CHARACTERIZATION AND MOLECULAR ANALYSIS OF FRAGILYSIN: THE *BACTEROIDES FRAGILIS* TOXIN

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(ABSTRACT)

Bacteroidesfragilis is a gram negative, anaerobic rod, that is a member of the normal colonic microflora of most mammals, and it is the anaerobe most commonly isolated from human soft-tissue infections. During the past decade, strains of *B. fragilis* that produce an enterotoxin have been implicated as the cause of diarrhea in a number of animals, including humans. The extracellular enterotoxin has been purified and characterized as a single polypeptide (Mr~ 20,600) that causes rapid morphological changes in human colon carcinoma cell lines, particularly, HT-29. This dissertation research began in 1993 with the purpose of determining how this enterotoxin, termed fragilysin, causes diarrhea. The deduced amino acid sequence revealed a signature zinc-binding consensus motif (His-Glu-Xx-Xxx-His-Xxx-Xxx-Gly-Xxx-Xxx-His/Met) characteristic of metalloproteinases. Sequence analysis showed close identity with metalloproteinases within the zinc-binding and Met-turn regions. Purified fragilysin contained 1 gram atom of zinc per molecule, and it hydrolyzed a number of proteins, including gelatin. Optimal proteolytic activity occurred at 37°C and pH 6.5. Activity was inhibited by metal chelators but not by inhibitors of other classes of proteinases. When fragilysin is injected into ligated ileal and colonic loops of animals, there is significant tissue damage and a subsequent dose dependent fluid response. Histological examination revealed mild necrosis of epithelial cells, crypt elongation, villus attenuation, and hyperplasia. There was extensive detachment and rounding of surface epithelial cells and an infiltration of neutrophils. Enterotoxic activity was inhibited by the metal chelators EDTA and 1,10-phenanthroline; and, to some degree, the enterotoxic activity could be reconstituted by the addition of zinc to chelated toxin.   Fragilysin rapidly increased the permeability of the paracellular barrier of epithelial cells to ions (decrease in electrical resistance across monolayers) and to larger molecules (increase in mannitol flux across monolayers). Furthermore, there is a direct effect on the tight junction proteins. Fragilysin appears to cause diarrhea by proteolytically degrading the paracellular barrier of epithelial cells. Fragilysin is a recently discovered virulence factor that could contribute to the pathogenesis of *B. fragilis* in both intestinal and soft tissue infections.

FOREWORD

This dissertation contains eight sections (I-VIII). The first section is a review of the literature, which provides an introduction to bacterial enterotoxins, bacterial metalloproteinases, and *Bacteroides fragilis*. Sections II through VI are written in a format for submission for publication. Of these sections, sections II-IV have been published (1-3); whereas section V will be published in Clinical Infectious Diseases, July 1997 (4). Section VI is written as a possible manuscript. Section VII will be published in "The Handbook of Proteolytic Enzymes" (5). Section VIII is a conclusion and discussion section. Where appropriate, each reference below lists my contribution to the manuscripts.

1. **Moncrief, J.S., R.J. Obiso, Jr., L.A. Barroso, J.J. Kling, R.L. Wright, D.M. Lyerly, R.L. Van Tassell, and T.D. Wilkins.** 1995. The enterotoxin from *Bacteroides fragilis* is a metalloproteinase. Infection and Immunity. **63**:175-181.

   In this research article, I contributed most of the protein biochemistry, protein purification, and protein analysis. J.S. Moncrief, L. Barroso, and R.L. Wright developed the SSP-PCR, cloned a portion of the toxin, and sequenced the cloned portion of the gene for the toxin.

2. **Obiso, R.J., Jr., R.L. Van Tassell, D.M. Lyerly, and T.D. Wilkins**. 1995. Proteolytic activity of the *Bacteroides fragilis* enterotoxin causes fluid secretion and intestinal damage in vivo. Infection and Immunity. **63**:3820-3826.

   In this research article, most of the work described was completed by me. The other authors contributed by assisting with the experimental design and assisting with writing of the manuscript.

3. **Obiso, R.J., Jr., A.O. Azghani, and T.D. Wilkins**. 1997 *Bacteroides fragilis* toxin fragilysin disrupts the paracellular barrier of epithelial cells. Infection and Immunity. **65**:1431-1439

   In this research article, most of the work described was completed by me. Dr. Azghani contributed the data on the Rat type II lung cells because they are a primary cell line in his laboratory.

4. **Obiso, R.J., Jr., D.R. Bevan, and T.D. Wilkins**. 1997. Molecular modeling and analysis of the *Bacteroides fragilis* toxin. Clinical Infectious Diseases. In press for July.

   In this research article, Dr. Bevan and I worked together to develop the molecular model of fragilysin, including amino acid alignments, molecular similarities, secondary structure predictions, and modeling of fragilysin.

5. **Obiso, R.J., Jr. and T.D. Wilkins.** The *Bacteroides fragilis* toxin, fragilysin. *In*, F. Woessner and A. Barrett (eds.), Handbook of Proteolytic Enzymes, Academic Press Publishing Co., London. In press for January 1998.

This is a brief summary of fragilysin that I wrote with Dr. Wilkins. This will become a chapter in, "The Handbook of Proteolytic Enzymes".

In addition, I contributed to the following publications:

1. **Lawrence, J.P., L.Brevetti, R.J. Obiso, Jr., K. Kimura, R. Soper, a n d T.D. Wilkins**. 1997. Effects of epidermal growth factor and *Clostridium difficile* Toxin B in a model of mucosal injury. Journal of Pediatric Surgery. **32**:430-435.

   I provided purified Toxin A and Toxin B from *Clostridium difficile* and fragilysin f r o m *B. fragilis*. I also helped design the experimental approach for this paper.

2. **Wells, C.L., E.M.A. Van de Westerlo, R.P. Jechorek, B.A. Feltis, T . D . Wilkins, and S.L. Erlandsen**. 1996. *Bacteroides fragilis* Enterotoxin Modulates Epithelial   Permeability and Bacterial Internalization by HT-29 Enterocytes. Gastroenterology. **110**:1429-1437.

   In this paper I provided purified fragilysin, chelated fragilysin, and zinc-   reconstituted fragilysin.

3. **Donelli, G., A. Fabbri, C. Fiorentini.** 1996. *Bacteroides fragilis* e n t e r o t o x i n Induces Cytoskeletal Changes and Surface Blebbing in HT-29 cells. Infection and Immunity **64**:113-119.

   In this paper I provided purified fragilysin, chelated fragilysin, and zinc-reconstituted fragilysin.

4. **Koshy, S.S., M.H. Montrose, and C.L. Sears**. 1996. Human intestinal epithelial cells swell and demonstrate actin rearrangement in response to the metalloproteinase toxin of *Bacteroides fragilis*. Infection and Immunity **64**:5022- 5028.

   In this paper I provided purified fragilysin, chelated fragilysin, and zinc-reconstituted fragilysin.

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This work was accomplished at the Anaerobe Lab and at the Fralin Biotechnology Center at Virginia Polytechnic Institute and State University. I would like to acknowledge and express my appreciation to the co-authors and others who appear on these publications, which make up a majority of this dissertation.

I would especially like to express my appreciation to Dr. Tracy Wilkins for providing space, support, and encouragement; appreciation is also expressed for allowing me to determine the nature and direction of my research, for teaching me how to "think", and for allowing me to be involved with my dream, teaching. For this I am grateful. I would also like to thank Tracy for his guidance; I learned a great many things about life, business, making money in science, entrepreneurism, teaching, and research. Tracy has helped me develop into who I am today, and for this I am truly grateful.

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I would like to thank Dr. Scott Moncrief, Dr. David Lyerly, and Roger VanTassell for their help, their ideas, their advice and support, and their encouragement over the last four years. These individuals are reputable scientists and good teachers. I would like to thank the members of my committee: Dr. J.S. Chen, Dr. D. Dean, Dr. G. W. Claus, Dr. E.M. Gregory, and Dr. B. Storrie for their interest, patience, and assistance during the past four years. I would also like to thank other faculty, who have contributed their knowledge and energy to my learning and understanding this field: Dr. D.R. Bevan, Dr. P.J. Kennelly, Dr. N.R. Krieg, Dr. D.M. Moore, Dr. G.Saunders, and the late Dr. W.E.C. Moore. I would also like to thank the many friends and colleagues that I have made during the course of this research: Lisa B., Jim K., Sam P., Len C., Mark T. Paul G., Valerie C., Limin Z., Richard J., John P., Kim J., Ghada A., Dave G., the animal technicians, the veterinary medicine histologist and pathologist technicians, and Greg Wagner. Each of these individuals has added to my education and has made it a fun experience. I would also like to acknowledge the many undergraduates that I have taught in classes and especially in the research lab; I may have learned more from them then they learned from me.

I would like to thank my family for their encouragement and guidance. This dissertation is dedicated to my wife, Melissa, who has provided emotional support, encouragement, understanding, and a sense of perspective through my education, but, most of all, who was there to listen.

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CHAPTER 1:   LITERATURE REVIEW

A.   INTRODUCTION
At one point in time, the Earth was anaerobic. A few billion years ago, when the world was cooling and life was evolving in the oceans, all of the oxygen was bound in molecules such as carbon dioxide and water. As the Earth developed, so did life; as the world became aerobic, most living things evolved to utilize gaseous oxygen. However, not all of the world became aerobic. Anaerobic conditions still exist in the sediments of the sea and land, and in the intestines of animals.

Anaerobic bacteria evolved during the period when there was no oxygen in the atmosphere. However, the existence of anaerobes has only been known for a little over a century and a half, when Pasteur noticed that certain microbes were motile only when oxygen was absent. Anaerobic bacteria were frequently ignored for the first fifty years following their discovery. This was not because they are rare, actually, anaerobic bacteria outnumber aerobes in many environments, but was probably due to the inability to culture them (27, 46, 124, 151).

The study of anaerobes, however, is now a well-established field in microbiology. Over the past thirty-five years, technical advances in the collection and cultivation of these anaerobic bacteria have helped to define the importance of these organisms in the environment and in human disease.

The normal flora of the gastrointestinal tract forms an important and protective barrier against infection by pathogens. Under normal conditions, the concentration of bacteria in the human small intestine ranges from 10^5 to 10^8 organisms per mL, which includes both aerobes and anaerobes (27, 46, 74). In the colon, however, there is a logarithmic increase in the number of bacteria (10^{10} to 10^{11} per mL) in which the number of anaerobes outnumber the aerobes approximately 100:1 (27, 46, 151). Moore and Holdeman (92) reported that there are over 400 different species of bacteria in the intestinal tract of humans, but most are present at less than 10^8 cells per gram of feces. Therefore, the colonic environment of mammals is one of the most complex anaerobic ecosystems known today.

The following is a compilation of relevant information that should provide some background to help the reader better understand this dissertation. The review of the literature will encompass an analysis of some of the "classical" bacterial enterotoxins, some bacterial metalloproteinases, *Bacteroides fragilis*, and enterotoxigenic *Bacteroides fragilis*.

B.   BACTERIAL ENTEROTOXINS AND DIARRHEAL DISEASE

Gastrointestinal diseases are a major concern in developing areas of the world and a disturbing problem in industrialized regions. Diarrheal disease is the most common acute infectious disease in the world and is responsible for significant mortality in developing countries (154). Acute diarrhea is estimated to kill upwards of five million children under five years of age each year. Death rates from diarrhea often exceed 20 per thousand in the early years of life (40). Microorganisms that cause human diarrhea include a variety of bacterial, viral, and protozoan species; some helminths can also cause diarrheal disease (109).

With microorganisms such as *Clostridium difficile*, *Shigella* species, *Salmonella enteritidis*, enteroinvasive *Escherichiacoli*, and *Entamoebahistolytica*, invasion of epithelial cells and/or the effects produced by toxins on the epithelium, results in an inflammatory diarrheal disease. Leukocytes appear in the stool, indicating that polymorphonuclear leukocytes have been recruited to the area and have escaped into the intestinal lumen. Noninflammatory diarrheas occur with *V. cholerae*, rotaviral disease, and enterotoxigenic *E. coli* infections. These infections are characterized by a watery diarrhea with little to no tissue damage (40).

1.   Detecting Bacterial Enterotoxins

One challenge to researchers in this field is to develop ways to characterize bacterial enterotoxins. Enterotoxins have been studied in a number of different ways, including ligated intestinal loops, in vivo perfusion, RITARD, Ussing chambers, and tissue culture assays.

Intestinal loop assays. In 1959, De et al. were the first group to invent the intestinal loop assay. In this experiment, this group ligated ileal and colonic intestinal segments and inoculated these segments with sterile culture filtrates of *V. cholerae* in rabbit intestinal loops and found that *V. cholerae* stimulates intestinal secretions. Since then, the assay has been used as a gold standard to study the effects of bacteria and toxins in the intestines. This method provides data on the amount and type of fluid that is secreted into the intestines, as well as, data on the histology of the intestinal lining.

In vivo perfusion assays. In vivo perfusion is a modification of the intestinal loop assay that allows for more precise measurements of the change in ions and the type of secretions in the intestine (3). For this experiment, a ligated intestinal segment is cannulated with a multiperforated tube. Intestinal measurements are made in under 5 hours, which yields a more accurate analysis of the changes in water and ion transport caused by bacteria and their toxins.

Oral inoculation of animals. This approach establishes whether a particular bacterium or toxin can cause diarrhea or cause the accumulation of fluid in the intestine (59, 97, 100, 122). This method is also used to establish an infection with enteric pathogens (59).

RITARD model. The reversible-ileal-tie-rabbit-diarrheal-disease model (RITARD) is useful in studying enteric bacteria and their toxins because it more closely mimics the native disease than intestinal loops. In this model, the intestinal tract is ligated at the cecum and the samples are injected into the intestine at a site proximal to the ligature. This provides a chance for organisms to attach or establish an infection in the intestine without being washed out by the flow of intestinal contents. After a short incubation time, the ligature is removed and the infection is allowed to proceed over the course of hours or days (95, 141). This method is used mainly when oral

inoculation is not sufficient to establish an infection.

Ussing chambers. The Ussing chamber is used to identify specific changes in active ion transport caused by enteric bacteria and their toxins. Either intestinal epithelial explants or monolayers of cultured epithelial cells are mounted on permeable (0.4 µm pore size) chambers that are neutral to ions and osmolarity. Once epithelial cells attach to the chamber, toxins or bacteria are added to the system and any change in the ionic, osmotic, or electrical flux can be measured (78, 147).

Tissue culture assays. Cell lines of intestinal and nonintestinal cells have been used extensively to detect enteric toxins. Most often, the activity is detected by a change in shape of the cells (For reviews, see: 82, 108, 134, 140).

2. Bacterial Virulence and Mechanism of Action of Enterotoxins

Recently, two unique but prevalent themes have emerged in the field of bacterial virulence: type III secretion systems and pathogenicity islands. Type III secretion systems, which are found in various gram negative organisms, are specialized for the export of virulence factors to host cells. The genes encoding several type III secretion systems reside on pathogenicity islands. These are inserted DNA segments within the bacterial chromosome that contain virulence traits, such as the ability to acquire iron, adhere to or enter host cells, or to produce toxins. Type III secretion systems and pathogenicity islands must have played important roles in the evolution of pathogens and are likely to aid in the emergence of new pathogens (87). Two other specialized secretions systems, type I and type II, transport molecules to the bacterial cell surface (119). Proteins secreted by the Type I system cross directly from the cytoplasm to the cell surface. Type II secreted proteins use the general secretory pathway to reach the periplasmic space and then transverse the outer-membrane through porins.

Understanding the virulence factors and pathogenesis of some enteric diseases has advanced markedly at the cellular level within the last two decades. A description of a few of the "classical" enterotoxins is presented in the pages that follow.

Vibrio cholerae. *V. cholerae* is a curved gram-negative rod that is the cause of cholera, a severe and often lethal diarrheal disease (9). The disease has been recognized since ancient times (10). Historically, cholera has been associated with explosive outbreaks, including the pandemic of Indonesia in 1961, Peru and South America in 1991, India in 1992, and in Zaire in 1994 (71, 130). Man is the only known natural host for *V. cholerae*. The organism is usually acquired from contaminated food or water (60).

During growth, *V. cholerae* produces cholera toxin, which is an A-B-type toxin. The genes that encode the A and B subunits of cholera toxin are part of the *ctx*AB operon, which is part of a bacteriophage encoded pathogenicity island (87, 110, 153, 159) The B subunits bind the toxin to the eukaryotic cell receptor, the ganglioside GM_1, and allow the A subunit to enter the cell. The A subunit is an enzyme that catalyzes the ADP-ribosylation of the G_{sa} subunit of a G protein, leading to the activation of adenylate cyclase. This results in an increase in intracellular cAMP concentrations. Elevated cAMP levels, in turn, leads to an increase in the secretion of chloride by intestinal crypt cells and a decreased reabsorption of chloride and water by villus cells (37). All of this causes a very large net secretion of water and death can ensue if the ionic balance is not

maintained with oral rehydration therapy.

Recently, Mekalanos et al. (153) sequenced the *V. cholerae* pathogenicity island and found that some of the genes within the island had sequence similarity with reported bacteriophage genes. They also found that a small percentage of nontoxigenic *V. cholerae* could become toxic without directly contacting toxigenic strains. They proved that the pathogenicity island was an inserted bacteriophage that was able to transfer to nontoxigenic strains. They purified the virus, and by electron microscopy were able to see the curved bacteriophage.

This island of DNA also encodes a number of other toxins (e.g. zot, ace, cep) and phage structural proteins, as well as, proteins involved in a Type III secretion system (87, 153). The *zot* gene encodes the zonula occludens toxin (8). Zot toxin has been shown to disorganize the cytoskeleton of eukaryotic cells, which results in weak enterotoxic activity (8, 34). Recently, zot was shown to have significant homology with proteins that assemble bacteriophage. The *ace* gene, which encodes the protein called "accessory cholera toxin", results in a mild diarrhea in the absence of other toxins (144); this protein also functions as an assembly component of the bacteriophage. The *cep* gene, which encodes a pilus-like intestinal colonization factor, also functions as a capsid protein that makes up a major component of the filamentous phage (117). *V. cholera* also produces a hemolysin and a metalloproteinase (16, 57, 61) that are not part of the bacteriophage island. The metalloproteinase nicks the hemolysin to activate it (16). This hemolysin has also been shown to induce fluid accumulation in rabbit loops (61). So, *V. cholerae* contain a number of virulence factors that can be used to help cause disease in humans. Table 1 shows the genes involved in virulence along the pathogenicity island and their functions before and after the bacteriophage was discovered.

The results from the study by Mekalanos et al. provide insight into how virulence genes can be transferred in pathogens. This information may challenge vaccine researchers who use live, nontoxic strains of *V. cholerae* and other pathogens. Bacteriophage that carry virulence genes could infect live nonvirulent bacteria that are used for vaccines. This is particularly important in the case of *V. cholerae*, because there is a large research effort to develop a vaccine for cholera.

TABLE 1. Genes located in the toxigenic element of *V. cholerae*.

GENE	DESCRIBED FUNCTION BEFORE PHAGE DISCOVERY	DESCRIBED FUNCTION AFTER PHAGE DISCOVERY (153)
zot	cytoskeletal rearrangement, disruption of tight junction - - weak enterotoxin (34)	protein required to assemble bacteriophage
ace	accessory cholera toxin - - enterotoxin (144)	bacteriophage assembly component
cep	pilus-like protein - - adherence (117)	bacteriophage capsid protein
ctxA	cholera toxin - - ADP-ribosylating activity	cholera toxin - - ADP-ribosylating activity
ctxB	cholera toxin - - binding domain	cholera toxin - - binding domain
rstA, B, C, R	repetitive sequences - unknown (117)	repetitive sequences required for phage insertion/excision
orfU	unknown (144)	bacteriophage receptor binding protein

Shigella   dysenteriae. *Shigelladysenteriae* was discovered by Kiyoshi Shiga in 1898 during an epidemic of especially severe dysentery in Japan (136). *S. dysenteriae* is not the only pathogenic species of *Shigella*. *S. flexneri*, *S. sonnei*, and *S. boydii* all contain similar toxins and cause enteric disease (125). Infection by *Shigella* species can occur with a very small inoculum, which permits rapid person-to-person spread (30).

Shiga toxin is part of a family of structurally and functionally related toxins, including the *E. coli* shiga-like toxins, or verotoxins. A typical infection by a *Shigella* species starts out with a watery diarrhea and progresses into a bloody diarrhea. When the organism grows, the toxin is produced and accumulates, forming lesions in the distal colon with death of epithelial cells. When this tissue damage occurs, the host sends a large number of neutrophils to the colonic epithelium, resulting in leukocytes in the stool (108).

Shiga toxin consists of two covalently linked peptides, a 32-kDa enzymatically active A subunit and five 8-kDa B subunits that are responsible for binding toxin to the cell surface glycolipid receptor, globotriaosylceramide (Gb$_3$) (62, 63). The A subunit possesses the same N-glycosidase action as the toxic plant lectin, ricin. These two toxins permanently inactivate the eukaryotic ribosome by hydrolyzing adenine from a single specific adenosine in the 28S component of the 60S ribosomal subunit. This stops protein synthesis, resulting in cell death (33).

Shigella species, like some *Listeria* species and other intracellular pathogens, invade the host epithelial, endothelial, or macrophage cells by a process resembling phagocytosis. *Shigella* species bind to the cell and are internalized by the formation of an intracellular vacuole leading to complex changes in the actin cytoskeleton. Shigella multiply in the cell soon after invasion and migrate from cell to cell by utilizing the host cytoskeleton. The shigella direct actin polymerization and create an actin tail behind the bacterium. The rates of actin assembly and bacterial migration are rapid, reaching speeds of up to 1.4 μm/sec (108). When the bacterium reaches the cell membrane, the actin polymerization forces it against the cell membrane, which makes a finger-like projection that allows invasion into the adjacent cell (132). This mechanism allows shigella to spread from cell to cell without coming in contact with the host's extracellular defenses.

Escherichia coli. Although most strains of *E. coli* exist as harmless facultative anaerobes in the human gut, several distinct groups are armed with virulence factors capable of causing serious illness. These *E. coli* strains include several types that can cause diarrhea by different mechanisms (79).

The first evidence that *E. coli* caused diarrhea was reported in the 1940s (18), and since then, six distinct categories of *E. coli* that cause diarrhea have been named: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). Strains belonging to these groups usually contain specific diarrheic genes located on plasmids (121). Table 2 provides a brief overview of each *E. coli* type and their associated characteristics.

Enterotoxigenic *E. coli* (ETEC) produce two groups of toxins. One group of toxins are antigenically similar to cholera toxin and are called the heat-labile, or LT toxins, and the other are known as the heat-stable, or ST toxins. Several forms of each type of toxin have been characterized (43, 139). Two types of stable toxins, STI and STII have been described (93, 155). These are 18 and 19 amino acids in length, respectively, and contain 6 cysteine residues that are required for activity. The STI and STII toxins are homologous to the hormone guanylin, which regulates fluid and electrolyte secretion in the intestine. The hormone and the toxins activate guanylate cyclase, which increases cGMP and increases chloride efflux into the intestinal lumen (93, 155). Two types of labile toxins, LTI and LTII, have also been characterized (35, 41). The heat labile toxins are analogous to cholera toxin in sequence, structure, and activity (44). The LT toxins and cholera toxin were probably derived from the same ancestral gene: LTI and LTII are 76% and 78% similar to cholera toxin, respectively. The A subunit catalyzes the transfer of ADP-ribose from NAD to a G-protein in the cell. This locks the G-protein in the stimulatory mode and causes the activation of adenylate cyclase, which in turn leads to elevated cAMP. This increase in cAMP increases chloride secretion and water efflux into the intestinal lumen (44).

Infection by enteroinvasive *E. coli* (EIEC) causes an illness similar to shigellosis (58). These organisms harbors a large plasmid that contains several genes that allow this *E. coli* to act like *Shigella* (45). EIEC cause diarrhea by penetration into enterocytes followed by multiplication, leading to cell death.

Enterohemorrhagic *E. coli* (EHEC) produce a cytotoxin called the verotoxin or Shiga-like toxin (SLT). The serotype O157:H7 is mostly associated with EHEC, which is now recognized as the cause of hemorrhagic colitis (73). These *E. coli* produce hemorrhagic colitis by attaching to intestinal enterocytes and producing the shiga-like toxins SLT1 and SLT2. These toxins are

composed of A-B subunits similar to shiga toxins and work by the same mechanism (123). Virulence also is dependent on adhesion, which is mediated by a 60-MDA plasmid (72).

Enteropathogenic *E. coli* (EPEC) attach to and destroy microvilli of enterocytes, but do not produce high levels of shiga-like toxin (72). For a review of this recently discovered type of pathogenic *E. coli*, see Donnenberg et al (26).

Enteroaggregative *E. coli* (EAEC) are defined as having an aggregative adherence phenotype. These *E. coli* contain a large plasmid that encodes for a fimbrial protein, toxins similar to the heat stable toxins, and a newly described toxin, called contact hemolysin (47).

TABLE 2. Pathogenic *E. coli* types and characteristics (26).

E. COLI TYPE	EPIDEMIOLOGY	CLINICAL SYNDROME
ETEC	Travelers; children	Watery diarrhea
EIEC	Occasional epidemics; children	Dysentery
EPEC	Infants in developing countries	Watery diarrhea
EHEC	Outbreaks in all ages	Bloody diarrhea
EAEC	Children	Persistent diarrhea
DAEC	Children	Persistent diarrhea

Clostridium difficile. *C. difficile* causes gastrointestinal disease ranging from mild diarrhea to life-threatening pseudomembranous colitis (PMC). The disease, which is almost always nosocomial in origin, occurs primarily following the disruption of the normal protective microflora by antibiotics (82). The role of *C. difficile* in diarrheal disease became established in the 1970s, when reports of PMC and death were observed in patients receiving clindamycin (42, 69). Patients that had died of the resulting antibiotic-related diarrhea had severe inflammation of the colonic mucosa, including a sheath covering the colon that was composed of fibrin, epithelial cells and leukocytes (81).

C. difficile produces the two largest single polypeptide toxins, A and B. Toxin A has an Mr of 308,000 and toxin B has an Mr of 269,000 (7, 32). Table 3 summarizes the biological activities of these toxins. Both toxins have an overall similarity of over 45% at the amino acid level (7). Both are composed of three major domains: a multiple repeating region, a hydrophobic region, and a nucleotide binding region that may bind UDP-glucose (2, 67, 68). Both toxins are cytotoxic and cause eukaryotic cells to become round and eventually die; toxin A also has enterotoxic activity, toxin B does not (81). It is thought that toxin B does not bind to the receptors on the lumen side of the intestine, which is probably why toxin B gives different clinical effects. Recently, the mechanism of action for these toxins has been determined (31, 67). They bind to the eukaryotic cell and are taken up by receptor mediated endocytosis and general pinocytosis (52). The toxins then monoglucosylate a small group of GTP-binding proteins, including rho, by using

UDP-glucose as a cofactor (2, 67, 68). Rho is a major cytoskeletal regulatory protein that controls actin polymerization and depolymerization (21, 116, 158). When the toxins catalyze the addition of glucose to rho at threonine 37 it becomes inactivated and the actin cytoskeleton falls apart, resulting in the cells becoming round (31, 67). The lack of epithelial cell structure leads to disruption of the paracellular barrier and an increase in the permeability of the intestinal epithelium, which causes the diarrhea (50, 51). There are also several other secondary effects caused by the toxins' action on the intestinal cells, including cytokine production, inflammation, and cell death.

TABLE 3. Biological activity of the *Clostridium difficile* toxins A and B (82).

TOXIN	ENTEROTOXIC ACTIVITY (rabbit loop assay)	CYTOTOXIC ACTIVITY (tissue cultured cells)	LETHAL ACTIVITY (I.P. injection mice)
TOXIN A	1 μg	10 ng	30 ng
TOXIN B	none	1 pg	30 ng

All of the bacterial enterotoxins have one thing in common; they all possess a mechanism to cause a fluid response in the intestines. There are many toxins produced by many different species of bacteria that go far beyond the scope of this dissertation. In general, bacterial toxins are classified as either membrane damaging, intracellular acting, or extracellular damaging. The membrane damaging toxins seem to act by a non-specific action on the cell membrane. Examples of these are phospholipases and hemolysins. The intracellular acting toxins share a common mechanism involving binding to a receptor, internalization, and interaction with the intracellular target. Examples of these are the *Clostridium difficile* toxins, cholera toxin, and shiga toxin. The extracellular acting toxins include proteinases that degrade the extracellular matrix and tight junction proteins, most of which relay a secondary action into the cell.

C.   BACTERIAL METALLOPROTEINASES AND VIRULENCE

Proteinases are enzymes that catalyze the hydrolysis of peptide bonds in proteins or peptides. They are either exopeptidases, whose actions are restricted to the amino- or carboxy-termini of proteins, or endopeptidases, which cleave internal peptide bonds. Proteinases are present in all living organisms and have a variety of physiological functions (14).

Microbial proteinases are predominantly extracellular and can be classified into four groups based on the essential catalytic residue at their active site. They include serine proteinases (EC 3.4.21.X), cysteine proteinases (EC 3.4.22.X), aspartate proteinases (3.4.23.X), and the metalloproteinases (EC 3.4.24.X) (14). Most metalloproteinases contain zinc. The majority of zinc endopeptidases contain a characteristic His-Glu-Xxx-Xxx-His sequence integrated into an 'active-site'. The two histidine residues serve as zinc ligands, and the glutamic acid polarizes a water molecule involved in the nucleophilic attack at the peptide bond (94).

Extracellular bacterial metalloproteinases are produced in both gram-positive and gram-negative organisms and some play important roles in virulence (48, 49). Bacterial metalloproteinases contribute to virulence by damaging host tissues to aid in invasion and translocation. In some cases they release nutrients for further bacterial growth (48, 49, 64).

The zinc-proteinases are classified into families based on the zinc-binding site. Currently, there are two superfamilies: the thermolysins and the metzincins (64). Within the metzincins, there are four subfamilies: the astacin family, the serratial family, the adamalysin/snake venom family, and the matrixin family (13).

The thermolysin superfamily, or "neutral proteinases", contain the sequence His-Glu-Xxx-Xxx-His, with a glutamic acid residue located approximately 25 residues toward the C-terminal of the enzyme. This glutamic residue serves as the third zinc ligand (64). These enzymes can be found in a number of gram-positive bacteria. The general term for these enzymes are the neutral proteinases (thermolysin or thermolysin-like); all of these enzymes have a high amino acid sequence identity. Neutral proteinases have been identified from over 20 bacterial species (for a review, see 49). They degrade a number of extracellular matrix proteins, as well as other proteins in milk and serum (49). Also, some of the neutral proteinases nick enzyme precursors that are needed for bacterial metabolism (15). Interestingly, most of the neutral proteinases are not virulence factors. One notable exception is *Pseudomonasaeruginosa* elastase, which is a member of the thermolysin family of metalloproteinases. This enzyme increases the permeability of epithelial cells by proteolytically degrading the tight junctions between cells and the basement membranes (4, 5, 11, 118).

The metzincin superfamily contains a number of zinc endopeptidases that contain an extended zinc binding motif: His-Glu-Xxx-Xxx-His-Xxx-Xxx-Gly-Xxx-Xxx-His-Zzz. These peptidases include the astacins, the adamalysins/snake venoms, the serralysins, and the matrixins (13). These enzymes have a similar zinc binding motif and a common "met-turn'' or methionine turn. This methionine serves as a hydrophobic base beneath the three zinc ligands and preserves the structural integrity of the active site of these enzymes (142). The last residue in this extended signature,

'Zzz', which immediately follows the third histidine zinc ligand, differs in all four subfamilies, but

seems to be somewhat conserved within each subfamily (Glu in the astacins, Asp in the adamalysins, Ser in the matrixins, and Pro in the serralysins). This residue served as a label to identify and differentiate proteinases within each subfamily (13). A few of the metzincins, however, contain sequences at the 'Zzz' that do not match any of the subfamilies described (Wright, unpublished data).

Several members of the metzincin family have been characterized, including astacin and adamalysin. Astacin is a major collagenolytic enzyme found in the digestive system of crayfish (*Astacus astascus*). Astacin's main role is to degrade tissue and aid in the digestive process (12). Adamalysin is a highly active proteinase found in the venom of rattlesnakes (*Crotalus adamanteus*), and is responsible for the degradation of tight junctions. Adamalysin also inactivates several host proteinase inhibitors. This results in an increase in the activity of a number of host proteinases, which causes damage to the host (76, 77). It is thought that Adamalysin and other venom proteinases act on the extracellular matrix proteins to cause tissue damage and to allow neurotoxins to rapidly invade the victim (39, 86).

The serralysins include serralysin from *Serratia marcesens* (104) and *P. aeruginosa* alkaline proteinase (29). These proteinases are responsible for the extensive damage that occurs in the eye and lung infections, respectively, produced by these opportunistic pathogens. Serralysin degrades a number of physiologically important proteins, including fibronectin, collagen, and serum proteins; it enhances vascular permeability and suppresses the complement system (70, 104). Alkaline proteinase degrades elastin, collagen, laminin, fibrin, and several complement proteins (29).

Six collagenases are produced by the anaerobe, *Clostridium histolyticum.* These collagenases have a very similar sequence and they all cross-react immunologically (15). Many other clostridia also produce virulence-related metalloproteinases, including *C. sporogenes*, *C. bifermentens*, *C. perfringens* , *C. tetani*, and *C. botulinum* (1, 83, 115).

The clostridial neurotoxins are also metalloproteinases. These include the tetanus toxin and seven serotypes (A to G) of the botulinum toxin produced by *C. tetani* and *C. botulinum*, respectively. Each toxin type has a heavy and light chain, linked by disulfide bonds (38). Recently, the deduced amino acid sequences of the tetanus and botulinum toxins have been determined; they are homologous in five regions, including a segment with the metalloproteinase signature motif His-Glu-Xxx-Xxx-His. Zinc was shown to bind to the toxins and to be essential for inhibiting neurotransmitter release (23). All known tetanus and botulinum toxins cleave one or more key components in the synaptic vesicle docking and fusion protein complex (80). Tetanus toxin and botulinum toxin types B, D, F and G cleave VAMP (vesicle-associated membrane protein) and synaptobrevin, which are integral membrane proteins of the synaptic vesicles. Botulinum types A, C, and E cleave two other synaptic proteins from the plasma membrane, SNAP-25 and syntaxin (133, 80).

D. *BACTEROIDES* SPECIES AND *B. FRAGILIS*

The *Bacteroides* genus, comprising 38 species, is nonsporeforming rods that are gram-negative, obligately anaerobic chemoorganotrophs (55). The species in this genus metabolize many carbohydrates, peptones, and metabolic intermediates. Fermentation products include combinations of succinate, acetate, lactate, formate, propionate, short chain alcohols, isobutyrate, and isovalerate (84). These gram-negative rods form terminal vacuoles or swellings under certain conditions and do not contain KDO (2-keto-3-deoxyoctonate) in the lipopolysaccharide (56). Carbon dioxide is utilized and incorporated into succinic acid (19). All species of *Bacteroides* require, to some extent, hemin and vitamin K, which are used to make cytochromes b and o (85) and NADH:fumarate oxidoreductase (84) . There are currently 29 fermentative species and 9 non-fermentative species (55).

Bacteroides fragilis was first described by Veillon et al. in 1898 (152). In 1970, Holdeman and Moore published a report that grouped all the strains that fit the general description of *B. fragilis* into the general species "fragilis" with various subspecies (56). Subsequent DNA homology studies by Johnson (65) demonstrated that the *B. fragilis* subspecies were genetically distinct. Therefore, a species rank was reinstated by Cato and Johnson in 1976 (20). Babb and Johnson went on to identify DNA homology groups within the species, *B. fragilis* (6). The four groups are designated as I, II, 3452-A, and 4664.

Bacteroides fragilis is the anaerobe most commonly isolated from human clinical specimens (138). It is also a normal inhabitant of the human colon where it comprises about 1-2% of the flora, or about 10^9 cells/gram of fecal matter (91). When *B. fragilis* escapes from the colon, it causes abscesses, soft tissue infections, and bacteremias (53). It is considered by many to be "the most important of all anaerobes because of its frequency of occurrence in clinical infections and its resistance to antimicrobial agents" (36). Most members of the normal intestinal flora are not infective when they are released into the body from the colon during trauma or surgery. So, *B. fragilis* must have pathogenic features that are absent in other organisms. The most studied pathogenic mechanism is a thin capsular polysaccharide layer that appears to protect the cells from phagocytosis (146). The capsular material is composed of two distinct polysaccharides that are designated as PS A and PS B (114). Each polysaccharide is composed of repeating units of carbohydrates that contain positively charged amino groups and negatively charged carboxyl groups and phosphate groups (145). Interestingly, the purified capsular material can induce abscesses when injected into mice; the charged groups are required for this effect (145). However, most researchers feel that the presence of this capsule does not sufficiently explain the invasive nature of this species because non-capsulated *Bacteroides fragilis* are also virulent (145, 146).

B. fragilis has been shown to produce several enzymes that are involved in its pathogenesis, including several proteinases, catalase, neuraminidase, hyaluronidase, phosphatase, DNase and beta-lactamase (66, 105). Although each of these enzymes can also be produced by many normal bowel bacteria, none has been demonstrated to be a dominant virulence factor for this organism (66). Many strains of *B. fragilis* produce zinc-containing Beta-lactamases; these strains are relatively resistant to most penicillins and cephalosporins (55). Also, resistance to tetracycline, erythromycin, and clindamycin is carried on a plasmid that is commonly found in clinical isolates (137). This antibiotic resistance may also partly explain the prevalence of *B. fragilis* infections.

It seems the combined effect of a number of different characteristics is the key to

understanding the pathogenicity of *B. fragilis*.   The species is very tolerant to oxygen, and this may help the cells to survive their escape from the bowel to the body's more aerobic environment immediately after trauma or surgery (143). The capsular material then induces the body to wall off the bacterial cells to produce large abscesses. The organism kills human cells during this period, and many investigators have searched for the production of cytotoxins. Ironically, the proof of a *B. fragilis* toxin came not from studies by clinical researchers on human soft tissue infections, but from studies by veterinarians on diarrheas of unknown origin in lambs, calves, and foals.

E. ENTEROTOXIGENIC *BACTEROIDES FRAGILIS*

1. Discovery and Animal Studies.

The enterotoxigenic nature of *B. fragilis* was originally described by Myers et al. in the early 1980s, who isolated *B. fragilis* in large numbers from the feces of diarrheic animals in the absence of any recognized pathogens. These strains caused the accumulation of fluid in ileal loops of lambs, but large numbers (2 - 4 x 10^9 CFU) of viable cells had to be inoculated into the loops (96). Culture filtrates from enterotoxigenic strains did not give very large fluid responses (96, 102). Nonetheless, by using basic anaerobic microbiology to isolate the *B. fragilis* strains, and the lamb ileal loop assay to look for enterotoxigenic activity, Myers et al. concluded that some strains of *B. fragilis* produced an enterotoxic factor (96, 97, 102). More circumstantial evidence was accumulated for the involvement of these enterotoxigenic strains in diarrheas of lambs (96), rabbits (101), and calves (17). Enterotoxigenic strains were also isolated from piglets that were 1-4 weeks in age (22, 97). These piglets developed a severely mucoid and hemorrhagic diarrhea that was different from *B. fragilis* diarrhea found in other animals. Later, enterotoxigenic strains of *B. fragilis* were found in infant diarrheic foals (99). The data from this series of papers provided strong evidence that a pathogenic organism was causing disease. Research in this area then focused on identifying the enterotoxic factor that was responsible for causing the disease.

Enterotoxigenic strains caused a profuse watery diarrhea indicative of an enterotoxin. Because of the symptoms of the animals and the central dogma of bacterial enterotoxins, a *V. cholerae-* or *E. coli* -like enterotoxin was immediately suspected. However, an assay that is used to test the secretory effect of toxins dismissed this idea (96). These enterotoxigenic strains were producing a watery fluid response in intestinal loops that included tissue damage to the intestinal lining.

Myers concluded that an extracellular enterotoxin was responsible for the fluid accumulated in loops treated with these enterotoxigenic strains (102). His research group was able to partially purify a toxin that was heat labile and about 20,000-30,000 in molecular weight (101). This work was a great achievement, considering that the only assay that worked well at the time was the ileal loop assay. Myers's group had hoped to find a better way to detect the toxin so that this enterototoxic factor could be characterized. A report in 1989 (98) attempted to distinguish enterotoxigenic strains from nonenterotoxigenic strains using an agglutination assay with whole cells. However, there was too much variability between the strains to differentiate between them. The main research focus of this group then turned to developing alternative animal models that could be used to more easily detect and analyze enterotoxigenic strains and this enterotoxic factor.

They could induce lethal diarrhea in infant rabbits (101) and neonatal germ-free pigs (28) by oral challenge with live enterotoxigenic strains. In this case, the disease was characterized by watery diarrhea and dehydration. In another model, adult rabbits would not get diarrhea when given organisms orally but, adult rabbits with ligated ceca developed fatal diarrhea following intracecal injection with live organisms (95, 100). The disease was characterized by mucoid, often hemorrhagic, diarrhea with the histopathology resembling moderate to severe necrotizing colitis. The main physical characteristics of the diseased intestine were severe histologic lesions with inflammation, exfoliation, and crypt hyperplasia. These animal models were useful in implicating the enterotoxigenic strains in diarrheal disease. In retrospect, this series of reports not only pioneered the role of enterotoxigenic *B. fragilis* in diarrheal disease in animals, but it also

provided a broad foundation to study this organism. However, the ileal loop assay was too costly and complicated for use in characterization and mechanism studies. So, most research in this area was done to implicate the organism in "disease" and not necessarily to study the biochemistry and mechanism of this toxic factor. Although enterotoxigenic *B. fragilis* had been implicated as a cause of diarrhea disease in animals,the reports described below by Myers and others, that indicate that the disease can be significantly associated with humans, are less than desirable.

2.   Human studies

There were two initial reports by Sack et al. (127) and Myers et al. (103) that identified a few enterotoxigenic strains of *B. fragilis* in the stools of some children and adults with an unexplained diarrheal disease. These two studies did not provide enough evidence to implicate *B. fragilis* as a cause of diarrhea in humans. Later, other researchers' examined the role of enterotoxigenic *B. fragilis* in human diarrheal disease. These studies were case controlled and involved fecal sample collection from adults and children in Oklahoma City (131) and Bangladesh (126).

These case-controlled studies provide no convincing data that clearly shows that enterotoxigenic *B. fragilis* cause human diarrhea. Although enterotoxigenic strains were isolated from children under one year of age in one of the studies, these researchers could not show that these strains were significantly associated with diarrheal disease in this age group. Conversely, children with diarrhea in the one to five year old age group were the only group that had any significant association with enterotoxigenic strains (131). However this association was only slightly significant (P   0.03). In all of these studies, the symptoms were like that from the animal studies; the patients had a self limiting, watery diarrhea in the absence of any other enteric pathogen. These studies also show that enterotoxin producers are part of the indigenous flora.   In spite of this, several other reports were published, all of which do not clearly show an association of enterotoxigenic *B. fragilis* with diarrhea.

Several of these reports were from Italy (111, 112, 113), Poland (88, 89), and India (120). These groups have shown that enterotoxigenic strains exist in these countries, and that they are present in the normal indigenous flora of humans. However, no detailed case studies were performed in these countries by the research groups and there is no data that shows that these strains cause diarrhea.

3.   Characterization of the toxin

Our laboratory had been following the research from Myers's laboratory. However, as stated before, there was no good assay to detect the toxin; a good assay should be inexpensive, fast, and quantitative, and the established animal assays met none of these criteria. So, research that was needed to make significant research progress could not be done. However, in 1992, Weikel et al. tested enterotoxigenic *B. fragilis* on several different tissue cultured cell lines and showed that the toxic culture filtrates caused "specific and striking morphological changes" on certain colon carcinoma cell lines, particularly HT-29 (156). This effect on HT-29 cells included disruption of junctional barriers, rounding, swelling, and pyknosis (129, 156). Recently, Donelli et al. (25) found that when the HT-29 cells are treated with toxin, they round, swell, and blebbing along the cell membranes also occurs. Later, Weikel et al. (now C.L. Sears) and co-workers presented two abstracts indicating that the enterotoxigenic *B. fragilis* and "partially purified" *B*.

fragilis enterotoxin had a significant effect on other human colonic cell lines, including Caco-2 and T-84. These effects resembled the effects on HT-29 cells (128, 135). The contribution of the cell rounding assay cannot be overemphasized. This model has permitted the purification of the toxin and studies of its mechanism of action.

Weikel's discovery of a cell rounding assay opened the way for our laboratory to become more involved. Within 6 months after the publication of the cell rounding assay, Van Tassell et al. (148) purified the enterotoxin from culture filtrates of enterotoxigenic *Bacteroidesfragilis* strains. Purification of the toxin is a complex process, and involves several steps. *B. fragilis* is grown in a very rich medium of brain heart infusion (BHI), with added hemin and vitamin K, to mid-to-late log phase at 37°C and then pelleted. Culture supernatants are then precipitated with ammonium sulfate to concentrate the culture and prevent the degradation of the toxin. The toxigenic sample is then purified by anion exchange chromatography, hydrophobic interaction chromatography, and high resolution anion exchange chromatography. The purified toxin is an acidic protein (pI 4.5) with a molecular weight of 20,600 that induces the same fluid accumulation and cytotoxic response as crude culture filtrates.

In 1994, Van Tassell et al. prepared a monospecific neutralizing antiserum against the toxin. The sera was used to develop a cytotoxicity neutralization assay (149) for confirming the cytotoxic activity observed on HT-29 cells and to develop an enzyme linked immunosorbant assay (ELISA) for screening isolates (150). There was almost a 100% correlation between cytotoxicity and the ELISA response with about 50 strains of *B. fragilis* and showed that other intestinal *Bacteroides* species do not produce this toxin (Van Tassell, unpublished data).

The ELISA also provided evidence that the toxin was being rapidly degraded in the cultures and that cultural conditions had to be controlled carefully to enable us to detect biologically active toxin in the culture filtrates from many strains. The ELISA also reacted strongly with cell membranes from toxic isolates. This led to the discovery that much of the toxin is attached to the membrane. Kling et al. found that the deduced amino acid sequence of the toxin contains a motif for lipoprotein attachment (75). Experimentation in this area provided proof that the proteinase is a lipoprotein that must be processed off the membrane to be toxic (Kling, unpublished data).

This dissertation will explore the effects of the purified toxin in vitro and in vivo. I believe that the action of this toxin follows a simple mechanism: the toxin is a metalloproteinase(90) that proteolytically degrades the proteins that create a barrier between epithelial cells, which leads to cellular damage (106). This disruption not only allows bacterial internalization as shown by Wells et al (157), but also allows damage to individual cells (25), to tissues, and allows fluid to leak out into the intestines (107).

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CHAPTER 2: **Fragilysin is a metalloprotease.**

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CHAPTER 6: **Detection of enterotoxigenic*B.fragilis* by the Polymerase Chain Reaction**

Obiso, R.J., Jr. A.J. Duncan, and T.D. Wilkins. 1997. Detection of enterotoxigenic *Bacteroides fragilis* strains. in preparation

CHAPTER 7: **Fragilysin: the toxin from *Bacteroides fragilis***

Obiso, R.J., Jr. and T.D. Wilkins. The *Bacteroides fragilis* toxin, fragilysin. *In*, F. Woessner and A. Barrett (eds.), Handbook of Proteolytic Enzymes, Academic Press Publishing Co., London. In press.

CHAPTER VIII   OVERALL DISCUSSION AND SUMMARY

The role of *Bacteroidesfragilis* as a major clinical pathogen has been known for many years. This anaerobe is commonly isolated from a number of different infection sites, including, soft tissue, abscesses, and bacteremias. The capsule, a complex of two distinct polysaccharides, is thought to be the main virulence factor, aiding in its prevalence in clinical samples (Section I). During the last 13 years, however, the results from several researchers suggested that this organism also causes diarrheal disease (Section I). When I began this project, Van Tassell et al. (37) had recently purified the enterotoxin; and, our research group was trying to clone and sequence the toxin gene. Little was known about the toxin's biological activity, its properties, or its mechanism of action.   The central dogma about bacterial enterotoxins was, and still is, that they follow one of only a few distinct pathways to cause diarrhea in the host. (34) Preliminary evidence with whole cells or culture filtrates from enterotoxigenic strains, however, showed that this toxin was not a "classical" enterotoxin that stimulated intestinal secretion (26). The main focus of my research, therefore, was to determine how the *B. fragilis* toxin, fragilysin, causes diarrhea.

Biological Activity of Fragilysin

Probably the most significant result to come from this research was the discovery that the toxin (fragilysin) is a metalloproteinase that coordinates zinc at the active site (Section II). Our research group was the first to clone fragilysin, and J. S. Moncrief found a zinc-binding motif in the deduced amino acid sequence of the toxin, which is characteristic of metalloproteinases (23). The first part of my research was to determine if this enterotoxin had proteolytic activity. I found that the toxin is a metalloproteinase that hydrolyzes a number of proteins. The proteinase activity is specific for a few peptide bonds (usually between two nonpolar amino acids like Gly-Leu, Met-Leu, or Cys-Leu). Fragilysin is most active at 37°C and at physiological pH; inhibitors of metalloproteinases also inhibited fragilysin's proteolytic activity and cell rounding activity (23). The finding that fragilysin was a proteinase was a breakthrough that left a puzzling question. It was not clear how a proteinase could cause diarrhea, especially in the intestinal environment that is a virtual haven of proteinases, which include trypsin, chymotrypsin, and many bacterial proteinases.

Previous studies using whole cells and culture filtrates from enterotoxigenic strains had resulted in some characterization of the histological effects in the intestine (7, 25, 27, 28); however, there were no detailed studies on the action of purified fragilysin in the intestine, particularly in the colon where the organism resides. Because we did not know what fragilysin did in the intestine, I characterized the histological and pathological effects of purified fragilysin in the intestine (Section III).

The purified toxin reproduced the effects of whole cultures in the intestine, characterized by sloughing of the epithelial cells (exfoliation) and crypt hyperplasia. The fluid response elicited by fragilysin is greater in the colon than in the ileum; and, analysis of this fluid revealed a net accumulation of chloride, sodium, and potassium, as well as, albumin and total protein. However, this fluid is unlike that elicited by either *C. difficile* toxin A or cholera toxin. Typically, fluid from intestinal loops

treated with cholera toxin contains an abnormally high amount of chloride (millimolar increases) with low amounts of protein and albumin as compared to controls (9, 10). Conversely, intestinal loops treated with *C.difficile* toxin A may contain up to ten fold higher amounts of albumin and total protein with lower amounts of chloride or other electrolytes, as compared to controls (18, 19). In fragilysin treated intestinal loops, there is both an increase in chloride and total protein. However, the chloride amounts are significantly lower than in cholera toxin-treated loops; and, the total protein and albumin amounts are considerably lower than toxin A-treated loops (30). I suspected that the increase in electrolytes was due to the tissue damage rather than active transcellular chloride secretion. Furthermore, I postulated that the increases in protein, albumin, and chloride concentration were probably due to leakage of serum proteins into the intestine and from sloughed epithelial cells. Further experimentation supported this idea. I found that the fluid from the fragilysin-treated intestine had the same banding pattern on SDS-PAGE as the serum from that animal (Obiso, unpublished data).

The most important finding from this series of studies was that fragilysin's proteolytic activity is responsible for the tissue damage and fluid secretion in the intestines. Chelated fragilysin consistently gave no responses in the intestine and some biological activity could be restored with the addition of zinc. These results provided direct evidence that the proteinase activity of fragilysin was causing the effects in the intestine. Other proteinases, like *P. aeruginosa* elastase (16) and *V. mimicus* metalloproteinase (6) can cause slight fluid responses in intestine, however, the doses required are extremely high and are not biologically relevant, nor are they consistent with the effective dose of fragilysin (10 µg of fragilysin *vs*. 2.5 mg of elastase). This study did not explain how fragilysin caused these effects in the intestine; however, it provided enough evidence to support the fact that proteolysis in the intestine could cause diarrhea.

Mechanism of action

The paracellular barrier of epithelial cells is composed of the tight junctions (zonula occludens), which hold cells to each other and play an integral role in cellular architecture. There is a direct link between the tight junction and the cytoskeleton at an attachment site on the plasma membrane (15, 20, 21, 22). *P.aeruginosa* elastase, and the *C. difficile* cytotoxins, disrupt this paracellular barrier through different mechanisms. *C. difficile* toxins cause the disorganization of the actin cytoskeleton through the UDP-glucosylation of rho (See section I); this disruption causes the actin cytoskeleton to rearrange, which then causes the tight junctions between cells to open, in turn increasing the intestinal permeability (1, 13, 14). Tight junctions also can be opened by the direct effect of proteinases; this is how elastase from *Pseudomonas aeruginosa* exerts its effects on tissue in the lungs. This metalloproteinase degrades the tight junctions and basement membranes of the epithelial cells (2, 3, 4).

I found that fragilysin increases the paracellular permeability of epithelial cells and metal chelators prevent these effects. This indicates that the proteinase activity is responsible for this result. Fragilysin most likely does not enter the epithelial cells, since inhibitors of receptor mediated endocytosis do not inhibit the rounding of HT-29

cells. Fragilysin is not lethal to the cells; trypan blue dye exclusion showed that >90% of the cells remained viable, even at high toxin doses. My data agrees with Saidi and Sears (33) who report that the *B. fragilis* toxin (fragilysin) intoxication does not involve pH sensitive endocytosis.

B. fragilis fragilysin decreases the transepithelial electrical resistance in cultured monolayers of epithelial cell lines from the colon, lung, and kidney in a dose and time dependent manner. Changes in the electrical resistance could have meant that either there was a direct effect on the cell membrane, or there was an alteration at the tight junctions between the cells. My results from loading HT-29 cells with radiolabeled chromium showed that there was no change in the integrity of the cell membranes treated with the *B. fragilis* metalloproteinase. Furthermore, the change in the electrical resistance was accompanied by an increase in mannitol flux across cell monolayers, which only occurs when intercellular spaces are opened between the cells (29).

My data agrees with Wells et al. (38) who also found that fragilysin increases the epithelial permeability of HT-29 cells. I provided purified fragilysin to this group, who found that fragilysin increases the permeability of HT-29 cells, which increased bacterial internalization into these enterocytes. My data also agrees with the preliminary studies done by Sears et al., who used HT-29 cells (35) and T-84 cells (32) (both intestinal cell lines) in Ussing chambers to show that fragilysin effected the paracellular barrier function. However, I disagree with the interpretations presented by Sears et al. on the mechanism of action of fragilysin. This group originally postulated that the toxin acts intracellularly, solely by degrading the actin cytoskeleton (17, 24); however, in a recent report by this group, it has been suggested that the toxin may contain a hydrophobic "membrane-spanning" region that would allow the toxin to insert or punch holes in the cell membrane (11, 33). My data (summarized below), disagrees with the interpretations by this group.

First, I developed a molecular model of fragilysin in order to see if the toxin contained a unique structure or certain characteristics that differ from other metzincins. Fragilysin does contain several similarities in amino acid sequence and in secondary structure relationships with other metzincins, especially with the snake venom toxin, adamalysin (36)**.**   I found that the modeled structure of fragilysin does not contain any membrane spanning regions, nor does its modeled structure suggest that fragilysin contains an amphipathic domain, which could form a pore or channel in a eukaryotic membrane (Section V). The model of fragilysin clearly shows that fragilysin is a typical metzincin, and that the amino acid sequence of the mature toxin does not contain extraneous sequences that could lead to another biological activity other than the proteolytic activity. This suggests to me that fragilysin's mechanism is probably similar to the other metzincins.   For the *B. fragilis* toxin, the degradation of the tight junctions and extracellular matrix proteins would lead to cytoskeletal rearrangements and cellular damage to epithelial cells.

Secondly, that fragilysin has a direct effect on the tight junction; I saw that epithelial cell monolayers that were treated with fragilysin, resisted recovery of their tight junctions. Furthermore, HT-29 cells loaded with radiolabeled chromium showed no evidence of membrane damage when they were treated with fragilysin. I also

observed a rapid and direct effect on the tight junction protein ZO-1. By using immunofluorescence, I was able to follow a decrease in the amount of this tight junction protein between the cells; this decrease correlated with the amount of fragilysin and length of exposure (29).

Based on this data, the most obvious mechanism of action for fragilysin would be direct proteolytic degradation of the intestinal tight junctions and cell-to-cell contacts. Virtually all tight junctions in epithelial cells are the same (20, 22), they only vary in the "tightness" of the barrier (21). The tightness of the barrier is directly controlled by the amount of extracellular matrix proteins present and the presence of calcium (22). My data shows that effects on the tight junction occur in minutes, whereas, cell rounding on HT-29 cells occurs in hours (29). Since the tight junction is linked to the cytoskeleton, the "cytoskeleton altering" observation, suggested by Sears et al. (34), is probably a secondary effect.

Interestingly, other proteinases in the metzincin family, like adamalysin, also degrade tight junctions and extracellular matrix proteins, as well as inactivate host serine proteinase inhibitors (36). Because of the wide variety of effects caused by this class of metalloproteinases, and because there is so much damage to the intestine (Section III), I thought that the toxin may have a secondary effect, such as activation of cytokines or host proteinases. If this were true, it would exacerbate the tissue damage and spur an inflammatory response. Because neutrophils are recruited to the fragilysin-treated intestinal loop (30), I thought that the neutrophils may actually play a role in the tissue damage. I found that neutrophils are not directly activated by fragilysin (Obiso, unpublished data). They are, however, recruited to fragilysin-treated intestinal loops, where they degranulate and become active when they "see" the normal intestinal flora in the lumen of "non-germ-free" rats. I do think that the neutrophil degranulation effect contributes to the tissue damage in intestine because "germ-free" rats treated with fragilysin give the same responses as non-germ-free animals (Obiso and Duncan, unpublished data). Furthermore, rats treated with high doses of antiinflammatory drugs and fragilysin gave the same responses as rats that did not receive antiinflammatory drugs (Obiso, unpublished data). Further experimentation in this area, and in the area of cytokines should be pursued to gain a further understanding of the different effects this proteinase may have in the intestines.

In conclusion, bacteria have evolved highly specialized molecules that interact with host tissues and contribute to virulence (5, 12, 31). In some cases, the enzymes are toxins that interfere with cellular functions, such as ADP-ribosylating and UDP-glucosylating toxins (1, 8, 9, 34). Other enzymes, like, fragilysin, destroy host tissues or neutralize host defense mechanisms (3, 4, 5, 12). The results of the research presented in this dissertation provide the framework for further studies on *B. fragilis* as a cause of disease in intestinal and extraintestinal infections. The intestinal permeability effects by this enzyme may have other potential uses in microbiology and immunology, particularly in the field of immuno-adjuvants. Hopefully the work included in this dissertation will be helpful to other investigators interested in this area.

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