 genes controlled by the in vivo-expressed regulator HlyU and identified *rtxA1*. Upon further examination, they demonstrated that the CMCP6 *rtxA1* mutant was noncytotoxic and was attenuated in i.p. inoculated, iron dextran-treated mice. They observed a 10³-fold increase in LD₅₀ (50% lethal dose) in this mouse model and concluded that RtxA1 was a major virulence factor. Lee, *et al.* (88) and Kim, *et al.* (87) independently identified transposon mutants of *V. vulnificus* MO6-24/O with reduced cytotoxicity. These mutants had insertions in genes within the *rtx1* locus. Both groups examined their respective MO6-24/O *rtxA1* mutants for virulence and cytotoxicity. The *rtxA1* mutant constructed by Lee, *et al.* had a 10³-fold increase in i.p. LD₅₀ in iron-treated mice (88). Kim, *et al.* (87) observed an LD₅₀ increase of 10²-fold in i.p. or intragastric (i.g.) inoculated, non-iron-treated mice. Each group also concluded that RtxA1 is major cytotoxic factor, although the *rtxA1* mutants exhibited some residual cytotoxicity with increasing MOIs and with longer infection times (87,88), and one group determined that the residual cytotoxicity was due to the hemolysin/cytolysin, VvhA (87). Lee, *et al.* later demonstrated that RtxA1 caused apoptosis in INT-407 cells (90). Subsequent studies provided evidence that *rtxA1* is secreted by a T1SS encoded by *rtxBDE* at the same locus (81).
All of the previous studies were performed in either i.p. or i.g. inoculated mice. These mouse models examine the ability to cause systemic disease, but do not produce a skin infection. From these studies, one proposed function in virulence is that RtxA1 was to assist the bacteria in invading through the intestinal barrier into the bloodstream (87); however, a function for this cytotoxic factor in tissue damage during skin infection has not yet been studied. We investigated the role of RtxA1 after s.c. inoculation in mice, which allows us to examine the ability to cause skin infection as well as systemic infection. Our examination of RtxA1 yielded cytotoxicity results similar to those of others. However, our rtxA1 mutants were not as attenuated in the s.c. inoculated, iron dextran-treated mouse model as were the rtxA1 mutants of the other groups in the i.p inoculated mouse model. We initially thought that this discrepancy was due to the location of our original mutation within the rtxA1 gene, and so we deleted the entire gene. However, deletion of the entire rtxA1 gene still yielded less attenuation in our mouse model than that observed by others in their mouse models. Despite their noncytotoxic phenotype, the rtxA1 mutants were still able to cause extensive tissue damage and lethality in s.c. inoculated mice.

**Results**

**Disruption of rtxA1 by aph Insertion**

To examine the role of RtxA1 in the pathogenesis of *V. vulnificus*, an insertion mutation had previously been constructed in the in the rtxA1 gene of the clinical virulent strain, MO6-24/O. The 3.8-kb rtxA1 DNA of the MO6-24/O sequencing clone described above was cloned into pCVD442 and subsequently disrupted by insertion of an aph cassette, encoding kanamycin resistance. The resulting plasmid, pGTR653, was conjugated into the sequenced, clinical strain, CMCP6, for insertion of aph into the rtxA1
gene. The mutants were selected on LB-N containing kanamycin and verified to have undergone allelic exchange by PCR with the primers rtxA-test-a and rtxA-test-b. The resulting CMCP6 mutant with the \textit{aph} insertion approximately 10.6-kb downstream in the \textit{rtxA1} gene is called FLA554. The \textit{rtxA1} gene is very large, making complementation in trans, and therefore fulfillment of the molecular Koch’s postulates (54,55), difficult. Instead of complementation with the wild-type allele on a plasmid, the \textit{aph} mutation of FLA554 was reverted back to wild-type by allelic exchange with the MO6-24/O \textit{rtxA1} sequence carried on the allelic exchange plasmid pGTR1119. The reversion of the \textit{rtxA1::aph} mutation in FLA554 yielded strain FLA590.

**Cytotoxicity of \textit{rtxA1::aph} mutant**

Previous studies in the laboratory demonstrated that the RtxA1 toxin is a major cytotoxic factor of MO6-24/O. To determine the role of RtxA1 in cytotoxicity of CMCP6, in vitro characterization was carried out in tissue culture using the intestinal epithelial cell line INT-407. Two in vitro assays were used to measure the ability of the \textit{rtxA1} mutant to cause monolayer detachment/destruction and lysis of epithelial cells.

A crystal violet staining assay (61) was used to assess ability of the strains to cause monolayer detachment/destruction of INT-407 cells. Confluent monolayers of INT-407 cells were infected with \textit{V. vulnificus} at an MOI of 10 and stained as described in the Materials and Methods. Similar to the observations with MO6-24/O, there was a significant decrease in the ability of the CMCP6 \textit{rtxA1::aph}, FLA554, to cause monolayer detachment/destruction as compared to the parental strain (5.6% vs. 76.6%, \( P=0.0001 \)) (Figure 3-2). Reversion of the mutation back to wild type restored cytotoxicity for CMCP6 mutants (\( P = 0.09 \)).
The crystal violet assay detects general detachment of the monolayers, but does not indicate the method of cell death. RTX toxins are typically pore-forming toxins, resulting in lysis of the host cells. Members of the MARTX subfamily have different methods for cytotoxicity due to the variability in the activity domains. The toxins insert into the membrane of host cells and translocate the internal enzymatic domains. A method used to assess lysis of host cells is by measurement of lactate dehydrogenase (LDH) release. Infection of INT-407 monolayers was carried out exactly as for the crystal violet assay. LDH release was measured using the Cytotoxicity Detection Kit (Roche Boehringer Manheime, Indianapolis, IN). Upon infection of INT-407 cells, the rtxA1::aph mutant, FLA554, caused a significantly lower amount of LDH to be released by the cells than the parental strain, CMPC6 (17.8% lysis vs. 71.3% lysis, \( P = 0.002 \)) (data not shown). These defects in lysis were restored by reversion of the mutation back to wild type. This indicates that the RtxA1 toxin causes lysis of epithelial cells in vitro, likely due to pore-formation and disruption of the membrane, as with many other RTX toxins. Due to the significant reduction of cytotoxicity caused by the mutant strain, RtxA1 is considered to be one of the major cytotoxic factors produced by V. vulnificus.

**Virulence of rtxA1::aph mutant in mice**

The attenuated virulence of the rtxA1::aph mutant of MO6-24/O seen in previous studies in the laboratory gave indications that RtxA1 has a minor role in virulence. The rtxA1::aph mutant of CMCP6 was examined for virulence using the iron dextran-treated, s.c. inoculated mouse model. This infection model allows a quantitative assessment of the ability of a strain to cause both localized and systemic infection, as measured by enumeration of bacteria recovered from skin lesion and liver tissues, respectively. Mice are treated with iron dextran at least 1 hour prior to infection to mimic the predisposition
for elevated serum iron levels in the susceptible host. Mice are then inoculated by s.c. injection of bacteria into the lower right flank. Infection with FLA554, CMCP6 \( \text{rtxA1::aph} \), resulted in decreased virulence at the minimum lethal dose of 300 CFU (Figure 3-3). Five out of five mice had visible skin lesions that looked similar to wild-type skin lesions (Figure 3-4); however, only four out of five mice yielded detectable CFU from the skin, resulting in a mean of \( 10^{7.4} \) CFU/g skin tissues. Of five mice infected, only three had detectable CFU isolated from the liver, yielding a significantly lower level of bacteria compared to the wild type-infected mice (\( 10^{2.9} \) CFU/g vs. \( 10^{5.7} \) CFU/g, \( P = 0.0005 \)). At a 10-fold higher inoculum, 3,000 CFU, the \( \text{rtxA1} \) mutant was able to cause wild-type levels of skin infection in all five mice infected. All of the mice had systemic infection, indicated by bacteria isolated from the liver, albeit at a lower level than the wild type infected at 300 CFU (\( 10^{3.7} \) CFU/g vs. \( 10^{5.7} \) CFU/g, \( P = 0.007 \)). Infection with the reversion strain restored virulence to levels comparable to the wild type at the minimum lethal dose of 300 CFU/mouse. These results are in agreement with what has been observed for the \( \text{rtxA1} \) mutation in MO6-24/O, suggesting that \( \text{rtxA1} \) does have a minor role in virulence, because increasing the inoculum by only 10-fold overcomes the attenuation of the \( \text{rtxA1} \) mutants.

**Deletion of \( \text{rtxA1} \)**

Our results from the \( \text{rtxA1::aph} \) mutant strains indicated that RtxA1 is a major cytotoxic factor of \( V. \text{vulnificus} \); however, it is not a major virulence factor. While our cytotoxicity data agree with other reports in the literature for \( V. \text{vulnificus} \text{ rtxA1} \), other laboratories observed a larger attenuation of virulence using other \( \text{rtxA1} \) mutations in \( V. \text{vulnificus} \) (87-89). These groups reported a \( 10^2 \) to \( 10^3 \)-fold increase in i.p. LD\(_{50}\), whereas we observed full virulence after increasing the inoculum by 10-fold in our s.c.
injected mouse model. The *aph* insertion described above is approximately 10 kb into the *rtxA1* open reading frame, leaving over half of the gene undisrupted. We considered the possibility that a portion of the RtxA1 protein was being produced and secreted, resulting in less attenuation than observed with the *rtxA1* mutations of others.

To resolve this discrepancy, we constructed a mutant strain in which the entire *rtxA1* open reading frame was deleted and replaced with an *aph* cassette. The Δ*rtxA1::aph* mutant, FLA943, was expected to have similar phenotypes as the *rtxA1::aph* mutant in both cytotoxicity assays and in the mouse model for virulence.

Three-way USER cloning was used to construct the allelic exchange plasmid to delete the 15.6-kb *rtxA1* gene. The oligonucleotide pairs *rtxA1*-up-3'/*rtxA1*- up-5' and *rtxA1*-down-3'/*rtxA1*-down-5' were used to amplify 1.0-kb upstream and downstream sequences of *rtxA1* with the USER friendly cloning sequences complementary to the USER vector on the outside ends and the sequence ACCCGGGU on the inside ends where the two fragments were to be joined to create a common *Sma*I restriction site. These upstream and downstream *rtxA1* fragments were cloned into the USER friendly allelic exchange vector pGTR1129 exactly as described for three-way USER cloning in the Materials and Methods, yielding pGTR272 (Δ*rtxA1*). To enable the selection of *rtxA1* deletion mutants of *V. vulnificus*, a blunt-ended *aph* kanamycin resistance cassette was cloned into the *Sma*I site between the upstream and downstream sequences of pGTR272, yielding plasmid pGTR274 carrying Δ*rtxA1::aph*. This allelic exchange plasmid was moved into *V. vulnificus* CMCP6 by conjugation and used to recombine the mutation into CMCP6 in the two-step *sacB*-assisted allelic exchange process, resulting in two independent isolates, FLA900 and FLA901. The correct
recombination events were confirmed by PCR using the oligonucleotides RtxA1-deletion up and RtxA1-deletion down and Southern blot analyses.

**Cytotoxicity of ΔrtxA1::aph mutants**

As a confirmation of the mutant phenotype, the ΔrtxA1::aph isolates FLA900 and FLA901 were tested for cytotoxicity to INT-407 cells in vitro. The mutants had a great reduction in the ability to cause detachment/destruction of the monolayers of INT-407 cells (1.4% for FLA900 and 11.3% FLA901; \( P = 0.0001 \) and 0.0003, compared to wild-type, respectively) (Figure 3-5). This reduction in cytotoxicity was the equivalent to that observed for the original rtxA1::aph mutant.

**Virulence of ΔrtxA1::aph mutants in mice**

To examine if deletion of the entire rtxA1 gene caused greater attenuation than the rtxA1::aph mutation, the first ΔrtxA1::aph isolate, FLA900, was inoculated into mice at the wild-type minimum lethal dose of 300 CFU. At this inoculum, FLA900 caused a detectable skin lesion in three of the five mice, and the mean CFU recovered from the skin tissues was \( 10^5 \) CFU/g skin lesion. Bacteria were not recovered from the livers of any of the mice, indicating that FLA900 was unable to cause systemic infection at this dose. Interestingly, this experiment showed that this ΔrtxA1::aph isolate was more attenuated than the rtxA1::aph mutant, which caused liver infection in three of five mice at this inoculum. To substantiate this result, we infected mice with FLA900 and a second independent ΔrtxA1::aph isolate, FLA901, each at an inoculum of 5,000 CFU. At this inoculum, we would expect that the mice would succumb to the infection with either of these mutants, similar to the infection with the original rtxA1::aph mutation. Surprisingly, the two ΔrtxA1::aph isolates had different virulence phenotypes (Figure 3-6). The first isolate, FLA900, remained attenuated at this dose, causing skin lesions in
three of five mice and liver infections in only two of five mice. The amount of bacteria isolated from the skin lesions caused by FLA900 was approximately 100-fold lower than the amount of bacteria isolated from the skin lesions of mice infected with only 300 CFU of the wild type ($10^{5.7}$ CFU/g vs. $10^{7.9}$ CFU/g, respectively, $P = 0.009$). Systemic infection by FLA900 was nearly undetectable, with a bacterial load of $10^{2.0}$ CFU/g in the liver ($P = 10^{-6}$). Interestingly, the second $\Delta rtxA1::aph$ isolate, FLA901, was virulent when inoculated at the same inoculum (5,000 CFU) at which the first isolate was attenuated. The amount of bacteria recovered from the skin and liver was not significantly different from wild-type levels ($10^{8.4}$ CFU/g skin lesion and $10^{5.5}$ CFU/g liver); however, it was significantly higher than the amount of bacteria recovered from FLA900 ($P = 0.002$ for skin; $P = 10^{-6}$ for liver). Based on these results, the level of attenuation caused by the $\Delta rtxA1::aph$ mutations remained questionable.

**Reconstruction of the $\Delta rtxA1::aph$ Mutation by Chitin-Induced Natural Transformation**

It is possible that one of the two $\Delta rtxA1::aph$ isolates, most likely the more attenuated FLA900, had a secondary mutation elsewhere in the chromosome causing an additional defect in virulence. To resolve this issue, each mutation was recreated in the wild-type CMCP6 background. We took advantage of the chitin-induced natural transformation of *V. vulnificus* to move the mutation from each $\Delta rtxA1::aph$ isolate into CMCP6. Genomic DNA was extracted from each mutant and incubated with CMCP6 growing in seawater in the presence of crabshell. Transformed bacteria were selected by plating on LB-N agar containing kanamycin to select for allelic exchange of *aph* for the *rtxA1*. The $\Delta rtxA1::aph$ mutant recreated from more attenuated FLA900 was named FLA943, and the mutant recreated from more virulent FLA901 was named FLA923.
Deletion of the \textit{rtxA1} gene was confirmed by PCR, and the noncytotoxic phenotype of each isolate was verified (Figure 3-7).

**Virulence of reconstructed $\Delta rtxA1::aph$ mutants**

Each reconstructed $\Delta rtxA1::aph$ mutant was examined for virulence in mice. If the cause for the attenuation in the original FLA900 was due to a secondary mutation, then both of the mutants recreated from the original $\Delta rtxA1::aph$ isolates would have the same virulence phenotype as the more virulent of the two original isolates, FLA901. Iron dextran-treated mice were inoculated with 6,000 CFU of each mutant, and FLA943 and FLA923 were similarly attenuated (Figure 3-8). FLA943 caused a visible skin lesion in four of the five mice, with a bacterial load of $10^{7.4}$ CFU/g. FLA923 caused a visible infection in five out of five mice; however, the bacterial yield was slightly lower $10^{6.9}$ CFU/g tissue. Each mutant caused systemic infections in three of five mice, yielding $10^{4.4}$ CFU/g and $10^{4.3}$ CFU/g from the livers of mice infected with FLA943 and FLA923, respectively. This was lower than what was observed for the wild-type CMCP6 at a 10-fold lower inoculum. The virulence phenotypes of these two reconstructed mutants lie between the two original $\Delta rtxA1::aph$ mutants, for which one was avirulent and the other was fully virulent at 5,000 CFU. Because the two mutants behaved similarly, we chose to use FLA943 $\Delta rtxA1::aph$ for the remainder of the studies.

**Attempted complementation of $\Delta rtxA1::aph$ with the wild-type $rtxA1$ allele**

The two $rtxA1$ mutants, $rtxA1::aph$ FLA554 and $\Delta rtxA1::aph$ FLA943 differed slightly in their virulence in mice. The $rtxA1::aph$ infected every mouse at a 10-fold higher inoculum than the minimum lethal dose for wild-type CMCP6. The $\Delta rtxA1::aph$ mutant, FLA943, did not consistently infect every mouse when the inoculum was as high as 20-fold above the wild-type minimum lethal dose. While two and sometimes three of five
mice succumbed to infection by ΔrtxA1::aph with wild-type levels of bacteria in the skin and liver, each time there was one or two mice without any detectable bacterial load in the liver. To fulfill the molecular version of Koch’s postulates (54,55) and confirm that this defect in virulence is indeed due to the ΔrtxA1::aph mutation, the wild-type allele must be expressed in trans to complement the mutation. The rtxA1 open reading frame was PCR amplified using iProof High-Fidelity DNA polymerase (Bio-Rad, Hercules, CA) with the oligonucleotides rtxA1 clone 5’ and rtxA1-clone 3’, containing the SacI and XbaI restriction sites at the 5’ ends of the oligonucleotide, respectively. The 15.6-kb rtxA1 amplicon was digested with the restriction endonucleases SacI and XbaI and cloned into the plasmid pGTR1204 (pCOS5::lacZ-USER) which had been digested with Sacl and XbaI. The rtxA1 gene was captured in a directional manner, so as to be expressed by the lacZ promoter. The resulting rtxA1 complementation plasmid, pGTR1227, was moved into the ΔrtxA1::aph mutant FLA943 via conjugation.

Cytotoxicity was restored to FLA943 by expressing rtxA1 in trans on pGTR1227. Cytotoxicity to INT-407 cells was verified by both crystal violet stain to measure detachment/destruction of the monolayers and by measuring lysis by detecting LDH release (Figure 3-9). This result confirmed that the rtxA1 gene was being expressed from the plasmid and was able to form a cytotoxic RtxA1 toxin capable of killing INT-407 cells in vitro.

Next, it was imperative to test the complementation strain for virulence in the iron dextran-treated mouse. Unfortunately, the complementation was not as straightforward in vivo as it was in vitro. An initial infection with FLA943 and its complementation counterpart carrying pGTR1227 showed that the complementing plasmid was able to
restore virulence to the mutant (Figure 3-10(a)). The complemented mutant infected the skin and livers of five out of five mice, and the bacterial load in the liver was restored to wild-type levels (10^{5.1} CFU/g liver). Unfortunately, this result was not reproduced in subsequent infections; sometimes virulence was restored and sometimes it was not (Figure 3-10(b)).

**Histopathology of s.c. lesions of ΔrtxA1::aph infected mice**

The gross appearance of the s.c. skin lesions formed in mice infected with the rtxA1 mutants resembled those of mice infected with CMCP6. We were interested to see if there was a difference in tissue damage at the histological level. Samples of the s.c. lesion from infected mice were fixed in 10% (v/v) buffered formalin, embedded in paraffin, and cut into 5 μm sections. Histological sections were stained with hematoxylin-eosin.

FLA943 (ΔrtxA1::aph) caused similar damage as CMCP6 at the histological level (Figure 3-11). Bacteria were present throughout the s.c. layer, and the s.c. muscle was fragmented similar to a wild type infection. Damage extending into the dermis was also observed. It has been suggested that RtxA1 may have a role in evasion/killing of immune defense cells, and while we observed an influx of neutrophils or polymorphonuclear cells (PMNs) in some sections, the majority of the PMNs observed were damaged or dead. There was also evidence of perivascular infection in the ΔrtxA1::aph lesions; however, this does not rule out the possibility of a defect in breaching the vasculature to invade into the bloodstream. Overall, despite the abolishment of cytotoxicity in cell culture, deletion of rtxA1 had little effect on tissue damage in mice. These observations indicate that perhaps RtxA1 has some other role
in virulence other than damage or killing of leukocytes, and that there are other cytotoxic factors involved in the tissue damage.

**Insertion of Ωaph at 5’ End of rtxA1**

A slight disagreement remained between results obtained with the original rtxA1::aph mutant FLA554 and the ΔrtxA1::aph mutant FLA943. Unfortunately, the failure of the complementation to fully restore virulence some of the time left this disagreement unsettled. To substantiate the virulence results, we decided to construct yet another mutation in the rtxA1 gene. Three-way USER cloning was used to construct a plasmid to insert the Ωaph cassette 100-bp into the rtxA1 open reading frame. The Ωaph cassette (67) consists of transcriptional termination signals in both directions and translational stop codons in every reading frame flanking an aph kanamycin resistance gene. By inserting the Ωaph into the 5’ end of rtxA1 gene, transcription and translation would be blocked and the RtxA1 protein would not be made. The resulting mutant CMCP6rtxA1::Ωaph, named FLA904, should have behaved similarly to the ΔrtxA1 FLA943.

**In vitro characterization of rtxA1::Ωaph**

FLA904 was examined for cytotoxicity for INT-407 cells by assessing detachment/destruction of the monolayers and LDH release, as described above. Similar to the previous rtxA1 mutants, FLA904 (rtxA1::Ωaph) was significantly reduced in its ability to cause destruction/detachment of the monolayer compared to the parent CMCP6 (11% vs. 89%, \( P = 0.001 \)) (Figure 3-12).

**Virulence of rtxA1::Ωaph in mice**

FLA904 was examined for virulence using the iron dextran-treated, s.c. injected mouse model. The rtxA1::Ωaph mutant was examined at the minimum lethal dose for
wild-type CMCP6, 300 CFU, and in 10-fold increments, 3,000 CFU and 30,000 CFU (Figure 3-13). At the lowest inoculum, FLA904 was attenuated compared to CMCP6. Three of five mice inoculated with the mutant at this dose had detectable skin infections, giving a mean of $10^{5.9}$ CFU/g isolated from the skin lesion. Of the three mice with detectable skin infection, only one had a temperature drop below 33°C, indicating the mouse was moribund. This was the only mouse with bacteria isolated from liver tissue, and at $10^{6.8}$ CFU/g liver, this amount of bacteria signified the potential of this mutant to cause systemic disease. We took into consideration the mice with no detectable CFU in the liver and assigned them a minimum detectable level, resulting in a mean liver CFU of $10^{3.2}$ CFU/g liver tissue. At a 10-fold higher inoculum (3,000 CFU), FLA904 was able to cause skin lesions in all of the mice, with a mean bacterial load of $10^{7.8}$ CFU/g in the skin. Two of the five mice remained healthy, with no detectable liver infection. The other three mice became moribund, resulting in a mean of $10^{4.7}$ CFU/g of liver, not significantly different than wild-type CMCP6. An increase in the inoculum to 30,000 CFU resulted in nearly full virulence, where four of the five mice became moribund and had a mean of $10^{5.0}$ CFU/g in liver.

**Verification of the virulence defect of the rtxA1::Ωaph mutant**

To fulfill the molecular version of Koch’s postulates and confirm that the attenuation and decreased cytotoxicity was due to the $Ωaph$ insertion in rtxA1, we attempted to revert the mutation by allelic exchange with the wild type rtxA1 5’ sequence. This proved to be technically challenging, and was unsuccessful. Taking into account that this mutant behaved similarly in vitro and in vivo as the ΔrtxA1::aph mutant, we believe that the defect seen in each of these mutants was, in fact, due to mutation of rtxA1 mutations, and not a secondary mutation elsewhere.
**RtxA1 Causes Apoptosis**

*V. vulnificus* induces apoptosis in host cells both in vitro and during infection of mice (90-92). Until recently, no specific factors have been implicated in causing apoptosis. RtxA1 and VvhA can induce apoptosis in INT-407 cells and in HUVEC cells, respectively (45,90). The rtxA1 mutants described above were tested for their ability to cause apoptosis in J774 murine macrophage-like cells. We measured apoptosis using the Apo-ONE Caspase-3/7 assay (Promega). Caspase-3 and caspase-7 are effector caspases activated during the process of apoptosis. The Apo-ONE kit utilizes a profluorescent caspase-3/7 substrate, rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide) (Z-DEVD-R110). Upon cleavage of the DEVD substrate by caspase-3 or caspase-7, the rhodamine 110 can be detected at an excitation wavelength 498 nm and emission wavelength 521 nm. The amount of fluorescence represents the amount of caspase activity in the sample.

Monolayers of J774 cells established in black 96-well tissue culture plates were infected with *V. vulnificus* at an MOI of 10. Each strain was infected in triplicate, and cells treated with gliotoxin were used as a positive apoptotic control. Each rtxA1 mutant induced significantly less apoptosis in J774 cells than CMCP6 and gliotoxin (Figure 3-14). Whereas wild-type CMCP6 induced 76% apoptosis in J774 cells, rtxA1::aph FLA554 caused 39% apoptosis (*P* = 0.0001), rtxA1::Ωaph FLA904 caused 40% apoptosis (*P* = 10^-5), and ΔrtxA1::aph FLA943 caused 27% apoptosis (*P* =10^-6). Reversion of the rtxA1::aph mutation and complementation of the ΔrtxA1::aph mutation restored apoptotic ability to these mutants. It is noteworthy that rtxA1 mutants retained the ability to cause approximately 30% apoptosis, suggesting that *V. vulnificus* has additional means of causing apoptosis in host cells.
A Combination of RtxA1 and VvhA Contributes to Cytotoxicity

Based on the above results, RtxA1 is considered to be one of the major cytotoxic factors. However, it is not the only factor contributing to cytotoxicity. Cytotoxicity of rtxA1 mutants increases with increasing MOI and increased infection times (87,88). At a MOI of 10, the rtxA1::Ωaph mutant FLA904 is cytotoxic to INT-407 cells at infection times longer than one hour (Figure 3-15). It is possible that other cytotoxic factors are produced by V. vulnificus during infection of INT-407 cells. One intriguing protein to examine is the hemolysin/cytolysin VvhA. The role of VvhA in virulence has been a source of contention for years. Originally, it was considered to be a major hemolysin and cytotoxin. Purified VvhA is highly cytotoxic in vitro, and when injected into mice, VvhA causes skin damage similar to infection with V. vulnificus (43,44). Despite the activity of the purified toxin, a vvhA mutant of V. vulnificus is as virulent in mice as is the wild-type strain (47).

Construction of a double mutation of rtxA1::Ωaph and ΔvvhA

To determine if VvhA accounts for the residual cytotoxicity observed in the rtxA1 mutants, a double mutant in vvhA and rtxA1 was created. The vvhA gene was deleted by three-way USER cloning combined with chitin-based transformation to replace the vvhA gene with tetAR encoding tetracycline resistance. The ΔvvhA mutant was named FLA912. The genomic DNA from rtxA1::Ωaph FLA904 was added to FLA912 growing in the presence of crabshell. The chitin-transformed rtxA1::Ωaph/ΔvvhA mutant was called FLA916.

Cytotoxicity of rtxA1::Ωaph/ΔvvhA

FLA912, FLA904, and FLA916 were tested for cytotoxicity to INT-407 cells. The cells were infected as before, except gentamicin was added either at 1 hr or 3 hr
postinfection to allow for a longer infection times. The cells were washed and stained with crystal violet at 4 hr postinfection to assess the detachment/destruction of the monolayers (Figure 3-15). The rtxA1 mutant FLA904 was cytotoxic when the infection proceeded for longer than 1 hr without the addition of gentamicin (17.2% destruction at 1 hr vs. 103.8% destruction at 3 hr, P=0.0008). Deletion of vvhA had no effect on cytotoxicity compared to CMCP6 (55.8% vs. 53.9% at 1 hr, P = 0.9; 82.5% vs. 101.7% destruction at 3 hr, P = 0.1). Interestingly, the double mutant FLA916 maintained reduced cytotoxicity up to 3 hr postinfection and was significantly less cytotoxic than the rtxA1 mutant alone (27.5% destruction by FLA916 vs. 103.8% destruction by FLA904, P=0.0001). Infection times longer than three hours have not been examined. Therefore, VvhA contributes to the residual cytotoxicity caused by the rtxA1 mutants during long term infections. The cytotoxicity observed when vvhA is deleted by itself is probably due to the presence of the potent cytotoxin RtxA1.

**Virulence of rtxA1::Ωaph, ΔvvhA**

The greater reduction in cytotoxicity of the double rtxA1, vvhA mutant over the rtxA1 mutant raised the question if the double mutant would be more attenuated in the mouse model. The rtxA1 mutants are still able to cause lethal infection at 3,000 CFU. Additionally, skin lesions caused by these mutants are as severe as ones caused by wild-type infection, suggesting that, despite its cytotoxicity, RtxA1 alone does not account for the tissue damage observed during infection. Most likely a combination of toxic factors contributes to this damage; therefore, it is relevant to examine the double rtxA1/vvhA for virulence in the mouse model.

FLA904, FLA912, and FLA916 were examined for virulence in mice and the ability to cause tissue damage in the skin lesion (Figure 3-16). FLA912 was as virulent as
wild-type at the low inoculum of 300 CFU (10^{7.8} CFU/g skin and 10^{5.5} CFU/g liver). FLA904 was slightly attenuated at 3,000 CFU, causing skin infections in all five mice (10^{7.9} CFU/g skin) and lethal systemic infection in two of five mice (10^{3.1} CFU/g liver). The double mutant FLA916 was not more attenuated than the FLA904 at the same inoculum of 3,000 CFU. FLA916 caused visible skin lesions in four of five mice (10^{6.6} CFU/g skin) and systemic infections in three of five mice (10^{4} CFU/g liver). The histopathology of skin lesions caused by these mutants indicated that there was still extensive tissue damage (Figure 3-17). FLA916 caused less damage in the dermis than FLA904 or FLA912; however, it was still able to cause tissue edema and necrosis in the subcutis, with few live PMNs present. These results indicate the presence of other factors involved in tissue damage.

**Prevalence of rtxA1 in V. vulnificus**

Previous studies in the laboratory have demonstrated that cytotoxicity does not necessarily correlate with virulence. We have a collection of environmental and clinical V. vulnificus strains whose genotypes, virulence in mice, and cytotoxicity phenotypes have been determined. There are several strains with little or no cytotoxicity in cell culture, and many of these strains are still virulent in mice. On the opposite end of the spectrum, we have several attenuated or avirulent strains that are as cytotoxic as CMCP6 or MO6-24/O in cell culture. Since RtxA1 is the major cytotoxic factor, we wanted to determine if the presence of RtxA1 correlated with the cytotoxicity potential of the strain.

Southern blot analysis was used to determine the prevalence of rtxA1 in our collection of strains. Four digoxigenin-labeled probes (A, B, C, D) spanning the rtxA1 gene were generated. A selection of 41 strains, encompassing clade 1 and clade 2,
with varying virulence and cytotoxicity phenotypes, were analyzed. Surprisingly, the \textit{rtxA1} probes hybridized to all strains, regardless of genetic clade, virulence, or cytotoxicity (Table 3-1). There was some variation in the sizes of the bands to which the probes A, B, and D hybridized, but with the exception of probe B, the sizes did not correlate to any specific group. Probes A and D are at each end of the gene, and the hybridization to different sized bands may be attributed to sequence variation flanking the gene. Probe B, designed to detect an internal portion of the gene upstream of the C-terminal repeats, hybridized to two different sized bands in clade 1 (environmental-type) and clade 2 (clinical-type) strains. In 15 of 20 clade 1 strains, probe B hybridized to a smaller band. In 13 of 14 clade 2 strains, probe B hybridized to a larger band. The band sizes did not correlate with cytotoxicity or virulence potential, only with genotype. The difference in size could be due to a few nucleotide changes resulting in an additional restriction site, or it could be indicative of different domains present in the \textit{rtxA1} gene among the \textit{V. vulnificus} clades.

These Southern blot analyses indicate that \textit{rtxA1} is widespread among \textit{V. vulnificus} strains; however, there is some sequence variation that may influence the potential for cytotoxicity and virulence. We have simply analyzed the presence of the \textit{rtxA1} gene, and it is possible that RtxA1 is not produced or secreted at all in the less cytotoxic strains.

\textbf{Discussion}

\textit{V. vulnificus} is highly cytotoxic in cell culture, and studies to identify the key cytotoxic factor(s) have been ongoing for more than twenty years. Initial reports of the cytotoxic activity of culture supernatants (42) initiated the interest in the VvhA cytolysin (43,44,47) and the VvpE metalloprotease (49,50,93). Treatment of cell culture or
injection of the purified proteins in mice indicated that these were cytotoxic factors that were able to cause tissue damage similar to what is observed during infection (43,44,49,93). In contradiction to studies with the purified toxins, mutation of either one or both of the genes encoding these proteins in V. *vulnificus* has no effect on virulence in mice (47,52,53). These studies left an unanswered question: What is causing the extensive tissue damage during infection?

**RtxA1 is the Major Cytotoxic Factor of V. *vulnificus***

Our laboratory first identified a gene, now known as *rtxA1*, during a pilot genomic sequencing project of MO6-24/O. Initial studies concluded that RtxA1 is one of the major cytotoxic factors of V. *vulnificus* MO6-24/O. We continued examining RtxA1 in the *V. vulnificus* strain CMCP6. An insertion of the *aph* cassette about two-thirds into the *rtxA1* gene nearly abolished cytotoxicity as it did for V. *vulnificus* MO6-24/O. Subsequent mutations, including an Ω insertion in the 5’ end of the *rtxA1* gene and a complete deletion of the gene, emphasized the importance of RtxA1 for cytotoxicity to the intestinal epithelial cell line INT-407. *V. vulnificus* causes rapid detachment/destruction of the cell monolayer within one hour postinfection, which is nearly abolished in the *rtxA1* mutants.

Most typical RTX toxins disrupt cell membranes and cause lysis of the host cells. The *V. cholerae* RtxA toxin does not form pores or cause lysis. Instead it induces cell rounding via actin crosslinking which leads to cell death (84). The RtxA1 of *V. vulnificus* does not contain the ACD causing actin crosslinking but contains other domains such as the RID, which may target other signaling pathways in the host cell. Each of the *rtxA1* mutants had a reduced ability to cause lysis of INT-407 cells, and these results were similar to what we observed for monolayer detachment/destruction. These results
indicated that RtxA1 ultimately is able to disrupt membrane permeability resulting in lysis of the cells.

While the exact mechanism of cell death by RtxA1 remains unknown, we have demonstrated that RtxA1 induces apoptosis in J774 murine macrophage-like cells (Figure 3-14). The rtxA1 mutants were defective in activating the caspase 3 and caspase 7, leading to decreased apoptosis. Lee, et al. (90) also observed the ability of RtxA1 to induce apoptosis in INT-407 cells. Interestingly, RtxA1 contains a domain that with similarity to a portion of the MCF toxin of Photorhabdus luminescens. The MCF toxin causes apoptosis in mammalian cells; however, it is uncertain if the portion of the toxin with similarity to the RtxA1 toxin is necessary for the apoptotic activity (94,95). It is important to note that RtxA1 is not the only apoptotic factor of V. vulnificus. Infection with rtxA1 mutants resulted in decreased apoptosis, but there was still residual apoptosis above background levels. Further examination into the other apoptotic factors is needed.

**RtxA1 has a Role in Virulence of V. vulnificus**

While we and others have definitively demonstrated that RtxA1 is the major cytotoxic factor of V. vulnificus, its precise role in virulence is still uncertain. Our initial mutation by insertion of the aph cassette into rtxA1 of CMCP6 resulted in attenuation of virulence in iron dextran-treated mice at a low inoculum; however, increasing the inoculum 10-fold resulted in all of the mice developing systemic infection (Figure 3-3). Despite, the noncytotoxic phenotype of the mutants (Figure 3-2), the rtxA1 mutants were still able to cause visible tissue damage in the skin lesion. At the same time, other groups reported that rtxA1 mutants caused a $10^2$- to $10^3$-fold increase in LD$_{50}$ (87-89). While this difference could be due to the use of two different mouse models of infection,
i.p. vs. s.c., we also considered that we may not have had a complete knockout mutant in rtxA1. The insertion disrupted rtxA1 approximately 10-kb downstream in the gene, just upstream of the CPD and C-terminal repeats. This meant that nearly two-thirds of the protein could have been produced. If the 5' end region of rtxA1 carried domains essential for virulence, we would see less attenuation with our mutant than if the whole gene was inactivated. Therefore, we constructed two other mutations, a complete deletion (ΔrtxA1::aph) and a 5' insertion of the Ωaph element (rtxA1::Ωaph). These mutants were slightly more attenuated in mice than the rtxA1::aph mutant. At 3,000 CFU and 5,000 CFU (10-fold to 17-fold higher than the minimum lethal dose), the new rtxA1 mutants were able to cause skin infection, with lesions similar to wild type. However, the mutants were defective at causing lethal, systemic infection, only causing systemic infection in some of the mice (Figures 3-8, 3-10, and 3-13).

These results indicated that RtxA1 was involved in virulence, more so than we originally thought. We attempted to fulfill the molecular Koch’s postulates, by cloning the wild-type rtxA1 gene into a plasmid and expressing it in the ΔrtxA1::aph mutant to complement the mutation. The complementing plasmid was able to restore cytotoxicity (Figure 3-9), but this result was not consistent. The fact that it did not consistently restore virulence does not negate the previous results (Figure 3-10). We cloned only the rtxA1 gene, but not the surrounding accessory genes. It is possible that the RtxA1 expressed in trans on a plasmid may not be properly exported by the T1SS encoded on the chromosome. In fact, not one of the other groups publishing about RtxA1 showed complementation of their mutation by expressing rtxA1 in trans during infection of mice. Kim, et al. (87) were able to complement their mutant in cytotoxicity assays by
expressing the *rtxA1*, *rtxC1*, and *rtxB1* genes in a cosmid vector, but this group did not show complementation of virulence in mice.

What is most interesting is that the deletion of *rtxA1* has no effect on tissue damage. Histopathology of the skin lesions of mice infected with the Δ*rtxA1::aph* showed similar damage in the subcutis and dermis as that caused by wild type (Figure 3-11). We were able to observe a reduction in tissue damage in lesions of Δ*rtxA1::aph* infected mice only when the amount of bacteria isolated from the lesion was low (approximately 10⁵ CFU/g skin) (not shown). This decrease in damage may be due to the reduced level of bacteria and a lower production of other factors, rather than being an effect of the absence of RtxA1. Unfortunately, we have not examined histopathology of wild-type lesions yielding low levels of bacteria to compare the damage. It is clear, however, that when the infection produces high levels of bacteria in the skin, the Δ*rtxA1::aph* mutants are capable of causing tissue damage similar to wild type.

**Are Other Factors Involved in Cytotoxicity and Tissue Damage?**

While we can conclude that RtxA1 is the major cytotoxin of *V. vulnificus*, this does not rule out presence of other accessory cytotoxins. Infection of cell culture for longer than one hour with *rtxA1* mutants causes increasing cytotoxicity (Figure 3-15) (87,88). The bacteria are likely growing rapidly during the infection of cell culture, so the cytotoxicity could be due to the increased number of bacteria in the well. Supporting this hypothesis is the fact that at increasing MOIs, the *rtxA1* mutants become more cytotoxic (87,88). It is possible that the expression of other cytotoxins will eventually lead to cytotoxicity. VvhA is the hemolysin/cytolysin previously identified to be a major cytotoxic factor of *V. vulnificus*; however, deletion of *vvhA* alone did not attenuate virulence in mice or cytotoxicity in our cell culture model, possibly because the presence
of RtxA1 causes such rapid cytotoxicity. Evidence supporting the role of vvhA as an accessory cytotoxin was provided by a vvhA/rtxA1 double mutant. The double mutant was noncytotoxic up to 3 hours postinfection in our cell culture assay (Figure 3-15), and Kim, et al. (87) reported a reduction in cytotoxicity up to 6 hours postinfection. These cytotoxicity results were intriguing, suggesting that VvhA may be contributing to the tissue damage observed in the rtxA1 mutant. Upon s.c. inoculation of iron-treated mice, the double mutant was able to cause systemic infection similar to the rtxA1 mutant (Figure 3-16). Despite its noncytotoxic phenotype, the double mutant was able to cause skin lesions with gross tissue damage that resembled lesions caused by the wild type. The histopathology of the lesions revealed that the double mutant causes slight less damage in the dermis, although it is by far not at causing damage throughout the dermis (Figure 3-17).

**Presence of the rtxA1 Gene is Widespread Among V. vulnificus Strains**

The fact that Southern blot analysis revealed that the rtxA1 gene was present in all V. vulnificus strains examined, regardless of source of isolation or genotype, is very interesting. Some of these strains are less cytotoxic and some are even non-cytotoxic compared to CMCP6 and MO6-24/O. We had expected that at least a portion of the rtxA1 gene would be absent in these strains; however, all four probes for rtxA1 hybridized to all strains examined (Table 3-1). Additionally, not all of the strains encoding rtxA1 have the potential to cause skin infection and lethal infection in mice. This result indicates that RtxA1 is not sufficient to cause virulence that perhaps RtxA1 is not expressed in these strains during infection, or that these RtxA1 toxins are missing some critical domains necessary for virulence and/or cytotoxicity. It is also likely that
the presence of RtxA1 alone is not sufficient for virulence, and that these strains are missing other key virulence factors.

**What is the Function of RtxA1 in Virulence?**

What is interesting from these studies is that in our s.c. mouse model of infection, we see less attenuation than that observed during i.p. inoculation of mice. During s.c. inoculation, the bacteria must be able to establish a local skin infection, and then invade through the tissues to reach systemic sites. Our *rtxA1* mutants are able to establish a local infection and cause tissue damage, indicating that there are other factors involved in damage. The defect of the *rtxA1* mutants, both in our s.c. mouse model and in the i.p. mouse model, is that they are not able to cause lethal infection. Kim, *et al.* (87) demonstrated a defect in the *rtxA1* mutants invading into the bloodstream after inoculation into ligated ileal loops. This result suggested that the RtxA1 toxin aids in invasion through the intestinal wall. This invasion defect could explain our results in the s.c. mouse. While *rtxA1* mutants are still able to cause establish a local infection capable of causing damage in the s.c. tissue, perhaps they cannot cause the vasodilation or damage the vasculature that may be necessary to invade into the bloodstream. Another possible role for RtxA1 may be in evading/killing host immune defenses. We have demonstrated that the presence RtxA1 is induces apoptosis in J774 macrophage-like cells (Figure 3-14). We have previously shown that very little intact PMN response occurs during s.c inoculation of mice (28), and there are of RTX toxins targeting leukocytes (96). However, the histopathology of skin lesions of Δ*rtxA1::aph* infected mice show destruction of the subcutaneous tissue and evidence of PMN killing occurring, resembling wild-type infections, suggesting that RtxA1 is not targeting PMNs. Finally, we must not disregard that RtxA1 may not have a role in tissue
damage. The presence of other rtxA genes and other putative destructive toxins suggests that a combination of these factors most likely necessary to cause destruction, and deletion of one of the factors may not be sufficient to eliminate damage.
Figure 3-1. Schematic of rtx gene clusters of *Vibrio vulnificus* strain CMCP6. The prototypical rtx operon from *E. coli* hlyA is shown at the top, with a ruler for size comparison. In CMCP6, there are two rtx gene clusters on chromosome II and one on chromosome I. rtxA encodes the RtxA toxin protein, rtxC encodes an acyltransferase, rtxB and rtxE encode ABC-transport proteins, and rtxD encodes a membrane fusion protein.
Figure 3-2. Detachment/destruction of INT-407 monolayers by \textit{rtxA1::aph} mutant. Confluent INT-407 monolayers were infected with wild-type, the \textit{rtxA1::aph} mutant FLA554, or the \textit{rtxA1::aph} reversion FLA590. Each strain was infected in triplicate for one hour prior to treatment with gentamicin. After overnight incubation, the attached cells were stained with crystal violet, and the percent of destruction/detachment caused by infection was calculated as described in Materials and Methods. FLA554 had significantly reduced cytotoxicity compared to wild-type. Cytotoxicity was restored by reversion with the wild-type allele ($P = 0.09$, reversion compared to wild type). *, $P = 0.0001$; ** $P = 0.0005$ by Fisher’s LSD for difference in mean % destruction by \textit{rtxA1::aph} compared to wild type or the reversion.
Figure 3-3. Virulence of rtxA1::aph mutant in iron dextran-treated mice. Mice were inoculated subcutaneously with either 300 CFU of the wild-type CMCP6, 300 or 3,000 CFU of the rtxA1::aph mutant FLA554, or 300 CFU of the rtxA1::aph reversion FLA590. Mice were euthanized when temperatures dropped below 33°C or at 23 hours postinoculation, and samples of the skin lesion and liver were homogenized for quantification of bacteria. All mice had skin lesions. Fractions beneath the bars indicate the numbers of mice that yielded detectable numbers of bacteria from the skin or liver samples over the number of inoculated mice. Bars without fractions indicate that bacteria were recovered from 5/5 mice. FLA554 was attenuated for systemic infection in mice compared to the wild type. Virulence was restored by reversion with the wild-type allele ($P = 0.58$, reversion vs. CMCP6). Asterisks indicate statistical significance of CFU/g liver tissue in mutant infections by Fisher’s LSD (*, $P = 0.007$; **, $P = 0.002$; ***, $P = 0.0005$; ****, $P = 0.0002$).
Figure 3-4. Skin lesions of mice infected with $rtxA::aph$. After s.c. inoculated mice were euthanized, the skin was peeled back to reveal the s.c. lesions. Lesions of mice infected with 300 CFU of (A) CMCP6, (B) FLA554 ($rtxA1::aph$), or (C) FLA590 ($rtxA1::aph$ reversion) are shown. There was no visible difference in lesions caused by FLA554 and wild type CMCP6.
Figure 3-5. Detachment/destruction of INT-407 monolayers by the ΔrtxA1::aph mutant. Confluent INT-407 monolayers were infected with wild-type, the rtxA1::aph mutant FLA554, or the ΔrtxA1 isolates, FLA900 (#1) or FLA901 (#2). After infection, the attached cells were stained with crystal violet, and % destruction/detachment was calculated. Deletion of rtxA1 caused a similar reduction in cytotoxicity as the mutation by aph insertion. Mutant strains were not significantly different from each other (P ≥ 0.5). Asterisks indicate statistical significance of mean % destruction by mutant strains compared to wild-type by Fisher’s LSD (*, P = 0.0003; **, P = 0.0002; ***, P = 0.0001).
Figure 3-6. Virulence of ΔrtxA1::aph mutants in mice. Iron dextran-treated mice were inoculated s.c. with 300 CFU of wild-type CMCP6, or 5,000 CFU of either FLA900 (ΔrtxA1 #1) or FLA901 (ΔrtxA1 #2). The two ΔrtxA1 isolates had different virulence phenotypes. Fractions beneath the bars indicate the proportion of samples that yielded bacteria. Bars without fractions indicate that bacteria were recovered from 5/5 mice. Samples were not taken from mice with no visible skin lesion, and the minimum detectable CFU/g was used for these mice for statistical analysis (10^4 CFU/g skin and 10^{2.5} CFU/g liver). Asterisks indicate statistical significance of CFU/g tissue or temperature ΔrtxA1 infections compared to wild-type infections or each other by a Fisher’s LSD (*, P = 0.05; **, P = 0.01; *** P = 0.002; ****, P = 0.0004; ***** P =10^{-6}). A dagger indicates statistical significance of number of samples yielding bacteria in the mutant infection compared to wild-type by χ² test (†, P = 0.04).
Figure 3-7. Detachment/destruction of INT-407 cell monolayers by chitin-recreated $\Delta rtxA1$ mutants. Confluent INT-407 monolayers were infected with wild-type or the recreated $\Delta rtxA1$ mutants, FLA943 ($^\#1$) or FLA923 ($^\#2$). After infection, the attached cells were stained with crystal violet and % destruction/detachment was calculated. The chitin recreated $\Delta rtxA1$ mutants had reduced cytotoxicity. *, $P = 0.005$; **, $P = 0.003$ by Fisher’s LSD for difference in mean % destruction by mutant strains compared to wild-type.
Figure 3-8. Virulence of ΔrtxA1::aph mutants recreated by chitin transformation. Iron dextran-treated mice were s.c. inoculated with 5,000 CFU of either ΔrtxA1 #1 chitin recreated mutant FLA943 or ΔrtxA1 #2 chitin-recreated mutant FLA923. The two chitin-recreated had similar virulence in mice ($P > 0.7$). Fractions beneath the bars indicate the proportion of samples that yielded bacteria. Bars without fractions indicate bacteria were recovered from 5/5 mice. Samples were not taken from mice with no visible skin lesion, and the minimum detectable CFU/g was used for these mice for statistical analysis.
Figure 3-9. Cytotoxicity to INT-407 cells by ΔrtxA1::aph complemented with wild-type rtxA1 in trans. INT-407 monolayers were infected with either wild-type CMCP6, the ΔrtxA1 mutant FLA943, or FLA943 carrying the complementation plasmid pGTR1227. Cytotoxicity was restored to FLA943 by expressing rtxA1 in trans on pGTR1227. (A) The amount of detachment/destruction of the cell monolayers was determined by crystal violet assay. (B) Lysis of INT-407 cells was measured by LDH release. Asterisks indicate statistical significance by Fisher’s LSD comparing ΔrtxA1 to CMCP6 or to complemented mutant (*, $P = 0.003$; **, $P \leq 10^{-6}$). The complemented mutant was not significantly different from CMCP6 ($P > 0.7$).
Figure 3-10. Complementation of virulence of ΔrtxA1::aph by expressing wild-type rtxA1 in trans on pGTR1227. Iron dextran-treated mice were inoculated with 1,000 CFU of either CMCP6, ΔrtxA1 mutant FLA943, or FLA943 complemented with pGTR1227. Fractions beneath the bars indicate the proportion of samples that yielded bacteria. (A) Virulence was restored in the initial test of complemented mutant. *, P = 0.03; **, P = 0.02; ***, P = 0.0002; ****, P < 0.0001 by Fisher's LSD comparing ΔrtxA1 to CMCP6 or to the complemented mutant. The complemented mutant was not significantly different from CMCP6. (B) Virulence was not restored in a separate experiment. *, P = 0.007; **, P = 0.005; ***, P < 0.0002 by Fisher's LSD comparing ΔrtxA1 or complemented mutant to wild-type. Daggers indicate statistical significance of numbers of samples yielding bacteria in the mutant or complemented infection compared to wild-type by χ² test (†, P = 0.04; ††, P = 0.01).
Figure 3-11. Histopathology of s.c. lesions of ΔrtxA1-infected mice. Mice were s.c. inoculated with either CMCP6 or FLA943 (ΔrtxA1). After infection, tissue samples were collected, fixed in buffered formalin, embedded in paraffin, and cut into 5-µm sections. Sections were stained with hematoxylin and eosin. Magnifications, x100 (A through C) and x400 (D through E). (A) Uninfected mouse skin. Epidermis (e), dermis (d), and subcutis (s) with adipocytes, blood vessels, and muscle layer. (B) Skin of mouse infected with CMCP6. Extensive edema throughout subcutis layer (*), and edema and necrosis extending into dermis (arrow). Fragmentation and destruction of muscle layer (arrowhead). (C) Skin of mouse infected with FLA943 (ΔrtxA1). Severe infection and edema in subcutis (*), with damage extending into dermis (arrow). Muscle layer is fragmented, similar to the CMCP6 infected mouse (arrowhead). (D) CMCP6-infected mouse skin showing very few live neutrophils (PMNs) present (arrowhead). Most PMNs are necrotic or degenerated (arrows). (E) Skin of mouse infected with FLA943. More PMNs are present in subcutis (arrowheads); however, many of them are dying or necrotic (arrows). (F) Perivascular infection caused by FLA943. Clot forming within blood vessel (arrow). Staining indicates suggests presence of bacteria surrounding the blood vessel (*).
Figure 3-12. Detachment/destruction of INT-407 monolayers by \rtxA1::\Omegaaph\ mutant.
INT-407 monolayers were infected with CMCP6, the \Delta\rtxA1 mutant FLA901, or the \rtxA1::\Omegaaph mutant FLA904. The amount of destruction of the cell monolayers was determined by staining with crystal violet. FLA904 had a similar reduction in cytotoxicity as FLA901, and the two strains were not significantly different from each other. Asterisks indicate statistical significance by Fisher’s LSD comparing FLA901 or FLA904 to wild-type (*, \( P = 0.002 \); **, \( P = 0.001 \)).
Figure 3-13. Virulence of the rtxA1::Ωaph mutant at increasing inocula. Iron dextran-treated mice were inoculated s.c. with wild-type CMCP6 or rtxA1::Ωaph FLA904 at inocula of 300 CFU, 3,000 CFU, or 30,000 CFU. Virulence of FLA904 increased at higher inocula. Fractions beneath the bars indicate the proportion of samples that yielded bacteria. Bars without fractions indicate that bacteria were recovered from 5/5 mice. Samples were not taken from mice with no visible skin lesion, and the minimum detectable CFU/g was used for these mice for statistical analysis. *, \( P = 0.03 \) by Fisher’s LSD comparing FLA904 (300 CFU) to CMCP6 or FLA904 (3,000 CFU) and **, \( P = 0.01 \) by Fisher’s LSD comparing FLA904 (300 CFU) to FLA904 (30,000 CFU). FLA904 inoculated at 3,000 CFU or 30,000 CFU caused no significant difference in CFU/g of skin compared to wild type. There was no significant difference in liver or systemic infection caused by FLA904 compared to wild type.
Figure 3-14. Apoptosis of J774 cells infected with rtxA1 mutants. J774 monolayers seeded in black 96-well tissue culture plates were infected with either wild-type CMCP6, FLA554 (rtxA1::aph), FLA590 (rtxA1::aph reversion), FLA904 (rtxA1::Ωaph), FLA943 (∆rtxA1::aph), or FLA943(pGTR1227) (complemented ∆rtxA1). Caspase-3/7 activity was measured using the Apo-ONE Homogeneous Caspase-3/7 assay. % apoptosis was calculated by dividing the normalized relative fluorescence units (RFU) for each sample by the mean normalized RFU for gliotoxin treated wells (considered 100% apoptosis). The rtxA1 mutants induced less apoptosis than the wild type and gliotoxin (positive control). The ability to cause apoptosis was restored by reversion or complementation. FLA590 caused more apoptosis than CMCP6 \( P \leq 0.0001 \); however, the complemented FLA943 (pGTR1227) caused significantly lower apoptosis than CMCP6 \( P = 0.002 \). Asterisks indicate statistical significance by Fisher’s LSD comparing each strain to wild type or to the strain indicated by lines (*, \( P = 0.002 \); **, \( P \leq 0.0001 \); ***, \( P < 10^{-5} \); ****, \( P < 10^{-6} \)).
Figure 3-15. Detachment/destruction of INT-407 monolayers infected with rtxA1, vvhA, or rtxA1/vvhA mutants. INT-407 monolayers were infected with wild-type CMCP6, the rtxA1::Ωaph mutant FLA904, the ΔvvhA mutant FLA912, or the double rtxA1/vvhA mutant FLA916 at an MOI of 10. Each strain was infected in triplicate. Gentamicin was added either 1 hour or 3 hours postinfection, and monolayer detachment/destruction was assessed by crystal violet staining at 4 hours postinfection. FLA904 was cytotoxic after 3 hours. This cytotoxicity was reduced upon inactivation of rtxA1 and vvhA. Asterisks indicate statistical significance by Fisher’s LSD of mutant to either wild-type or the strain indicated by lines (*, \(P = 0.02\); ** \(P = 0.001\); *** \(P = 0.0001\)).
Figure 3-16. Virulence of double *rtxA*1::Ωaph, Δ*vvhA*::*tet* mutant. Iron dextran-treated mice were inoculated s.c. with either 300 CFU of wild type CMCP6, 3,000 CFU of *rtxA*1::Ωaph mutant FLA904, 3,000 CFU of *rtxA*1::Ωaph/Δ*vvhA* mutant FLA916, or 300 CFU of Δ*vvhA* mutant FLA912. FLA916 was not more attenuated than FLA904, indicating that *vvhA* was not necessary for virulence. Fractions beneath the bars indicate the proportion of samples that yielded bacteria. Bars without fractions indicate that bacteria were recovered from 5/5 mice. Samples were not taken from mice with no visible skin lesion, and the minimum detectable CFU/g was used for these mice for statistical analysis. Statistical significance is calculated by a Fisher’s LSD (*, *P* = 0.01 for FLA904 to CMCP6; **, *P* = 0.02 for FLA916 to FLA912).
Figure 3-17. Histopathology of s.c. lesions of mice infected with ΔvvhA and rtxA1::Ωaph mutants. Mice were s.c. inoculated with FLA912 (ΔvvhA), FLA904 (rtxA1::Ωaph), or FLA916 (rtxA1::Ωaph, ΔvvhA). After infection, tissue samples were collected, fixed in buffered formalin, embedded in paraffin, and cut into 5-µm sections. Sections were stained with hematoxylin and eosin. Magnification x100 (A) Skin of mouse infected with FLA912. Edema and destruction of the tissues through the subcutis and extending into the dermis (*). Heavy staining of subcutis beneath muscle layer indicates massive bacterial infection in this region (arrow). (B) Skin of mouse infected with FLA904. Extensive destruction in the subcutis layer, including fragmentation of the muscle layer indicated by an arrows. Necrosis extending into dermis indicated by an arrowhead. (C) Skin of mouse infected with FLA916. Infection and edema in subcutis (arrow) with damage to the muscle (arrow). Dermis was mostly intact, with very little edema (arrow). The double rtxA1::Ωaph, ΔvvhA mutant was still able to cause significant tissue damage, although damage was less severe in the dermis than the rtxA1::Ωaph mutant.
Table 3-1. Southern blot to detect *rtxA1* in *V. vulnificus* isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>16s rRNA</th>
<th>RAPD</th>
<th>MLST</th>
<th>Skin CFU</th>
<th>Liver CFU</th>
<th>Cytotoxicity (%)</th>
<th>Probe A</th>
<th>Probe B</th>
<th>Probe C</th>
<th>Probe D</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMCP6</td>
<td>B</td>
<td>C</td>
<td>2</td>
<td>7.2</td>
<td>4.3</td>
<td>93.9</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA101</td>
<td>AB</td>
<td>E</td>
<td>1</td>
<td>8.0</td>
<td>4.9</td>
<td>62.9</td>
<td>+</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA102</td>
<td>B</td>
<td>C</td>
<td>2</td>
<td>8.3</td>
<td>7.3</td>
<td>59.1</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA104</td>
<td>A</td>
<td>E</td>
<td>1</td>
<td>6.8</td>
<td>3.4</td>
<td>100.0</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA105</td>
<td>B</td>
<td>C</td>
<td>2</td>
<td>5.9</td>
<td>2.6</td>
<td>96.0</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA106</td>
<td>AB</td>
<td>E</td>
<td>1</td>
<td>6.1</td>
<td>3.2</td>
<td>95.7</td>
<td>+</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA107</td>
<td>A</td>
<td>E</td>
<td>1</td>
<td>8.3</td>
<td>4.7</td>
<td>43.6</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA108</td>
<td>A</td>
<td>E</td>
<td>ND</td>
<td>6.8</td>
<td>2.5</td>
<td>100.0</td>
<td>+</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA109</td>
<td>AB</td>
<td>E</td>
<td>1</td>
<td>6.0</td>
<td>3.7</td>
<td>99.0</td>
<td>+</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA111</td>
<td>A</td>
<td>E</td>
<td>1</td>
<td>7.2</td>
<td>3.0</td>
<td>100.0</td>
<td>+</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FLA112</td>
<td>B</td>
<td>C</td>
<td>2</td>
<td>6.1</td>
<td>3.4</td>
<td>100.0</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA113</td>
<td>B</td>
<td>C</td>
<td>2</td>
<td>5.9</td>
<td>3.7</td>
<td>100.0</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA114</td>
<td>AB</td>
<td>E</td>
<td>1</td>
<td>8.1</td>
<td>6.7</td>
<td>100.0</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>FLA115</td>
<td>AB</td>
<td>E</td>
<td>1</td>
<td>8.4</td>
<td>4.5</td>
<td>100.0</td>
<td>+</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FLA116</td>
<td>B</td>
<td>C</td>
<td>2</td>
<td>8.4</td>
<td>3.3</td>
<td>62.9</td>
<td>+</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FLA117</td>
<td>B</td>
<td>C</td>
<td>2</td>
<td>8.5</td>
<td>5.4</td>
<td>99.7</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA118</td>
<td>A</td>
<td>E</td>
<td>1</td>
<td>4.6</td>
<td>2.5</td>
<td>100.0</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FLA119</td>
<td>A</td>
<td>E</td>
<td>1</td>
<td>6.0</td>
<td>2.5</td>
<td>38.1</td>
<td>ND</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA120</td>
<td>B</td>
<td>E</td>
<td>1</td>
<td>7.3</td>
<td>2.5</td>
<td>85.0</td>
<td>+</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA121</td>
<td>A</td>
<td>E</td>
<td>1</td>
<td>6.3</td>
<td>2.5</td>
<td>90.3</td>
<td>ND</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA122</td>
<td>A</td>
<td>E</td>
<td>1</td>
<td>7.0</td>
<td>2.6</td>
<td>2.0</td>
<td>ND</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA125</td>
<td>A</td>
<td>E</td>
<td>1</td>
<td>8.3</td>
<td>5.4</td>
<td>100.0</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FLA128</td>
<td>A</td>
<td>E</td>
<td>1</td>
<td>7.7</td>
<td>2.5</td>
<td>87.9</td>
<td>+</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA136</td>
<td>B</td>
<td>C</td>
<td>2</td>
<td>8.1</td>
<td>3.5</td>
<td>66.7</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA137</td>
<td>AB</td>
<td>E</td>
<td>1</td>
<td>7.9</td>
<td>2.5</td>
<td>100.0</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA139</td>
<td>B</td>
<td>C</td>
<td>2</td>
<td>4.9</td>
<td>2.8</td>
<td>99.9</td>
<td>ND</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA140</td>
<td>B</td>
<td>C</td>
<td>2</td>
<td>5.9</td>
<td>2.5</td>
<td>91.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FLA141</td>
<td>AB</td>
<td>E</td>
<td>1</td>
<td>6.9</td>
<td>2.7</td>
<td>20.8</td>
<td>+</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA142</td>
<td>A</td>
<td>E</td>
<td>1</td>
<td>7.9</td>
<td>2.8</td>
<td>82.3</td>
<td>ND</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FLA143</td>
<td>AB</td>
<td>E</td>
<td>1</td>
<td>6.5</td>
<td>2.5</td>
<td>98.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FLA144</td>
<td>AB</td>
<td>E</td>
<td>1</td>
<td>7.8</td>
<td>5.7</td>
<td>39.8</td>
<td>+</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA145</td>
<td>A</td>
<td>E</td>
<td>1</td>
<td>7.7</td>
<td>3.6</td>
<td>41.4</td>
<td>+</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FLA146</td>
<td>B</td>
<td>C</td>
<td>2</td>
<td>6.1</td>
<td>2.5</td>
<td>30.6</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA147</td>
<td>B</td>
<td>C</td>
<td>2</td>
<td>7.9</td>
<td>5.3</td>
<td>26.4</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLA148</td>
<td>A</td>
<td>E</td>
<td>ND</td>
<td>6.7</td>
<td>4.8</td>
<td>22.8</td>
<td>+</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MO6-24/O</td>
<td>B</td>
<td>C</td>
<td>2</td>
<td>9.8</td>
<td>5.7</td>
<td>93.9</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LL728</td>
<td>B</td>
<td>C</td>
<td>7.5</td>
<td>4.8</td>
<td>37.3</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MLT367</td>
<td>B</td>
<td>C</td>
<td>3.5</td>
<td>2.5</td>
<td>84.4</td>
<td>ND</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>YJ016</td>
<td>B</td>
<td>C</td>
<td>2</td>
<td>7.7</td>
<td>5.7</td>
<td>ND</td>
<td>+</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>302/99</td>
<td>AB</td>
<td>E</td>
<td>ND</td>
<td>7.8</td>
<td>5.7</td>
<td>91.3</td>
<td>ND</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>313/98</td>
<td>AB</td>
<td>E</td>
<td>ND</td>
<td>7.0</td>
<td>2.8</td>
<td>3.1</td>
<td>ND</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*+, hybridization; ND, No data/probe not tested; 1, hybridization to small band; 2, hybridization to large band.*
CHAPTER 4
THE ROLE OF THE OTHER RTX LOCI IN VIRULENCE OF V. vulnificus AND THE IMPORTANCE OF ACTIVATION OF THE RtxA TOXINS

Rationale for Study

Deletion of rtxA1 nearly abolishes cytotoxicity; however, the RtxA1 toxin does not appear to be as important for virulence in the s.c. model of infection as it is for virulence after i.p. inoculation studied by other laboratories (87,88). In the s.c. injected mouse model, the rtxA1 mutants are still able to cause skin lesions comparable to the wild-type and they are able to cause systemic infection with an inoculum of 3,000 CFU (Figures 3-7 and 3-11). To provide a more complete analysis of the RTX toxins of V. vulnificus we wanted to examine the other two RTX-like proteins, RtxA2 and RtxA3, as well as the necessity of RtxC1 for activity of the RtxA toxins. There was a possibility that the RtxA toxins encoded at these other loci could account for residual virulence in the absence of RtxA1. We investigated the role of RtxC1 and the putative RTX toxins through mutagenesis, followed by cell culture analysis and in vivo infections.

Introduction

V. vulnificus encodes three rtx gene loci. The RtxA1 toxin proved to be the major cytotoxic factor in vitro, and it contributes to virulence in s.c. inoculated, iron dextran-treated mice. This locus encodes a complete MARTX system (56) (Figure 3-1). Upstream in the rtxA1 operon is rtxC1, annotated as a hemolysin acyltransferase. RtxC1 has 96% identity to the V. cholerae RtxC and 32% identity to the HlyC of E. coli. HlyC activates the E. coli HlyA protein by posttranslational fatty acylation on lysine residues of the HlyA (77). Other previously studied RTX toxins also require activation by acylation of the RtxA protein (75,76,78), but this has not been shown for V. cholerae RtxA.
The other two rtx loci are incomplete by consensus RTX standards. The second locus encompasses the 14-kb gene VV2_1514 encoding RtxA2, a putative RTX toxin. Included in this locus are genes encoding RtxB2 and RtxD2, which are annotated to be involved in secretion of RtxA2. The third rtx3 locus includes only the 8.8-kb gene VV1_2715 encoding an RTX-like protein, RtxA3, which at 2,937 amino acids is smaller than RtxA1 and RtxA2.

To address if there is redundancy in the functions of the RtxA toxins, we constructed an insertional mutation in the rtxA2 gene and deleted the rtxA3 gene. The mutations either were combined or are in the process of being combined with rtxA1 mutations to produce double and triple rtxA mutants. Given that rtxA1 mutants alone had a vast reduction in cytotoxicity to INT-407 cells, we did not expect mutations in rtxA2 and rtxA3 to have an effect on cytotoxicity. However, there remained the possibility that the RTX toxins act in concert and that mutation of any one of them could affect cytotoxicity or virulence. If the RtxA1 toxin is not the only RTX toxin contributing to virulence, we would observe attenuation of the rtxA2 and rtxA3 mutants in the s.c. inoculated mouse.

We also investigated the role of the RtxC1 acyltransferase in activation of the RtxA toxins by deletion of the rtxC1 gene. Typical RtxA toxins are activated by the RtxC enzyme encoded in the same operon, so we anticipated that RtxC1 would be necessary for RtxA1 activity and probably not involved in activation of the other two RtxA toxins. However, because there are no rtxC homologs in the regions surrounding rtxA2 and rtxA3, we considered the possibilities that these RTX toxins were either not active RTX toxins, or that the RtxC1 in the rtx1 locus could also activate these RTX toxins.
Results

Identification and Examination of RtxA2

Upon examining the published genomic sequence of CMCP6, we were immediately interested in the gene VV2_1514 due to its large size comparable to the VV2_0479 rtxA1 gene. The VV2_1514 gene is 14-kb, about 1.6 kb smaller than the rtxA1 gene, which is the largest known gene of V. vulnificus. According to the NCBI Conserved Domain search, glycine-rich peptide repeats similar to RTX-repeats are located in the C-terminus of the RtxA2 protein. The repeat region and the large size of the protein indicated that it may be a member the RTX family. Just downstream of rtxA2 are the putative secretion genes VV2_1515, annotated as a toxin secretion ATP-binding protein, and VV2_1516, annotated as an HlyD family protein. BLAST analysis revealed that the amino acid sequences of the two proteins have approximately 25% identity to the RtxB1 and RtxD1 proteins, respectively. The similarities with these and other T1SS proteins provide support that the products of these genes should be called RtxB2 and RtxD2, and that these proteins are components of a secretion system for RtxA2.

Despite the evidence that the rtx2 locus encodes a putative RtxA2 toxin secreted by a T1SS, BLAST analysis revealed very low sequence similarity between the amino acid sequence of RtxA2 and either the V. vulnificus RtxA1 or V. cholerae RtxA. While RtxA2 contains RTX-like repeats in the C-terminus, the N-terminal repeats of the MARTX family have not yet been identified in RtxA2. The lack of similarity, our inability to identify the conserved N-terminal repeats, and the absence of nearby accessory genes rtxC and rtxH support that the RtxA2 may not be a true member of the MARTX
subfamily. However, RtxA2 could still be a member of the RTX family, and therefore warranted study as a potential virulence factor.

**Mutation of rtxA2 to Examine a Role in Virulence**

**Construction of rtxA2::Ω**

A mutation in gene VV2_1514, rtxA2, was constructed by insertion of the translational stop sequences of the Ω-element (67). For insertion of the Ω into the 5’ end of the rtxA2 gene, a 1-kb fragment was PCR amplified using the oligonucleotides rtxA2-A and rtxA2-B and cloned into pCR2.1 by TOPO-TA Cloning (Invitrogen) to create pGTR256. The Ωaph element was excised from pBR322:Ωaph by BsmHI digestion and subcloned at a unique SspI restriction site within the rtxA2 fragment. The rtxA2::Ωaph was excised using restriction endonucleases XbaI and SstI and was subcloned into pGTR260 (pUC19 with the HindIII restriction site destroyed). The resulting plasmid was named pGTR261. Because one of the ultimate goals was to combine the rtxA2 mutation with the rtxA1 mutation selected by kanamycin resistance, the kanamycin resistance cassette of the Ωaph needed to be removed. The transcriptional stop signal and the kanamycin resistance cartridge were removed by HindIII digestion, gel extraction, and religation of the plasmid to yield pGTR262, carrying the 1-kb rtxA2 region interrupted by 35-bp of the Ω element with translation stop codons in all six reading frames. The rtxA2::Ω fragment was excised by XbaI and SstI and inserted into the allelic exchange vector pGTR1122 (pCVD442::lacZ, cat) yielding pGTR263. The allelic exchange vector was moved into V. vulnificus FLA399 (a spontaneous rifampicin-resistant isolate of CMCP6) via conjugation. rtxA2 mutants were selected by the sacB-assisted allelic exchange process, and insertion of the 35-bp Ω sequence was
confirmed by PCR using the primers rtxA2-A and rtxA2-B. The FLA399rtxA2 mutant was named FLA441.

**Cytotoxicity of the rtxa2::Ω mutant**

The RtxA1 toxin of *V. vulnificus* has an obvious role in cytotoxicity, and the large reduction observed during infection of cells with *rtxA1* mutants implied that the other RTX toxins produced by *V. vulnificus* do not have the same level of cytotoxicity as Rtxa1 in this cell culture model. However, if all of the RTX toxins are essential for cytotoxicity, then deleting any one of them would abolish cytotoxicity. Therefore, it was possible that the *rtxA2* mutant would have a reduction in cytotoxicity to INT-407 cells. INT-407 monolayers were seeded in 24-well plates and infected with either wild type FLA399 or the *rtxA2* mutant FLA441. Each strain was infected in triplicate, and uninfected wells and wells containing media alone served as positive and negative controls, respectively. FLA399 *rtxA1::aph* was used as a control for a less cytotoxic strain. It should be noted that FLA399 *rtxA1::aph* behaves similarly to FLA554 (CMCP6 *rtxA1::aph*) during infection of cell culture and mice. Interestingly, mutation of the *rtxA2* gene caused no reduction in the detachment or destruction of the monolayers compared to FLA399 (77.8% vs. 82.6%, respectively, \( P = 0.6 \)), whereas the *rtxA1::aph* was less cytotoxic (16.4%, \( P = 0.0002 \) compared to FLA399) (Figure 4-1). These results implied that RtxA1 was the main RTX toxin causing cytotoxicity in INT-407.

**Virulence of the rtxA2::Ω mutant in iron-treated mice**

The iron dextran-treated mouse model was used to assess the virulence of the *rtxA2* mutant. S.c. inoculations of 300 CFU of FLA441 caused all mice to become moribund. The levels of bacteria isolated from the skin lesion (10^6 CFU/g) and the liver (10^5 CFU/g) were comparable to what is observed with the wild-type (Figure 4-2).
Therefore, RtxA2 is not necessary for virulence, but may be a redundant factor to RtxA1. If this is the case, construction a double mutation in rtxA1 and rtxA2 would be more attenuated than the single rtxA mutants.

**Double Mutation of rtxA1 and rtxA2**

To investigate the role of RtxA2 as an accessory toxin involved in virulence, a double mutant with the rtxA1 mutation combined with the rtxA2::Ω mutation was constructed. We constructed a double mutant by moving the pGTR263 (rtxA2::Ω allelic exchange plasmid) into FLA399 rtxA1::aph by conjugation, followed by sacB-assisted allelic exchange. The resulting rtxA1::aph, rtxA2::Ω double, named FLA439, was attenuated in cytotoxicity similar to the rtxA1::aph mutant (data not shown), confirming that RtxA2 was not involved in cytotoxicity. The mutant was similarly attenuated in virulence, similarly to the rtxA1::aph mutant; however, there was experimental variation in virulence.

Due to concerns over the virulence of the FLA399 background in relation to other mutant strains in the laboratory, we discontinued the use of mutants created in FLA399 and reconstructed all of the mutations in the wild-type CMCP6 background. The CMCP6 rtxA1::aph, named FLA554, is discussed in detail in Chapter 3. The rtxA2::Ω mutant of CMCP6, named FLA561, behaved similarly to FLA441 (FLA399 rtxA2::Ω) in cell culture. The double mutant was constructed as before and the resulting strain, FLA558, was examined.

Similar to results from the mutants in the FLA399 background, RtxA2 was not necessary for cytotoxicity (Figure 4-3). The double mutant FLA558 caused 13% destruction of INT-407 monolayers, which was not significantly different from the 9%
destruction caused by the \textit{rtxA1} mutant FLA554. As expected, CMCP6 and the \textit{rtxa2::\Omega} FLA561 were highly cytotoxic to INT-407 cells.

\textbf{Virulence of double \textit{rtxA1}/\textit{rtxA2} mutant in mice}

FLA554 (\textit{rtxA::aph}) or FLA558 (\textit{rtxA::aph, rtxA2::\Omega}) were s.c. inoculated into iron dextran-treated mice to determine if the double mutant was more attenuated than the single mutant (Figure 4-4). When inoculated at 300 CFU, FLA554 caused skin lesions in four of four mice and systemic infection in two of four mice. Means of $10^{7.4}$ CFU/g and $10^{3.3}$ CFU/g of bacteria were isolated from the skin and liver, respectively. The double mutant, FLA558, was more attenuated than the FLA554 mutant at this inoculum. Of the five mice infected, three had a detectable skin infection, but only one of these mice had a wild-type skin lesion with $10^8$ CFU/g. The other two mice had small skin lesions with very low levels of bacteria in the skin lesion ($10^{3.8}$ CFU/g and $10^{3.3}$ CFU/g). Not one of the five mice infected with FLA558 had a detectable infection of the liver, indicating that the double mutant was not able to cause systemic infection at an inoculum of 300 CFU (Figure 4-4 (A)). Based on these results, RtxA2 appeared to be contributing to the initial local infection in the skin lesion, since only one of the mice had a wild-type skin infection. If the double mutant is less able to cause a local infection, then it would also be unable to cause a systemic infection, which is the result we observed in this experiment.

We increased the inoculum of FLA554 and FLA558 to 3,000 CFU (Figure 4-4 (B)) to further test the level of attenuation of these mutants. The results of this higher inoculum reflected the inconsistency in the virulence of the \textit{rtxA1} mutant. Four of the five FLA554-infected mice developed skin lesions similar to the wild-type ($10^{7.1}$ CFU/g of skin); however, only one of these mice developed a detectable systemic infection,
although much lower (10^{2.6} CFU/g of liver) than what is observed during a typical wild-
type infection (approximately 10^5 CFU/g). In contrast to results from infection with 300 
CFU, infection with 3,000 CFU caused the double mutant FLA558 to be slightly more 
virulent than FLA554. Every mouse infected with FLA558 developed a skin lesion 
containing a high level of bacteria (10^{7.9} CFU/g of skin). Two of the five mice became 
moribund, with temperatures below 33°C and a high amount of bacteria in their livers 
(10^{4.5} CFU/g and 10^{3.5} CFU/g). The other three mice had no detectable liver infection. 
The mean liver infection of FLA558-infected mice was not significantly different from the 
level infection in FLA554-infected mice (10^{3.0} CFU/g vs. 10^{2.3} CFU/g, P = 0.15). 
Therefore, inoculation of a higher dose of the mutants gave the impression that RtxA2 is 
disposable for virulence in mice.

**Construction of ΔrtxA1::aph, rtxA2::Ω**

The inconsistency in the virulence of the rtxA1::aph mutant at low and high inocula 
could be a reason for the attenuation observed for the double mutant at 300 CFU. The 
attenuation may not be due to the rtxA2 mutation; instead, it is more likely due to 
experimental fluctuation observed with rtxA1::aph. After examining the other rtxA1 
mutant constructs, discussed in Chapter 3, we felt that the deletion of rtxA1 provided a 
more consistent virulence phenotype in mice. Therefore, the deletion was a better rtxA1 
mutant background in which to study the effect of the rtxA2 mutation. Genomic DNA of 
the ΔrtxA1::aph mutant FLA943 was used to move the ΔrtxA1::aph mutation into 
FLA561 via chitin-induced natural transformation. Transformants were selected on LB-
N with kanamycin and verified by PCR. The ΔrtxA1::aph, rtxA2::Ω mutant strain was 
named FLA947.
Cytotoxicity of ΔrtxA1::aph, rtxA2::Ω

The mutant was first examined for its ability to cause lysis in INT-407 cells (Figure 4-5). Infection with FLA947 caused 12.4% LDH release, which was not significantly lower than 16.4% LDH release by cells infected with the rtxA1 mutant FLA943 ($P = 0.3$). Therefore, as we expected from the previous results, RtxA2 was not contributing to cytotoxicity to INT-407 cells.

Virulence of ΔrtxA1::aph, rtxA2::Ω

We next examined the double mutant for virulence in mice. FLA943 typically caused infection in some of the mice at an inoculum of 3,000 CFU, and so if FLA947 was more attenuated than the FLA943, we would observe complete attenuation at this inoculum. In agreement with the double mutant FLA558 described above, the FLA947 was not more attenuated than the ΔrtxA1::aph FLA943 (Figure 4-6). At an inoculum of 3,000 CFU, FLA943 caused detectable skin and liver infection in three of five mice, with mean yields of $10^{6.6}$ CFU/g from skin and $10^{3.1}$ CFU/g from liver. FLA947 was able to cause detectable infection in five of five mice, although the amount of bacteria recovered was not significantly higher than the amount recovered from FLA943 ($10^{7.7}$ CFU/g skin, $P = 0.3$; and $10^{4.3}$ CFU/g liver, $P = 0.1$). Although the double mutant FLA947 caused skin lesions resembling those of wild type infections, it would be interesting to examine the histopathology of the skin lesions of mice infected with the ΔrtxA1::aph, rtxA2::Ω mutant and compare it to the lesions of mice infected with ΔrtxA1::aph or rtxA2::Ω mutants. This would reveal if there are any differences in damage caused in the skin lesion.
Identification and Examination of RtxA3

In addition to the \textit{rtx1} and \textit{rtx2} loci, CMCP6 encodes a third, putative \textit{rtxA} gene, VV1\_2715, which we have designated \textit{rtxA3}. Unlike the other two \textit{rtx} loci, it appears that \textit{rtxA3} is the only \textit{rtx} gene in the region. The typical type I secretion proteins, \textit{rtxB} and \textit{rtxD}, are not found in the surrounding region as they are in the \textit{rtx1} and \textit{rtx2} gene clusters. The size of the \textit{rtxA3} gene is 8.8 kb, which is much shorter than the other two \textit{rtxA} genes, yet still larger than most \textit{V. vulnificus} genes. There is very little sequence similarity between the CMCP6 RtxA toxins at the amino acid level. The C-terminal region of RtxA3 has 36\% similarity with the C-terminal repeat region of RtxA1, but these sequences are not as conserved in RtxA2. The internal portion of the RtxA3 sequences has less than 40\% similarity with the RtxA2 sequence. The RtxA3 sequence was used in a BLAST search of the \textit{E. coli} sequences in the NCBI database, and although there was low similarity, every hit was an RTX protein including the prototypical HlyA and larger putative RTX toxins of \textit{E. coli}. The most conserved region of these proteins is at the C-terminal end. A Prosite scan through ExPASy identified a series of Hemolysin Ca\textsuperscript+\,-Binding repeats, also known as the RTX repeats, in the C-terminal end of RtxA3. These repeats are also used to predict type I secretion of proteins. Evidence of part of a T1SS is found just downstream of \textit{rtxA3}. Gene VV1\_2752 encodes an outer membrane protein with homology to the TolC family of proteins involved in TISS. As mentioned above, the other TISS proteins, ABC transport protein and membrane fusion protein, are not encoded near to the \textit{rtxA3} gene.

The presence of the RTX glycine-rich repeats indicates RtxA3 is accurately labeled as an RTX toxin. The absence of the \textit{rtxCBD} genes suggests that RtxA3 may not be activated or secreted; therefore, it may not be a functional RTX toxin. To provide
a more complete analysis of RTX toxins of *V. vulnificus*, we examined an *rtxA3* mutant for its role in pathogenesis.

**Deletion of the *rtxA3* Gene to Examine a Role in Virulence**

**Construction of the Δ*rtxA3* mutant**

An allelic exchange vector for deletion of *rtxA3* was constructed by three-way USER cloning. Upstream and downstream sequences of *rtxA3* were PCR amplified using oligonucleotides with USER-friendly ends. A USER-Smal site was used to join the upstream and downstream fragments. The PCR products were captured in pGTR1129, resulting in the plasmid pGTR268. The plasmid was conjugated into CMCP6, and deletion of *rtxA3* was selected by the *sacB*-assisted allelic exchange process. Deletion of *rtxA3* was verified by PCR, and the resulting mutant was named FLA899. There was no antibiotic resistance cassette used for allelic exchange, so that the Δ*rtxA3* mutation could be used in combination with any antibiotic resistance-labeled mutation.

**Cytotoxicity of the Δ*rtxA3* mutant**

Because our hypothesis was that RtxA1 was the major cytotoxin, we did not expect that FLA899 would exhibit a significant reduction in cytotoxicity compared to wild type CMCP6. FLA899 caused 95.8 ± 2.6 % destruction/detachment of the INT-407 cell monolayer, which was comparable to the 92.5 ± 3.8 % destruction/detachment of the monolayer caused by CMCP6 (\(P = 0.28\)). These results substantiated our belief that RtxA3 is not a cytotoxic factor of CMCP6.

**Virulence of the Δ*rtxA3* mutant**

Although deletion of *rtxA3* did not have an effect on cytotoxicity to INT-407 cells, the possibility of a role for RtxA3 in virulence was not ruled out. FLA899 was examined
for virulence at the minimum lethal inoculum of 300 CFU. All of the mice infected with this mutant became moribund within 16 hours postinoculation. $10^{7.8}$ CFU/g was harvested from the skin lesions, and $10^{6.4}$ CFU/g was harvested from the livers of mice infected with FLA899 (Figure 4-7). We concluded that RtxA3 is not necessary for virulence of *V. vulnificus* in mice or for cytotoxicity to INT-407 cells.

**Future Direction: Construction of a Triple rtxA Mutant**

A triple rtxA1/rtxA2/rtxA3 mutant will be the key construct to confirm that the only RTX protein that has role in virulence in mice is RtxA1. To take advantage of the chitin-induced natural transformation of *V. vulnificus* to move mutations, each mutation must be labeled with a selectable marker. Because the original ΔrtxA3 construct did not have an antibiotic resistance marker, a new rtxA3 deletion plasmid construct was created by capturing the upstream and downstream PCR products in pGTR1113 by three-way USER cloning and inserting the *cat* gene (for chloramphenicol resistance) at the *SmaI* site between the upstream and downstream sequences. This plasmid, designated pGTR288, was moved into CMCP6 by conjugation, and the rtxA3 allele was replaced by *cat*. The chloramphenicol resistant ΔrtxA3 mutant was named FLA969, and genomic DNA from this mutant will be used to move the ΔrtxA3 mutation into FLA947 (ΔrtxA1::aph, rtxA2::Ω) to construct a triple rtxA mutant. Chloramphenicol resistant mutants will be verified by PCR, and confirmed mutants will evaluated for cytotoxicity to INT-407 cells, ability to cause apoptosis J774, and virulence in mice.

**Examination of RtxC1 in Virulence**

RTX toxins often require activation by an acyltransferase, typically named RtxC. While most studies of this activation have been carried out using *E. coli* HlyA and HlyC (77), the importance of acylation for activation has also been demonstrated for other
RTX toxins, including *B. pertussis* CyaA (78) and *Pasteurella haemolytica* LktA (76). *V. vulnificus* encodes multiple annotated acyltransferases, but only one has similarity to the *E. coli* HlyC protein, and it is designated RtxC1. The *rtxC1* gene of *V. vulnificus* is located directly upstream of *rtxA1* in the operon. We hypothesized that if RtxC1 is activating RtxA1, which is essential for cytotoxicity and full virulence, then a deletion of *rtxC1* would have a similar phenotype to the *rtxA1* mutants. Since there is no *rtxC* nearby in the clusters encoding *rtxA2* and *rtxA3*, our first thought was they may be activated by the RtxC1. However, the results from the *rtxA2* and *rtxA3* mutants indicated that they may not be active RTX toxins, may have a phenotype we have not yet identified, or may be activated by a different mechanism.

**Deletion of VV2_0480 encoding RtxC1**

The *rtxC1* gene is located directly upstream of *rtxA1* in an operon. We designed a clean deletion of the *rtxC1* open reading frame to ensure that the *rtxC1* mutation would not have a polar effect on the downstream *rtxA1*. The allelic exchange plasmid for the *rtxC1* deletion was designed to be constructed by crossover PCR (97). Approximately 500 bp upstream and downstream of *rtxC1* were amplified by PCR. The inside primers (*rtxC-up3'* and *rtxC-down5'*) contained 33 bp of overlapping sequences including a *NotI* site. The outside primers (*rtxC-up5'*and *rtxC-down3'*) included the USER friendly cloning sites at the 5’ ends that corresponded to the USER vector. In crossover PCR, a second PCR reaction is performed to anneal the upstream and downstream fragments to form a single joined fragment. This step was unsuccessful for the *rtxC1* upstream and downstream fragments. As an alternative, the upstream and downstream amplicons were digested with *NotI* to create compatible 5’ overhangs. The two *NotI*-digested PCR products were USER cloned into the allelic exchange vector pGTR1113,
and the two NotI ends were annealed during the ligation step. The resulting plasmid, pGTR265, was used for two-step sacB-assisted allelic exchange mutagenesis to create the CMCP6 ΔrtxC1 mutant, which was named FLA591. The mutation was confirmed by PCR using the outside primers rtxC-up5’ and rtxC-down3’.

**In vitro analysis of the ΔrtxC1 mutant**

We expected the rtxC1 mutant, FLA591, to be as attenuated in cytotoxicity assays as the rtxA1 mutants were. Surprisingly, the results were not as expected. Unlike the rtxA1 mutations, deletion of rtxC1 had no effect on monolayer detachment/destruction or lysis of INT-407 cells. Infection with FLA591 caused 58.6% detachment/destruction, whereas the rtxA1 mutant FLA554 caused only 5.6% detachment/destruction of the INT-407 cell monolayers (\(P = 0.002\)) (Figure 4-8 (A)). The destruction caused by FLA591 was comparable to that caused by wild type (76.5% detachment/destruction, \(P = 0.12\)). Because the rtxC1 mutation had no effect on detachment or destruction, we did not expect it to affect lysis. Indeed, CMCP6 and FLA591 caused high levels of lysis of INT-407 cells (71.3% and 81.4% lysis, respectively) compared to 17.6% lysis caused by the FLA554 (\(P < 0.001\)) (Figure 4-7 (B)).

**Virulence of ΔrtxC1 in iron-treated mice**

The unattenuated cytotoxic phenotype of the ΔrtxC1 mutant suggested that RtxA1 activity was not dependent on fatty acylation by RtxC1. However, it remained possible that activation of RtxA1 by RtxC1 was required for virulence in mice. We inoculated iron dextran-treated mice with the ΔrtxC1 mutant to determine if RtxC1 was required for RtxA1 activity and virulence. We inoculated the mice with 300 CFU of FLA591 or CMCP6, an inoculum at which rtxA1 mutants are consistently attenuated. Similar to the cytotoxicity results, the deletion of rtxC1 had no effect on virulence. All mice infected
with FLA591 developed skin lesions and became moribund. The amounts of bacteria recovered from FLA591-infected mice \((10^{8.1} \text{ CFU/g of skin lesion and } 10^{4.8} \text{ CFU/g of liver})\) were comparable to the amounts recovered after infection with CMCP6 \((10^{9.0} \text{ CFU/g of skin, } P = 0.4; 10^{4.9} \text{ CFU/g of liver, } P = 0.9)\) (Figure 4-9). It is evident from the mouse-virulence data and the cytotoxicity in cell culture that the RtxA1 toxin does not require activation by RtxC1 to cause lethality in mice.

**Discussion**

The RtxA1 toxin of *V. vulnificus* has an obvious role in cytotoxicity, and the reduction in cytotoxicity observed during infection of INT-407 cells with *rtxA1* mutants implies that the other RTX toxins produced by *V. vulnificus* either do not have similar functions or are not active during these growth conditions. Another possibility was that the RTX toxins act in concert, and that deletion of any one of them would affect the cytotoxicity phenotype. In mice, there is an even more intriguing story. The potent cytotoxicity of the RtxA1 in vitro suggested that, if expressed, it would be contributing to tissue damage during infection in vivo. However, as illustrated in Chapter 3, mutation of the *rtxA1* gene did attenuate virulence of CMCP6, but there was no visible effect on tissue damage. The skin lesions looked very similar to those caused by the wild-type, both at the macroscopic level and the histological level. It was possible that either RtxA2 or RtxA3 by themselves could be factors in virulence or that the presence of any of the three toxins, RtxA1, RtxA2 and/or RtxA3, was sufficient to contribute to virulence and perhaps tissue damage. For this reason, we examined RtxA2 and RtxA3 through mutagenesis and analysis of virulence and cytotoxicity.
RtxA2 is Not Essential for Virulence

The size of the rtxA2 gene and the genes in the surrounding region drew our attention to rtxA2. At 14-kb, rtxA2 is one of the largest genes of V. vulnificus. The C-terminal region of the RtxA2 protein contains a series of glycine-rich repeats, similar to the conserved RTX repeats. The genes directly downstream of rtxA2 encode an ABC-transport protein and a membrane fusion protein. This gene arrangement is similar to other RTX toxin loci, in which the genes for their T1SS are encoded downstream of the toxin. The C-terminal RTX repeats and the evidence for T1SS suggests that RtxA2 is a secreted RTX toxin. Other than the large size (over 400 kDa), there is no indication that RtxA2 is a member of the MARTX subfamily of RTX toxins. MARTX toxins, such as RtxA1 of V. vulnificus and RtxA of V. cholerae, are characterized by their large size, conserved repeats in the N-terminus, RTX repeats in the C-terminus, central enzymatic domains, and a cysteine protease domain. RtxA2 does not follow this pattern. Despite numerous bioinformatics searches and scanning by eye, N-terminal MARTX repeats could not be identified in RtxA2. The enzymatic domains are a bit more ambiguous, as there is very little conservation within the MARTX family. One domain identified in RtxA2 was a Von Willebrand Factor (VWF) domain. VWFs are eukaryotic glycoproteins that mediate adhesion of platelets and binding of the blood clotting factor VIIIa. While VWF domains are present in bacteria, there have been no reports identifying a function for them.

We constructed a mutation in rtxA2 by inserting the translational stop codons of the Ω element (67). Interestingly, mutation of rtxA2 had no effect on the cytotoxicity of V. vulnificus towards INT-407 cells or virulence in mice (Figures 4-1 and 4-2). The
rtxA2 mutants were able to cause severe skin lesions in the s.c. inoculated mice, indicating that RtxA2 is not essential for tissue damage.

It is possible that RtxA1 and RtxA2 could have redundant functions during infection of mice. If RtxA1 is the more potent cytotoxin, then its presence in the rtxA2 mutant could compensate for the lack of RtxA2. Hence, we constructed a double mutation of rtxA1::aph and rtxA2::Ω. The double mutant, FLA558, had a similar noncytotoxic phenotype as the single rtxA1 mutant, FLA554 (Figure 4-3). When inoculated into mice, FLA558 appeared more attenuated than FLA554 at an inoculum of 300 CFU (Figure 4-4 (A)). At this inoculum, FLA558 was defective at causing a wild-type skin infection in four of the five mice, and was therefore unable to cause lethal infection in the mice. This was an interesting result because it signified that both RtxA1 and RtxA2 were necessary for skin infection, and perhaps tissue damage. However, when we increased the inoculum to 3,000 CFU, FLA558 was able to cause skin lesions in all mice and was not more attenuated than the rtxA1 mutant (Figure 4-4 (B)). Therefore, it is still possible that rtxA2 is contributing to virulence. However, it is more likely that the discrepancy in the results at the two different inocula could be a reflection of the experimental fluctuation observed with the rtxA1 mutant. Deletion of the rtxA1 gene causes more consistent attenuation than the rtxA1::aph mutation; therefore, a double mutant was constructed by deletion of rtxA1 and Ω insertion in rtxA2. It had a similar reduced cytotoxicity phenotype as the previous double mutant (Figure 4-5). At an inoculum of 3,000 CFU, it was more virulent than the ΔrtxA1::aph mutant (Figure 4-6), and so it confirms our initial conclusion that RtxA2 is not essential for virulence in the s.c. inoculated mouse model.
Very recently, Chou, et al., (98) characterized the \textit{rtxA2} gene of \textit{V. vulnificus} YJ016 and reported that an \textit{rtxA2} mutant yielded similar results to our own mutant. In addition, expression of the \textit{rtxA2} gene was decreased at 37°C compared to 30°C. Iron also seemed to affect expression, with higher expression levels in iron-limiting conditions than during growth in media supplemented with iron. These data argue against a pivotal role of \textit{rtxA2} in virulence.

The only domain identified in RtxA2 is the VWF domain, which could mediate adhesion of the toxin to cells. When Chou, \textit{et al.} examined the adherence of a YJ016 \textit{rtxA2} mutant for adherence to human laryngeal epithelial cell (HEp-2) monolayers, there was no significant difference in adherence to HEp-2 cells compared to the wild type (98).

\textbf{RtxA3 is Not Essential for Virulence}

The third \textit{rtxA} locus in CMCP6 encodes only one gene, \textit{rtxA3}. The RtxA3 protein is predicted to be 2,937 amino acids, much smaller than the other two RtxA proteins of CMCP6. Analysis of the RtxA3 protein sequence revealed the presence of the C-terminal Hemolysin Ca$^{+}$-Binding repeats, but not the N-terminal MARTX repeats. RtxA3, like RtxA2, is not a MARTX toxin, but still may be classified as a member of the RTX family.

We deleted the \textit{rtxA3} gene and evaluated its role in virulence and cytotoxicity. Similar to results that we obtained with RtxA2, deletion of \textit{rtxA3} had no effect on virulence mice or cytotoxicity in cell culture (Figure 4-7).

BLAST analysis revealed little similarity to well studied proteins. Similar proteins are encoded by other \textit{Vibrio} species, as well as \textit{E. coli}, \textit{Burkholderia amifaria}, \textit{Pectobacterium} species, \textit{Shewanella} species, and \textit{Acinetobacter baumanii}, to name a
few. Most of the proteins identified from alignments are annotated as putative RTX toxins, calcium-binding hemolysin proteins, or outer membrane adhesin-like proteins. The presence of a VWF domain in the C-terminal end of the protein, indicates that RtxA3 may be involved in adhesion. The fact that most of the similar proteins identified by BLAST alignments are present in environmental bacteria, as opposed to known human or animal pathogens, suggests that RtxA3 may have a role in survival and adherence in the environment and may not be important for virulence. It should be noted that *V. vulnificus* has evolved to be marine organism and not a human pathogen, and most likely any factor we identify as involved in virulence also has a function for survival or growth in its ecological niche.

**RtxC is Not Required for Virulence Caused by RtxA1**

We also examined possible activation the RtxA toxins by an RtxC protein. RtxC1, encoded by a gene upstream of *rtxA1*, is the only acyltransferase with homology to *E. coli* HlyC. Therefore, we proposed that if RtxC-mediated acylation of the RtxA toxins is required for activity of the toxin, then deletion of *rtxC1* should have the same effect on virulence as we observed for the *rtxA* mutants.

Of the three RTX toxins, only RtxA1 appeared to be necessary for cytotoxicity in cell culture or virulence in mice. Therefore, we expected to see a similar phenotype of Δ*rtxC* in our cell culture assays and mouse infection as we saw with the *rtxA1* mutants. Instead, the Δ*rtxC* mutant was similar to the wild type for cytotoxicity (Figure 4-8) and virulence in mice (Figure 4-9). This result agreed with cytotoxicity results by Liu, *et al.* (89) who noted that their *rtxC* mutant was fully cytotoxic. These researchers observed a 10-fold increase in i.p. LD$_{50}$ compared to the wild type; however, we observed no attenuation in of Δ*rtxC* in s.c. inoculated mice. The *rtxC* mutant constructed by Liu, *et
*al.* was more virulent than the *rtxA1* mutant they studied, which had a 10²-fold increase in LD₅₀.

These results were surprising because it had always been assumed that the RTX toxin, RtxA, requires activation by RtxC (75). Although this requirement has not been confirmed in all RTX-producing bacteria, it has been demonstrated for well studied RTX toxins, such as *E. coli* HlyA, *B. pertussis* CyaA, and *P. haemolytica* LktA (76,77,99). Our study and that of Liu, *et al.* are the only known reports of the function of *rtxC* in the MARTX family of toxins.

Activation by RtxC should not be discounted, though. It is possible that RtxC has a role in activating RtxA1 to produce an uncharacterized phenotype. Along the same thought, it is possible that RtxA2 and RtxA3 may still require activation by RtxC, but we have not yet determined a measurable phenotype to use in analysis.
Figure 4-1. Detachment/destruction of INT-407 monolayers infected with *rtxA2::Ω*. Confluent INT-407 monolayers were infected with wild-type FLA399, *rtxA2* mutant FLA441, or the *rtxA1::aph* mutant FLA413. The cells remaining attached after incubation were stained with crystal violet. The *rtxA2::Ω* mutant caused similar levels of detachment/destruction as the wild-type did (*P* = 0.6). *, *P* < 0.0003 by Fisher’s LSD comparing % destruction by *rtxA1::aph* to FLA399 or the *rtxA2::Ω*. 
Figure 4-2. Virulence of rtxA2::Ω in mice. Iron dextran-treated mice were s.c. inoculated with 300 CFU of either FLA399 or the rtxA2::Ω mutant. The rtxA2::Ω mutant was not significantly attenuated compared to the wild type. Skin lesions were observed for all mice. Fractions below bars indicate proportion of mouse samples that yielded detectable CFU of bacteria. Bars without fractions indicate that bacteria were recovered from 5/5 mice.
Figure 4-3. Cytotoxicity to INT-407 cells caused by the rtxA1::aph, rtxa2::Ω double mutant. INT-407 monolayers were infected with CMCP6, FLA554 (rtxA1::aph), FLA558 (rtxA1::aph, rtxa2::Ω), or FLA561 (rtxA2::Ω) at an MOI of 10. Cells remaining attached to the well after infection were stained with crystal violet to assess destruction of the monolayers. Inactivation of rtxA2 had no additional effect on cytotoxicity of the rtxA1 mutant ($P = 0.2$). Asterisks indicate significance of the mutants to wild-type or the parent mutant strain determined by Fisher's LSD (*, $P = 10^{-6}$; **, $P = 10^{-7}$).
Figure 4-4. Virulence of *rtxA1::aph*, *rtxA2::Ω* mutant in mice. Iron dextran-treated mice were s.c. inoculated with either FLA554 (*rtxA1::aph*) or FLA558 (*rtxA1::aph*, *rtxA2::Ω*) at an inoculum of (A) 300 CFU or (B) 3,000 CFU. Fractions beneath the bars indicate the proportion of mouse samples that yielded bacteria. (A) FLA558 was more attenuated than FLA554 at 300 CFU. *, *P* = 0.05 by Student’s *t*-test comparing FLA554 to FLA558. †, *P* = 0.002, comparing proportion mice infected with FLA558 to FLA554 by *χ*²-test. (B) However, FLA558 was not more attenuated than FLA554 at 3,000 CFU (*P* = 0.1 comparing liver CFU).
Figure 4-5. Lysis of INT-407 cells infected with ΔrtxA1::aph or ΔrtxA1::aph, rtxA2::Ω mutants. INT-407 cell monolayers were infected with CMCP6, the ΔrtxA1 mutant FLA943, or the ΔrtxA1, rtxA2::Ω mutant FLA947. Lysis was measured by detection of LDH release. The double mutant FLA947 and the single mutant FLA943 have reduced lytic ability compared to wild-type; however, they were not different from each other (P = 0.3). Asterisks indicate statistical significance compared to wild type CMCP6 by a Fisher’s LSD (*, P = 10^{-5}).
Figure 4-6. Virulence of ΔrtxA1::aph, rtxA2::Ω mutant in mice. Iron dextran-treated mice were s.c. inoculated with either FLA943 (ΔrtxA1) or FLA947 (ΔrtxA1, rtxA2::Ω) at an inoculum of 3,000 CFU. Fractions beneath the bars indicate the proportion of mouse samples that yielded bacteria. FLA943 was slightly more attenuated at this inoculum, indicating rtxA2 is not essential for virulence. (P = 0.3, comparing skin CFU/g; P = 0.1, comparing liver CFU/g; determined by Student’s t-test)
Figure 4-7. Virulence of the ΔrtxA3 mutant in mice. Iron dextran-treated mice were inoculated s.c. with 300 CFU of either CMCP6 or FLA899 (ΔrtxA3). Deletion of rtxA3 had no effect on the ability to cause systemic infection ($P = 0.1$). *, $P = 0.003$ by Student’s $t$-test comparing FLA899 to CMCP6.
Figure 4-8. Cytotoxicity to INT-407 cells caused by the ΔrtxC mutant. INT-407 cell monolayers were infected CMCP6, the ΔrtxC1 mutant FLA591, or the rtxA1::aph mutant FLA554. (A) Detachment/destruction of the monolayers was assessed by crystal violet stain. FLA591 was not significantly different from CMCP6 ($P = 0.12$). Significance determined by Fisher’s LSD. (*, $P = 0.002$ comparing FLA554 to FLA591; **, $P = 0.0004$ comparing FLA554 to CMCP6) (B) Lysis was measured by LDH release. FLA591 was not significantly different from CMCP6 ($P = 0.35$). Significance determined by Fisher’s LSD. (*, $P = 0.002$; **, $P = 0.001$ comparing mutant FLA554 to either the wild type or FLA591).
Figure 4-9. Virulence of \( \Delta \)rtxC1 mutant in mice. Iron-treated mice were s.c. inoculated with 300 CFU of either wild type CMCP6 or the \( \Delta \)rtxC1 mutant FLA591. Mice were euthanized when their temperatures dropped below 33°C, a sign that they were moribund. FLA591 was as virulent as CMCP6 was in mice, indicating RtxC is not necessary for RtxA1-mediated virulence. \( P = 0.43 \), comparing skin CFU/g; \( P = 0.9 \), comparing liver CFU/g)
CHAPTER 5
EXAMINING THE ROLE OF THE TYPE VI SECRETION SYSTEM IN PATHOGENESIS OF Vibrio vulnificus

Rationale for Study

Deletion of rtxA1, encoding one of the major cytotoxic factors of V. vulnificus, had little effect, if any, on tissue damage in the subcutaneous lesion of s.c. infected mice. This result indicated that V. vulnificus produces other cytotoxic factors that are involved in the destruction of host tissues. Cytotoxic factors are often extracellular proteins that are exported by one of the secretion systems bacteria. Knocking out the secretion of the proteins can have the same, if not greater, effect as knocking out the cytotoxic effectors themselves. Genomic sequence analysis of V. vulnificus revealed the presence genes with homology to the type VI secretion system (T6SS) genes of V. cholerae. The T6SS is a recently characterized secretion system in gram-negative bacteria (57), and it is believed to play a role in the virulence. Two secreted substrates are conserved among the bacteria possessing a T6SS: a hemolysin-coregulated protein (Hcp) and a valine-glycine repeat protein (VgrG). Not only are these proteins secreted via the T6SS, there is evidence that they are required to form a functional T6SS. We hypothesized that the effectors secreted by the V. vulnificus T6SS could contribute to virulence and tissue damage, and thus we constructed deletions of these two genes, hcp and vgrG, and examined them for cytotoxicity in vitro and virulence in the mouse model of disease.

Introduction

Gram-negative bacteria have developed numerous ways to transport proteins across the inner membrane, through the periplasm, and across the outer membrane into the extracellular milieu or into nearby eukaryotic cells. These secretion systems
play an important role in pathogenesis of many bacterial pathogens. The recently identified T6SS is suspected to contribute to virulence of pathogens including *V. cholerae* (100), *Pseudomonas aeruginosa* (101), *Aeromonas hydrophila* (102), *Francisella tularensis* (103), *Edwardsiella tarda* (104), *Burkholderia mallei* (105), and *Agrobacterium tumefaciens* (106). In addition to pathogenic interactions, the T6SS also contributes to symbiotic interactions of bacteria with their eukaryotic hosts, exemplified by studies of the *Rhizobium*-legume symbiotic relationship (107).

Due to the recent discovery of this secretion system and variability in the genetic composition in the T6SS regions, little is known about the genomic organization, the structure of the apparatus, and the exact mechanism of secretion. *In silico* analysis identified a set of 13 conserved proteins defined as the T6SS "core components" (108). Some of these components share homology with the type IV secretion system (T4SS) components, although the two secretion systems are distinct from one another. The core components included the hemolysin-coregulated protein (Hcp), valine-glycine repeat protein (VgrG), ClpB ATPase homologs (ClpV), and the T4SS homologs IcmF and DotU (108). Boyer, *et al.* (108) have identified one or more T6SS loci present in the genomes of over 90 gram-negative bacteria, many of which are pathogens or symbionts.

Despite attempts to identify components involved in this new secretion system, numerous questions remain as to the exact function and mechanism of secretion. Two known secreted substrates of the T6SS, Hcp and VgrG, are encoded by all T6SS-containing bacteria. There is some debate as to if these proteins are secreted effector proteins, structural components of the secretion apparatus itself, or both. In 1996, Hcp
was first examined in *V. cholerae* by Williams, *et al.* (109). At that time, the T6SS was unknown and Hcp was thought to be regulated and secreted in a manner similar to the hemolysin, HlyA. Deletion of the two *hcp* genes had no effect on virulence in the infant mouse model or on cytotoxicity in vitro (109). A decade later, Pukatzki, *et al.* (100) used transposon mutagenesis of a *V. cholerae* non-01/non-0139 strain to identify factors causing virulence towards the amoeba *Dictyostelium discoideum*. *Dictyostelium*-attenuated mutants contained transposon insertions in *vas* (*virulence associated secretion*) genes, some of which had similarity to the *icm* genes of the *L. pneumophila* type IV secretion system. Secretion of four proteins (Hcp, VgrG-1, VgrG-2, VgrG-3, and VgrG-4) was affected by the transposon mutations. One of the mutations in *vasH* (*a σ^54 activator*) affected transcription of *hcp-1* and *hcp-2*, encoding identical Hcp proteins. Additionally, an *hcp* double mutant was avirulent towards *D. discoideum* and was defective in secretion of the three VgrG proteins, indicating that Hcp has an integral role in the T6SS.

Other studies have confirmed that Hcp and VgrG are secreted factors and that their secretion is mutually dependent on each other. In *E. tarda*, the three known T6SS-secreted proteins are the Hcp homolog EvpC, a VgrG homolog EvpI, and an effector protein EvpP (not conserved among T6SSs). Inactivation of any of the 13 genes encoding T6SS core components, including *evpC* and *evpI*, disrupted secretion of the three secreted proteins EvpC, EvpI, and EvpP, and caused attenuation in virulence to fish (110). While EvpC (Hcp) and EvpI (VgrG) are required for a functional T6SS, EvpP is not required for secretion of the other proteins, indicating that Hcp and VgrG may be components of the secretion apparatus and that EvpP is a secreted effector protein.
Hcp is also a secreted substrate of *P. aeruginosa*. The crystal structure of the *P. aeruginosa* Hcp revealed the formation of hexameric rings (101), and these rings polymerize to form tubes up to 100 nm long (111). These nanotubes are proposed to form part of the secretion complex through which other effectors travel.

All T6SS-encoding bacteria have at least one VgrG, and many of them encode more than one. These proteins have domains sharing homology to structural features of the tail spike protein of the T4 bacteriophage (112). *V. cholerae* encodes three VgrG proteins, VgrG-1, VgrG-2, and VgrG-3, each containing domains with similarity to the gp44 protein of the bacteriophage Mu and gp5 protein of bacteriophage T4. The three proteins were predicted to form a trimeric complex, similar to the bacteriophage tail spike complexes used to puncture the bacterial membrane, and were demonstrated to interact with each other (112). The similarity to the bacteriophage tail spike proteins suggests that VgrG proteins could form a membrane-puncturing device at the tip of the secretion apparatus. In addition to the gp44 and gp5 domains, *V. cholerae* VgrG-1 contains a C-terminal extension with an actin-crosslinking domain, and VgrG-3 contains a peptidoglycan-binding domain. Deletion of VgrG-1 or VgrG-2 disrupts secretion of Hcp, VgrG-1, VgrG-2, and VgrG-3. VgrG-2 and its actin-crosslinking domain are also cytotoxic for J774 and Raw264.7 cell lines and *Dictyostelium* amoeba, indicating that VgrG proteins could be effector proteins as well as a component of the secretion apparatus (100,113). VgrG proteins containing C-terminal extensions were identified in many, but not all, T6SS-encoding bacteria and are classified as “evolved VgrG” proteins.
*V. vulnificus* contains a genomic region encoding a T6SS on chromosome 2. This region, although not well defined, contains 18 genes homologous to T6SS genes of other bacteria and includes all of the 13 T6SS “core components”. Considering the importance of the T6SS in virulence of several organisms, including *V. cholerae*, we speculated that the T6SS may have a role in virulence of *V. vulnificus*. Given that there is still little known about the T6SS and the specific proteins required for proper secretion, we planned to delete a 10-kb portion of the chromosomal locus encoding the *V. vulnificus* T6SS. Seven genes were encoded in the 10-kb region to be deleted, VV2_0428 through VV2_0434, including *vgrG* (VV2_0428) and *hcp* (VV2_0429). Other genes to be deleted included VV2_0430 encoding a ClpB homolog, an ATPase hypothesized to provide the energy necessary for secretion (101,114), and other genes predicted to be core components of the T6SS. Deletion of this region would have knocked out secretion via this pathway, and we would have been able to assess the importance of T6SS in pathogenesis of *V. vulnificus*. Had we seen an effect, we would have deleted individual genes to study their functions. Unfortunately, we were unsuccessful at deleting the 10-kb of this genetic locus. As an alternative plan, we decided to individually delete the genes *hcp* and *vgrG* to disrupt secretion.

**Results**

**Identification and Deletion of *V. vulnificus vgrG***

As described above, all T6SS-encoding bacteria have vgrG genes. *V. cholerae* and several other T6SS-encoding bacteria have more than one vgrG gene. *V. cholerae* encodes three VgrGs, two of which are “evolved VgrGs”, meaning they possess an extra domain at the C-terminal end of the protein. BLAST analysis using the amino acid sequence of VgrG-1, VgrG-2, and VgrG-3 of *V. cholerae* identified only one protein in
*V. vulnificus* CMCP6 encoded by the gene VV2_0428. *V. vulnificus* VgrG has 31% identity with the conserved N-terminal portion of the *V. cholerae* VgrGs; however, there is no additional C-terminal extension as is present in VgrG-1 and VgrG-2 of *V. cholerae*. VgrG encoded by *V. vulnificus* is predicted to be the type VI secretion protein VgrG according to the NCBI Conserved Domains database, and similarly to the other T6SS VgrGs, the VgrG sequence of CMCP6 has domains with similarity to the gp27/gp44 and the gp5 proteins of the bacteriophage tail spike proteins. The similarity of VgrG with other T6SS VgrGs indicates that VgrG should be essential for T6SS of effector proteins and may have cytotoxic activity itself.

The vector to delete *vgrG* was constructed by three-way USER cloning. 1-kb upstream and downstream of *vgrG* was PCR amplified with the primers vgrG-down-5'/vgrG-down-3' and T6SS-up-5'/T6SS-up-3'. The upstream and downstream fragments were USER cloned into the allelic exchange vector pGTR1113 (pCVD442::lacZ-USER), and the resulting plasmid was named pGTR1304. For selection of the deletion of *vgrG*, the *aph* gene (encoding kanamycin resistance) was inserted at the *Sma*I site engineered between the upstream and downstream fragments. The resulting Δ*vgrG::aph* plasmid, pGTR1307, was linearized and added to CMCP6 growing in the presence of crabshell to induce chitin-based natural transformation. Chitin transformants with the correct mutation were selected on LB-N containing kanamycin and were verified by PCR to have undergone allelic exchange of the *aph* for *vgrG*. The Δ*vgrG::aph* mutant was named FLA1035.

**Cytotoxicity of Δ*vgrG::aph* mutant**

Since VgrG proteins are suggested to form a membrane-puncturing device on the tip of the T6SS apparatus and potentially have cytotoxic activity, we tested FLA1035 for
cytotoxicity for both INT-407 cells and J774 cells. INT-407 monolayers were infected with FLA1035 for 1 hour, treated with gentamicin, and incubated overnight. The following day, the cells remaining attached to the wells were washed and stained with crystal violet to assess the level of detachment/destruction of the monolayer. FLA1035 did not have a decrease in cytotoxicity compared to CMCP6 (86.5% destruction vs. 88.7% destruction, respectively; \( P = 0.63 \)) (Figure 5-1).

The T6SS of *V. cholerae* mediates cytotoxicity towards phagocytic cell types, including Raw264.7 and J774 cells \((100,113)\). We therefore examined the T6SS mutants for cytotoxicity to J774 murine-macrophage like cells. Monolayer detachment/destruction was assessed for J774 cells in a similar manner to INT-407 cells. Monolayers were established and infected at an MOI of 10 for 1 hour. Detachment/destruction of the monolayers was measured as it was for INT-407 monolayers. Similar to the results from infection of the INT-407 cells, deletion of *vgrG* did not have a significant effect on cytotoxicity to J774 cells (79.2% destruction by FLA1035 vs. 88.2% destruction by CMCP6, \( P = 0.16 \)) (Figure 5-1).

Although deletion of *vgrG* had no effect on the ability to detach/destroy the monolayers of J774 cells, there was a reduction in the ability of FLA1035 to induce apoptosis of J774 cells. The level of caspase-3/7 activity was measured in infected J774 cells and was lower in FLA1035-infected cells than in CMCP6-infected cells. FLA1035 caused 38.2% apoptosis compared to 54% apoptosis caused by CMCP6 (\( P = 0.0001 \)) (Figure 5-2), indicating that VgrG or a protein secreted in a VgrG-dependent manner is an apoptotic factor. The defect of the \( \Delta vgrG::aph \) mutants was not as great as that observed with the \( \Delta rtxA1::aph \) mutants (38.2% compared to 20.5%, \( P = 10^{-5} \)).
The low level of apoptosis induced by each of these mutants is above the background level of uninfected cells, indicating that each protein contributes to apoptosis. Apparently, in the absence of either VgrG or RtxA1, the remaining factor can cause apoptosis.

**Virulence of ΔvgrG::aph in mice**

To determine the role of VgrG and potentially the T6SS in virulence, FLA1035 was s.c. inoculated in iron dextran-treated mice. The ΔvgrG::aph mutant was not attenuated at 1,000 CFU (three times the minimum lethal dose for wild type), although it did cause visible infection in only four of five mice. Means of $10^{6.7}$ CFU/g and $10^{3.8}$ CFU/g were recovered from the skin lesions and livers of the FLA1035-infected mice (Figure 5-3). These levels were not significantly lower than the level of bacteria in CMCP6-infected mice ($10^{7.8}$ CFU/g skin lesion, $P = 0.14$; and $10^{4.1}$ CFU/g liver, $P = 0.69$). In addition, the skin lesions caused by FLA1035 looked very similar at the gross level to the lesions caused by CMPC6. Therefore, VgrG and potentially the T6SS are not essential for virulence or tissue damage in our s.c. model.

**Identification and Deletion of V. vulnificus hcp**

As an additional evaluation of the T6SS and its role in virulence of *V. vulnificus*, we also examined the hemolysin coregulated protein, Hcp. Hcp is secreted in all T6SS-encoding bacteria and is predicted to be a part of the T6SS machinery and therefore necessary for proper secretion of other substrates (100,111). While a BLAST search using the HCP sequences from *V. cholerae* and *P. aeruginosa* did not reveal any hits in *V. vulnificus*, searching the NCBI published genome of CMCP6 revealed a gene annotated as a hemolysin-coregulated protein or hcp. This gene, VV2_0429, is directly downstream of the vgrG gene discussed above and within the locus encoding the T6SS
genes of *V. vulnificus*. *V. vulnificus* Hcp belongs to the DUF796 superfamily and the COG3157 according to the Conserved Domain database (NCBI), both of which are comprised of the T6SS Hcp proteins. The COG3157 group of proteins is specified by Boyer, *et al.* (108) as one of the essential T6SS components. An InterProScan search (EBI database) for protein function also groups the *V. vulnificus* Hcp into the family including the T6SS Hcp proteins. The Hcp of *V. vulnificus* shares 34% similarity and 62% identity to the *A. tumefaciens* Hcp protein. *V. vulnificus* Hcp also has 26% similarity and 40% identity to *E. tarda* EvpC (Hcp). Both of these proteins are secreted and are proposed to have similar functions to *V. cholerae* Hcp (100), although there is very little sequence similarity between them. Boyer, *et al.* (108) noted in their in silico analysis that there is a lot of sequence variation among the different T6SSs, especially in the secreted proteins. We therefore assumed that the protein encoded by VV2_0429 was indeed the T6SS Hcp protein, despite the lack of homology with *V. cholerae*. Since previous reports indicate that T6SS, including secretion of VgrG, is dependent on Hcp, we anticipated that inactivation of *hcp* would disrupt T6SS.

The plasmid for deletion *hcp*, pGTR1303, was constructed by three-way USER cloning of the PCR-amplified upstream and downstream regions of *hcp* into the USER-friendly allelic exchange vector, pGTR1113. The chloramphenicol acetyltransferase (*cat*) cassette from pCOS5 was inserted at the *Sma*I site engineered between the *hcp* upstream and downstream fragments. Once the deletion vector, pGTR1305, was confirmed, it was linearized with *Nde*I and moved into CMCP6 via chitin-induced transformation. Chitin-transformants were selected on LB-N agar plates containing
chloramphenicol and verified by PCR for deletion of the hcp gene. The Δhcp::cat mutant strain was named FLA1030.

**Cytotoxicity of Δhcp::cat**

FLA1030 was tested for cytotoxicity in vitro in J774 cells. Monolayers of J774 cells were infected with either CMCP6 or FLA1030 at MOI of 10. After one hour of infection, the bacteria were killed with gentamicin and the cells were incubated overnight. Monolayer detachment/destruction was assessed by staining with crystal violet. Deletion hcp of had no effect on cytotoxicity to J774 cells. FLA1030 caused a mean 89.6% destruction of J774 cell monolayer, compared to 88.2% destruction caused by CMCP6 (P = 0.73) (Figure 5-4). As with ΔvgrG::aph mutant, FLA1030 was similarly cytotoxic to INT-407 cells (Figure 5-4).

As detailed above, the ΔvgrG::aph mutant caused significantly less apoptosis in J774 cells than the wild-type parent. Therefore, we expected that if VgrG was secreted in an Hcp-dependent manner, then the Δhcp::cat mutant would have a similar reduction in ability to induce apoptosis. Interestingly, the Δhcp::cat mutant, FLA1030, caused apoptosis in J774 cells similar to that caused by CMCP6 (Figure 5-5). This result indicated that Hcp may not be necessary for T6SS-mediated secretion, as opposed to the current model, or that VgrG secretion is independent of Hcp.

**Virulence of Δhcp::cat**

We tested if Hcp was necessary for virulence by s.c. infection of iron dextran-mice with 1,000 CFU of FLA1030. We did not expect to see an effect on virulence, since the ΔvgrG::aph was virulent and we already speculated that the T6SS does not have an essential role in virulence. Confirming the previous results with VgrG, FLA1030 was as
virulent as the wild type in mice (Figure 5-6). Therefore, the T6SS does not appear to be essential for virulence.

**Deletion of the T6SS Factors in an rtxA1 Background**

It is possible that any cytotoxic defect of an hcp or vgrG mutant was covered up by the presence of the major cytotoxic factor, RtxA1. In many T6SS-encoding bacteria, including *V. cholerae* and *A. hydrophila* (100,102,113), the effects of T6SS effectors were examined in a parent strain lacking other major cytotoxic factors of these strains. For example, *V. cholerae* rtxA mutants were used as background for all of the T6SS mutants in the studies identifying the role in cytotoxicity towards macrophage-like cells and *D. discoideum* (100,113). To eliminate the cytotoxic effects of RtxA1, we decided to examine the T6SS mutations in an rtxA1-deficient strain. We constructed double ΔrtxA1::tetA, ΔvgrG::aph and ΔrtxA1::tetA, Δhcp::cat mutants by chitin-induced transformation. To construct the ΔrtxA1::tetA, ΔvgrG::aph mutant, genomic DNA from FLA1035 (CMCP6ΔvgrG::aph) was added to FLA954 (CMCP6ΔrtxA1::tetA) growing on crabshell to induce natural transformation. Chitin transformants were selected on LB-N containing kanamycin (to select for the deletion of vgrG) and were verified to have tetracycline resistance (due to the ΔrtxA1::tetA mutation). For construction of the ΔrtxA1::tetA, Δhcp::cat, genomic DNA of FLA954 (CMCP6ΔrtxA1::tetA) was added to FLA1030 (CMCP6Δhcp::cat) growing on a crabshell. Chitin-transformants were selected on LB-N agar supplemented with tetracycline and verified to have resistance to chloramphenicol. The double mutants were confirmed by PCR, and the resulting mutants were named FLA960 (ΔrtxA1::tetA, ΔvgrG::aph) and FLA965 (ΔrtxA1::tetA, Δhcp::cat).
Cytotoxicity of T6SS, rtxA1 double mutants

The ΔrtxA1::tetA, ΔvgrG::aph mutant, FLA960, was tested for the ability to cause destruction and detachment of INT-407 monolayers. As detailed above, the rtxA1 mutants are cytotoxic beyond one hour postinfection, so it was possible that VgrG or VgrG-dependent factors could contribute to the residual cytotoxicity. INT-407 cells were infected with the various mutants for 1 hour and 2 hours prior to addition of gentamicin. The cells were incubated for a total of 4 hours after infection, and the cells remaining attached to the wells were stained with crystal violet. At 1 hr postinfection, the ΔrtxA1::tetA mutant was reduced for cytotoxicity (-13.1% destruction), as expected, and the double ΔrtxA1::tetA, ΔvgrG::aph mutant did not have a significant additional reduction in cytotoxicity (7.7% destruction) (Figure 5-7). At two hours postinfection, the ΔrtxA1::tetA and the double ΔrtxA1::tetA, ΔvgrG::aph mutants caused similar levels of destruction of the monolayers (69.2% and 69.7% destruction, respectively) (Figure 5-7). These results indicate that VgrG is not essential for causing cytotoxicity to INT-407 cells, even in an rtxA1 background.

We also tested the ability of the double rtxA1 and T6SS mutants to cause destruction/detachment of J774 cell monolayers. ΔvgrG::aph FLA1035 and Δhcp::cat FLA1030 were cytotoxic towards J774 cells and, as was expected, the ΔrtxA1::tetA mutant, FLA954, had reduced cytotoxicity. Deletion of vgrG or hcp in the ΔrtxA1::tetA mutant had no additional effect on cytotoxicity compared to the ΔrtxA1::tetA (Figure 5-8).

The apoptosis of the double ΔrtxA1::tetA, ΔvgrG::aph mutant, FLA960, needs to be examined. Both the ΔrtxA1::tetA mutant and the ΔvgrG::aph mutant caused less apoptosis than the wild type in infected J774 cells, but each mutant caused some
apoptosis above the background levels. It will be interesting to see if FLA960 causes less apoptosis than either one of the two individual mutants. If RtxA1 and VgrG are the two major factors contributing to apoptosis in this cell culture model, we would expect to see very little apoptosis, similar to the background level in uninfected cells.

**Virulence of T6SS, rtxA1 double mutants**

We tested the virulence of the T6SS, rtxA1 double mutants by s.c. inoculation in iron dextran-treated mice. FLA960 (ΔrtxA1::tetA, ΔvgrG::aph) and FLA965 (ΔrtxA1::tetA, Δhcp::cat) each appeared to be slightly, although not significantly, attenuated compared to FLA954 (ΔrtxA1::tetA) (Figure 5-9). FLA960 caused visible lesions in five of five mice with a mean 10^{7.5} CFU/g in the skin. This was similar to the bacteria recovered from the skin of FLA954 (10^{6.1} CFU/g; P = 0.2). FLA960 caused systemic infection in only two of five mice compared to five of five mice with systemic infection by FLA954 (P = 0.4). Despite fewer mice with systemic infections, the amount of bacteria recovered from the livers of FLA960 infected mice was not significantly different from the amount of bacteria recovered from FLA954 infected mice (10^{3.6} CFU/g vs. 10^{4.7} CFU/g; P = 0.3). Similar results were observed for FLA965 (ΔrtxA1::tetA, Δhcp::cat) (Figure 5-9). FLA965 caused detectable skin infections in three of five mice, and a mean of 10^{6.3} CFU/g was recovered from the skins (P = 0.01, compared to FLA954). The three mice with detectable skin infection had detectable liver infection, resulting in a mean of 10^{3.5} CFU/g recovered from the livers (P = 0.1, compared to FLA954).

The similar decrease in bacteria recovered from mice infected with FLA960 and FLA965, though not significant from FLA954-infected mice, may suggest a small role for T6SS in virulence. These mutants should be examined at higher inocula to investigate
the level of attenuation and at lower inocula to detect a significant difference between the ΔrtxA1::tetA mutant and the double mutants. Interestingly, the skin lesions caused by the double mutants resembled those caused by FLA954 and wild type (Figure 5-10), although the lesions have not yet been examined by histopathology. At the histological level, there may be a difference in tissue damage or PMN response in these mice, that was not observed when rtxA1 was knocked out alone.

**Discussion**

The discovery and characterization of the T6SS in several well-known pathogens, as well as other gram-negative bacteria, suggested that it might be a provocative target for our studies in pathogenesis of *V. vulnificus*. Many reports suggest that the T6SS mediates cytotoxicity to host cells and contributes to virulence and survival during infection. The studies performed on the *V. cholerae* T6SS have proposed a role in cytotoxicity to phagocytic cells types, such as J774, Raw264.7, and *Dictyostelium* amoeba (100,113). The T6SS of *E. tarda*, *F. tularensis*, *A. hydrophila*, and *P. aeruginosa* have also been demonstrated or suggested to have a role in virulence.

*V. vulnificus* CMCP6 contains a locus of 18 genes with similarity to or annotated domains of T6SS of other bacteria, including the 13 core components identified by (108). During this investigation, we examined the role of the *V. vulnificus* T6SS in virulence by construction of vgrG and hcp mutants and testing them in the cell culture and mouse models of infection.

**VgrG Causes Apoptosis; However, it is Not Essential for Virulence**

The VgrG proteins of the T6SS share structural similarity with the T4-bacteriophage needle complex (gp27-gp5). The three VgrG proteins of *V. cholerae* are hypothesized to form a trimeric complex, either at the tip of a secretion apparatus or
exposed somewhere on the bacterial surface, and this complex is speculated to act as a membrane puncturing device (112). While *V. cholerae* encodes three VgrG proteins, two of which have C-terminal extensions, *V. vulnificus* encodes only one VgrG. The *V. vulnificus* VgrG has domains with homology to the gp27/gp44 and the gp5 proteins; however, it does not have a C-terminal extension. Possession of a single VgrG is not unusual, as there are other T6SS-encoding bacteria with a single VgrG that lacks C-terminal extensions (115). It is possible that the single VgrG is able to form into a homotrimer.

We expected that deletion of *vgrG* would knock out the T6SS, as is the case for other bacteria. Deletion of the single *vgrG* gene of *V. vulnificus* had no effect on destruction of INT-407 or J774 cell monolayers (Figure 5-1). We speculated that the lack of a cytotoxicity phenotype could be due to the activity of the major cytotoxic factor RtxA1, therefore we constructed a ΔrtxA1::tetA, ΔvgrG::aph mutant. The double mutant was not more attenuated in cell culture than the ΔrtxA1::tetA mutant (Figure 5-7 and 5-9), indicating that VgrG does not contribute to the residual cytotoxicity caused by rtxA1 mutants. Interestingly, the ΔvgrG::aph mutant had reduced ability to cause apoptosis in J774 cells (Figure 5-2), although it has not yet been determined if apoptotic activity is completely abolished in the ΔrtxA1::tetA, ΔvgrG::aph mutant. Despite this small effect in cell culture, the ΔvgrG::aph mutant had no significant effect on virulence in mice when deleted by itself (Figure 5-3). However, when vgrG was deleted in ΔrtxA1::tetA, the double mutant was slightly, though not significantly more attenuated than the ΔrtxA1::tetA, suggesting that VgrG may have a role in virulence, but the presence of the cytotoxic factor RtxA1 may compensate for its absence.
Hcp is Not Necessary for Cytotoxicity or Virulence

Hcp is another protein secreted by all known T6SSs, and it is proposed to form a tubular structure through which other T6SS-secreted proteins pass. In well studied T6SSs, the secretion of the VgrG proteins and other T6SS effectors is dependent on Hcp, adding to the evidence that Hcp is a part of the secretion apparatus. We expected that if T6SS has a role in virulence, then a mutation in Hcp would cause attenuation in the mouse model. First we characterized the Δhcp::cat mutant in vitro. As was expected based on the vgrG results, Δhcp::cat had no observable decrease in cytotoxicity towards J774 cells, even in the absence of the major cytotoxic factor, RtxA1 (Figure 5-7). Because we had observed a decrease in apoptosis of J774 cells infected with the ΔvgrG::aph mutants, we were surprised to discover that the Δhcp::cat mutants were not defective at causing apoptosis (Figure 5-5). Additionally, we observed no difference in virulence of the Δhcp::cat in mice; however, similar to the results from ΔrtxA1::tetA, ΔvgrG::aph mutant, the ΔrtxA1::tetA, Δhcp::cat mutant was slightly, though not significantly attenuated in mice compared to the ΔrtxA1::tetA mutant. Given that both of the double mutants behaved similarly suggests that there may be a small role for T6SS in virulence of V. vulnificus. It is possible that RtxA1 and T6SS factors have a similar function in pathogenesis, and although RtxA1 is more important in virulence, T6SS may have a smaller accessory role.

It is intriguing that the two T6SS mutants had different apoptosis phenotypes, since we expected Hcp to be necessary for secretion of VgrG. It is possible that V. vulnificus produces more than one Hcp, especially since V. cholerae encodes two hcp genes and deletion of both of them is necessary to disrupt secretion. However, close examination of the V. vulnificus genome confirmed that the hcp that was deleted
is the only identifiable \textit{hcp} gene with similarity to any other \textit{hcp} genes known. It is also notable that the Hcp sequence of \textit{V. vulnificus} is quite different from the well studied Hcp proteins of \textit{V. cholerae} and \textit{P. aeruginosa}, but has similarity to Hcp proteins of other bacteria (e.g., \textit{A. tumefaciens} Hcp, \textit{E. tarda} EvpC, \textit{B. mallei} Hcp, putative Hcp proteins of other \textit{Vibrio} species). While domain searches, structural motifs, and protein function searches classify it as belonging to the same family of proteins as T6SS Hcp proteins, it is possible that the \textit{V. vulnificus} Hcp may not be a true T6SS Hcp protein, it might not have the same function, or may not be secreted. Another possibility that could explain the two different apoptosis phenotypes is that VgrG may be secreted in an Hcp-independent manner and therefore a mutation in \textit{hcp} does not affect secretion or activity of VgrG.

\textbf{What is the Function of T6SS?}

It is not entirely surprising that T6SS mutants are not attenuated in mice, and it is possible that the T6SS is not expressed in the presence of mammalian cells. T6SS has been demonstrated to be tightly regulated in other bacteria by different mechanisms (115). The environmental cues signaling T6SS expression vary among the bacteria, and there is still a lot unknown. Temperature can be a factor in regulation. For instance, the T6SS of \textit{Yersinia pestis} is upregulated at 26°C compared to 37°C (116). Quorum sensing and growth phase influence expression of Hcp in \textit{V. cholerae} (117), and these factors also mediate T6SS expression in \textit{A. hydrophila} (118). Many of the T6SSs have a role in survival within or cytotoxicity to host cells, indicating that there are host factors that may mediate expression. For example, secretion of Hcp and VgrG proteins was increased in the plant pathogen \textit{Pectobacterium atrosepticum} when the bacteria were grown in the presence of potato tuber extracts (119). Signals during
infection also regulate the T6SS secretion of *P. aeruginosa*, as it is negatively regulated by RetS and positively regulated by LadR and is expressed during chronic infection (101). The T6SS of *P. aeruginosa* is also regulated by the serine/threonine kinase (PpkA) and phosphatase (PppA) encoded in the T6SS region, although the environmental cues for T6SS expression remain unknown (120).

It would be advantageous to know what factors regulate expression of T6SS in *V. vulnificus*. We did not evaluate the expression of the T6SS and do not know if it is active during growth in mammalian cell culture or in mice. *V. vulnificus* lives primarily in an estuarine environment, both in the water and cohabitating with shellfish, and it is possible that environmental cues such as temperature, salinity, or availability of nutrients may influence the expression of the T6SS. *V. vulnificus* can colonize and persist within the filter-feeding mollusks, such as oysters. During the colonization of oysters, *V. vulnificus* is able to resist being killed by the oyster defenses, such as the phagocytic hemocytes (121). Perhaps the T6SS has a role in persistence within the oyster and resistance to phagocytosis by hemocytes. This is not out of the realm of possibility given that many of the T6SSs of other bacteria influence survival and killing by phagocytic cell types. Since it appears that T6SS is not essential for virulence in mice, the role of the T6SS during growth in oysters is a potential path worth following in the future.
Figure 5-1. Detachment/destruction of cell monolayers by ΔvgrG::aph. INT-407 or J774 monolayers were infected with either wild type CMCP6 or FLA1035 (ΔvgrG) at an MOI of 10 for 1 hour. The cells remaining attached to the wells were stained with crystal violet and the percent destruction of the monolayer was calculated. Deletion of vgrG had no effect on the ability to destroy the monolayers of infected INT-407 or J774 cells.
Figure 5-2. Apoptosis induced by ΔvgrG::aph. J774 cells were infected with wild-type CMCP6, FLA1035 (ΔvgrG), or FLA943 (ΔrtxA1::aph) at an MOI of 10. Apoptosis was measured using the Apo-ONE kit to detect activity of caspase-3 and caspase-7. Gliotoxin was used as a positive apoptotic control and was considered to induce 100% apoptosis. % apoptosis for each infection was calculated by dividing the normalized RFU of each sample by the average normalized RFU from the gliotoxin treated wells and multiplying that number by 100. FLA1035 caused less apoptosis than wild type; however, it causes more apoptosis than ΔrtxA1. Asterisks indicate statistical significance by a Fisher’s LSD. (*, \( P = 0.0001 \); **, \( P = 10^{-5} \); ***, \( P = 10^{-6} \))
Figure 5-3. Virulence of ΔvgrG::aph in s.c. inoculated mice. Iron dextran-treated mice were s.c. inoculated with 1,000 CFU of either wild-type CMCP6 or FLA1035 (ΔvgrG). Mice were euthanized once their temperatures dropped below 33°C. Samples of the skin lesion and liver were homogenized, diluted, and plated. Fractions below the bars indicate the proportion of mice with detectable bacteria in the samples over the total number of mice infected. While FLA1035 caused detectable infections in only 4 of 5 mice, it was as virulent as wild type in the remaining mice, indicating VgrG is not essential for virulence.
Figure 5-4. Detachment/destruction of cell monolayers by Δhcp::cat. J774 or INT-407 monolayers were infected with either wild type CMCP6 or FLA1030 (Δhcp) at an MOI of 10 for 1 hour. The cells remaining attached to the wells were stained with crystal violet, and the percent destruction of the monolayer was calculated. Similar to the observations with VgrG, deletion of hcp had no effect on the ability to destroy the monolayers of infected J774.
Figure 5-5. Apoptosis of J774 cells infected with Δhcp::cat. J774 cells were infected with wild type CMCP6, FLA1030 (Δhcp), or FLA1035 (ΔvgrG). Apoptosis was measured as before using the Apo-ONE kit. Apoptosis over background level was calculated by dividing the normalized RFU of each sample by the normalized RFU of uninfected cells. Unlike infection with ΔvgrG, infection with Δhcp caused an increase in apoptosis of J774 cells. *, \( P = 0.05 \); **, \( P = 0.03 \); ***, \( P = 0.003 \) determined by Fisher’s LSD.
Figure 5-6. Virulence of Δhcp::cat in s.c. inoculated mice. Iron dextran-treated mice were s.c. inoculated with 1,000 CFU of either wild-type CMCP6 or FLA1030 (Δhcp). Mice were euthanized once their temperatures dropped below 33°C. FLA1030 was as virulent as wild type at this inoculum in all mice, although FLA1030 infected mice had higher temperatures than CMCP6-infected mice, although the average temperature was still below 33°C. (*, P = 0.02; **, P = 0.002 comparing FLA1030 to CMCP6 by Student’s t-test)
Figure 5-7. Cytotoxicity of ΔrtxA1::tetA, ΔvgrG::aph double mutant. INT-407 monolayers were infected with either wild-type CMCP6, FLA954 (ΔrtxA1), FLA960 (ΔrtxA1,ΔvgrG), or FLA1035 (ΔvgrG) at an MOI of 10. Gentamicin was added either at 1 hour or 2 hours postinfection. At 4 hours postinfection, the attached cells were stained with crystal violet and the percent of detachment/destruction of the monolayer was calculated. Deletion of vgrG in the ΔrtxA1 mutant caused no additional reduction of cytotoxicity at 1 hour ($P = 0.08$) or at 2 hours ($P = 0.94$), indicating VgrG is not essential for cytotoxicity. Asterisks indicate statistical significance by Fisher’s LSD. (*, $P = 0.03$ comparing FLA954 or FLA960 to wild-type or FLA1035; **, $P = 0.0001$ comparing FLA960 to FLA1035 or CMCP6; ***, $P = 10^{-5}$ comparing FLA954 to CMCP6).
Figure 5-8. Cytotoxicity of rtxA1 and T6SS double mutants to J774 cells. J774 cell monolayers were infected with CMCP6, FLA954 (ΔrtxA1), FLA960 (ΔrtxA1, ΔvgrG), FLA1035 (ΔvgrG), FLA965 (ΔrtxA1, Δhcp), or FLA1030 (Δhcp) at an MOI of 10 for 1 hour. After overnight incubation, the cells remaining attached to the wells were stained with crystal violet and the level of detachment/destruction of the monolayer was calculated. As expected based on the results during INT-407 cell infections, deletion of either of the T6SS genes had no additional effect on cytotoxicity in the ΔrtxA1 mutant (P = 0.64 for ΔrtxA1, ΔvgrG; P = 0.3 for ΔrtxA1, Δhcp). Asterisks indicate statistical significance by Fisher’s LSD. (*, P = 0.001; **, P = 0.0001; ***, P = 10⁻⁵)
Figure 5-9. Virulence of rtxA1, T6SS double mutants in mice. Iron dextran-treated mice were s.c. inoculated with 3,000 CFU of either FLA954 (ΔrtxA1), FLA960 (ΔrtxA1, ΔvgrG), or FLA965 (ΔrtxA1, Δhcp). While it appeared that the double mutants are more attenuated than the ΔrtxA1 FLA954, deletion of vgrG or hcp had no significant effect on amount of bacteria recovered from the skin compared to FLA954 by Student’s t-test (P = 0.2, FLA960; P = 0.1, FLA965). Similarly, there was no significant difference in bacteria recovered from the liver compared to FLA954 (P = 0.3, FLA960; P = 0.1, FLA965). Fractions below the bars indicate the proportion of mice with detectable bacteria in the samples over the total number of mice infected. FLA960 had a significant difference in the number of mice with systemic disease compared to FLA954 (†, P = 0.04, FLA960 compared to FLA954 by χ² test).
Figure 5-10. Subcutaneous lesions of mice infected with T6SS, rtxA1 mutants. Iron dextran-treated mice were s.c. inoculated with 3,000 CFU of (A) FLA954 (ΔrtxA1), (B) FLA960 (ΔrtxA1, ΔvgrG), or (C) FLA965 (ΔrtxA1, Δhcp). After euthanization, the skin was peeled back to reveal the s.c. lesion at the site of injection.
Cutaneous lesions with extensive tissue damage occur during both primary septicemia and wound infection by *V. vulnificus*. The wound infections and secondary bullous lesions that form during primary septicemia can become necrotic, often requiring surgical debridement of the tissue or amputation of the limb (2). *V. vulnificus* causes extensive damage and is highly invasive, reflected by its ability to invade through the intestinal wall or through the tissues at the site of the wound, causing a systemic infection and eventually death (2).

What is causing the tissue damage observed during infection? This is a question that has remained unanswered, despite more than twenty years of research on the pathogenesis of *V. vulnificus*. *V. vulnificus* is highly cytotoxic in cell culture, and studies to identify the key cytotoxic factor(s) are ongoing. Initial reports of the cytotoxic activity in culture supernatants initiated the interest in secreted toxic factors (42). *V. vulnificus* produces a spectrum of extracellular enzymes that could have potential cytotoxic activity, including the cytolysin (VvhA) (44), metalloprotease (VvpE) (50,93), the RTX toxins (This study, (87-89), type VI secreted factors (108), other hemolysins (38,39), and phospholipases (40,41).

VvhA and VvpE are the best-characterized of the putative toxins. Injection of purified VvhA or VvpE induces extensive dermonecrosis in the skin tissues mice comparable to infection by *V. vulnificus* (44,49). These studies concluded that VvhA and/or VvpE were the cause of the extensive tissue damage. However, strains with mutations constructed in either *vvhA*, *vvpE*, or both genes were still able to cause severe tissue damage and lethal infection, similar to the wild type (47,52,53). It became
clear that while VvhA and VvpE may contribute tissue damage, they are not essential for it and other factors must be contributing to it.

**What Role Do the RTX Toxins Play?**

The RTX toxins represented a promising direction in identifying the destructive factors of *V. vulnificus*. For years, RTX toxins have been recognized for their hemolytic and leukotoxic activities in other bacterial pathogens (70). During this investigation, we have characterized the three putative RTX toxins of *V. vulnificus* and determined that RtxA1 is the major cytotoxic factor of *V. vulnificus*. Despite the potent cytotoxicity of RtxA1 and attenuation of an *rtxA1* mutant for virulence, *rtxA1* mutants are still able to cause tissue damage at the site of the s.c. inoculation in mice. We speculated that the other RTX toxins, RtxA2 and RtxA3, may act in concert with RtxA1. Upon mutation of the *rtxA2* and *rtxA3* genes, we discovered that these toxins were not necessary for cytotoxicity or virulence. It still remains possible that the presence of any one of the toxins may cause damage and lethal infection. Therefore, we are in the process of constructing a triple *rtxA1/rtxA2/rtxA3* mutant.

Interestingly, an *rtxA1* mutant had cytotoxic activity when incubated with INT-407 cells for longer time periods than the usual cytotoxicity assay (>1 hour). This result hinted that RtxA1 may not be the only important cytotoxic factor. Deletion of *vvhA* in combination with *rtxA1* abolished this residual cytotoxicity, confirming previous reports (43) that *vvhA* does have a role in cytotoxicity in vitro. Studies in our laboratory demonstrated that cytotoxicity in vitro does not correlate with virulence in vivo, and the *rtxA1/vvhA* mutant strain provides supports this finding. Despite the noncytotoxic phenotype, the double mutant was not more attenuated than the *rtxA1* mutant, and it
was still able to cause severe skin infections and lethality in mice at a 10-fold higher inoculum than the minimum lethal dose for the wild-type parent.

What, then, is the function of RtxA1 during infection? The defect of the bacteria to cause a lethal infection at low inocula and the increase in i.p. LD<sub>50</sub> observed by other groups (87-89) indicate that RtxA1 may be involved in invasion into the bloodstream to cause systemic disease. Kim, et al. (87) demonstrated that an rtxA1 mutant is defective at invading from the intestine into the bloodstream. RtxA1 may also aid in evasion of the host innate immune response. There are RTX toxins classified as leukotoxins (96); however, examination of the s.c. lesions in the mice suggests that the <i>V. vulnificus</i> ΔrtxA1 mutant is still capable of killing PMNs. This does not rule out a role for RtxA1 in PMN killing, but it indicates that there are other important factors involved in the killing and evasion of immune defenses that are still active in the absence of RtxA1.

**Type VI Secreted Factors are Not Essential for Virulence**

The newly identified T6SS has been implicated in virulence of several pathogens, including <i>V. cholerae</i> (100), <i>P. aeruginosa</i> (101), <i>F. tularensis</i> (103), and <i>E. tarda</i> (104). We identified a region on chromosome II of CMCP6 that encodes the 13 conserved proteins defined as the T6SS "core components" (108). Hcp and VgrG are believed to be the key factors for type VI secretion in other T6SS-encoding bacteria (57), so we targeted these genes for deletion. Deletion of either gene had no effect on cytotoxicity to INT-407 cells or virulence in mice. The vgrG mutant induced less apoptosis than did the wild type, although this effect was not observed for the hcp mutant. This result indicates that hcp may not be necessary for vgrG secretion. Studies with <i>V. cholerae</i> suggest that phenotypes of T6SS mutants may not be observable in the presence of the RtxA toxin (113), so we constructed double mutants.
of ΔrtxA1::tetA, ΔvgrG::aph and ΔrtxA1::tetA, Δhcp::cat. While the deletion of hcp or vgrG in the ΔrtxA1 background has no effect on cytotoxicity compared to the ΔrtxA1, there may be a small effect on virulence, although further studies are needed to confirm this. It is possible that RtxA1 compensates for the loss of T6SS in the single ΔvgrG::aph and Δhcp::cat mutants, and that is why we were able to observe any effect in the absence of rtxA1. Despite these results, it remains clear that T6SS is not essential for virulence in mice, and that it has a very small role, if any, in pathogenesis.

**If Not RTX Toxins or T6SS, What is Causing Damage?**

Other putative toxins such as the HlyIII, VlY, and phospholipases have been proposed to contribute virulence, although little experimental evidence exists. Chen, et al. (38) identified the HlyIII as a putative hemolytic-protein that confers hemolytic activity in *E. coli*. HlyIII has similarity to the hemolysin III (*hlyIII*) of *Bacillus cereus*. This *V. vulnificus* HlyIII is also highly similar to other putative hemolysins, including those of *V. cholerae*, *Y. pestis*, and *S. enterica*. HlyIII of *B. cereus* is a pore-forming hemolysin; however, similar genes in other species have not been examined. *V. vulnificus* HlyIII is indicated to be a hemolysin, although the pore-forming ability has not been observed (35). The *V. vulnificus* hlyIII mutant analyzed by Chen, et al. (38) remained hemolytic, although the mutant was attenuated in i.p. inoculated mice. During our analysis of toxins of *V. vulnificus*, we also deleted hlyIII. Similar to observations made by Chen, et al., our *V. vulnificus* ΔhlyIII mutant was hemolytic on both rabbit blood agar plates and sheep blood agar plates, and the ΔhlyIII mutant was cytotoxic in cell culture. The ΔhlyIII mutant was reduced for virulence at the minimum lethal dose of 300 CFU in s.c. inoculated mice, causing systemic infection in only three of five mice. However, the
mutant was able to cause wild-type levels of skin infection in the mice, suggesting HlyIII does not have a role in tissue damage.

Chang, et al. (39) characterized another putative hemolysin, VIIY, with similarity to the legiolysin (Lly) of *Legionella pneumophila*. Recombinant expression of VIIY in *E. coli* caused the bacteria to become hemolytic and pigmented; however, a *vllY* mutant in *V. vulnificus* was not constructed by Chang, et al. (39). During this study, we constructed a Δ*vllY* mutant, and observed that it was as hemolytic as the wild type on rabbit blood agar plates and sheep blood agar plates, and was fully cytotoxic in cell culture. The *vllY* mutant was slightly attenuated, causing lethal infection in only three of five mice when inoculated at 300 CFU, yet it was able to cause wild-type levels of skin infection.

VIIY and Lly have similarity to the family of 4-hydroxyphenylpyruvate dioxygenase (HppD) proteins, which are eukaryotic and prokaryotic proteins involved in the catabolism of tyrosine (39,122). HppD is involved in the production of pyomelanin and results in pigmentation of the bacteria (48). An *hppD* mutant of *Burkholderia cepacia* is non-pigmented and more susceptible to oxidative stress and killing by macrophages (123). *L. pneumophila* Lly is involved in the production of melanin; however, a mutation in *lly* has no effect on hemolysis or on the survival of the intracellular survival of the bacteria in macrophages (124). In *V. cholerae*, melanin production is induced during osmotic and temperature stress, and the HppD/tyrosine catabolism pathway has recently been attributed to expression of certain virulence factors (125). A *V. cholerae* mutant in homogentisate 1,2-dioxygenase (*hmgA*), downstream of *hppD*, was hyperpigmented due to overproduction of melanin. The
hmgA mutant had elevated expression of cholera toxin and the toxin-coregulated pilus and had an increased ability to colonize mice compared to wild type (125). While differences in pigmentation of the V. vulnificus ΔvllY have not been observed, this does not rule out a role in melanin production. V. vulnificus VllY may have a role in virulence, perhaps through regulation of other virulence factors, as observed for V. cholerae, or by enhancing survival during oxidative stress and other environmental stresses.

The phospholipase activity of V. vulnificus has also been proposed to have a role in virulence. Testa, et al. (40) demonstrated that V. vulnificus possesses phospholipase A₁/A₂ and lysophospholipase activities, but not phospholipase C activity. Koo, et al. (41) subsequently proposed that this phospholipase A (PLA) activity was important for virulence in a mouse model. The researchers used tetracycline to inhibit phospholipase activity during infection and claimed that it was the inhibition of PLA activity that caused attenuation in virulence (41). Unfortunately, there were many gaps in the study by Koo, et al. (41), including the lack of construction and analysis of a phospholipase mutant. PLA has a role in virulence of other bacterial pathogens (126). A lecithin-dependent hemolysin with PLA activity contributes to virulence of Yersinia enterocolitica by promoting colonization and by inducing inflammation and necrosis of Peyer’s patches and mesenteric lymph nodes (127). PLA enzymes can also be hemolytic/cytolytic by causing destabilization of cell membranes (126). For example, the lecithin-dependent hemolysin (LDH) V. parahaemolyticus with PLA activity causes lysis of erythrocytes (128).

In light of this unresolved issue of the V. vulnificus PLA, we followed up on the report by Koo, et al., to determine if PLA activity of V. vulnificus was important for
We examined the annotated genome of *V. vulnificus* CMCP6 and discovered that CMCP6 encodes two genes with annotated PLA activity, phospholipase/lecithinase/hemolysin (*tlh*) and an outer membrane phospholipase A (*ompla*). We deleted these genes from CMCP6 individually and analyzed the mutants for hemolytic ability, crude phospholipase activity, and virulence. Both mutants were hemolytic on rabbit or sheep blood agar plates, despite the fact that the phospholipase/lecithinase/hemolysin protein has 73% identity to the thermolabile hemolysin TLH (also known as the lecithin-dependent hemolysin, LDH) of *V. parahaemolyticus*. We observed lecithinase activity on tryptic soy agar plates supplemented with 5% (vol/vol) egg yolk. The *tlh* mutant had a very slight difference in lecithinase/phospholipase activity compared to the wild type, but no difference in activity of the *ompla* mutant compared to wild-type. Both mutants were as virulent as the wild type in s.c. inoculated, iron-treated mice. Until a mutant strain lacking phospholipase activity is isolated, the claims made by Koo, *et al.* cannot be completely disregarded. Phospholipase activity may still contribute the invasiveness or the tissue damage caused by *V. vulnificus*.

*V. vulnificus* is an Accidental Human Pathogen

By simply looking at the annotated functions of the proteins described above, one would speculate that those proteins would be involved in virulence of *V. vulnificus*. Hemolysins, proteases, RTX toxins, type VI-secreted effectors, or phospholipases do contribute to virulence in many pathogens. *V. vulnificus* is first and foremost an environmental organism residing in shellfish and seawater that predominantly causes disease in people with underlying health conditions (19). The rapid nature of the disease and the fact that humans are a dead-end host are reflective of the fact that *V.
*V. vulnificus* has not evolved to be a human pathogen, and infection of humans is not a requirement for *V. vulnificus* to maintain its lifestyle. Why, then, would *V. vulnificus* possess so many destructive enzymes? *V. vulnificus* lives in an environment in which it must compete for nutrients and colonize filter-feeding shellfish, all the while avoiding predatory organisms which may feed on bacteria. The hemolysins and proteases, which we classify as toxins, may be important in acquiring and breaking down nutrients or avoiding predation by other organisms. The RtxA2 and RtxA3 toxins with the putative VWF domain may be involved in adherence and colonization of shellfish or other surfaces. The higher expression of *rtxA2* at 20°C and 30°C, compared to 37°C, provides evidence for a role of RtxA2 in the environment, as opposed to human disease (98). The T6SS may also be an environmental survival factor. The T6SS was identified in *V. cholerae* by identifying mutants defective at killing the predatory amoeba *D. discoideum* (100). Perhaps the T6SS of *V. vulnificus* has a similar role in avoiding predatory organisms. The *V. vulnificus* T6SS genes resemble the T6SS genes of symbiotic bacteria, more so than the genes of pathogenic bacteria, indicating T6SS effectors may have role in colonizing or survival within shellfish. In summary, each of the toxins described here most likely has an important role for survival and growth in the environment and by chance happen to be destructive to host cells and tissue.

**Final Remarks**

We have not been able to determine what is causing the destructive tissue damage during infection with *V. vulnificus*, although we have provided evidence that it is most likely multifactorial. *V. vulnificus* produces many extracellular enzymes with potential destructive activity, but deletion of any one of them individually has little or no effect on virulence. It is possible that the production of the other toxins will compensate
for the loss of one or that the key destructive factor has not yet been examined. Damage of the tissues may also be a side effect of the rapid growth of the bacteria and the inability of the host immune response to handle the infection. A fadR mutant exhibits slow growth in vitro and is highly attenuated in vivo (129). This mutant is defective at causing skin infections in mice at inocula as high as $10^5$ CFU, despite evidence suggesting FadR has no role in cytotoxicity to host cells in vitro. The most definitive virulence factors of V. vulnificus identified to date contribute mainly to growth and survival of the organism during infection (i.e., iron acquisition, capsule). Despite numerous attempts by many researchers, the factors causing destructive tissue damage and invasion during infection remain elusive.
REFERENCE LIST


Choy, H.E., Progulske-Fox, A., Hillman, J.D. et al. 2003. Characterization and
pathogenic significance of *Vibrio vulnificus* antigens preferentially expressed in

positive regulator of the M protein gene of *Streptococcus pyogenes* with similarity


vector for use in *Vibrio cholerae* and other gram-negative bacteria. *Gene* 153:85-
87.

cloning vectors and host strains: nucleotide sequences of the M13mp18 and

70. Welch, R.A. 2001. RTX toxin structure and function: a story of numerous
257:85-111.

The calmodulin-sensitive adenylate cyclase of *Bordetella pertussis*: cloning and

meningitidis* produces iron-regulated proteins related to the RTX family of

73. Lin, W., Fullner, K.J., Clayton, R., Sexton, J.A., Rogers, M.B., Calia, K.E.,
cholerae* RTX toxin gene cluster that is tightly linked to the cholera toxin

74. Welch, R.A., Forestier, C., Lobo, A., Pellett, S., Thomas, W., Jr., and Rowe, G.
1992. The synthesis and function of the *Escherichia coli* hemolysin and related


76. Forestier, C. and Welch, R.A. 1990. Nonreciprocal complementation of the *hlyC*
and *lktC* genes of the *Escherichia coli* hemolysin and *Pasteurella haemolytica*


BIOGRAPHICAL SKETCH

Jennifer Joseph was born and raised in Jacksonville, FL. After graduating from Stanton College Preparatory School, she attended Florida State University in Tallahassee, FL. She graduated *cum laude* with a B.S. in biological sciences and moved to Gainesville, FL to work at the United States Department of Agriculture for a short while before pursuing a graduate degree through the Interdisciplinary Program in Biomedical Sciences at the University of Florida. During her graduate career, Jennifer has been funded by the Alumni Fellowship and an NIH-funded Training Grant in Biodefense and Emerging Infectious Disease.