Studies of Three Human Intestinal Opportunistic Pathogens

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(Abstract)

Opportunistic bacterial pathogens are present in the intestines of all mammals. These bacteria are symbionts to a certain extent, but under certain conditions these organisms can be deadly. Intestinal opportunistic pathogens encompass many genera and include organisms such as those in the *Bacteroides fragilis* group (i.e. *B. fragilis* and *B. thetaiotaomicron*), *Escherichia coli*, and *Clostridium perfringens*, resulting in an array of diseases and serious health risks. Typically these diseases affect individuals in poor or weakened health (elderly, immuno-compromised, neonates, etc.) but can affect healthy individuals as well. The intestinal tract is the main area of infection for these bacteria, however some of these organisms can be involved in wound infections, septicemia, urinary tract infections, and meningitis. This study focused on three areas: 1) Analysis of differences in gene expression between *Bacteroides* and *Escherichia coli*, in order to learn more about promoter structure, 2) Establishment of a diabetic mouse model for use in examining bacterial synergy during a polymicrobial infection, and 3) Characterization of *Escherichia coli* 360A and evaluation of the role of several virulence factors and environmental modulators in the pathogenesis of this strain.

We used a newly developed *lux* gene reporter to evaluate gene expression in *Bacteroides*. We observed that there are barriers in both transcription and translation initiation that appear to limit the expression of foreign genes in *Bacteroides*. We were able to establish a mouse model for studying synergy during a polymicrobial infection and observed that *E. coli* 360A provided synergy towards *B. fragilis* NCTC 9343. These experiments also showed that the longer a

mouse is afflicted with the complications of diabetes the more susceptible it is to polymicrobial infections. Systemic infections were used to evaluate the contribution of several virulence factors and environmental modulators in the pathogenesis of *E. coli* 360A. The results showed that a strain lacking both virulence factors CNF1 and HlyA, the terminal oxidase cytochrome *o*, or a double *cyo/cyd* mutant were, deficient in survival in the spleen, but not the liver of BALB/c mice.

Dedication

I would like to dedicate this to my family, my wife and my son, for being understanding about the long days and late nights and being there when I needed a good laugh. I love you both, thank you for being there for me. To my parents for instilling a good work ethic on me early in life and for being there through all the little things.

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Chapter One

Literature review

Polymicrobial infections in diabetics.

Diabetes is a group of diseases that has been associated with high levels of glucose in the blood, which is a result of defects in either the production of insulin, insulin action, or both (51). Diabetes can be classified into several groups including: type 1, type 2, and gestational diabetes. Each of these syndromes has been seen to affect different ages and situations of a person's life. The Centers for Disease Control and Prevention (CDC) estimates that at least 18.2 million people in the U.S., approximately 6.3% of the population, have diabetes (51). CDC statistics show that more than 60% of the non-traumatic lower-limb amputations in the U.S. occur among people with diabetes. Between 2001-2002, approximately 82,000 non-traumatic lower-limb amputations were performed each year on people with diabetes (51). The side effects of these amputations not only include physical limb loss and the long-term effects on personal health, but also a financial burden on these individuals for the remainder of their lives. In a study done in 1998, a typical diabetic patient undergoing limb amputation had an average hospital stay of approximately 16 days, with a total cost of over $27,000 (7). With the added cost of prosthetics the total expense can range up to a lifetime cost of over $403,000 (329). These estimated figures do not account for factors such as lost wages due to extensive hospital stays and rehabilitation.

Type 2 diabetes is a late onset disease induced by physical inactivity, old age, obesity, and other such complications. This debilitating disease has many serious side effects such as delayed wound healing and increased susceptibility to localized and systemic infections, which could ultimately lead to limb amputations. At the systemic level, obesity and type 2 diabetes have been linked to increased levels of the pro-inflammatory molecules: tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) (65-67). Evidence indicates that obesity leads to both insulin

resistance and oxidative stress, which results in an inflammatory response (68, 209). Insulin has an anti-inflammatory effect, but the increased levels of both TNF-α and IL-6 appear to suppress the insulin signal transduction, which counteracts the anti-inflammatory response (3). This cascade of events results in a systemic long-term inflammatory response during diabetes (65). At the local level the inflammatory response to a wound is an overactive one with heavy infiltration of polymorphonuclear leukocytes (PMNs) and macrophages, resulting in localized tissue damage and slow healing of the infected area (325). Sores that develop will remain for a significantly longer time in diabetics than non-diabetics due to delayed wound healing, which can often lead to infections in the extremities.

Diabetics are highly susceptible to lower limb infections, especially in the foot (6). Diabetes-associated manifestations that contribute to the increased susceptibility to these infections include peripheral vascular disease (with accompanying ischemia), neuropathy, and a dysfunctional immune system (169). These syndromes can lead to the development of ulcers on the lower limbs, which can become infected (169). Lower-limb infections in diabetics are most often polymicrobial in nature involving aerobic, facultative anaerobic, and obligate anaerobic microorganisms (267, 268). These bacterial infections account for approximately 85% of circumstances that require lower limb amputations in diabetic patients (6). These polymicrobial infections are often difficult to treat due to the nature of the variety of species present, which have various natural and acquired resistances to a range of antibiotics (108). Methods such as the use of broad-spectrum antibiotics intravenously and the amputation of limbs are used to stop the spread of these infections and help save the life of the patient (6). Therefore determining the mechanisms of action of these bacteria, in establishing these infections will help doctors better

understand these organisms and aid in the formation of new and better treatments for combating this debilitating disease.

An animal model for studying polymicrobial infections in diabetics.

Diabetic mice have been used to study certain diseases that occur most frequently in diabetic patients such as periodontitis (99), urinary tract infections (225), septicemia (160), and lung infections (224). Bessman, *et. al.* (20) used a strain of mice, C57B1.Ks-J-db-m (now named BKS.Cg-*m* +/+ *Lepr*^{db}/J), which is homozygous for the diabetes spontaneous mutation in the leptin receptor and is a model for type 2 diabetes [(54) and http://jaxmice.jax.org/jaxmice-cgi/jaxmicedb.cgi?objtype=pricedetail&stock=000642 for a description of the strain] to examine abscess formation and bacterial load after subcutaneous inoculation of *Escherichia coli*, *Bacteroides fragilis*, and *Enterococcus*. In that study, 9-11 week old BKS.Cg-*m* +/+ *Lepr*^{db}/J and their non-diabetic littermates were infected with each possible two-organism combination of *E. coli*, *B. fragilis* or *Enterococcus* (20). They observed that abscesses formed in the diabetic mice inoculated with those organisms were more persistent than those abscesses formed in non-diabetic mice (20).

The leptin receptor gene, *Lepr* (also known as *db*), which is mutated in the BKS.Cg-*m* +/+ *Lepr*^{db}/J (137) strain of mice normally functions to control body weight by the hormone leptin (15, 92, 235). Therefore mice lacking the receptor become morbidly obese by 3 to 4 weeks of age with a drastic increase in blood glucose levels at 4 to 8 weeks (137). These mice show delayed wound healing and have a shortened lifespan, totaling approximately 10 months (137).

Bacterial synergy in polymicrobial infections.

The pathogenic bacteria involved in polymicrobial infections exhibit synergism in their collective ability to survive and cause an infection (39). These pathogens frequently exhibit a distinct synergistic activity when inoculated together in animal models that develop abscesses. Bacterial synergism is based on the role of each bacterium's contribution to causing the infection. The term synergism was first defined by Kämmer in 1924, as the sum activity of two or more microorganisms (152). This definition was further modified by Bjornson, as the cooperative interaction of two or more bacterial species that produces a result not achieved by the individual bacterium acting alone (25). Other researchers at the time had observed that the combination of several bacteria in mixed infections lead to greater numbers of bacteria in the host than a single species bacterial infection (4, 121, 202).

Synergism in virulence has been seen in experiments where the formation of an abscess in non-diabetic mice or rats was used as the *in vivo* assay model (39, 260). Studies have shown that *B. fragilis* was synergistic for the facultative anaerobe *E. coli* (39) and the obligate anaerobe *Clostridium perfringens* (42), and the presence of *E. coli* was also seen to be synergistic towards *C. perfringens* (42). Based on clinical and experimental results in animal models, it has been hypothesized that different types of bacteria promote synergy with polymicrobial infections via different mechanisms (260). The mechanisms of this synergism have been hypothesized to fall into four categories (38, 39, 189): 1) The improvement of the local micro-environment of the host, which includes the ability to lower the ambient oxygen concentration/redox potential and to decrease the pH of the host tissue to allow anaerobic organisms to grow [e.g., *E. coli* terminal oxidases (111-113, 128, 205, 260)]. 2) The resistance to host defenses such as the synthesis of a

bacterial capsule that affects immune functions via the inhibition of phagocytosis [e.g., *B. fragilis* (132, 217, 279)]. 3) The provision of essential nutrients that enhance bacterial growth, which include growth factors and other compounds such as heme, [*e.g., E. coli* (128, 230)]. 4) The production of harmful cytotoxins that damage eukaryotic cells such as hemolytic toxins (*e.g., E. coli* and *C. perfringens* (300)). A study done by Verweij-Van Vught, *et. al.* 1985, compared four strains, two *E. coli* and two *B. fragilis*, in various combinations/concentrations with each other (306). They looked for the formation of abscesses and concluded that different strains of the same species cannot complement the same strain of another organism (306). This means that no two strains are alike in their pathogenic capabilities and that synergy provided by these organisms discriminates between different bacterial species and strains. Other researchers have noted that *Clostridium tetani* and several other spore forming anaerobes cannot germinate during an infection of mice without the aid of oxygen reducing organisms (111-113).

Pathogenic bacteria used to study polymicrobial infections

The human intestinal tract is comprised of multitudes of bacteria that are both beneficial and non-beneficial. When opportunities arise, such as during an infection (especially following a traumatic event), during the administration of antibiotics, or when the immune system becomes compromised, certain normal symbiotic microbial flora can become a problem. Opportunistic pathogens such as those in the *Bacteroides fragilis* group (i.e. *B. fragilis* and *B. thetaiotaomicron*), *Escherichia coli*, and *Clostridium perfringens* can cause an array of diseases, such as wound infections, intestinal infections, and intra-abdominal abscesses, and pose a serious health risk.

***Bacteroides fragilis*:** *B. fragilis* are Gram-negative, aerotolerant anaerobic, non-sporulating rods, and are members of the *Bacteroidetes* phylum (185). They are a major component of the natural microflora of the human intestinal tract. *B. fragilis* produces a pro-inflammatory capsule that has been seen to be an essential player in the formation of abscesses in animal models (226). Pathogenic strains can produce up to eight different types of capsular polysaccharides, termed PS A-H (166). Phase switching of these polysaccharides occurs by inversion of a DNA region containing promoters lying upstream of the capsule biosynthesis genes (166). Two of these polysaccharides PS A and PS B have been seen to induce abscesses in the absence of the bacterium (298). Interestingly, mutations in the genes involved in the biosynthesis of PS A led to a greatly decreased level of abscess formation by live bacteria *in vivo* (64), while the mutations in the PS B (63) and the PS C loci did not (59). The mechanism of abscess formation by the capsule involves stimulation of lymphoid T-4 cells. The zwitterionic capsular material binds directly to T-cells and stimulates the production of pro-inflammatory chemokines, such as interleukin 8 [IL-8 (57, 151, 285, 296)]. IL-8 then leads to induction of intercellular adhesion molecule ICAM-1 on endothelial cells, which lead to the recruitment of PMNs and the establishment of abscesses (296). Interestingly, if mice are pre-inoculated or co-inoculated with capsular material they become resistant to the formation of abscesses (297, 299).

B. fragilis species may also possess toxins that are involved in the disruption of intestinal epithelial cells. One such toxin has been characterized as a heat-labile metalloprotease toxin, which is called *B. fragilis* toxin or BFT (210). BFT acts by stimulating the rearrangement of the F-actin filaments in epithelial cells causing them to undergo dramatic conformational changes in structure and detachment from the intestinal wall (79). Strains carrying this toxin have been seen

to cause noninvasive diarrhea in animals and young children (214, 264, 265). Other studies have found isolates from blood and other extra-intestinal sites, such as abscesses, may also contain BFT, but conclusive results have not been documented (154, 212, 237). The formation of abscesses is often due to the presence of more than just one isolate of the *B. fragilis* group. Other bacteria such as *E. coli* and *C. perfringens* can also be found in these abscesses.

***Escherichia coli*:** *E. coli* is a Gram-negative, facultative anaerobe that is a versatile pathogen and is the causative agent of an assortment of diseases from urinary tract infections to food borne illnesses. Extra-intestinal pathogenic *E. coli* or ExPEC (263) are a group of *E. coli* strains that can cause urinary tract infections, meningitis, pneumonia, bacteremia, wound infections, and intra-abdominal abscesses. These ExPEC strains are the leading cause of urinary tract infections (100, 339) and neonatal meningitis (in newborns less than one month old) (72). These debilitating diseases, in addition to being a nuisance, can be deadly if left untreated.

E. coli strains have been categorized into separate pathotypes based on many characteristics. These groups are based on toxin typing, presence of adhesins, host specificity, area of infection, serotype (both O and H antigens), and capsule typing (K antigen). The O antigen is a component of the lippopolysaccaride layer (LPS) of the cell while the H antigen is a component of the flagella.

Pathogenic *E. coli* isolates can carry an arsenal of toxins and other virulence factors that aid in the infection of both mammalian and avian hosts. Some of these toxins are detrimental to immune functions and disrupt natural processes of the human body. Two such toxins are the α-hemolysin (HlyA) and cytotoxic necrotizing factor (CNF). These two toxins are common in ExPEC isolates, especially uropathogenic *E. coli* (UPEC) strains (334). HlyA is a membrane-

binding toxin that forms small pores in the cytoplasmic membrane of host cells making them leaky and ultimately leading to cell death (204). It is encoded as part of the *hlyCABD* operon (120), with its promoter lying upstream of *hlyC* (323). Several transcription factors such as HlyT and RfaH are involved in activation and enhancement of transcription of the *hlyCABD* operon (11, 174, 175). RfaH is also involved in the transcription of TolC (315), a protein involved in the secretion of HlyA. HlyA is produced as a pro-HlyA protein that is approximately 107 kDa in size before being converted into its active form in the cytoplasm by the 18-kDa protein HlyC. HlyC is a fatty-acid acyltransferase (109, 183, 283) that works in conjunction with a cellular acyl carrier protein (134) to convert HlyA to its active form. This protein complex acylates the side chains of two lysine residues (K564 and K690) of HlyA (183, 283). The other two gene products from *hlyB* and *hlyD* form a complex, HlyBD, which is required for the transport of HlyA (107, 273, 312, 316). HlyB is an integral membrane ATPase that is a member of the ATP-binding cassette (ABC) superfamily of protein transporters (107, 125, 270, 316). HlyD is a member of the membrane fusion protein family (78, 273). HlyD is essential for the folding of a functional HlyA protein (240). The HlyBD complex interacts with the outer membrane protein TolC to transport the active hemolysin out of the bacterial cell through the type I secretion system (312, 314).

HlyA is a member of the repeat structural toxin (RTX) family, characterized by a nine amino acid repeat in the C-terminal portion of the protein (61, 94). This nine amino acid repeat is comprised of glycine, which is found 13 times within the C-terminal portion of the protein (322). Alterations or deletions of any set of these amino acid repeats of the C-terminal region affects both secretion, calcium binding, and erythrocyte binding of HlyA (31, 94, 184). The

ability of HlyA to bind calcium is directly related to the hemolytic activity of the protein (30). HlyA has been shown to be important to ExPEC strains *in vitro* (261) and *in vivo* (82, 156, 198, 223, 261, 274, 304, 311, 321). The HlyA toxin is able to lyse red blood cells (RBCs), leukocytes (22, 49, 147, 261), other mammalian cells (155, 288), and damage chicken embryos (130, 208). A study done by Nagy, *et. al.* (216) examined the *E. coli* strain 536 and found that the gene products from both of its *hlyA* genes, *hlyAI* and *hlyAII*, contribute to the full virulence of the strain in both cell culture and in an animal model (216).

Therefore, HlyA may play a pivotal role promoting synergy to bacteria in the *Bacteroides* group in mixed infections by providing protection from immune cells and lysing RBCs to release hemoglobin. The hemoglobin then acts as a source of hemin, a necessary growth factor for organisms such as *Bacteroides* (328). A study done by Ushijima, *et. al.* (300) using non-isogenic strains of *E. coli* suggests that the production of the hemolysin toxin by *E. coli* might play a role in the formation of subcutaneous abscesses. These results however, have not been further examined by using isogenic strains of *E. coli*. Another study done by Otto, *et. al.* (230), showed that an enzyme called hemoglobin-binding protease (Hbp), which has the ability to bind hemoglobin (232), promotes the growth of *B. fragilis* strain BE1. These studies suggest that *E. coli* can provide mechanisms of synergism towards anaerobes in addition to lowering the oxygen concentration of the host tissue.

Another group of toxins, CNF, was first identified by Caprioli, *et. al.* (45). There are two types of CNF toxins, CNF1 and CNF2, which are both heat-labile proteins approximately 115 kDa and 110 kDa in size, respectively (44, 45, 75). These toxins are members of the A:B toxin family (14, 34), which includes the cholera toxin. The *cnf1* gene is located on the bacterial

chromosome (89), while the *cnf2* gene is located on a plasmid (228). These gene, *cnf1* and *cnf2*, sequences are approximately 85% homologous (90, 229). Recently a third member of the CNF toxin group has been discovered and is encoded by the *cnf3* gene, which was shown to be associated with two other genes, *eae* and *ehxA* (227). *E. coli* strains that produce these toxins, CNF1 and CNF2, can be isolated from humans as well as animals (28, 44-46, 73, 75, 76, 89, 290). These toxins have slightly different host specificities: CNF2 is lethal to chickens (74), lambs (76) and necrotic for mouse footpads (75) while both CNF1 and CNF2 can kill mice (74) and are necrotic for rabbit skin (73). In a study done by Blanco, *et. al.* (28), over 300 *E. coli* isolates were examined in order to detect if either the *cnf1* or *cnf2* genes were present. Of these isolates 107 were identified to carry the *cnf1* gene of which 105 were isolated from humans, while all but 1 of the 202 isolates that were *cnf2* positive were isolated from cows (28). Other studies have shown that ExPEC strains responsible for neonatal meningitis produce the CNF1 toxin and are K1 encapsulated (33, 144). Since the *E. coli* strains producing CNF1 are more prevalent in human infections and have been studied in greater detail than strains producing CNF2, the remainder of the section will focus on CNF1.

Studies that have examined ExPEC strains have noted that a large number of CNF1 producing strains also produce HlyA (23, 24, 45-47, 56, 75). Studies looking at the association of these two genes, *hlyA* and *cnf1*, also showed that they are encoded on the same pathogenicity island in close proximity to one another (89). It was also determined that the *hlyCABD* operon is upstream of the *cnf1* gene (26, 27, 170). Further investigation of this linkage showed that the *hly* operon is linked to the *cnf1* gene by an intergenic (*igs*) region that is approximately 950 to 1000 bp in size (90, 171). CNF1 has been shown to be excreted from the cell by outer membrane

vesicle (70) and the receptor for CNF1 has been identified as the 37-kDa laminin receptor precursor (58, 199).

The CNF1 toxin is a three domain protein composed of a cell-binding, a membrane translocation, and a catalytic domain (34). The N-terminus of the toxin, amino acid residues from 53-190, contains the cell binding region (88, 177). The membrane translocation domain consists of amino acid residues from approximately 190-720 and contains two putative membrane spanning alpha-helices (34, 90, 177). Substitutions of the amino acid pairs, D373/D379 or E382/383, as well as single substitutions in either D373 or D379 to lysine in the membrane translocation domain, dramatically alters the multinucleation effect on Hep-2 cells (238). These mutations affect the toxin's ability to translocate the membrane, but do not affect the cell binding or endocytosis of the toxin (238). The C-terminus of the toxin, amino acid residues at approximately 720-1014, contains the catalytic domain (177, 272). CNF1 is a member of the catalytic triad superfamily of deamidases/transglutaminases, where the amino acid residues C866 and H881 are two members of the triad required for the deamidase activity of the toxin (272). The third member of the catalytic triad T884 seems to be required specifically for the induction of multinucleation (200). The substitution of this threonine residue with lysine decreases the amount of multinucleated cells as compared to the wild type toxin (200). Once the CNF1 toxin has been endocytosed by a target cell, the catalytic domain is translocated through the membrane following acidification, allowing the catalytic domain to gain access to small GTPase binding proteins in the host cell cytosol (60).

The cytotoxic activity of the CNF1 toxin is due to its ability to deamidate glutamine residues into glutamic acid of the small GTPase binding proteins Rho (Q63), Rac (Q61), and

Cdc42 (Q61) (98, 178, 271). The glutamine residues of Rho, Rac, and Cdc42 are important for the GTPase activity (251). The deamination of the glutamine residues results in the constitutively active proteins, which causes formation of distinct morphological changes due to continuous actin polymerization (34). The toxin has been shown to induce enlargement and multinucleation of various eukaryotic cell lines (45, 133, 200, 206) and is cytotoxic to HeLa and 5637 human bladder cells (45, 206). A study done by Rippere-Lampe, *et. al.* (250), which involved a UPEC isolate CP9 (262) and a *cnf1* isogenic mutant, showed that the wild-type strain CP9 was more resistant to neutrophil killing than its mutant counterpart.

In addition to these findings, the CNF1 toxin has been shown to inhibit antibacterial activity and phagocytosis of leukocytes (43, 70, 123), affect PMN morphology (123), and the outer membrane vesicles harboring CNF1 have been seen to negatively affect chemotaxis of PMNs in a dose-dependent manner (70). CNF1 can also induce phagocytosis in both epithelial and endothelial cell lines (45, 58, 87, 91). In a further study by Khan, *et. al.* (157), they showed that the activation of RhoA either by CNF1 or a constitutively active RhoA protein induces higher levels of invasion than a strain lacking *cnf1*. The requirement of CNF1 to induce cell invasion was further verified by a study by Doye, *et. al.* (80) in which they determined that the constitutive activation of Rho GTPases targets them for ubiquitin-proteasomal degradation, however this was not seen in all cell lines tested (36, 80). These modifications of the immune system may allow for an increased formation of abscesses by blocking immune cell functions in response to bacterial infections. Therefore, CNF1 may be another virulence factor that could aid in bacterial synergism in mixed infections.

Since *E. coli* is a versatile pathogen, various strains can infect different animals and several areas of the human body. These strains have been seen to produce over 12 different types of adhesins and 20 different types of fimbriae (10, 142, 153, 164, 182, 310). These adhesins and fimbriae are produced by various pathotypes of *E.* coli and no one strain produces all of these types (153). The ability of cells to bind to human and other animal tissue may give them an advantage in a polymicrobial infection in order to colonize an area by binding to human tissue and possibly allow for attachment of other bacteria leading to the formation of biofilms or abscesses.

Two well-studied fimbrial operons, *fim* and *pap*, are involved in attachment to different cell types and aid in the establishment of infections. The *fim* operon is composed of 8 genes, *fimBEACDFGH*, which encode for type I fimbriae (1, 161, 162). The tip protein/adhesion, FimH, is encoded by *fimH* (1). FimH has been shown to bind mannose-containing receptors on the surface of eukaryotic cells (13, 191, 195, 211). The role of type I fimbriae in urinary tract infections is controversial with studies both verifying and disproving their role in this type of infection, since the *fim* operon is present in both virulent and non-virulent strains [reviewed in (19, 173)].

The *pap* (pyelonephritis-associated pili) pili/fimbriae are associated with kidney infections and is encoded by 11 genes (*papA* to *K*) on the bacterial chromosome (129, 131). The PapG is the adhesion which binds to the Galα1→Galβ4-containing receptors expressed by uroepithelial cells (176). The *papG* gene is divided into three major subgroups *papGI* through *III* and their gene products have slightly different specificities (141, 187, 286). *pap* positive strains are a majority of the isolates found in bladder infections (cystitis) and kidney infections

(pyelonephritis) (241). Following infection of the kidneys bacteria would have better access to the blood since the kidneys are blood filtering organs. Several studies have shown a strong statistical correlation between *pap*+ isolates from kidney infections and the *hlyA* gene (163, 301), as well as urosepsis isolates carrying the *papGIII* allele and both the *hlyA* and *cnf1* genes (146). Since there is a high incidence of *pap*+ strains that cause pyelonephritis there is no surprise that a majority of septicemic isolates have been shown to carry the *pap* operon (32, 37, 145, 233, 234).

As a facultative anaerobe, *E. coli* possesses three terminal oxidases, which act as electron transport chains and as environmental modulators. These three terminal oxidases are encoded by the *cydAB*, the *cyoABCDE*, and the *cyxAB-appA* (also known as *appCBA*) operons (106). Both the *cyd* and *cyo* operons are regulated by the transcriptional activator Fnr in a negative manner when the cells are in low oxygen to anaerobic conditions (62). The ArcA and ArcB proteins also regulate these two operons. These proteins work as a two-component regulatory system, where ArcB senses the oxygen concentrations and subsequently activates ArcA via phosphorylation under anaerobic conditions (135, 136).

It has been shown that both cytochrome bo_3 and *bd* I oxidases (encoded by the *cyo and cyd* operons, respectively) are functionally equivalent, meaning that eliminating the function of either enzyme maintains the wild-type phenotype under laboratory conditions (105). The *cyo* operon is activated in the presence of high amounts of oxygen, whereas the *cyd* operon is activated when oxygen is removed from the environment (microaerophillic conditions) (62, 246, 295). Biochemical assays have confirmed those results (62, 246, 295), and show that cytochrome *bd* I has a higher affinity for oxygen, while cytochrome bo_3 has a lower affinity for

oxygen (246). Studies have shown that strains lacking either functional cytochrome *bo*$_3$ or *bd* I oxidases are sensitive to hydrogen peroxide (181, 313) as well as mutants lacking functional cytochrome *bd* II oxidase (encoded by the *cyx* operon) (181). Strains lacking a functional cytochrome *bo*$_3$ oxidase seem to be more sensitive to hydrogen peroxide than strains lacking a functional cytochrome *bd* I oxidase, which may be due to the 2-fold decrease in catalase production by the *cyo* mutants (181).

The two terminal oxidases are predicted to contribute to synergy during polymicrobial infections by decreasing the oxygen content of the surrounding tissue and thereby enable the colonization of the tissue by anaerobes (260). These gene products may not directly contribute to virulence, but could aid in bacterial synergy. If the terminal oxidases were not functional, this could affect the strain's ability to affectively colonize a particular area or indirectly protect itself from reactive oxygen species. Therefore, the elimination of the function of both terminal oxidases could severely hinder *E. coli's* pathogenic abilities and its contribution to synergy.

***Clostridium perfringens*:** *C. perfringens* are Gram-positive, aerotolerant anaerobic spore-forming rods that are found in soil and water sediments, as well as in the human intestinal tract and the skin (257). Strains of *C. perfringens* cause a series of diseases, from food poisoning to gas gangrene (clostridial myonecrosis). The most severe of these diseases is gas gangrene, which occurs as an infection in soft tissue. Gas gangrene occurs when there is insufficient blood supply to an area of tissue due to some sort of trauma or blockage (222). *C. perfringens* also produces 13 potent cytotoxins, which are major virulence factors (257), and are hypothesized to contribute to the synergy in polymicrobial infections. Two of the most important of these toxins are: 1) PLC (alpha toxin), a phospholipase C and sphingomyelinase; and 2) PFO (theta toxin), a

member of the thiol-sensitive pore-forming toxin family (256). Studies have been done with strains containing mutations in the genes encoding PLC and PFO. These studies showed that *plc* mutants were unable to produce gangrene in a mouse model (8), while *pfoA* mutants remained virulent, but there was altered pathology in the regions infected (8, 9, 81).

Septicemic infections

Septicemia is a significant and deadly disease. The Centers for Disease Control and Prevention (CDC) reported that in 2004 and 2005 it was the 10th leading cause of death in the U.S., responsible for approximately 30,000 people dying from this disease annually (168, 207). Many different bacterial pathogens can cause this disease. *E. coli* is the cause of majority of Gram-negative systemic infections including cases seen in children (especially newborns) (158, 159), in the age group over 65 years of age (mainly in nursing homes) (215), and in hospital patients (278). In a study done by Wisplinghoff, *et. al.* (330), which involved the analysis of nosocomal blood infections, their data showed that *E. coli* was the 5th leading cause of these infections in both patients in and out of the intensive care unit (ICU). *Bacteroides* species were the 9th leading cause of non-ICU blood stream infections (330). Wisplinghoff, *et. al.* (330) also observed that out of all the major players that caused these infections, *E. coli* has the shortest incubation period from admission of patients to onset of the infection. These types of infections may be the result of transfer of bacteria from the fecal matter into the urinary tract, since both *E. coli* and *Bacteroides* are normal fecal flora. From the urinary tract these organisms could gain access to the blood. Other *E. coli* strains, such as avian pathogenic *E. coli*, can also cause extraintestinal diseases in birds, which leads to a high mortality and morbidity in turkeys and chickens (336).

Bacterial interactions with the host immune system

Bacteria are able to evade the immune system through the production of a capsular polysaccharide or cytotoxins that can either block phagocytosis or lyse immune cells, respectively. However, the ability of bacteria to invade and survive inside immune cells is also a desirable trait for these organisms. Some *E. coli* strains can invade and survive in phagocytic cells such as PMNs (71, 219) and macrophages (16, 95, 287). *E. coli* strains have also been shown to invade HeLa cells via curli fibers (110) and afimbrial adhesins (104, 149). A study performed by Sukumaran, *et. al.* 2003. (287) showed that outer membrane protein A is needed for invasion and survival in the RAW264.7 murine macrophage-like cell line. The fact that some strains of *E. coli* can invade and survive in these different professional phagocytes is a legitimate pathogenic trait and possible virulence factor.

Bioluminescence as a tool to study gene expression and *in vivo* infections

Bioluminescent reporters have been used to study disease progression *in vivo* (101, 116, 138, 139, 192, 252). Bioluminescence has evolved naturally as a means to allow certain bacteria to produce light under advantageous conditions. The most well-studied bioluminescent bacterium is *Vibrio fischeri* (83-85, 201, 281, 284, 294). *V. fischeri* is a marine bacterium that lives in small cell groups in the environment or at high population densities as a symbiont of aquatic organisms. The *lux* operon of *V. fischeri* is comprised of two divergent genetic elements that include the *luxR* gene and the *lux* operon, which is composed of 7 genes, *luxICDABEG* (83-85). *luxI* encodes the autoinducer synthase, which is responsible for the production of the acyl-homoserine lactone autoinducer, 3-oxohexanoyl-L-homoserine lactone (84). The *luxR* gene encodes the transcriptional activator LuxR which, when bound to an autoinducer, acts as a

homodimer to activate the *lux* operon (84). The three genes, *luxCDE*, encode the three proteins of the fatty acid reductase complex that is involved in the reduction of long-chain fatty acids to the aldehyde substrate [Figure 1.1B, (247, 253, 254)]. The genes, *luxAB*, encode the α and β subunits of a mixed function oxidase, which is responsible for catalyzing the production of light in the presence of $FMNH_2$, oxygen, and a long-chain aldehyde [Figure 1.1B, (12)]. *luxG* encodes a flavin reductase, which supplies reduced FMN needed to drive the light reaction (220).

Since *V. fischeri* is a marine bacterium, it grows best at temperatures below 30ºC meaning the Lux proteins cannot function at temperatures above 30ºC. This poses a problem when expressing these genes in pathogenic organisms that typically grow at temperatures around 37°C. To overcome this problem, another Lux system from the bacterium *Photorhabdus luminescens* can be used because it functions at temperatures up to 45°C (289). *P. luminescens* is a terrestrial bacterium that lives in the gut of nematodes in the group *Heterorhabditis bacteriophora* and acts as a parasite to insects infected by the nematode (127).

The *P. luminescens lux* operon is made of five essential genes, *luxCDABE* (Fig. 1.1A). These gene products have the same function as their counterparts in *V. fischeri*. The two genes *luxAB* encode the luciferase enzyme while the remaining three genes, *luxCDE*, produce a multienzyme fatty acid reductase complex (52). Light output may be measured quantitatively over several orders of magnitude, making it an ideal reporter under aerobic conditions only. The *P. luminescens lux* operon can be used as an effective gene reporter for use *in vitro* (239, 252) as well as *in vivo* (77, 101, 102, 138, 139, 150, 192, 252, 332) in a number of bacteria. Most of the *in vivo* studies have focused on treatment strategies, with bactericidal compounds such as

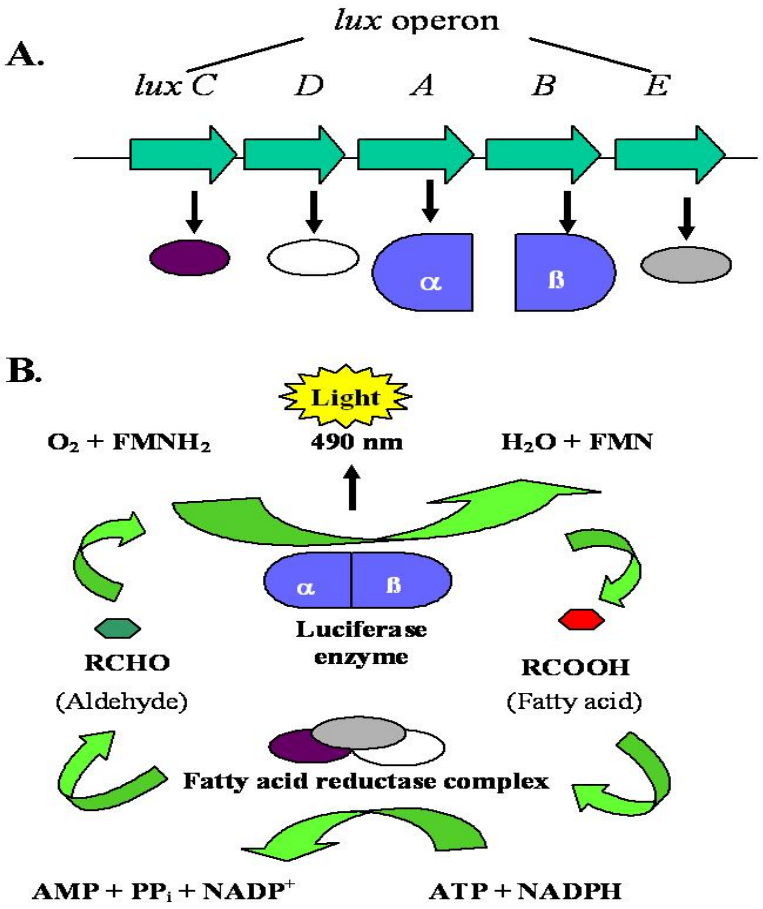

Figure 1.1. Cartoon model of the steps involved in the production of light during bacterial bioluminescence. A) Shows the *lux* operon of *P. luminescens* (*luxCDABE*) and the genes involved in the production of five protein subunits. Genes *luxAB* encode the two α and β subunits of the luciferase enzyme, respectively. The genes *luxCDE* encode the three subunits of the fatty acid reductase. B) Shows the process by which the light reaction is carried out. The fatty acid reductase complex converts a long chain fatty acid to a long chain aldehyde. This long chain aldehyde is then used by the enzyme luciferase in the presence of oxygen and an energy source in the form of $FMNH_2$ to produce light at the wavelength of 490 nm (blue-green light). The other by-products are water, an oxidized FMN, and a long chain fatty acid.

antibiotics. Chapters Two and Six describe the use of a *lux* gene reporter to examine gene expression in *Bacteroides* (Chapter Two) and *in vivo* modeling of an *Escherichia coli* infection (Chapter Six).

Gene expression in *Bacteroides*

There are barriers that impede successful expression of foreign bacterial genes in *Bacteroides* that are not fully understood. To date most studies in the area of gene expression of *Bacteroides* have focused on metabolic reporter systems. The four reporter systems that have previously been used in *Bacteroides* make use of β-glucuronidase (GUS) (93), xylosidase/arabinosidase (326), chloramphenicol acetyltransferase (18, 282), and catechol 2,3-dioxygenase (XylE) (53, 166).

The β-glucuronidase, encoded by the *uidA* gene, is used in the metabolism of certain carbohydrates (93). Using the *uidA* gene as a reporter system has its limitations because *Bacteroides* produces the enzyme under normal conditions. Since various *Bacteroides* species often have multiple copies of genes that code for metabolic enzymes, a GUS-minus strain has not yet been constructed. In addition, the GUS reporter system has another limitation; this reporter system cannot be used in viable whole cell assays (93). The xylosidase/arabinosidase gene is involved in the metabolism of polysaccharides into fermentable sugars (326). This gene reporter system can be used with either whole living cells or lysed cells. The problem involved with the use of this reporter system is that the substrate has to be supplied to the cell and these enzymes are coded for in a few *Bacteroides* species (280). The chloramphenicol acetyltransferase (*cat*) gene reporter system was used to characterize the relative activity of multiple mutant *cepA* promoters as compared to the wild-type promoter (18, 196, 255). The drawback of the *cat*

system is that the substrate is very expensive for use in large-scale assays. The fourth system is the *xylE* reporter system, which was recently developed, is a colorimetric system enabling differential (yellow/white) screening (53, 166).

In one previous study that examined the differences in transcription between *E. coli* and *Bacteroides*, they looked at a unique sigma factor from *B. fragilis*, σ^{ABfr}, and its ability to bind to *E. coli* RNA polymerase (309). It was observed that this σ factor could bind *E. coli* RNA polymerase, but did not allow for the initiation of transcription *in vitro* (309). Purified *Bacteroides* core RNA polymerase with the addition of σ^{ABfr} was able to bind *Bacteroides* promoters, but not *E. coli* promoters *in vitro* (309). Interestingly, it was observed that the *Bacteroides* core RNA polymerase with *E. coli* σ^{70} could form a complex and promote expression of both *E. coli* and *Bacteroides* promoters, but with less efficiency than the native holoenzymes (309). In a further study the structure of the sigma factor σ^{A} and its interactions with the DNA was evaluated (308). They observed that three amino acids, in particular F61, H179, and K265, are involved in promoter binding, with F61 and K265 playing more important roles (308).

Besides the differences in the promoter regions and specificity of the sigma factors of *E. coli* and *Bacteroides*, there is also a difference in the ribosomal binding site (RBS) between the two organisms. Based on complementary to the 16S ribosomal RNA, the typical *E. coli* RBS is AGGAGGU (318). Although the ribosomal machinery does not demand a precise distance, the optimal spacing between the Shine-Delgarno sequence and the initiation codon is eight nucleotides (248). A potential *B. fragilis* RBS, AGAAAGGAG, was published by Tribble, *et. al.* (293). The Shine-Delgarno sequence was deduced by determining the complement to the 3' end

of the *B. fragilis* 16S rRNA. Based on that sequence (AGAAAGGAG), potential RBS were found within three to nine nucleotides upstream of the translation start site of a number of *B. fragilis* genes (293). The differences in both transcription and translation between *E. coli* and *Bacteroides* were evaluated in Chapter Two.

Research Goals

The purpose of this research is to examine bacterial pathogenesis through a combination of molecular-based and classical microbiological techniques. In addition, differences in promoter structure, gene expression, and bacterial interaction were examined with the use of a *lux* gene reporter system. Mouse models were established to examine bacterial synergy during a polymicrobial infection and to evaluate virulence during a systemic infection. This project focused on three main areas: 1) First, promoter structure and function in *Bacteroides* was examined using a newly developed bioluminescent gene reporter system to quantitatively measure gene expression levels. Two promoters, the well studied *cepA* promoter (196, 255) from *Bacteroides fragilis* and a *Bacteroides thetaiotaomicron* 16S ribosomal RNA promoter, were studied. The remainder of the project has focused on bacterial pathogenesis. 2) Polymicrobial infections were used to determine how different bacteria contributed to bacterial synergy in these infections using a diabetic mouse model. 3) From this study, the decision was made to focus on the primary pathogen in the interactions, *Escherichia coli* 360A. We investigated how specific virulence factors and environmental modulators of this strain play a role in its pathogenesis and survival in a mouse model during a systemic infection and interactions with two murine macrophage-like cell lines. These experiments have helped further our knowledge and understanding of the role of *E. coli*, *B. fragilis*, and *Clostridium perfringens* as intestinal symbionts and intestinal pathogens.

Chapter Two

Characterization of a *Bacteroides thetaiotaomicron* 16S rRNA Promoter with a new *lux* gene reporter

Manuscript in preparation

Supplemental material is located in Appendix I

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Running Title: Expression of a *Bacteroides* 16S rRNA promoter using a *lux* reporter

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Some of the material contained in this chapter is also described in Mary Thorson's masters thesis.

Key words: *lux*, luminescence, *Bacteroides*, gene expression, reporter system

Abstract

There is no cross expression of genes between *Bacteroides sp.* and *Escherichia coli*. In this study, the promoter structure and function of a strong housekeeping *B. thetaiotaomicron* 16S rRNA promoter region was examined and compared to one from *E. coli in vivo* using a newly developed *Bacteroides lux* reporter system. Analysis of the *B. thetaiotaomicron* sequences upstream of the 16S rRNA gene has revealed the same overall structure known for *E. coli* 16S rRNA promoters in that there are two putative promoters separated by approximately 150 bp. However, the *B. thetaiotaomicron* 16S rRNA promoter contains the proposed *Bacteroides* –7 and –33 consensus sequences instead of the well known *E. coli* –10 and –35 consensus sequences. The biological activity of various configurations of the *B. thetaiotaomicron* 16S rRNA promoter was analyzed. In addition, by pairing the *B. thetaiotaomicron* 16S rRNA promoter with an *E. coli* ribosomal binding site, and vice-versa, we further confirmed that the difference in gene expression between the two species lies at the level of transcription. However, in *Bacteroides*, barriers to both transcription and translation initiation appear to limit expression of foreign genes.

Introduction

Bacteroides sp. make up a large portion of the microbial flora in the human intestinal tract, and some species are the primary cause of anaerobic infections in humans (324). They are as phylogenetically distinct from other Gram-negative enterics, including *E. coli* which is the most well-studied proteobacteria, as they are from Gram-positive bacteria. There are barriers that impede successful expression of genes from *E. coli*, and other Gram-negative species in *Bacteroides* (282). It is thought that this difference in gene expression lies in part at the level of transcription initiation and is due to the sequences within the promoter region itself. In *Bacteroides*, two conserved regions analogous to the -10 and -35 housekeeping consensus sequences in *E. coli* have been found, but their sequences were significantly different (Figure 2.1A). One region, TTTG, is centered at -33, and the other, TAnnTTTG, is centered at -7 (18).

In this study, the structure and function of a *B. thetaiotaomicron* 16S ribosomal RNA promoter was compared to that of an *E. coli* 16S ribosomal RNA promoter from the *rrnB* operon. In *E. coli*, transcription starts from two promoters (P1 and P2), which are separated by approximately 150 bp and each contain the -10 and -35 consensus sequences. P1 is located approximately 350 bp upstream, while P2 is about 200 bp upstream, from the 5' end of the mature 16S rRNA. Three antitermination sequences are located downstream of P2 and are thought to allow efficient transcription of long, highly structured untranslated rRNA.

E. coli rRNA synthesis is regulated by the collective response of the dual promoters to growth rate (114), amino acid starvation (48, 103), and rRNA gene dose (114). However, P1 and P2 are not regulated in the same way, thus providing the two with different functions. P1 is considered the stronger of the two promoters since P1 promoters account for the majority of

(A) **(B)**

	-35	-10	
E. coli	- - - TTGACA - - - - - -	-TATAAT	- - AGGAGGU - -
	-33	**-7**	
B. fragilis	- - - - TTTG - - - - - - -	-TAnnTTTG	- - AGAAAGGAG - -

(C)

	-33	-7
P1	CGATAAAGTTTGGAAGATAAAGCTAAAAGTTCTTATCTTTGCAG	
P2	CGAAAACATTTGGTAGTTAAAATAAAACCTCTTACCTTTGCACC	
P1 -33	CGATAAAG**AAAC**GAAGATAAAGCTAAAAGTTCTTATCTTTGCAG	
P1 -7 downstream	CGATAAAGTTTGGAAGATAAAGCTAAAAGTTCTTATC**AAAC**CAG	
P1 -7 upstream	CGATAAAGTTTGGAAGATAAAGCTAAAAGTTCT**AT**TCTTTGCAG	
P1 no upstream sequence	GTTTGGAAGATAAAGCTAAAAGTTCTTATCTTTGCAG	

Figure 2.1. Comparison of *E. coli* and *B. fragilis* promoter consensus sequences (A), Shine-Delgarno sequences (B), and promoter substitutions (C). A) Promoter consensus sequences of *E. coli* and *B. fragilis* were taken from Hawley and McClure, 1983 (118) and Bayley, *et. al.*, 2000 (18), respectively. B) Shine-Delgarno sequences of *E. coli* and *B. fragilis* were taken from Weaver, 1999 (318) and Tribble, *et. al.*, 1999 (293), respectively. See text for details. C) The sequences of the -33 and -7 regions of the P1 and P2 promoters from *B. thetaiotaomicron* as well as the substitutions made in the P1 region of the 16S rRNA promoter. The -33 and -7 regions of the P1 and P2 promoters are underlined. The spacing between the -33 and -7 of P1 is 21 bp and P2 is 20 bp. The substitutions made in these two regions are in bold. The P1 promoter with no upstream sequence starts at 1 base pair upstream of the first base of the -33 region.

rRNA transcription at all but the slowest growth rates (269). It has been suggested that the activities of P2 promoters are inhibited by transcription from P1 (103). The P2 promoters are low-level constitutive promoters, responsible for most of the rRNA expression at low growth rates when transcription from the P1 promoters is much reduced (213, 269). The P1 promoter of *E. coli* contains sequences upstream of its -35 hexamer, including an UP element and Fis-binding site that are responsible for strong stimulation of promoter activity, but are not required for growth rate regulation. The UP element is an A+T rich region from -40 to -60, which binds the carboxy-terminal region of the α subunit of RNA polymerase and stimulates the activity of the P1 promoter about thirty-fold (244, 259). Further upstream, between positions -61 and -121 of P1, lie three binding sites for the Fis protein, which stimulates transcription from P1 as much as ten-fold (221). The P2 promoter also contains an UP element; however, it has a much smaller effect on promoter activity than that exerted by the P1 UP element (259). Both P1 and P2 promoters also contain a G+C rich region (292) between the -10 consensus sequence and the transcription start site that is required for stringent control (148, 213).

In the examination of the differences in transcription between *E. coli* and *Bacteroides*, one previous study involved a unique *Bacteroides fragilis* sigma factor, σ^{ABfr}, and looked at its ability to bind to *E. coli* RNA polymerase. It was observed that (1) the σ^{ABfr} factor could bind *E. coli* core RNA polymerase, but did not allow for the initiation of transcription *in vitro*, (2) purified *Bacteroides* core RNA polymerase plus σ^{ABfr}, was able to bind *Bacteroides* promoters and not *E. coli* promoters *in vitro*, (3) *Bacteroides* core RNA polymerase with *E. coli* σ^{70} could form a complex and promote expression of both *E. coli* and *Bacteroides* promoters, but with less efficiency than the native holoenzymes (309). In a further *in vitro* study the structure of the

sigma factor σ^A and its interactions with the DNA was evaluated (308). That study identified three amino acids involved in the promoter binding (308).

Besides the differences in the promoter regions and specificity of the sigma factors of *E. coli* and *Bacteroides*, there is also a difference in the ribosomal binding sites (RBS) between the two organisms. A canonical *E. coli* RBS is AGGAGGU (Figure 2.1B (318)). Although the ribosomal machinery does not demand a precise distance, the optimal spacing between the Shine-Delgarno sequence and the initiation codon is eight nucleotides (248). A potential *B. fragilis* RBS was published by Tribble, *et. al.* (Figure 2.1B (293)). The Shine-Delgarno was deduced by determining the complement to the 3' end of the *B. fragilis* 16S rRNA. Based on that sequence, potential RBS were found within three to nine nucleotides upstream of the translation start site of a number of *B. fragilis* genes.

Our *in vivo* study complements the work done by Vingadassalom, *et. al.* (308, 309) and further demonstrates that the difference in gene expression between *Bacteroides* and *E. coli* lies in differences in the promoter structure. In our study the role of the RBS of *E. coli* and *Bacteroides* has also been analyzed. We developed two luminescent reporter systems that utilize the *lux* genes from the symbiotic luminescent soil bacterium *Photorhabdus luminescens* for use in *Bacteroides* species to comparatively examine the role of promoter elements and the RBS in controlling gene expression.

Material and Methods

Bacterial strains, plasmids, media, and growth conditions. Table 2.1 lists pertinent strain and plasmid information. *E. coli* DH5α (117) was the host for all cloning steps. *E. coli* JM109 (335) was used as the host for analysis of the activity from plasmids containing *lux* genes. *E. coli* S17-1 (280) was used for conjugating all the *E. coli-Bacteroides* shuttle vectors into the *Bacteroides* host strain, *Bacteroides thetaiotaomicron* strain 4001 (276). All pMDM-series *lux* vectors contain two genes that confer resistance to tetracycline (Tc) in *E. coli* and erythromycin (Em) in *Bacteroides*. All pMMT1 and pMMT2 vectors contain two genes, conferring resistance to kanamycin (Kan) in *E. coli* and Em in *Bacteroides*. All *E. coli* strains were grown aerobically in Luria-Bertani (LB) broth at 37^{o}C with shaking (250 RPM) and the appropriate antibiotics required for maintenance of the plasmids, either 100 μg/mL ampicillin (Ap100), 10 μg/mL Tc (Tc10), or 100 μg/mL Kan (Kan100). All *B. thetaiotaomicron* carrying the *lux* reporters were grown anaerobically at 37^{o}C in 10 mL culture tubes containing Tryptocase Yeast Extract Glucose broth (TYG, (124)), 10 μg/mL Em (Em10) and 200 μg/mL gentamicin (Gm200). Stock tubes of *B. thetaiotaomicron* carrying the *lux* reporters were kept in 7 mL PRAS chopped meat broth (Remel, Inc.) with Em10 and Gm200 for up to two weeks.

Isolation of a *Bacteroides thetaiotaomicron* 16S rRNA promoter. As this work was initiated prior to publication of the genome sequence (333), partial sequence data for a *B. thetaiotaomicron* 16S rRNA gene was obtained via nucleotide sequencing of two isolates of a cloned PCR product (pJMP4) amplified with the universal primers 27f and 907r ((172), Table 2.2).

Table 2.1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevance to Study and Strain Genotype[a]	Reference[b]
Strains		
E. coli		
JM109	Host strain for *lux* containing constructs, *endA1*, *glnV44*, *thi-1*, *relA1*, *gyrA96*, *recA1*, *mcrB*$^+$, Δ(*lac-proAB*), *e14-* [F' *traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*] *hsdR17*($r_K^-m_K^+$)	(335)
DH5α	Host strain for cloning of terminator constructs, F$^-$, *endA1*, *glnV44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *deoR*, *nupG*, Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsdR17*(r_K^- m_K^+), λ–	(117)
S17-1	Strain used in di-parental matings with *Bacteroides*, *recA*, *pro*, *hsdR*, Tp^R, Spc^R, Str^R	(280)
B. thetaiotaomicron		
4001	Spontaneous Rif^R isolate of *B. thetaiotaomicron* VPI 5842, Gm^R; used as host strain for the bioluminescent gene reporters pMDM and pMMT system	(276)
Plasmids		
pGEM-T	TA cloning vector used for sequencing, Ap^R	Promega
pVAL1	*E. coli-Bacteroides* shuttle vector, Tc^R / Ap^R / Em^R	(302)
pJT205	*P. luminescens lux* operon, Ap^R	(305)
pJMP2	*E. coli rrnB* P1 (300 bp) flanked by 5' *Pst*I and 3' *Kpn*I sites	J. Meador-Parton
pJMP4	Internal coding sequence of a *B. thetaiotaomicron* 16S rRNA gene	J. Meador-Parton
pJMP6	1300 bp inverse PCR product, amplified from *B. thetaiotaomicron* 4001 chromosomal DNA digested with *Ava*I	J. Meador-Parton
pJMP20	*cepA* promoter in pJT205	J. Meador-Parton
pMLT2	Full length *B. thetaiotaomicron* 16S rRNA promoter	M. Thorson
pMLT7	Full length *B. thetaiotaomicron* 16S rRNA promoter, flanked by 5' *Pst*I and 3' *Bam*HI sites	M. Thorson
pMLT9	*B. thetaiotaomicron* strain 4001 full length 16S ribosomal RNA promoter upstream of *P. luminescens lux* operon from pJT205 Ap^R	M. Thorson
pMDM1	Wild type *cepA* promoter construct, with entire *lux* operon	This Study
pMDM4	Promoterless *lux* operon reporter construct	This Study
pMDM5A-2	T_1T_2 *E. coli* transcription terminators in pUC19	This Study
pMDM7A-7	T_1T_2 *E. coli* transcription terminators in pGWA48.3	This Study
pMDM8	*B. thetaiotaomicron* strain 4001 16S ribosomal RNA promoter-*lux* operon reporter construct	This Study
pMDM9-14	phage lambda transcription terminator t_0 flanked by *Eco*RI sites in pGEM-T	This Study
pMLT10	*B. thetaiotaomicron* 16S rRNA P2 (160 bp) flanked by 5' *Pst*I and 3' *Bam*HI sites in pGEM-T	M. Thorson
pMLT11	*B. thetaiotaomicron* 16S rRNA P1P2 with no upstream sequence (260 bp) flanked by 5' *Pst*I and 3' *Bam*HI sites in pGEM-T	M. Thorson
pMLT12	*B. thetaiotaomicron* 16S rRNA P1 (150 bp) flanked by 5' *Pst*I and 3' *Bam*HI sites in pGEM-T	M. Thorson
pMLT13	*P. luminescens luxAB* with a *Bacteroides* RBS flanked by 5' *Kpn*I site and 3' *Eco*RI sites in pGEM-T	M. Thorson
pMLT14	*P. luminescens luxAB* with an *E. coli* RBS flanked by 5' *Kpn*I site and 3' *Eco*RI sites in pGEM-T	M. Thorson
pMLT15	*P. luminescens luxAB* with a *Bacteroides* RBS downstream from T1T2 in pMDM7A-7	M. Thorson
pMLT16	*P. luminescens luxAB* with an *E. coli* RBS downstream from T1T2 in pMDM7A-7	M. Thorson
pMDM15-1	-7 downstream sequence variant of *B. thetaiotaomicron* 16S rRNA full length promoter P1 in pGEM-T	This Study
pMDM16	-7 upstream sequence variant of *B. thetaiotaomicron* 16S rRNA full length promoter P1 in pGEM-T	This Study
pMDM50-5	Truncated *B. thetaiotaomicron* 16S ribosomal RNA promoter P1 in pGEM-T	This Study
pMDM51	-33 variant of *B. thetaiotaomicron* 16S ribosomal RNA full length promoter P1 in pGEM-T	This Study
pMMT1	*luxAB* reporter system with the *Bacteroides* consensus RBS, Kan^R / Em^R	This Study
pMMT1A	*E. coli rrnB* P1 promoter in pMMT1	M. Thorson
pMMT1B	*B. thetaiotaomicron* full length 16S rRNA promoter in pMMT1	M. Thorson
pMMT1C	*B. thetaiotaomicron* 16S rRNA P1 promoter in pMMT1	M. Thorson
pMMT1D	*B. thetaiotaomicron* 16S rRNA P2 promoter in pMMT1	M. Thorson
pMMT1E	contains a *B. thetaiotaomicron* P1P2 16S rRNA promoter with no upstream sequence in pMMT1	M. Thorson
pMMT1F	*cepA* promoter construct in pMMT1	This Study
pMMT1G	variant *cepA* promoter construct (0% activity) in pMMT1	This Study
pMMT1H	variant *cepA* promoter construct (33% activity) in pMMT1	This Study
pMMT1I	*B. thetaiotaomicron* 16S rRNA P1 promoter with a substituted -7 downstream region in pMMT1	This Study
pMMT1J	*B. thetaiotaomicron* 16S rRNA P1 promoter with a substituted -7 upstream region in pMMT1	This Study
pMMT1K	*B. thetaiotaomicron* 16S rRNA P1 promoter with a substituted -33 region in pMMT1	This Study
pMMT1L	*B. thetaiotaomicron* 16S rRNA truncated P1 promoter in pMMT1	This Study
pMMT2	*luxAB* reporter system with the *E. coli* consensus RBS, Kan^R / Em^R	M. Thorson
pMMT2A	*E. coli rrnB* P1 promoter in pMMT2	M. Thorson
pMMT2B	*B. thetaiotaomicron* full length 16S rRNA promoter in pMMT2	M. Thorson
pMMT2C	*B. thetaiotaomicron* 16S rRNA P1 promoter in pMMT2	M. Thorson

[a]For use in *Escherichia coli*: Tetracycline resistance (Tc^R); Ampicillin resistance (Ap^R); Trimethoprim resistance (Tp^R); Kanamycin resistance (Kan^R), For use in *Bacteroides*: Rifampicin resistance (Rif^R); Gentamicin resistance (Gm^R); Erythromycin resistance (Em^R); Spectinomycin (Spc^R); Streptomycin (Str^R).

[b]The primers referenced to M. Thorson are located in her master's thesis (291).

Table 2.2. Primers used to characterize a *B. thetaiotaomicron* 16S rRNA promoter.

Primer Name	**Primer Sequence** (5' to 3')[a, b]	**Reference**[c]
LuxABREcoR2	GGGAATTCCTATTAGGTATATTCCATGTGG	This Study
LuxABFKpn	GGGGTACCAGAAAGGAGACTCTCTATGAAATTTGGAAAC	This Study
LuxABEcRBS	GGGGTACCAGGAGGGACTCTCTATGAAATTTGGAAAC	M. Thorson
T1T2F3	GCATGCAAGCTTGGATGAGAGAAGATTTTCAGC	This Study
T1T2R3	TGTCGGAAGCTTAAGAGTTTGTAGAAACGC	This Study
T0F	CGGAATTCCCAGTAATGACCTCAGAACTCC	This Study
T0R	CGGAATCGCCCCATACGATATAAGTTG	This Study
MLT2A	CCGGATCCAAAAGTATTGTTAGTCAGC	M. Thorson
MLT2B	AACTGCAGTGGTACTTTGCCCCGCTTTC	M. Thorson
MLT7C	CGGGATCCCTTTTTCTATCGTTTCTCAGAC	M. Thorson
MLT3A	AACTGCAGGTCTGAGAAACGATAGAAAAAG	M. Thorson
MLT37B	CGGGATCCCTTGTACTACTTGTATTG	M. Thorson
MLT7A	AACTGCAGGTTTGGAAGATAAAGC	M. Thorson
BtPE1	CTTTTTCTATCGTTTCTCAGAC	M. Thorson
33P1	CCGATAAAG**AAAC**GAAGATAAAGCTAAAGGTTC	This Study
7P1U	GTTCT**AT**TCTTTGCAGTCCGATTCG	This Study
7P1D	GTTCTTATC**AAAC**CAGTCCGATTCG	This Study
27f	AGAGTTTGATCMTGGCTCAG	(172)
907r	CCGTCAATTCMTTTRAGTTT	(172)
RRNF1	GCAAGTCGAGGGGCATCAT	J. Meador-Parton
RRNR1	GCCTGTAGCTAGCGTTCATCC	J. Meador-Parton
89R	CTGAGCCAGGATCAAACTCTTC	M. Thorson
599F	GGTACTTTTGCCCCGCTTTCC	M. Thorson

[a]Key to Symbols: M = A or C; R = A or G.

[b]The highlighted region indicates the region being replaced in the 16S rRNA promoter.

[c]The primers referenced to M. Thorson are located in her master's thesis (291).

To isolate the promoter region, chromosomal DNA was digested to completion with *Ava*I. The digested DNA fragments were religated and PCR amplified using *Taq*2000 DNA Polymerase (Stratagene, La Jolla, Ca) using primers RRNF1 and RRNR1 (Table 2.2). This inverse PCR product was cloned into pGEM-T (Promega, Madison, WI) to generate the plasmid pJMP6.

Primers 89R and 599F (Table 2.2) were designed from this sequence data in order to amplify the putative *B. thetaiotaomicron* 4001 16S rRNA promoter from chromosomal DNA. Recombinant plasmid pMLT2 is composed of the PCR fragment cloned into pGEM-T (Promega). In a similar manner, pMLT7 was constructed when a PCR product, generated from pMLT2 using the primers MLT2B and MLT2A (Table 2.2), was cloned into pGEM-T.

Development of a bioluminescent gene reporter. Two reporters were constructed, pMDM and pMMT series vectors (Figure 2.2), containing the entire *lux* operon (*luxCDABE*) or just containing *luxAB*, respectively. The pMDM-series vectors were constructed using the entire *P. luminescens lux* operon from pJT205 (Biotechnical Resources, Manitowoc, WI (305)). Promoters were first cloned directly into pJT205 using *Pst*I and *Bam*HI (New England Biolabs, Ipswich, MA). The promoter and *lux* operon were ligated into the *E. coli-Bacteroides* shuttle vector pVAL1 (302) using *PstI* and *Eco*RI sites. The pMDM system was first tested by examining the expression of the *Bacteroides* wild-type (wt), *cepA* (196, 255) and 16S rRNA promoters. These vectors were named pMDM1 (wt *cepA* promoter, Figure 2.2A), pMDM4 (promoterless control), and pMDM8 (16S rRNA promoter from *B. thetaiotaomicron*).

A second series of vectors was created, which gives an easy one step cloning of promoters into the *lux* reporter. The pMMT-series *luxAB* constructs (Figure 2.2B) were made

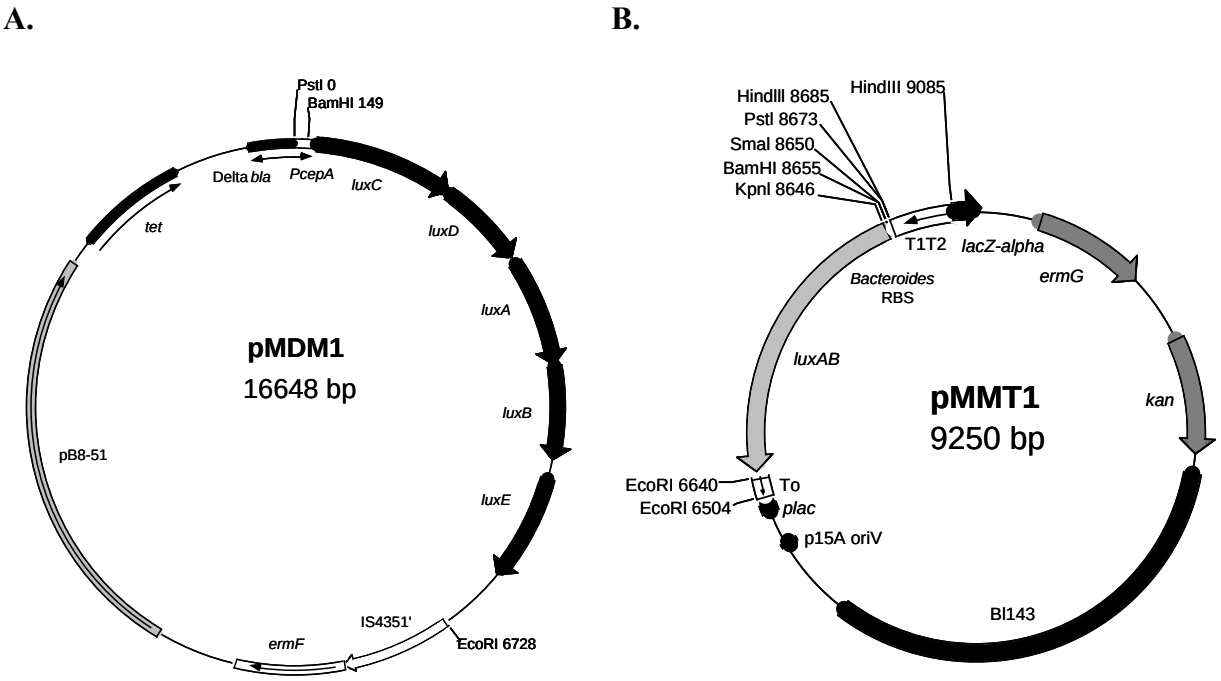

Figure 2.2. Maps of two *E. coli* – *Bacteroides* shuttle/expression vectors. A) The plasmid pMDM1 is a fusion of pVAL1 with pJMP20; the plasmids were fused using the restriction sites *Eco*RI and *Pst*I. The *cepA* promoter was cloned directly into the pJT205 via *Pst*I and *Bam*HI creating the plasmid pJMP20. **B)** The plasmid pMMT1 contains the *luxAB* with flanking *Kpn*I (5' end) and *Eco*RI (3' end). The t_o transcription terminator and the T_1T_2 transcription terminator are flanked by *Eco*RI and *Hin*dIII restriction sites, respectively. The MCS contains 4 unique sites: *Pst*I, *Sma*I, *Bam*HI, and *Kpn*I, which may be used for cloning additional promoters. See text for details of the vector construction.

via the cloning of transcription terminators and the *luxAB* cassette, into the plasmid pGWA48.3 (327). The *E. coli rrnB* T_1T_2 transcription terminators were amplified from the plasmid pKK223-3 (5) using the primers T1T2F3 and T1T2R3 (Table 2.2) with flanking *Hin*dIII restriction sites. This upstream terminator is needed to block any read through transcription from sequences upstream of the *luxAB* cassette in the final reporter construct. The T_1T_2 transcription terminators (~450 bp) were amplified via PCR, digested with *Hin*dIII, cloned into pUC19 (335), creating pMDM5A-2. The terminator was subsequently cloned into pGWA48.3 (327), creating pMDM7A-7.

The *luxAB* fragment (~2000 bp) was amplified from pJT205 (305) using the primers LuxABFKpn or LuxABEcRBS (with the *Bacteroides* and *E. coli* ribosomal binding sites, respectively) and LuxABREcoR2 (Table 2.2) and sequenced, creating pMLT13 and pMLT14, respectively. Both of these fragments were cloned into pMDM7A-7 using the restriction enzymes *Kpn*I and *Eco*RI (New England Biolabs), creating pMLT15 (*Bacteroides* RBS) and pMLT16 (*E. coli* RBS). A second transcription terminator was needed in the reporter to prevent read through transcription into the downstream region, where the origin of replication is located. The λ phage t_0 transcription terminator was amplified from the plasmid pZA3P$_{lac/ara\text{-}1}$ (188) using the primers T0F and T0R (Table 2.2), which amplified the region with flanking *Eco*RI sites. The t_0 transcription terminator (~150 bp) was cloned into the plasmids pMLT15 and pMLT16 creating pMMT1 and pMMT2, which contain the *Bacteroides* and *E. coli* ribosomal binding sites, respectively. The pMMT constructs contain four unique restriction sites (*Pst*I, *Sma*I, *Bam*HI, and *Kpn*I) for cloning promoters (Figure 2.2B).

Cloning of 16S rRNA promoters and derivatives. *B. thetaiotaomicron* 16S rRNA promoters P1, P2, and both P1 and P2 with no upstream sequence of P1 were isolated from pMLT7 via PCR, using primers listed in Table 2.2, which introduced a *Pst*I site upstream and a *Bam*HI site downstream. Primers MLT2B and MLT7C were used to amplify P1. Primers MLT3A and MLT37B were used to amplify P2. Primers MLT7A and MLT37B were used to amplify P1 and P2 with no upstream sequence of P1. All products were first cloned into pGEM-T (Promega), to generate pMLT10 [P2], pMLT11 [P1P2 with no upstream sequence of P1], and pMLT12 [P1].

Substitutions made to the -33 and two portions of the -7 consensus sequence of the P1 subunit of the 16S rRNA *Bacteroides* promoter as well as the P1 promoter with no upstream sequence (Figure 2.1C) were done using the primers outlined in Table 2.2. The template for the substitutions was the plasmid pMLT12. The PCR was done in two steps. The first round of PCR was done using DeepVent polymerase (New England Biolabs) with the forward primers 33P1, 7P1U, and 7P1D, used for the -33, -7 upstream, and -7 downstream substitutions, respectively, and the reverse primer MLT7C, which adds a *Bam*HI site to the 3' end. The P1 promoter with no upstream sequence was amplified using the primers MLT7A and MLT7C (Table 2.2). The products were subsequently gel extracted using a QIAquick Gel Purification kit (QIAGEN, Valencia, CA) and used for a second round of PCR with the primer MLT2B, which adds a *Pst*I site to the 5' end, to obtain the full length P1 promoter, approximately 200 bp in size. The resulting PCR product was gel purified a second time. A-overhangs were added using *Taq* polymerase (New England Biolabs) the reactions were purified using a QIAquick PCR Purification kit (QIAGEN) and ligated into pGEM-T (Promega). The resultant plasmids verified

by DNA sequencing are as follows: pMDM15-1 (-7 downstream), pMDM16 (-7 upstream), pMDM50-5 (P1 truncated), and pMDM51 (-33).

The *B. thetaiotaomicron* full-length 16S rRNA promoter and all of the isolated P1 and P2 fragments were cloned into pMMT1 via the *Pst*I and *Bam*HI sites. The *B. thetaiotaomicron* full length 16S rRNA promoter and P1 were also subcloned into pMMT2 via *Pst*I and *Bam*HI. *E. coli rrnB* P1 was subcloned from pJMP2 into both pMMT1 and pMMT2 via *Pst*I and *Kpn*I. See Table 2.1 for descriptions and designations of these promoter-reporter fusions.

The wt *cepA* promoter and two variant forms were also cloned into pMMT1 via *Pst*I and *Bam*HI. This was used to evaluate the sensitivity of the vector. The activity of these variant promoters has been documented previously by Bayley, *et. al.* (18). These plasmids were named pMMT1F (wt *cepA*), pMMTG (0% activity) and pMMTH (33% activity).

Conjugation procedure. All of the *E. coli-Bacteroides lux* reporter/shuttle vectors were transferred into *B. thetaiotaomicron* strain 4001 by di-parental mating via *E. coli* strain S17-1 (280) using standard filter mating protocols (275).

***In vitro* luciferase assays.** *E. coli* JM109 containing plasmids pMMT1 and pMMT2 constructs were grown at 37°C in LB Kan100 medium with shaking. Likewise, *B. thetaiotaomicron* 4001 strains (276) containing pMMT1 and pMMT2 constructs were grown at 37°C anaerobically in pre-reduced TYG (124), Gm200 and Em10. All of these samples were standardized by growing each strain to a final OD_{600} of 0.5 regardless of the genus. One ml aliquots of each culture were sedimented by centrifugation and the pellets were stored at -70°C. Luciferase assays using the harvested crude cell extracts were performed as described by Finney, *et. al.* (97). Activities are expressed as relative light units (RLU) using a TD-20/20 luminometer

(Turner Designs, Sunnyvale, CA). The measurements are shown as fold induction, which is defined as the amount of relative light units of the promoter construct divided by the relative light units of the promoterless negative control.

***In vivo* luminescence assays.** Cellular luminescence was measured from *B. thetaiotaomicron* 4001 strains carrying the pMDM-series as well as the pMMT1 constructs with the *cepA* promoter and two variant forms. Assays were then performed as described by Phillips-Jones (239), over a period of ten minutes (Appendix I, FigureAI.1 to 3). In short, the cells were grown overnight in 10 mL of TYG with the appropriate antibiotics and then subcultured into fresh medium. Then each strain was grown to an OD_{600} of 0.5. Measurements were made in relative light units (RLU) using a TD-20/20 luminometer (Turner Designs).

Primer extension. The primer extension work was done by Mary Thorson and Dr. Ann Stevens. Primers BtPE1 and MLT37B (Table 2.2) were used to examine the transcription start site of the P1 and P2 promoters, respectively. Each primer was 5′ end labeled using [γ-^{32}P] dATP (Amersham, Piscataway, NJ) and a Primer Extension System kit (Promega). Primer extension was performed using this kit according to the manufacturer's instructions, except that an ethanol precipitation step was used to remove unincorporated [γ-^{32}P] dATP from the primer. For ethanol precipitation 90 µL RNase-free water, 11 µL 3 M RNase-free sodium acetate, and 220 µL ethanol were added, mixed by vortexing, and kept at room temperature for 1 hour. Sequencing reactions of purified plasmids were performed using an *fmol* DNA cycle sequencing system kit (Promega) according to the manufacturer's instructions.

Statistics. The results were analyzed using the Instat 3 software (Graphpad, Inc.) with an unpaired Student's T-test. A two-tailed *P* value < 0.0500 was considered statistically significant.

Results

Development of the *lux* gene reporter system. First the pMDM-series reporter with the entire *lux* operon, encoding both luciferase and the fatty acid reductase complex, was used to measure the production of light using both whole cell luminescence assays and crude cell extract luciferase assays (Appendix I, FigureAI.1 and 2). These results showed that *B. thetaiotaomicron* 4001 is able to produce a functional luciferase enzyme and produce light in the presence of oxygen using a *lux* operon reporter system, but only when exogenous decanal substrate is added (Appendix I, FigureAI.1A and 2A). The output of the negative control pMDM4 is negligible with respect to the background production of light in these two assay systems (Appendix I, Figures AI.1 and AI.2). However, there are concerns when using the pMDM-series vectors. First, promoters are not easily cloned, because each promoter has to be cloned into pJT205 before the constructs can be fused with pVAL1. Second, the size of the vectors is an issue for concern, since these constructs are approximately 17 kilobases in size. Third, strains carrying these large vectors had roughly a 25-fold increase in generation times. Finally, the decanal substrate had to be supplied exogenously to the cells.

The development of a second reporter was needed due to: 1) the amount of growth time required, 2) the multiple steps involved in cloning of promoters into pMDM-series constructs, and 3) the requirement of the addition of the decanal substrate to the assay samples. Therefore, the pMMT1-series vector system is an improved reporter for *Bacteroides* because a shorter one-step cloning method may be used to introduce promoters into the vector using the unique *Bam*HI, *Kpn*I, *Pst*I, and *Sma*I sites. Efforts were also made to improve the system in order to create a higher through-put screening method for detection of light production (Appendix I).

pMMT reporter sensitivity. To establish the sensitivity of the new pMMT *lux* reporter, expression levels were measured from fusions to the wt *cepA* promoter and two variants, which have decreased activity (0% and 33% activity) as demonstrated by the chloramphenicol acetyltransferase gene reporter system (18). The strains carrying pMMT1F (wt), pMMT1G (0%), and pMMT1H (33%) were used for both luciferase and luminescence assays. The results obtained from both the luciferase and luminescence assays (Appendix I, Figure AI.3) showed similar results to the chloramphenicol acetyltransferase gene reporter system mentioned above and indicate that the *luxAB* reporter can show subtle differences in promoter strength (18). Hence, the new reporter was used to analyze expression from the 16S rRNA constructs.

Promoter expression in *E. coli*. Luciferase assays in *E. coli* demonstrated the activity of the *E. coli rrnB* P1 promoter in that it permitted initiation of expression of the *lux* reporter at 250 to 160-fold higher levels than the negative controls, pMMT1 and pMMT2, (pMMT1A and pMMT2A [Figure 2.3A and B]). *rrnB* P1 allowed expression of the *lux* reporter in the presence of either the *Bacteroides* or *E. coli* RBS, but expression was slightly enhanced in the presence of the *Bacteroides* RBS. In pMMT1, the *Bacteroides* RBS (Figure 2.1B) was designed to be seven nucleotides upstream from the translation start site of the *lux* reporter whereas in pMMT2, the *E. coli* RBS (Figure 2.1B) was designed to be eight nucleotides upstream of the translation start site. Even though in *E. coli* the optimal spacing between the Shine-Delgarno sequence and the initiation codon is eight nucleotides (248), the spacing of the RBS does vary from gene to gene. Differences in the spacing between the RBS and start codon being recognized in *E. coli* might explain the enhanced levels of expression observed from the pMMT1A construct when

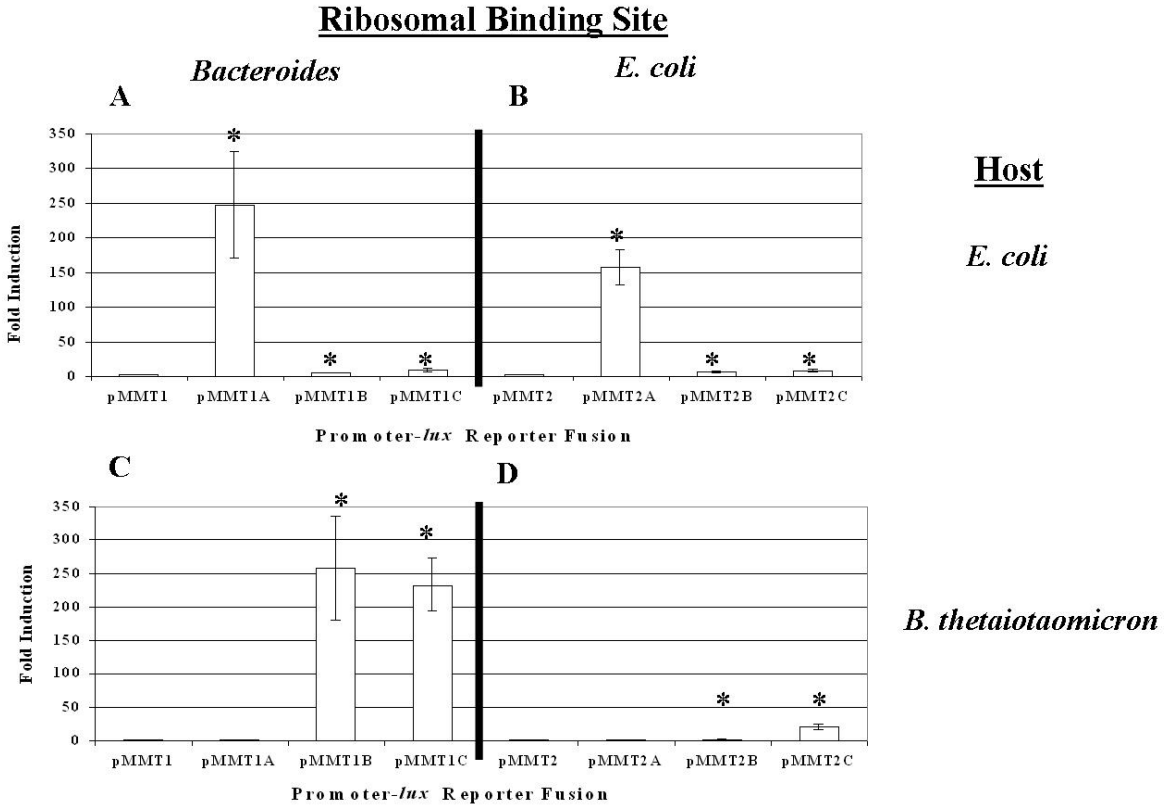

Figure 2.3. Luciferase assays using both *E. coli* and *B. thetaiotaomicron* whole cell lysates. Panels A and B, indicate transcription initiation from *E. coli* and *B. thetaiotaomicron* 16S rRNA promoters using *E. coli* whole-cell lysates with the *Bacteroides* RBS (A) and the *E. coli* RBS (B). Panels C and D, indicate transcription initiation from *E. coli* and *B. thetaiotaomicron* 16S rRNA promoters using *B. thetaiotaomicron* whole cell lysates with the *Bacteroides* RBS (C) and *the E. coli* RBS (D). The graphs from left to right: pMMT1/2, promoterless controls with the *Bacteroides* RBS or *E. coli* RBS respectively; pMMT1/2A, *E. coli rrnB* P1 promoter; pMMT1/2B, *B. thetaiotaomicron* full length 16S rRNA promoter; pMMT1/2C, *B. thetaiotaomicron* 16S rRNA P1 promoter. Results shown are the mean (± SD) of two independent trials, performed in triplicate as described in the Materials and Methods. Measurements of luciferase activity were made in relative light units (RLU). The average RLU for the promoterless controls, for panels A and B, pMMT1 and pMMT2 were 0.71 and 0.13, respectively. The average RLU for the promoterless controls, for panels C and D, pMMT1 and pMMT2 were 0.062 and 0.081, respectively. Statistically significant values as compared to the promoterless controls, were determined using the Student's unpaired *t*-test (two-tailed); *, $p < 0.001$.

compared to pMMT2A (Figure 2.3A and B). Despite the difference of expression between the two constructs, transcription was strongly induced by the recognition of the *E. coli rrnB* P1 in the presence of either RBS. Luciferase assays in *E. coli* also showed that, despite the presence of the *E. coli* RBS, the *B. thetaiotaomicron* full-length 16S rRNA promoter and *B. thetaiotaomicron* 16S rRNA P1 were unable to permit expression of the *lux* reporter at biologically significant levels (pMMT2B, pMMT2C [Figure 2.3B]). This supports the hypothesis that the difference in gene expression between *E. coli* and *Bacteroides* species lies at the level of promoter recognition.

Promoter expression in *B. thetaiotaomicron*. Luciferase assays in *B. thetaiotaomicron* demonstrated the strength of the *B. thetaiotaomicron* 16S rRNA promoter in that, in the presence of its own RBS (pMMT1B), it permitted initiation of expression of the *lux* reporter at approximately 260-fold higher levels than the promoterless control, pMMT1 (Figure 2.3C). Likewise, *B. thetaiotaomicron* 16S rRNA P1 (pMMT1C) permitted initiation of expression of the reporter at 230-fold higher levels than the negative control (Figure 2.3C). *E. coli rrnB* P1 was unable to allow expression of the *lux* reporter in *B. thetaiotaomicron*, even in the presence of the *Bacteroides* RBS (pMMT1A [Figure 2.3C]. Similarly, the *Bacteroides* promoters were unable to permit initiation of luciferase expression at biologically significant levels in the presence of the *E. coli* RBS (pMMT2B, pMMT2C [Figure 2.3D]). In the presence of the *E. coli* RBS, *B. thetaiotaomicron* 16S rRNA P1 allowed expression of the *lux* reporter at twenty-fold higher levels than the promoterless control (pMMT2C [Figure 2.3D]). When comparing this to the strength of activity of the *B. thetaiotaomicron* 16S rRNA P1 promoter in the presence of the *Bacteroides* RBS (pMMT1C [Figure 2.3C]), this level of induction is significantly lower. These

results suggest that in *Bacteroides* species, translation initiation might also play a role in the disparity of gene expression between the two species.

The *lux* reporter also allowed us to analyze the role of different regions of the *B. thetaiotaomicron* 16S rRNA promoter to evaluate the role they play in initiating transcription. The *B. thetaiotaomicron* full-length 16S rRNA promoter, putative P1, putative P2, and full-length promoter with no upstream sequences of the P1 promoter (pMMT1B, pMMT1C, pMMT1D, and pMMT1E, respectively) were also examined (Figure 2.4). Luciferase assays demonstrated that the regions believed to contain *B. thetaiotaomicron* 16S rRNA P1 and P2 contained a functional promoter and that each was capable of permitting initiation of gene expression in the absence of the other (pMMT1C and pMMT1D, respectively, Figure 2.4). This suggests that, as in *E. coli*, the two promoters might work together in order to maintain the proper amount of rRNA in a cell at a given growth condition. Also, *B. thetaiotaomicron* full-length 16S rRNA promoter without any upstream sequences of the P1 promoter was capable of being expressed at levels close to that of the same promoter with upstream sequences (pMMT1E vs. pMMT1B, respectively, Figure 2.4). This demonstrates that an UP element is not necessary for enhanced expression of this 16S rRNA full-length promoter in *B. thetaiotaomicron*.

Primer extension was done in order to examine the start site of transcription in *Bacteroides*. The results of these experiments showed that the transcription start site from the P1 promoter is a cytosine (Figure 2.5B). The dominant start site of transcription for the P2 promoter is guanine with a secondary adjacent start site at a cytosine (Figure 2.5A). These results indicate that the spacing between the -7 region and the transcription start site is 8 bp.

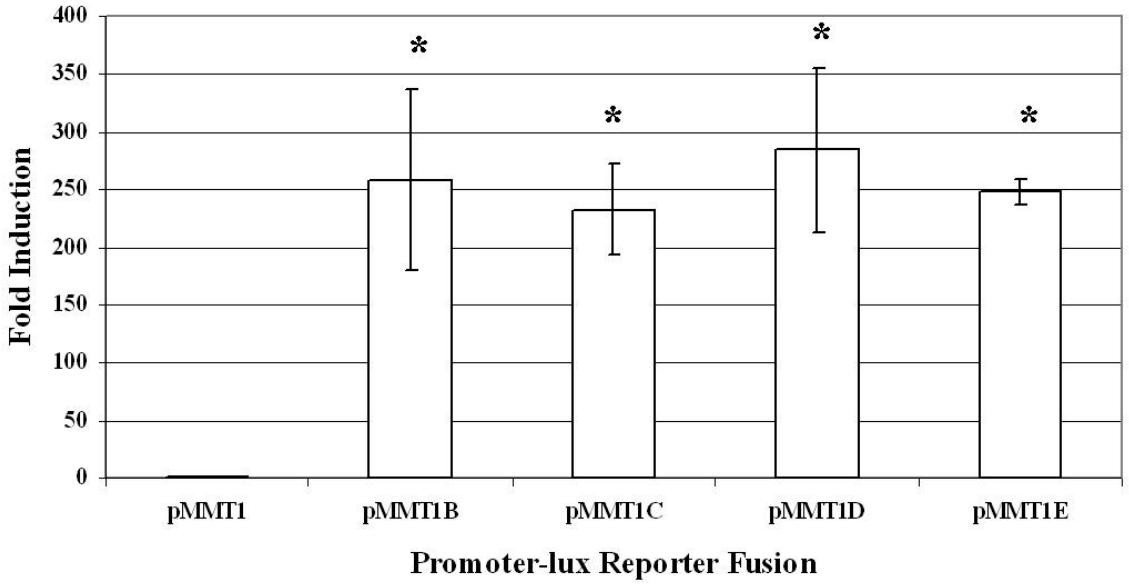

Figure 2.4. Luciferase assays demonstrate transcription initiation from the different regions of the *B. thetaiotaomicron* 16S rRNA promoter. Results shown are the mean (± SD) of two independent trials, performed in triplicate as described in the Materials and Methods. The graph from left to right: pMMT1, promoterless control; pMMT1B *B. thetaiotaomicron* full length 16S rRNA promoter; pMMT2C, *B. thetaiotaomicron* 16S rRNA P1 promoter; pMMT1D *B. thetaiotaomicron* 16S rRNA P2 promoter; pMMT1E *B. thetaiotaomicron* 16S rRNA P1P2 promoter, with no sequence upstream of P1. Measurements of luciferase activity were made in relative light units (RLU). Average RLU for the promoterless control pMMT1 was 0.062. Statistically significant from promoterless control, using the Student's unpaired *t*-test (two-tailed); *, $p < 0.001$.

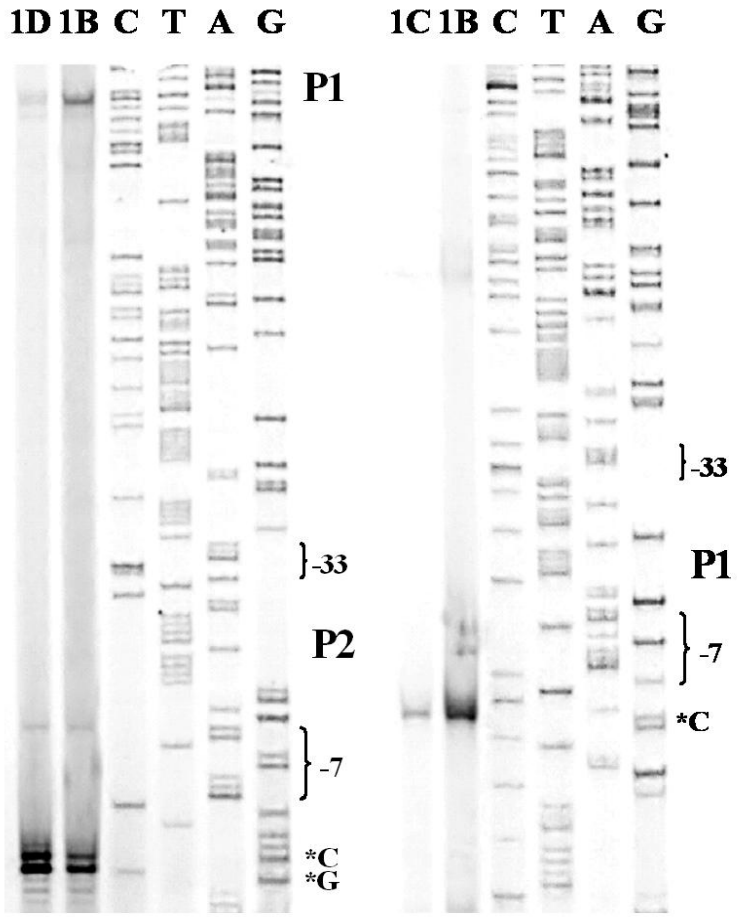

Figure 2.5. Primer extension reactions mapping the transcription initiation sites of the P1 and P2 promoters. The lanes labeled 1B, 1C and 1D correspond to primer extension products produced from transcripts derived from pMMT1B, pMMT1C and pMMT1D, respectively. A) Indicates the expression from the P2 promoter and B) Indicates the expression from the P1 promoter. Sites of transcription initiation are highlighted by an asterisk and the nucleotide at that position on the coding strand is indicated. The sequence for both of the P1 and P2 promoters is located in Figure 2.1C. The brightness/contrast of the lanes showing results of the primer extension reactions and sequencing reactions were differentially enhanced using Adobe Photoshop.

Examination of the putative P1 -33, -7, and upstream sequences. Various substitutions were made in the *Bacteroides* P1 promoter to examine their effect on the promoter strength. Luciferase assays (Figure 2.6) showed that crude cell extracts from cells that contained the P1 promoter (pMMT1C) had approximately 550-fold higher relative light units than the promoterless control (pMMT1). The activity levels of the other promoters as compared to the wt P1 (percentage of wt activity) are: 11.2% for the -33 substituted, 4.0% for the P1 promoter no upstream sequence, 1.8% for the -7 upstream substituted region, and 0.44% for the -7 downstream substituted region (pMMT1K, pMMT1L, pMMT1J, and pMMT1I respectively). These results indicate that the -7 upstream and downstream sequences are both equally important to the functionality of this promoter more so than the -33 region. It also showed that the upstream sequence of this P1 promoter, is important to its functionality.

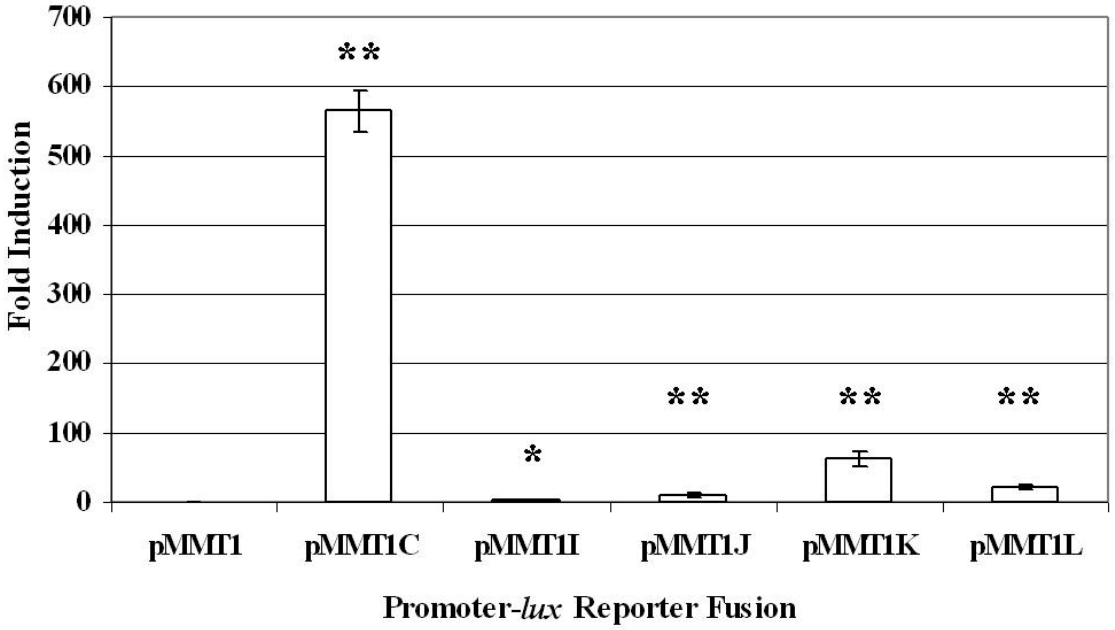

Figure 2.6. Luciferase assays of crude cell extracts of *B. thetaiotaomicron* 4001 containing plasmid constructs of interest. The graph from left to right: pMMT1, promoterless negative control; pMMT1C, wild type P1 16S *Bacteroides* rRNA promoter construct; pMMT1I, substituted -7 downstream; pMMT1J, substituted -7 upstream sequence; pMMT1K, -33 substituted sequence; and pMMT1L no upstream sequence of the P1 16S *Bacteroides* rRNA promoter. Data is based on fold induction as compared to the expression levels of pMMT1. Average RLU for the promoterless control pMMT1 was 0.047 from the three independent trials performed in replicates of six. Statistically significant from promoterless control, using the Student's unpaired *t*-test (two-tailed); *, $p < 0.008$; **, $p<0.0001$.

Discussion

At the present time there are barriers that impede successful expression of foreign bacterial genes in *Bacteroides* that are not fully understood. To date, most of the studies in the area of gene expression of *Bacteroides* have focused on metabolic reporter systems. The four reporter systems that have previously been used in *Bacteroides* make use of β-glucuronidase (GUS) (93), xylosidase/arabinosidase (326), chloramphenicol acetyltransferase (18, 282), and catechol 2,3-dioxygenase (XylE) (53, 166).

The new approach that we have used here involves the luminescence or *lux* operon, as a tool for evaluating levels of gene expression in *Bacteroides*. There is only one other study that has focused on using bioluminescence as a reporter system for an anaerobic bacterium. This study was performed on another human pathogen, *Clostridium perfringens*, using the *lux* operon from the marine bacterium *Vibrio fischeri* (239). The problem with using the *V. fischeri lux* operon for studies in *Bacteroides* is that the Lux proteins are not fully active at temperatures above 30^{o}C, while *Bacteroides* has an optimal growth temperature of 37^{o}C. To overcome this problem the Lux system from *Photorhabdus luminescens*, which functions up to 45^{o}C (289), was used for this study. Light output may be measured quantitatively over several orders of magnitude, making it an ideal reporter, but only under aerobic conditions.

Although *Bacteroides* are anaerobes, they are aerotolerant, capable of surviving in an oxygenated environment for a period of time, but are incapable of maintaining the activities of key enzymes necessary for energy production via central metabolism (236). Therefore, whole cell luminescence assays were found to be feasible on a limited time frame, where sufficient energy/reduced coenzymes were available to produce the substrates $FMNH_2$ and decanal

necessary for luciferase activity. This problem was eliminated by adding the substrates exogenously during *in vitro* luciferase assays performed on crude cell extracts as described above. These reporters should be useful to examine promoter activity and gene expression in other species of *Bacteroides*, *Cytophaga*, and *Flavobacterium*. This study focused on the analysis of a *Bacteroides* 16S rRNA promoter.

Analysis of the sequence of the *Bacteroides* 16S rRNA promoter region amplified via inverse PCR reveals the same overall rRNA operon promoter structure as in *E. coli*. There are two possible *B. thetaiotaomicron* 16S rRNA promoters, both containing the -7 and -33 *Bacteroides* consensus sequences, and are approximately 150 bp apart. Overall, there are five rRNA operons in *B. thetaiotaomicron* (333), four of which are 94% to 98% identical in sequence. In those four, both P1 and P2 promoters are 100% conserved. The fifth rRNA operon is 88% identical to this sequence and only contains putative P2.

The results from the luciferase assays showed that *B. thetaiotaomicron* 16S rRNA P1 and P2 promoters were each capable of initiating gene expression in the absence of each other and that the full-length 16S rRNA promoter was able to initiate gene expression in the absence of any sequences upstream of the P1 promoter (Figure 2.4). Estrem, *et. al.* (86) identified a consensus UP element sequence in *E. coli* by using *in vitro* selection for upstream sequences that promote rapid RNAP binding to the *E. coli rrnB* P1 promoter, followed by *in vivo* screening for high promoter activity. The consensus is: nnAAAWWTWTTTTnnnAAAnnn (W = A or T). UP elements are not as highly conserved as the -10 and -35 elements. Their effects on promoter activity differ, correlating generally with the degree of similarity to the UP element consensus sequence (258). A+T rich upstream sequences were also identified in compilations of *Bacillus*

subtilis (119) and *Clostridium* promoters (115). Even though there is no sequence upstream from *B. thetaiotaomicron* 16S rRNA P1 and P2 similar to the *E. coli* consensus UP element sequence, there appears to be A+T rich regions. It has been demonstrated that sequences out to -54 of certain regulated *B. fragilis* promoters are required for optimal activity (18), but this may reflect a requirement for activator binding. Our results showed that the upstream portion of the P1 promoter is directly involved in the activity of this promoter (Figure 2.6).

The Fis protein can bind DNA in a non-specific manner as well as at specific sites, making determination of a clear consensus sequence difficult (21). The consensus is: GntyRaWWWtYranCn (R = A or G, Y = C or T, W = A or T, and lower case letters represent less well conserved bases (96). Fis binding sites could be present upstream of the *B. thetaiotaomicron* rRNA operon, but due to a weak consensus, further studies will need to be done to determine if they are present. However, sequence analysis of the published *B. thetaiotaomicron* genome sequence (333) reveals no obvious Fis homologs based on amino acid sequence alignments (N. Shoemaker, personal communication). This suggests that Fis may not play a role in regulation of 16S rRNA promoters in *Bacteroides* species.

A comparison of the P1 16S rRNA promoter variants to that of the *B. fragilis cepA* promoter in the study by Bayley, *et. al.* (18) confirmed the role of the -7 and -33 consensus sequences. With respect to the -7 downstream region both the *cepA* and P1 substitutions show a lack of expression when this region, has been changed ((18), Figure 2.6). The P1 promoter with no upstream sequence and -33 substituted sequence help confirm that the -33 region and the bases upstream of it are both important to the overall expression of the promoter. However, it seems that the upstream sequence has a larger impact on the activity of the promoter than the -33

region (Figure 2.6). These results also agree with previous studies using the *cepA* promoter (18). One discrepancy was seen in comparing the results from the -7 upstream region of the P1 promoter to that of the *cepA* promoter. We observed that the substitution of these two bases, resulted in a 1.8% activity as compared to wt P1 as opposed to the results that were seen using the *cepA* promoter, where a 38% activity was observed (18). The only rationale for this result is that possibly the -7 upstream region for the P1 promoter is critical to the activity of this promoter, and not others, since the promoters examined by Bayley, *et. al.* (18) do not all share the consensus -7 upstream region.

Overall, the results of this study expand on the work of others (18, 308, 309) to provide additional insights into the requirements for transcription initiation in *Bacteroides*. Characterization of the P1 and P2 promoters, and P1 promoter variants confirm the importance of the -33 and -7 sites for promoter activity *in vivo*. In *E. coli*, promoter recognition is the limiting factor in expressing *Bacteroides* genes (Figure 2.3A and B). However, in *Bacteroides*, it seems that translation and transcription work together to create a barrier to efficient gene expression of foreign genes (Figure 2.3C and D). Furthermore, it appears that the RBS sequence and spacing recognition by the *Bacteroides* ribosome is more selective than in *E. coli*. Both of the *lux* reporters developed in this study could be used for further investigation of promoters in organisms related to *Bacteroides* such as: *Flavobacterium* or *Cytophagae*.

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Chapter Three

Synergy in Polymicrobial Infections in a Mouse Model of Type 2 Diabetes

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Supplemental material is located in Appendix II and at http://iai.asm.org/.

Abstract

Human diabetics frequently suffer delayed wound healing, increased susceptibility to localized and systemic infections, and limb amputations as a consequence of the disease. Lower limb infections in diabetic patients are most often polymicrobial, involving mixtures of aerobic, facultative anaerobic, and anaerobic bacteria. The purpose of this study is to determine if these organisms contribute to synergy in polymicrobial infections using diabetic mice as an *in vivo* model. The model was the obese diabetic mouse BKS.Cg-*m* +/+ *Lepr*^{db}/J, a model of human type 2 diabetes. Young (5- to 6-week-old) pre-diabetic mice and aged (23- to 24-week-old) diabetic mice were compared. The diabetic mice were injected subcutaneously with mixed cultures containing *Escherichia coli*, *Bacteroides fragilis*, and *Clostridium perfringens*. Progression of the infection (usually abscess formation) was monitored by examining mice for bacterial populations and numbers of white blood cells at 1, 8, and 22 days post-infection. Synergy in the mixed infections was defined as a statistically significant increase in the number of bacteria at the site of injection when co-infected with a second bacterium, compared to when the bacterium was inoculated alone. *E. coli* provided strong synergy to *B. fragilis*, but not *C. perfringens*. *C. perfringens* and *B. fragilis* provided moderate synergy to each other, but only in young mice. *B. fragilis* was anergistic (antagonistic) to *E. coli* in co-infections in young mice at 22 days post-infection. When aged–matched non-diabetic mice (C57BLKS/J) were used as controls, the diabetic mice exhibited 5 to 35 times the number of CFU as did the non-diabetic mice, indicating diabetes was a significant factor in the severity of the polymicrobial infections.

Introduction

The Centers for Disease Control (CDC) estimates that 18.2 million people in the United States, 6.3% of the population, have diabetes (51). Lower-limb amputations are a common and severe side effect of diabetes. This is shown in statistics from the CDC: more than 60% of nontraumatic lower-limb amputations in the United States occur among people with diabetes. From 2001 to 2002, about 82,000 nontraumatic lower-limb amputations were performed each year among people with diabetes (51). Bacterial infections account for ~85% of circumstances that require lower-limb amputations in diabetic patients (6).

Diabetes-associated manifestations that contribute to the increased susceptibility to infections include peripheral vascular disease (with accompanying ischemia), neuropathy, and a dysfunctional immune system (169). These syndromes lead to the development of foot ulcers, which then become infected (169). The infections are most often polymicrobial, with mixtures of aerobes, facultative anaerobes, and obligate anaerobes (267, 268). These polymicrobial infections are difficult to cure, since the bacterial species present often have a wide variety of natural and acquired resistance to antibiotics (108). Broad-spectrum antibiotics delivered intravenously, surgical debridement, and limb amputation are the methods most often used to treat the infected diabetic limb (6).

The pathogenic bacteria present in polymicrobial infections exhibit synergistic effects in their ability to cause infections (39). Synergy was originally defined as a significant increase in the number of bacteria in a wound when co-infected with a second bacterium, compared to when the bacterium was inoculated alone. This definition was modified over time, to define synergy as the cooperative interaction of two or more bacterial species that produces a result not achieved

by the individual bacterium acting alone (25). Synergy has been demonstrated in experiments where the formation of an abscess in a mouse or rat was used as the *in vivo* assay model (39, 260). For example, *Bacteroides fragilis* was synergistic for the facultative anaerobe *Escherichia coli* (38) and the obligate anaerobe *Clostridium perfringens* (42). The presence of *E. coli* was also synergistic for *C. perfringens* and vice versa (42).

Based on clinical and experimental results in animal models, it has been hypothesized that different types of bacteria promote synergy within polymicrobial infections via different mechanisms (260). Aerobes and facultative anaerobes (e.g., *E. coli*) are thought to enhance the ability of anaerobes to grow by lowering the ambient oxygen concentration and redox potential of the infected tissue (260). Encapsulated bacteria, such as the anaerobe *B. fragilis*, act as antiphagocytic and proinflammatory agents, increase the likelihood of abscess formation (38), and protect other organisms from being phagocytosed (132). Others, such as the anaerobe *C. perfringens*, are thought to contribute to synergy by secreting powerful cytotoxins that disrupt the immune system (260).

Diabetic mice have been used to study the effects of diabetes on periodontitis (99), urinary tract infections (225), septicemia (160), and lung infections (224). Bessman et al. (20) used a strain of mice, C57B1.Ks-J-*db*-*m* (now named BKS.Cg-*m* +/+ *Lepr*^{db}/J), which is homozygous for the diabetes spontaneous mutation in the leptin receptor and is a model for type 2 diabetes (see reference (54) and http://jaxmice.jax.org/jaxmice-cgi/jaxmicedb.cgi?Objtype=pricedetail&stock=000642 for a description of the strain) to examine abscess formation and bacterial load after subcutaneous inoculation of *E. coli*, *B. fragilis*, and enterococcus. In the study by Bessman et al., 9- to 11-week-old BKS.Cg-*m* +/+ *Lepr*^{db}/J and their non-diabetic

littermates were infected with each possible two-organism combination of *E. coli*, *B. fragilis*, and enterococcus (20). Bessman et al. demonstrated that abscesses in the diabetic mice were more persistent than and harbored a higher number of CFU than abscesses induced in nondiabetic mice (20). We used the same strain of mice, BKS.Cg-*m* +/+ *Lepr*^{db}/J, and the pathogens, *E. coli*, *B. fragilis* and *C. perfringens* in this study to ask two questions: (i) Do these bacteria exhibit synergy towards each other in an abscess model? (ii) Does long-term exposure to the symptoms of diabetes predispose the mice towards lower resistance to controlling the growth and persistence of these bacteria in an abscess model? We used multiple combinations of bacteria to infect young prediabetic mice (5- to 6-weeks-old) and aged diabetic mice (23- to 24-weeks-old) in an abscess model and found that there are significant differences in synergistic pattern and susceptibility to the infection between the young and aged diabetic mice.

Materials and Methods

Bacterial strains and growth media. Three strains of bacteria were used in this study. *E. coli* strain 360A was obtained from S. Finegold (Veterans Administration Medical Center, Los Angeles, CA), and is a clinical isolate taken from the leg ulcer of a male diabetic patient. Strain 360A was sent to the Pennsylvania State University Gastroenteric Disease Center, Wiley Laboratory, Orchard Road, University Park, PA for serotyping and genotyping. The results are shown in Table 3.1. The strain has O type 6, a non-typeable H antigen, as well as the gene encoding cytotoxic necrotizing factor 1 (CNF-1). Also, we detected hemolysis on blood agar plates with strain 360A and the *hlyA* gene, encoding hemolysin HlyA, was detected using PCR methods (data not shown). Preparations of cell suspensions in India ink showed the presence of a capsule around strain 360A (Chapter Four, Figure 4.3), but this was a non-K1 type capsule (Table 3.1). *B. fragilis* NCTC9343 is the type strain for *B. fragilis*, which was originally isolated from an appendix abscess from a human patient (143). *C. perfringens* strain 13 was isolated from a human gangrene infection (190).

E. coli cultures were grown aerobically at 37^{o}C in Luria-Bertani broth (LB). The anaerobes *B. fragilis* and *C. perfringens* were grown at 37°C in a Coy anaerobic chamber (Coy Laboratory Products., Grass Lake, MI), *C. perfringens* in peptone glucose yeast extract medium (203) and *B. fragilis* in trypticase yeast extract glucose medium (TYG), which contained, per liter: 10 g trypticase, 5 g yeast extract, 2 g glucose, 0.04 g vitamin K (menadione), 0.004 g of hemin, 0.001 g of resazurin, 0.004 g of $FeSO_4.7\ H_2O$, 0.5 g cystine (free base), 0.02 g of $MgSO_4.7H_2O$, 0.4 g $NaHCO_3$, 0.08 g NaCl, 0.008 g $CaCl_2$, 0.1 M $KHPO_4$ (pH 7.2).

Table 3.1. Serotyping and genotyping results for the *E. coli* strain (360A) used in this study.[a]

Strain	O Type	H Type	STa	STb	LT	SLT1	SLT2	**CNF1**	CNF2	EAE	K1	BFP	**HlyA**
360A	6	+	-	-	-	-	-	+	-	-	-	-	+

[a]STa and STb, heat stable toxin a and b; LT, heat labile toxin; SLT1 and SLT2, Shiga-like toxins 1 and 2; EAE, intimin; BFP, bundle forming pili.

Diabetic and non-diabetic control mice. Three- to four-week-old female mice of strain BKS.Cg-*m* +/+ *Lepr*^{db}/J were obtained from The Jackson Laboratories (Bar Harbor, Maine). Mice were quarantined for a week in isolator cages with filter tops. Two groups of mice were used in this study: (i) young (5- to 6-week-old) pre-diabetic mice and (ii) aged (23- to 24-week-old) diabetic mice. Mice were randomly assigned to one age group and were housed in cages of two to three mice until the appropriate age was attained. Mouse feed necessary to maintain the health of the diabetic mice was provided ad libitum. On a bi-weekly basis, the mice were weighed and blood glucose levels were determined for the assessment of hyperglycemia using a handheld glucometer. The mean values of mass and blood glucose levels for all of the mice used in this study are shown in Fig. 3.1. A glucose level of >200 mg/dl indicated hyperglycemia and this occurred between the ages of 5 and 6 weeks for these mice.

Non-diabetic C57BLKS/J, the parent strain of BKS.Cg-*m* +/+ *Lepr*^{db}/J, were obtained from Jackson Labs at either 4 to 5 weeks of age or 22 to 23 weeks of age. The older mice were from a group of retired breeders. The mice were maintained on the same diet as the diabetic mice and used as age-matched controls for the polymicrobial infections in a manner identical to that of the diabetic mice. These mice did not show signs of hyperglycemia or obesity (Fig. 3.1).

Polymicrobial infections in mice. Bacterial cultures were grown overnight and then subcultured the following day into fresh media appropriate for each bacterium. Three milliliters of each culture was pelleted and washed three times with phosphate-buffered saline (PBS) to remove residual medium and toxins. Bacterial suspensions were diluted in PBS to give a final concentration of 2 x 10^6 to 3 x 10^7 CFU/ml (as determined by serial dilutions and plating of the PBS suspensions of bacteria). The polymicrobial infections for the young and aged diabetic

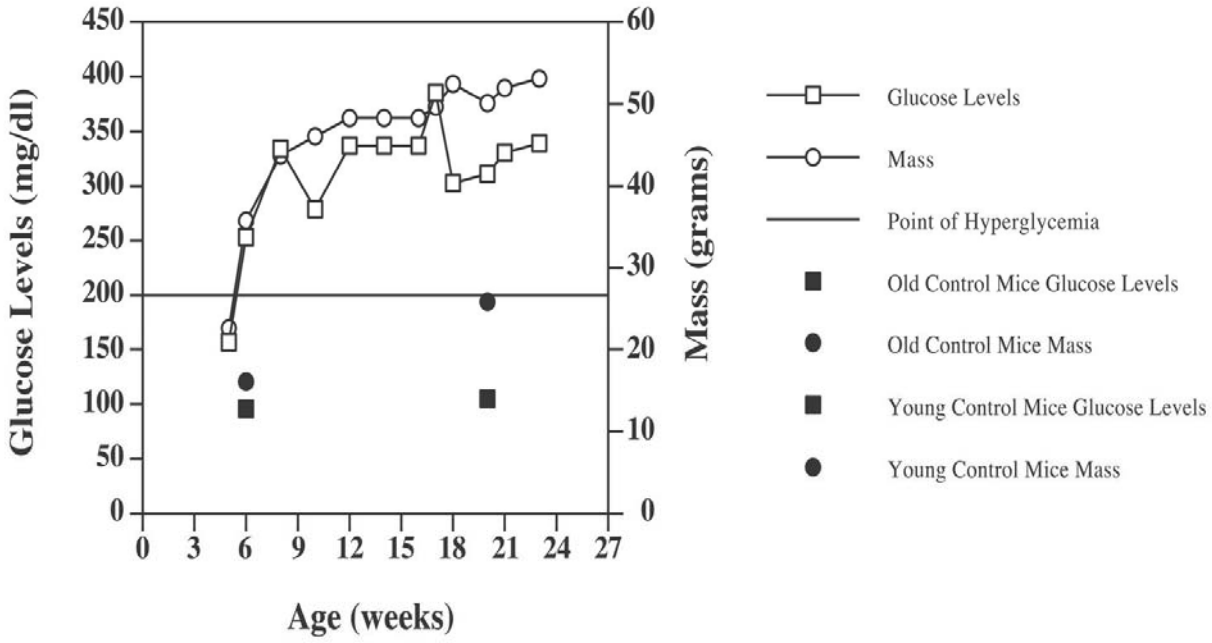

Figure 3.1. **Graph showing the mean values of serum glucose levels (open squares) and mass (open circles) with increasing age in diabetic BKS.Cg-*m* +/+ *Lepr*^{db}/J mice.** Also shown are the serum glucose levels (filled squares) and mass (filled circles) of the young and aged nondiabetic C57BLKS/J mice. Blood glucose levels above 200 mg/dl (horizontal bar) were considered evidence of hyperglycemia.

mice were separated into eight experimental groups, as shown in Table 3.2. One hundred-microliter samples of each bacterial suspension (2×10^5- 3×10^6 CFU), singly or in combination, were injected subcutaneously into the inner thighs of the mice, as previously described (41). Each experimental group (Table 3.2) was comprised of 24 mice. At 1, 8, and 22 days post-injection, eight mice from each experimental group were euthanized by cervical dislocation following the induction of deep anesthesia with 100% CO_2 gas. Six mice were used for determination of the number of CFU in the injection area; the remaining two mice were used to assess the pathology of the injection site. Blood from all mice was removed from the caudal vena cava and/or directly from the heart and placed in blood vials containing EDTA. These samples were sent to a reference laboratory (Ani Lytics, Inc., Gaithersburg, MD) for determining complete blood cell counts.

From six of the mice, the tissue surrounding the area at the site of injection was excised and the spleens removed. Each sample was homogenized using a tissue homogenizer and resuspended in a final volume of 1 ml of PBS. Appropriate dilutions were plated on a selective medium specific for growth of each of the three organisms used. *E. coli*-containing samples were plated on Eosin Methylene Blue (EMB) agar (Becton-Dickinson and Company, Cockeysville, MD) and incubated aerobically at 37^{o}C overnight. *B. fragilis*-containing samples were plated on *Bacteroides* Bile Esculin (BBE) agar (Becton-Dickinson Microbiology Systems, Cockeysville, MD) and incubated anaerobically at 37^{o}C for approximately 2 days. *C. perfringens*-containing samples were plated on either Tryptose-sulfate cycloserine (TSC) agar base (EM Science, Gibbstown, NJ) or Shahidi Ferguson Perfringens (SFP) agar base (Becton-Dickinson and Company, Sparks, MD) and incubated anaerobically at 44 ^{o}C for 8 to 12 h.

Table 3.2. Combinations of bacteria used to infect the diabetic mice.[a]

Bacterium	Injection combination for experimental group no.[c]							Control[b]
	1	2	3	4	5	6	7	
E. coli	X			X	X		X	
B. fragilis		X		X		X	X	
C. perfringens			X		X	X	X	

[a]The same pattern was used for both the young (5- to 6-week-old) and aged (23- to 24-week-old) mice.
[b]The control group was injected only with PBS.
[c]An X indicates that the bacterium was injected.

The remaining two mice were necropsied and the area of injection and spleens were examined for pathological changes in the infection. Five-micron-thick sections of the abscess area were stained with hematoxylin and eosin and mounted on microscope slides.

Experimental protocols involving mice were examined and approved by the Virginia Tech Institutional Animal Care and Use Committee.

Statistical methods. For CFU counts of each species (*E. coli*, *B. fragilis*, and *C. perfringens*) using the diabetic mice alone (Table 3.3), the SAS System (version 8.2, SAS Institute Inc., Cary, NC) was used to perform analysis of variance (ANOVA) to test for the main effects of age and challenge with each of the other two organisms as well as all of their two- and three-way interactions. All CFU data was log-transformed before analysis to stabilize variances. Model adequacy was assessed using standardized residual plots. Other statistical analyses were done using Instat 3 software (Graphpad, Inc.). For all statistical analyses, P values of <0.05 were considered significant.

Results

Polymicrobial infections in young (prediabetic) mice. Mice were infected with the combinations of bacteria (or PBS for the control mice) listed in Table 3.2. At 1, 8, and 22 days post-infection, the abscesses (or infected area if no abscess was present) were excised and the number of CFU determined for each bacterium. We chose these time points to represent early, middle, and late stages of abscess formation. The infected mice formed abscesses in 2 to 3 days. The abscesses then matured in one of two ways during the final 19 days they were monitored: either they were retained by the mice or they penetrated the skin and drained. The mean numbers of CFU in the abscesses formed in the young (5- to 6-week-old) mice are shown in Fig. 3.2. Some mice succumbed to systemic infections, and all of these cases involved mice infected with *E. coli*. In experimental groups 1, 5, and 7, 12%, 4% and 39% of the mice died, respectively, almost always by 2 to 3 days post-infection (Table AII.1). Apparently, the injection of all three bacteria together leads to the highest rate of mortality. *E. coli*-dependent lethal systemic infections have been previously reported for this strain of mouse (20).

With single infections, the *E. coli* strain exhibited the highest number of CFU over the course of each experiment (Fig. 3.2). The *B. fragilis* strain that was used, NCTC9343, has been shown to cause intra-abdominal abscesses in a mouse model when 1 X 10^6 CFU number of CFU were injected intraperitoneally along with sterile rat fecal contents (63, 64). This strain did not establish visible abscesses when injected subcutaneously and by itself at the dosage used in these experiments (1 X 10^6 CFU) in the absence of sterile rat fecal contents (data not shown). However, this was a deliberate part of our experimental design in which we wanted to observe synergistic effects between the bacteria in the absence of adjuvants such as the sterile rate fecal

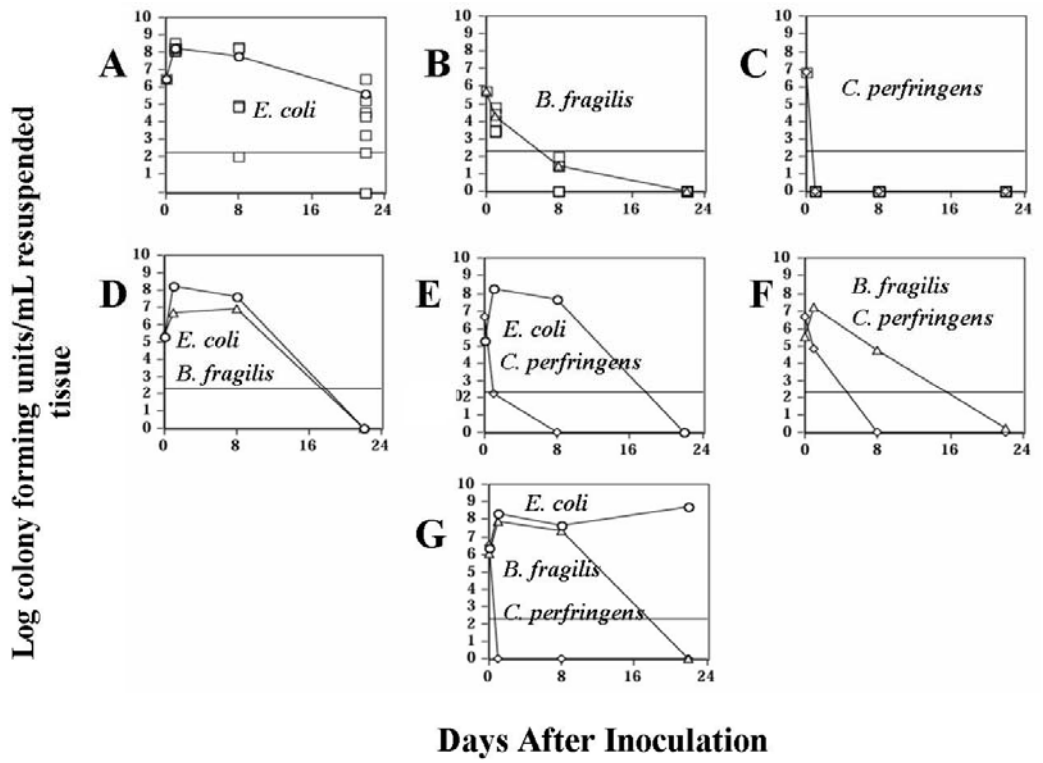

Figure 3.2. Number of CFU isolated from abscesses in young (5- to 6-week-old) prediabetic BKS.Cg-*m* +/+ *Lepr*^{db}/J mice. One hundred-microliter samples of bacterial suspensions (2 x 10^5 to 3 x 10^6 CFU), singly or in combination, were injected subcutaneously into the inner thighs of the mice, as described in Materials and Methods. Control mice (data not shown) were injected with PBS alone. Panels A to G correspond to the infection protocols listed in Table 4.2 as experimental groups 1 to 7, respectively. For panels A to C, the numbers of CFU for individual mice (squares) are shown along with the mean values for *E. coli* (panel A, circles), *B. fragilis* (panel B, triangles), and *C. perfringens* (panel C, diamonds). In panels D to G, only the mean values are shown, with the same symbols used in panels A to C. The horizontal lines represent the lower limit of detection; for some panels levels less than the lower limit are shown to illustrate the mean calculated values. *B. frag*, *B. fragilis*; *C. per*, *C. perfringens*.

contents. High numbers of CFU in the abscesses formed by injecting the single bacteria would have made synergistic effects difficult to observe.

With the experiments in which multiple bacteria were injected, distinct synergy was observed. In particular, *E. coli* was synergistic towards *B. fragilis* in the experiments where they were co-injected, providing about a 6 log increase in *B. fragilis* CFU at 8 days post-infection (Fig. 3.2, compare panels B, D, and G). *B. fragilis* provided a moderate level of synergy to *C. perfringens* but at day 1 only, while *C. perfringens* provided moderate synergy to *B. fragilis* at days 1 and 8 (Fig. 3.2, compare panels B, C, and F).

Young mice infected with *E. coli* exhibited an unusual effect at 22 days post-infection where the presence of *B. fragilis* and/or *C. perfringens* led to a decrease in the number of CFU of *E. coli* (Fig. 3.2, compare the 22 day time points in panel A to those in D and E). This negative synergy, or "anergy" was seen only in the young mice late in the infection.

Polymicrobial infections in aged (long term diabetic) mice. The mean numbers of CFU in the abscesses formed in the aged (23- to 24-week-old) diabetic mice are shown in Fig. 3.3. The formation and progression of abscesses were difficult to measure in these mice due to the presence of thick layers of subcutaneous adipose tissue, which masked the swelling seen in abscesses formed in the young mice. However, no abscesses were seen to drain through the skin, suggesting that the abscesses (if formed) in the aged mice did not migrate to the skin surface as they did in the young mice.

Some of the aged mice died 2 to 3 days post-infection due to systemic infection and all of these deaths involved mice infected with *E. coli*. In experimental groups 1, 5, and 7, 7%, 15% and 14% of the mice died, respectively (Table AII.1). For the single infections, *E. coli* was

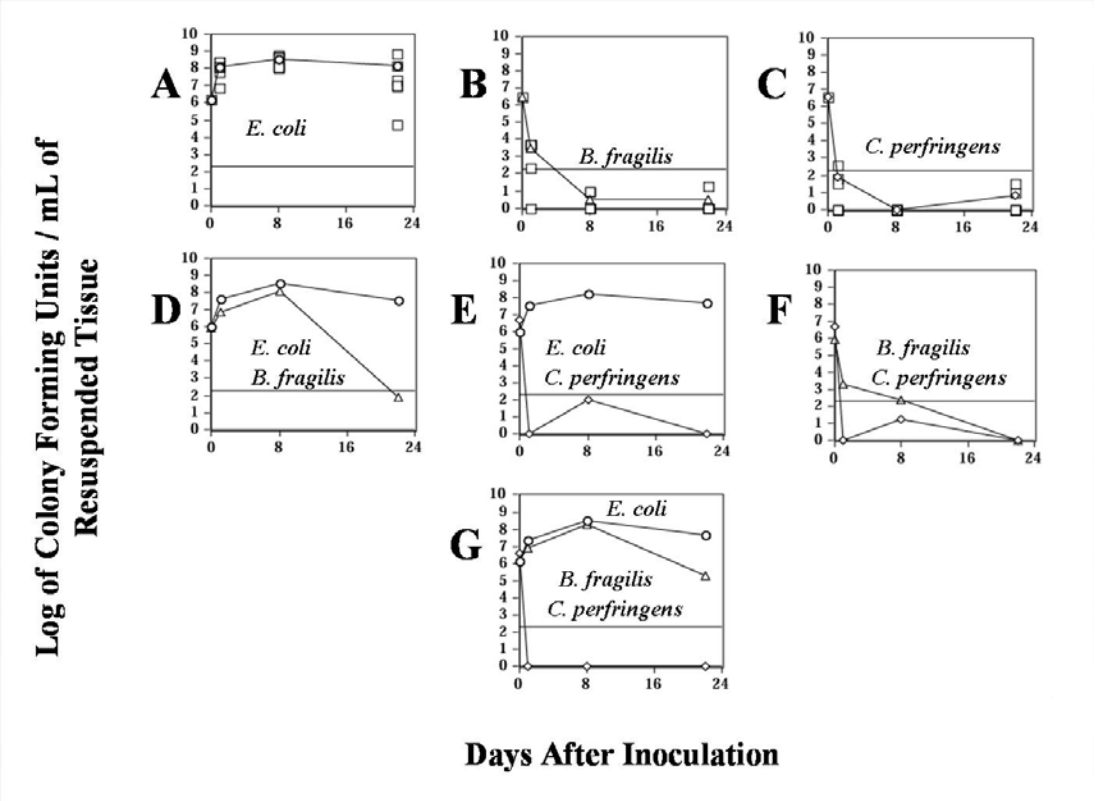

Figure 3.3. Number of CFU isolated from abscesses in aged (23- to 24-week-old) diabetic BKS.Cg-*m* +/+ *Lepr*^{db}/J mice. The symbols used are described in the legend for Fig. 3.2.

found at much higher levels than were *B. fragilis* or *C. perfringens* over the course of the experiments. The aged mice infected with *E. coli* alone exhibited higher numbers of CFU at 8 and 22 days post-infection than did the young mice (Fig. 3.2A and 3.3A; Table 3.3). For the mixed infections, the highest level of synergy was provided by *E. coli* to *B. fragilis* (Fig. 3.3 compare panels B, D, and G) at day 8, as was seen in the young mice. For the aged mice, however, there did not appear to be synergistic effects between *B. fragilis* and *C. perfringens*, as was observed with the young mice (Figs. 3.2 and 3.3, compare panels B, C, and F).

Synergy and age-dependent factors in the bacterial infections. To determine whether synergistic effects and/or age-dependent effects were responsible for changes in CFU for each bacterium during the course of the experiment, all of the CFU results were compared using ANOVA. For each bacterium, the results at each day post-infection (i.e., 1, 8, or 22) were analyzed to determine if age or the presence of other bacteria had a significant effect on the number of CFU we observed at that time. A summary of the results from this analysis are shown in Table 3.3. The complete statistical analyses are attached as supplemental material (http://iai.asm.org/). For *C. perfringens*, the large majority of samples from days 8 and 22 did not contain statistically significant numbers of CFU (Fig. 3.2 and 3.3), so comparisons with those data are less robust than with the rest of the experimental data. Therefore, statistical analyses from these times were not analyzed further. When age was tested as a variable, sometimes the aged mice had higher numbers of CFU than did the young mice and at other times the reverse was true. The direction of the age-dependent effects can be seen in the supplemental material by examining the sign (positive of negative) of the mean difference between each group.

In all cases except one, *E. coli* at day 1, age was a statistically significant source of variability for the number of CFU observed (Table 3.3). For *E. coli*, the number of CFU was affected only by *B. fragilis* at day 1, age at day 8 and all effects except (i) *B. fragilis* and (ii) age plus *C. perfringens* at day 22 (Table 3.3). *B. fragilis* was the bacterium most affected by synergistic and age-dependent effects, in particular at day 22 post-infection (Table 3.3). As described above, *B. fragilis* provided synergy in an age-dependent manner to *C. perfringens* at day 1 (Table 3.3) and received synergy from *C. perfringens* at all three times post-infection.

To determine if one of the species used could inhibit the growth of others in vitro, all possible pairs of bacteria were cross-streaked on TYG agar, incubated anaerobically at 37^{o}C, and the growth pattern at the streak junctions examined (Fig. AII.2). TYG agar was used because all three organisms exhibited normal growth rates in this medium. Examination of the cross streaks revealed that none of the bacteria tested showed any growth inhibition on the other species.

Diabetic mice have higher levels of bacteria in abscesses than comparably aged nondiabetic mice. Age was a common effect leading to changes in the number of CFU in the abscesses of diabetic mice (Table 3.3). However, the comparison between the young and the aged diabetic mice was actually composed of two separate variables: age and exposure to diabetes. To compensate for age-dependent factors, we tested 5- to 6-week-old and 23- to 24-week-old mice of strain C57BLKS/J, the parent strain of BKS.Cg-*m* +/+ *Lepr*^{db}/J. The C57BLKS/J were used instead of the heterozygous *Lepr*^{db}/+ mice because the heterozygotes exhibit increased metabolic efficiency and survive fasting longer than controls (http://jax mice.jax.org/jaxmice-cgi/jaxmicedb.cgi?objtype=pricedetail&stock=000642). The C57BLKS/J mice were infected with all 3 pathogens for 8 days, and the number of CFU in the injection area

Table 3.3. Factors affecting bacterial populations in polymicrobial infections.[a]

E. coli		**B. fragilis**		**C. perfringens**	
Day 1		**Day 1**		**Day 1**	
Effect	***P***	**Effect**	***P***	**Effect**	***P***
Age	0.2875	**Age**	**0.0007**	**Age**	**<0.0001**
Fragilis	**0.0352**	**Ecoli**	**<0.0001**	**Ecoli**	**0.0002**
Age*Fragilis	0.5910	**Age*Ecoli**	**<0.0001**	Age* Ecoli	0.1044
Perfring	0.3825	**Perfring**	**0.0123**	Fragilis	0.1044
Age* Perfring	0.1676	**Age* Perfring**	**0.0001**	**Age* Fragilis**	**0.0002**
Fragilis* Perfring	0.6595	Ecoli* Perfring	0.8645	**Ecoli* Fragilis**	**<0.0001**
Age* Fragilis* Perfring	0.5528	Age* Ecoli* Perfring	0.4973	**Age* Ecoli* Fragilis**	**<0.0001**
Day 8		**Day 8**		**Day 8**	
Effect	***P***	**Effect**	***P***	**Effect**	***P***
Age	**<0.0001**	**Age**	**0.0055**	Age	0.1064
Fragilis	0.8206	**Ecoli**	**<0.0001**	Ecoli	0.9333
Age*Fragilis	0.9802	**Age*Ecoli**	**0.0079**	Age* Ecoli	0.9333
Perfring	0.3966	**Perfring**	**0.0012**	Fragilis	0.9333
Age* Perfring	0.3188	Age* Perfring	0.4214	Age* Fragilis	0.9333
Fragilis* Perfring	0.1266	Ecoli* Perfring	0.6650	Ecoli* Fragilis	0.1064
Age* Fragilis* Perfring	0.2091	Age* Ecoli* Perfring	0.0548	Age* Ecoli* Fragilis	0.1064
Day 22		**Day 22**		**Day 22**	
Effect	***P***	**Effect**	***P***	**Effect**	***P***
Age	**<0.0001**	**Age**	**0.0003**	Age	0.1152
Fragilis	0.3407	**Ecoli**	**0.0013**	Ecoli	0.1152
Age*Fragilis	**0.0204**	**Age*Ecoli**	**0.0004**	Age* Ecoli	0.1152
Perfring	**0.0157**	**Perfring**	**0.0157**	Fragilis	0.1152
Age* Perfring	0.3970	**Age* Perfring**	**0.0387**	Age* Fragilis	0.1152
Fragilis* Perfring	**<0.0001**	**Ecoli* Perfring**	**0.0122**	Ecoli* Fragilis	0.1152
Age* Fragilis* Perfring	**<0.0001**	**Age* Ecoli* Perfring**	**0.0045**	Age* Ecoli* Fragilis	0.1152

[a]The data illustrated in Figs. 3.2 and 3.3 were analyzed by ANOVA. The results are listed as the probability a variable, shown under the "source" heading, had had a statistically significant effect on the number of CFU of each species of bacteria at the number of days post-infection that are indicated in the table. *P* values of < 0.05 were considered statistically significant. For example, for all mice in which *E. coli* was injected, there was not a statistically significant difference between the young and aged mice on Day 1 (i.e., $P > 0.05$). However, for all mice injected with *E. coli* there was a statistically significant difference on Day 1 if *B. fragilis* was present (i.e., $P < 0.05$). The *P* values that were considered significantly different are shown in bold.

were determined. These results were compared by ANOVA to those from the young and aged diabetic mice that had been infected in the same manner (see experimental group no. 7 in Table 3.2). There was no statistically significant difference between the young and aged mice within the control or diabetic groups, but there were differences between the control and diabetic mouse groups (Table 3.4). For *E. coli*, the young and aged diabetic mice had, on average, 5-fold- and 27-fold-higher levels of CFU, respectively, than did the comparably aged control mice (Table 3.4). For *B. fragilis*, the young and aged diabetic mice had, on average, 20-fold- and 35-fold-higher levels of CFU, respectively, than did the comparably aged control mice (Table 3.4). *C. perfringens* cells were not detected at a statistically significant level for any mice examined.

Inflammatory responses to polymicrobial infections. The systemic inflammatory responses to the bacterial infections were evaluated by measuring the white blood cell (WBC) counts in all mice. The WBC counts of the mice infected with the bacteria were compared to those of mock (PBS)-infected controls. For the mock infected young mice, the mean WBC counts were 5,700, 10,700, and 13,100 per mm^3 at 1, 8, and 22 days post-injection, respectively. For the aged mice, the mean WBC counts were 5,500, 3,500, and 3,400 per mm^3 at 1, 8, and 22 days post-injection, respectively. These values suggest that, for unknown reasons, the young mice exhibited a consistently higher level of inflammation during the course of the experiment than did the aged mice. The WBC from the infected mice were compared to those of the mock-infected mice by using ANOVA. There was a statistically significant difference between some of the age-matched and mock-infected groups at the corresponding post-infection times, and these are listed in Table 3.5. For *B. fragilis*- and *C. perfringens*-infected young mice, there was a significant decrease in the WBC counts in comparison to those for the mock-infected control

Table 3.4. Comparison of the number of CFU recovered from non-diabetic C57BLKS/J mice, the parent strain of the diabetic BKS.Cg-*m* +/+ *Lepr^{db}*/J mice[a].

		Mean no. of CFU (Range) recovered from:	
Mouse strain(s)	**Inoculum**	**5- to 6-wk-old mice**	**23- to 24-wk-old mice**
C57BLKS/J	***E. coli***	9.5×10^6 (6.0×10^2-5.6×10^7)	1.2×10^7 (2.6×10^4-5.2×10^7)
	B. fragilis	1.1×10^6 (0-6.2×10^6)	5.1×10^6 (1.5×10^2-3.6×10^7)
BKS.Cg-*m* +/+ *Lepr^{db}*/J	***E. coli***	4.5×10^7(2.1×10^5-1.6×10^8)	3.3×10^8(1.8×10^8-5.6×10^8)
	B. fragilis	2.2×10^7(1.4×10^6-1.1×10^8)	1.8×10^8(8.2×10^7-3.1×10^8)
		Ratio of mean CFU	
BKS.Cg-*m* +/+ *Lepr^{db}*/J and C57BLKS/J	***E. coli***	5	27
	B. fragilis	20	35

[a]For the non-diabetic mice, young or aged mice were infected with *E. coli*, *B. fragilis*, and *C. perfringens* in the abscess model. At 8 days post-infection, the number of CFU in the abscesses were determined. No significant levels of *C. perfringens* were recovered from either age group.
[b]The values for the diabetic BKS.Cg-*m* +/+ *Lepr^{db}*/J mice at 8 days post-infection for experimental group 7 (from Table 3.2) are also shown for comparison.

mice (Table 3.5). For the aged mice, five different infections led to a significant decrease in WBC counts, and in all cases the WBC counts increased when *E. coli* was part of the infectious group (Table 3.5). Therefore, the young and aged mice exhibited a clear difference in their responses to the bacterial challenge: the young mice exhibited a decreased inflammatory response, while the aged mice showed an increased inflammatory response.

The local inflammatory responses to the infections were measured using tissue sections prepared from the injection sites of 85 animals. This represented sampling of 33 young mice and 52 aged mice. Injection sites were evaluated blindly by a veterinary pathologist. Lesions were scored on a semiquantitative scale, noting incidence and severity (minimal-mild-moderate-marked-severe). No significant acute or chronic inflammatory lesions were noted in PBS-injected control mice. Infected mice showed formation of microabscesses or acute steatitis and cellulitis at injection sites as acute lesions, or developed mixed inflammatory cell lesions with fibrosis. In general, more fibrosis was seen with older lesions. Higher percentages of inflammatory lesions at injection sites were seen associated with *E. coli* infections and with mixed bacterial infections containing *E. coli* than other single or combination treatments. For several mice, granuloma formation was noted, with typical infiltrating populations of macrophages, epithelioid cell formation, and giant cells (Fig. 3.4).

Table 3.5. Infections which led to a statistically significant difference in total WBC count in infected mice compared to that of the mock (PBS)-infected control[a]

Mean WBC count							
Inoculum[b]	Mouse age (wk)	Days post-infection	Mock-infected Mice	Treated mice	*P* value	Mean difference	95% confidence level (range)
B. fragilis	5-6	8	10,700	5,100	<0.01	-5,600	-9,700 to -1,600
C. perfringens	5-6	22	13,100	5,000	<0.001	-8,100	-12,300 to -3,800
E. coli	23-24	8	3,500	8,800	<0.05	5,200	780 - 9,600
Ec-Bf-Cp	23-24	8	3,500	12,100	<0.001	8,600	4,000 - 13,200
E. coli	23-24	22	3,400	18,800	<0.001	15,400	7,900 - 22,900
Ec-Bf	23-24	22	3,400	10,300	<0.05	6,900	250 - 13,600
Ec-Bf-Cp	23-24	22	3,400	10,200	<0.05	6,800	150 - 13,500

[a]All other infections did not lead to a difference between the infected and mock-infected controls. The number of mice in each sample group ranged from five to eight. The statistical analysis was done using one-way ANOVA, with the Tukey-Kramer posttest.
[b]Ec, *E. coli*; Bf, *B. fragilis*; Cp, *C. perfringens*.

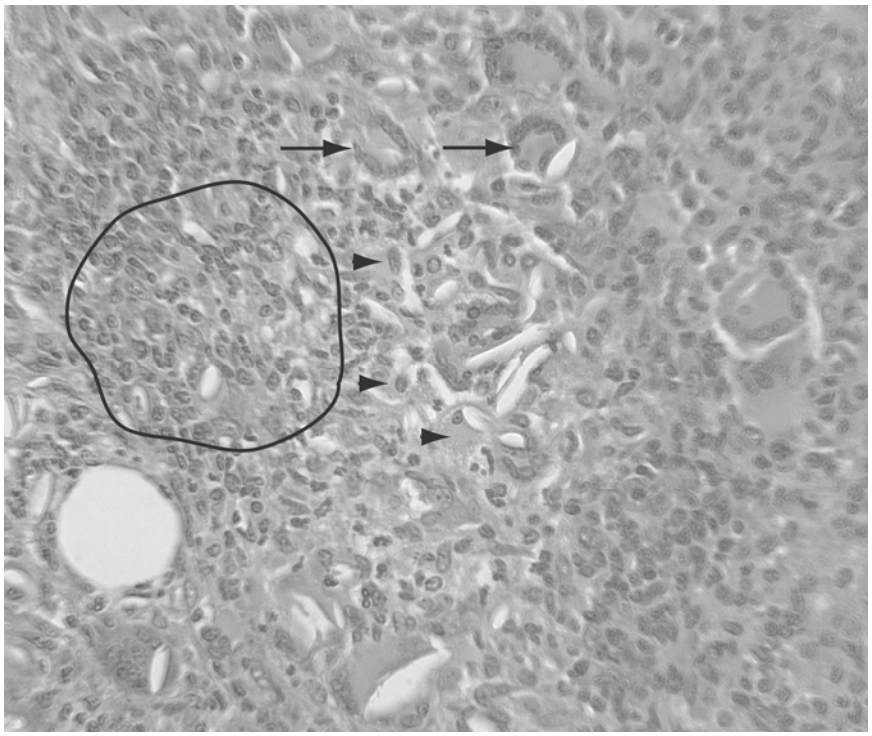

Figure 3.4. Representative image showing a lesion with granulomatous characteristics from an infected diabetic mice. A 5-µm-thick section from an abscess area of a BKS.Cg-*m* +/+ *Lepr*^{db}/J mouse was stained with hematoxylin and eosin. The young (5- to 6-week-old) mouse was infected with a combination of *E. coli* and *B. fragilis* and euthanized 22 days post-infection, when the area around the lesion was removed and processed to obtain the section shown. Prominent in the figure are giant cells (arrows), epithelioid cells (arrowheads), and high levels of infiltrating polymorphonuclear leukocytes, macrophages, and lymphoid cells (area inside the black boundary line).

Discussion

We undertook the present study to determine if *E. coli*, *B. fragilis* and *C. perfringens* exhibited synergy to each other in a mouse model for type 2 diabetes. We used two different age groups of mice to determine whether long-term exposure to the symptoms of diabetes affects the ability of diabetic mice to resist bacterial infections. The synergistic properties of the pathogens were different for young prediabetic mice and aged diabetic mice. This is reflected in the frequency with which age was a determining factor in the number of CFU isolated from most of the infections (Table 3.3).

Our results suggest that the highest level of synergy was provided by *E. coli* to *B. fragilis* (Figs. 3.2 and 3.3, Table 3.3). *B. fragilis* also received synergy from *C. perfringens*, but the effect was not as strong as that seen with *E. coli*. *B. fragilis* provided moderate levels of synergy to *C. perfringens*, but only in young mice at day 1 post-infection. It appeared that *B. fragilis* was the species that obtained the most benefit from the presence of the other bacteria, particularly in the young mice. This has been reported previously for abscess models using nondiabetic mice, and this ability may be a major factor in *B. fragilis* being identified at high frequencies in diabetic infections (38, 40, 42). While there are numerous reports of *B. fragilis* providing synergy to other organisms in cutaneous abscess models (38, 40, 306, 307), we did not observe this except in the case of *C. perfringens* in the young mice at day 1 post-infection (Fig. 3.2).

In young mice, the number of *E. coli* CFU at 22 days post-infection was reduced if *B. fragilis* and/or *C. perfringens* were also present. This effect has been observed in previous studies (38). We are calling this effect "anergy" to represent the effects that are opposite to those seen in synergistic interactions. This effect may be due to localized stimulation of the immune

system by the other pathogens earlier in the infection, since neither *B. fragilis* nor *C. perfringens* were detected in these 22-day-old abscesses (Fig. 3.2). *B. fragilis* produces a proinflammatory capsule, which has been shown to be essential for abscess formation in animal models (226, 298). Pathogenic strains can produce up to eight types of capsular polysaccharides (PS), termed PS A to PS H (166). Phase switching of these polysaccharides occurs by inversion of a DNA region containing promoters lying upstream of the capsule biosynthesis genes (166). While both purified PS A and PS B can induce abscesses in the absence of bacteria (298), a mutation in the loci encoding genes involved in the biosynthesis of PS A led to a greatly decreased level of abscess formation by live bacteria *in vivo* (64), but mutations in the PS B (63) and PS C loci did not (59). It is possible that the immune response to the *B. fragilis* capsule, while favoring the formation of an abscess and persistence of *B. fragilis* in the wound, inhibited the survival of *E. coli* in the abscess environment.

The molecular mechanisms for the strong synergistic effect *E. coli* provided to *B. fragilis* may be due to a combination of environmental modulation effects and production of toxins. The facultative anaerobe *E. coli* has been hypothesized to lower the ambient oxygen concentration and redox potential via aerobic respiration, allowing growth of obligate anaerobes (260). The respiratory chain in *E. coli* has two major terminal oxidases, cytochrome ***bo*$_3$** and cytochrome ***bd***, and a minor terminal oxidase, encoded by the genes *cyxAB*, that contributes only a small percent of the aerobic respiratory activity (106). Oxygen depletion through the use of these terminal oxidases may be an important factor for the growth of *B. fragilis* in the abscesses in which *E. coli* was present. This hypothesis is currently being tested in our laboratory by introduction of

mutations into the terminal oxidase-encoding genes and examination of synergy by the mutant *E. coli* strains.

Extraintestinal pathogenic *E. coli*, including the strain used in this study (360A), can produce a range of virulence factors, including the cytotoxins CNF1 and HlyA. CNF1 is cytotoxic due to its ability to deamidate glutamine residues in the small G proteins Rho, Rac and Cdc42 (98, 178, 271). Deamidation results in constitutive activity of these G proteins and the formation of pronounced morphological changes due to aberrant actin polymerization control (34). Also, CNF1 has been shown to reduce transmigration of polymorphonuclear leukocytes across an epithelial-cell layer and inhibit bacterial phagocytosis by leukocytes (43, 122, 123). These effects on phagocytes may be important in providing protection for both *E. coli* and *B. fragilis* in a coinfection model. HlyA is a membrane binding toxin that forms small pores in the cytoplasmic membrane of host cells, leading to cell death (204). HlyA not only lyse red blood cells but also can prove very cytotoxic to leukocytes (204). Therefore, HlyA may provide dual functions in promoting synergy: protecting *B. fragilis* from phagocytic cells and lysing red blood cells, which release hemoglobin. The hemoglobin can then act as a source of hemin, a necessary growth factor for *B. fragilis* (231). Based on results with nonisogenic strains in a mouse model, Ushijima, et al. (300) suggested that the production of hemolysin by *E. coli* might play a major role in the synergy during the formation of subcutaneous abscesses. The role that CNF1 and HlyA play in the contribution to synergy is currently under investigation.

The diabetic mouse model we have used in these studies, which is homozygous for the leptin receptor ($Lepr^{db}$ or *db/db*), has been described as having defects in T-cell-mediated functions but is hypersensitive to monocyte/macrophage stimulation (92). However, to our

knowledge, whether the age of the mice contributes to these immune effects has not been demonstrated. As measured by the number of WBC in the circulation in our experiments, the systemic immune response to the infections differed between the young and aged mice. The young mice had a consistently higher level of WBC even in the absence of a bacterial challenge. The observation that infections with *B. fragilis* and *C. perfringens* actually lowered the number of WBC at 8 and 22 days post-infection in the young mice may be due to recruitment of the host immune cells to the site of infection, thereby leading to lower levels in the general circulation, but this remains to be proven. The aged diabetic mice responded to *E. coli* infections by raising the number of CFU in a typical response to a bacterial challenge. The high mortality rates due to *E. coli* infections in the young mice suggests that the *E. coli* strain we used has the ability to cause a systemic infection, so the response of the aged mice would be that expected if the bacteria enter the bloodstream.

The local immune response to the infections also indicated that *E. coli* was responsible for eliciting the highest level of immune cell infiltration into the infected area. In fact, some of the abscesses formed granulomatous lesions, which indicates that a long-term infection state may become established in the abscesses formed in the diabetic mice. This would have consequences for infections of human diabetic patients, which have been shown to be chronic and last for months in some patients (6).

Acknowledgments

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Chapter Four

Characterization of the Extraintestinal Pathogenic *Escherichia coli* (ExPEC) strain 360A

Supplemental material is located in Appendices III through VI

Introduction

Escherichia coli is a facultative anaerobe that is the cause of an array of diseases from gastroenteritis to urinary tract infections. In order to establish a mouse model to examine bacterial synergy during a polymicrobial infection, we used pre-diabetic and diabetic mice, of the strain BKS.Cg-m+/+*Lepr*^{db}/J. These mice were injected with various combinations of *Escherichia coli* 360A, *Clostridium perfringens* strain 13, and *Bacteroides fragilis* NCTC 9343 and examined at 1, 8, and 22 days post infection (Chapter Three). Bacterial counts were performed on the area of infection and the spleens of these mice (Chapter Three and Appendix II). The results from these experiments showed that *E. coli* 360A exhibits synergy towards *B. fragilis* in both pre-diabetic and diabetic mice. This *E. coli* strain, 360A, is a clinical isolate that was obtained from a leg abscess of a male diabetic patient (Chapter Three and Table 4.1). In order to learn more about this strain, we wanted to examine its antimicrobial resistance profile and its possible virulence factors.

The detection of genes that encode environmental modulators and possible virulence factors in this strain would allow us to pick targets to examine in future experiments involving strain 360A. Our known targets are the terminal oxidases, cytochrome *o* and *d* oxidase, of *E. coli* 360A. *E. coli* possesses three terminal oxidases encoded by the *cydAB*, *cyoABCDE*, and the *cyxAB-appA* (also known as *appCBA*) operons (106). The terminal oxidases have been shown to protect cells from oxygen radicals such as hydrogen peroxide (181, 313). Mutants lacking a functional cytochrome *o* oxidase are more sensitive to hydrogen peroxide than mutants lacking either cytochrome *bd* I or *bd* II oxidases, by indirectly inducing catalase production (181). Knockouts of both the *cyo* and *cyd* operons are being sought in order to examine the role of their

gene products in synergism during polymicrobial infections, the formation of abscesses, and systemic infections. The terminal oxidases are predicted to contribute to synergy by decreasing the oxygen content of the surrounding tissue and thereby enabling the colonization by anaerobes (260). We used different molecular techniques, plate screens, and microscopy to observe and identify possible virulence factors and their associated genes. PCR was used to identify the presence of each of the oxidase operons as well as the two genes *hlyA* and *cnf1*. A wet mount using India ink was used to observe the presence of an exterior polysaccharide capsule via phase contrast microscopy. Serotyping and genotyping of this strain was done by the Gastroenteric Disease Center, The Pennsylvania State University.

Materials and Methods

Bacterial strains and growth conditions. All strains used for these experiments are listed in Table 4.1. *Escherichia coli* 360A is a clinical isolate from a diabetic patient (197). The two *E. coli* strains: DH5α (117) and DH10B (Gibco BRL, Gaithersburg, MD) were used for cloning purposes. All strains were grown at 37ºC with shaking in Luria-Bertani (LB) broth with the presence of appropriate antibiotics.

Characterization of the clinical isolate *E. coli* 360A. Metabolic testing was performed on several isolated colonies of *E. coli* 360A using an API 20E test strip from bioMérieux (Durham, NC) according to the manufacturer's instructions. (This was done in order to determine if the sample we received was a pure culture.) Also, sugar utilization tests were performed in test tubes that were provided by Laura Link (Microbiology Preproom Staff). A plasmid was purified from the strain to identify if any endogenous plasmids were present in the strain using a QIAquick Plasmid miniprep kit (Qiagen, INC., Valencia, CA) and then the samples were examined by gel electrophoresis using a 0.8% agarose gel. Assays were also done to test for the production of β-glucoronidase and siderophores; these were done by John Varga and Deric Learman, respectively.

Analysis of virulence determinants of *E.* coli 360A. A wet mount using India ink stain was used to assess the presence of an exterior polysaccharide capsule using phase-contrast microscopy. For this experiment, wild type *C. perfringens* strain 13 and a natural capsular mutant strain EMS1 were both used as an encapsulated and non-encapsulated control, respectively. A sheep's blood agar plate was used to test for the production of the hemolysin toxin, which is involved in the lysis of erythrocytes. Toxin, capsule typing, presence of fimbriae,

Table 4.1. Strains and plasmids used to characterize the clinical isolate *E. coli* 360A.

Strain or plasmid	Relevance to Study and Strain Genotype[a]	Reference
***E. coli* strains**		
360A	clinical isolate under investigation	(197)
JM109	used as a host strain for cloning, *endA1*, *glnV44*, *thi-1*, *relA1*, *gyrA96*, *recA1*, *mcrB*$^+$, Δ(*lac-proAB*), *e14-* [F' *traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*] *hsdR17*($r_K^-m_K^+$)	(335)
DH10B	used as a host strain for cloning, F$^-$, *endA1*, *recA1*, *galE15*, *galK16*, *nupG*, *rpsL*, *ΔlacX74*, Φ80*lacZΔM15*, *araD139*, Δ(*ara, leu*)7697, *mcrA*, λ$^-$, Δ(*mrr-hsdRMS-mcrBC*)	Gibco/BRL
Plasmids		
pGEM-T	AT cloning vector, Ap^R	Promega
pIGS2 / pIGS3	vector containing *igs* region for sequencing	This study
phlyA2 / phlyA3	vector used in sequencing 2.2 kb internal region of *hlyA*	This study
pMDM48	vector used in sequencing 3' end of *hlyA*	This study
pMDM49	vector used in sequencing 5' end of *hlyA*	This study
pMDM70	vector used in sequencing 5' end of *cnf1*	This study
pMDM71	vector used in sequencing 3' end of *cnf1*	This study
pMDM90	vector used in sequencing 2.2 kb internal fragment of *cnf1*	This study
pBluescript II SK +	used for determining the electocompetancy of strain 360A	(277)

[a]Ampicillin resistance (Ap^R)

and analysis for the presence of O and H antigens were also evaluated at The Gastroenteric Disease Center, The Pennsylvania State University (Wiley Laboratory, University Park, PA) via PCR and serum testing. Screening for the presence of several other genes, *hlyA*, *hbp*, *fimH*, and *papG*, was done via PCR. A detailed description of the PCR screening is described below.

Sequence analysis of target genes in *E. coli* 360A. Preliminary PCR reactions were needed to identify if each target gene (*hlyA* and *cnf1*) or operon (*cydAB* and *cyoABCDE*) was present in *E. coli* strain 360A. All primers are listed in Table 4.2. The primers targeting the oxidase operons were made by using known sequences of the *cydAB* (cydABF and cydABR) and *cyoABCDE* (cyoABF and cyoABR) operons from three *E. coli* strains: K12, CFT073 (a uropathogenic strain) and O157:H7 (a pathogenic gastrointestinal strain) found on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/sites/entrez ?db=pubmed). Degenerate primers, hlyAF and hlyAR, designed to detect the *hlyA* gene were designed based on partial homology between two known *E. coli hlyA* sequences from strains CFT073 and O157:H7. Alignment of the published sequences was done using The Biology WorkBench (http://workbench.sdsc.edu/). PCR reactions were carried out as follows: using 2.5 units of *Taq* DNA polymerase (New England Biolabs, Ipswich, MA) in a 100 µL reaction containing approximately 500 ng of *E. coli* 360A chromosomal DNA and 0.6 pmol of primers, in a 1X ThermoPol reaction buffer (New England Biolabs), 0.2 mM dNTPs, and 2 mM $MgSO_4$. The reactions were run under the following conditions: 94^{o}C for 2 minutes; 30 cycles of 94^{o}C for 1 minute, 60^{o}C for 2 minutes, and 72^{o}C for 2 minutes; and one cycle at 72^{o}C for 10 minutes. The amplification of *hlyA* was done with an annealing temperature of 48^{o}C and for the oxidase operons at an annealing temperature of 56^{o}C. The expected sizes of these PCR products are as

Table 4.2. Primers used in analysis of sequences of target genes/operons for chromosomal disruption of *E. coli* 360A.[a]

Primer Name	Primer Sequence (5' to 3')[b, c]
cydABF	CTGCTGGCCATTATGGAAACGGTCTACGTG
cydABR	CAGTAACACCAGGCGGTGTAGAGCAGAATG
cyoABF	CTGCGCTGTTAGATCCCAAAGGACAGATTG
cyoABR	CCCTGCCTTAGTAATCTCATCGAAATGCAG
hlyAF	CACCACAAYTWGACAAAYTWCTGC[a]
hlyAR	SATAAATATCATTACCAWAWCCACC[a]
Hly1	GTATTGAGTCACACCTGGGAGACGG
Hly3	CAGATAAAAAGACCTTATCATCCCC
Hly5	GGCTTCAGTATCTTCAGGTATTAG
Hly6	GGGACTAATTGCTAATGTCACTACAG
hlyCA1	CCGCGGGGTTCATTGACTGGATTGCTCCTTTCG
hlyCA2	TCTAGAGTATCTGCATCTGCATTGCTCAGAATG
hem3	AAGCTTCGTTATCTTTCAGGATATGGCCATC
hlyAB2	CTCGAGATGGCAGGGATAAACCAGGTAAAGTC
CNF1-A1	GCGGCTTACCCTTAAAGAACTTATTAAAG
CNF1-B1	CTGCCCTCATTATTTATAACGCTGC[b]
CNF1	GGTGAAGGGAAATTTGATACCAACTC
HLYD	GCGWAGYGTWATCAGCTATCTTCTTAGTCC[a]
CNF1F	CGACATGATTGCTAACCTCTCGCCAG
CNF1R	GCGATTTAAGGCGTTCATGGAG
IGS1	CCGCGGGAGGAAGAGGTAAGCTTCAGAATGG
IGS2	GGATCCGGTCGGGCCAGCAGGTTAAGTATTATCTG
Cnf1B2	GCAGCGTTATAAATAATGAGGGCAGG
cnf1Bam	GGATCCCAGACTCATCTTCACTCGGCTCAAC
CNF1-1	ATCGATCACTGGAGGAAGTCCAGGAGGGTAAACC
CNF1-2	CTCGAGCCTTACGACAACATTGCCGTCCACTCTCACC
cnf1I1	CCATGAACGCCTTAAATCGCAGTG
cnf1I2	TACCCTCCTGGACTTCCTCAAGTGC
cnf1down	GTTGTTGATTACGCCGTCGCTTTCCCGG
prsGF	CCTGTCAGGCTGTAATGATGCTCTGGCTG
prsGR	GGCAATATCATGAGCAGCGTTGCTG
HBPF	CTATTCTCTGCAGGAAGTCTTGC
HBPR	CTGCCCGATAAAATCCAGTGGAATAAC
T1PF	GGGCTGGTCGGTAAATGCCTGGTCATTC
T1PR	CGTAATTTGCCGTTAATCCCAGACTTACC

[a]All of the primers were designed for use in this study.
[b]Degenerate base descriptions: S = G or C, W = A or T, Y = C or T.
[c]These primers were adapted from Blanco, *et. al.* (28).

follows: *cyoAB*, 2600 bp; *cydAB*, 2900 bp; and *hlyA* 2200 bp and these were verified on a 0.8% agarose gel.

The *hlyA* PCR product was cloned into pGEM-T vector (Promega, Madison, WI). Transformants were selected for on LB containing ampicillin 100 μg/mL (Ap100), 5-bromo-4-chloro-3indolyl-β-D-galactosidase (X-Gal, 40 μg/mL), and isopropyl thio-β-D-galactoside (IPTG, 0.2 M). Clones were verified by *SacI* and *SphI* (New England Biolabs) digests and compared to the predicted restriction profile that was expected based on sequence analysis of the known *E. coli hlyA* sequences. Two constructs phlyA2 and phlyA3 (Table 4.1) were analyzed via primer walking using four primers: hly1, hly3, hly5, and hly6. The relative binding sites of these primers are shown in Figure 4.1A. The remainder of the gene (the 5' and 3' ends) was sequenced using the primers hlyCA1 and hlyCA2 (5' end) and primers hem3 and hlyAB2 (3' end) from the plasmids pMDM49 and pMDM48 (Table 4.1), respectively.

The presence of the *cnf*1 gene was verified by The Gastroenteric Disease Center, Wiley Laboratory, The Pennsylvania State University. An independent verification was also performed using similar primers as described by Blanco, *et. al.*, 1996 (28). These primers (CNF1-A1 and CNF1-B1, Table 4.2) were modified using sequence alignments from two previously sequenced *cnf1* genes from *E. coli* strains 96-1913 and 5383-2. The primers CNF1F, CNF1R, cnf1I1, cnf1I2, CNF1-B1, cnf1B2, cnf1Bam, Cnf1-1, Cnf1-2, and cnf1down (Table 4.2) were used to sequence the entire gene using the 3 constructs pMDM70, pMDM71, and pMDM90 (Table 4.1).

All of the sequencing reactions were carried out as follows: 450 ng of template, 3.2 pmol of primers, the primers listed above and the two universal primers T7 and SP6, and 4 μL of Big Dye Terminator Ready Reaction Mix (purchased from Virginia Bioinformatics Institute [VBI]

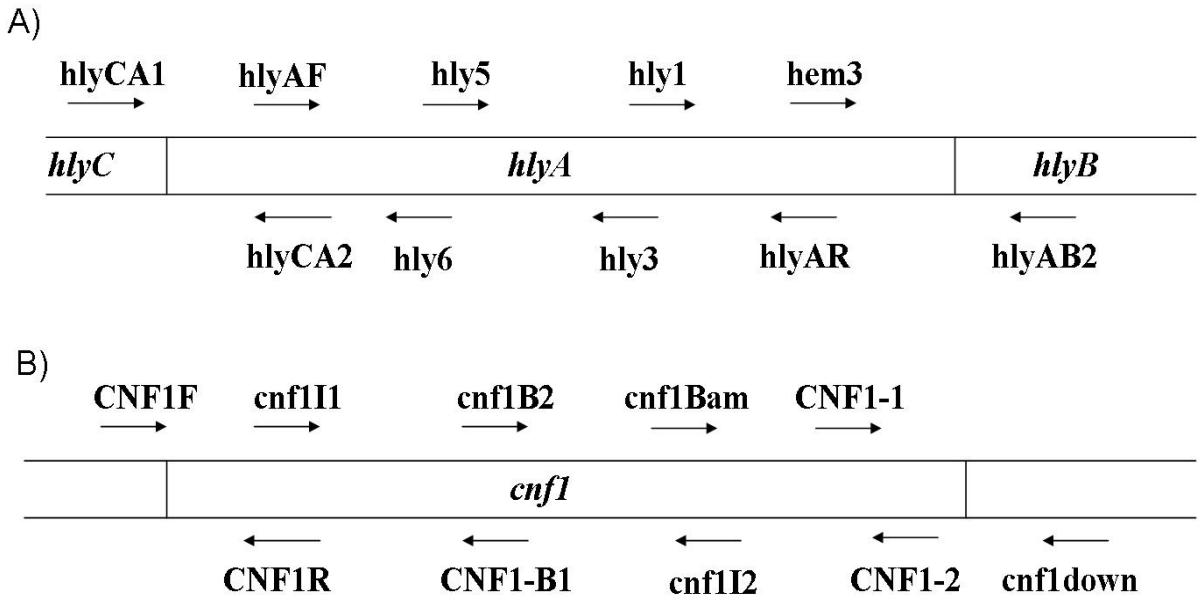

Figure 4.1. Cartoon depiction of relative binding sites primers used to sequence the *hlyA* and *cnf1* genes. The arrows indicate the relative binding sites and the direction of the primers used in sequencing several constructs of A) *hlyA* and B) *cnf1*.

Core Laboratory Facility, Blacksburg, VA) in a 20 μL final volume. The reactions were performed under the following conditions: 35 cycles of 94°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes. The samples were sent to the VBI Core Lab for sequencing.

Evaluation of the putative *hlyCABD-cnf1* operon structure of *E. coli* 360A. Further analysis was done using the primer pair CNF1 and HLYD (Table 4.2) to examine a possible link between the *hlyCABD* operon and *cnf1*. Primers were designed using known sequences of *hlyD* and *cnf1* to determine if the *hlyCABD* operon was linked to *cnf1* as found by other researchers (90, 171). The primer CNF1 binds 42 bp downstream of the *cnf1* start codon and the primer HLYD binds 37 bp upstream of the *hlyD* stop codon (Figure 4.2). If these genes are linked then the resulting PCR product would be approximately 1 kb, which includes approximately 945 bp of the intergenic sequence. The PCR reaction was performed in the following manner: using 2.5 units of Deep Vent DNA polymerase (New England Biolabs) in a 100 μL reaction containing approximately 500 ng of *E. coli* 360A chromosomal DNA and 60 pmol of primers in a 1X ThermoPol reaction buffer (New England Biolabs), 0.2 mM dNTPs, and 1 mM $MgSO_4$. The reactions were run under the following conditions: 94°C for 4 minutes; 30 cycles of 94°C for 1 minute, 53°C for 1 minute, and 75°C for 1 minute; 75°C for 10 minutes. The PCR reaction was examined using an 0.8% agarose gel. The resultant PCR product was cloned into pGEM-T (Promega) following the manufacturer's instructions (with the help of Rebecca Starr). These clones, pIGS2 and pIGS3 (Table 4.1) were sequenced by the VBI Core Laboratory Facility. The primers IGS1 and IGS2 (Table 4.2) were also used in order to get a complete sequence of the products.

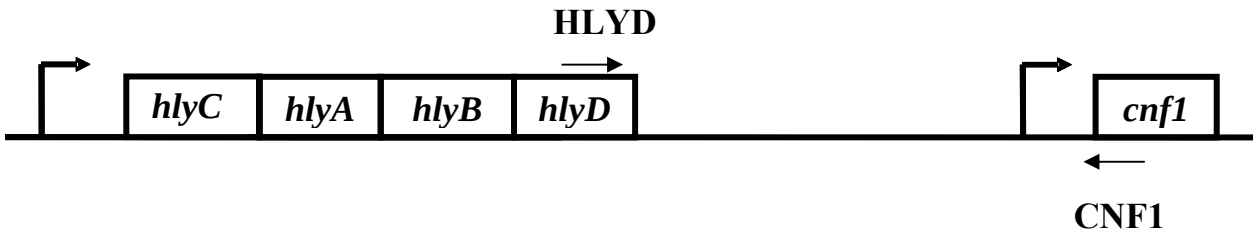

Figure 4.2. *E. coli* 360A *hlyCABD-cnf1* operon structure. This operon structure is present in many extraintestinal isolates of *E. coli* (90, 171). The two independent units, the *hlyCABD* operon and *cnf1* posses their own promoter (171). The primers HLYD and CNF1 were used to determine if these genes were linked in strain 360A and to amplify this region in between the two genes for sequence.

PCR screening for additional virulence associated genes. Other genes such as *papG*, *fimH*, and *hbp* were also screened for as mentioned above. The *papG* or *prsG* gene encodes for a protein that is the receptor binding portion of P-fimbriae/P-pili (167, 180, 186). These P-pili are termed pyelonephritis-associated pili, since they have been observed to be involved with kidney infections (234). Identification of this gene was done using the primer pair PrsGF and PrsGR (Table 4.2) via PCR with the same reaction conditions as mentioned above with an annealing temperature of 55ºC. A positive result for this PCR reaction would produce a product of approximately 970 bp in size. Detection of the *fimH* gene was done using the primers T1PF and T1PR (Table 4.2) with the same PCR conditions mentioned above with an annealing temperature of 53ºC. A positive PCR result would be approximately 900 bp in size. The presence or absence of the *hbp* gene was examined using the primers HBPF and HBPR.

Analysis of *E. coli* 360A antibiotic susceptibility. A Kirby-Bauer disk diffusion antibiotic susceptibility test was performed using twelve different antibiotics on Müller-Hinton agar plates. The procedure for this experiment and the concentration of antibiotics were used according to standard methods outlined by the Clinical Laboratory Standards Institute (218). A minimum inhibitory concentration (MIC) assay was also done on *E. coli* 360A by the Clinical Bacteriology Laboratory, Veterinary Teaching Hospital, Virginia-Maryland Regional College of Veterinary Medicine (Blacksburg, VA). *E. coli* 360A was grown overnight in LB broth without antibiotics. The following day the strain was subcultured into fresh medium to an OD_{600} of 0.5. The cells were serially diluted and cultured on plates containing kanamycin at concentrations of 10, 20, 25, 50, and 100 µg/mL to check for resistance. Strain 360A was also cultured on LB (without antibiotics), which served as a control.

Determination of electrocompetancy of *E. coli* 360A. Electrocompetent *E. coli* 360A cells were prepared by standard electrocompetent methods (Invitrogen Corporation, Carlsbad, California). Aliquots of these cells (50 μL) were stored at -80°C. Cells were electroporated with 1 ng of plasmid DNA, pBluescript II SK+ (277) using a Personal Electroporation Pak (BTX Technologies, INC., Hawthorne, NY), with 0.2 cm pathlength cuvettes (Bio-Rad Laboratories, Hercules, CA). The cells were resuspended in 1 mL of SOC (266) and incubated for 1 hour at 37ºC with shaking. Serial dilutions were made of the cells and they were plated on LB containing Ap100. The amount of CFU per μg of DNA was then calculated.

Results and Discussion

Characterization of the clinical isolate *E. coli* 360A. *E. coli* 360A was isolated by Dr. Sydney Finegold (Veterans Administration Medical Center, Los Angeles, CA), from the leg ulcer of a male diabetic patient (Chapter Three). Metabolic testing using an API 20E test strip (bioMérieux) was used to confirm that the bacterial strain we received was in fact a pure culture of *E. coli*. Table 4.3 shows the sugars that were used in these experiments. We were able to identify that this strain was able to utilize 11 of the 13 carbon sources tested. These results are consistent with a majority of the *E. coli* strains (126). In examining mutagenesis methods for use on strain 360A there were several issues to consider: 1) strain 360A is able to utilize sucrose as a carbon source, so *sacB* could not be used as a negative selection marker. 2) strain 360A can also utilize arabinose, which is needed to induce the λ red recombinase genes on pKD46 (69). Arabinose is used to induce the expression of the arabinose inducible promoter during the competent cell procedure. The required amount used for laboratory strains is 10 mM of arabinose; we attempted to use 100 mM, since 360A has the ability to utilize the sugar. The λ red recombinase procedure (69) was used in attempts to generate allelic replacement mutants in strain 360A, but was not successful.

Gel electrophoresis results showed the presence of a plasmid in this strain. The β-glucoronidase assay showed a negative result and the presence of siderophore was verified by using a plate assay.

Analysis of virulence determinants of *E.* coli 360A. A bacterial capsule was observed using an India ink staining procedure. Figure 4.3 shows the transparent bacterium surrounded by a clear zone around the cells indicating the presence of a capsule. Though encapsulated, the

Table 4.3. Metabolic testing of the clinical isolate *E. coli* 360A using API tests strips and other carbon sources.

Carbon Source	Utilization
Amylose	-
L-Arabinose	+
Ducitol	+
Glucose	+
Lactose	+
Malonate	-
Maltose	+
Mannitol	+
Melibiose	+
Raffinose	+
Sorbitol	+
Sucrose	+
Xylose	+

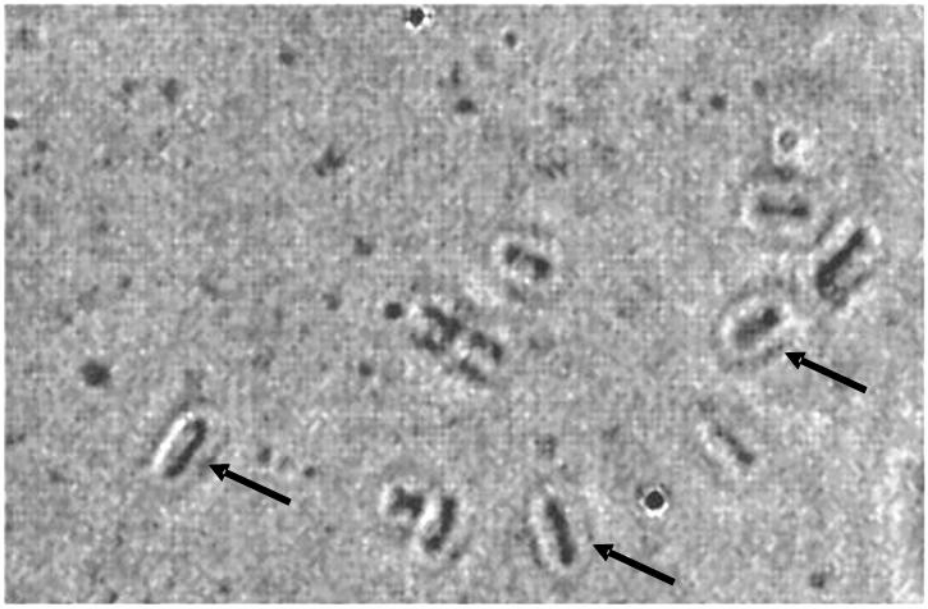

Figure 4.3. The presence of a bacterial capsule around the clinical isolate *E. coli* 360A. The above picture is a digital image of an India ink wet mount taken on a phase-contrast microscope. The arrows in the above image indicate a clear zone around the *E. coli* cell. This image was enhanced using Adobe Photoshop.

capsule was of a non-K1 type (Table 4.4). The presence of the hemolysin toxin was determined by using a sheep's blood agar plate. The results obtained from The Gastroenteric Disease Center are shown in Table 4.4. These results indicated that this strain is of O type 6, has a serum-positive but unidentified H type, and contains the gene *cnf1*, which encodes for the virulence factor, cytotoxic necrotizing factor 1 (CNF1). Independent PCR-based sequencing methods were done to verify that this strain also carries the genes encoding the hemolysin (HlyA).

The genetic profile of strain 360A shows similarities to that of extra-intestinal pathogenic strains of *E. coli*, especially uropathogenic *E. coli* strains (334). The results of these tests suggest that knockouts of the *hlyA* gene and *cnf1* gene as well as the *cydAB* and *cyoABCDE* operons might be beneficial in examining the role of these gene products in the overall pathogenesis of this strain in a systemic model. The *hlyA* gene has been found in a majority of the isolates from systemic infections (32, 233). The *cnf1* gene has been shown to be genetically linked to the *hly* operon (171). The two virulence factors CNF1 and HlyA may play a role in defense against the immune system during a systemic infection, which has not been investigated. Cytochrome bo_3 and *bd* I oxidases are involved in aerobic respiration and aid in the protection from oxygen radicals (181, 313), which is due to the induction of catalase production which responds to intracellular concentrations of H_2O_2 (181). Ultimately, our lab wishes to use these mutants in a polymicrobial infection to examine the role of the terminal oxidases and virulence factors (CNF1 and HlyA) in the formation of abscesses and their involvement in the survival of bacteria. HlyA has been seen to contribute to bacterial synergy, but has not been tested in a strain with an isogenic mutant (300). The cytochrome oxidases are thought to decrease oxygen in the tissue and allow anaerobes to grow (260).

Table 4.4. The examination of *Escherichia coli* 360A virulence factors using serotyping and PCR.

Virulence attribute and name[a]	Function/description of virulence factor	Result of PCR Analysis
O Antigen	Polysaccharide side chains on LPS used for typing of serogroups	**6**
H Antigen	Flagella antigen used for typing of serogroups	+[b]
Heat labile toxin (LT)	Binds to a receptors on intestinal epithelial cells triggering loss of Chloride ions	-
Heat stable toxin a (STa)	Induces chloride ion secretion and sodium chloride uptake in intestinal epithelial cells	-
Heat stable toxin b (STb)	Induces chloride ion secretion and sodium chloride uptake in intestinal epithelial cells	-
Shiga-like toxin type I (SLTI)	Interferes with protein synthesis in host cells	-
Shiga-like toxin type II (SLTII)	Interferes with protein synthesis in host cells	-
Cytotoxic Necrotizing Factor 1 (CNF1)	Targets epithelial cells and induces the increase of fibers and enlargement of the cells and causing death	+
Cytotoxic Necrotizing Factor 2 (CNF2)	Targets epithelial cells and induces the increase of fibers and enlargement of the cells and causing death	-
Intimin (EAE)	Responsible for attachment to intestinal epithelial cells	-
K1 Capsular antigen	Polysaccharide antigen group of *E. coli* capsule	-
Bundle forming pili (Bfp)	Responsible for bacterium to bacterium adherence	-
Hemoglobin Binding Protease (HBp)	Binds to hemoglobin and degrades it and subsequently binds the freed heme groups	-
Hemolysin (HlyA)	Targets and lyses erythrocytes	+
papG-III allele (pili associated with pyelonephritis)	Receptor binding portion of P-fimbriae/P pili	+
fimH-Type 1 pili	Type 1 pili tip adhesion protein	+

[a]All virulence factors excluding the hemoglobin binding protease, α-hemolysin, *papGIII*, and *fimH* genes were examined by The Gastroenteric Disease Center, Wiley Laboratory, The Pennsylvania State Institute.
[b]This result indicates the presence of a flagella but it is either a novel group or does not fall into any known standard.

Sequence analysis of target genes/operons in *E. coli* 360A. Preliminary sequence analysis of known oxidase operons from three sequenced *E. coli*: K-12, O157:H7, and CFT073, showed an approximately 98-99% identity, when comparing either *cydAB* or *cyoABCDE* in each strain. When examining the hemolysin genes of O157:H7 and CFT073 the sequences were only approximately 62% identical. The primers, hlyAF and hlyAR (Table 4.5), were designed based on the highest conserved regions found by sequence alignment. The results of the gel analysis showed PCR products expected based on the size of known sequences. Primer walking across the remainder of the 2200 bases of the *hlyA* internal fragment was done with the help of Rebecca Starr. She also helped perform sequence alignments of the sequence information obtained from the *hlyA* internal fragment constructs, phlyA2 and phlyA3. The remainder of the gene was sequenced using primer pairs hlyCA1/hlyCA2 and hem3/hlyAB2 to analyze the 5' and 3' regions using plasmids pMDM49 and pMDM48, respectively. The sequence analysis of the entire hemolysin gene showed that this sequence was approximately 97% identical to that of 3 published sequences of strains CFT073 (320), J96, and UTI89 (55), which are all uropathogenic isolates. This sequence alignment is located in Appendix III. The sequence analysis that was done on *cnf1* from *E. coli* 360A revealed that it was 99% identical to that of 4 known *cnf1* sequences 96-1913, 5383-2, O83/K24/H31, and UTI89 (55) which were found using the NCBI website and aligned using The Biology Workbench. The results of this sequence analysis are located in Appendix IV.

Evaluation of the putative *hlyCABD-cnf1* operon structure of *E. coli* 360A. The PCR analysis of the putative *hlyCABD-cnf1* operon structure produced approximately a 1 kb band leading to the conclusion that these genes are linked. We further examined this region by

sequencing it and comparing it to the published sequences of *E. coli* strain J96 (171) and UTI89 (55). These sequences were seen to be approximately 99% identical (Appendix V). Since these two genes are linked and involved in the virulence of *E. coli* strains this region is categorized as a pathogenicity island (PAI), $PAI_{360A}II$, which is named according to previous descriptions by Blum, *et. al.* (29) due to its structure.

PCR screening for additional virulence genes. Two other genes were identified by PCR screening, the *papG* and *fimH* genes (Table 4.4). There was no PCR product seen during the screen for the *hbp* gene. There are three variant forms of the *papG* gene: *papGI*, *papGII*, and *papGIII* (also known as *prsG*) (194). The PCR product was cloned into pGEM-T (Promega) and sequenced. The analysis of the sequences obtained by comparing the *papG* of strain 360A to known gene sequences characterizes this gene as a *papGIII* allele (Appendix VI). The sequences from all the sequenced genes were assembled using The Biology WorkBench.

Analysis of *E. coli* 360A antibiotic susceptibility. The susceptibility tests were done using a total of thirty-three antibiotics to determine which antibiotic resistance genes could be used as markers in the creation of mutants. First, a Kirby-Bauer disk diffusion antibiotic susceptibility test was done, and the antibiotics used are listed in Table 4.5. *E. coli* 360A was seen to be resistant to vancomycin (30 μg/mL) and erythromycin (15 μg/mL). A second round of testing was done by MIC analysis, which showed that this strain was resistant to seven of the thirty antibiotics tested, and confirmed that the strain was resistant to vancomycin and erythromycin (Table 4.5 and 4.6). This strain also displays resistance to are clindamycin, clarithromycin, oxacillin + 2% NaCl, penicillin, and rifampin. The results from the kanamycin

Table 4.5. Kirby-Bauer disk diffusion antibiotic susceptibility test on the clinical isolate *Escherichia coli* 360A.

Antibiotic	Concentration of Antibiotic (µg/mL)	Zone of Inhibition (mm)	Resistance
Amoxicillin / Clavulanic Acid	30	18	**Moderate**
Ampicillin	10	22	Susceptible
Carbenicillin	100	28	Susceptible
Cefoxitin	30	28	Susceptible
Chloramphenicol	30	26	Susceptible
Ciprofloxacin	5	39	Susceptible
Erythromycin	15	11	**Resistant**
Gentamicin	10	20	Susceptible
Imipenem	10	31	Susceptible
Naladixic Acid	30	28	Susceptible
Tetracycline	30	24	Susceptible
Vancomycin	30	0	**Resistant**

*Resistant and susceptibility ranges taken from Becton-Dickinson Microbiology Systems - BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs (218).

Table 4.6. Minimum inhibitory concentration assay on the clinical isolate *Escherichia coli* 360A.

Antimicrobial Agent	Concentration	Result	Target
Amikacin	≤ 4 μg/mL	Susceptible	Binds 30S ribosomal subunit of bacterial ribosome inhibiting translation
Amoxicillin / Clavulanic Acid	2/1 μg/mL	Susceptible	Inhibition of bacterial cell wall synthesis
Ampicillin	2 μg/mL	Susceptible	Inhibition of bacterial cell wall synthesis
Ampicillin / Sulbactam	8/4 μg/mL	Susceptible	Inhibition of bacterial cell wall synthesis
Cefazolin	≤ 1 μg/mL	Susceptible	Inhibition of bacterial cell wall synthesis
Cefoxitin	≤ 2 μg/mL	Susceptible	Inhibition of bacterial cell wall synthesis
Ceftazidime	≤ 1 μg/mL	Susceptible	Inhibition of bacterial cell wall synthesis
Ceftriaxone	≤ 4 μg/mL	Susceptible	Inhibition of bacterial cell wall synthesis
Cefuroxime sodium	2 μg/mL	Susceptible	Inhibition of bacterial cell wall synthesis
Cephalothin	4 μg/mL	Susceptible	Inhibition of bacterial cell wall synthesis
Chloramphenicol	4 μg/mL	Susceptible	Binds peptidyl transferase component of 50S ribosomal subunit, blocking peptide elongation
Ciprofloxacin	≤ 0.06 μg/mL	Susceptible	DNA gyrase, blocking DNA replication
Clarithramycin	> 4 μg/mL	**Resistant**	Binds 50S ribosomal subunit of bacterial ribosome inhibiting translation
Clindamycin	> 2 μg/mL	**Resistant**	Binds 50S ribosomal subunit, blocks peptide elongation; Inhibits peptidyl transferase by interfering with binding of amino acid-acyl-tRNA complex
Erythromycin	> 4 μg/mL	**Resistant**	Binds 50S ribosomal subunit of bacterial ribosome inhibiting translation
Gentamicin	≤ 0.25 μg/mL	Susceptible	Binds 30S ribosomal subunit of bacterial ribosome inhibiting translation
Gentamicin	500 μg/mL	Susceptible	Binds 30S ribosomal subunit of bacterial ribosome inhibiting translation
Lomefloxacin	≤ 0.5 μg/mL	Susceptible	DNA gyrase, blocking DNA replication
Mezlocillin	≤ 4 μg/mL	Susceptible	Inhibition of bacterial cell wall synthesis
Nitrofurantoin	32 μg/mL	Susceptible	Binds 30S ribosomal subunit of bacterial ribosome inhibiting translation
Norfloxacin	4 μg/mL	Susceptible	DNA gyrase, blocking DNA replication
Ofloxacin	≤ 0.25 μg/mL	Susceptible	DNA gyrase, blocking DNA replication
Oxacillin + 2% NaCl	> 2 μg/mL	**Resistant**	Inhibition of bacterial cell wall synthesis
Penicillin	8 μg/mL	**Resistant**	Inhibition of bacterial cell wall synthesis
Peperacillin	≤ 4 μg/mL	Susceptible	Inhibition of bacterial cell wall synthesis
Rifampin	2 μg/mL	**Resistant**	Binds bacterial RNA polymerase inhibiting transcription
Sulfizoxazole	256 μg/mL	Susceptible	Interferes with enzyme systems essential to normal metabolic and growth patterns of bacteria
Tetracycline	1 μg/mL	Susceptible	Blocks tRNA binding to 30S ribosome subunit-mRNA complex
Ticarcillin / Clavulanic Acid	≤ 4/2 μg/mL	Susceptible	Inhibition of bacterial cell wall synthesis
Trimethoprim / Sulphamethoxazole	0.5/9.5 μg/mL	Susceptible	Inhibits dihydrofolate reductase preventing synthesis of folic acid
Vancomycin	> 16 μg/mL	**Resistant**	Inhibition of bacterial cell wall synthesis

plates showed that strain 360A was resistant to concentrations of kanamycin up to 50 μg/mL. No growth occurred on the LB plates containing 100 μg/mL of kanamycin.

Determination of electrocompetancy of *E. coli* 360A. The amount of colony forming units per micrograms of DNA for electrocompetent cells of this strain was found to be 1.6×10^9 CFU/μg of plasmid DNA.

Conclusions

Strain 360A has several virulence associated genes and the O serotype (O6), which groups this strain with extra-intestinal pathogenic strains of *E. coli*, especially uropathogenic strains (334). We have identified and sequenced portions of four target genes to generate disruptions, *cnf1*, *hlyA*, *cydAB*, and *cyoAB*. We then used these gene knockouts to examine the role of their gene products in a systemic infection (Chapter Five). Using these mutants in these types of infections will give us new insights on the importance of these gene products in the pathogenesis of *E. coli* that cause extra-intestinal infections.

Chapter Five

The Role of Aerobic Respiration, CNF1, and HlyA in the Pathogenesis of *Escherichia coli* 360A During a Systemic Infection Using a Mouse Model

Manuscript in preparation

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Supplemental material is located in Appendix VII

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Abstract

Systemic infections cause over 30,000 deaths per year in the United States. These infections can be caused by many different bacteria including *Escherichia coli*. In this study we investigated if aerobic respiration and two virulence factors, CNF1 and HlyA, have an affect on the colonization and survival of a pathogenic *E. coli* strain in the liver and spleen of mice. We infected BALB/c mice intravenously in the tail vein with *E. coli* 360A (O6:K+:H+) or 5 isogenic mutants and the infection was allowed to go for twelve hours. The outcome of these infections showed that strains lacking either cytochrome *o* terminal oxidase, or both the cytochrome *o* and cytochrome *d* terminal oxidases are deficient in survival in the spleen of a mouse during this type of infection. A strain lacking both a functional *cnf1* and *hlyA* gene show the same decreased survival as the oxidase mutants. Single mutants lacking either a functional *cnf1* or *hlyA* gene do not show any differences in the number of CFUs compared to strain 360A. The cytotoxicity and ability of *E. coli* 360A to interact and invade two murine macrophage-like cell lines J774a.1 and RAW264.7 was evaluated. Strain 360A was able to invade and survive in both murine macrophage-like cell lines for up to 4 hours. Mutants lacking both functional genes of either *cnf1*, *hlyA*, or both, had higher numbers of CFUs in the two murine macrophage-like cells tested compared to strain 360A.

Introduction

Septicemia is a significant and deadly disease that is responsible for thousands of death a year. In a report by the Centers for Disease Control and Prevention (CDC) between the years 2004 and 2005 it was the 10th leading cause of death in the U.S. when approximately 30,000 people died from this disease each year (168, 207). Studies over the past three decades have shown a rise in the number of systemic infections. These infections are usually secondary in nature. The most prevalent organisms in these infections are the Gram-positive bacteria *Staphylococcus aureus* and coagulase-negative staphylococci or the Gram-negative bacteria *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas sp.* (215, 319, 337). Of these, *E. coli* and *S. aureus* are usually involved in the highest number of infections.

E. coli is the cause of a large number of septicemia cases seen in the very young and the very old: in children (especially newborns) (158, 159), in the age group over 65 years of age (mainly in nursing homes) (215), and in hospitals (278). Some *E. coli* strains have the ability to evade the immune system by producing a capsule or potent cytotoxins. Others can invade and even survive in phagocytic cells such as polymorphonuclear leukocytes (71, 219) and macrophages (16, 95, 287). The fact that some *E. coli* strains can invade and even survive in these different professional phagocytes makes these strains better geared to cause infections than other less virulent strains. This attribute gives these strains a competitive advantage, hence it is considered to be a pathogenic trait and possible virulence factor that could aid strains in causing septicemia.

Strains of *E. coli* often carry the ColV plasmids, which have been shown to encode genes involved in resistance to serum and are responsible for iron uptake systems (303, 317, 338).

Other factors such as P fimbriae, hemolysin production, and K1 encapsulation occur more frequently in isolates from septicemia (32, 233). Toxins such as CNF1 do not occur commonly in isolates that cause septicemia (32). The role of several of these virulence factors in systemic infections, including the toxins, has not been evaluated. In this study we were interested in the ability of *E. coli* 360A to cause a systemic infection in BALB/c mice. We have detected bacteria in the spleen of both diabetic and non-diabetic mice in a previous study (Chapter Three and Appendix II, Figure AII.3). In that study, we used a subcutaneous infection to examine bacterial synergy. The area of injection and the spleen from each of the mice were removed and examined for bacterial counts. We observed that greater than 50% of the mice, diabetic or non-diabetic, infected with *E. coli* 360A had bacteria in the spleen (unpublished data, Appendix II, Figure AII.3).

In this study we examined: 1) the cytotoxic affects of strain 360A on two murine macrophage-like cell lines, 2) the ability of strain 360A to invade and survive in murine macrophage-like cell lines, 3) if any virulence factor or environmental modulator has an effect on the ability of strain 360A to invade and survive in or escape from both murine macrophage-like cell lines, and 4) which genes are involved in this strain's ability to cause a systemic infection. Our goals were to 1) construct a mutant form of *E. coli* 360A that lacks both functional terminal oxidase operons and 2) construct mutants in the two virulence genes, *hlyA* and *cnf1* and a double (*cnf1/ hly*) toxin mutant. These targets are outlined in Figure 5.1. Experiments involving strain 360A and the 5 isogenic mutants should allow us to establish if the presence of functional environmental modulators and virulence factors contribute to the early stages of an infection and in the pathogenesis of *E. coli* 360A. Ultimately, our lab wishes to

examine the contribution of these different *E. coli* environmental modulators and virulence factors toward survivability and synergy, singly and in combination with a strain of *B. fragilis* that was used in our previously established mouse model (Chapter Three).

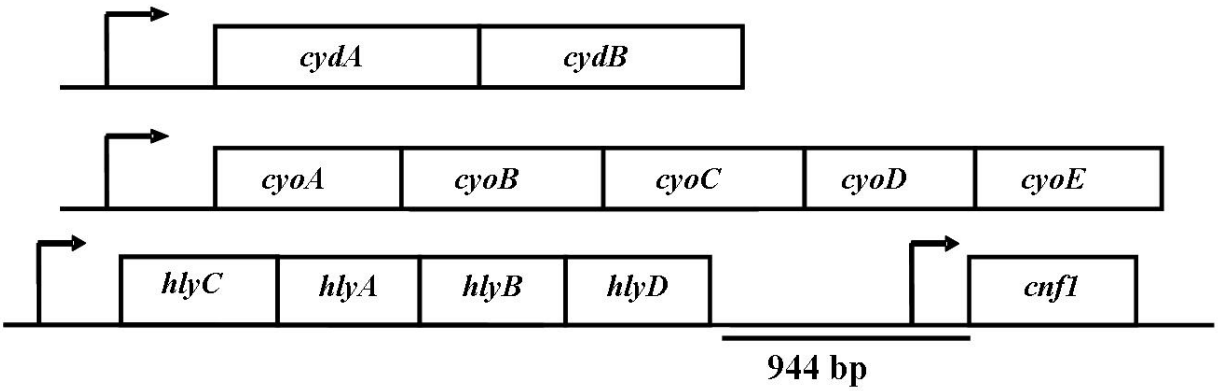

Figure 5.1. *E. coli* 360A genes/operons that are the target of this study. From top to bottom: *cydAB* and *cyoABCDE* operons that encode the two terminal oxidases of *E. coli*; the third operon is the *hlyCABD-cnf1* operon that encodes for the hemolysin toxin (*hlyA*), accessory genes needed for HlyA activation and secretion, and the cytotoxic necrotizing factor 1 (*cnf1*). Both the *hlyCABD* operon and *cnf1* gene have their own promoters. The arrows indicate the direction of transcription. (Note: these genes and operons are not drawn to scale.)

Materials and Methods

Bacterial strains, macrophages, and growth conditions. All *E. coli* strains used in this study are listed in Table 5.1. *E. coli* S17 λ *pir* (280) was used as the host strain for the suicide vectors used in the mutagenesis procedure. All strains were grown in Luria-Bertani (LB) broth in the presence of the appropriate antibiotics (Table 5.1) at 37ºC with shaking (250 RPM). Antibiotics used in this experiment include: 100 μg/mL ampicillin (Ap100), 25 μg/mL chloramphenicol (Cm25), 30 μg/mL gentamicin (Gm30), and 10 μg/mL tetracycline (Tet10).

Murine macrophage-like cell lines J774a.1 (243) and RAW264.7 (242, 245) were obtained from the American Type Culture Collection (Manassas, VA). Both macrophage cell lines were cultured in Dulbecco's Modified Eagle's Medium with L-glutamine, 4.5 g/L glucose and sodium pyruvate (DMEM, Mediatech, Inc., Manassas, VA) containing 10% fetal bovine serum (Lonza, Inc., Allendale, NJ) at 37ºC in a 5% CO_2 modified environment. Cultures were grown for up to two weeks with replenishment of fresh medium every 2-3 days. These cells were subcultured every 7 days with a 1:4 dilution of fresh DMEM.

Chromosomal disruption of target genes in *E. coli* 360A. Two fragments approximately 500 to 1400 base pairs in size of each target gene/operon (Figure 5.1) were PCR amplified from the *E. coli* 360A chromosome. (A detailed description of the constructs is located in Appendix VII.) These fragments were cloned into the pKNOCK-Tc (2) suicide vector along with a resistance marker. A four fragment ligation was done utilizing the four unique restriction sites, which were engineered on each of the fragments. These constructs (Table 5.1) were transformed by electroporation into electrocompetent *E. coli* S17 λ *pir* cells (280). The suicide vectors were transferred into *E. coli* 360A using bacterial matings with *E. coli*

Table 5.1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevance to Study and Strain Genotype[a]	Reference
***E. coli* strains**		
360A	wild-type strain used to make gene disruptions	(197)
MM1	360A *hly* mutant: Cm^R, Tc^R	This study
MM2	360A *cyo* mutant: Gm^R	This study
MM3	360A *cyo/cyd* mutant: Gm^R, Cm^R, Tc^R	This study
MM4	360A *cnf1* mutant, Gm^R, Tc^R	This study
MM5	360A *cnf1/hly* mutant, Gm^R, Cm^R, Tc^R	This study
S17 λ *pir*	strain used in di-parental matings, *recA*, *pro*, *thi*, *hsdRM*$^+$, RP4::2-Tc::Mu::Kan Tn7, λ *pir* phage lysogen, Tp^R	(280)
Plasmids		
pGEM-T	AT cloning vector, Ap^R	Promega
pKNOCK-Tc	suicide vector, Tc^R	(2)
pMDM56	suicide vector used for *cyo* knockout, Gm^RTc^R	This study
pMDM63	suicide vector used for *hly* knockout, Cm^RTc^R	This study
pMDM64	suicide vector used for *cyd* knockout, Cm^RTc^R	This study
pMDM64	suicide vector used for *cyd* knockout, Cm^RTc^R	This study
pMDM99	vector used for *cnf1* knockout, Gm^RTc^R	This study
pHLK102	*cnf1* complementation vector, Ap^R	(249)
pSF4000	contains *E. coli* J96 *hlyCABD* operon, Cm^R	(321)
pBBR1MCS4	medium copy vector used in cloning *hlyCABD* operon, Ap^R	(165)
pMDM103	*hlyCABD* complementation vector, Ap^R	This study

[a]Tetracycline resistance (Tc^R); Ampicillin resistance (Ap^R); Trimethoprim resistance (Tp^R); Gentamicin resistance (Gm^R), Kanamycin resistance (Kan^R).

S17 λ *pir* in a 2:1 ratio of recipient to donor. The matings were set up as follows: An overnight culture of each strain was grown in LB at 37°C with shaking (250 RPM) and the appropriate antibiotics. Samples from each strain were taken and 200 μL of S17 λ *pir* cells were mixed in a microcentrifuge tube with 400 μL of 360A cells. The cells were then sedimented by centrifugation and the supernatant was removed. The cell pellet was resuspended in 1 mL of LB to wash the cells and sedimented by centrifugation a second time. The supernatant was then removed again and the cells were resuspended in 200 μL of LB. The matings were plated on a 0.45 μm HA filter on an LB plate and allowed to incubate at 37ºC overnight. Following the incubation period the bacteria were removed from the filters by resuspending them in 3 mL of M9 minimal medium containing glucose. A 0.5 to 1 mL sample of the resuspended mating was sedimented by centrifugation and the pellets were then resuspended in 100 μL of the same medium. These samples were cultured on M9 minimal medium containing glucose and the appropriate antibiotics to select for both single and double crossover events (Figure AVII.2). Colonies were allowed to recover for up to three days at 37ºC and were subsequently transferred to fresh medium. Double mutants were constructed in the same manner following verification of the single mutants. This procedure is also outlined in Appendix VII (Figure AVII.2).

Screening of possible virulence factor mutants. Following the isolation of several colonies on M9 minimal medium, possible *hly* mutants were subcultured onto sheep's blood agar plates (BAP) containing Cm25. This was done in order to screen for the loss of hemolytic activity. These colonies were also compared to *E. coli* S17 λ *pir* for differences in colony morphology. Possible *cnf1* mutants were transferred to fresh M9 minimal medium containing glucose and Gm30. Following the preliminary plate-screening of possible mutants, the isolates

were screened via PCR to determine the site of insertion. These PCR products were cloned into the AT cloning vector pGEM-T (Promega, Madison, WI) and then sequenced by the Virginia Bioinformatics Institute Core Laboratory Facility (Blacksburg, VA). This PCR screening procedure is described in more detail in Appendix VII (Table AVII.3 and Figure AVII.3). Hemolysin-negative colonies were then evaluated using hemolytic protein assays as described elsewhere (17). The assays were performed with 150 μL of cell free supernatant was used from each of the samples. The amount of red blood cell lysis in distilled water was set as 100% red blood cell lysis. In the case of the *cnf1* mutants, multinucleation assays using Hep-2 cells were performed as previously described (206).

Screening of possible oxidase mutants. The oxidase mutants, *cyo* and *cyo/cyd*, were screened using growth assays. Single *cyo* mutants were screened on M9 minimal medium containing glucose and Gm30 and then verified by PCR and sequencing. Possible double oxidase mutants were patched on plates using M9 minimal medium containing either glucose or succinate and Gm30/Cm25. Following the preliminary plate screening, possible mutants were screened via PCR to determine the sites of insertion. These PCR products were cloned into the AT cloning vector pGEM-T (Promega), then sequenced. Following this, several oxidase mutants were screened in liquid culture. These strains were grown overnight in M9 minimal medium containing glucose with shaking (250 RPM) and the appropriate antibiotics: *cyo* mutants in Gm30 and *cyo/cyd* mutants in Gm30/Cm25. Samples from all the strains were sedimented by centrifugation and washed with M9 minimal medium containing either glucose or succinate and resuspended in a final volume of 200 μL of M9 minimal medium. The strains were all inoculated at the similar OD_{600} of approximately 0.040 into 25 mL of M9 minimal medium

containing succinate or glucose and the appropriate antibiotics. The samples were grown at 37ºC with shaking (350 RPM) and monitored for a 24 hour period. The mutants were compared to the growth of strain 360A under the same conditions.

Vector construction for complementation of the virulence factor mutants. The plasmid pSF4000 (321), was provided by Rodney Welch (The University of Wisconsin-Madison School of Medicine and Public Health, Madison, WI). This plasmid contains a 11.7 kb fragment of chromosomal DNA from *E. coli* strain J96 that contains the *hly* operon flanked by *Sal*I restriction sites. This fragment was cloned into the medium copy plasmid pBBR1MCS4 (165) via the *Sal*I restriction sites and named pMDM103. The plasmid pMDM103 was electroporated into all *hly* minus strains. The *cnf1* mutants were complimented with the *cnf1* gene from *E. coli* strain J96, expressed from the plasmid pHLK102 (250), which was provided by Alison O'Brien (Uniformed Services University of the Health Sciences).

Stability of chromosomal insertions. Cultures of all 5 mutants were grown overnight in 5 mL of LB with the appropriate antibiotics at 37ºC. All the strains were subcultured into fresh LB medium and grown for 5 to 10 days without antibiotics. Each strain was subcultured twice a day into fresh LB medium. Serial dilutions were also done on each culture once a day at an OD_{600} between 0.6 and 1.0 and the samples were cultured on LB plates with and without antibiotics. Colony forming units were calculated and the results were compared.

Cytotoxicity assays. The effects of strain 360A and five isogenic mutants on two murine macrophage-like cell lines J774a.1 and RAW264.7 were analyzed. The bacteria were prepared as mentioned above. The macrophages were subcultured in DMEM with 10% fetal bovine serum in 96-well tissue culture plates for 18 to 24 hours prior to infection and incubated at 37°C

with a 5% CO_2 modified atmosphere. The macrophages were washed with 100 µL of fresh DMEM. These wells contained approximately 8 x 10^4 macrophages. Macrophage cells were infected with a multiplicity of infection (MOI) of 0.1, 1, and 10 CFU to 1 macrophage in a total volume of 200 µL of DMEM in 96 well tissue culture plates. The samples were incubated for 1, 2, and 4 hours at 37ºC with 5% CO_2. The samples were then evaluated using the CytoTox 96 kit (Promega), according to the manufacturer's instructions. The samples were examined at 492 nm using a Tecan SpectraFlour Plus (Tecan US, Inc, Durham, NC). These assays were performed in three independent trials in sets of four.

Macrophage invasion assays. Both J774a.1 and RAW264.7 murine macrophage-like cell lines were used in these experiments. The bacterial cultures were prepared as mentioned above. The macrophage samples were also prepared as mentioned above. The macrophages were infected with a multiplicity of infection (MOI) of 0.1, 1, and 10 CFU to 1 macrophage in a total volume of 200 µL of DMEM. Each MOI was done in duplicate and incubated at 37°C with 5% CO_2 for 4 hours. The wells were washed 3 times with 1X phosphate buffered saline (PBS) to remove any non-adherent bacteria. Fresh medium either with or without 100 µg/mL of streptomycin was added to each well for 1 hour at the same growth conditions to kill any extracellular bacteria. Again the wells were washed 3 times with 1X PBS and treated with 200 µL of 0.5% Triton X-100 for 10 minutes at room temperature to lyse the macrophages and release any intracellular bacteria. The number of viable bacteria was enumerated by plating serial dilutions of the lysate on LB. The number of bacteria recovered from the wells treated with streptomycin was compared to the total number of bacteria present recovered from wells without streptomycin treatment. Controls were performed to examine: 1) the Triton X-100

toxicity on strain 360A, 2) the inhibition of phagocytosis of strain 360A by cytochalasin D, and 3) if strain 360A could adhere to the tissue culture plates. These control assays and the results are described in detail in Appendix VII. All of the assays were performed in three independent trials in duplicate.

Gentamicin is typically used for killing extracellular bacteria for invasion assays. However, other antibiotics such as streptomycin have been used (50). Assays were performed side by side using strain 360A and the *hly* mutant (MM1) with gentamicin and streptomycin. The number of bacteria recovered from these independent trials were similar (data not shown). This result showed that streptomycin is a viable substitution for gentamicin in these assays.

Systemic infections. Overnight cultures of either strain 360A or the five isogenic mutants (Table 5.1) were grown for 12 hours in LB broth with appropriate antibiotics at 37ºC with shaking (250 RPM). Each strain was subcultured (50 μL) into 6 mL of fresh medium and grown under the same conditions mentioned above to an OD_{600} of between 0.58 and 0.60. The entire culture was sedimented by centrifugation and the cell pellet was washed 3 times with 1X PBS. The samples were resuspended in 1 mL of 1X PBS and diluted to obtain approximately 1 x 10^8 CFU/mL. Six female BALB/c mice were injected intravenously in the tail vein with approximately 1 x 10^7 CFU of strain 360A or the five isogenic mutants in 100 μL. Twelve hours after inoculation the mice were then euthanized as described in Chapter Three. Both the spleens and livers from each mouse were removed, homogenized, and viable plate counts were done on eosin-methylene blue agar with or without antibiotics in order to evaluate retention of the mutations.

Statistics. The results were analyzed using Instat 3 software (Graphpad, Inc.) with a paired Student's T-test. A two-tailed *P* value < 0.0500 was considered statistically significant.

Results

Chromosomal disruption of the virulence and oxidase operons in *E. coli* 360A. Five mutant derivatives of 360A were verified via PCR and sequencing of the fragments (Table 5.1 and Figure AVII.4). These mutants were then evaluated for the appropriate phenotypes. Hemolysin mutants were examined for the inability to lyse red blood cells using a hemolytic protein assay [(17), Figure 5.2]. Both of the mutants MM1 (*hly*) and MM5 (*cnf1/hly*) showed a statistically significant decrease ($P<0.0050$) in red blood cell lysis, which is 2% of strain 360A. The wild-type phenotype was restored to the mutants by complementation using the plasmid pMDM103 (Figure 5.2). The percent red blood cell lysis of the complemented strains were statistically higher than that of strain 360A ($P<0.0070$). A plausible explanation for this difference may be due to the high copy number of the plasmid.

The examination of the *cnf1* mutants MM4 (*cnf1*) and MM5 (*cnf1/hly*) showed a lack of multinucleation as compared to strain 360A and the positive control carrying pHLK102 (Figure 5.3 panels C, D, A, and B, respectively). The wild-type phenotype was restored to both the mutants, MM4 and MM5, using the plasmid pHLK102 (Figure 5.3E and F). All *cnf1*+ strains showed between 2 and 5 nuclei per cell as shown by the arrows in Figure 5.3. The *hly* mutant was also examined and the results were very similar to that of strain 360A (data not shown).

The oxidase mutants, MM2 (*cyo*) and MM3 (*cyo/cyd*), were evaluated for growth in M9 minimal medium with high aeration (350 RPM) and in the presence of the non-fermentable carbon source succinate (Figure 5.4A). Both of the oxidase mutants showed a defect in growth in the presence of succinate. In this medium, the double mutant MM3 was unable to utilize

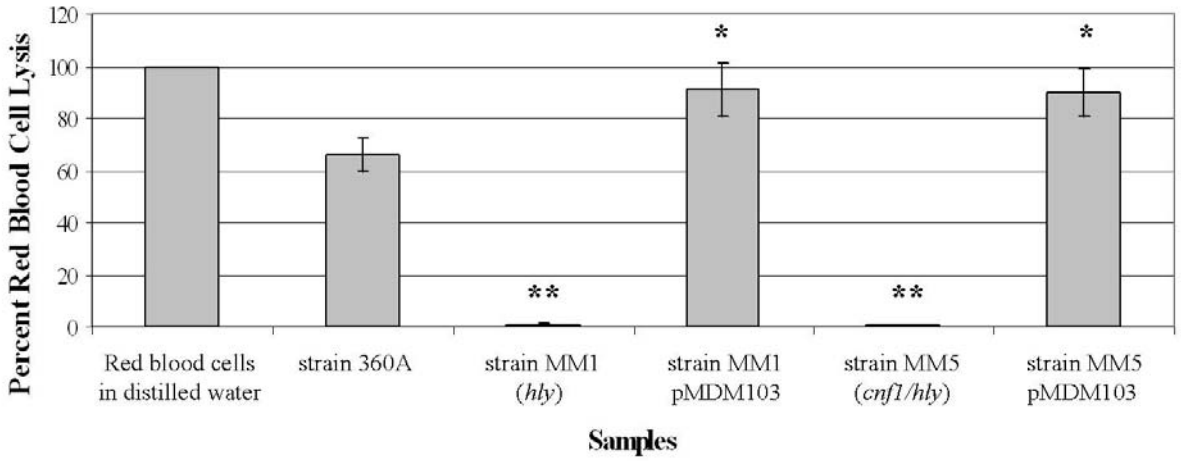

Figure 5.2. Hemolytic activity of cell free supernatants from *E. coli* 360A and several isogenic toxin mutants. Cell free supernatants from strain 360A and two *hly* mutants, MM1 (*hly*) and MM5 (*cnf1/hly*) and the complemented strains containing pMDM103 were analyzed. The assays were performed in three independent trials in replicates of three. The red blood cell lysis, which is statistically significant from strain 360A, are as follows: *, $P<0.0070$; **, $P<0.0050$.

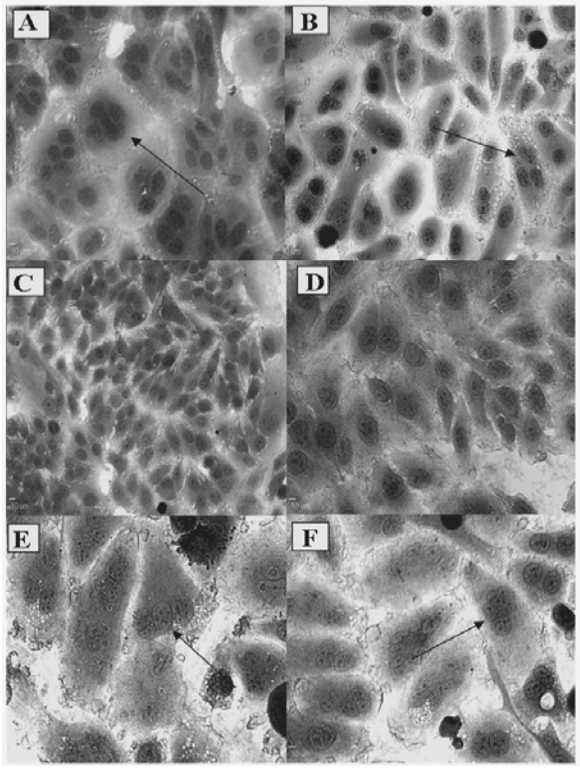

Figure 5.3. Examination of multinucleation of Hep-2 cells via several *E. coli* strains. Hep-2 cells were incubated for 72 hours with cell free lysates at a dilution of 1:32. The samples are as follows: A) *E. coli* 360A, B) *E. coli* XL1Blue containing the complementation vector pHLK102, C) MM4 (*cnf1*), D) MM5 (*cnf1/hly*), E) MM4 complemented with pHLK102, F) MM5 complemented with pHLK102. In the *cnf*-positive samples, Hep-2 cells contained between 2 and 5 nuclei per cell as indicated by the arrows. These assays were performed in two independent trials in duplicate.

succinate as the sole carbon source. Succinate is an integral part of the citric acid cycle and can only be utilized by organisms with intact electron transport chains. Since ATP would not be generated directly from the consumption of succinate, these strains would not be able to grow without the generation of energy in the form of ATP. The *cyo* mutant, MM2, grew slower in the medium compared to strain 360A. The *cyo* operon is expressed at high oxygen concentrations (62, 246, 295). So, in the absence of a functional cytochrome *o* oxidase, the cytochrome *d* oxidase could not fully compensate for this defect. When these strains were grown in the presence of glucose under the same aeration conditions they exhibit growth rates very similar to that of strain 360A, but exhibit a longer lag phase (Figure 5.4B).

Stability of chromosomal insertions. Results from all 5 mutants examined showed greater than 72% of the colonies remained antibiotic resistant for at least 5 to 10 days of growth without antibiotics (data not shown). These results indicated that the mutations were very stable in the absence of antibiotics.

Cytotoxicity of strain 360A. Strain 360A and all five mutants were evaluated for their ability to lyse murine macrophage-like cells. The single oxidase mutant MM2 (*cyo*-) showed no noticeable differences from strain 360A in either cell line (Figure AVII.7 and 8 Panel B, Table AVII.5). However, the double oxidase mutant has decreased cytotoxicity at 4 hours post-infection with J774a.1 macrophages (Figure AVII.7 panel C, Table AVII.5). This may be due to decreased growth rate in DMEM. Experiments were performed in both LB (at 37ºC with shaking, 250 RPM) and the cell culture medium DMEM at 37ºC with 5% CO_2. There was no change in the growth rate of either MM2 or MM3 compared to strain 360A (data not shown).

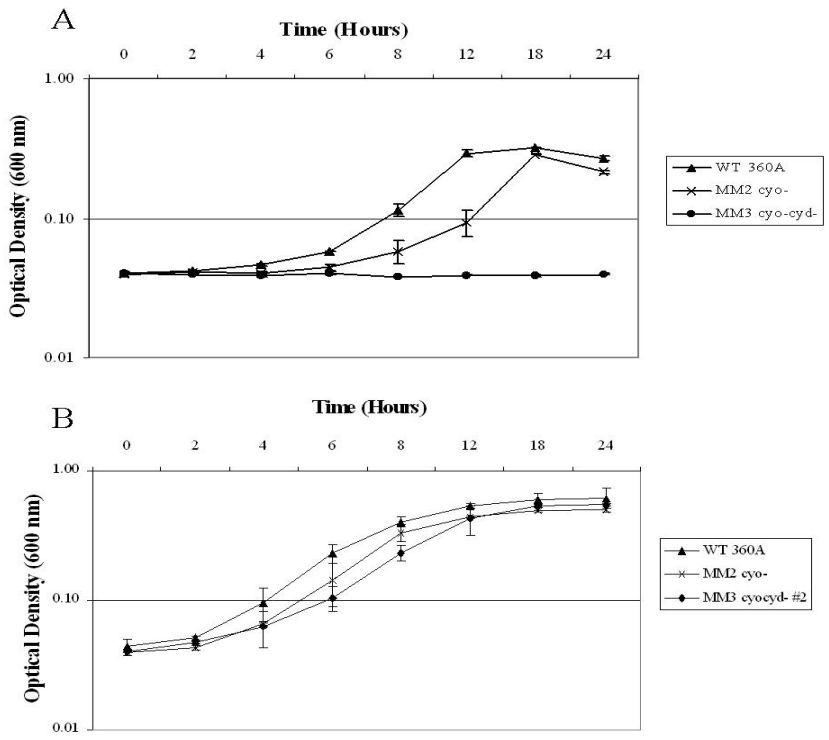

Figure 5.4. Growth of *E. coli* 360A and two isogenic oxidase mutants in M9 minimal medium containing either succinate (A) or glucose (B). Cells were grown in 25 mL cultures of M9 minimal medium containing either succinate (A) or glucose (B) at 37ºC with shaking at 350 RPM. The cultures were monitored for 24 hours of growth. These graphs are representative of two independent trials done in duplicate. The error bars indicate the standard deviation of the four samples.

Since no major growth defects were noted in the double mutant the most likely conclusion is that the strain is deficient in cytotoxicity for these cells.

Both *hly* mutants, MM1 (*hly*) and MM5 (*cnf1/hly*), showed statistically significant decreases in cytotoxicity in both strains (Figure 5.5 and 5.6, panels B and D). This effect was expected since HlyA is a pore-forming toxin that can lyse red blood cells (RBCs), leukocytes (22, 49, 147, 261), and other mammalian cells (155, 288). The *cnf* mutant (MM4) did not show any noticeable decrease in cytotoxicity (Figure 5.5 and 5.6, panel C). This is in accordance with another study showing that CNF1 does not have a significant affect on cell lysis (261). Our assays show that strain 360A is cytotoxic to both murine macrophage-like cell lines with greater than 50% cytotoxicity at all MOI tested at 4 hour post infection (Figure 5.5 and 5.6, panel A). Higher levels of cytotoxicity were seen at 2 hours in the RAW264.7 experiments than in the J774a.1 experiments (Figure 5.5 and 5.6, panel A). However, these levels were very similar at 4 hours post infection.

Macrophage invasion assays. The results from these assays indicated that strain 360A was able to invade and survive in both murine macrophage-like cell lines for at least 4 hours post infection (Figures 6.7A and 6.8A). In all the strains tested we observed that as the MOI increased the percent CFU recovered decreased (Figures 5.7 and 5.8). There was little difference in the cell numbers of the double oxidase mutant MM3 (*cyo/cyd*) compared to strain 360A in the J774a.1 infections (Figure AVII.9B). Only the MOI of 10:1 in the presence of streptomycin showed any significant difference (Table AVI.7, P=0.0410). In the RAW264.7 infection strain 360A had significantly higher recovery in the lower MOIs of 0.1 and 1:1 treated with streptomycin (0.1, P=0.0057; 1, P=0.0005) or the MOI 1:1 of total cell-associated bacteria

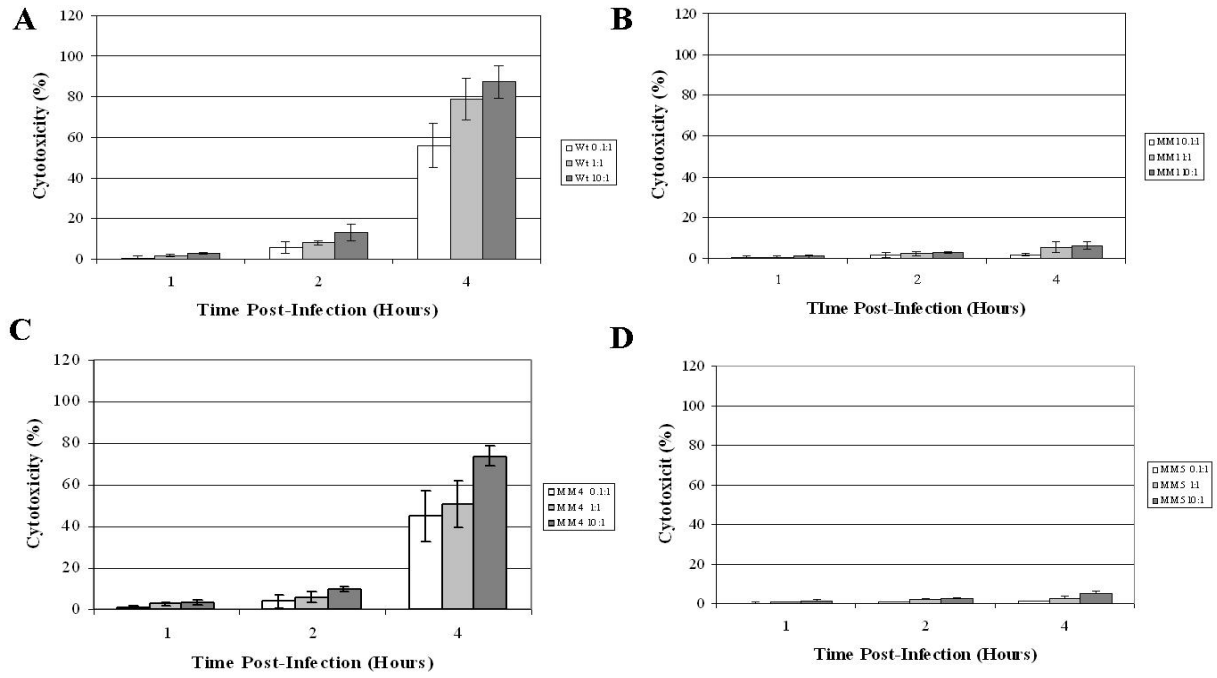

Figure 5.5. Cytotoxicity of *E. coli* 360A and three isogenic toxin mutants for J774a.1 murine macrophage-like cells. Macrophages were infected with MOIs of 0.1, 1, and 10 to 1 CFU/cell with strain 360A (A), MM1 (*hly*) (B), MM4 (*cnf1*) (C), or MM5 (*cnf1/hly*) (D) using 96-well plates. At 1, 2, and 4 hours post infection the samples were evaluated for lactate dehydrogenase release. Only mutants lacking functional *hly* operons showed significant decreases in cytotoxicity compared to stain 360A (refer to Table 5.2, left side). These assays were performed in three independent trials in sets of four.

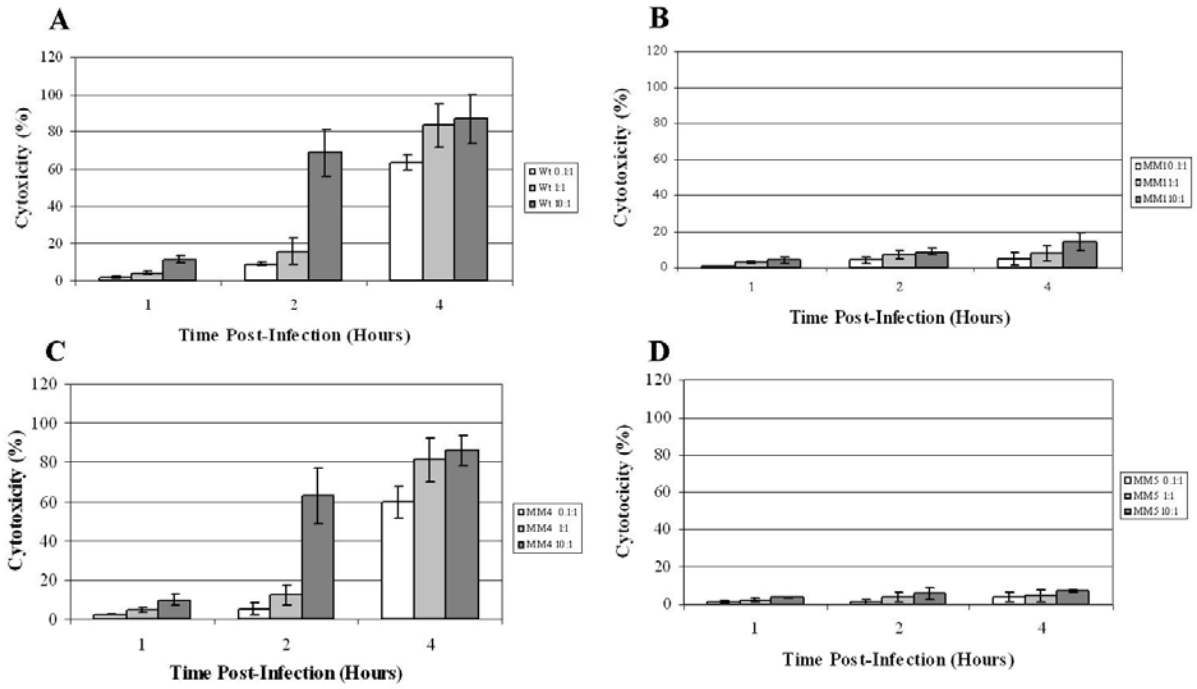

Figure 5.6. Cytotoxicity of *E. coli* 360A and three isogenic toxin mutants for RAW264.7 murine macrophage-like cells. Macrophages were infected with MOIs of 0.1, 1, and 10 to 1 CFU/cell with strain 360A (A), MM1 (*hly*) (B), MM4 (*cnf1*) (C), or MM5 (*cnf1/hly*) (D) using 96 well plates. At 1, 2, and 4 hours post infection the samples were evaluated for lactate dehydrogenase release. Only mutants lacking functional *hly* operons showed significant decreases in cytotoxicity compared to stain 360A (refer to Table 5.2, right side). These assays were performed in three independent trials in sets of four.

Table 5.2. Comparative statistical analysis of three isogenic toxin mutants to strain 360A using a cytotoxicity assays with J774a.1 and RAW264.7 murine macrophage-like cells.

J774a.1	***P* values[a]**			**RAW264.7**	***P* values[a]**		
1 hr	Wt 0.1	Wt 1	Wt 10	**1 hr**	Wt 0.1	Wt 1	Wt 10
MM1 (*hly*)	0.2685	0.0547	0.1587	MM1 (*hly*)	**0.0437**	**0.0455**	**0.0441**
MM4 (*cnf1*)	0.4968	0.0948	0.3617	MM4 (*cnf1*)	0.6335	0.4852	0.0607
MM5 (*cnf/hly)*	0.4253	0.0617	0.0882	MM5 (*cnf/hly)*	**0.0355**	**0.0079**	**0.0314**
2 hrs	Wt 0.1	Wt 1	Wt 10	**2 hrs**	Wt 0.1	Wt 1	Wt 10
MM1 (*hly*)	0.1036	**0.0109**	**0.0408**	MM1 (*hly*)	**0.0046**	**0.0166**	**0.0154**
MM4 (*cnf1*)	0.1994	0.1226	0.3056	MM4 (*cnf1*)	0.0852	0.1185	0.2455
MM5 (*cnf/hly)*	0.0877	**0.0151**	**0.0378**	MM5 (*cnf/hly)*	**0.0026**	**0.0219**	**0.0107**
4 hrs	Wt 0.1	Wt 1	Wt 10	**4 hrs**	Wt 0.1	Wt 1	Wt 10
MM1 (*hly*)	**0.0139**	**0.0082**	**0.0040**	MM1 (*hly*)	**0.0044**	**0.0103**	**0.0142**
MM4 (*cnf1*)	0.1695	0.0553	0.2051	MM4 (*cnf1*)	0.2265	0.1492	0.9532
MM5 (*cnf/hly)*	**0.0119**	**0.0077**	**0.0042**	MM5 (*cnf/hly)*	**0.0012**	**0.0108**	**0.0087**

[a] Statistically significant values ($P<0.0500$) are in bold.

(*P*=0.0090) (Figure AVII.10B). The reverse was true for the MOI of 10:1 where MM3 (*cyo/cyd*) had significantly higher recovery (*P*=0.0343) in the total number of cell-associated bacteria that had interacted with the macrophages (Table AVII.6).

Greater numbers of the toxin mutants were recovered from both macrophage cell lines in a majority of the samples we evaluated. Figure 5.7 shows the infection of J774a.1 murine macrophage-like cell line with strain 360A, MM1 (*hly*), MM4 (*cnf1*), and MM5 (*cnf1/hly*). Recovery of both MM1 and MM4 was significantly higher in all infection experiments except for the total cell-associated bacteria at three MOI of 10:1 (refer to Table 5.3, Figure 5.7B and C). The double toxin mutant MM5 (*cnf1/hly*) shows similar results as the *hly* mutant at both a MOI of 1:1 and 10:1 (Figure 5.7B and D). However, in the MOI of 0.1:1 the numbers recovered are very similar to strain 360A (Figure 5.7A and D). In the examination of the infection of RAW264.7 (Figure 5.8) similar results as the J774a.1 infections were seen. Both single toxin mutants MM1 and MM4 showed statistically higher recovery of CFUs than strain 360A at each sample tested (refer to Table 5.3, Figure 5.8B and C, respectively). The double toxin mutant MM5 (*cnf1/hly*) showed slightly different results as compared to the single toxin mutants, MM1 (*hly*) and MM5 (*cnf1*), in both of the MOIs 0.1:1 and 1:1 (Figure 5.8D). These results at those two MOIs were very similar to strain 360A (Figure 5.8D). It was seen that the recovery of MM5 in the MOI of 10:1 either treated with streptomycin or total cell-associated showed significantly higher numbers (*P*=0.0021 and *P*=0.0005, respectively) than strain 360A (Figure 5.8D).

Systemic infections. It was seen that the *cnf1* (MM4) and *hly* (MM1) single mutants do not have an affect on the colonization of the spleen (Figure 5.9). However, when a strain lacking functional copies of both of these genes, in the case of MM5 (*cnf/hly*), these mutations had a

dramatic affect on the ability of the strain to cause an infection (Figure 5.9). The decrease in the number of CFU is statistically significant (P=0.0110). Both the single [MM2 (*cyo*)] and double [MM3 (*cyo/cyd*)] oxidase mutants were also deficient in survival in the spleen (Figure 5.9). These mutants may either be deficient in growth or protection from oxidative radicals. Our growth data would indicate the former is not the case (data not shown). Studies have shown that *E. coli* cells lacking either cytochrome *o* or cytochrome *d* oxidases are more susceptible to H_2O_2 (181, 313). A study has shown that cytochrome *o* seems to be more important than cytochrome *d* oxidase, which may be due to the 2-fold decrease in catalase production by the *cyo* mutant (181). Bacterial cell counts were also determined for the liver and the results showed no significant differences between strain 360A and any of the 5 mutants tested (data not shown).

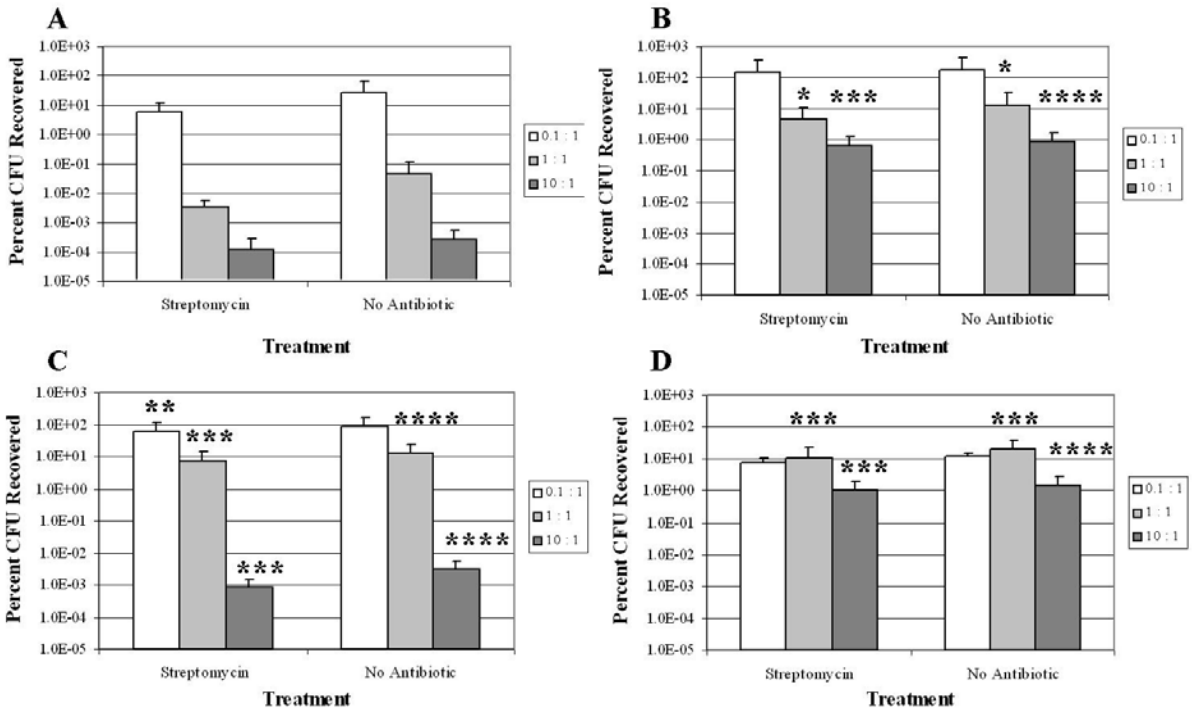

Figure 5.7. Infection of J774a.1 murine macrophage-like cells by *E. coli* 360A and three isogenic toxin mutants. Macrophages were infected with strain 360A (A), MM1 (*hly*) (B), MM4 (*cnf1*) (C), or MM5 (*cnf1/hly*) (D) at three MOI 0.1, 1, and 10 CFU per cell. The macrophages were incubated for 4 hours to allow for invasion and then streptomycin was added for an additional hour to kill all extracellular bacteria. These assays were performed in three independent trials in duplicate. The bacterial counts, which are statistically significant from strain 360A, are as follows: *, $P<0.0400$; **, $P<0.0300$; ***, $P<0.0090$; ****, $P<0.0030$.

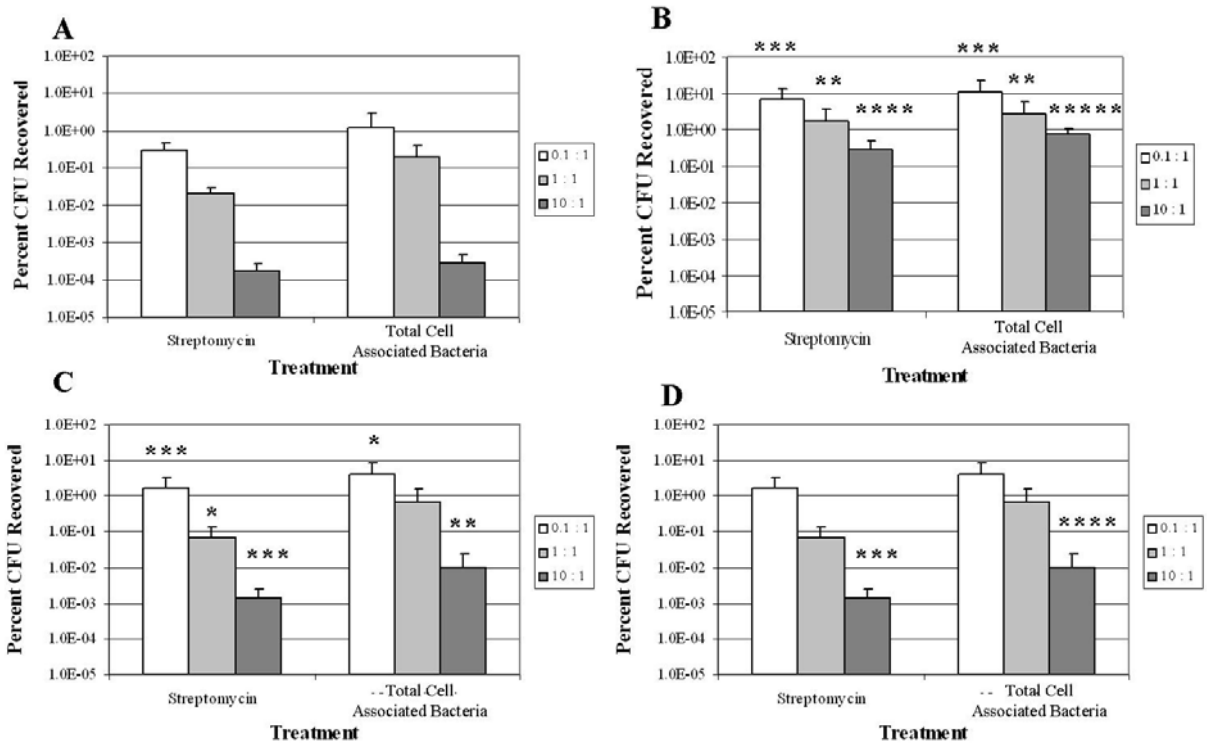

Figure 5.8. Infection of RAW264.7 murine macrophage-like cells by *E. coli* 360A and three isogenic toxin mutants. Macrophages were infected with strain 360A (A), MM1 (*hly*) (B), MM4 (*cnf1*) (C), or MM5 (*cnf1/hly*) (D) at three MOI 0.1, 1, and 10 CFU per cell. The macrophages were incubated for 4 hours to allow for invasion and then streptomycin was added for an additional hour to kill all extracellular bacteria. These assays were performed in three independent trials in duplicate. The bacterial counts, which are statistically significant from strain 360A, are as follows: *, $P<0.0500$; **, $P<0.0200$; ***, $P<0.0060$; ****, $P<0.0006$; *****, $P<0.0001$.

Table 5.3: Comparative statistical analysis of three isogenic toxin mutants to strain 360A using invasion assays with J774a.1 and RAW264.7 murine macrophage-like cells.

[a] Statistically significant values (P<0.0500) are in bold.

J774a.1	***P* values[a]**			**RAW264.7**	***P* values[a]**		
Streptomycin	Wt 0.1	Wt 1	Wt 10	**Streptomycin**	Wt 0.1	Wt 1	Wt 10
MM1 (*hly*)	**0.0390**	**0.0284**	**0.0038**	MM1 (*hly*)	**0.0020**	**0.0187**	**0.0004**
MM4 (*cnf1*)	**0.0126**	**0.0088**	**0.0068**	MM4 (*cnf1*)	**0.0051**	**0.0330**	**0.0020**
MM5 (*cnf/hly)*	0.5374	**0.0085**	**0.0032**	MM5 (*cnf/hly)*	0.2257	0.0755	**0.0021**
Total cell associated bacteria	Wt 0.1	Wt 1	Wt 10	**Total cell associated bacteria**	Wt 0.1	Wt 1	Wt 10
MM1 (*hly*)	0.0979	**0.0396**	**0.0018**	MM1 (*hly*)	**0.0029**	**0.0169**	**<0.0001**
MM4 (*cnf1*)	0.0675	**0.0018**	**0.0012**	MM4 (*cnf1*)	**0.0414**	0.0515	**0.0311**
MM5 (*cnf/hly)*	0.1990	**0.0033**	**0.0021**	MM5 (*cnf/hly)*	0.1656	0.0606	**0.0005**

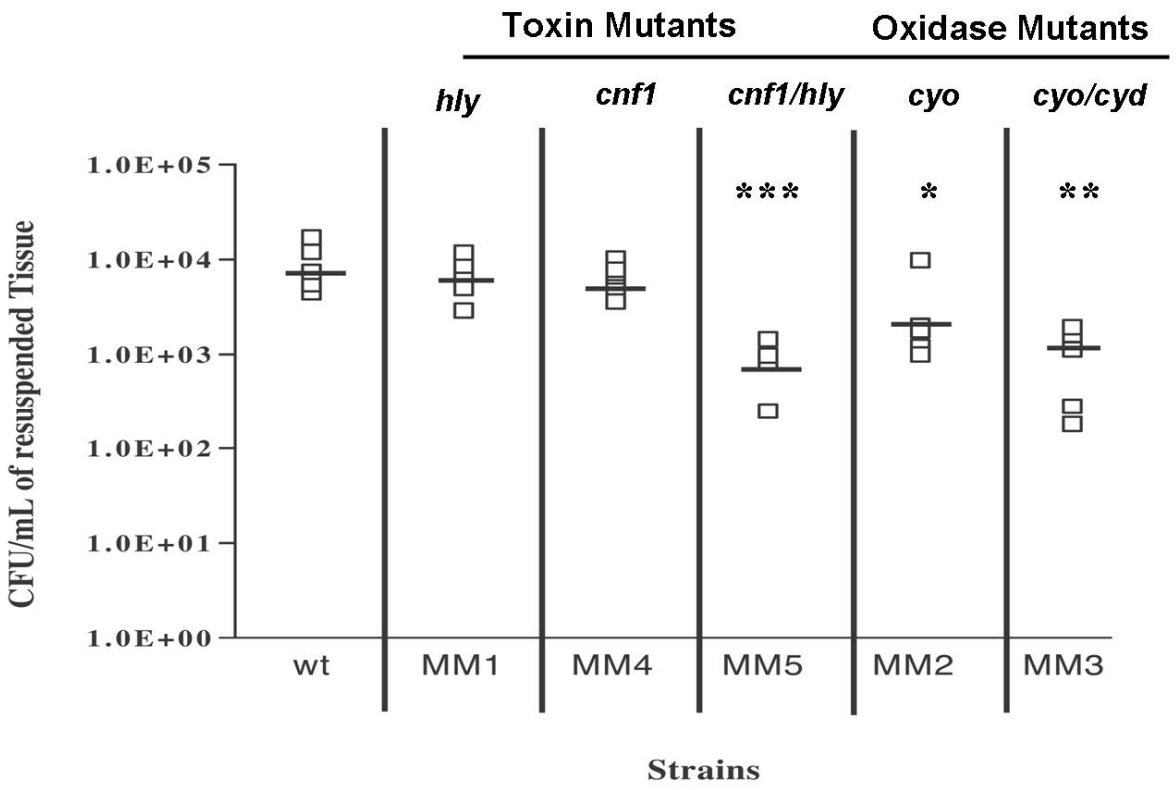

Figure 5.9. Intravenous infections of BALB/c with strain 360A and five isogenic mutants. Six female BALB/c mice were infected with approximately $1x10^7$ CFU of either strain 360A, the toxin mutants: MM1 (*hly*), MM4 (*cnf1*), or MM5 (*cnf1/hly*) and the oxidase mutants: MM2 (*cyo*), or MM3 (*cyo/cyd*) in the tail vein. After 12 hours of infection the spleens were recovered from the mice. Bacterial counts from the spleens of the mice infected with each of the mutants were compared to strain 360A. The bacterial counts of strain 360A were significantly higher than the double toxin mutant, MM5 (*cnf1/hly*), *** (P=0.0110), the double oxidase mutant MM3 (*cyo/cyd*), ** (P=0.0142), and the single oxidase mutant MM2 (*cyo*), * (P=0.0294).

Discussion

In this study we have examined the roles played by two virulence factors and the two terminal oxidases of *E. coli* 360A in the invasion of murine macrophage-like cell lines and in pathogenesis during a systemic infection. The invasion assay showed that both of the single toxin mutants MM1 and MM4 have higher invasion rates than strain 360A. First, the *hly* mutant, MM1, has decreased cytotoxicity of the two macrophage cell lines tested when compared to strain 360A (Figures 5.5 and 5.6 compare panels A and B). The *cnf1* mutant, MM4, showed similar levels of cytotoxicity as strain 360A (Figures 5.5 and 5.6 compare panels A and C). A statistically significant decrease in red blood cell lysis was seen by the *cnf1* mutant when compared to strain 360A (data not shown). This decreased hemolytic activity of MM4 (*cnf1*), may contribute in part to the increased invasion of the strain. CNF1 is able to modify small Rho GTPases (98, 178, 271), which can lead to both dramatic effects on the morphology of eukaryotic cells and negatively affects phagocytosis of immune cells (43, 70, 123). These modification of the Rho GTPases leads to continuous actin polymerization and multinucleation, which might lead to the cells detaching from the wells and possibly contribute to the decreased number of strain 360A CFU recovered. Also, both toxins could be directly involved in a bacterium's ability to escape the phagosome once inside these murine macrophage-like cell lines. In the case of the double mutant [MM5, (*cnf1/hly*)] the results differ when comparing the two murine macrophage-like cell lines. MM5 may actually be killed more efficiently by both murine macrophage-like cell lines at least at the MOI of 0.1:1 than either of the single mutants (Figures 5.7 and 5.8). It was also observed that the double oxidase mutants had decreased numbers of cells recovered as compared to strain 360A mainly in the RAW264.7 murine macrophage-like

cell line infections. This decrease in the percent CFU recovered may be due to the increased sensitivity to oxygen radicals.

The mouse data indicates that a less fit/virulent strain is not able to survive in the harsh environment of the animal host. Studies that involve *hlyA* mutants have shown the production of HlyA is crucial in the establishment of extraintestinal infections and mutants lacking a functional *hlyA* gene have a dramatic increase in their LD_{50} (216, 321). In this study we did not examine the lethal dose of the strain; we wanted to evaluate the ability of each strain to cause a systemic infection by examining the bacterial numbers in both the liver and spleen. The single toxin mutants MM1 (*hly*) and MM4 (*cnf1*) did not show a significant decrease in cell numbers in the spleen as did the double mutant. However, the double toxin mutant was at a disadvantage in the mouse model since it lacked both virulence factors: CNF1 and HlyA. Since MM5 lacks both of these virulence factors it could be inferred that both CNF1 and HlyA must act separately in some sort of manner to aid in survival in the spleen of a mouse. If one virulence factor is missing, the other virulence factor may aid the strain in establishment of the infection. We also have data showing that at least a functional cytochrome *o* oxidase is required for full virulence of this strain *in vivo*. The double oxidase mutant does not show any significant difference from the single oxidase mutant (liver, P=0.9829 and spleen P=0.2871). From this data we could infer that cytochrome *o* (and not cytochrome *d*) may be more critical to the establishment of an infection and for survival in the host. In both cell culture and rich growth media the two oxidase mutants grew at a similar growth rate as strain 360A and did not show any major growth defects (data not shown). However, in an animal, nutrients would be limiting in a struggle between the mouse and the bacterium to compete for their share of nutrients to survive. The animal will sequester

certain nutrients to help combat the infection, such as iron, while the bacterium tries to grow. The macrophages and polymorphonuclear leukocytes of the immune system would play a crucial role in the removal of these bacteria from the blood and tissues.

To conclude, the ability to respire using oxygen enhances the survival of *E. coli* 360A *in vivo*. Additionally, the production of both CNF1 and HlyA enables strain 360A to better survive *in vivo* and escape from murine macrophage-like cell lines. Finally the hemolysin is the main virulence factor involved in the cytotoxicity of strain 360A. Hence this work establishes the role of these environmental modulators and virulence factors in permitting a systemic infection caused by *E. coli* strain 360A.

Chapter Six

In vivo imaging of *Escherichia coli* 360A using a mouse model for systemic and subcutaneous infections

Introduction

Bioluminescence has been used in several studies to track the progression of infections caused by both Gram-negative and Gram-positive bacteria (101, 116, 138, 139, 192, 252). The goals of most of these studies were to validate the technology and to track the effects of antibiotics on bacterial survival in host tissues. One of these studies, by Rocchetta, *et. al.* (252) involved the use of the *Photorhabdus luminescens lux* genes, in the vector pCGLS1.UC, in an *Escherichia coli* strain EC14 infection using a neutropenic mouse thigh infection model. In the report by Rocchetta, *et. al.* (252) the *E. coli* strain was injected into the inner thigh muscles of the mouse and light emission was collected by an intensified charge-coupled device camera (252). The light output from the *E. coli* strain increased over the first 8 hours post-infection in the untreated (without antibiotics) mice (252).

In our study, mice infected with *E. coli* 360A carrying a bioluminescent reporter, pMDM89, were followed to monitor the progression of the infection at different time points. Our study had two specific goals: First we developed a bioluminescent reporter (*luxCDABE* from *P. luminescens*) for use in *E. coli* 360A to examine oxygen tension in a developing abscess. Second, we attempted to establish a mouse model using two different light sensing systems: 1) the Xenogen IVIS™ system and Living Image® Software and 2) the Multi-view Bioluminescence Tomography System, developed by Dr. Ge Wang (Virginia Tech). Both of these methods would allow us to monitor the progression of subcutaneous as well as systemic infections. The work done by Rocchetta, *et. al.* (252) was only carried out for 12 hours, which may not be enough time to examine the differences in abscess formation between *E. coli* 360A and our isogenic mutants. In our previous experiment (Chapter Three), abscess formation was

not seen in any groups of mice before 48 hours and the abscesses drained by 5 to 6 days post infection. We used the Xenogen IVIS™ system to observe 360A during a systemic infection. Other experiments were performed using the Multi-view Bioluminescence Tomography System to examine both systemic infections and abscess formation using strain 360A. By using these systems to examine strain 360A we would gain valuable information in the infection process. After establishing this model we could further evaluate the role of CNF1, HlyA, and the terminal oxidases in their ability to contribute to the pathogenesis of strain 360A in both systemic and subcutaneous infections.

Materials and Methods

Bacterial strains and plasmids. The strains used in this section are located in Table 6.1. *E. coli* DH10B was used for all cloning purposes as a host for recombinant plasmids. All *E. coli* strains were grown in Luria-Bertani (LB) broth at 37ºC with shaking in the appropriate antibiotics.

Animal care. Mice were housed and cared for in accordance with the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech. Sites of animal housing were the animal facility in Derring Hall, Department of Biological Sciences (Blacksburg, VA) or the Center for Molecular Medicine and Infectious Disease (Virginia-Maryland Regional School of Veterinary Medicine, Blacksburg, VA).

Bacterial dosage experiments for systemic infections. Four groups of BALB/c mice (3 mice per group) were injected in the tail vein with 100 μL of *E. coli* 360A at a concentration of 8.2×10^3 to 8.2×10^6 CFU/mL suspended in phosphate buffered saline. This range of inoculums was used to determine the appropriate dosage for use in the systemic infection. In a previous study we used both BKS.Cg-m+/+*Lepr*^{db}/J (diabetic) and C57BLKS/J (non-diabetic) mice injected subcutaneously and observed that 15% of the mice infected with strain 360A succumbed to the infection in 2-4 days post-infection (Chapter Three). The inoculum size used for those infections ranged from 2×10^5 to 3×10^6.

Construction of a bioluminescent (*lux*) reporter for *E. coli* 360A. All plasmids that were used are described in Table 6.1. The P_{spc} promoter (179) was amplified from *E. coli* chromosomal DNA using the primers SPCF and RIGHTSPC (Table 6.1), which add *Pst*I and

Table 6.1. Bacterial strains, plasmids, and primers used in constructing a stable bioluminescent (*lux*) reporter.

Strain or plasmid	Relevance to Study and Stain Genotype[a]	Reference
***E. coli* strains**		
360A	parent strain of the mutants:MM1 through MM4	(197)
MM1	360A *hly* mutant: Cm^R, Tc^R	This study
MM2	360A *cyo* mutant: Gm^R	This study
MM3	360A *cyo/cyd* mutant: Gm^R, Cm^R, Tc^R	This study
MM4	360A *cnf1* mutant, Gm^R, Tc^R	This study
DH10B	used as a host strain for cloning, F^-, *endA1*, *recA1*, *galE15*, *galK16*, *nupG*, *rpsL*, *ΔlacX74*, Φ80*lacZΔM15*, *araD139*, Δ(*ara,leu*)7697, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), λ^-	Gibco/BRL
Plasmids		
pJT205	Contains the *P. luminescens lux* operon	(305)
pDHB60	Plasmid used for λ InCh procedure, contains the P_{tac} promoter	(35)
pDHB60Not+P_{tac}-	Backbone of the *lux* reporter without the P_{tac} promoter, Ap^R	J. Williams
pMDM89	*lux* reporter construct for use in strain 360A and derivatives, Ap^R	This study
Primers	**Sequence 5' to 3[b]**	**Reference**
SPCF	AA**CTGCAG**ACTGGTGTTCGGGTTGTCAT	This study
RIGHTSPC	CGC**GGATCC**TACACACCCGGCCTATC	X. Cui

[a]Tetracycline resistance (Tc^R); Ampicillin resistance (Ap^R); Gentamicin resistance (Gm^R)
[b]The bases highlighted in bold indicate the restriction sites.

*Bam*HI restriction sites, respectively. The PCR fragment was digested using *Pst*I and *Bam*HI (New England Biolabs, Ipswich, MA) and cloned directly into the plasmid pJT205, which carries the *lux* operon from *P. luminescens* [Biotechnical Resources, Manitowoc, WI, (305)]. This P_{spc}-*lux* reporter fusion was digested with *Pst*I and *Eco*RI (New England Biolabs). The fragment was blunt ended with T4 DNA polymerase (New England Biolabs) as described by the manufacturer's instructions. The fragment was cloned into the plasmid pDHB60Not+P_{tac}-, constructed by Josh Williams [the plasmid is a modified version of pDHB60, (35)], which was digested with *Sma*I and then dephosphorylated with calf intestinal phosphatase (New England Biolabs) as described by the manufacturer's instructions. pDHB60 was the primary vector used in the λ InCh procedure for chromosomal insertions as described by Boyd, *et al.* 2000. (35). This plasmid construct was named pMDM89 and was electroporated into electrocompetent strain 360A and four isogenic mutants MM1, MM2, MM3, and MM4 (Table 6.1). Recombinant cells were selected on LB containing 100 μg/mL ampicillin (Ap100) and the appropriate antibiotic for the chromosomal insertion (Table 6.1).

Stability of the bioluminescent (*lux*) reporter in *E. coli* 360A and four isogenic mutants. Strain 360A and its four mutant derivative were grown overnight in LB containing Ap100 and appropriate antibiotics for retention of the chromosomal insertions. The bacteria were then diluted 1,000-fold into fresh LB containing no antibiotics. The cultures were grown to an optical density between 0.9 and 1.3, serially diluted, and cultured on LB and LB plus appropriate antibiotics. These plate counts were used to examine the retention of the plasmid and the chromosomal insertions. This was done in order to determine if the bacteria were able to retain the plasmid and to evaluate if the burden of the bioluminescent reporter would have

detrimental effects on the chromosomal insertions. Light readings (in relative light units [RLU]) were also taken at the same time as optical density readings, and RLU/OD were calculated to examine the amount of light output for each culture. RLU were taken using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). Cells were then subcultured using a 1,000-fold dilution approximately 10-12 hours after the initial inoculation and grown overnight. The process was repeated for 5 days, which is more than two times the length of the proposed mouse infections. The stability of the *lux* reporter *in vivo* was examined using both subcutaneous and systemic infections, as described below.

Infection model for a systemic infection using the Xenogen IVIS™ system. BALB/c mice were used to establish the infection model. First, eight mice were injected intravenously in the tail vein with 100 μL of either strain 360A (2 mice) or strain 360A (pMDM89) carrying the P_{spc}-*lux* reporter (6 mice), and light output was examined in these mice. Two different inoculum sizes were tested approximately 5.0 x 10^5 CFU/mL and approximately 1.0 x 10^6 CFU/mL. Two mice succumbed to the infection using the larger inoculum of 1.0 x 10^6, so a lower inoculum 5.0 x 10^5 CFU/mL was used for the next assays. The mice were monitored at set time intervals, which include 4, 24, 48, 72, and 96 hours post-infection. These mice were used to help develop our model using the Xenogen IVIS™ system.

Infection model for subcutaneous (abscess) and systemic infections using the Multi-view Bioluminescence Tomography System. BALB/c mice were used to establish both infection models. First, strain 360A carrying the P_{spc}-*lux* reporter was examined for light output in BALB/c mice using the subcutaneous model. The mice were injected subcutaneously in the inner thigh region as previously described (41) with strain 360A carrying the *lux* reporter,

pMDM89, at approximately 1 x 10^5 CFU/mL. The mice were anesthetized with a ketamine/xylosine (50 μL/75 μL) mixture and light was imaged from the mice with an integration time of 5 to 10 minutes for 4, 24, 54, and 76 hours post infection. The mice were then euthanized as described in Chapter Three. The spleens, livers, and area of injection were removed and homogenized, and viable plate counts were performed on EMB with and without Ap100.

Mice systemically infected were injected as described in Chapter Five with approximately 1 x 10^5 CFU of strain 360A (pMDM89). The light output was imaged in the mice with an integration time of 10 to 15 minutes at 4, 24 and 48 hours post-infection. The mice were anestitized as described above. The mice were euthanized at 48 hours post-infection as described in Chapter Three. The liver and spleens were removed, homogenized, and viable plate counts were performed on EMB with and without Ap100.

Statistics. Statistics were done using either Microsoft Excel (Microsoft Corporation, Redmond, WA) or Cricket Graph III (Cricket Software).

Results and Discussion

Examination of dosage. The results of the mice injected with 8.2 x 10^3 to 8.2 x 10^6 CFU/mL dosage of strain 360A, only one animal succumbed to the infection at the highest dosage 8.2 x 10^6 (Table 6.2). These results show that at these dosages strain 360A is not very lethal to BALB/c mice.

Construction of a bioluminescent (*lux*) reporter for use in a mouse model. Several different luminescent vectors were constructed in an attempted to create a stable *lux* vector for use in strain 360A. Plasmids were constructed using three different promoters: an *E. coli* rRNA promoter; the hybrid promoter P_{tac}; and the promoter P_{spc} a promoter from an operon encoding ribosomal proteins (179). Three origins of replication were also used: pUC (140), pBR322, and from the medium copy pBBR1MCS (165) vectors. The *lux* containing plasmids with the pUC and pBBR1MCS were not stable in strain 360A or MM1 (*hly*). A 2-log decrease of resistant colonies was seen with the pUC based plasmid in strain 360A grown overnight at 37ºC in LB containing Ap100. Both the high and medium copy plasmids showed a low amount of light output when examined during log phase growth in the presence of antibiotics (data not shown).

This led to the construction of a λ InCh phage strain. The results of the phage insertion experiment showed a lack of colonies even after 2 days of growth following the phage infection. A modified plasmid used in the first step of the λ InCh procedure pDHB60Not+P_{tac}-, constructed by Josh Williams [a modified version of pDHB60 (35)] was used. Since this plasmid was constructed for the λ InCh procedure it was examined for light production in strain 360A. This plasmid, pMDM89, showed strong light output in a growth dependent manner for all 5 strains tested (Figure 6.1).

Table 6.2. Preliminary systemic infections to determine bacterial dosage of *E. coli* 360A.

	Number of dead mice/number of total mice injected for total number of bacteria			
Strain	8.2 x 10^3	8.2 x 10^4	8.2 x 10^5	8.2 x 10^6
360A	0/3	0/3	0/3	1/3

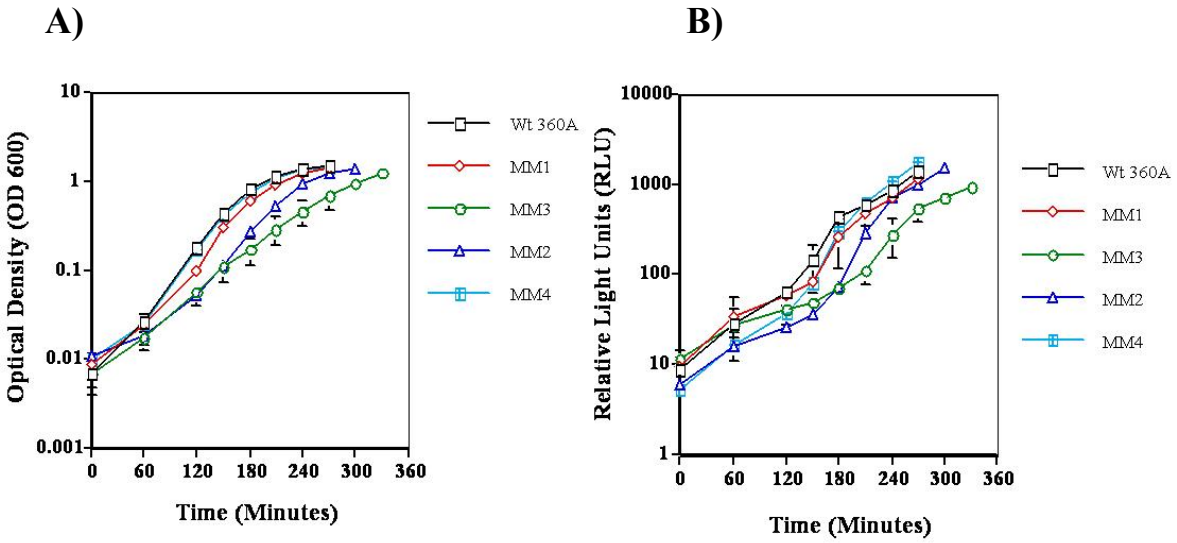

Figure 6.1. Growth curve and luminescence assay of *E. coli* 360A and four isogenic mutants carrying the *lux* plasmid pMDM89. Strain designations MM1, *hly*; MM3 *cyo/cyd*; MM2, *cyo*; and MM4, *cnf1*. **A)** Optical density: cells were initially subcultured to an OD between 0.007 and 0.010 and samples were then taken at 60 minute intervals for the first 2 hours and then 30 minutes after that for up to 330 minutes to monitor cell growth. **B)** Light output: samples were taken and light readings in relative light units (RLU) were evaluated using a TD-20/20 Luminometer (Turner Designs). The standard deviation for both graph A and B are indicated by the error bars.

Stability of the bioluminescent (*lux*) reporter in *E. coli* 360A and four isogenic mutants. Cells carrying the plasmid pMDM89 were grown in LB medium without antibiotics for five days. The light output and number of CFU/mL from Day 0 to Day 2 were very similar (Figure 6.2A and Figure 6.2B, respectively). At Day 3 the light output from the cultures was between 55-72% of the starting culture. By Day 4 the amount of light output dropped to between 9-43% of the starting culture, and by Day 5 the light output was only 7-30% of the starting culture (Figure 6.2A). The results from the viable plate counts showed that between days 0 and 2 approximately 70% of the cells possessed the *lux* plasmid (Figure 6.2B). Between Day 3 and 5 the amount of cells possessing the *lux* reporter dropped to less than 20% of the total population. These results indicated that the amount of light produced by each of the strains is stable for up to 54 hours, which includes 6 hours of growth before the first light reading and approximately 48 hours between the baseline reading and the second day (Figure 6.2A). The light output is variable for each culture, as noted by the large standard deviations. We wanted to examine the initial colonization of the mouse, which would be within 24 hours post-infection. The amount of time the strains retained the plasmid above 70% was between 48 and 72 hours post-inoculation of LB without antibiotics, which is more than sufficient than the 24 hours proposed for the systemic infections.

If our experiments were designed to go longer than 48 hours there would be no guarantee that the reporter would to be detected *in vivo*. The only possible problem that we could foresee is that we do not know how the growth in mice will have an affect on: 1) the amount of light produced per cell and 2) the amount of cells that are present at the specific time points during the

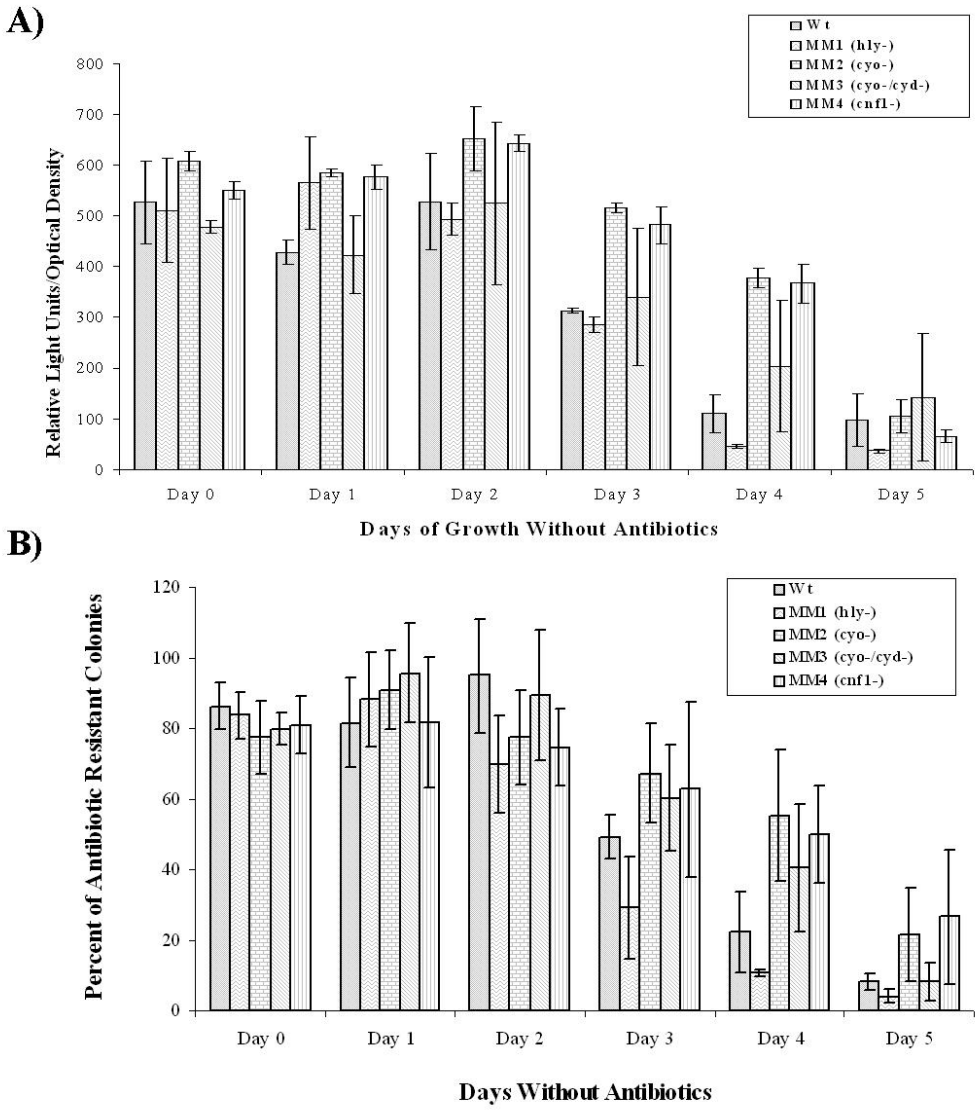

Figure 6.2. Stability of light output from *E. coli* 360A and four isogenic mutants carrying the *lux* plasmid pMDM89 for 5 days without antibiotic selection. A) represents the relative light units / optical density of the three strains strain 360A, MM1, *hly*; MM2, *cyo*; MM3, *cyo/cyd*; and MM4, *cnf1*, during a 5 day period of growth without antibiotics. B) represents the stability of the *lux* reporter over time. The results show stability is not a problem for the first two days without antibiotics. Cells were subcultured by a 1,000-fold dilution twice a day. At the end of the exponential growth phase, which was between OD 0.98 and 1.30, the cells were examined for light output and samples were taken to determine total CFU/mL of cells carrying pMDM89. The assays were performed in three independent trials.

experiment. By evaluating the amount of light output during the course of a 4 to 5 day infection we can hope to answer these questions before going on with the full study.

Infection model for a systemic infection using the Xenogen IVIS™ system. A set of eight mice were injected intravenously in the tail vein with approximately 1 x 10^6 CFU of either strain 360A (2 mice) or strain 360A carrying the P_{spc}-*lux* reporter (6 mice), pMDM89. The infection was allowed to proceed for 72 hours. Of the six mice injected with strain 360A carrying pMDM89, two died, one in less than 24 hours and the other in less than 72 hours there were no fatalities among the mice infected with strain 360A without pMDM89. The spleens and livers were removed from all surviving mice and serial dilutions and viable plate counts were performed on Eosin Methylene Blue (EMB) agar (Becton-Dickinson and Company, Cockeysville, MD) and EMB containing Ap100. Samples were also taken and evaluated using a TD-20/20 Luminometer (Turner Designs). Very weak light output was seen from all of the mice infected with strain 360A carrying pMDM89 (data not shown). From these samples only two of the mice infected with strain 360A (pMDM89), showed any CFU numbers and the bacteria were found mainly in the liver (Figure 6.4A). These two mice also had liver samples that were positive for light output, and one of those mice also had a blackened tail. The number of colonies on both EMB and EMB containing Ap100 were virtually identical, suggesting that unlike the *in vitro* experiments, the plasmid pMDM89 was stable in strain 360A during *in vivo* experiments. However, the amount of bacteria at 72 hours was low in both the mouse liver and spleen of the mice sampled.

The results from the tissue sampling lead us to change the protocol since two of the mice infected with strain 360A carrying pMDM89 succumbed to the infection; we lowered the dosage

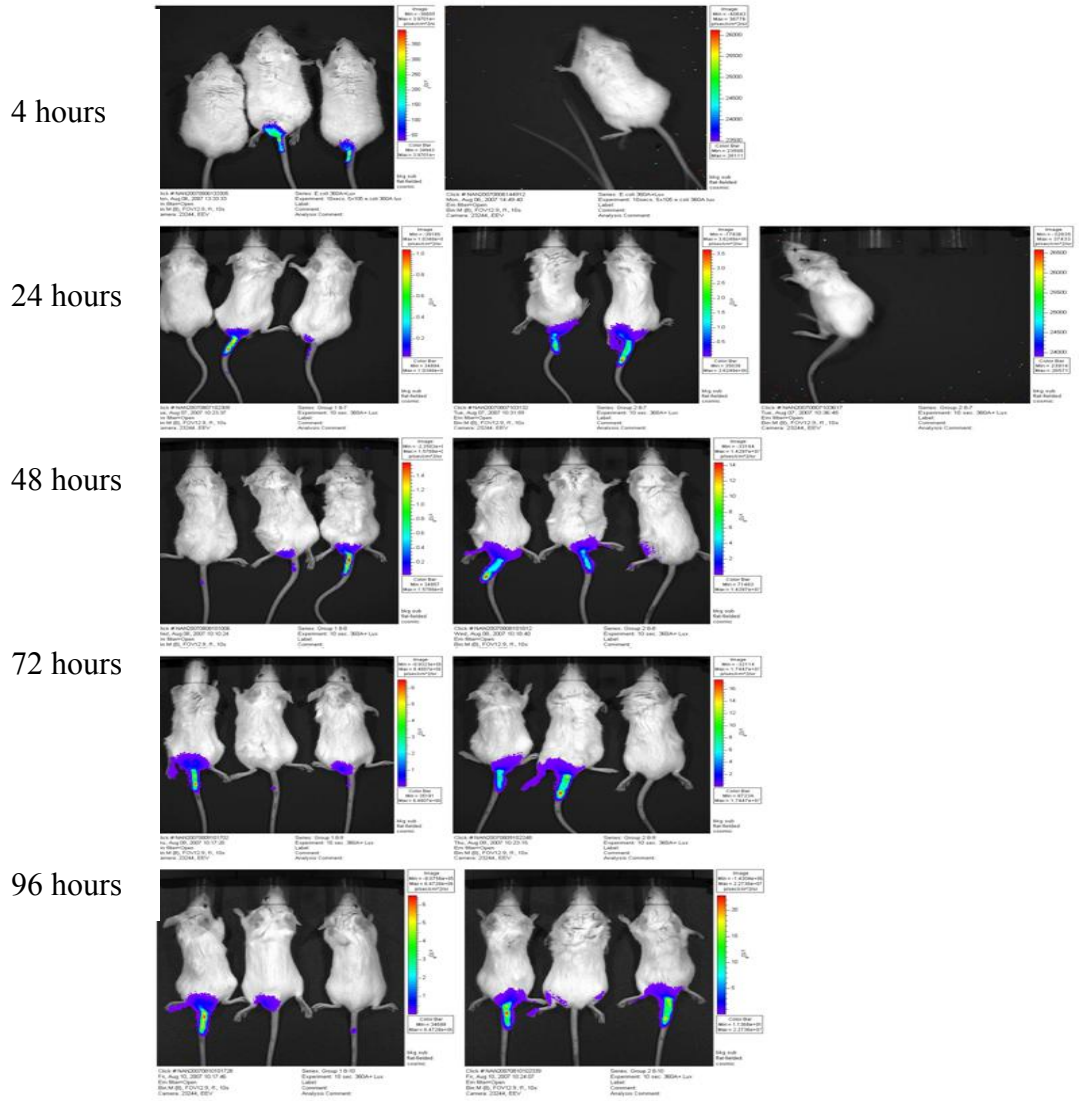

Figure 6.3 Detection of *in vivo* luminescence using the Xenogen IVIS™ system. Six BALB/c mice were injected intravenously in the tail vein with approximately 5 x 10^5 CFU. Light output was monitored from all of the mice for 96 hours. Light output was observed from 3 of the mice for all of the time points examined.

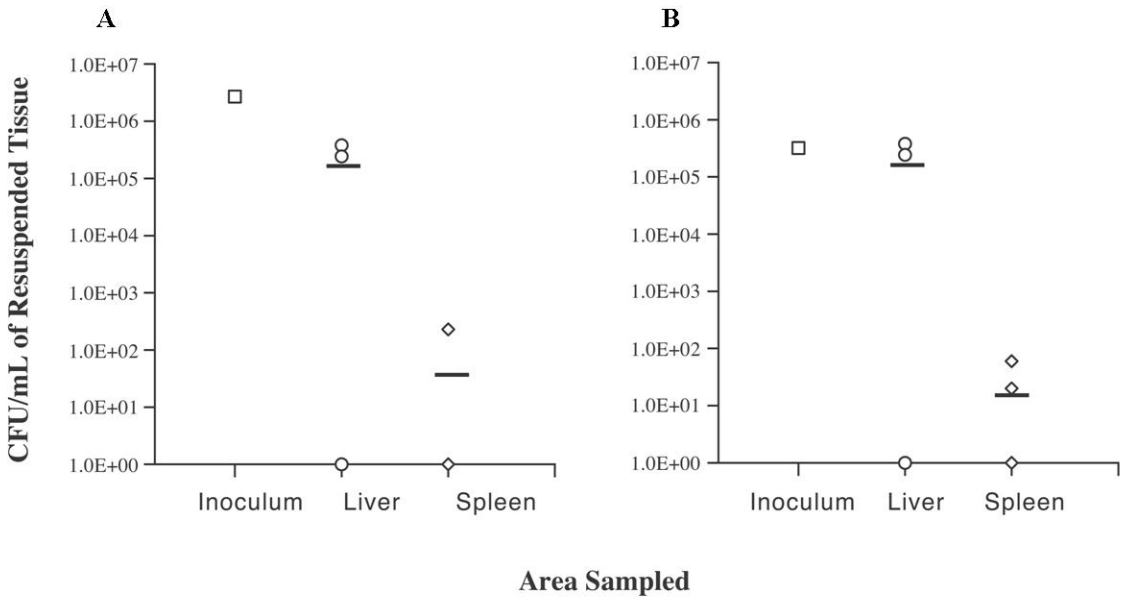

Figure 6.4. Recovery of *E. coli* 360A carrying the *lux* reporter, pMDM89, from a systemic infection, monitored by the Xenogen IVIS™ system. A) Samples taken from 4 BALB/c mice infected with strain 360A, pMDM89, for 72 hours without monitoring light output. B) Samples taken from 6 BALB/c mice infected with *E. coli* 360A, pMDM89, for 96 hours with the monitoring of light output using the Xenogen IVIS™ system. The samples analyzed in B do not correlate with the light output from Figure 6.3. Figure 6.3 did not show light output from any portion of the mice except the immediate area of injection.

from 1 x 10^6 to 5.0 x 10^5. The lower dosage of strain 360A carrying pMDM89 did not cause any deaths of the mice injected; however one mouse injected with strain 360 (without pMDM89) was euthanized after 24 hours. The remaining mice were imaged for 96 hours and are shown in Figure 6.3. At 4 hours three mice were imaged and two of them showed light output from the area of injection. Light output at 24 hours was seen from the tail of 4 of the 6 mice infected with strain 360A carrying pMDM89. For the next 3 days the amount of light output increased in these four mice, but never spread from the initial area of inoculation. The mice were euthanized at 96 hours post-infection and both the liver and spleen were removed, and cultured. The results of the viable cell counts showed high bacterial counts in two of the mice in the liver (Figure 6.4B). The amount of bacteria in the spleen was very low, indicating that 96 hours post-infection, which may be too long for the infection to go since the number of CFUs recovered from the spleen was low. The comparison of the resistant colonies to the total number of colonies in these data showed approximately 100% resistance to Ap. These data correlates with the previous experiment in that the *lux* reporter, pMDM89, is stable *in vivo*. What we did not observe using the IVIS™ system was light from the spleen. This may be due to the short 3-5 second exposure time. A longer exposure time may be required to see the light from the spleen unless the light output is too faint to detect in deeper tissue.

Infection model for subcutaneous (abscess) and systemic infections using the Multi-view Bioluminescence Tomography System. We examined the use of a second system developed by Dr. Ge Wang (Virginia Tech) for both a subcutaneous and a systemic murine infection. This system utilizes multiple images and the use of a 3D model to help more accurately pinpoint the light. The subcutaneous infections proceeded for 76 hours (Figure 6.5).

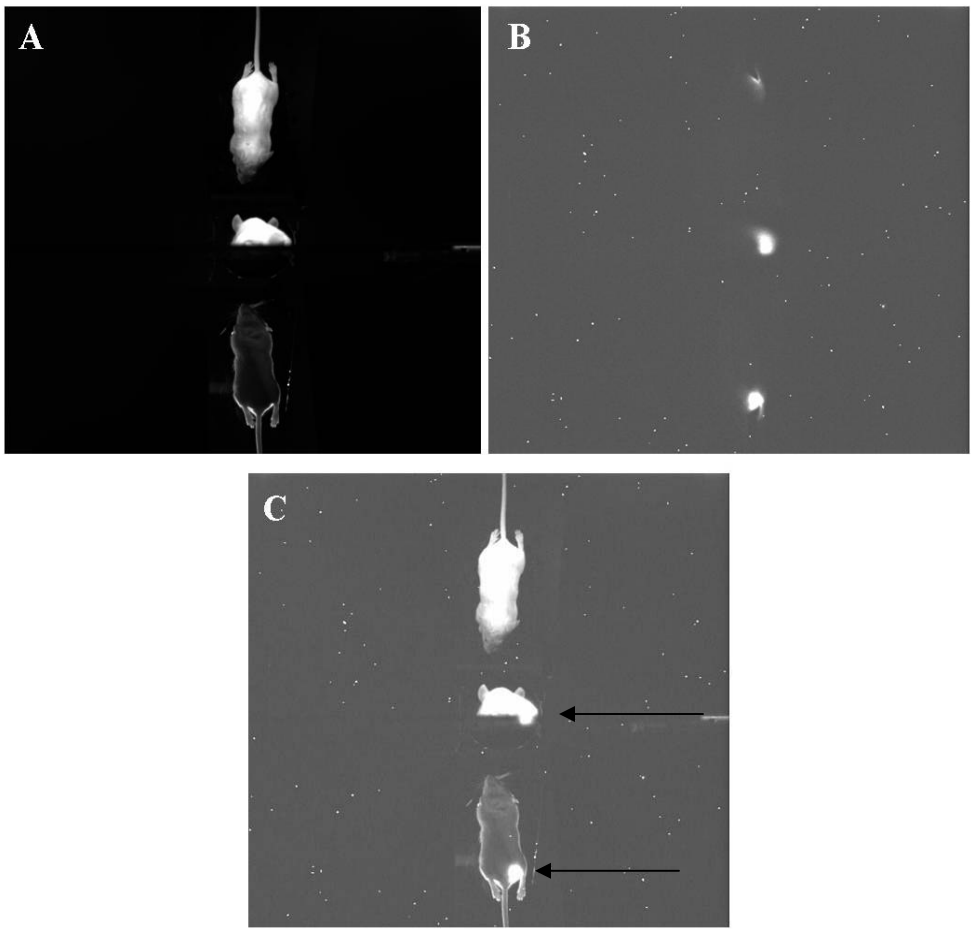

Figure 6.5. Detection of *in vivo* luminescence using the Multi-view Bioluminescence Tomography System. Mice were injected subcutaneously with approximately $1x10^5$ strain 360A carrying pMDM89. The mice were imaged at 4 time points; 76 hours post-infection is shown above. Panel C is a merged image of panels A and B. Light output is clearly seen in the area of injection as indicated by the two arrows.

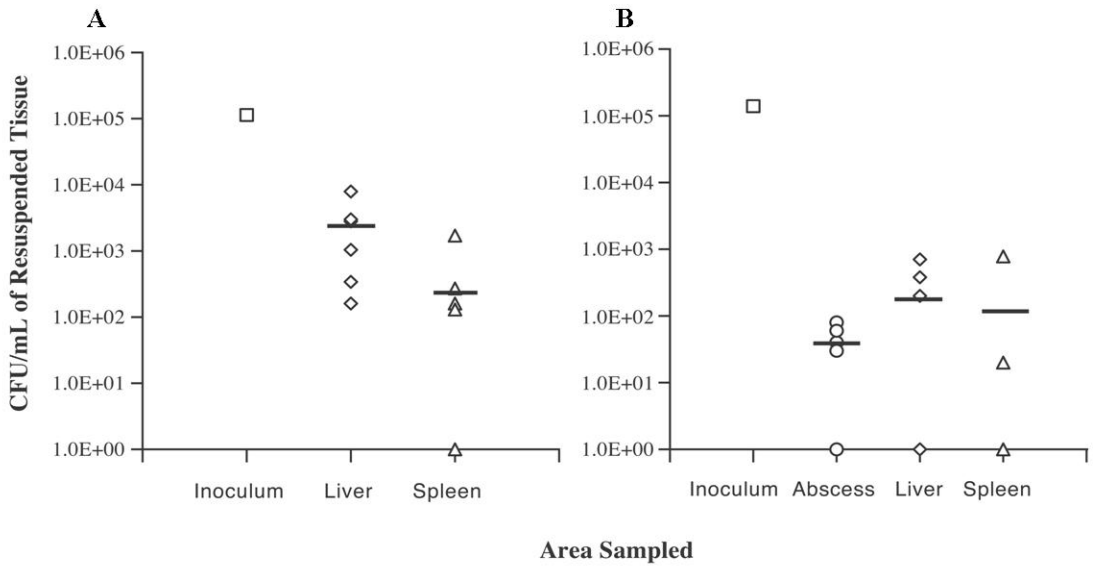

Figure 6.6. Recovery of *E. coli* 360A carrying the *lux* reporter, pMDM89, from a systemic infection monitored by the Multi-view Bioluminescence Tomography System. A) The results from 6 BALB/c mice injected intravenously in the tail vein with approximately $1x10^5$ CFU of strain 360A. These mice were monitored for 76 hours. B) The results from 6 BALB/c mice injected subcutaneously in the left groin region with approximately $1x10^5$ CFU. These mice were monitored for 48 hours.

There was noticeable light output from all mice in the area of injection, but no light was seen anywhere else in the mouse (Figure 6.5). Necropsies indicated there were no abscesses in the mice infected with very low levels of bacteria in the area of the injection, liver, and spleen (Figure 6.6B). In addition, the retention of the plasmid was low in this strain, with 38%, 9%, and 35% antibiotic resistant colonies from the liver, spleen, and area of injection, respectively. These data are different from those obtained in the IVIS experiment, where approximately a 100% of the colonies were still antibiotic resistant.

The results of the light output from the intravenous infections examined using the Multi-view Bioluminescence Tomography System did not show any noticeable light anywhere in the mouse that was distinctly different from non-inoculated control mice (Data not shown). These mice were monitored for 48 hours. The culture results from this experiment are shown in Figure 6.6A. Bacterial numbers were relatively high from both the liver and the spleen at an average of approximately 5×10^4 and 2×10^2, respectively. However, only 1% of the colonies were still antibiotic resistant from the liver and 0% from the spleen. This low level of resistant colonies is most likely the reason for the lack of light output seen during the experiment. Another factor contributing to the low recovery of resistance may be due to only approximately 80% resistant colonies seen in the original inoculum.

Conclusions

In this study we were able to construct a *lux* gene reporter for use in *E. coli* 360A and visualize light output both *in vitro* and *in vivo*. Unfortunately, several things may have gone wrong: 1) the intensity of the light output was not strong enough for us to detect it in the internal organs of the mice using either of the systems described above and 2) the light output pattern from the tail of the mice using the Xenogen IVIS™ system may have been due to improper injection. The bacteria were not successfully injected in the tail vein, either they were injected in the tail itself or leaked out of the vein following injection. Further investigation into this model would have to be done in order to create a *lux* reporter with stronger expression to allow for us to visualize the bacteria deep inside a mouse.

Chapter Seven

Overall Conclusions

The overall goals of my project were to: 1) examine promoter structure and function in *Bacteroides* using a bioluminescent gene reporter system to examine expression levels, 2) utilize polymicrobial infections to help determine how different bacteria contribute to bacterial synergy in an infection using a diabetic mouse model, and 3) further examine the ability of *Escherichia coli* 360A to cause a systemic infection.

Promoter expression in *Bacteroides*. In conjunction with Mary Thorson, two promoters: the well studied *cepA* promoter (196, 255) from *Bacteroides fragilis* and a *Bacteroides thetaiotaomicron* 16S ribosomal RNA promoter were used to examine gene expression. The *B. thetaiotaomicron* 16S rRNA promoter was analyzed to further determine the consensus sequence for *Bacteroides* promoters (Figure 2.1A). We determined that both the P1 and P2 promoters were able to independently initiate transcription and the upstream sequence of P1 is important for the activity of the P1, but not the full-length (both P1 and P2) 16S rRNA promoter. We further verified the -33 and -7 regions of the P1 promoter are both important for its activity. We were able to determine that the differences in gene expression between *E. coli* and *Bacteroides* occur during both transcription and translation.

Polymicrobial infections. A diabetic mouse model for examining bacterial synergy during a polymicrobial infection was established. We observed that the diabetic mice were more susceptible to polymicrobial infections than their non-diabetic counterparts. We determined that *B. fragilis* strain NCTC 9343 benefited most from the presence of other organisms, especially *E. coli* 360A. *E. coli* 360A did not benefit from the presence of the other organisms, and was able to produce abscesses leading to a 15% mortality rate. *C. perfringens* seemed to make no contribution in this infection and would have to be examined in further studies to see if in fact it

could play a role as a secondary colonizer at the site of infection. Due to the importance of *E. coli* 360A in this study, the remainder of my thesis research focused on evaluating its virulence and ability to cause infections.

Virulence of *E. coli* 360A during a systemic infection. I focused on how two virulence factors (CNF1 and HlyA) and environmental modulators (terminal oxidases) were involved in the interactions with two murine macrophage-like cell lines and the role they play in the pathogenesis and survival in a mouse model for a systemic infection. It was observed that the absence of either a functional *hlyA* or *cnf1* gene increases the number of internalized bacteria in both murine macrophage lines tested. These results may indicate a decrease in the virulence of the strain, by affecting the strains' ability to escape the phagosome. Since the intracellular cell numbers of strain 360A were significantly lower than both of the single toxin mutants, one can infer that both CNF1 and HlyA were involved in the bacteria escaping from the phagosome and then the macrophage. Hence, strain 360A would come into contact with streptomycin, which is used to kill extracellular bacteria. It was also observed that the double mutant (*cnf1/hly*) seemed to be killed more effectively by the macrophages than either of the single toxin mutants. The decreased virulence of the (*cnf1/hlyA*) strain was seen in the systemic infection, where this double mutant had a statistically significant decrease in cell numbers as compared to strain 360A. In contrast, the single *cnf1* and *hlyA* mutants did not show any differences in cell numbers compared to strain 360A. It would seem that a strain lacking both of these virulence factors (CNF1 and HlyA) is significantly affected in its ability to colonize or survive in the spleen of a mouse.

Compared to strain 360A both the single (*cyo*) and double (*cyo/cyd*) oxidase mutants showed similar cytotoxicity. The double oxidase mutant was compared to strain 360A using invasion assays involving murine macrophage-like cell lines, these results also showed similar numbers of CFU recovered from the macrophages. However, when tested in a mouse, both the single (*cyo*) and the double (*cyo/cyd*) oxidase mutants have a statistically significant decrease in cell numbers, compared to strain 360A recovered from the spleen. The factor common to these two mutants is the *cyo* mutation, which indicates that the gene products of the *cyo* operon seems to be more important to the survival in a mouse spleen than those of the *cyd* operon. One possible explanation for these results may be the effect of the mutation on growth-rate. However, both of the oxidase mutants tested showed similar growth rates in rich laboratory medium, cell culture medium, and only a slight difference in M9 minimal medium containing glucose, compared to strain 360A. Another possibility is the increased sensitivity of the mutants to oxygen radicals, which is expected to impair the cell's ability to survive. Further studies are necessary to examine this phenomenon. Additional experiments to measure the growth rate of these cells in mouse blood culture or to examine the number of bacteria present in the blood of infected mice after the 12 hour experiment would also provide insights into the development of a systemic infection. Examining the virulence of oxidase mutants (*cyo* or *cyo/cyd*) and virulence factor mutants (*hlyA*, *cnf1*, or *cnf1/hlyA*) in further mouse experiments using *in vivo* imaging methods beyond those we have attempted could further allow us to determine the interactions between *E. coli* and its mammalian host. The ability to track these infections would provide more insights on how these organisms cause infections, as well as where they can reside during the progression of the disease. Using mutants that have altered virulence properties in these

types of infections could lead to better understanding the way in which these organisms cause disease.

Concluding remarks. Extra-intestinal infections caused by enteric organisms pose serious health risks to humans. Many of these infections can become recurring, such as urinary tract infections and wound infections, especially in immuno-compromised people. In order to better understand these infections, we must understand the molecular basis of pathogenesis. Determining which virulence factors are responsible for causing the infection could lead to the development of vaccines or other intervention therapies to prevent these diseases. In our experiments that examined a systemic infection, we observed that the loss of functional oxidases or two key virulence factors hindered the ability of *E. coli* strain 360A to survive in the spleen of a mouse. These key gene products are likely involved in both the establishment of the infection or survival in the blood. Additional factors may also be involved in the ability of *E. coli* strains to cause this type of infection. Therefore, further investigation of other factors, such as adhesins, may lead to better understanding of this pathogen.

Virulence factors may also play a critical role in polymicrobial infections. When examining bacterial synergy during a polymicrobial infection several different interactions are happening at the same time: 1) bacterial-bacterial interactions and 2) bacterial host interactions. In examining synergy during these types of infections researchers first have to determine who the major players are in these interactions. Some organisms have been seen to provide synergy to some organisms and not others [Chapter Three and Verweij-van Vught, *et. al.*, 1985. (306)]. Identification of the bacteria responsible for providing synergy to other bacteria (i.e. *E. coli* in this dissertation work) could help guide treatment strategies. Different drug regimens could be

tailored for various combinations of bacterial pathogens in order to treat the infections. Doctors and other medical professionals would have to work together to formulate these regimens by: 1) examining samples from patients to determine the bacteria present in the infection, 2) prescribing a drug regimen for patients that are infected with organisms that have been shown to play a major role in these infections, and 3) monitoring the infections.

This would be the ideal scenario, but we do not yet have a good understanding of the complexities associated with polymicrobial infections. First, the organisms in the infection may not be identified easily, such as those that have long generation times or specific growth requirements. Second, antibiotic susceptibility tests may have to be performed on some organisms due to multiple drug resistant strains. Both of these points would lead to a delay in treatment of the patient and may increase the risk of death due to the seriousness of the infection. Finally, laboratory experiments with respect to synergy performed on mice or other small mammals may not mimic the true nature of the infection in a human. Since not all people have the same physiological make-up, health issues, and immune systems, some individuals would be able to fight off these different pathogens better than others. However, studies such as those present here, are important first steps in leading researchers to a better insight into how different bacterial species interact with one another.

Chapter Eight

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Appendix I

Supplemental Material for Chapter Two:

Characterization of a *Bacteroides thetaiotaomicron* 16S rRNA Promoter with a new *lux* gene reporter system

Materials and Methods

Efforts to improve the *lux* reporters. In an attempt to make the system more amenable to rapid screening of a large number of isolates, other methods were used to observe the production of light. These included the growth of cells containing the pMMT1F vector (wt *cepA* promoter), on TYG grid plates (Em and Gm) at 37^{o}C anaerobically for 2-3 days, followed by saturation of the plate with 10% sonicated decanal. These plates were observed and then exposed to x-ray film for 5 minutes at the point of decanal addition and again ten minutes later.

TYG plates of *B. thetaiotaomicron* containing pMDM-series and pMMT-series constructs were removed from an anaerobic environment, isolated colonies were then scraped off of these plates every 5 to 10 minutes for one hour and resuspended in a luminometer cuvette in 50 µL of luciferase assay buffer (10 mM KPO_4, 1 mM dithiothreitol, and 0.1 g bovine serum albumin per 100 mL). One microliter of 10% sonicated decanal was added to the cell suspension and light output was monitored in a luminometer with an integration time of 4 seconds.

Finally, 96-well plate assays were performed with both, 200 µL TYG agar plugs and 200 µL TYG broth cultures plus Gm200 and Em10. Colonies of the pMDM- and pMMT-series vectors were removed from TYG plates and inoculated into the pre-reduced TYG medium in an anaerobic environment. Broth cultures were incubated for 24 hours and agar cultures were incubated for 48 hours. Both plates were covered in parafilm to prevent evaporation, and incubated at 37^{o}C in a COY anaerobic chamber (Coy Laboratory Products). Light output was evaluated using a SpectraFlour Plus plate reader (Tecan US), with a gain of either 100 or 125 and an integration time of 500 ms.

Results

Efforts to improve the *lux* reporters. The TYG plates flooded with the decanal substrate were exposed to x-ray film showed a very faint emittance of light that could be seen by the naked eye, but lasted for less than a minute. Unfortunately, the images on the x-ray film were too diffuse to visualize individual colonies. When examining colonies resuspended in assay buffer, some light output above background was observed for up to 30 minutes from cells containing pMDM1 and up to 60 minutes for cells containing pMMT1F and pMMT1H. These time limitations were presumably due to the decreased energy/reduced coenzyme levels of the cells upon exposure to oxygen. Overall, the cells containing these constructs qualitatively produced less light when scrapped off a plate than the levels seen using cells containing these constructs grown to mid-logarithmic phase in broth culture.

Ninety-six well plates were inoculated with *B. thetaiotaomicron* carrying *cepA* promoter constructs, pMDM1, pMMT1F, and pMMT1H, for 2 days. The plates were taken from the chamber and 2 to 5 μL of a 10% decanal was added to broth and agar cultures, respectively. Fortunately, the amount of light output from the broth cultures containing the pMMT1F and pMMT1H constructs was 2 to 15 times above the background levels for approximately 30 minutes. The background levels from the negative control cultures were only 2 to 5 RLU. However, with broth cultures containing pMDM1 the maximum light output was only 4 to 6 times above background that lasted for only 5 minutes and the amount of light output from these strains was very weak.

When the cultures containing the pMMT1F and pMMT1H were examined grown in the agar plugs, the RLU from these strains were above background for only about 10 minutes.

Therefore, high throughput qualitative measurements to score for the presence of light output are possible for a short amount of time following the addition of the substrate. The best results were achieved using the pMMT-series vectors with the wt *cepA* promoter grown anaerobically in broth cultures on microtiter plates. However, these assays might not be applicable for detecting promoter strength since subtle differenced in expression between the wt *cepA* promoter (pMMT1F) and a variant with 33% activity (pMMT1H) showed little difference in these assays. These assays would have to be evaluated on a promoter by promoter basis using appropriate controls.

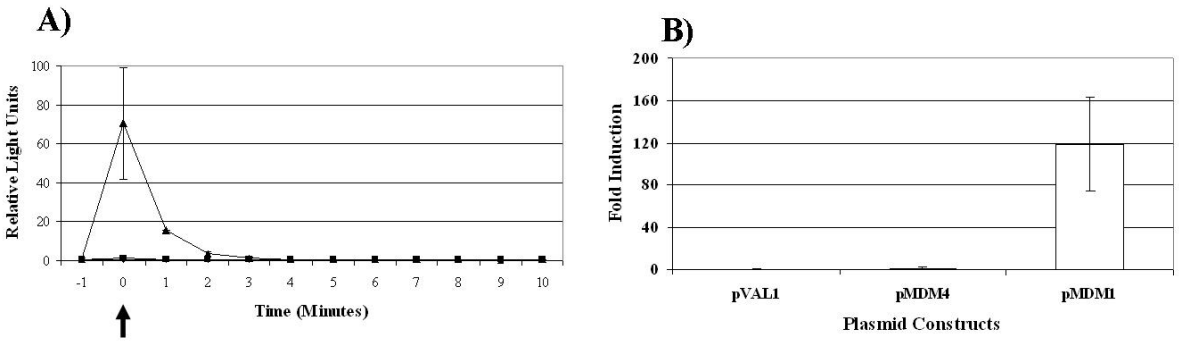

Figure AI.1. Luminescence and luciferase assays of *B. thetaiotaomicron* 4001 using the pMDM-series *cepA* promoter construct. A) The graph illustrates qualitative studies of whole cell luminescence assays performed on *B. thetaiotaomicron* 4001 strains containing the pMDM-series constructs (refer to Table 2.1). pVAL1 (♦), pMDM4 (■), and pMDM1 (▲). Data shown is representative of experiments performed in two independent trials done in replicates of two. The black arrow at time point 0 indicates the addition of decanal. **B)** The graph illustrates *in vitro* luciferase assays performed on crude cell extracts from strains containing the indicated vectors. From left to right: pVAL1, vector control with no *lux* operon; pMDM4, promoterless control; pMDM1, *cepA* promoter. Data is based on fold induction as compared to the expression levels of pMDM4. The average values in relative light units for pMDM4 containing cultures was 0.113 +/- 0.008 from three independent trials performed in replicates of six.

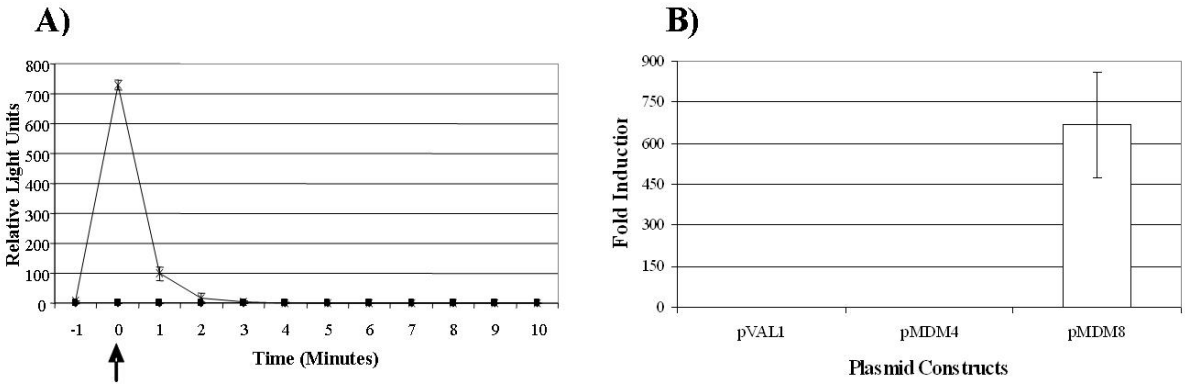

Figure AI.2. Luminescence and luciferase assays of *B. thetaiotaomicron* 4001 using the pMDM-series 16S rRNA promoter construct. **A)** The graph illustrates qualitative studies of whole cell luminescence assays performed on *B. thetaiotaomicron* 4001 strains containing the pMDM-series constructs (refer to Table 2.1). pVAL1 (♦), pMDM4 (■), and pMDM8 (▲). Data shown is representative of experiments performed in two independent trials done in replicates of two. The black arrow at time point 0 indicates the addition of decanal. **B)** The graph illustrates *in vitro* luciferase assays performed on crude cell extracts from strains containing the indicated vectors. From left to right: pVAL1, vector control with no *lux* operon; pMDM4, promoterless control; pMDM8, full length 16S rRNA promoter from *B. thetaiotaomicron*. Data is based on fold induction as compared to the expression levels of pMDM4. The average values in relative light units for pMDM4 containing cultures was 0.113 +/- 0.008 from three independent trials performed in replicates of six.

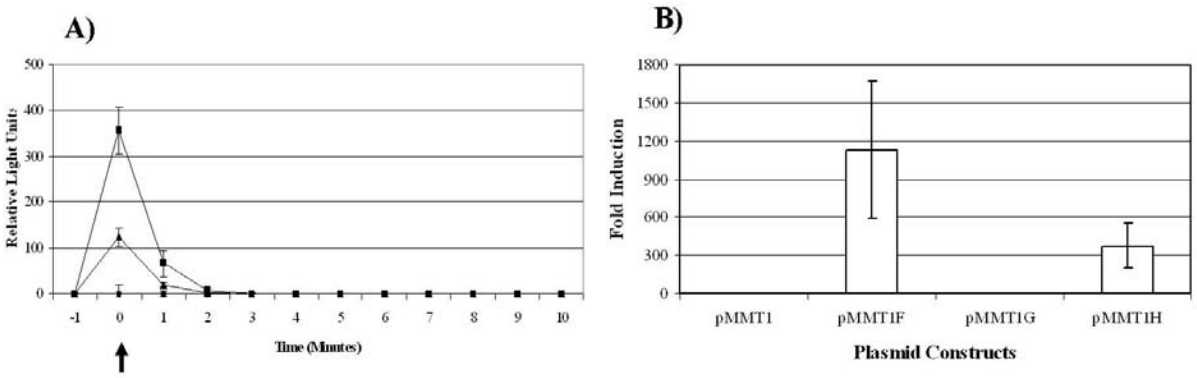

Figure AI.3. Luminescence and luciferase assays of *B. thetaiotaomicron* 4001 containing the wt and *cepA* promoter variants. **A)** The graph illustrates qualitative studies of whole cell luminescence assays performed on *B. thetaiotaomicron* 4001 strains containing the pMMT-series constructs (refer to Table 2.1). pMMT1 (♦), pMMT1F (■), pMMT1G (**x**), and pMMT1H (▲). Data shown is representative of experiments performed in two independent trials done in replicates of two. The black arrow at time point 0 indicates the addition of decanal. **B)** The graph illustrates *in vitro* luciferase assays performed on crude cell extracts from strains containing the indicated vectors. From left to right: pMMT1, promoterless control; pMMT1F wt *cepA* promoter; pMMT1G, *cepA* promoter variant (0% activity); pMMT1H, *cepA* promoter variant (33% activity). Data is based on fold induction as compared to the expression levels of pMMT1. The average values in relative light units for pMMT1 containing cultures was 0.048 +/- 0.025 from three independent trials performed in replicates of six.

Appendix II

Supplemental Material for Chapter Three:

Synergy in Polymicrobial Infections in a Mouse Model of Type 2 Diabetes

Materials and Methods

Examination of *E. coli* 360A inhibition of bacterial growth in laboratory media. Although some synergy was seen between *C. perfringens* and *B. fragilis*, no synergy was observed between *C. perfringens* and *E. coli* even in the presence of *B. fragilis*. To address the issue of whether *E. coli* was impairing the ability of *C. perfringens* to grow in different types of laboratory culture media. Each culture was grown up overnight in the following media: *B. fragilis* in 10 mL of TYG at 37^{o}C anaerobically, *C. perfringens* in 3 mL of PGY at 37^{o}C anaerobically, and *E. coli* in 6 mL of LB at 37^{o}C aerobically. Samples of each, *B. fragilis*, *C. perfringens*, and *E. coli*, were streaked on TYG agar, where two strains were allowed to grow in contact with each other (Figure AII.1). These plates were incubated at 37^{o}C anaerobically for one to two days and were subsequently examined for growth. Samples were taken from the mixed regions and from regions where the organisms grew by themselves. The samples were Gram stained and examined under a microscope. Next, cultures of *C. perfringens*, and *E. coli* were grown together in 7 mL of Brain Heart Infusion broth (BHI, Difco Laboratories, Detroit, MI) to determine if these cultures are able to grow together in liquid culture. These cultures were grown at 37^{o}C anaerobically for two days. Samples of these cultures at one and two days after inoculation were also Gram stained and examined under a microscope. Finally, *C. perfringens* was grown in a 3 mL of PGY at 37^{o}C anaerobically with varying amounts cell free supernatant from an *E. coli* 360A overnight culture to examine if this strain 360A produces any bacteriostatic or bactericidal compounds that are harmful to *C. perfringens*.

Examination of trends seen in experiments comparing control mouse strain of C57BLKS/J to the diabetic mouse strain of BKS.Cg-*m* +/+ *Lepr^{db}*/J. 5- to 6-week-old and

23- to 24-week-old BKS.Cg-*m* +/+ *Lepr*^{db}/J, C57BLKS/J and their diabetic littermates were compared at the height of the bacterial infections at 8 days post-infection. Mice were examined for CFU/mL of resuspended tissue to examine if diabetes influenced bacterial survival during an infection.

Evaluation of secondary infections due to complications during a bacterial infection. Mice were examined to see if bacteria were present in the spleen. Mice were also monitored for complications do to the bacterial infections. Records were kept of mice that died due to the complications from the bacterial infections and are outlined in Table AII.1 and AII.2. The spleens of at least 6 mice in each experimental group were taken and the tissue was homogenized in the same manner as outlined in Chapter Three. Serial dilutions and plate counts were done to determine the presence of bacteria in the spleen. The limit of detection was set at 200 CFU/mL and any number below this value was not included in further statistical analysis. Exact numbers of bacteria were not able to be taken due to the small amount of dilutions plated, only qualitative data was able to be done.

Results and Discussion

Examination of *E. coli* 360A inhibition of bacterial growth. Samples of the cross-streaked regions of the organisms from the TYG were Gram stained and examined under a microscope. When examining the *Clostridial* cells we observed elongated bacteria, when they were grown in the presence of either *E. coli* or *B. fragilis*. Further examination was done using *C. perfringens* and *E. coli* in BHI broth culture. Some *C. perfringens* cells looked elongated as in the crossed streaked regions on the TYG plates, but for the most part they resembled normal box car shaped cells. This growth was allowed for two days and similar results were seen. *C. perfringens* was also grown with *E. coli* 360A supernatant the effect seen on TYG was also not seen. These results suggest that *C. perfringens* does not grow as well on TYG medium, due to the lack of proteose peptone #3, as they due on plates containing this ingredient, which is part of its standard growth medium, PGY.

Examination of trends seen in experiments comparing control mouse strain of C57BLKS/J to the diabetic mouse strain of BKS.Cg-*m* +/+ *Lepr*^{db}/J. A comparison of control and experimental mice was done using day 8 post infection as a comparison point (Figure AII.2). Day 8 was used since both *E. coli* and *B. fragilis* were at the height of their bacterial concentrations in the infection and the following days saw decreases in one or both of the cultures. There was no difference in the CFUs between the young and aged control mice, indicating that the age of the mice was not a factor in the susceptibility to polymicrobial infections. Comparably aged diabetic mice had higher levels of CFUs than control mice, indicating that diabetic mice were more susceptible to polymicrobial infections than non-diabetic

mice. What is also seen from this comparison is that diabetes contributes to the susceptibility of a polymicrobial infection in this strain of mice.

Evaluation of secondary infections do to complications during a bacterial infection. Records were kept outlining any deaths that occurred during the course of the infection and what infectious agent the mice were infected with (Table AII.1 and AII.2). Out of all the pre-diabetic and diabetic mice injected with *E. coli* 360A 18% of them died from this infection. The highest mortality was seen in the infection involving all three pathogens, where 39% of the prediabetic mice died from the infection (Table AII.1). It was also noted that the mortality rate of the 5- to 6-week-old control mice was 46% (Table AII.2). The number of mice sampled that showed bacterial loads greater than 200 CFU/mL are shown in Figure AII.3. The pre-diabetic and diabetic groups of mice infected with *E. coli* either in singly or in combination with other organisms had the highest incidence of bacteria in the spleen, 64% and 89%, respectively. Both of the groups of control mice were seen to have *E. coli* in 50% of spleens from the mice sampled. *B. fragilis* was also found in the spleens mainly in mixed infections with *E. coli* at a frequency of 27%, 32%, and 45% for the pre-diabetic, diabetic, and 23- to 24-week-old control mice, respectively. These numbers may indicate either the ability of these two pathogens: 1) to cause a secondary infection in the mice by escaping the area of injection or 2) an attempt by the host immune system to clear the infection. In either case *B. fragilis* seems to directly benefit from the presence of *E. coli* 360A in the form of overall numbers and the length of time that *B. fragilis* is present in the mice during the infection.

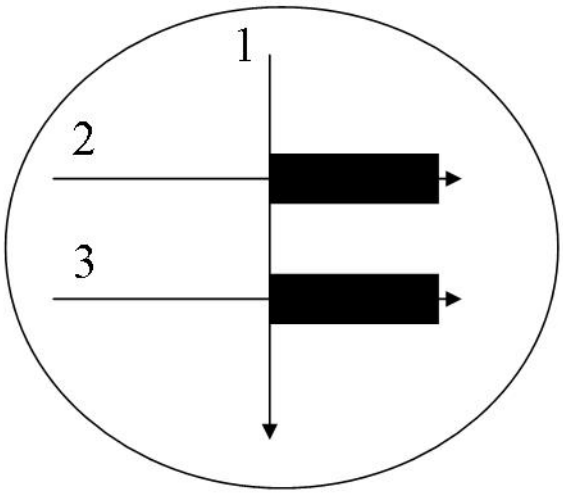

Figure AII.1. Cartoon depiction of cross streaking of bacterial strains. The plate above shows three arrows in which the bacteria were streaked. 1) the first organism was streaked down the middle of the plate, 2) the second organism was streaked across the plate, and 3) the third organism was then streaked across the bottom of the plate. Each individual organism grows across the streak (the portion that is not highlighted). In the highlighted region two organisms grow together. These plates were incubated anaerobically for one to two days after inoculation. The samples from the black regions were Gram stained and examined under a microscope for any differences in cell morphology compared to the regions were only one organism grew.

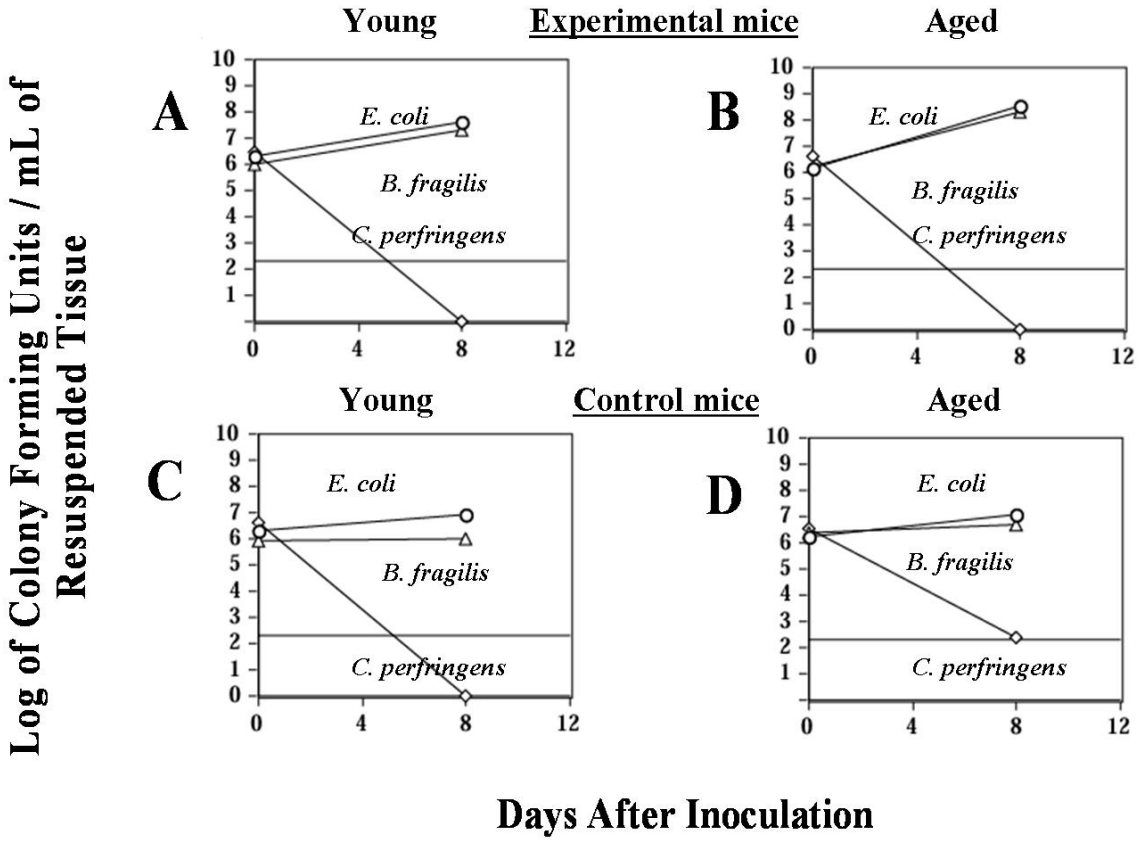

Figure AII.2. Comparison of experimental and control mice of the same age. This is a comparison of the tri-infection at day 8 between experimental and control mice. For all panels A to D, the number of average CFU for each group are shown as *E. coli* (circles), *B. fragilis* (triangles), and *C. perfringens* (diamonds). *E. coli* and *B. fragilis* CFU levels in all the panels either stays the same or increases in numbers from the initial inoculum, while the number of *C. perfringens* CFU declined below the lower limit of detection. When comparing experimental to control mice of the same age, both *E. coli* and *B. fragilis* CFU levels in the experimental mice 1 to 2 logs from the original inoculum. Panel B, the aged experimental mice showed the greatest increase in CFU of both *E. coli* and *B. fragilis*.

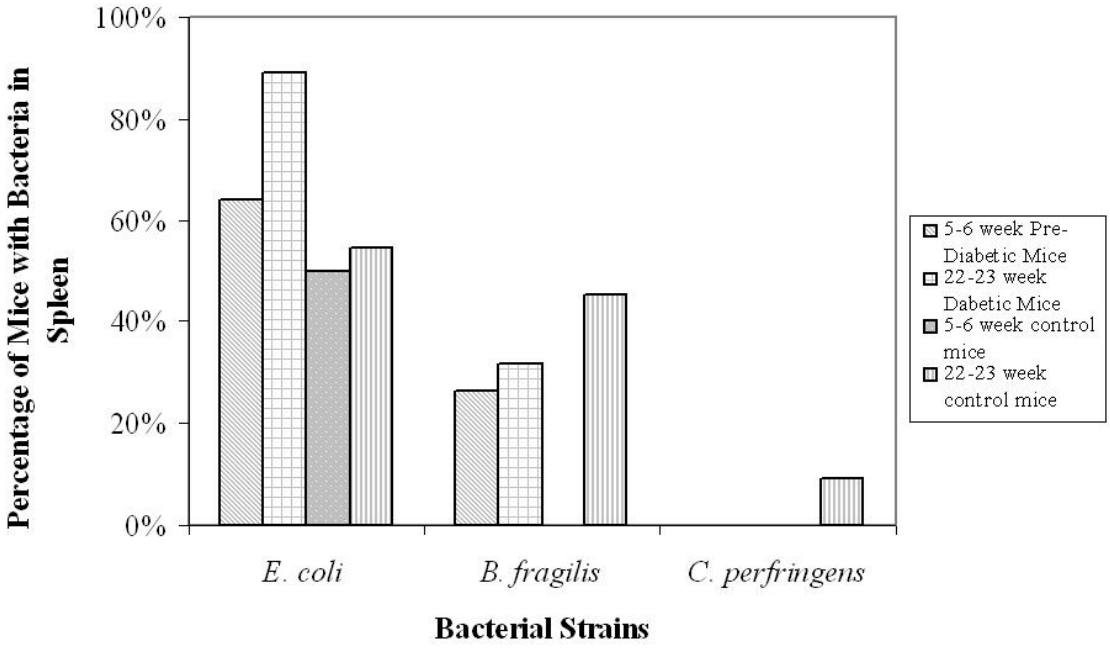

Figure AII.3. Statistical analysis of bacterial loads in the spleens of BKS.Cg-*m* +/+ *Lepr*^{db}/J and C57BLKS/J mice. Spleens were taken from mice at 1, 8, and 22 days post infection from both control and experimental groups. A bacterial count of >200 CFU/mL of resuspended tissue was set as the lower limit of detection. *E. coli* was recovered from approximately 50% or greater of all the mice sampled in each group. *Bacteroides* was recovered from ~20-45% of the mice sampled in each group with the exception of the 5- to 6-week-old control mice. *Clostridium* was found in a very small amount of mice from the 23- to 24-week-old control group.

Table AII.1. Summary of the number of mice used of the strain BKS.Cg-*m* +/+ *Lepr^{db}*/J for the subcutaneous polymicrobial bacterial infections.

	5- to 6-week-old mice			23- to 24-week-old mice		
Experiment	Total mice that died	Total mice injected	Percentage (%)	Total mice that died	Total mice injected	Percentage (%)
1	4	32	13	2	26	8
2	0	24	0	0	24	0
3	0	24	0	0	24	0
4	0	24	0	0	24	0
5	1	27	4	4	27	15
6	0	24	0	1	25	4
7	18	46	39	4	29	14
Control	0	24	0	0	24	0
Total	23	225	10	11	203	5

*Note: That 14% of all mice injected with *E. coli* 360A died.

Table AII.2. Summary of the number of mice used of the strain C57BLKS/J for the subcutaneous polymicrobial bacterial infections.

	5- to 6-week-old mice			23- to 24-week-old mice		
Experiment	Total mice that died	Total mice injected	Percentage (%)	Total mice that died	Total mice injected	Percentage (%)
7	6	13	46	0	13	0

Appendix III

Sequence analysis of *hlyA*

hlyA alignment of *Escherichia coli* strain 360A (clinical isolate) and three uropathogenic strain isolates CFT073, J96, and UTI189:

```
CFT073          ATGCCAACAATAACCACTGCACAAATTAAAAGCACACTACAGTCTGCAAAGCAATCCTCT
J96             ATGCCAACAATAACCGCTGCACAAATTAAAAGCACACTGCAGTCTGCAAAGCAATCCGCT
360A            ATGCCAACAATAACCACTGCACAAATTAAAAGCACACTACAGTCTGCAAAGCAATCCGCT
UTI89           ATGCCAACAATAACCACTGCACAAATTAAAAGCACACTACAGTCTGCAAAGCAATCCGCT
                *************** ********************** ****************** **

CFT073          GCAAATAAATTGCACTCAGCAGGACAAAGCACGAAAGATGCATTAAAAAAAGCAGCAGAG
J96             GCAAATAAATTGCACTCAGCAGGACAAAGCACGAAAGATGCATTAAAAAAAGCAGCAGAG
360A            GCAAATAAATTGCACTCAGCAGGACAAAGCACGAAAGATGCATTAAAAAAAGCAGCAGAG
UTI89           GCAAATAAATTGCACTCAGCAGGACAAAGCACGAAAGATGCATTAAAAAAAGCAGCAGAG
                ************************************************************

CFT073          CAAACCCGCAATGCGGGAAACAGACTCATTTTACTTATCCCTAAAGATTATAAAGGACAG
J96             CAAACCCGCAATGCGGGAAACAGACTCATTTTACTTATCCCTAAAGATTATAAAGGGCAG
360A            CAAACCCGCAATGCGGGAAACAGACTCATTTTACTTATCCCTAAAGATTATAAAGGACAG
UTI89           CAAACCCGCAATGCGGGAAACAGACTCATTTTACTTATCCCTAAAGATTATAAAGGACAG
                ************************************************************ ***

CFT073          GGTTCAAGCCTTAATGACCTTGTCAGGACGGCAGATGAACTGGGAATTGAAGTCCAGTAT
J96             GGTTCAAGCCTTAATGACCTTGTCAGGACGGCAGATGAACTGGGAATTGAAGTCCAGTAT
360A            GGTTCAAGCCTTAATGACCTTGTCAGGACGGCAGATGAACTGGGAATTGAAGTCCAGTAT
UTI89           GGTTCAAGCCTTAATGACCTTGTCAGGACGGCAGATGAACTGGGAATTGAAGTCCAGTAT
                ************************************************************

CFT073          GATGAAAAGAATGGCACGGCGATTACTAAACAGGTATTCGGCACAGCAGAGAAACTCATT
J96             GATGAAAAGAATGGCACGGCAATTACTAAACAGGTATTCGGCACAGCAGAGAAACTCATT
360A            GATGAAAAGAATGGCACGGCGATTACTAAACAGGTATTCGGCACAGCAGAGAAACTCATT
UTI89           GATGAAAAGAATGGCACGGCGATTACTAAACAGGTATTCGGCACAGCAGAGAAACTCATT
                ******************** ****************************************

CFT073          GGCCTCACCGAACGGGGAGTGACTATCTTTGCACCACAATTAGACAAATTACTGCAAAAG
J96             GGCCTCACCGAACGGGGAGTGACTATCTTTGCACCACAATTAGACAAATTACTGCAAAAG
360A            GGCCTCACCGAACGGGGAGTGACTATCTTTGCACCACAATTAGACAAATTACTGCAAAAG
UTI89           GGCCTCACCGAACGGGGAGTGACTATCTTTGCACCACAATTAGACAAATTACTGCAAAAG
                ************************************************************
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CFT073          TATCAAAAAGCGGGTAATAAATTAGGCGGCAGTGCTGAAAATATAGGTGATAACTTAGGA
J96             TATCAAAAAGCGGGTAATAAATTAGGCGGCAGTGCTGAAAATATAGGTGATAACTTAGGA
360A            TATCAAAAAGCGGGTAATAAATTAGGCGGCAGTGCTGAAAATATAGGTGATAACTTAGGA
UTI89           TATCAAAAAGCGGGTAATAAATTAGGCGGCAGTGCTGAAAATATAGGTGATAACTTAGGA
                ************************************************************

CFT073          AAGGCAGGCAGTGTACTGTCAACGTTTCAAAATTTTCTGGGTACTGCACTTTCCTCAATG
J96             AAGGCAGGCAGTGTACTGTCAACGTTTCAAAATTTTCTGGGTACTGCACTTTCCTCAATG
360A            AAGGCAGGCAGTGTACTGTCAACGTTTCAAAATTTTCTGGGTACTGCACTTTCCTCAATG
UTI89           AAGGCAGGCAGTGTACTGTCAACGTTTCAAAATTTTCTGGGTACTGCACTTTCCTCAATG
                ************************************************************

CFT073          AAAATAGACGAACTGATAAAGAGACAAAAATCTGGTAGCAATGTCAGTTCTTCTGAACTG
J96             AAAATAGACGAACTGATAAAGAAACAAAAATCTGGTGGCAATGTCAGTTCTTCTGAACTG
360A            AAAATAGACGAACTGATAAAGAAACAAAAATCTGGTAGCAATGTCAGTTCTTCTGAACTG
UTI89           AAAATAGACGAACTGATAAAGAAACAAAAATCTGGTAGCAATGTCAGTTCTTCTGAACTG
                ********************* ************ ************************

CFT073          GCAAAAGCGAGTATTGAGCTAATCAACCAACTCGTGGACACAGCTGCCAGCATTAATAAT
J96             GCAAAAGCGAGTATTGAGCTAATCAACCAACTCGTGGACACAGCTGCCAGCCTTAATAAT
360A            GCAAAAGCGAGTATTGAGCTAATCAACCAACTCGTGGACACAGCTGCCAGCATTAATAAT
UTI89           GCAAAAGCGAGTATTGAGCTAATCAACCAACTCGTGGACACAGCTGCCAGCATTAATAAT
                ************************************************* ********

CFT073          AATGTTAACTCATTTTCTCAACAACTCAATAAGCTGGGAAGTGTATTATCCAATACAAAG
J96             ---GTTAACTCATTTTCTCAACAACTCAATAAGCTGGGAAGTGTATTATCCAATACAAAG
360A            AATGTTAACTCATTTTCTCAACAACTCAATAAGCTGGGAAGTGTATTATCCAATACAAAG
UTI89           AATGTTAACTCATTTTCTCAACAACTCAATAAGCTGGGAAGTGTATTATCCAATACAAAG
                   ************************************************************

CFT073          CACCTGACCGGTGTTGGTAATAAGTTACAGAATTTACCTAACCTTGATAATATCGGTGCA
J96             CACCTGAACGGTGTTGGTAATAAGTTACAGAATTTACCTAACCTTGATAATATCGGTGCA
360A            CACCTGAACGGTGTTGGTAATAAGTTACAGAATTTACCTAACCTTGATAATATCGGTGCA
UTI89           CACCTGAACGGTGTTGGTAATAAGTTACAGAATTTACCTAACCTTGATAATATCGGTGCA
                ******* ********************************************************

CFT073          GGGTTAGATACTGTATCGGGTATTTTATCTGCGATTTCAGCAAGCTTCATTCTGAGCAAT
J96             GGGTTAGATACTGTATCGGGTATTTTATCTGCGATTTCAGCAAGTTTCATTCTGAGCAAT
360A            GGGTTAGATACTGTATCGGGTATTTTATCTGCGATTTCAGCAAGCTTCATTCTGAGCAAT
UTI89           GGGTTAGATACTGTATCGGGTATTTTATCTGCGATTTCAGCAAGCTTCATTCTGAGCAAT
                ********************************************* ***************

CFT073          GCAGATGCAGATACCGGAACTAAAGCTGCAGCAGGTGTTGAATTAACAACGAAAGTACTG
J96             GCAGATGCAGATACCGGAACTAAAGCTGCAGCAGGTGTTGAATTAACAACGAAAGTACTG
360A            GCAGATGCAGATACCGGAACTAAAGCTGCAGCAGGTGTTGAATTAACAACGAAAGTACTG
UTI89           GCAGATGCAGATACCGGAACTAAAGCTGCAGCAGGTGTTGAATTAACAACGAAAGTACTG
                ************************************************************
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CFT073          GGTAATGTTGGAAAAGGTATTTCTCAATATATTATCGCACAGCGCGCTGCACAGGGATTA
J96             GGTAATGTTGGAAAAGGTATTTCTCAATATATTATCGCACAGCGTGCAGCACAGGGGTTA
360A            GGTAATGTTGGAAAAGGTATTTCTCAATATATTATCGCACAGCGCGCTGCACAGGGGTTA
UTI89           GGTAATGTTGGAAAAGGTATTTCTCAATATATTATCGCACAGCGCGCTGCACAGGGGTTA
                ****************************************** ** ******** ***

CFT073          TCTACATCTGCTGCTGCTGCCGGTTTAATTGCTTCTGTAGTGACATTAGCAATTAGTCCC
J96             TCTACATCTGCTGCTGCTGCCGGTTTAATTGCTTCTGTTGTGACACTGGCAATTAGTCCC
360A            TCTACATCTGCTGCTGCTGCCGGTTTAATTGCTTCTGTAGTGACATTAGCAATTAGTCCC
UTI89           TCTACATCTGCTGCTGCTGCCGGTTTAATTGCTTCTGTAGTGACATTAGCAATTAGTCCC
                ************************************ ****** * ************

CFT073          CTCTCATTCCTGTCCATTGCCGATAAGTTTAAACGTGCCAATAAAATAGAGGAGTATTCA
J96             CTCTCATTCCTGTCCATTGCCGATAAGTTTAAACGTGCCAATAAAATAGAGGAGTATTCA
360A            CTCTCATTCCTGTCCATTGCCGATAAGTTTAAACGTGCAAATAAAATAGAGGAGTATTCA
UTI89           CTCTCATTCCTGTCCATTGCCGATAAGTTTAAACGTGCAAATAAAATAGAGGAGTATTCA
                ************************************ ********************

CFT073          CAACGATTCAAAAAACTTGGATACGATGGTGACAGTTTACTTGCTGCTTTTCACAAAGAA
J96             CAACGATTCAAAAAACTTGGATACGATGGTGACAGTTTACTTGCTGCTTTCCACAAAGAA
360A            CAACGATTCAAAAAACTTGGATACGATGGTGACAGTTTACTTGCTGCTTTCCACAAAGAA
UTI89           CAACGATTCAAAAAACTTGGATACGATGGTGACAGTTTACTTGCTGCTTTCCACAAAGAA
                ************************************************** **********

CFT073          ACAGGAGCTATTGATGCATCGTTAACAACGATAAGCACTGTTCTGGCTTCAGTATCTTCA
J96             ACAGGAGCTATTGATGCATCGTTAACAAGGATAAGCACTGTTCTGGCTTCAGTATCTTCA
360A            ACAGGAGCTATTGATGCATCATTAACAACGATAAGCACTGTACTGGCTTCAGTATCTTCA
UTI89           ACAGGAGCTATTGATGCATCATTAACAACGATAAGCACTGTACTGGCTTCAGTATCTTCA
                ******************** ******* ************ ******************

CFT073          GGTATTAGTGCTGCTGCAACGACATCTCTGGTTGGTGCACCGGTAAGCGCGCTGGTAGGG
J96             GGTATTAGTGCTGCTGCAACGACATCTCTGGTTGGTGCACCGGTAAGCGCGCTGGTAGGG
360A            GGTATTAGTGCTGCTGCAACGACATCTCTTGTTGGTGCACCGGTAAGCGCACTGGTAGGT
UTI89           GGTATTAGTGCTGCTGCAACGACATCTCTTGTTGGTGCACCGGTAAGCGCACTGGTAGGT
                ****************************** ******************** ********

CFT073          GCTGTTACGGGGATAATTTCAGGCATCCTTGAGGCTTCAAAACAGGCAATGTTTGAACAT
J96             GCTGTTACGGGGATAATTTCAGGCATCCTTGAGGCTTCAAAACAGGCAATGTTTGAACAT
360A            GCTGTTACGGGGATAATTTCAGGTATCCTTGAGGCTTCAAAGCAGGCAATGTTTGAACAT
UTI89           GCTGTTACGGGGATAATTTCAGGTATCCTTGAGGCTTCAAAGCAGGCAATGTTTGAACAT
                ************************ ***************** ******************

CFT073          GTCGCCAGTAAAATGGCCGATGTTATTGCTGAATGGGAGAAAAAACACGGCAAAAATTAC
J96             GTCGCCAGTAAAATGGCCGATGTTATTGCTGAATGGGAGAAAAAACACGGCAAAAATTAC
360A            GTTGCCAGTAAAATGGCTGATGTTATTGCTGAATGGGAGAAAAAACACGGTAAAAATTAC
UTI89           GTTGCCAGTAAAATGGCTGATGTTATTGCTGAATGGGAGAAAAAACACGGTAAAAATTAC
                ** ************** ********************************** **********
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CFT073          TTTGAAAATGGATATGATGCCCGCCATGCTGCATTTTTAGAAGATAACTTTAAAATATTA
J96             TTTGAAAATGGATATGATGCCCGCCATGCTGCATTTTTAGAAGATAACTTTAAAATATTA
360A            TTTGAAAATGGATATGATGCCCGCCATGCTGCATTTTTAGAAGATAACTTTAAAATATTA
UTI89           TTTGAAAATGGATATGATGCCCGCCATGCTGCATTTTTAGAAGATAACTTTAAAATATTA
                ************************************************************

CFT073          TCTCAGTATAATAAAGAGTATTCTGTTGAAAGATCAGTCCTCATTACCCAGCAACATTGG
J96             TCTCAGTATAATAAAGAGTATTCTGTTGAAAGATCAGTCCTCATTACCCAGCAACATTGG
360A            TCTCAGTATAATAAAGAGTATTCTGTTGAAAGATCAGTCCTCATTACTCAACAACATTGG
UTI89           TCTCAGTATAATAAAGAGTATTCTGTTGAAAGATCAGTCCTCATTACTCAACAACATTGG
                ********************************************** ** *********

CFT073          GATACGCTGATAGGTGAGTTAGCGGGTGTCACCAGAAATGGAGACAAAACACTCAGTGGT
J96             GATACGCTGATAGGTGAGTTAGCTGGTGTCACCAGAAATGGAGACAAAACACTCAGTGGT
360A            GATATGCTGATAGGTGAGTTAGCTAGTGTCACCAGAAATGGAGACAAGACACTCAGTGGT
UTI89           GATATGCTGATAGGTGAGTTAGCTAGTGTCACCAGAAATGGAGACAAGACACTCAGTGGT
                **** ******************  ********************** ************

CFT073          AAAAGTTATATTGACTATTATGAAGAAGGAAAACGTCTGGAGAAAAAACCGGATGAATTC
J96             AAAAGTTATATTGACTATTATGAAGAAGGAAAACGTCTGGAGAAAAAACCGGATGAATTC
360A            AAAAGTTATATTGACTATTATGAAGAGGGAAAGCGGCTGGAAAGAAGGCCAAAAGAGTTC
UTI89           AAAAGTTATATTGACTATTATGAAGAGGGAAAGCGGCTGGAAAGAAGGCCAAAAGAGTTC
                *************************** ***** ** ***** * **  **  * ** ***

CFT073          CAGAAGCAAGTCTTTGACCCATTGAAAGGAAATATTGACCTTTCTGACAGCAAATCTTCT
J96             CAGAAGCAAGTCTTTGACCCATTGAAAGGAAATATTGACCTTTCTGACAGCAAATCTTCT
360A            CAGCAACAAATCTTTGATCCATTAAAAGGAAATATTGACCTTTCTGACAGCAAATCTTCT
UTI89           CAGCAACAAATCTTTGATCCATTAAAAGGAAATATTGACCTTTCTGACAGCAAATCTTCT
                *** * *** ******* ***** ************************************

CFT073          ACGTTATTGAAATTTGTTACGCCATTGTTAACTCCCGGTGAGGAAATTCGTGAAAGGAGG
J96             ACGTTATTGAAATTTGTTACGCCATTGTTAACTCCCGGTGAGGAAATTCGTGAAAGGAGG
360A            ACGTTATTGAAATTTGTTACGCCATTGTTAACTCCCGGTGAGGAAATTCGTGAAAGGAGG
UTI89           ACGTTATTGAAATTTGTTACGCCATTGTTAACTCCCGGTGAGGAAATTCGTGAAAGGAGG
                ************************************************************

CFT073          CAGTCCGGAAAATATGAATATATTACCGAGTTATTAGTCAAGGGTGTTGATAAATGGACG
J96             CAGTCCGGAAAATATGAATATATTACCGAGTTATTAGTCAAGGGTGTTGATAAATGGACG
360A            CAGTCCGGAAAATATGAATATATTACCGAGTTATTAGTCAAGGGTGTTGATAAATGGACG
UTI89           CAGTCCGGAAAATATGAATATATTACCGAGTTATTAGTCAAGGGTGTTGATAAATGGACG
                ************************************************************

CFT073          GTGAAGGGGGTTCAGGACAAGGGGTCTGTATATGATTACTCTAACCTGATTCAGCATGCA
J96             GTGAAGGGGGTTCAGGACAAGGGGTCTGTATATGATTACTCTAACCTGATTCAGCATGCA
360A            GTGAAGGGGGTTCAGGACAAGGGGTCTGTATATGATTACTCTAACCTGATTCAGCATGCA
UTI89           GTGAAGGGGGTTCAGGACAAGGGGTCTGTATATGATTACTCTAACCTGATTCAGCATGCA
                ************************************************************
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CFT073          TCAGTCGGTAATAACCAGTATCGGGAAATTCGTATTGAGTCACACCTGGGAGACGGGGAT
J96             TCAGTCGGTAATAACCAGTATCGGGAAATTCGTATTGAGTCACACCTGGGAGACGGGGAT
360A            TCAGTCGGTAATAACCAGTATCGGGAAATTCGTATTGAGTCACACCTGGGAGACGGGGAT
UTI89           TCAGTCGGTAATAACCAGTATCGGGAAATTCGTATTGAGTCACACCTGGGAGACGGGGAT
                ************************************************************

CFT073          GATAAGGTCTTTTTATCTGCCGGCTCAGCCAATATCTACGCAGGTAAAGGACATGATGTT
J96             GATAAGGTCTTTTTATCTGCCGGCTCAGCCAATATCTACGCAGGTAAAGGACATGATGTT
360A            GATAAGGTCTTTTTATCTGCCGGCTCAGCCAATATCTACGCAGGTAAAGGACATGATGTT
UTI89           GATAAGGTCTTTTTATCTGCCGGCTCAGCCAATATCTACGCAGGTAAAGGACATGATGTT
                ************************************************************

CFT073          GTTTATTATGATAAAACAGACACCGGTTATCTGACCATTGATGGCACAAAAGCAACCGAA
J96             GTTTATTATGATAAAACAGACACCGGTTATCTGACCATTGATGGCACAAAAGCAACCGAA
360A            GTTTATTATGATAAAACAGACACCGGTTATCTGACCATTGATGGCACAAAAGCAACCGAA
UTI89           GTTTATTATGATAAAACAGACACCGGTTATCTGACCATTGATGGCACAAAAGCAACCGAA
                ************************************************************

CFT073          GCGGGTAATTACACGGTAACACGTGTACTTGGTGGTGATGTTAAGATTTTACAGGAAGTT
J96             GCGGGTAATTACACGGTAACACGTGTACTTGGTGGTGATGTTAAGGTTTTACAGGAAGTT
360A            GCGGGTAATTACACGGTAACACGTGTACTTGGTGGTGATGTTAAGGTTTTACAGGAAGTT
UTI89           GCGGGTAATTACACGGTAACACGTGTACTTGGTGGTGATGTTAAGGTTTTACAGGAAGTT
                ********************************************** **************

CFT073          GTGAAGGAGCAGGAGGTTTCAGTTGGAAAAAGAACTGAAAAAACGCAATATCGGAGTTAT
J96             GTGAAGGAGCAGGAGGTTTCAGTTGGAAAAAGAACTGAAAAAACGCAATATCGGAGTTAT
360A            GTGAAGGAGCAGGAGGTTTCAGTCGGAAAAAGAACTGAAAAAACGCAATATCGGAGTTAT
UTI89           GTGAAGGAGCAGGAGGTTTCAGTCGGAAAAAGAACTGAAAAAACGCAATATCGGAGTTAT
                ************************ ************************************

CFT073          GAATTCACTCATATCAATGGTAAAAATTTAACAGAGACTGATAACTTATATTCCGTGGAA
J96             GAATTCACTCATATCAATGGTAAAAATTTAACAGAGACTGATAACTTATATTCCGTGGAA
360A            GAATTCACTCATATCAATGGTAAAAATTTAACAGAGACAGATAACTTATATTCCGTGGAA
UTI89           GAATTCACTCATATCAATGGTAAAAATTTAACAGAGACAGATAACTTATATTCCGTGGAA
                ************************************ **********************

CFT073          GAACTTATTGGGACCACGCGTGCCGACAAGTTTTTTGGCAGTAAATTTACTGATATCTTC
J96             GAACTTATTGGGACCACGCGTGCCGACAAGTTTTTTGGCAGTAAATTTGCTGATATCTTC
360A            GAACTTATTGGGACCACGCGTGCCGACAAGTTTTTTGGCAGTAAATTTACTGATATCTTC
UTI89           GAACTTATTGGGACCACGCGTGCCGACAAGTTTTTTGGCAGTAAATTTACTGATATCTTC
                ************************************************ ************

CFT073          CATGGCGCGGATGGTGATGACCATATAGAAGGAAATGATGGGAATGACCGCTTATATGGT
J96             CATGGCGCGGATGGTGATGACCATATAGAAGGAAATGACGGGAATGACCGCTTATATGGT
360A            CATGGCGCGGATGGTGATGACCATATAGAAGGAAATGATGGGAATGACCGCTTATATGGT
UTI89           CATGGCGCGGATGGTGATGACCATATAGAAGGAAATGATGGGAATGACCGCTTATATGGT
                ************************************ **********************
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CFT073          GATAAAGGTAATGATACGCTGAGGGGCGGAAACGGGGATGACCAGCTCTATGGCGGTGAT
J96             GATAAAGGTAATGACACACTGAGTGGTGGAAACGGAGATGACCAGCTCTATGGCGGTGAT
360A            GATAAAGGTAATGATACGCTGAGGGGCGGAAACGGGGATGACCAGCTCTATGGCGGTGAT
UTI89           GATAAAGGTAATGATACGCTGAGGGGCGGAAACGGGGATGACCAGCTCTATGGCGGTGAT
                ************** ** ***** ** ******** ************************

CFT073          GGCAATGATAAGTTAATTGGGGGGACAGGTAATAATTACCTTAACGGCGGTGACGGAGAT
J96             GGCAACGATAAGTTAATTGGGGGAGCAGGTAATAATTACCTGAACGGCGGAGATGGCGAT
360A            GGCAATGATAAGTTAATTGGGGGGACAGGTAATAATTACCTTAACGGCGGTGACGGAGAT
UTI89           GGCAATGATAAGTTAATTGGGGGGACAGGTAATAATTACCTTAACGGCGGTGACGGAGAT
                ***** *****************  ***************** ******** ** ** ***

CFT073          GATGAGCTTCAGGTTCAGGGGAATTCTCTTGCTAAAAATGTATTATCCGGTGGAAAAGGT
J96             GATGAGCTTCAGGTTCAGGGAAATTCTCTTGCAAAAAATGTATTATCCGGTGGAAAAGGT
360A            GATGAGCTTCAGGTTCAGGGGAATTCTCTTGCTAAAAATGTATTATCCGGTGGAAAAGGT
UTI89           GATGAGCTTCAGGTTCAGGGGAATTCTCTTGCTAAAAATGTATTATCCGGTGGAAAAGGT
                ******************** *********** ****************************

CFT073          AATGACAAGTTGTACGGCAGTGAGGGAGCAGATCTGCTTGATGGCGGAGAAGGGAATGAT
J96             AATGACAAGCTGTACGGCAGTGAGGGAGCAGATCTGCTTGATGGCGGAGAAGGGAATGAT
360A            AATGACAAGTTGTACGGCAGTGAGGGAGCAGACCTGCTTGATGGCGGAGAAGGGAATGAT
UTI89           AATGACAAGTTGTACGGCAGTGAGGGAGCAGACCTGCTTGATGGCGGAGAAGGGAATGAT
                ********* ********************** ****************************

CFT073          CTTCTGAAAGGTGGATATGGTAATGATATTTATCGTTATCTTTCAGGATATGGCCATCAT
J96             CTTCTGAAAGGTGGATATGGTAATGATATTTATCGTTATCTTTCAGGATATGGCCATCAT
360A            CTTCTGAAAGGTGGATTTGGTAATGATATTTATCGTTATCTTTCAGGATATGGCCATCAT
UTI89           CTTCTGAAAGGTGGATATGGTAATGATATTTATCGTTATCTTTCAGGATATGGCCATCAT
                ***************** ******************************************

CFT073          ATTATTGACGATGATGGGGGGAAAGACGATAAACTCAGTTTGGCTGATATTGATTTCCGG
J96             ATTATTGACGATGATGGGGGGAAAGACGATAAACTCAGTTTGGCTGATATTGATTTCCGG
360A            ATTATTGACGATGAAGGGGGGAAAGACGATAAACTCAGTTTAGCTGATATAGATTTCCGG
UTI89           ATTATTGACGATGAAGGGGGGAAAGACGATAAACTCAGTTTAGCTGATATAGATTTCCGG
                *************** ************************** ******** *********

CFT073          GATGTGGCCTTCAGGCGAGAAGGTAATGACCTCATCATGTATAAAGCTGAAGGTAATGTT
J96             GATGTGGCCTTCAGGCGAGAAGGTAATGACCTCATCATGTATAAAGCTGAAGGTAATGTT
360A            GACGTTGCCTTTAAGCGAGAAGGGAATGACCTCATTATGTATAAAGCTGAAGGTAATGTT
UTI89           GACGTTGCCTTTAAGCGAGAAGGGAATGACCTCATTATGTATAAAGCTGAAGGTAATGTT
                ** ** ***** * ********* ************ ************************

CFT073          CTTTCCATTGGTCATAAAAATGGTATTACATTCAGGAACTGGTTTGAAAAAGAGTCAGGT
J96             CTTTCCATTGGCCACAAAAATGGTATTACATTTAAAAACTGGTTTGAAAAAGAGTCAGGT
360A            CTTTCTATTGGCCACAAAAATGGTATTACATTTAAAAACTGGTTTGAAAAAGAGTCAGAT
UTI89           CTTTCTATTGGCCACAAAAATGGTATTACATTTAAAAACTGGTTTGAAAAAGAGTCAGAT
                ***** ***** ** ***************** *  ********************** *
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```
CFT073          GATATCTCTAATCACCAGATAGAGCAGATTTTTGATAAAGACGGCAGGGTAATCACACCA
J96             GATATCTCTAATCACCAGATAGAGCAGATTTTTGATAAAGACGGCAGGGTAATCACACCA
360A            GATCTCTCTAATCATCAGATAGAGCAGATTTTTGATAAAGACGGCAGGGTAATCACACCA
UTI89           GATCTCTCTAATCATCAGATAGAGCAGATTTTTGATAAAGACGGCAGGGTAATCACACCA
                *** ********** ****************************************

CFT073          GATTCCCTTAAAAAGGCACTTGAGTATCAACAGAGTAATAATAAGGCAAGTTATGTGTAT
J96             GATTCCCTTAAAAAGGCACTTGAGTATCAACAGAGTAATAATAAGGCAAGTTATGTGTAT
360A            GATTCTCTTAAAAAAGCATTTGAATATCAGCAGAGTAATAACAAGGTAAGTTATGTGTAT
UTI89           GATTCTCTTAAAAAAGCATTTGAATATCAGCAGAGTAATAACAAGGTAAGTTATGTGTAT
                ***** ******** *** **** ***** *********** **** *************

CFT073          GGGAATGATGCATTAGCCTATGGAAGTCAGGATAATCTTAATCCATTAATTAATGAAATC
J96             GGGAATGATGCATTAGCCTATGGAAGTCAGGGTAATCTTAATCCATTAATTAATGAAATC
360A            GGACATGATGCATCAACTTATGGGAGCCAGGACAATCTTAATCCATTAATTAATGAAATC
UTI89           GGACATGATGCATCAACTTATGGGAGCCAGGACAATCTTAATCCATTAATTAATGAAATC
                **  ********* * * ***** ** ****  ****************************

CFT073          AGCAAAATCATTTCAGCTGCAGGTAATTTTGATGTTAAAGAGGAAAGAGCTGCAGCTTCT
J96             AGCAAAATCATTTCAGCTGCAGGTAATTTTGATGTTAAAGAGGAAAGAGCTGCAGCTTCT
360A            AGCAAAATCATTTCAGCTGCAGGTAACTTCGATGTTAAGGAGGAAAGATCTGCCGCTTCT
UTI89           AGCAAAATCATTTCAGCTGCAGGTAACTTCGATGTTAAGGAGGAAAGATCTGCCGCTTCT
                ************************** ** ******** ********* **** ******

CFT073          TTATTGCAGTTGTCCGGTAATGCCAGTGATTTTTCATATGGACGGAACTCAATAACTTTG
J96             TTATTGCAGTTGTCCGGTAATGCCAGTGATTTTTCATATGGACGGAACTCAATAACTTTG
360A            TTATTGCAGTTGTCCGGTAATGCCAGTGATTTTTCATATGGACGGAACTCAATAACTTTG
UTI89           TTATTGCAGTTGTCCGGTAATGCCAGTGATTTTTCATATGGACGGAACTCAATAACTTTG
                ************************************************************

CFT073          ACAGCATCAGCATAA
J96             ACAGCATCAGCATAA
360A            ACAGCATCAGCATAA
UTI89           ACAGCATCAGCATAA
                ***************
```

The regions are:
* - single, fully conserved residue
  - no consensus

The sequence analysis was done using The Biology WorkBench (http://workbench.sdsc.edu/).

Appendix IV

Sequence analysis of *cnf1*

cnf1 alignment of five *Escherichia coli* strains 360A, 5383-2, 91-1913, UTI89, and O83/K24/H31:

```
O83/K24/H31     ATGGGTAACCAATGGCAACAAAAATATCTTCTTGAGTACAATGAGTTGGTATCAAATTTC
96-1913         ATGGGTAACCAATGGCAACAAAAATATCTTCTTGAGTACAATGAGTTGGTATCAAATTTC
5383-2          ATGGGTAACCAATGGCAACAAAAATATCTTCTTGAGTACAATGAGTTGGTATCAAATTTC
360A            ATGGGTAACCAATGGCAACAAAAATATCTTCTTGAGTACAATGAGTTGGTATCAAATTTC
UTI89           ATGGGTAACCAATGGCAACAAAAATATCTTCTTGAGTACAATGAGTTGGTATCAAATTTC
                ************************************************************

O83/K24/H31     CCTTCACCTGAAAGAGTTGTCAGCGATTACATTAAGAATTGTTTTAAAACTGACTTGCCG
96-1913         CCTTCACCTGAAAGAGTTGTCAGCGATTACATTAAGAATTGTTTTAAAACTGACTTGCCG
5383-2          CCTTCACCTGAAAGAGTTGTCAGCGATTACATTAAGAATTGTTTTAAAACTGACTTGCCG
360A            CCTTCACCTGAAAGAGTTGTCAGCGATTACATTAAGAATTGTTTTAAAACTGACTTGCCG
UTI89           CCTTCACCTGAAAGAGTTGTCAGCGATTACATTAAGAATTGTTTTAAAACTGACTTGCCG
                ************************************************************

O83/K24/H31     TGGTTTAGTCGGATTGATCCTGATAATGCTTATTTCATCTGCTTTTCTCAAAACCGGAGT
96-1913         TGGTTTAGTCGGATTGATCCTGATAATGCTTATTTCATCTGCTTTTCTCAAAACCGGAGT
5383-2          TGGTTTAGTCGGATTGATCCTGATAATGCTTATTTCATCTGCTTTTCTCAAAACCGGAGT
360A            TGGTTTAGTCGGATTGATCCTGATAATGCTTATTTCATCTGCTTTTCTCAAAACCGGAGT
UTI89           TGGTTTAGTCGGATTGATCCTGATAATGCTTATTTCATCTGCTTTTCTCAAAACCGGAGT
                ************************************************************

O83/K24/H31     AATAGCAGATCTTATACTGGATGGGATCATCTTGGGAAATATAAAACAGAAGTACTGACA
96-1913         AATAGCAGATCTTATACTGGATGGGATCATCTTGGGAAATATAAAACAGAAGTACTGACA
5383-2          AATAGCAGATCTTATACTGGATGGGATCATCTTGGGAAATATAAAACAGAAGTACTGACA
360A            AATAGCAGATCTTATACTGGATGGGATCATCTTGGGAAATATAAAACAGAAGTACTGACA
UTI89           AATAGCAGATCTTATACTGGATGGGATCATCTTGGGAAATATAAAACAGAAGTACTGACA
                ************************************************************

O83/K24/H31     CTCACTCAAGCCGCTCTTATTAATATTGGTTATCGTTTTGATGTTTTTGATGATGCAAAT
96-1913         CTCACTCAAGCCGCTCTTATTAATATTGGTTATCGTTTTGATGTTTTTGATGATGCAAAT
5383-2          CTCACTCAAGCCGCTCTTATTAATATTGGTTATCGTTTTGATGTTTTTGATGATGCAAAT
360A            CTCACTCAAGCCGCTCTTATTAATATTGGTTATCGTTTTGATGTTTTTGATGATGCAAAT
UTI89           CTCACTCAAGCCGCTCTTATTAATATTGGTTATCGTTTTGATGTTTTTGATGATGCAAAT
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O83/K24/H31     TCAAGCACAGGAATTTATAAAACAAAGAGTGCAGATGTGTTTAACGAAGAAAATGAAGAA
96-1913         TCAAGCACAGGAATTTATAAAACAAAGAGTGCAGATGTGTTTAACGAAGAAAATGAAGAA
5383-2          TCAAGCACAGGAATTTATAAAACAAAGAGTGCAGATGTGTTTAACGAAGAAAATGAAGAA
360A            TCAAGCACAGGAATTTATAAAACAAAGAGTGCTGATGTGTTTAACGAAGAAAATGAAGAA
UTI89           TCAAGCACAGGAATTTATAAAACAAAGAGTGCAGATGTGTTTAACGAAGAAAATGAAGAA
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O83/K24/H31     AAAATGCTCCCGTCGGAATACCTGCATTTTTTACAAAAGTGTGATTTTGCAGGTGTTTAT
96-1913         AAAATGCTCCCGTCGGAATACCTGCATTTTTTACAAAAGTGTAATTTTGCAGGTGTTTAT
5383-2          AAAATGCTCCCGTCGGAATACCTGCATTTTTTACAAAAGTGTGATTTTGCAGGTGTTTAT
360A            AAAATGCTCCCGTCGGAATACCTGCATTTTTTACAAAAGTGTGATTTTGCAGGTGTTTAT
UTI89           AAAATGCTCCCGTCGGAATACCTGCATTTTTTACAAAAGTGTGATTTTGCAGGTGTTTAT
                **************************************** ****************

O83/K24/H31     GGAAAAACTCTGTCAGATTACTGGTCGAAATACTATGATAAATTTAAGCTTTTACTAAAA
96-1913         GGAAAAACTCTGTCAGATTACTGGTCGAAATACTATGATAAATTTAAGCTTTTACTAAAA
5383-2          GGAAAAACTCTGTCAGATTACTGGTCGAAATACTATGATAAATTTAAGCTTTTACTAAAA
360A            GGAAAAACTCTGTCAGATTACTGGTCGAAATACTATGATAAATTTAAGCTTTTACTAAAA
UTI89           GGAAAAACTCTGTCAGATTACTGGTCGAAATACTATGATAAATTTAAGCTTTTACTAAAA
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O83/K24/H31     AATTATTATATTTCTTCTGCTTTGTATCTTTATAAAAATGGAGAGCTTGATGAGCGTGAA
96-1913         AATTATTATATTTCTTCTGCTTTGTATCTTTATAAAAATGGAGAGCTTGATGAGCGTGAA
5383-2          AATTATTATATTTCTTCTGCTTTGTATCTTTATAAAAATGGAGAGCTTGATGAGCGTGAA
360A            AATTATTATATTTCTTCTGCTTTGTATCTTTATAAAAATGGAGAGCTTGATGAGCGTGAA
UTI89           AATTATTATATTTCCTCTGCTTTGTATCTTTATAAAAATGGAGAGCTTGATGAGCGTGAA
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O83/K24/H31     TATAATTTCTCCATGAACGCCTTAAATCGCAGTGATAATATATCACTATTCTTCTTTGAT
96-1913         TATAATTTCTCCATGAACGCCTTAAATCGCAGTGATAATATATCACTATTCTTCTTTGAT
5383-2          TATAATTTCTCCATGAACGCCTTAAATCGCAGTGATAATATATCACTATTCTTCTTTGAT
360A            TATAATTTCTCCATGAACGCCTTAAATCGCAGTGATAATATATCACTATTATTCTTTGAT
UTI89           TATAATTTCTCCATGAACGCCTTAAATCGCAGTGATAATATATCACTATTATTCTTTGAT
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O83/K24/H31     ATTTATGGATATTACTCATCTGATATTTTTGTAGCCAAAAATAATGATAAGGTAATGCTT
96-1913         ATTTATGGATATTACTCATCTGATATTTTTGTAGCCAAAAATAATGATAAGGTAATGCTT
5383-2          ATTTATGGATATTACTCATCTGATATTTTTGTAGCCAAAAATAATGATAAGGTAATGCTT
360A            ATTTATGGATATTACGCATCTGATATTTTTGTAGCCAAAAATAATGATAAGGTAATGCTT
UTI89           ATTTATGGATATTACGCATCTGATATTTTTGTAGCCAAAAATAATGATAAGGTAATGCTT
                *************** ********************************************

O83/K24/H31     TTCATTCCTGGTGCAAAAAAACCTTTTTTATTCAAGAAGAATATCGCTGATTTGCGGCTT
96-1913         TTCATTCCTGGTGCAAAAAAACCTTTTTTATTCAAGAAGAATATCGCTGATTTGCGGCTT
5383-2          TTCATTCCTGGTGCAAAAAAACCTTTTTTATTCAAGAAGAATATCGCTGATTTGCGGCTT
360A            TTCATTCCTGGTGCAAAAAAACCTTTTTTATTCAAGAAGAATATCGCTGATTTGCGGCTT
UTI89           TTCATTCCTGGTGCAAAAAAACCTTTTTTATTCAAGAAGAATATCGCTGATTTGCGGCTT
                ************************************************************

O83/K24/H31     ACCCTTAAAGAACTTATTAAGGATAGTGACAACAAACAATTACTTTCCCAACATTTTTCA
96-1913         ACCCTTAAAGAACTTATTAAGGATAGTGACAACAAACAATTACTTTCCCAACATTTTTCA
5383-2          ACCCTTAAAGAACTTATTAAGGATAGTGACAACAAACAATTACTTTCCCAACATTTTTCA
360A            ACCCTTAAAGAACTTATTAAGGATAGTGACAACAAACAATTACTTTCCCAACATTTTTCA
UTI89           ACCCTTAAAGAACTTATTAAGGATAGTGACAACAAACAATTACTTTCCCAACATTTTTCA
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O83/K24/H31     TTATATAGTCGTCAAGATGGAGTTTCCTATGCAGGAGTAAATTCAGTTCTACATGCAATA
96-1913         TTATATAGTCGTCAAGATGGAGTTTCCTATGCAGGAGTAAATTCAGTTCTACATGCAATA
5383-2          TTATATAGTCGTCAAGATGGAGTTTCCTATGCAGGAGTAAATTCAGTTCTACATGCAATA
360A            TTATATAGTCGTCAAGATGGAGTTTCCTATGCAGGAGTAAATTCTGTTCTACATGCAATA
UTI89           TTATATAGTCGTCAAGATGGAGTTTCCTATGCAGGAGTAAATTCTGTTCTACATGCAATA
                ****************************************** ***************

O83/K24/H31     GAAAATGATGGTAATTTTAATGAGTCTTACTTTCTGTATTCCAATAAGACACTTAGCAAT
96-1913         GAAAATGATGGTAATTTTAATGAGTCTTACTTTCTGTATTCCAATAAGACACTTAGCAAT
5383-2          GAAAATGATGGTAATTTTAATGAGTCTTACTTTCTGTATTCCAATAAGACACTTAGCAAT
360A            GAAAATGATGGTAATTTTAATGAGTCTTACTTTCTGTATTCGAATAAGACACTTAGCAAT
UTI89           GAAAATGATGGTAATTTTAATGAGTCTTACTTTCTGTATTCCAATAAGACACTTAGCAAT
                ****************************************** ******************

O83/K24/H31     AAAGATGTTTTTGATGCTATAGCTATTTCTGTTAAGAAACGCAGTTTCAGTGATGGTGAT
96-1913         AAAGATGTTTTTGATGCTATAGCTATTTCTGTTAAGAAACGCAGTTTCAGTGATGGTGAT
5383-2          AAAGATGTTTTTGATGCTATAGCTATTTCTGTTAAGAAACGCAGTTTCAGTGATGGTGAT
360A            AAAGATGTTTTTGATGCTATAGCTATTTCTGTTAAGAAACGCAGTTTCAGTGATGGTGAT
UTI89           AAAGATGTTTTTGATGCTATAGCTATTTCTGTTAAGAAACGCAGTTTCAGTGATGGTGAT
                ************************************************************

O83/K24/H31     ATCGTTATAAAATCAAACAGTGAAGCTCAACGAGACTATGCTCTGACTATACTCCAGACG
96-1913         ATCGTTATAAAATCAAACAGTGAAGCTCAACGAGACTATGCTCTGACTATACTCCAGACG
5383-2          ATCGTTATAAAATCAAACAGTGAAGCTCAACGAGACTATGCTCTGACTATACTCCAGACG
360A            ATCGTTATAAAATCAAACAGTGAAGCTCAACGAGACTATGCTCTGACTATACTCCAGACG
UTI89           ATCGTTATAAAATCAAACAGTGAAGCTCAACGAGACTATGCTCTGACTATACTCCAGACG
                ************************************************************

O83/K24/H31     ATTTTATCAATGACCCCTATATTTGATATCGTAGTCCCGGAGGTATCTGTTCCGCTTGGA
96-1913         ATTTTATCAATGACCCCTATATTTGATATCGTAGTCCCGGAGGTATCTGTTCCGCTTGGA
5383-2          ATTTTATCAATGACCCCTATATTTGATATCGTAGTCCCGGAGGTATCTGTTCCGCTTGGA
360A            ATTTTATCAATGACCCCTATATTTGATATCGTAGTCCCGGAGGTATCTGTTCCGCTTGGA
UTI89           ATTTTATCAATGACCCCTATATTTGATATCGTAGTCCCGGAGGTATCTGTTCCGCTTGGA
                ************************************************************

O83/K24/H31     CTGGGGATTATTACTTCCAGTATGGGGATCAGTTTTGATCAACTGATTAATGGTGATACT
96-1913         CTGGGGATTATTACTTCCAGTATGGGGATCAGTTTTGATCAACTGATTAATGGTGATACT
5383-2          CTGGGGATTATTACTTCCAGTATGGGGATCAGTTTTGATCAACTGATTAATGGTGATACT
360A            CTGGGGATTATTACTTCCAGTATGGGGATCAGTTTTGATCAACTGATTAATGGTGATACT
UTI89           CTGGGGATTATTACTTCCAGTATGGGGATCAGTTTTGATCAACTGATTAATGGTGATACT
                ************************************************************

O83/K24/H31     TATGAAGAACGTCGTTCTGCTATACCTGGTTTGGCGACAAATGCAGTACTGCTTGGTCTG
96-1913         TATGAAGAACGTCGTTCTGCTATACCTGGTTTGGCGACAAATGCAGTACTGCTTGGTCTG
5383-2          TATGAAGAACGTCGTTCTGCTATACCTGGTTTGGCGACAAATGCAGTACTGCTTGGTCTG
360A            TATGAAGAACGTCGTTCTGCTATACCTGGTTTGGCGACAAATGCAGTATTGCTTGGTCTG
UTI89           TATGAAGAACGTCGTTCTGCTATACCTGGTTTGGCGACAAATGCAGTATTGCTTGGTCTG
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O83/K24/H31     TCTTTTGCAATTCCACTCTTGATTAGTAAGGCAGGAATAAACCAGGAGGTACTTAGCAGC
96-1913         TCTTTTGCAATTCCACTCTTGATTAGTAAGGCAGGAATAAACCAGGAGGTACTTAGCAGC
5383-2          TCTTTTGCAATTCCACTCTTGATTAGTAAGGCAGGAATAAACCAGGGGGTACTTAGCAGC
360A            TCTTTTGCAATTCCACTCTTGATTAGTAAGGCAGGAATAAACCAGGAGGTACTTAGCAGC
UTI89           TCTTTTGCAATTCCACTCTTGATTAGTAAGGCAGGAATAAACCAGGAGGTACTTAGCAGC
                ********************************************** ************

O83/K24/H31     GTTATAAATAATGAGGGCAGGACTCTGAATGAAACAAATATCGATATATTTTTGAAGGAA
96-1913         GTTATAAATAATGAGGGCAGGACTCTGAATGAAACAAATATCGATATATTTTTGAAGGAA
5383-2          GTTATAAATAATGAGGGCAGGACTCTGAATGAAACAAATATCGATATATTTTTGAAGGAA
360A            GTTATAAATAATGAGGGCAGGACTCTGAATGAAACAAATATCGATATATTTTTGAAGGAA
UTI89           GTTATAAATAATGAGGGCAGGACTCTGAATGAAACAAATATCGATATATTTTTGAAGGAA
                ************************************************************

O83/K24/H31     TATGGAATTGCTGAAGATAGTATATCCTCAACTAATGTGTTAGACGTTAAGCTTAAAAGT
96-1913         TATGGAATTGCTGAAGATAGTATATCCTCAACTAATGTGTTAGACGTTAAGCTTAAAAGT
5383-2          TATGGAATTGCTGAAGATAGTATACCCTCAACTAATGTGTTAGACGTTAAGCTTAAAAGT
360A            TATGGAATTGCTGAAGATAGTATATCCTCAACTAATTTGTTAGACGTTAAGCTTAAAAGT
UTI89           TATGGAATTGCTGAAGATAGTATATCCTCAACTAATTTGTTAGACGTTAAGCTTAAAAGT
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O83/K24/H31     TCCGGGCAGCATGTCAATATTGTAAAGCTTAGTGATGAAGATAATCAAATTGTCGCTGTA
96-1913         TCCGGGCAGCATGTCAATATTGTAAAGCTTAGTGATGGAGATAATCAAATTGTCGCTGTA
5383-2          TCCGGGCAGCATGTCAATATTGTAAAGCTTAGTGATGAAGATAATCAAATTGTCGCTGTA
360A            TCCGGGCAGCATGTCAATATTGTAAAGCTTAGTGATGAAGATAATCAAATTGTCGCTGTA
UTI89           TCCGGGCAGCATGTCAATATTGTAAAGCTTAGTGATGAAGATAATCAAATTGTCGCTGTA
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O83/K24/H31     AAAGGGAGTTCTCTGAGCGGCATCTACTATGAAGTGGACATTGAAACAGGATATGAGATT
96-1913         AAAGGGAGTTCTCTGAGCGGCATCTACTATGAAGTGGACATTGAAACAGGATATGAGATT
5383-2          AAAGGGAGTTCTCTGAGCGGCATCTACTATGAAGTGGACATTGAAACAGGATATGAGATT
360A            AAAGGGAGTTCTCTGAGCGGCATCTACTATGAAGTGGACATTGAAACAGGATATGAGATT
UTI89           AAAGGGAGTTCTCTGAGCGGCATCTACTATGAAGTGGACATTGAAACAGGATATGAGATT
                ************************************************************

O83/K24/H31     TTATCCCGAAGAATTTATCGTACCGAATATAATAATGAAATTCTCTGGACTCGAGGTGGT
96-1913         TTATCCCGAAGAATTTATCGTACCGAATATAATAATGAAATTCTCTGGACTCGAGGTGGT
5383-2          TTATCCCGAAGAATTTATCGTACCGAATATAATAATGAAATTCTCTGGACTCGAGGTGGT
360A            TTATCCCGAAGAATTTATCGTACCGAATATAATAATGAAATTCTCTGGACTCGAGGTGGT
UTI89           TTATCCCGAAGAATTTATCGTACCGAATATAATAATGAAATTCTCTGGACTCGAGGTGGT
                ************************************************************

O83/K24/H31     GGTCTAAAAGGGGGGCAGCCATTTGATTTTGAAAGTCTCAATATTCCTGTATTTTTTAAA
96-1913         GGTCTAAAAGGGGGGCAGCCATTTGATTTTGAAAGTCTCAATATTCCTGTATTTTTTAAA
5383-2          GGTCTAAAAGGGGGGCAGCCATTTGATTTTGAAAGTCTCAATATTCCTGTATTTTTTAAA
360A            GGTCTAAAAGGGGGGCAGCCATTTGATTTTGAAAGTCTCAATATTCCTGTATTTTTTAAA
UTI89           GGTCTAAAAGGGGGGCAGCCATTTGATTTTGAAAGTCTCAATATTCCTGTATTTTTTAAA
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O83/K24/H31     GATGAACCCTATTCTGCAGTGACCGGATCTCCGTTATCATTTATTAATGATGACAGCTCA
96-1913         GATGAACCCTATTCTGCAGTGACCGGATCTCCGTTATCATTTATTAATGATGACAGCTCA
5383-2          GATGAACCCTATTCTGCAGTGACCGGATCTCCGTTATCATTTATTAATGATGACAGCTCA
360A            GATGAACCCTATTCTGCAGTGACCGGATCTCCGTTATCATTTATTAATGATGACAGCTCA
UTI89           GATGAACCCTATTCTGCAGTGACCGGATCTCCGTTATCATTTATTAATGATGACAGCTCA
                ************************************************************

O83/K24/H31     CTTTTATATCCTGATACAAACCCAAAATTACCGCAACCAACGTCAGAAATGGATATTGTT
96-1913         CTTTTATATCCTGATACAAACCCAAAATTACCGCAACCAACGTCAGAAATGGATATTGTT
5383-2          CTTTTATATCCTGATACAAACCCAAAATTACCGCAACCAACGTCAGAAATGGATATTGTT
360A            CTTTTATATCCTGATACAAACCCAAAATTACCGCAACCAACGTCAGAAATGGATATTGTT
UTI89           CTTTTATATCCTGATACAAACCCAAAATTACCGCAACCAACGTCAGAAATGGATATTGTT
                ************************************************************

O83/K24/H31     AATTATGTTAAGGGTTCTGGAAGTTTTGGGGATAGATTTGTAACTTTGATGAGAGGAGCT
96-1913         AATTATGTTAAGGGTTCTGGAAGTTTTGGGGATAGATTTGTAACTTTGATGAGAGGAGCT
5383-2          AATTATGTTAAGGGTTCTGGAAGTTTTGGGGATAGATTTGTAACTTTGATGAGAGGAGCT
360A            AATTATGTTAAGGGTTCTGGAAGCTTTGGGGATAGATTTGTAACTTTGATGAGAGGAGCT
UTI89           AATTATGTTAAGGGTTCTGGAAGCTTTGGGGATAGATTTGTAACTTTGATGAGAGGAGCT
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O83/K24/H31     ACTGAGGAAGAAGCATGGAATATTGCCTCTTATCATACGGCTGGGGGAAGTACAGAAGAA
96-1913         ACTGAGGAAGAAGCATGGAATATTGCCTCTTATCATACGGCTGGGGGAAGTACAGAAGAA
5383-2          ACTGAGGAAGAAGCATGGAATATTGCCTCTTATCATACGGCTGGGGGAAGTACAGAAGAA
360A            ACTGAGGAAGAAGCATGGAATATTGCCTCTTATCATACGGCTGGGGGAAGTACAGAAGAA
UTI89           ACTGAGGAAGAAGCATGGAATATTGCCTCTTATCATACGGCTGGGGGAAGTACAGAAGAA
                ************************************************************

O83/K24/H31     TTACACGAAATTTTGTTAGGTCAGGGCCCACAGTCAAGCTTAGGTTTTACTGAATATACC
96-1913         TTACACGAAATTTTGTTAGGTCAGGGCCCACAGTCAAGCTTAGGTTTTACTGAATATACC
5383-2          TTACACGAAATTTTGTTAGGTCAGGGCCCACAGTCAAGCTTAGGTTTTACTGAATATACC
360A            TTACACGAAATTTTGTTAGGTCAGGGCCCACAGTCAAGCTTAGGTTTTACTGAATATACC
UTI89           TTACACGAAATTTTGTTAGGTCAGGGCCCACAGTCAAGCTTAGGTTTTACTGAATATACC
                ************************************************************

O83/K24/H31     TCAAATGTTAACAGTGCAGATGCAGCAAGCAGACGACACTTTCTGGTAGTTATAAAAGTG
96-1913         TCAAATGTTAACAGTGCAGATGCAGCAAGCAGACGACACTTTCTGGTAGTTATAAAAGTG
5383-2          TCAAATGTTAACAGTGCAGATGCAGCAAGCAGACGACACTTTCTGGTAGTTATAAAAGTG
360A            TCAAATGTTAACAGTGCAGATGCAGCAAGCAGACGACACTTTCTGGTAGTTATAAAAGTG
UTI89           TCAAATGTTAACAGTGCAGATGCAGCAAGCAGACGACACTTTCTGGTAGTTATAAAAGTG
                ************************************************************

O83/K24/H31     CACGTAAAATATATCAACAATAATAATGTTTCATATGTTAATCATTGGGCAATTCCTGAT
96-1913         CACGTAAAATATATCAACAATAATAATGTTTCATATGTTAATCATTGGGCAATTCCTGAT
5383-2          CACGTAAAATATATCAACAATAATAATGTTTCATATGTTAATCATTGGGCAATTCCTGAT
360A            CACGTAAAATATATCACCAATAATAATGTTTCATATGTTAATCATTGGGCAATTCCTGAT
UTI89           CACGTAAAATATATCACCAATAATAATGTTTCATATGTTAATCATTGGGCAATTCCTGAT
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O83/K24/H31     GAAGCCCCGGTTGAAGTACTGGCTGTGGTTGACAGGAGATTTAATTTTCCTGAGCCATCA
96-1913         GAAGCCCCGGTTGAAGTACTGGCTGTGGTTGACAGGAGATTTAATTTTCCTGAGCCATCA
5383-2          GAAGCCCCGGTTGAAGTACTGGCTGTGGTTGACAGGAGATTTAATTTTCCTGAGCCATCA
360A            GAAGCCCCGGTTGAAGTACTGGCTGTGGTTGACAGGAGATTTAATTTTCCTGAGCCATCA
UTI89           GAAGCCCCGGTTGAAGTACTGGCTGTGGTTGACAGGAGATTTAATTTTCCTGAGCCATCA
                ************************************************************

O83/K24/H31     ACGCCTCCTGATATATCAACCATACGTAAATTGTTATCTCTACGATATTTTAAAGAAAGT
96-1913         ACGCCTCCTGATATATCAACCATACGTAAATTGTTATCTCTACGATATTTTAAAGAAAGT
5383-2          ACGTCTCCTGATATATCAACCATACGTAAATTGTTATCTCTACGATATTTTAAAGAAAGT
360A            ACGCCTCCTGATATATCAACCATACGTAAATTGTTATCTCTACGATATTTTAAAGAAAGT
UTI89           ACGCCTCCTGATATATCAACCATACGTAAATTGTTATCTCTACGATATTTTAAAGAAAGT
                *** ********************************************************

O83/K24/H31     ATCGAAAGCACCTCCAAATCTAACTTTCAGAAATTAAGTCGCGGTAATATTGATGTGCTT
96-1913         ATCGAAAGCACCTCCAAATCTAACTTTCAGAAATTAAGTCGCGGTAATATTGATGTGCTT
5383-2          ATCGAAAGCACCTCCAAATCTAACTTTCAGAAATTAAGTCGCGGTAATATTGATGTGCTT
360A            ATCGAAAGCACCTCCAAATCTAACTTTCAGAAATTAAGTCGCGGTAATATTGATGTGCTT
UTI89           ATCGAAAGCACCTCCAAATCTAACTTTCAGAAATTAAGTCGCGGTAATATTGATGTGCTT
                ************************************************************

O83/K24/H31     AAAGGACGGGGAAGTATTTCATCGACACGTCAGCGTGCAATCTATCCGTATTTTGAAGCC
96-1913         AAAGGACGGGGAAGTATTTCATCGACACGTCAGCGTGCAATCTATCCGTATTTTGAAGCC
5383-2          AAAGGACGGGGAGGTATTTCATCGACACGTCAGCGTGCAATCTATCCGTATTTTGAAGCC
360A            AAAGGACGGGGAAGTATTTCATCGACACGTCAGCGTGCAATCTATCCGTATTTTGAAGCC
UTI89           AAAGGACGGGGAAGTATTTCATCGACACGTCAGCGTGCAATCTATCCGTATTTTGAAGCC
                ************ ************************************************

O83/K24/H31     GCTAATGCTGATGAGCAACAACCTCTCTTTTTCTACATCAAAAAAGATCGCTTTGATAAC
96-1913         GCTAATGCTGATGAGCAACAACCTCTCTTTTTCTACATCAAAAAAGATCGCTTTGATAAC
5383-2          GCTAATGCTGATGAGCAACAACCTCTCTTTTTCTACATCAAAAAAGATCGCTTTGATAAC
360A            GCTAATGCTGATGAGCAACAACCTCTCTTTTTCTACATCAAAAAAGATCGCTTTGATAAC
UTI89           GCTAATGCTGATGAGCAACAACCTCTCTTTTTCTACATCAAAAAAGATCGCTTTGATAAC
                ************************************************************

O83/K24/H31     CATGGCTATGATCAGTATTTCTATGATAATACAGTGGGGCTAAATGGTATTCCAACATTG
96-1913         CATGGCTATGATCAGTATTTCTATGATAATACAGTGGGGCTAAATGGTATTCCAACATTG
5383-2          CATGGCTATGATCAGTATTTCTATGATAATACAGTGGGGCTAAATGGTATTCCAACATTG
360A            CATGGCTATGATCAGTATTTCTATGATAATACAGTGGGGCTAAATGGTATTCCAACATTG
UTI89           CATGGCTATGATCAGTATTTCTATGATAATACAGTGGGGCTAAATGGTATTCCAACATTG
                ************************************************************

O83/K24/H31     AACACCTATACTGGGGAAATTCCATCAGATTCATCTTCACTCGGCTCAACTTATTGGAAG
96-1913         AACACCTATACTGGGGAAATTCCATCAGATTCATCTTCACTCGGCTCAACTTATTGGAAG
5383-2          AACACCTATACTGGGGAAATTCCATCAGATTCATCTTCACTCGGCTCAACTTATTGGAAG
360A            AACACCTATACTGGGGAAATTCCATCAGACTCATCTTCACTCGGCTCAACTTATTGGAAG
UTI89           AACACCTATACTGGGGAAATTCCATCAGACTCATCTTCACTCGGCTCAACTTATTGGAAG
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O83/K24/H31     AAGTATAATCTTACTAATGAAACAAGCATAATTCGTGTGTCAAATTCTGCTCGTGGGGCG
96-1913         AAGTATAATCTTACTAATGAAACAAGCATAATTCGTGTGTCAAATTCTGCTCGTGGGGCG
5383-2          AAGTATAATCTTACTAATGAAACAAGCATAATTCGTGTGTCAAATTCTGCTCGTGGGGCG
360A            AAGTATAATCTTACTAATGAAACAAGCATAATTCGTGTGTCAAATTCTGCTCGTGGGGCG
UTI89           AAGTATAATCTTACTAATGAAACAAGCATAATTCGTGTGTCAAATTCTGCTCGTGGGGCG
                ************************************************************

O83/K24/H31     AATGGTATTAAAATAGCACTTGAGGAAGTCCAGGAGGGTAAACCAGTAATCATTACAAGC
96-1913         AATGGTATTAAAATAGCACTTGAGGAAGTCCAGGAGGGTAAACCAGTAATCATTACAAGC
5383-2          AATGGTATTAAAATAGCACTTGAGGAAGTCCAGGAGGGTAAACCAGTAATCATTACAAGC
360A            AATGGTATTAAAATAGCACTTGAGGAAGTCCAGGAGGGTAAACCAGTAATCATTACAAGC
UTI89           AATGGTATTAAAATAGCACTTGAGGAAGTCCAGGAGGGTAAACCAGTAATCATTACAAGC
                ************************************************************

O83/K24/H31     GGAAATCTAAGTGGTTGTACGACAATTGTTGCCCGAAAAGAAGGATATATTTATAAGGTA
96-1913         GGAAATCTAAGTGGTTGTACGACAATTGTTGCCCGAAAAGAAGGATATATTTATAAGGTA
5383-2          GGAAATCTAAGTGGTTGTACGACAATTGTTGCCCGAAAAGAAGGATATATTTATAAGGTA
360A            GGAAATCTAAGTGGTTGTACGACAATTGTTGCCCGAAAAGAAGGATATATTTATAAGGTA
UTI89           GGAAATCTAAGTGGTTGTACGACAATTGTTGCCCGAAAAGAAGGATATATTTATAAGGTA
                ************************************************************

O83/K24/H31     CATACTGGTACAACAAAATCTTTGGCTGGATTTACCAGTACTACCGGGGTGAAAAAAGCA
96-1913         CATACTGGTACAACAAAATCTTTGGCTGGATTTACCAGTACTACCGGGGTGAAAAAAGCA
5383-2          CATACTGGTACAACAAAATCTTTGGCTGGATTTACCAGTACTACCGGGGTGAAAAAAGCA
360A            CATACTGGTACAACAAAATCTTTGGCTGGATTTACCAGTACTACCGGGGTGAAAAAAGCA
UTI89           CATACTGGTACAACAAAATCTTTGGCTGGATTTACCAGTACTACCGGGGTGAAAAAAGCA
                ************************************************************

O83/K24/H31     GTTGAAGTACTTGAGCTACTTACAAAAGAACCAATACCTCGCGTGGAGGGAATAATGAGC
96-1913         GTTGAAGTACTTGAGCTACTTACAAAAGAACCAATACCTCGCGTGGAGGGAATAATGAGC
5383-2          GTTGAAGTACTTGAGCTACTTACAAAAGAACCAATACCTCGCGTGGAGGGAATAATGAGC
360A            GTTGAAGTACTTGAGCTACTTACAAAAGAACCAATACCTCGCGTGGAGGGAATAATGAGC
UTI89           GTTGAAGTACTTGAGCTACTTACAAAAGAACCAATACCTCGCGTGGAGGGAATAATGAGC
                ************************************************************

O83/K24/H31     AATGATTTCTTAGTCGATTATCTGTCGGAAAATTTTGAAGATTCATTAATAACTTACTCA
96-1913         AATGATTTCTTAGTCGATTATCTGTCGGAAAATTTTGAAGATTCATTAATAACTTACTCA
5383-2          AATGATTTCTTAGTCGATTATCTGTCGGAAAATTTTGAAGATTCATTAATAACTTACTCA
360A            AATGATTTCTTAGTCGATTATCTGTCGGAAAATTTTGAAGATTCATTAATAACTTACTCA
UTI89           AATGATTTCTTAGTCGATTATCTGTCGGAAAATTTTGAAGATTCATTAATAACTTACTCA
                ************************************************************

O83/K24/H31     TCATCTGAAAAAAAACCAGATAGTCAAATCACTATTATTCGTGATAATGTTTCTGTTTTC
96-1913         TCATCTGAAAAAAAACCAGATAGTCAAATCGCTATTATTCGTGATAATGTTTCTGTTTTC
5383-2          TCATCTGAAAAAAAACCAGATAGTCAAATCACTATTATTCGTGATAATGTTTCTGTTTTC
360A            TCATCTGAAAAAAAACCAGATAGTCAAATCACTATTATTCGTGATAATGTTTCTGTTTTC
UTI89           TCATCTGAAAAAAAACCAGATAGTCAAATCACTATTATTCGTGATAATGTTTCTGTTTTC
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O83/K24/H31     CCTTACTTCCTTGATAATATACCTGAACATGGCTTTGGTACATCGGCGACTGTACTGGTG
96-1913         CCTTACTTCCTTGATAATATACCTGAACATGGCTTTGGTACATCGGCGACTGTACTGGTG
5383-2          CCTTACTTCCTTGATAATATACCTGAACATGGCTTTGGTACATCGGCGACTGTACTGGTG
360A            CCTTACTTCCTTGATAATATACCTGAACATGGCTTTGGTACATCGGCGACTGTACTGGTG
UTI89           CCTTACTTCCTTGATAATATACCTGAACATGGCTTTGGTACATCGGCGACTGTACTGGTG
                ************************************************************

O83/K24/H31     AGAGTGGACGGCAATGTTGTCGTAAGGTCTCTGTCTGAGAGTTATTCTCTGAATGCAGAT
96-1913         AGAGTGGACGGCAATGTTGTCGTAAGGTCTCTGTCTGAGAGTTATTCTCTGAATGCAGAT
5383-2          AGAGTGGACGGCAATGTTGTCGTAAGGTCTCTGTCTGAGAGTTATTCTCTGAATGCAGAT
360A            AGAGTGGACGGCAATGTTGTCGTAAGGTCTCTGTCTGAGAGTTATTCTCTGAATGCAGAT
UTI89           AGAGTGGACGGCAATGTTGTCGTAAGGTCTCTGTCTGAGAGTTATTCTCTGAATGCAGAT
                ************************************************************

O83/K24/H31     GTCTCCGAAATATCGGTATTGAAGGTATTTTCAAAAAAATTTTGA
96-1913         GTCTCCGAAATATCGGTATTGAAGGTATTTTCAAAAAAATTTTGA
5383-2          GTCTCCGAAATATCGGTATTGAAGGTATTTTCAAAAAAATTTTGA
360A            GCCTCCGAAATATCGGTATTGAAGGTATTTTCAATAAAATTTTGA
UTI89           GCCTCCGAAATATCGGTATTGAAGGTATTTTCAAAAAAATTTTGA
                * ******************************** **********
```

The regions are:
* - single, fully conserved residue
  - no consensus

The sequence analysis was done using The Biology WorkBench (http://workbench.sdsc.edu/).

Appendix V

Sequence analysis of *igs* region between *hlyD* and *cnf1*

An alignment of the intergenic (*igs*) region between the genes *hlyD* and *cnf1* of *Escherichia coli* strains 360A (clinical isolate) and two uropathogenic strains UTI189 and J96:

```
360A            GCGAAGCGTAATCAGCTATCTTCTTAGTCCTCTGGAAGAGTCTGTAACAGAAAGTTTACA
UTI89           GCGAAGCGTAATCAGCTATCTTCTTAGTCCTCTGGAAGAGTCTGTAACAGAAAGTTTACA
J96             GCGAAGCGTAATCAGCTATCTTCTTAGTCCTCTGGAAGAGTCTGTAACAGAAAGTTTACA
                ************************************************************

360A            TGAGCGTTAAGTCTCAGAGCCGCGGTATCCGGCTCATATCTTCTCCTGTCATGTGTGACT
UTI89           TGAGCGTTAAGTCTCAGAGCCGCGGTATCCGGCTCATATCTTCTCCTGTCATGTGTGACT
J96             TGAGCGTTAAGTCTCAGAGCAGCGGTATCCGGCTCATATCTTCTCCTGTCATGTGT-ACT
                ****************** ********************************** ***

360A            GCACTGTTTTGTGGCAAACCAGTACTGACTATCCACTGGAATTCGGTAGTCATTAACTAT
UTI89           GCACTGTTTTGTGGCAAACCAGTACTGACTATCCACTGGAATTCGGTAGTCATTAACTAT
J96             GCACTGTTTTGTGGCAAACCAGTACTGACTATCCACCGGAATTCGGTAGTCATTAACTAT
                ********************************** ***********************

360A            GGCATTGATGTCCTTTATAACTGTCTTACAGGGCGTATAACACATCCCGCGACGGAAGCT
UTI89           GGCATTGATGTCCTTTATAACTGTCTTACAGGGCGTATAACACATCCCGCGACGGAAGCT
J96             GGCATTGATGTCCTTTATAACTGTCTTACAGGGCGTATAACACATCCCGCGACGGAAGCT
                ************************************************************

360A            TATCACAGAGGAAGAGGTAAGCTTCAGAATGGAAAGTATATGAGAAGACTGTGATTGTTT
UTI89           TATCACAGAGGAAGAGGTAAGCTTCAGAATGGAAAGTATATGAGAAGACTGTGATTGTTT
J96             TATCACAGAGGAAGAGGTAAGCTTCAGAATGGAAAGTATATGAGAAGACTGTGATTGTTT
                ************************************************************

360A            AAAAATACACAATCCGCTGTGAGGTGGAGTCAGGGGAGGTACTGGCCCGCAGAAGCGGAG
UTI89           AAAAATACACAATCCGCTGTGAGGTGGAGTCAGGGGAGGTACTGGCCCGCAGAAGCGGAG
J96             AAAAATACACAATCCGCTGTGAGGTGGAGTCAGGGGAGGTACTGGCC-GCAGAAGCG-AG
                ********************************************** ********* **

360A            AATGGTTGATGTATGAGGCGAGTGTGACATTTTTCATGAAAGTAAAACAGGTGATAACCT
UTI89           AATGGTTGATGTATGAGGCGAGTGTGACATTTTTCATGAAAGTAAAACAGGTGATAACCT
J96             AATGGTTGATGTATGAGGCGAGTGTGACATTTTTCATGAAAGTAAAACAGGTGATAACCT
                ************************************************************

360A            CCTTTGGTTAGGGGGATTCACAGGTATTATTACAGAACAATGTTGTTTCTGGCTTGTTCT
UTI89           CCTTTGGTTAGGGGGATTCACAGGTATTATTACAGAACAATGTTGTTTCTGGCTTGTTCT
J96             CCTTTGGTTAGGGGGATTACACGGTATTATTACAGAACAATGTTGTTTCTGGCTTGTTCT
                ******************    ************************************

360A            TTTTTTGCAACTGGAATAGCCTCTTTTAATATGGGGCACCCCGAAATACTGTATTTCTCA
UTI89           TTTTTTGCAACTGGAATAGCCTCTTTTAATATGGGGCACCCCGAAATACTGTATTTCTCA
J96             TTTTTTGCAACTGGAATAGCCTCTTTTAATATGGGGCACCC-GAAATACTGTATTTCTCA
                **************************************** ******************
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360A            GCCATCAGTACAGCACTTTCTCCGTTCTTTGCCTGGTGTTTGCGGTATCCTGATGAGGAG
UTI89           GCCATCAGTACAGCACTTTCTCCGTTCTTTGCCTGGTGTTTGCGGTATCCTGATGAGGAG
J96             GCCATCAGTACAGCACTTTCTCCGTTCTTTGCCTGGTGTTTGCGGTATCCTGATGAGGAG
                ************************************************************

360A            ATTAATGAAGGTATATGGGGATATAATGCTGTTCTGTATGGCATAGCCTGTGGCATGCTT
UTI89           ATTAATGAAGGTATATGGGGATATAATGCTGTTCTGTATGGCATAGCCTGTGGCATGCTT
J96             ATTAATGAAGGTATATGGGGATATAATGCTGTTCTGTATGGCATAGCCTGTGGCATGCTT
                ************************************************************

360A            GTCCCTGTGTCAGTCTCCGGAATTGCTGTACTTATTGTCGGAACACTGGAGATGCTCCTT
UTI89           GTCCCTGTGTCAGTCTCCGGAATTGCTGTACTTATTGTCGGAACACTGGAGATGCTCCTT
J96             GTCCCTGTGTCAGTCTCCGGAATTGCTGTACTTATTGTCGGAACACTGGAGATGCTCCTT
                ************************************************************

360A            TTAATGGGGTTCAGATAATACTTAACCTGCTGGCCCGACCTGTCCTGATGATGCCAGGTT
UTI89           TTAATGGGGTTCAGATAATACTTAACCTGCTGGCCCGACCTGTCCTGGTGATGCCAGGTT
J96             TTAATGGGGTTCAGATAATACTTAACCTGCTGGCCCGACCTGTCCTGGTGATGCCAGGTT
                ********************************************** ************

360A            TACCAGCTTTCTTTCAATGTGGAATATA-TTAATAGAAATCCAGTGGTTTTGCATACCGG
UTI89           TACCAGCTTTCTTTCAATGTGGAATATA-TTAATAGAAATCCAGTGGTTTTGCATACCGG
J96             TACCAGCTTTCTTTCAATGTGGAATATAATTAATAGAAATCCAGTGGTTTTGCATACCGG
                **************************** ********************************

360A            GATGTTGTGTAGTGCAAAATTTATACTACAGGGATCGGAGTAGTGATGTTTAAGATTCAT
UTI89           GATGTTGTGTAGTGCAAAATTTATACTACAGGGATCGGAGTAGTGATGTTTAAGATTCAT
J96             GATGTTGTGTAGTGCAAAATTTATACTACAGGGATCGGAGTAGTGATGTTTAAGATTCAT
                ************************************************************

360A            TAAATAGTTTCAAAGACGCTTTGTATACGACATGATTGCTAACCTCTCGCCCAGTGATTA
UTI89           TAAATAGTTTCAAAGACGCTTTGTATACGACATGATTGCTAACCTCTCGCCCAGTGATTA
J96             TAAATAGTTTCAAAGACGCTTTGTATACGACATGATTGCTAACCTCTCGCCCAGTGATTA
                ************************************************************

360A            GGTATTCTGATAAGGTGTAGTAAAATATTAATCTTCACAGAGGAGTTAAAAATATTATGG
UTI89           GGTATTCTGATAAGGTGTAGTAAAATATTAATCTTCACAGAGGAGTTAAAAATATTATGG
J96             GGTATTCTGATAAGGTGTAGTAAAATATTAATCTTCACAGAGGAGTTAAAAATATTATGG
                ************************************************************

360A            GTAACCAATGGCAACAAAAATATCTTCTTGAGTACAATGAGTTGGTATCAAATTTCCCTT
UTI89           GTAACCAATGGCAACAAAAATATCTTCTTGAGTACAATGAGTTGGTATCAAATTTCCCTT
J96             GTAACCAATGGCAACAAAAATATCTTCTTGAGTACAATGAGTTGGTATCAAATTTCCCTT
                ************************************************************
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An asterisk signifies a fully conserved residue. A black letter indicates a non-conserved residue. The green letters indicate the 3' end of the *hlyD* gene, the stop codon is in bold face type. The red letters indicate the 5' region of the *cnf1* gene, the underlined portion is the start codon of the *cnf1* gene. The underlined portions in blue are the -35 and -10 of the *cnf1* promoter and the ribosomal binding site in bold as defined by Landrad et. al. 2003. (171). The sequence analysis was done using The Biology WorkBench (http://workbench.sdsc.edu/).

Appendix VI

Sequence analysis of *papGIII*

Sequence alignment of *papGIII* (also known as *prsG*) alleles from eight *Escherichia coli* isolates:

```
5438        ATGAAAAAATGGCTCCCTGCTTTTTTATTTTTATCCCTGTCAGGCTGTAATGATGCTCTG
536         ATGAAAAAATGGTTCCCTGCTTTTTTATTTTTATCCCTGTCAGGCTGTAATGATGCTCTG
UTI19       ATGAAAAAATGGCTCCCTGCTTTTTTATTTTTATCCCTGTCAGGCTGTAATGATGCTCTG
5557        ATGAAAAAATGGCTCCCTGCTTTTTTATTTTTATCCCTGTCAGGCTGTAATGATGCTCTG
1442        ATGAAAAAATGGCTCCCTGCTTTTTTATTTTTATCCCTGTCAGGCTGTAATGATGCTCTG
CP9         ATGAAAAAATGGCTCCCTGCTTTTTTATTTTTATCCCTGTCAGGCTGTAATGATGCTCTG
360A        ----------------------------------CCTGTCAGGCTGTAATGATGCTCTG
83972       ATGAAAAAATGGTTCCCTGCTTTTTTATTTTTATCCCTGTCAGGCTGTAATGATGCTTTG
                                               ********************** **

5438        GCTGCAAACCAGAGTACAATGTTTTACTCGTTTAATGATAACATTTATCGTCCTCAACTT
536         GCTGCAAACCAGAGTACAATATTTTACTCGTTTAATGATAACATTTATCATCCTCAACTT
UTI19       GCTGCAAACCAGAGTACAATGTTTTACTCGTTTAATGATAACATTTATCGTCCTCAACTT
5557        GCTGCAAACCAGAGTACAATGTTTTACTCGTTTAATGATAACATTTATCGTCCTCAACTT
1442        GCTGCAAACCAGAGTACAATGTTTTACTCGTTTAATGATAACATTTATCGTCCTCAACTT
CP9         GCTGCAAACCAGAGTACAATGTTTTACTCGTTTAATGATAACATTTATCGTCCTCAACTT
360A        GCTGCAAACCAGAGTACAATGTTTTACTCGTTTAATGATAACATTTATCGTCCTCAACTT
83972       GCT---ATCCAGAGTACAATGTTTTACTCGTTTAATGATAACATTTATCGTCCTCGACTT
            ***   * ************ ***************************** ***** ****

5438        AGTGTTAAAGTAACCGATATTGTTCAATTCATAGTGGATATAAACTCCGCATCAAGTACG
536         AGTGTTAAAGTAACCGATATTGTTCAATTCATAGTGGATATAAACTCCGCATCAAGTACG
UTI19       AGTGTTAAAGTAACCGATATTGTTCAATTCATAGTGGATATAAACTCCGCATCAAGTACG
5557        AGTGTTAAAGTAACCGATATTGTTCAATTCATAGTGGATATAAACTCCGCATCAAGTACG
1442        AGTGTTAAAGTAACCGATATTGTTCAATTCATAGTGGATATAAACTCCGCATCAAGTACG
CP9         AGTGTTAAAGTAACCGATATTGTTCAATTCATAGTGGATATAAACTCCGCATCAAGTACG
360A        AGTGTTAAAGTAACCGATATTGTTCAATTCATAGTGGATATAAACTCCGCATCAAGTACG
83972       AGTGTTAAAGTAACCGATGTTATTCAAATTATAGTGGATATAAACTCTGCATCAAGTACG
            ****************** ** ***** * ***************** ************

5438        GCAACTTTAAGCTATGTGGCCTGCAATGGATTTACCTGGACTCATGGTCTTTACTGGTCT
536         GCAACTTTAAGCTATGTGGCCTGCAATGGATTTACCTGGACTCATGGTCTTTACTGGTCT
UTI19       GCAACTTTAAGCTATGTGGCCTGCAATGGATTTACCTGGACTCATGGTCTTTACTGGTCT
5557        GCAACTTTAAGCTATGTGGCCTGCAATGGATTTACCTGGACTCATGGTCTTTACTGGTCT
1442        GCAACTTTAAGCTATGTGGCCTGCAATGGATTTACCTGGACTCATGGTCTTTACTGGTCT
CP9         GCAACTTTAAGCTATGTGGCCTGCAATGGATTTACCTGGACTCATGGTCTTTACTGGTCT
360A        GCAACTTTAAGCTATGTGGCGTGCAATGGATTTACCTGGACTCATGGTCTTTACCGGTCT
83972       GCAACTTTAAGCTATGTGGACTGCAATGGATTTACATGGTCTCATGGTATTTACTGGTCT
            ******************  ************** *** ******** ***** *****
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5438        GAGTATTTTGCATGGCTGGTTGTTCCTAAACATGTTTCCTATAATGGATATAATATATAT
536         GAGTATTTTGCATGGCTGGTTGTTCCTAAACATGTTTCCTATAATGGATATAATATATAT
UTI19       GAGTATTTTGCATGGCTGGTTGTTCCTAAACATGTTTCCTATAATGGATATAATATATAT
5557        GAGTATTTTGCATGGCTGGTTGTTCCTAAACATGTTTCCTATAATGGATATAATATATAT
1442        GAGTATTTTGCATGGCTGGTTGTTCCTAAACATGTTTCCTATAATGGATATAATATATAT
CP9         GAGTATTTTGCATGGCTGGTTGTTCCTAAACATGTTTCCTATAATGGATATAATATATAT
360A        GAGTATTTTGCATGGCTGGTTGTTCCTAAACATGTTTCCTATAATGGATATAATATATAT
83972       GAGTATTTTGCATGGCTGGTTGTTCCTAAACGTGTTTCCTATAATGGATATGATATATAT
            ******************************** ****************** ********

5438        CTTGAACTTCAGTCCAAAGGAGGTTTTTCACTTGATGCAGAAGATAATGATAATTACTAT
536         CTTGAACTTCAGTCCAAAGGAGGTTTTTCACTTGATGCAGAAGATAATGATAATTACTAT
UTI19       CTTGAACTTCAGTCCAGAGGAAGTTTTTCACTTGATGCAGAAGATAATGATAATTACTAT
5557        CTTGAACTTCAGTCCAGAGGAAGTTTTTCACTTGATGCAGAAGATAATGATAATTACTAT
1442        CTTGAACTTCAGTCCAGAGGAAGTTTTTCACTTGATGCAGAAGATAATGATAATTACTAT
CP9         CTTGAACTTCAGTCCAGAGGAAGTTTTTCACTTGATGCAGAAGATAATGATAATTACTAT
360A        CTTGAACTTCAGTCCAGAGGAAGTTTTTCACTTGATGCAGAAGATAATGATAATTACTAT
83972       CTTGAACTTCAGTCCAGAGGAAGTTTTTCACTTGATGCAGAAGATAATGATAATTACTAT
            ***************** **** ************************************

5438        CTTACCAAGGGATTTGCATGGGATGAAGTAAACTCATCTGGACGGGTATGTTTCGATATC
536         CTTACCAAGGGATTTGCATGGGATGAAGTAAACTCATCTGGACGGGTATGTTTCGATATC
UTI19       CTTACCAAGGGATTTGCATGGGATGAAGTAAACTCATCTGGACGGGTATGTTTCGATATC
5557        CTTACCAAGGGATTTGCATGGGATGAAGTAAACCCATCTGGACGGACATGTTTCAATATC
1442        CTTACCAAGGGATTTGCATGGGATGAAGCAAACACATCTGGACAGACATGTTTCAATATC
CP9         CTTACCAAGGGATTTGCATGGGATGAAGCAAACACATCTGGACAGACATGTTTCAATATC
360A        CTTACCAAGGGATTTGCATGGGATGAAGCAAACACATCTGGACAGACATGTTTCAATATC
83972       CTTACCAAGGGATTTGCATGGGATGAAGCAAACACATCTGGACGGACATGTTTCAATATC
            **************************** **** ********* *  ******* *****

5438        GGAGAAAAAAGAAGTCTGGCATGGTCATTTGGTGGTGTTACCCTGAACGCCAGATTGCCT
536         GGAGAAAAAAGAAGTCTGGCATGGTCATTTGGTGGTGTTACCCTGAACGCCAGATTGCCT
UTI19       GGAGAAAAAAGAAGTCTGGCATGGTCATTTGGTGGTGTTACCCTGAACGCCAGATTGCCT
5557        GGAGAAAAAAGAAGTCTGGCATGGTCATTTGGTGGTGTTACCCTGAACGCCAGATTGCCT
1442        GGAGAAAAAAGAAGTCTGGCATGGTCATTTGGTGGTGTTACCCTGAACGCCAGATTGCCT
CP9         GGAGAAAAAAGAAGTCTGGCATGGTCATTTGGTGGTGTTACCTTGAACGCCAGATTGCCT
360A        GGAGAAAAAAGAAGTCTGGCATGGTCATTTGGTGGTGTTACCCTGAACGCCAGATTGCCT
83972       GGAGAAAAAAGAAGTCTGGCATGGTCATTTGGTGGTGTTACCCTGAACGCCAGATTTCCT
            ****************************************** ************* ***

5438        GTTGACCTTCCTAAGGGGGATTATACGTTTCCAGTTAAGTTCTTACGTGGCATTCAGCGT
536         GTTGACCTTCCTAAGGGGGATTATACGTTTCCAGTTAAGTTCTTACGTGGCATTCAGCGT
UTI19       GTTGACCTTCCTAAGGGGGATTATACGTTTCCAGTTAAGTTCTTACGTGGCATTCAGCGT
5557        GTTGACCTTCCTAAGGGGGATTATACGTTTCCAGTTAAGTTCTTACGTGGCATTCAGCGT
1442        GTTGACCTTCCTAAGGGGGATTATACGTTTCCAGTTAAGTTCTTACGTGGCATTCAGCGT
CP9         GTTGACCTTCCTAAGGGGGATTATACGTTTCCAGTTAAGTTCTTACGTGGCATTCAGCGT
360A        GTTGACCTTCCTAAGGGGGATTATACGTTTCCAGTTAAGTTCTTACGTGGCATTCAGCGT
83972       GTTGACCTTCCTAAGGGGGATTATACGTTTCCAGTCAAGTTCTTACGTGGCATTCAGCGT
            ********************************** ************************
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5438        AATAATTATGATTATATTGGTGGACGCTACAAAATCCCTTCCTCGTTAATGAAAACATTT
536         AATAATTATGATTATATTGGTGGACGCTACAAAATCCCTTCCTCGTTAATGAAAACATTT
UTI19       AATAATTATGATTATATTGGTGGACGCTACAAAATCCCTTCCTCGTTAATGAAAACATTT
5557        AATAATTATGATTATATTGGTGGACGCTACAAAATTCCTTCCTCGTTAATGAAAACATTT
1442        AATAATTATGATTATATTGGTGGACGCTACAAAATCCCTTCTTCGTTAATGAAAACATTT
CP9         AATAATTATGATTATATTGGTGGACGCTACAAAATCCCTTCTTCGTTAATGAAAACATTT
360A        AATAATTATGATTATATTGGTGGACGCTACAAAATTCCTTCCTCGTTAATGAAAACATTT
83972       AATAATTATGATTATATTGGTGGACGCTACAAAATTCCTTCCTCGTTAATGAAAACATTT
            ********************************** ***** ******************

5438        CCTTTTAATGGTACATTGAATTTCTCAATTAAAAATACCGGAGGATGCCGTCCTTCTGCA
536         CCTTTTAATGGTACATTGAATTTCTCAATTAAAAATACCGGAGGATGCCGTCCTTCTGCA
UTI19       CCTTTTAATGGTACATTGAATTTCTCAATTAAAAATACCGGAGGATGCCGTCCTTCTGCA
5557        CCTTTTAATGGTACATTGAATTTCTCAATTAAAAATACCGGAGGATGCCGTCCTTCTGCA
1442        CCTTTTAATGGTACATTGAATTTCTCAATTAAAAATACCGGAGGATGCCGTCCTTCTGCA
CP9         CCTTTTAATGGTACATTGAATTTCTCAATTAAAAATACCGGAGGATGCCGTCCTTCTGCA
360A        CCTTTTAATGGTACATTGAATTTCTCAATTAAAAATACCGGAGGATGCCGTCCTTCTGCA
83972       CCTTTTAATGGTACATTGAATTTCTCAATTAAGAATACCGGAGGATGCCGTCCTTCTGCA
            ******************************** ***************************

5438        CAGTCTCTGGAAATAAATCATGGTGATCTGTCGATTAATAGCGCTAATAATCATTATGCG
536         CAGTCTCTGGAAATAAATCATGGTGATCTGTCGATTAATAGCGCTAATAATCATTATGCG
UTI19       CAGTCTCTGGAAATAAATCATGGTGATCTGTCGATTAATAGCGCTAATAATCATTATGCG
5557        CAGTCTCTGGAAATAAATCATGGTGATCTGTCGATTAATAGCGCTAATAATCATTATGCG
1442        CAGTCTCTGGAAATAAATCATGGTGATCTGTCGATTAATAGCGCTAATAATCATTATGCG
CP9         CAGTCTCTGGAAATAAATCATGGTGATCTGTCGATTAATAGCGCTAATAATCATTATGCG
360A        CAGTCTCTGGAAATAAATCATGGTGATCTGTCGATTAATAGCGCTAATAATCATTATGCG
83972       CAGTCTCTGGAAATAAATCATGGTGATCTGTCGATTAATAGCGCTAATAATCATTATGCG
            ************************************************************

5438        GCTCAGACTCTTTCTGTGTCTTGCGATGTGCCTACAAATATTCGTTTTTTCCTGTTAAGC
536         GCTCAGACTCTTTCTGTGTCTTGCGATGTGCCTACAAATATTCGTTTTTTCCTGTTAAGC
UTI19       GCTCAGACTCTTTCTGTGTCTTGCGATGTGCCTACAAATATTCGTTTTTTCCTGTTAAGC
5557        GCTCAGACTCTTTCTGTGTCTTGCGATGTGCCTACAAATATTCGTTTTTTCCTGTTAAGC
1442        GCTCAGACTCTTTCTGTGTCTTGCGATGTGCCTACAAATATTCGTTTTTTCCTGTTAAGC
CP9         GCTCAGACTCTTTCTGTGTCTTGCGATGTGCCTACAAATATTCGTTTTTTCCTGTTAAGC
360A        GCTCAGACTCTTTCTGTGTCTTGCGATGTGCCTACAAATATTCGTTTTTTCCTGTTAAGC
83972       GCTCAGACTCTTTCTGTGTCTTGCGATGTGCCTACAAATATTCGTTTTTTCCTGTTAAGC
            ************************************************************

5438        AATACAACTCCGGCATACAGCCATGGTCAGCAATTTTCGGTTGGTCTGGGTCATGGCTGG
536         AATACAACTCCGGCATACAGCCATGGTCAGCAATTTTCGGTTGGTCTGGGTCATGGCTGG
UTI19       AATACAACTCCGGCATACAGCCATGGTCAGCAATTTTCGGTTGGTCTGGGTCATGGCTGG
5557        AATACAACTCCGGCATACAGCCATGGTCAGCAATTTTCGGTTGGTCTGGGTCATGGCTGG
1442        AATACAAATCCGGCATACAGCCATGGTCAGCAATTTTCGGTTGGTCTGGGTCATGGCTGG
CP9         AATACAAATCCGGCATACAGCCATGGTCAGCAATTTTCGGTTGGTCTGGGTCATGGCTGG
360A        AATACAACTCCGGCATACAGCCATGGTCAGCAATTTTCGGTTGGTCTGGGTCATGGCTGG
83972       AATACAGCTCCGGCATACAGCCATGGTCAGCAATTTTCGGTTGGTCTGGGTCATGGCTGG
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5438            GACTCCATTGTTTCGATTAATGGCGTGGACACAGGAGAGACAACGATGAGATGGTACAGA
536             GACTCCATTGTTTCGATTAATGGCGTGGACACAGGAGAGACAACGATGAGATGGTACAGA
UTI19           GACTCCATTGTTTCGATTAATGGCGTGGACACAGGAGAGACAACGATGAGATGGTACAGA
5557            GACTCCATTGTTTCGATTAATGGCGTGGACACAGGAGAGACAACGATGAGATGGTACAGA
1442            GACTCCATTATTTCGATTAATGGCGTGGACACAGGAGAGACAACGATGAGATGGTACAGA
CP9             GACTCCATTGTTTCGATTAATGGCGTGGACACAGGAGAGACAACGATGAGATGGTACAGA
360A            GACTCCATTGTTTCGGTTAATGGCGTGGACAAAGGAGAGACAACGATGAGATGGTACAGT
83972           GACTCCATTGTTTCGGTTAATGGCGTGGACAAAGGAGAGACAACGATGAGATGGTACAGA
                ********* ***** *************** ****************************

5438            GCAGGTACACAAAACCTGACCATCGGCAGTCGCCTCTATGGTGAATCTTCAAAGATACAA
536             GCAGGTACACAAAACCTGACCATCGGCAGTCGCCTCTATGGTGAATCTTCAAAGATACAA
UTI19           GCAGGTACACAAAACCTGACCATCGGCAGTCGCCTCTATGGTGAATCTTCAAAGATACAA
5557            GCAGGTACACAAAACCTGACCATCGGCAGTCGCCTCTATGGTGAATCTTCAAAGATACAA
1442            GCAGGTACACAAAACCTGACCACTGGCAGTCGCCTCTATGGTGAATCTTCAAAGATACAA
CP9             GCAGGTACACAAAACCTGACCATCGGCAGTCGCCTCTATGGTGAATCTTCAAAGATACAA
360A            GCAGGTACACAAAACCTGACCATCGGCAGTCGCCTCTATGGTGAATCTTCAAAGATACAA
83972           GCAGGTACACAAAACCTGACCATCGGCAGTCGCCTCTATGGTGAATCTTCAAAGATACAA
                ********************  ***********************************

5438            CCAGGAGTACTATCTGGTTCAGCAACGCTGCTCATGATATTGCCATAA
536             CCAGGAGTACTATCTGGTTCAGCAACGCTGCTCATGATATTGCCATAA
UTI19           CCAGGAGTACTATCTGGTTCAGCAACGCTGCTCATGATATTGCCATAA
5557            CCAGGAGTACTATCTGGTTCAGCAACGCTGCTCATGATATTGCCATAA
1442            CCAGGAGTACTATCTGGTTCAGCAACGCTGCTCATGATATTGCCATAA
CP9             CCAGGAGTACTATCTGGTTCAGCAACGCTGCTCATGATATTGCCATAA
360A            CCAGGAGTACTATCTGGTTCAGCAACGCTGCTCATGATATTGCCA---
83972           CCAGGAGTACTATCTGGTTCAGCAACGCTGCTCATGATATTGCCATAA
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Consensus key (see documentation for details)
* - single, fully conserved residue
 - no consensus

The sequence analysis was done using The Biology WorkBench (http://workbench.sdsc.edu/).

Appendix VII

Supplemental Material for Chapter 5:

The Role of Aerobic Respiration, CNF1, and HlyA in the Pathogenesis of *Escherichia coli* 360A During a Systemic Infection Using a Mouse Model

Materials and Methods

Bacterial strains and growth conditions. *E. coli* strains DH5α (117) and DH10B (Gibco BRL, Gaithersburg, MD) were used as host strains for cloning target genes and resistance genes (Table AVII.1). The *E. coli* strain GM2163 is a *dam*$^{-}$/*dcm*$^{-}$ strain (193), which was used as the host, when restriction enzymes that could be blocked by methlyation, *XbaI* and *ClaI* were used for cloning. Antibiotics used in the study were 100 μg/mL ampicillin (Ap100), 25 μg/mL chloramphenicol (Cm25), 30 μg/mL gentamicin (Gm30), and 10 μg/mL tetracycline (Tet10).

Suicide vector construction. All plasmids are listed in Table AVI.1. Preliminary work was attempted with both the *cyd* and *cyo* operons using a derivative of the plasmid pFRTK (331). pFRTK was obtained from Dr. Timothy Larson (Dept. of Biochemistry, Virginia Tech) (331). The plasmid contains FRT regions that can be used to remove the resistance gene following chromosomal insertion. The use of these FRT regions in removal of the resistance gene has been outlined elsewhere (69). The kanamycin resistance cassette was removed from the vector and replaced with a chloramphenicol (Cm) resistance gene. The Cm resistance gene was amplified from the plasmid pKD3 (69) using the primers CmP1F and CmP2R, which produce a product with *Eco*RI and *Hin*dIII restriction sites, respectively. The PCR product was cloned into the AT cloning vector pGEM-T (Promega, Madison, WI) according to manufacturer's instructions. The construct was transformed into DH5α competent cells and screened for both Cm and ampicillin (Ap) resistance. Constructs were sequenced by The Virginia Bioinformatics Institute Core Lab Facility (Blacksburg, VA) using both T7 and Sp6 universal primers. The correct construct was named pMDM18-1. The Cm resistance gene was cloned from pMDM18-1 using *Hind*III and

*Eco*RI (New England Biolabs, Ipswich, MA) into a double digested (*Hind*III/*Eco*RI) pFRTK, creating pFRTC.

Next, regions of the *cydAB* operon, one in the 5' region and one in the 3' region, approximately 500 bp in length were amplified from the chromosome. The 5' region of *cydA* was amplified by the primers cydA1 and cydA2 (Table AVII.2) with flanking *Sac*II and *Bam*HI restriction sites, respectively. The 3' region of *cydB* was amplified with the primers cydB1 and cydB2 (AVI.2), with flanking *Hin*dIII and *Xho*I restriction sites, respectively. These regions were cloned into pGEM-T (Promega). The fragments were then sequenced using the T7 primer. The correct fragments were excised from pGEM-T (Promega) using the restriction sites listed above. The samples were electrophoresed on a 0.8% agarose gel, excised from the gel and purified using a QIAquick Gel Purification kit (Qiagen, INC., Valencia, CA). These regions were cloned on either side of a Cm resistance gene in the plasmid pFRTC resulting in the plasmid pMDM21-1.

This same procedure was used to amplify the 5' and 3' regions of *cyoAB* (primers: cyoA1, cyoA2 and cyoB3, cyoB4) and the two genes *cnf1* (primers: IGS1, IGS2 and CNF1-1a, CNF1-3) and *hlyA* (primers: hem1, hem2 and hem3, hem4) (Table AVII.2). Following the cloning of the *cyo* fragments into the plasmid pFRTC, creating plasmid pMDM38-1, the Cm resistance gene was removed from the plasmid by *Xba*I (New England Biolabs) digest and replaced with a gentamicin (Gm) resistance gene. The Gm resistance gene was PCR amplified from the plasmid pBBR1MCS5 (165) using the primers FrtGnF and FrtGnR1, which both add *Xba*I sites. The PCR product was then cloned into pGEM-T (Promega). The construct was sequenced using both the T7 and SP6 primers. The plasmid containing the correct insert was

named pMDM41B. Both pMDM38-1, which contains the *cyoA* and *cyoB* fragments flanking a Cm resistance gene, and pMDM41B were electroporated into the *E. coli* methylase minus strain GM2163 (193) (New England Biolabs). This was done in order to digest the plasmids with the enzyme *Xba*I, due to the fact that this enzyme may be blocked by deoxyadenine methylase. These two fragments were ligated together using T4 DNA ligase (New England Biolabs) and the resultant plasmid was named pMDM43I. These plasmids, pMDM21-1 and pMDM43I, were digested with *Sac*II and *Xho*I, Gel purified and electroporated into electrocompetent *E. coli* 360A. None of these fragments ever generated any gene knockouts.

A second mutagenesis procedure was utilized using the pKNOCK-Tc vector (2). First, both pKNOCK-Tc and pMDM43I were digested with *Sac*II and *Xho*I (New England Biolabs). A digest of pKNOCK-Tc would produce a 2.2 kb, while the digest of pMDM43I would produce 2 bands 1) ~3 kb and 2) ~2 kb in size. The two ~2 kb were gel purified and ligated together to create pMDM56-1. The remainder of suicide vectors constructed for the target genes were remade using 1 kb fragments since the 500 bp regions never produced any gene knockouts. The regions of these target operons: *cydAB* and *hlyCABD* were PCR amplified using the primer pairs: the 5' end cydAup/cydA3 and the 3' end cydB1/cydBdown for *cydAB*; and the 5' end hlyCA1/hlyCA2 and the 3' end hem3/hlyAB2, for *hlyCABD*. The 5' regions of both target genes incorporated *Sac*II and *Xba*I restriction sites respectively. The 3' regions of both target genes incorporated *Hind*III and *Xho*I restriction sites, respectively. All of these fragments were cloned into pGEM-T and sequenced. The Cm resistance gene was amplified from pBBR1MCS1 using primers Cm1F and Cm1R, which add *Xba*I and *Hind*III sites, respectively. All of the fragments for the target operons along with a resistance cassette were cloned into the pKNOCK vectors (2)

via a four fragment ligation in a ratio of 6:1 of each fragment to plasmid. All plasmids were propagated in *E. coli* S17 λ *pir* (Table AVI.1, (280)). The resulting plasmids (Table AVII.3) were named pMDM63 and pMDM64, which contain the *hlyCA/AB* and *cydAB* fragments, respectively.

The *cnf1* gene construct was made in a different way. An approximately 2 kb fragment of the *cnf1* gene was PCR amplified from 360A chromosomal DNA using primers cnf1I1 and cnf1I2 (Table AVI.2). This fragment was ligated with pGEM-T and transformed into DH10B electrocompetent cells via electroporation. The plasmid was purified using a QIAprep miniprep kit (Qiagen), verified via sequencing, and named pMDM90. It was transformed into a *dam/dcm* minus strain GM2163 (New England Biolabs) via electroporation. The plasmid was then purified from an isolate of this strain using QIAprep miniprep kits (Qiagen) and digested with the restriction enzyme *Cla*I (New England Biolabs), which creates a 5' region of approximately 550 bp and a 3' region of approximately 1400 bp, making the plasmid linear. Following the *Cla*I digest of the plasmid, it was blunt ended using T4 DNA polymerase (New England Biolabs) and then dephosphorylated using Antarctic phosphatase (New England Biolabs) according to the manufacturer's instructions. The Gm resistance gene was amplified from pBBR1MCS5 (165) using the primers GmBam and GmPst, which add a *Bam*HI and *Pst*I restriction sites, respectively. The fragment was cloned into pGEM-T and sequenced. The construct was named pMDM73, the Gm resistant gene was removed using *Pst*I and *Bam*HI (New England Biolabs) and then blunt ended using T4 DNA polymerase. Both samples were electrophoresed on a 0.8% agarose gel and the fragments were excised from the gel and purified using a QIAquick Gel Purification kit (Qiagen). The fragments were ligated together and electroporated into

electrocompetent *E. coli* DH10B. Transformants were selected for on LB containing Gm30 and Ap100. Isolates were screened via PCR to determine the orientation of the gentamicin resistance gene, using the primer pairs cnf1I1/GmBam and cnf1I2/GmPst (Table AVII.2). A plasmid that showed products from both of these PCR reactions indicated the correct orientation. The correct plasmid was named pMDM92-2 (Table AVI.3) and digested with the restriction enzymes *Sac*II and *Spe*I (New England Biolabs). The sample was electrophoresed on a 0.8% agarose gel, excised, purified, and ligated into the *Sac*II and *Spe*I/dephosphorylated pKNOCK-Tc vector. This ligation product was transformed into electrocompetent *E. coli* S17 λ *pir* cells (280). The resulting plasmid was named pMDM93 (Table AVII.3).

This plasmid was used to knockout the *cnf1* gene in 360A with no success. An alternate strategy was used due to the short 500 bp 5' region of the *cnf1* vector. This region was replaced as shown in Figure AVII.1. A PCR fragment of the *cnf1* gene was amplified using the primers cnf1Sac and cnf1B1, which overlaps the cnf1I1/cnf1I2 fragment by 700 bp. The cnf1Sac/cnf1B1 fragment was cloned into pGEM-T, verified by sequencing, and named pMDM95. This plasmid was digested with *Sac*II and *Eco*RV (New England Biolabs), which produces approximately an 800 bp fragment. The plasmid pMDM92-2 was also digested with *Sac*II and *Eco*RV (New England Biolabs) removing approximately a 400 bp fragment. *Eco*RV cuts this *cnf1* fragment twice within 87 bp of each other. The 800 bp fragment from pMDM95 was ligated with pMDM92-2 creating the plasmid pMDM97 containing a region of approximately 1200 bp (5' of *cnf1)* and a region of 1400 bp (3' of *cnf1*). This *cnf1*/Gm resistance fragment was cloned into pKNOCK-Tc and the resultant plasmid was named pMDM99 (Table AVII.4).

Screening of chromosomal insertions. The single *cyo* and double *cyo/cyd* oxidase mutants were verified by Southern blot using probes generated from the primer pairs cyoSF/cyoSR and cydSF/cydSR (Table AVII.3) for verification of insertions in the *cyo* and *cyd* operons, respectively. Insertions in the *hly* operon at either the 5' or 3' end of *hlyA* were verified using the primer pairs hly6/hlyCA1 and hly6/hlyC or hly1/hlyAB2 and hly1/hlyB, respectively (Table AVII.2). The first primer pairs for each region hly6/hlyCA1 and hly1/hlyAB2 would result in a PCR product from either the parent strain 360A or a *hly* mutant. The second primer pairs for each region hly6/hlyC and hly1/hlyB would only result in a PCR product if the strain was the wild-type parent (strain 360A). The mutants were further verified by screening using the primer pairs located in Table AVII.3. These primer pairs included a primer that recognized the resistance gene and the other primer recognized a region approximately 100 bp outside of the region used to create the insertions. Each mutant was screened for insertions in both the 5' and 3' regions of the genes. The relative binding sites of these primers is shown in Figure AVII.3. Each fragment was cloned into pGEM-T and sequenced. These fragments were examined using The Biology workbench and Pubmed nucleotide blast searches.

Triton X-100 toxicity. We tested to see if 0.5% Triton X-100 would affect the amount of viable *E. coli* 360A present after 10 minutes of exposure. Strain 360A was grown overnight in 5 mL of LB. The following day the strain was subcultured (50 μL) into 6 mL of LB and allowed to grow for about 2 hours to an optical density of about 0.580-0.600. The culture was sedimented by centrifugation and washed three times with 1X PBS to remove dead cells and diluted into 1 mL of 1X PBS. Serial dilutions and plating was done to determine the number of CFU/mL. The original stock culture was approximately 10^8 CFU/mL. The cells were then

diluted into 900 mL of 0.5% Triton X-100 resulting in dilutions from a range of approximately 2 x 10^2 to 2 x 10^7 CFU/mL. The mixtures were incubated at room temperature for 10 minutes. Samples were serially diluted in 1X PBS after 10 minutes and cultured onto LB. The numbers were compared to samples not exposed to Triton X-100. These assays were plated in duplicate in three independent trials.

Macrophage invasion assay controls. Control assays were used to determine: 1) if *E. coli* strain 360A was able to adhere to the tissue culture plate and 2) if cytochalasin D could inhibit the invasion of 360A in both J774a.1 and RAW264.7 murine macrophage-like cell lines. First to evaluate if wt 360A could adhere to the tissue culture plates, the wells were seeded with the same concentrations as the invasion assays; 8 x 10^3, 10^4, and 10^5. The cells were allowed to incubate for 4 hours at 37ºC in a 5% CO_2 modified environment. The wells were treated the same as in the invasion assays, with washing with 1X PBS and incubation in 200 µL of DMEM for an additional hour with or without 100 µg/mL streptomycin (Str100). The wells were washed again with 1X PBS and then treated with 200 µL of 0.5% Trition X-100 for 10 minutes. The samples were then plated and evaluated for the presence of bacteria in the samples.

The cytochalasin D assays were also performed in the same manner as in Chapter Five. However, the two murine macrophage-like cell lines were incubated with a 1 µg/mL concentration of cytochalasin D for one hour prior to infection. The murine macrophage-like cells were then infected with an MOI of 0.1, 1, and 10 CFU per cell. Cytochalasin D (1 µg/mL) was present for the 4 hour incubation. Then the wells were washed three times with 1X PBS and incubated for an additional hour in 200 µL of DMEM either with or without Str100. Following treatment with streptomycin the murine macrophage-like cells were treated with 200 µL of 0.5%

Trition X-100 for 10 minutes. These samples were plated and evaluated for the presence of bacteria.

Results and Discussion

Screening of chromosomal insertions. All 5 of the mutants that were constructed in *E. coli* 360A using the pKNOCK-Tc vector. The oxidase mutants were verified by Southern blotting and the *hly* mutant was verified by PCR screening as mentioned above. The PCR method outlined in Figure AVII.3 was used to verify all five mutants. PCR and then sequencing of these regions that were cloned into pGEM-T was the preferred method of verifying the mutants. The site of insertion for each of these five mutants is shown in Figure AVII.4.

Triton X-100 toxicity. A toxicity assay was performed to determine if the concentration of Triton X-100 (0.5%) used to lyse the macrophages would also kill the intracellular bacteria. The results from these experiments are shown in Figure AVII.5. These results indicated that greater than 80% of the bacterial cells survived at each log interval (10^2 to 10^7) exposed to 0.5% Triton X-100.

Macrophage invasion assay controls. Assays were done to evaluate adherence of strain 360A to tissue culture plates in the absence of macrophages. The results showed that no bacteria were found in either the wells treated with or without Str100. These results indicated that the results observed in the presence of macrophages were not enhanced by the binding of the bacteria to the tissue culture plates.

Analysis of the J774a.1 experiments with cytochalasin D treatment showed bacteria only in the absence of streptomycin (Figure AVII.6), indicating that cytochalasin D inhibited J774a.1 cells from phagocytosing strain 360A. Also, it was observed that strain 360A was able to bind to the macrophages in the presence of cytochalasin D and were not completely removed from the macrophages even after vigorous washing with 1X PBS. The analysis of the

RAW264.7 cytochalasin D assays showed no bacteria either with or without streptomycin. Leading to the same conclusions as J774a.1 assays that cytochalasin D was able to inhibit the internalization of strain 360A. However, the lack of strain 360A in the absence of streptomycin may mean that strain 360A was not able to adhere to the RAW264.7 cells as efficiently as it was to J774a.1 cells.

Table AVII.1. Strains used in the cloning of chromosomal fragments and resistance genes.

Strain	Relevance to Study and Strain Genotype[a]	Reference
DH5α	Host strain for cloning of terminator constructs, F^-, *endA1*, *glnV44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *deoR*, *nupG*, Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsdR17*(r_K^- m_K^+), λ–	(117)
DH10B	used as a host strain for cloning, F^-, *endA1*, *recA1*, *galE15*, *galK16*, *nupG*, *rpsL*, Δ*lacX74*, Φ80*lacZΔM15*, *araD139*, Δ(*ara,leu*)7697, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), λ^-	Gibco/BRL
GM2163	Methylase minus strain used for cloning with enzymes blocked by methylation, *araC14*, *leuB6*, *fhuA13*, *lacY1*, *tsx-78*, *glnV44*, *galK2*, *galT22*, λ^-, *macrA0*, *dcm-6*, *hisG4*, *rfbC1*, *rpsL136*: Str^R, *dam-13*::Tn9, *xlyA5*, *mtl-1*, *thi-1*, *mcrB9999*, *hsdR2*, Cm^R	(193)

[a]Streptomycin resistance (Str^R), Chloramphenicol (Cm^R).

Table AVII.2. Primers used to construct vectors used for chromosomal disruption of *E. coli* 360A.[a]

Primer Name	Primer Sequence (5' to 3')[b, c]
CmP1F	**GAATTC**GTGTAGGCTGGAGCTGCTTC
CmP2R	**AAGCTT**GGTCCATATGAATATCCTCCTTAG
cydA1	**CCGCGG**GTTCCTGCTGGCCATTATGGAAACG
cydA2	**GGATCC**GGTTAAGCACCAGCTCGGAGAAGC
cydB1	**AAGCTT**GCACCTGCGTACCCGTGCAAC
cydB2	**CTCGAG**CAGAATGATCGGTACCAGAACCAC
cyoA1	**CCGCGG**GCAGGCACTGTATTGCTCAGTGGCTG
cyoA2	**GAATTC**CTGGCTACCCAGACGCGGAATGAAGAAGG
cyoB3	**AAGCTT**GCCAGCAGATTGACCCGCAGTTCCACACC
cyoB4	**CTCGAG**CACGATCCAGGTGATGATCATGCCTGCG
hem1	**CCGCGG**CCTCTGCAAATAAATTGCACTCAGC
hem2	**GGATCC**CCAGTTCAGAAGAACTGACATTGC
hem3	**AAGCTT**CGTTATCTTTCAGGATATGGCCATC
hem4	**CTCGAG**GCTGTCAAAGTTATTGAGTTCCGTCC
IGS1	**CCGCGG**GAGGAAGAGGTAAGCTTCAGAATGG
IGS2	**GGATCC**GGTCGGGCCAGCAGGTTAAGTATTATCTG
CNF1-1a	**AAGCTT**CACTTGAGGAAGTCCAGGAGGGTAAACC
CNF1-3	**GTYRAC**CCTTACGACAACATTGCCGTCCACTCTCAC
FrtFGnR1	**TCTAGA**GAATAGGAACTTCGGAAGCCGATCTCGGCTTGAACG
FrtRGnF	**TCTAGA**AAGTATAGGAACTTCGGACGCACACCGTGGAAACGG
Cm1F	**TCTAGA**GCGTATTGGGCGCATGCATAAAAACTG
Cm1R	**AAGCTT**GCACATTTCCCCGAAAAGTGCCACC
hlyCA1	**CCGCGG**GGTTCATTGACTGGATTGCTCCTTTCG
hlyCA2	**TCTAGA**GTATCTGCATCTGCATTGCTCAGAATG
hlyAB2	**CTCGAG**ATGGCAGGGATAAACCAGGTAAAGTC
hlyC	GCATGTATCCTGGCTCTGGGCCAGTTC
hlyB2	GAGATCGGTAGCGCCAGTAAATGCCGG
hly1	GTATTGAGTCACACCTGGGAGACGG
hly6	GGGACTAATTGCTAATGTCACTACAG
cydAup	**CCGCGG**CCTTGTTCCATCTGACTATACC
cydA3	**TCTAGA**GGTTAAGCACCAGCTCGGAGAAGC
cydBdown	**CTCGAG**GCTACTATACGCGCGCGATTGAGGTTTG
GmXba	**TCTAGA**GGAAGCCGATCTCGGCTTGAACG
GmBam	**CTGCAG**GGAAGCCGATCTCGGCTTGAACG
GmPst	**GGATCC**GGACGCACACCGTGGAAACGGATG
cnf1Sac	**CCGCGG**GAGTTGGTATCAAATTTCCCTTCAC
Cnf1Bam	**GGATCC**CAGACTCATCTTCACTCGGCTCAAC
cnf1I1	CCATGAACGCCTTAAATCGCAGTG
cnf1I2	TACCCTCCTGGACTTCCTCAAGTGC
cnf1Xba	**TCTAGA**TCCTGTGCTTGAATTTGCATCATC
Cnf1Pst	**CTGCAG**CCTGTGCTTGAATTGCATCATC
Cnf1B2	GCAGCGTTATAAATAATGAGGGCAGG

[a]All of these primers were designed for use in this study.
[b]Y = C or T, R = A or G
[c]The bases in bold indicate restriction sites.

Table AVII.3. Primers used to screen for chromosomal insertions.[a]

Primer Name	Primer Sequence (5' to 3')	Mutant screen
cyoSF	GCGGTACTGACCTGGAAAACCACTCAC	Used in Southern blot
cyoSR	CAGAATGCATCACGTTCGTGAACGTGCG	Used in Southern blot
cydSF	CTTCGGTTGGGATCGTCTGGGTAAAGTTCAGC	Used in Southern blot
cydSR	CCGCAACCCAGGTCATGACGTTAAGCGTC	Used in Southern blot
cyoupA1	CCGCGGGCTTGGTGGTTTGCTGGATTATCTG	*cyo* mutants, 5' insertion screen
GmBam	CTGCAGGGAAGCCGATCTCGGCTTGAACG	Used with both 5' *cyo* and 5' *cnf1* insertion screen
cyoBC2	CTCGAGGTACGGTTAGTGCTGGTCAGGCCG	*cyo* mutants, 3' insertion screen
GmXba	TCTAGAGGAAGCCGATCTCGGCTTGAACG	Used with 5' *cyo* insertion screen
upcyd	CGGTGTTTTCTAACGGGCTGGCAATCATTC	*cyd* mutants, 5' insertion screen
Cm1R	AAGCTTGCACATTTCCCCGAAAAGTGCCACC	Used with both 5' *cyd* and 5' *hly* insertion screen
downcyd	CGTTCAGCCATCAGCGCCTGTTGACTG	*cyd* mutants, 3' insertion screen
Cm1F	TCTAGAGCGTATTGGGCGCATGCATAAAAACTG	Used with both 3' *cyd* and 3' *hly* insertion screen
IGS1	CCGCGGGAGGAAGAGGTAAGCTTCAGAATGG	*cnf1* mutants, 5' insertion screen
CNF1-3	GTYRACCCTTACGACAACATTGCCGTCCACTCTCAC[b]	*cnf1* mutants, 3' insertion screen
GmPst	GGATCCGGACGCACACCGTGGAAACGGATG	Used with 3' *cyo* insertion screen
hlyC	GCATGTATCCTGGCTCTGGGCCAGTTC	*hly* mutants, 5' insertion screen
hlyB2	GAGATCGGTAGCGCCAGTAAATGCCGG	*hly* mutants, 3' insertion screen

[a]All of these primers were designed for use in this study.
[b]Y = C or T, R = A or G, CNF1-3 has a *Hinc*II restriction site at the 5' end.

Table AVII.4. Plasmids used in the construction of *Escherichia coli* 360A mutants.

Plasmid Name	Resistance Markers	Relevance to Study	Reference
pFRTK	Ap Kan	Vector for cloning PCR products directed at making chromosomal insertions	(331)
pFRTC	Ap Cm	Vector for cloning PCR products directed at making chromosomal insertions	This study
pGEM-T	Ap	AT cloning vector	Promega
pMDM18-1	Ap Cm	pGEM-T carrying Cm^R gene	This study
pMDM21-1	Ap Cm	Both 5'*cydA* and 3'*cydB* in pFRTC	This study
pMDM32	Ap Cm	Both 5'*hlyA* and 3'*hlyA* in pFRTC	This study
pMDM38-1	Ap Cm	Both 5'*cyoA* and 3'*cyoB* in pFRTC	This study
pMDM41B	Ap Gm	Gm resistance gene in pGEM-T	This study
pMDM42-2	Ap Cm	Both '*igs*' and 3'*cnf1* in pFRTC	This study
pMDM43I	Ap Gm	Both 5'*cyoA* and 3'*cyoB* of pMDM38-1 with Gm^R	This study
pBBR1MCS1	Cm	Source of the Cm resistance gene	(165)
pBBR1MCS5	Gm	Source of the Gm resistance gene and used in complementation experiments of *hlyA*	(165)
pKNOCK-Tc	Tc	Suicide cloning vector with R6K *ori* and RP4 *oriT*	(2)
pMDM56-1	Gm Tc	5'*cyoA* and 3'*cyoB* in pKNOCK-Tc	This study
pMDM63	Tc Cm	*hlyCA* and *hlyAB* in pKNOCK-Tc	This study
pMDM64	Tc Cm	upstream of *cydA* and downstream of *cydB* in pKNOCK-Tc	This study
pMDM73	Ap Gm	GmR gene from pBBR1MCS5 with *Pst*I/*Bam*HI restriction sites in pGEM-T	This study
pMDM90	Ap	2 kb fragment of *cnf1* from primers cnf1I1 to cnfI2	This study
pMDM92-2	Ap Gm	GmR gene from pMDM73 in pMDM90-*Cla*I blunt	This study
pMDM97	Ap Gm	cnf1Sac – cnfB1 *Sac*II/*Eco*RV replacement of the *Sac*II/*Eco*RV digest of the cnf1I1 to cnf1I2 fragment from pMDM92-2	This study
pMDM99	Tc Gm	*cnf1* fragments from pMDM97 in pKNOCK-Tc	This study

[a]Ampicillin resistance (Ap); Chloramphenicol resistance (Cm); Gentamicin resistance (Gm); Kanamycin resistance (Kan); Tetracycline resistance (Tc).

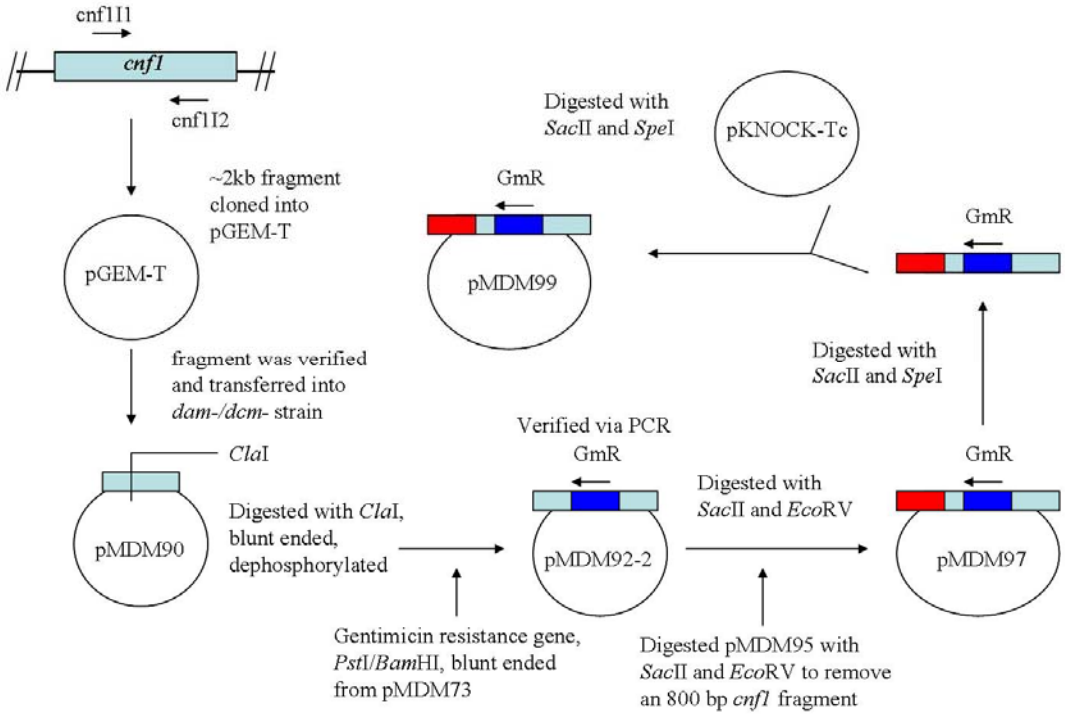

Figure AVII.1. Construction of the *cnf1* suicide vector pMDM99. A fragment of the *cnf1* gene was cloned from strain 360A chromosomal DNA and cloned into pGEM-T (Promega). The resultant plasmid pMDM90 was transferred into *E. coli* GM2163 *dam/dcm* minus strain. The plasmid was purified from this strain and digested with *Cla*I. A gentamicin resistance gene from the plasmid pMDM73 was subcloned into pMDM90 via a blunt ended ligation. Directionality of the resistance cassette was verified via PCR. The vector was named pMDM92-2. This vector was digested with *Eco*RV and *Sac*II, which removed a 400 bp fragment. This was replaced by an 800 bp fragment of the *cnf1* gene taken from pMDM95, creating pMDM97. From here the *cnf1*/Gm resistance gene fragment was cloned into pKNOCK-Tc via *Sac*II and *Spe*I. This final vector was named pMDM99, which was used in creating several *cnf1* mutants.

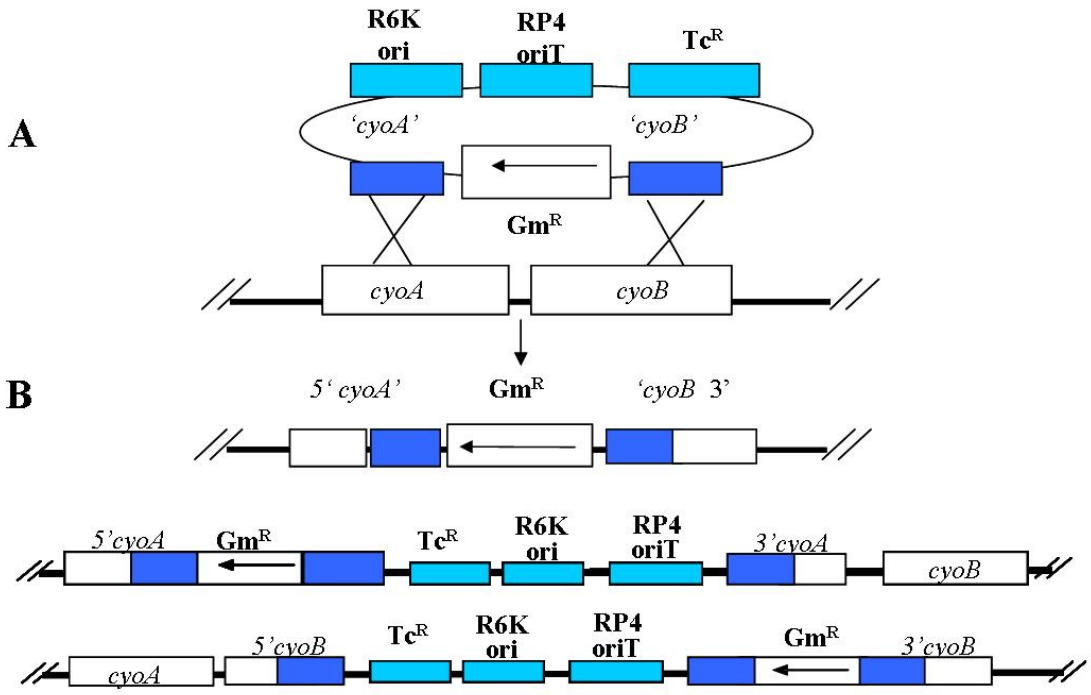

Figure AVII.2. Mutagenesis method and screening of insertions. **A**) Transfer of the pKNOCK suicide vector, containing fragments of the target gene flanking a resistance cassette, into strain 360A via mating or electroporation. Homologous recombination between plasmid DNA and chromosomal DNA. **B)** Selecting of both single and double crossover events was done using M9 minimal medium containing succinate and gentamicin (Gm), in this case. Following growth colonies would be culture further on M9 minimal medium containing succinate and gentamicin (Gm).

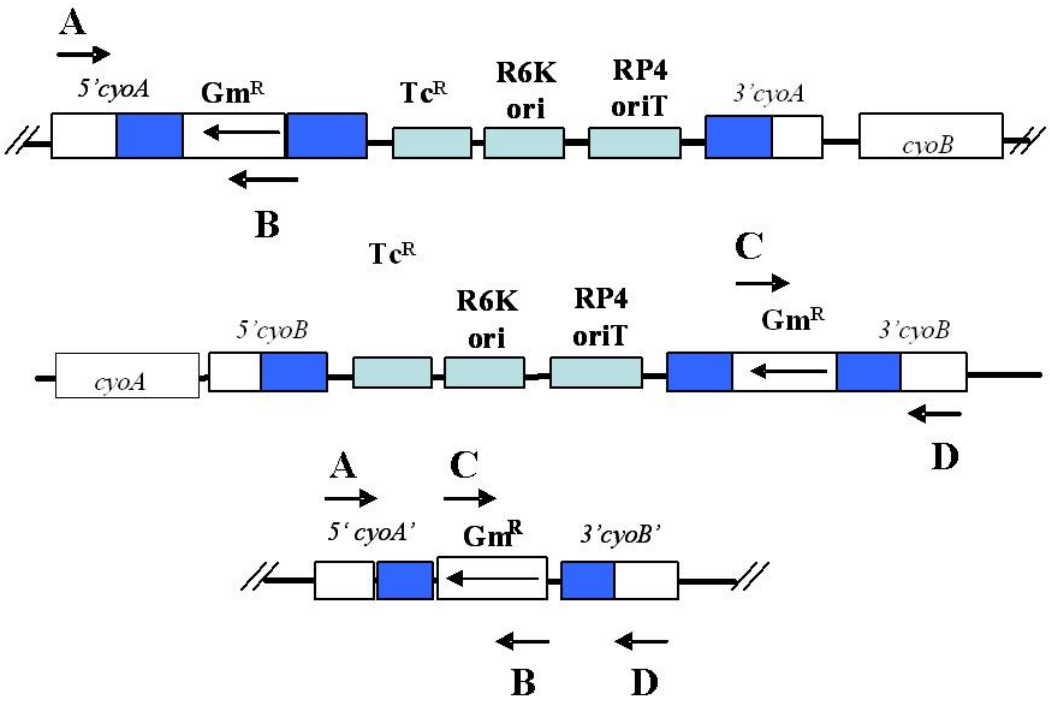

Figure AVII.3. Procedure used to screen chromosomal insertions for single and double crossovers. The arrows indicate the primers used to examine the possible *cyoAB* oxidase mutants for the insertion of the gentamicin resistance cassette and the flanking gene regions. Primers B and C are specific for the resistance gene. Primer pairs A and B or C and D could be used to examine which gene the insertion occurred, if these were single crossovers. Primer pair A and D could be used to see if there is a double crossover by comparing the size to a wild type product from the same region. This model is valid for the screening of both oxidase as well as toxin mutants.

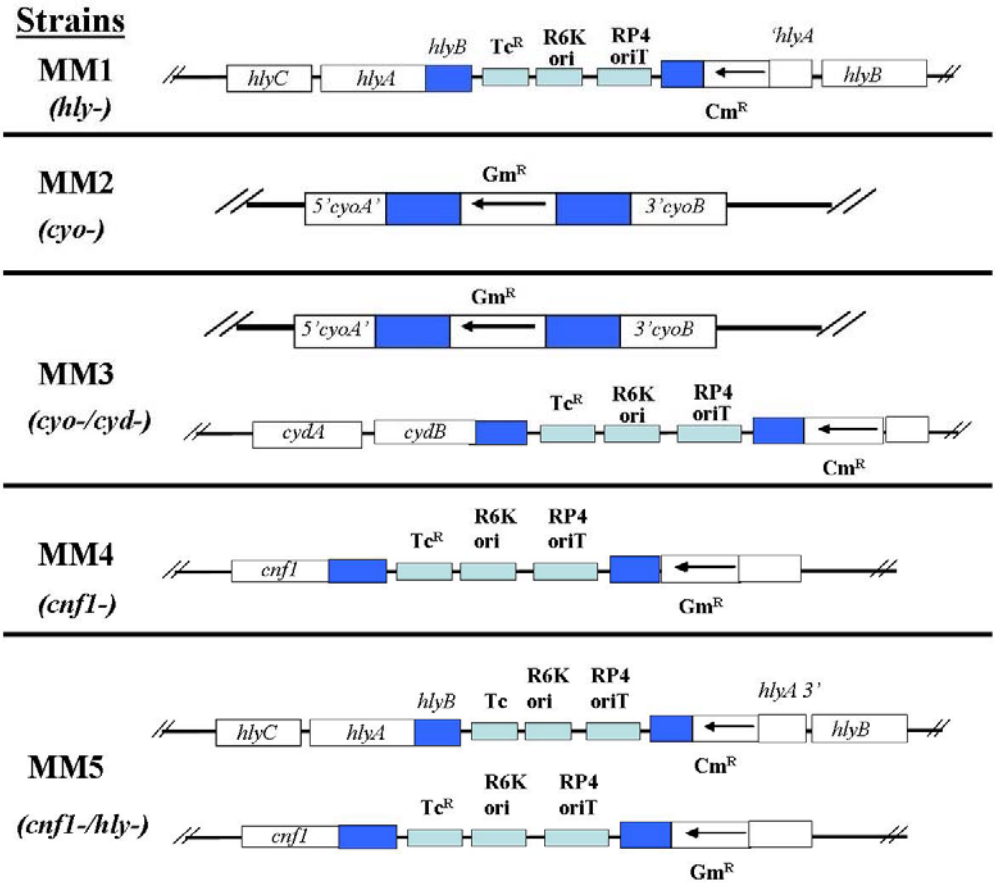

Figure AVII.4. Model of chromosomal insertions in 360A. PCR reactions were done as shown in Figure AVII.3. The fragments were cloned into the cloning vector pGEM-T (Promega) and sequenced by the Virginia Bioinformatics Institute Core Lab Facility (Blacksburg, VA). The outcome of the sequencing is shown above. MM1 is the hemolysin mutant, which has an insertion in between the *hlyAB* genes as a single crossover. MM2 is a *cyo* oxidase mutant, which has an insertion in the *cyoAB* genes as a double crossover. MM2 was used as the parent strain for MM3, which is a double *cyo/cyd* oxidase mutant and has the insertion in *cyo* as does MM2 as well as an insertion in the *cydB* gene that is a single crossover. MM4 is a *cnf1* mutant and is the parent strain for the double (*cnf1/hly*) mutant MM5. MM5 is a *cnf1/hly* double mutant, which has an insertion in the *cnf1* gene as dose MM4 as well as an insertion in between *hlyAB* genes.

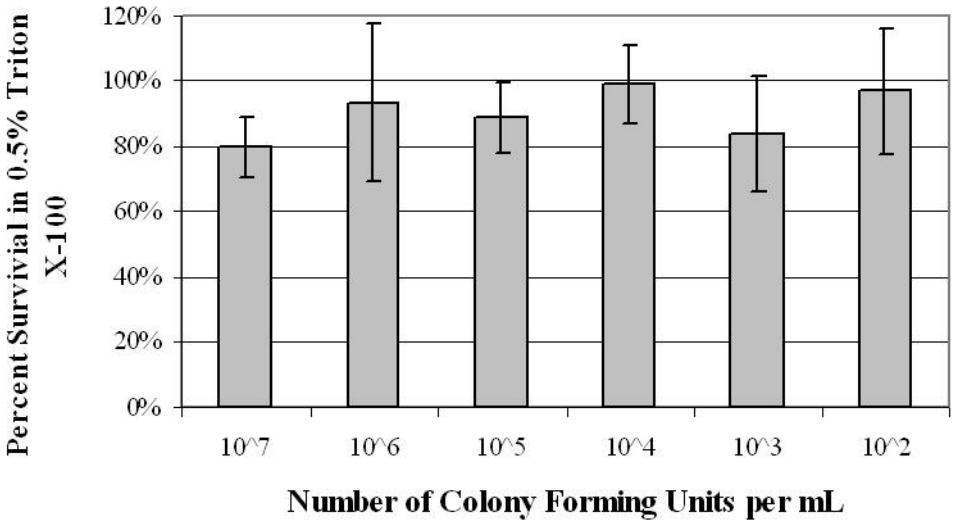

Figure AVII.5. Percent survival of *E. coli* 360A in 0.5% Triton X-100 using a log range of CFUs. Survival of wt 360A was examined in 0.5% Trition X-100 to evaluate cell viability following a 10 minute incubation time. These assays were performed in three independent trials plated in duplicate.

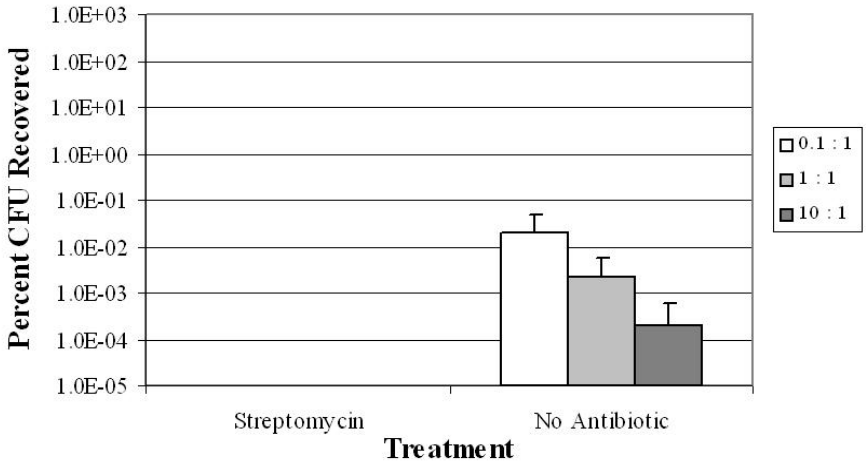

Figure AVII.6 Analysis of internalized strain 360A following cytochalasin D treatment. J774a.1 were treated with 1 µg/mL of cytochalasin D and then infected with three MOI 0.1, 1, and 10 CFU to 1 macrophage cell. The results indicate that no bacteria were able to invade the macrophages following cytochalasin D treatment. This assay was performed in three independent trials in duplicate.

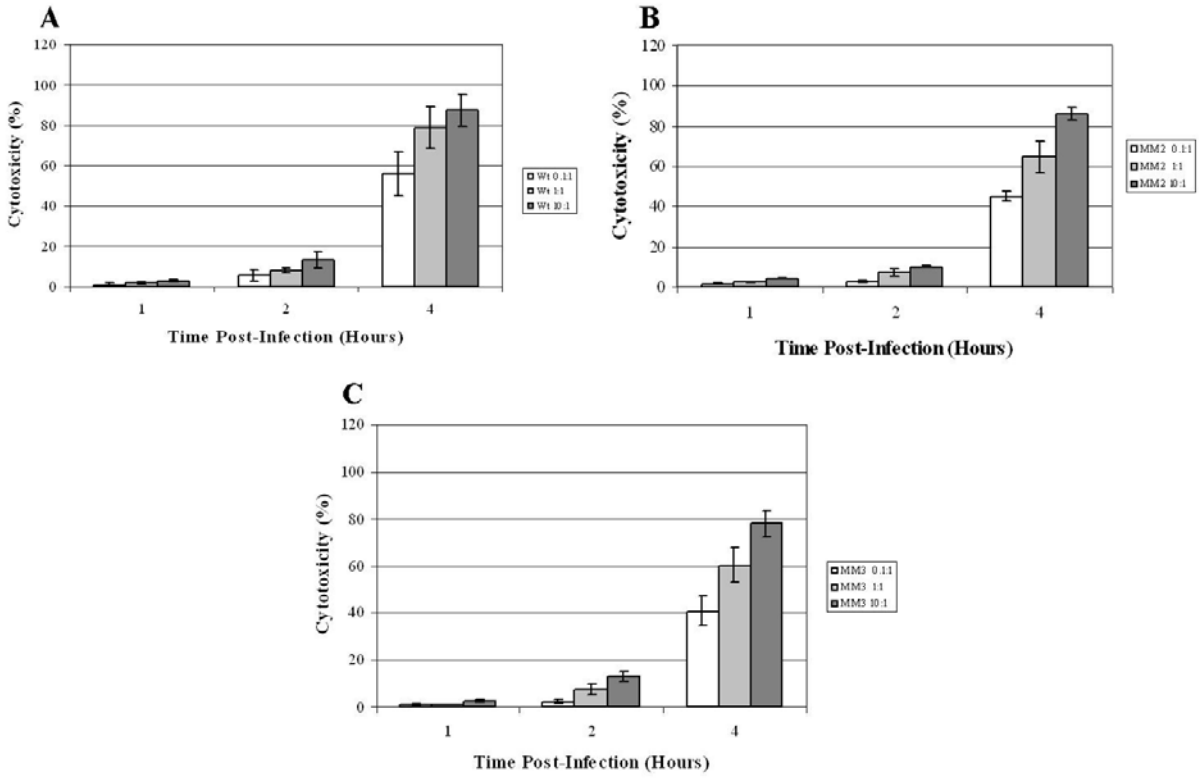

Figure AVII.7. Cytotoxicity of *E. coli* 360A and two isogenic oxidase mutants for J774a.1 murine macrophage-like cells. Macrophages were infected with three MOI of 0.1, 1, and 10 CFU per cell with strain 360A (A), MM2 (*cyo*) (B), and MM3 (*cyo/cyd*) (C) using 96 well plates. At 1, 2, and 4 hours post infection the samples were tested for lactate dehydrogenase activity. The cytotoxicity is shown as a percentage of total lysis as compared to completely lysed macrophage cells. These assays were performed in three independent trials in sets of four.

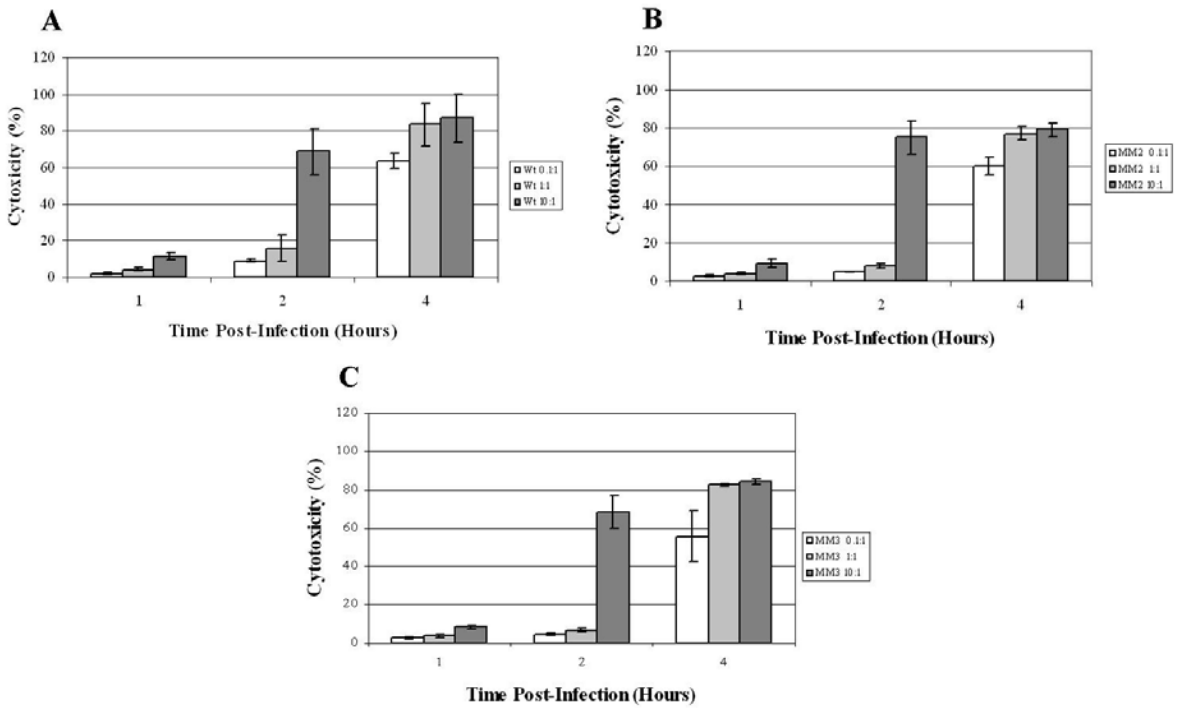

Figure AVII.8. Cytotoxicity of *E. coli* 360A and two isogenic oxidase mutants for RAW264.7 murine macrophage-like cells. Macrophages were infected with three MOI of 0.1, 1, and 10 CFU per cell with strain 360A (A), MM2 (*cyo*) (B), and MM3 (*cyo/cyd*) (C) using 96 well plates. At 1, 2, and 4 hours post infection the samples were tested for lactate dehydrogenase activity. The cytotoxicity is shown as a percentage of total lysis as compared to completely lysed macrophage cells. These assays were performed in three independent trials in sets of four.

Table AVII.5. Comparative statistical analysis of two isogenic oxidase mutants to strain 360A using a cytotoxicity assay with J774a.1 and RAW264.7 murine macrophage-like cells.

J774a.1	***P* values[a]**			**RAW264.7**	***P* values[a]**		
1 hr	Wt 0.1	Wt 1	Wt 10	**1 hr**	Wt 0.1	Wt 1	Wt 10
MM2 (*cyo*)	0.4309	0.5261	0.8362	MM2 (*cyo*)	0.1205	0.8271	0.3785
MM3 (*cyo/cyd*)	0.7750	0.1888	0.9013	MM3 (*cyo/cyd*)	0.1267	0.7037	0.0520
2 hrs	Wt 0.1	Wt 1	Wt 10	**2 hrs**	Wt 0.1	Wt 1	Wt 10
MM2 (*cyo*)	0.1910	0.3019	0.3427	MM2 (*cyo*)	**0.0382**	0.1405	0.5736
MM3 (*cyo/cyd*)	0.1827	0.3194	0.4086	MM3 (*cyo/cyd*)	**0.0376**	0.1483	0.9922
4 hrs	Wt 0.1	Wt 1	Wt 10	**4 hrs**	Wt 0.1	Wt 1	Wt 10
MM2 (*cyo*)	0.2056	0.3096	0.6146	MM2 (*cyo*)	0.4218	0.3167	0.6704
MM3 (*cyo/cyd*)	**0.0345**	**0.0125**	**0.0475**	MM3 (*cyo/cyd*)	0.5163	0.9100	0.7842

[a]Statistically significant values ($P<0.0500$) are in bold.

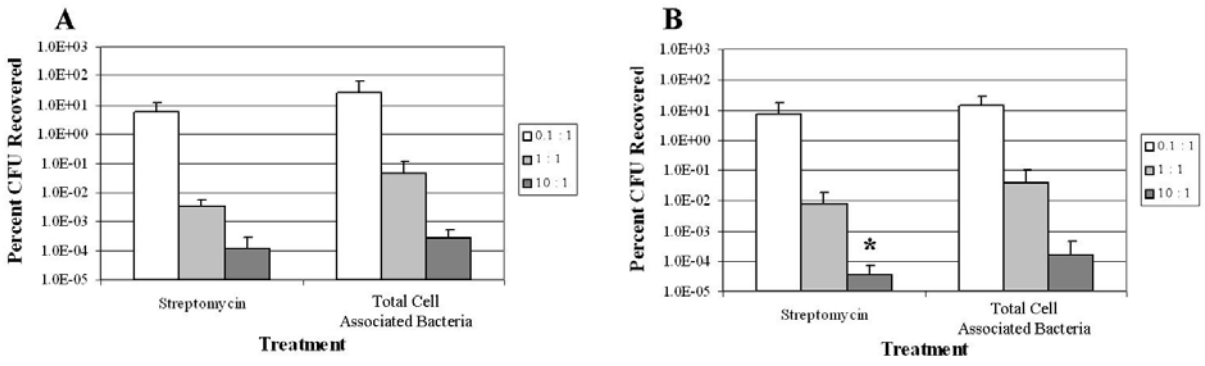

Figure AVII.9. Infection of J774a.1 murine macrophage-like cells by *E. coli* 360A and the isogenic double oxidase mutant (MM3). Macrophages were infected with strain 360A or the double oxidase mutant MM3 (*cyo/cyd*) at three MOI 0.1, 1, and 10 CFU per cell. Strain 360A (A) and MM3 (*cyo/cyd*) (B). A comparison of the bacterial numbers of strain 360A and MM3 showed similar results in most of the samples examined. The bacterial counts of strain 360A after the treatment with streptomycin indicates a statistically significant difference from that of MM3 (*, $P<0.0500$). These assays were performed in three independent trials in duplicate.

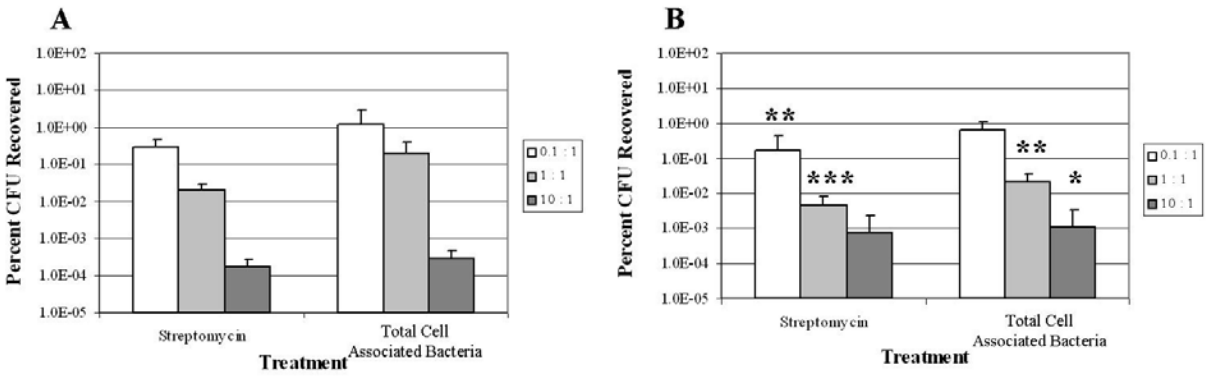

Figure AVII.10. Infection of RAW264.7 murine macrophage-like cells by *E. coli* 360A and the isogenic double oxidase mutant (MM3). Macrophages were infected with strain 360A or the isogenic double oxidase mutant MM3 (*cyo/cyd*) at three MOI 0.1, 1, and 10 CFU per cell. Strain 360A (A) and MM3 (*cyo/cyd*) (B). A comparison of the bacterial numbers of strain 360A and MM3 showed different results than J774a.1 in most of the samples examined. The bacterial counts of strain 360A show statistically significant difference from that of MM3 (**, $P<0.0100$; and ***, $P<0.0006$). However at one MOI, MM3 bacterial counts were actually significantly higher than wt (*, $P<0.0400$). These assays were performed in three independent trials in duplicate.

Table AVII.6. Comparative statistical analysis of the isogenic double oxidase mutants to strain 360A using invasion assay with J774a.1 and RAW264.7 murine macrophage-like cells.

J774a.1	***P* values[a]**			**RAW264.7**	***P* values[a]**		
Streptomycin	Wt 0.1	Wt 1	Wt 10	**Streptomycin**	Wt 0.1	Wt 1	Wt 10
MM3 (*cyo/cyd*)	0.7163	0.2726	**0.0410**	MM3 (*cyo/cyd*)	**0.0057**	**0.0005**	0.0776
Total cell associated	Wt 0.1	Wt 1	Wt 10	**Total cell associated**	Wt 0.1	Wt 1	Wt 10
MM3 (*cyo/cyd*)	0.3912	0.8267	0.3609	MM3 (*cyo/cyd*)	0.3908	**0.0090**	**0.0343**

[a]Statistically significant values ($P<0.0500$) are in bold.