Identification and characterization of a toxin-antitoxin system in the pVir plasmid of *Campylobacter jejuni*

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ABSTRACT

Toxin-antitoxin systems are prevalent in different bacterial organisms and are encoded in the chromosomal or plasmid DNA. The primary function of a plasmid toxin-antitoxin module is to stabilize the plasmid by eliminating plasmid-free daughter cells through a post segregation killing mechanism. In *Campylobacter jejuni*, a pathogen that causes human gastroenteritis, multiple plasmids have been identified, but there have not been any reports on toxin-antitoxin systems. In this study, a toxin-antitoxin system is identified and characterized in the pVir plasmid of *C. jejuni* IA 3902. pVir encodes a type IV secretion system and is found to be involved in natural transformation and virulence in *Campylobacter*. Comparative genomic analysis of the pVir45 and pVir46 genes in *C. jejuni* indicates that they encode a putative toxin-antitoxin system that belongs to the RelE/StbE family. Cloning and expression of the pVir46 gene alone in *Escherichia coli* inhibited bacterial growth, but co-expression of pVir45-46 led to the restoration of growth, confirming the functions deduced from comparative genomic analysis. The plasmid stability assay in *C. jejuni* showed that the toxin-antitoxin system is necessary for maintaining the stability of pVir because deletion of the pVir46 gene resulted in loss of the plasmid during passage in conventional media. qRT-PCR data demonstrated that expression of the pVir45-46 genes varied with growth phase, as early logarithmic phase had the highest level of gene expression. Together these results establish that pVir45-46 encode a functional toxin-antitoxin system in *C. jejuni*, which is required for ensuring the stability of the pVir plasmid. The stable maintenance of pVir may be necessary for optimal virulence of *C. jejuni* IA 3902.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

C. jejuni is a gram-negative, microaerophilic organism that causes human gastroenteritis. It is also increasingly associated with ovine abortions in the United States (75). *C. jejuni* is recognized as the leading cause of foodborne illnesses in the United States with approximately 2.4 million reported cases annually (19). *C. jejuni* infections are described as a self-limiting disease often resulting in abdominal cramping, diarrhea, and fever in patients (66). Severe diseases associated with *C. jejuni* often occur in infants, elderly, and HIV patients. A well-known complication of *C. jejuni* infection is Guillain-Barré syndrome, a neurological disorder resulting in the damage of the peripheral nervous system (1). As a foodborne pathogen, the incidence of *Campylobacter* infections is commonly linked to the consumption of contaminated water, unpasteurized milk, and poultry meat (22, 88).

C. jejuni contains no known identified putative toxin-antitoxin systems (86). The toxin-antitoxin system is considered to be an addictive genetic element in which the transcription of the two neighboring genes are auto regulated and the two loci are overlapping with each other (50). In this study, a toxin-antitoxin system is identified and characterized in the pVir plasmid of *C. jejuni* IA 3902 strain. Comparative genomic analysis of the pVir45 and pVir46 genes in *C. jejuni* indicates that the toxin-antitoxin system belong to the RelE/StbE family. Cloning and expression of the pVir46 gene alone in *Escherichia coli* inhibited bacterial growth, but when pVir45-46 is co-expressed, bacterial growth was restored. The plasmid

stability assay in *C. jejuni* showed that the entire toxin-antitoxin system is necessary for plasmid maintenance, but deletion of the pVir46 gene resulted in plasmid loss. The identified toxin-antitoxin system in pVir of *C. jejuni* IA 3902 may contribute to *Campylobacter* virulence by maintaining plasmid stability. This study represents the first report on a functional toxin-antitoxin system in *Campylobacter*.

Thesis Organization

The thesis is organized into three chapters. The first chapter of this thesis is a literature review about the characteristics of *C. jejuni* and the toxin-antitoxin stability system. The second chapter describes the identification and characterization of a toxin-antitoxin system in the pVir plasmid of *C. jejuni* IA 3902. The thesis concludes with a general conclusion of the experimental results.

Literature review

C. jejuni microbiology and genomics

C. jejuni is a gram-negative organism that has spiral or helical shaped morphology. *C. jejuni* is a motile bacterial organism consisting of either uni or bipolar flagella attached to its polar ends providing it with a corkscrew motion essential to its virulence. *C. jejuni* is grown in a microaerophilic environment for optimal growth at 5% O_2, 10% CO_2, and 85% N_2 at a temperature of 37 ^{o}C or 42 ^{o}C (87).

The pathogenic mechanisms of *C. jejuni* are still poorly defined, but more knowledge about the virulent determinants is being discovered with the advance of sequencing and annotation of *C. jejuni* genomes. Currently, there are 16 sequenced genomes of *C. jejuni* including RM 1221, 81-176, 81116, IA 3902, ICDCCJ07001, M1, NCTC 11168, S3, CG8486, CG8421, BH-01-0142, HB93-13, 260.94, CF93-6, 84-25 and doylei. *C. jejuni* genomes consist of a circular chromosome ranging from approximately 1.6 to 1.8 megabases in size. The sequence of *C. jejuni* NCTC 11168 reveals that 94.3% of the entire genome are coding sequences (61). The genome of *C. jejuni* has a relatively low G+C content (~30%), which is especially low in the lipooligosaccharide (LOS) and extracellular polysaccharide (EP) regions (61). Some unique features of the *C. jejuni* NCTC 11168 genome include the lack of insertion sequences and repeat sequences. The organization of genes in *C. jejuni* appears to be functionally unrelated and scattered randomly throughout the entire genome. The genome

of *C. jejuni* contains a high rate of hypervariable sequences particularly in the genes of biosynthesis or modification of surface structures (54).

C. jejuni 07001 was isolated from a stool sample obtained from an individual who suffered from Guillain-Barré syndrome (GBS) from an outbreak that occurred in China in 2007 (92). *C. jejuni* 07001 consists of a chromosome and a tetracycline resistant plasmid. The pTet plasmid is 41,742 base pairs in length and is similar to the 81-176 pTet plasmid. Genomic analysis of *C. jejuni* 07001 revealed high sequence variability in the regions encoding for LOS, flagella modification, and polysaccharide capsular biosynthesis. In *C. jejuni* 07001, there are 32 identified genes unique to this strain including McrB, which is part of the McrBC restriction endonuclease system, and FspA2, which is a flagellum-secreted protein inducing epithelial cell apoptosis (91). The genes of *cgtA*, *cgtB*, and *cst-II* in *C. jejuni* 07001 are involved with LOS ganglioside mimicry which are commonly present in strains associated with Guillain-Barré syndrome (55).

C. jejuni M1 was a strain isolated from a human patient who developed enteritis after visiting a poultry abattoir (26). *C. jejuni* M1 strain has similar genomic traits with *C. jejuni* 81116. However, the M1 strain has unique phenotypic characteristics compared to other *C. jejuni* strains as it has poor motility, is a poor colonization in chickens, and has straight morphology. *C. jejuni* M1 is identified to have *cadF* which is important for chicken colonization (46) and *porA*, which is a major outer membrane protein needed for adhesion (53). Interestingly, the *capA* gene is absent in the M1 strain which is associated with adherence in human cell lines and important in chicken colonization (4). The gene

CJM1_0038 encodes gamma-glutamyltranspeptidase, which is essential for *C. jejuni* colonization in the avian intestine and is conserved in *C. jejuni* 81116 and 81-176 (26).

C. jejuni subspecies *doylei* is different from the other *C. jejuni* strains in several aspects. It is primarily obtained from clinical samples associated with bacteremia (44). The *doylei* strain has some unique phenotypic characteristics that distinguish it from other *C. jejuni* strains as it does not readily grow at 42 ^{o}C because culturing methods for *C. jejuni* select against *C. jejuni doylei* and it is susceptible to cephalothin. *C. jejuni doylei* lacks L-arginine arylamidase and ɣ-glutamyl transferase activity. Comparative genomic analysis conducted by microarray technology indicates that *C. jejuni doylei* does not contain genes for nitrate reductase and cytolethal distending toxin (59).

C. jejuni 81-176 contains two plasmids approximately 35 kb in size. One plasmid known as the pTet plasmid contains the *tetO* gene, making it resistant to tetracycline. Another plasmid in 81-176 is known as the pVir plasmid, which contains significant homology to a *cag* pathogenicity island found in *Helicobacter pylori* (5). The pVir plasmid plays an important role in the invasion of *C. jejuni*. *C. jejuni* 81-176 contains an additional DMSO reductase system, which may be important for respiration in oxygen limited conditions of the intestinal tract. *C. jejuni* 81-176 encodes for homologs of C4-dicarboxylate carriers, which are active in metabolism under anaerobic conditions. Interestingly, 81-176 contains a gene for glycerol-3-phosphate transporter, allowing for the removal of glycerol-3-phosphate providing this strain with a metabolic advantage (39).

C. jejuni strain 81116 is a human isolate obtained in a 1982 waterborne outbreak (63). The strain 81116 contains 17 homopolymeric G tract regions, which are significantly fewer than other previously sequenced *C. jejuni* strains. *C. jejuni* strain 81116 has a 6.5-kb region that is duplicated in its genome, which is a distinguishing feature of this strain. This strain lacks some of the genes necessary for iron uptake as it doesn't have the CfrA and Cj0178 genes. This strain contains a single gene that encodes for TonB, while other *C. jejuni* strains have multiple copies of TonB (35).

C. jejuni strain S3 originated from the feces of a chicken and is the second poultry isolate to be sequenced and annotated. Some unique features of this strain are that it is nonmotile and considered to be a weak colonizer of poultry (16). The S3 strain consists of a circular chromosome and the pTET plasmid.

The genome size of *C. jejuni* strain RM1221 is larger than that of NCTC 11168. The distinguishing feature of this strain is that it contains four *C. jejuni*-integrated elements (CJIE) (60). CJIE1 is located upstream of *arg*C and encodes proteins that are similar in function to bacteriophage Mu. CJIE3 is most likely an integrated plasmid since it is located at the 3 prime end of the arginyl-tRNA. CJIE2 and CJIE4 encode for methylases, endonucleases, and repressors for phage related activities.

C. jejuni IA 3902 was isolated from the placenta of an aborted sheep. This strain is responsible for the majority of the ovine abortions occurring in the United States (75). *C. jejuni* IA 3902 is similar in genomic composition to NCTC 11168. The genome of IA 3902

consists of a chromosome with an inserted *tetO* gene, making it tetracycline resistant, and the pVir plasmid carrying a putative toxin-antitoxin stability system. A unique feature of this genome is that the *tetO* gene is inserted in the chromosome instead of being carried on a plasmid like in other *C. jejuni* strains.

Strain CG8486 was isolated in Thailand from an individual who experienced dysentery. CG8486 has a chromosome that is syntenic with the NCTC 11168 chromosome except for some random insertions and deletions scattered on the genome (68). The *tetO* gene is carried in the plasmid of CG8486, making it tetracycline resistant. The *C. jejuni* isolate has remnant of a degenerated virulence gene that shows significant sequence homology to the filamentous hemagglutinin in *C. lari* RM2100 (68). Additionally, CG8486 contains a cluster of genes that encode for arsenate resistance, such as the *arsB* and *arsC* genes.

The *C. jejuni* isolates of CG8421 and BH-01-0142 do not synthesize glycolipid mimics (69). Both of these strains were isolated from individuals after diarrheal episodes. Surprisingly, these strains have significantly lower levels of invasion in INT407 cell line compared with 81-176 (69). CG8421 contains the *tetO* gene in the chromosomal DNA, making it tetracycline resistant. CG8421 has the ability to survive in phosphate limited conditions as it contains phosphate utilization pathway.

C. jejuni 84-25 was initially isolated from the cerebrospinal fluid of a patient diagnosed with meningitis (8). *C. jejuni* 84-25 has a greater resistance to reactive nitrogen species than 81-176, suggesting its increased virulence and explaining why it caused a systemic clinical

manifestation (41). The sequence of this genome will likely facilitate the identification of the genes needed for antimicrobial defenses or resistance to nitrosative stress.

Recently, a greater emphasis has been placed on the virulent determinants of *C. jejuni* that cause Guillain-Barré syndrome. The following additional *C. jejuni* genomes have been sequenced: HB93-13, 260.94, and CF93-6. HB93-13 was isolated from an individual from China, and this isolate was shown to express GM1 and GD1a ganglioside structure types (38). HB93-13 has the *htrB* gene, which is needed for its survival under environmental stresses ensuring its survival (67). The CF93-6 strain was isolated from a patient with MFS in Japan and produces GT1a gangliosides that are cross reactive with anti-GQ1b antibodies (45). *C. jejuni* 260.94 was isolated from a patient in South Africa, which was associated with the most common type of GBS (89). These *C. jejuni* strains have similar genomic structures and sequence homology in the LOS region, further suggesting the association of unique LOS structures with GBS.

C. jejuni infections

C. jejuni infections are a significant cause of human gastroenteritis in the United States and other countries resulting in the symptoms of fever and diarrhea in patients. The *C. jejuni* infections are a common cause of bacterial diarrhea in both developing and industrial countries, but the nature of infection is different (2). In developing nations, children and adults infected by *C. jejuni* appear to have asymptomatic infections and outbreaks of the infection are rare compared to industrialized nations. In tropical climates, the infection is

hyperendemic among children under the age of two years (14). *C. jejuni* has been identified as the leading cause of foodborne illness primarily acquired from the consumption and handling of chickens in industrialized countries (21). *C. jejuni* infections are often self-limiting as antibiotic treatment is typically not needed for treatment. Clinical manifestations that can arise from *Campylobacter* are diarrhea from watery to bloody, abdominal cramps, fever, and bacteremia. Individuals with the infection have red blood cells and fecal leukocytes in their stool samples (7). Experimental studies have shown that increased cases of campylobacteriosis occur in individuals who are immunosuppressed like HIV patients, infants, and elderly (78, 79).

C. jejuni infections are often associated as a trigger in the development of Guillain-Barré syndrome (GBS). GBS is a debilitating disorder that impairs the peripheral nervous system resulting in severe neurological damage. The risk of actually contracting GBS from prior *C. jejuni* infections depends primarily on the particular *Campylobacter* serotypes as O:19 and O:41 are more frequently linked with GBS than others (3). The actual probability of contracting GBS after *Campylobacter* infection is relatively low as it only occurs in 1 out of 1000 reported cases infected by *Campylobacter* (1). After *C. jejuni* infection, GBS symptoms occur approximately one to three weeks after diarrheal symptoms (2). The pathogenesis of *C. jejuni* triggered GBS appears to be related to autoimmunity triggered by molecular mimicry between the LOS of *C. jejuni* and the peripheral nerve glycolipids (90).

Miller-Fisher Syndrome (MFS) is a less common variant of GBS and is known to cause ataxia and ophthalmoplegia (72). MFS is also associated with molecular mimicry between

C. jejuni infection and the neural gangliosides (32). Patients who are diagnosed with MFS have serum antibodies for GQ1b and GT1a present on motor nerve terminals (12).

A chronic disorder called Reiter's syndrome is also associated with *C. jejuni* infections. Reiter's syndrome causes inflammatory arthritis primarily in large joints like the knee or back of humans. Patients who have contracted Reiter's syndrome are positive for the Human Leukocyte Antigen B27 which is an inherited marker for the prevalence of rheumatoid diseases. Population studies have suggested that the prevalence of *C. jejuni* infection could have triggered reactive arthritis as approximately 7% of the patients with Reiter's syndrome in Finland hospital were positive for *C. jejuni* in their stool samples (36).

Epidemiologic features of *C. jejuni* infection

C. jejuni is a commensal organism which colonizes the normal gastrointestinal tracts of food producing animals. *C. jejuni* has a widespread animal reservoir including chickens, turkeys, sheep, cattle, swine, and wild birds such as geese (49) and seagulls (31). Transmission of *C. jejuni* to a human host occurs primarily through the consumption of contaminated foods of animal origins. Sporadic cases of *C. jejuni* infection in humans are a result of the ingestion of undercooked poultry, unpasteurized milk, and contaminated drinking water (9). *C. jejuni* infection can also be acquired through contact with pets or infected individuals, and travel to developing nations (6).

Experimental and epidemiological studies on the transmission of *C. jejuni* in broiler chickens support the theory that *C. jejuni* is horizontally transmitted on farms (74). Different from *Salmonella*, there is no solid evidence for vertical (or egg-borne) transmission of *C. jejuni* from breeder flocks to broiler flocks (77). The prevalence of *C. jejuni* in broiler chickens is primarily influenced by the environmental conditions on the poultry farms, indicating that horizontal transmission is the primary mode of spread. The source of contamination in poultry is attributable to multiple factors, such as contaminated drinking water, presence of domesticated animals, rodents, and poor hygiene of farm workers (9).

Treatment of *C. jejuni* infections

C. jejuni infections are often self-limiting and do not need antibiotics for treatment, but under certain circumstances, medication is needed to alleviate severe symptoms. Antibiotic therapy is often prescribed to patients who are immunocompromised or exhibit severe clinical symptoms from the infection (6). *C. jejuni* is known to be intrinsically resistant to several antibiotics, such as vancomyin, rifampin, trimethoprim, amoxicillin, ampicillin, metronidazole, and cephalosporin (2). Fluoroquinolone and erythromycin have been the antibiotics that are commonly prescribed for the treatment of *C. jejuni* infections. Fluoroquinolones are frequently used for the treatment of enteric infections, because it can alleviate the infection by different pathogens, such as *Salmonella* and *Campylobacter*. Recently, many *Campylobacter* strains have become fluoroquinolone resistant due to its widespread use in food animal production and human medicine, and due to the fact that *C. jejuni* is highly mutable to fluoroquinolone treatment (23, 40). In general, macrolide (e.g.

erythromycin) is now the most effective drug to treat *Campylobacter* infections and it is acid-resistant and incompletely absorbed in the gut (2). Erythromycin is easy and safe to be administered to immunosuppressed or immune-immature patients, such as children and pregnant women, and has low rates of resistance in *Campylobacter* species.

Prevention of *C. jejuni* transmission

To control *C. jejuni* transmission through the food chain, it is imperative to use a farm-to-folk approach that includes reducing its prevalence on farms, minimizing contamination of carcasses in processing plants, and ultimately educating consumers for proper handling and cooking poultry products. *C. jejuni* infections are known to occur from the consumption of undercooked poultry, unpasteurized milk, and untreated water (22, 56, 81). Overall, enhanced efforts are being made to develop intervention measures at both pre- and post-harvest stages.

The prevention of *C. jejuni* infections begins with limiting the bacterial load on poultry farms, but currently there are no effective and practical strategies that can be implemented to reduce *Campylobacter* colonization in live birds. Additionally, a number of studies have been performed to reduce carcass contamination in slaughter houses (83, 84). The implementation of disinfecting strategies during the processing steps can reduce the levels of *C. jejuni* on the carcasses. The use of chlorinated sprays have been used to clean the surfaces in slaughter facilities, which was reported to have up to a 100-fold reduction in *C. jejuni* contamination of the carcasses (52). A freeze-thaw chilling method is used to reduce the

contamination of pig and poultry processing by up to 100-fold reduction (57). Experimental studies have shown that poultry meat subjected to gamma radiation was successful in reducing *C. jejuni* (13, 62). However, the use of irradiation technology as a food safety measure is still not accepted by many consumers. Regardless, the precautions taken by the food industry during the slaughter and processing steps help to minimize the risk of *C. jejuni* transmission to consumers.

Currently, there are no vaccines available for the prevention of *C. jejuni* infection in animals or humans, but experimental studies have shown promises for vaccines against the disease. An oral vaccine of inactivated *C. jejuni* whole cell administered in broiler chickens resulted in reductions in the colonization of *C. jejuni* up to 93% compared with unvaccinated chickens (71). The whole cell vaccines increased the levels of anti *C. jejuni* secretory IgA antibodies in the vaccinated chickens compared to the non-vaccinated controls. An inactivated whole cell vaccine of 81-176 showed protection levels between 80 to 100% against *C. jejuni* infection in ferrets (11). An experimental study evaluated the effectiveness of a truncated recombinant flagellin subunit vaccine in mice which was administered by a nasal route and showed a high level of protection against *C. jejuni* (47). There are still no commercial vaccines developed against *C. jejuni*, but these experimental vaccines evaluated in model organisms provide hope for the development of effective vaccines.

Toxin-Antitoxin systems and plasmid maintenance

Toxin-antitoxin (TA) systems are commonly found on plasmid DNA and functions to maintain plasmid stability (42). TA genes are also present in chromosomal DNA, where their functions can vary significantly (50). TA systems are discovered as single or multiple copies in bacterial organisms, but no TA systems have been identified in *C. jejuni* (86). The organizations of a TA system in bacterial organisms are found in two possible arrangements. The most common organization of a TA system is where the antitoxin gene precedes the stable toxin gene. An alternative arrangement of the TA system is where the toxin gene precedes the antitoxin gene and has a second promoter before the antitoxin gene. A distinguishing feature of the TA stability systems is that the transcription of the genes are autoregulated by the protein products (50). The toxin and antitoxin genes have a region where the stop codon of the antitoxin gene and start codon of the toxin gene are overlapping with each other indicating translational coupling (93).

The regulation of plasmid stability by a TA module occurs when the antitoxin binds to the toxin to neutralize its toxic effects at the steady state. The antitoxin is synthesized at greater rates to inhibit the function of the toxin, but if there is a disruption to this continuous synthesis it would allow the toxin to be free and potentially kill the host (29). When the toxin is liberated, daughter cells that do not inherit the copy of the plasmid with an intact TA system will eventually be eliminated by a post-segregational killing mechanism by the deleterious activity of the toxin (86). Plasmid-encoded TA systems appear to increase the

fitness and prevalence of plasmids in the bacterial population through a post-segregational killing mechanism (86).

Diversity of plasmid addiction systems

There are three different types of TA systems prevalent in bacterial organisms. Type I TA systems consist of an antisense RNA antitoxin and a proteic toxin complex and was first represented by the Hok-Sok system in the R1 plasmid (30). The toxins of type I are primarily hydrophobic in nature and are small in size resulting in the damage of the cell membrane (86). The translation of the toxin is inhibited when the protein toxin is bound to the RNA antitoxin (37). The difference of having an antitoxin RNA from an antitoxin protein is that its interaction with the toxin results in an irreversible step as mRNA cleavage occurs. The antisense RNA is usually encoded on the antisense strand compared to its target toxin and has significant overlap with the toxin mRNA. Type I TA systems are not as prevalent as type II since the identification of the type I toxins are difficult due to its smaller size and having a shorter transmembrane helix (25).

Type II TA systems are composed of proteins for both the toxin and antitoxin components. Genetically, the antitoxin gene is located upstream of the toxin gene forming an operon. Type II TA systems are different in its regulation from the type I TA systems is regulated at the protein level. The type II TA systems are also widely distributed on bacterial chromosomal DNA with multiple copies, but the function of the chromosomally encoded complexes is not clearly defined. Type II TA systems have been determined to function in

post-segregation killing of plasmid-free cells (86). In type II TA systems, the antitoxin protein is more likely to be degraded by the host organism's protease than the toxin, resulting in growth impairment or cell death in the bacterial host lacking a functional TA system.

The third type of TA system consists of a direct interaction between an RNA antitoxin and toxin protein. The ToxIN system discovered in the phytopathogen *Erwinia carotovora* functions in the abortion of infections by different phages (24). The *toxI* gene suppresses the bacteriostatic effects of the ToxN protein as it prevents phage replication or synthesis.

Cellular targets of RelBE TA system

The RelBE family of TA system consists of antagonist RelB antitoxin and RelE toxin, which bind together to form the non-toxic RelBE TA system (27). The overexpression of the *relE* toxin gene results in bacteriostatic effects as it significantly reduces the cell growth and inhibits translation (33, 64). The *relBE* loci has been determined to function in maintaining plasmid stability (34). The RelE toxin inhibits translation by cleaving mRNA at the ribosomal A-site between the second and third bases of the A-codon site *in vitro*. Release factor I is known to control the amount of mRNA cleavage occurring as it binds to the ribosomal A-site (65) limiting the cleavage from the RelE protein. Under *in vivo* conditions, RelE cleavage occurs on the coding strands regardless if ribosomes are present.

Crystal structure analysis of an archaeal RelBE TA system in *Pyrococcus horikoshii* was compared to other families of TA systems. The experimental evidence shows that RelB

lacks a hydrophobic core and wraps around the surface of RelE (80). RelE has hydrophobic properties as it folds into an α/β structure while the RelB lacks any tertiary structure, making it susceptible to degradation from the cellular Lon proteases (85). As plasmid loss occurs, the Lon protease degrades the antitoxin, activating the effects of the RelE toxin. The Lon cellular protease degrades free and bound forms of the RelB allowing the inhibition of translation by the RelE. The interaction between RelB and RelE creates an inactive complex of RelBE.

Type II TA families

There have been nine type II TA families identified and characterized to date. The families of type II toxin-antitoxin systems in bacteria are the following: Ccd, RelBE, HigBA, HipAB, ParDE, Pem and MazEF, Phd/doc, VapBC, and ω-ε-ξ (86). The TA systems have been classified into two distinct groups of two component and three component gene families. These TA systems have been discovered on either plasmid or chromosomal DNA.

The *relBE* locus in the chromosome of *Escherichia coli* K-12 consists of a RelB antitoxin and RelE cytotoxin (33). The overexpression of RelE results in the inhibition of cell growth in the host organism while RelB prevents the toxic effect of RelE. RelE is degraded by Lon protease in order to form a tight binding with RelB to neutralize its toxic effects. The *relBE* locus is also discovered on the plasmids of *Escherichia coli*, *Plesiomonas shigelloides*, *Acetobacter europaeus*, and *Butyrivibrio fibrisolvens* (28). Experimental studies have

determined that plasmid and chromosomal *relBE* loci both function in plasmid stability by killing plasmid-free cells, making it an unique property of the RelBE TA family (33).

The *ccd* locus is located in the F plasmid of *E. coli* and functions in plasmid maintenance by killing plasmid-free daughter cells. The Ccd TA system is primarily discovered in plasmids and in the chromosomes of a few gram-negative bacteria (58). The *ccd* locus consists of the *ccdA* and *ccdB* gene pair, in which the CcdB prevents cell division while CcdA suppresses the activity of the CcdB toxin. The CcdB toxin inhibit DNA replication by the inactivation of the DNA gyrase (18). The binding of the *ccdA* and *ccdB* genes prevent the activation of the Lon-dependent proteolysis of CcdA (85).

The *higBA* locus was initially discovered in the Rts1 plasmid of *Proteus vulgaris* (82). The *higBA* TA system is found on both plasmids and chromosomes and the first chromosomal *higBA* was found in *Vibrio cholera* (10). The HigBA TA system consists of the HigA antitoxin and HigB toxin which inhibits cellular growth, but the specific inhibitory mechanisms of the toxin is still unknown. The gene organization of the *higBA* loci is different from other TA families as the *higB* toxin gene is upstream of the *higA* antitoxin gene, which controls the expression of *higB* (10).

The *parDE* TA system was originally found on the RK2 plasmid in which it functions in plasmid maintenance by a post-segregation killing mechanism. The *parDE* locus consists of the ParD antitoxin and ParE toxin, which form a tetrameric ParDE TA complex that autoregulates the transcription of the *parDE* locus (43). The ParE toxin inhibits DNA

replication by the inactivating of the DNA gyrase. The *parDE* homologues are found in both plasmid and chromosomal DNA.

The ω-ε-ξ TA family was initially discovered in the pSM19035 plasmid of *Streptococcal pyogenes* and is the only TA family that consists of three components (94). The ξ gene was determined to be a cytotoxin by its toxic activity in *Bacillus subtillis, E. coli*, and *Saccharomyces cerevisiae* (94). The ξ cytotoxin is unique compared to other toxins as it is made of 287 amino acids which is significantly larger than the average composition of 100 amino acids (28). The ε gene functions as an antitoxin which prevents the toxic effect of the ξ toxin through a direct protein interaction. The ω gene regulates transcription of the entire locus as it encodes for an autorepressor. The ω gene product strictly regulates the ω-ε-ξ locus, and it is not directly involved in the killing-anti-killing mechanism (20).

The *vapBC* locus was found in the virulence plasmid of *Salmonella dublin* (70). The experimental study characterized the function of the genes of *vapB* and *vapC* and found that VapB is an antitoxin while VapC is a toxin. The specific mechanism for the expression of the toxin gene is still unknown, but it was determined that VapB and VapC are involved in plasmid maintenance. The *vapBC* TA family has been discovered in archaeal species such as *Sulfolobus solfataricus* and hypothesized to function in microbial stress response (15).

The *phd/doc* locus was discovered in the P1 plasmid, which lysogenizes *E. coli*. Phd is the antitoxin that neutralizes the toxic effects of Doc by forming a Phd_2Doc trimeric complex. The Phd protein is responsible in autoregulating the *phd-doc* operon while the Doc toxin

functions in translational inhibition (51). The protease of ClpXP degrades the Phd antitoxin leading to the liberation of the toxin gene (48). The primary role of the Doc toxin is to eliminate plasmid-free cells.

The *pem* loci are located in the plasmid of R100 and function in the stabilization of the plasmids by eliminating the growth of plasmid-free cells. The *pem* loci are organized in the typical arrangement of other TA systems with the antitoxin gene preceding the toxin gene. The *pem* loci encode for the antitoxin genes of PemI and the toxin genes consist of PemK which inhibits the initiation of replication most likely in the DnaB helicase (73). Experimental evidence has indicated this class of toxin family is bacteriostatic in nature as it prevents the growth of cells and does not kill plasmid-free cells.

The *hipBA* loci are located in the *E. coli* chromosome. HipA is a toxin and HipB is antitoxin which binds to HipA represses the *hipBA* operon (17). The *hipBA* loci are organized in the typical arrangement of other TA systems with the antitoxin gene preceding the toxin gene. HipA contains a serine/threonine kinase which phosphorylates elongation factor-Tu indicating the possibility of a persistence mechanism (76).

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CHAPTER 2. Identification and characterization of a toxin-antitoxin system in the pVir plasmid of *Campylobacter jejuni*.

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Abstract

Toxin-antitoxin systems are prevalent in different bacterial organisms and are encoded in the chromosomal or plasmid DNA. The primary function of a plasmid toxin-antitoxin module is to stabilize the plasmid by eliminating plasmid-free daughter cells through a post segregation killing mechanism. In *C. jejuni*, a pathogen that causes human gastroenteritis, multiple plasmids have been identified, but there have not been any reports on toxin-antitoxin systems. In this study, a toxin-antitoxin system is identified and characterized in the pVir plasmid of *C. jejuni* IA 3902. pVir encodes a type IV secretion system and is found to be involved in natural transformation and virulence. Comparative genomic analysis of the pVir45 and pVir46 genes in *C. jejuni* indicates that they encode a putative toxin-antitoxin system that belongs to the RelE/StbE. Cloning and expression of the pVir46 gene alone in *Escherichia coli* inhibited bacterial growth, but co-expression of pVir45-46 led to the restoration of growth confirming the functions deduced from comparative genomic analysis.

The plasmid stability assay in *C. jejuni* showed that the toxin-antitoxin system is necessary for maintaining the stability of pVir because deletion of the pVir46 gene resulted in loss of the plasmid during passage in conventional media. qRT-PCR data demonstrated that expression of the pVir45-46 genes varied with growth phase, as early logarithmic phase had the highest level of gene expression. Together these results establish that pVir45-46 encode a functional toxin-antitoxin system in *C. jejuni*, which is required for ensuring the stability of the pVir plasmid. The stable maintenance of pVir may be necessary for optimal virulence of *C. jejuni* IA 3902.

Introduction

C. jejuni is a spiral, microaerophilic, gram-negative organism associated with human gastroenteritis and increasingly with ovine abortions (3, 36). *Campylobacter* is considered a highly infectious pathogen that causes diarrhea, headaches, and abdominal pain in humans and is mainly transmitted by the consumption of undercooked poultry meat, unpasteurized milk, and contaminated drinking water (5, 22, 28). *C. jejuni* infections also trigger GBS, which is an autoimmune disorder that adversely affects the peripheral nervous system (35). Recently, there has been a species change in the etiology of ovine abortions in the United States because *C. jejuni* has replaced *C. coli* as the predominant cause of sheep abortion (20). The pathogenic mechanisms of *C. jejuni* are still poorly understood, and the reported virulence factors associated with *C. jejuni* survival and campylobacteriosis are motility, invasion and adhesion to epithelial cells, cytolethal distending toxin production, antibiotic susceptibility, and oxidative stress defense (8, 12, 19, 21, 31).

Toxin-antitoxin (TA) systems are comprised of a pair of antagonistic genes that encode a stable toxin and an unstable antitoxin. The toxin product primarily inhibits bacterial metabolic activity while the antitoxin binds to the toxin preventing its toxic activity (18). There are three types of TA systems: type I systems are composed of RNA antitoxin, type II systems are composed of protein antitoxin, and type III systems are composed of RNA antisense and toxic protein (7, 16). TA systems have been identified on bacterial plasmids and chromosomes. The function of a plasmid-encoded TA system is for plasmid maintenance, in which a post-segregation killing mechanism is used to kill daughter cells that do not inherit a plasmid copy (9). TA systems are found in multiple copies on the chromosome of several bacterial organisms, which has led to the speculation about the functions of this complex, including antiaddiction module, programmed cell death, growth control, persistence, and stabilization of genomic parasites (6, 14, 15, 32).

Comparative genomic analysis of *C. jejuni* genomes conducted by RASTA-Bacteria has identified that there are two potential TA systems: one in the *relBE* and one in the *vapBC* family (34). An early study using exhaustive BLAST searches indicate that there are no identifiable groups of toxin-antitoxin systems present in *C. jejuni* (27). Recently, our laboratory has identified a gene (pVir46) on the pVir plasmid in *C. jejuni* IA3902, which has a significant sequence homology to *relE* that is a cytotoxic translational repressor of a TA system, suggesting the presence of a TA system in the pVir plasmid. pVir45 is immediately up stream of pVir46 and these two genes appear to form an operon. In this study, functional analysis of the pVir45 and pVir46 genes in *C. jejuni* IA 3902 is conducted by cloning and

expressing the genes in *E. coli* DH5α. In addition, plasmid stability assay of pVir in *C. jejuni* is performed using pVir46 knock-out mutant in comparison with the wild-type plasmid. Results from these experiments indicated that pVir45 and pVir46 indeed encode a TA system in *Campylobacter*.

Materials and Methods

Bacterial strains, plasmids, and culture conditions. The bacterial strains, plasmids, and mutant constructs used in this study are listed in Table 1. Mueller-Hinton (MH) agar or broth (Difco, Franklin Lakes, NJ) were used for the isolation and cultivation of *C. jejuni* strains. These *Campylobacter* strains were incubated at a temperature of 42 ^{o}C and grown under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2). *E. coli* strains are grown in Luria Bertani (LB) agar or broth (Difco) at 37 ^{o}C with shaking at 200 rpm. The selective media were supplemented with ampicillin (Amp) (100 μg/ml) or kanamycin (Km) (50 μg/ml) as needed for culturing *E. coli* or *C. jejuni* strains.

Nucleotide sequence database and software. The complete genome of the pVir plasmid in *C. jejuni* IA 3902 was obtained from the NCBI Nucleotide Database (http://www.ncbi.nlm.nih.gov/). The nucleotide sequence of pVir45 and pVir46 were analyzed by the Basic Local Alignment and Search Tool (BLAST) to determine their homology to previously annotated toxin-antitoxin systems in other bacterial organisms. Multiple sequence alignments were performed with ClustalW (Pathema, USA). The promoter of pVir45 and pVir46 are determined by BPROM (Softberry, Mount Kisco, NY).

Rapid Automated Scan for Toxin and Antitoxins in Bacteria (RASTA-Bacteria) (http://genoweb.univ-rennes1.fr/duals/RASTA-Bacteria/) was also used to identify potential toxin-antitoxin systems in the pVir plasmid of *C. jejuni* IA 3902.

Expression of pVir45-46 genes in *Escherichia coli* DH5α. The pVir45 and pVir46 genes were inserted downstream of the pBAD promoter in the multiple cloning site II of the arabinose inducible pBAD30 plasmid (Fig. 1). The PCR amplification of the genes of interest were accomplished by designing PCR primers that incorporate the restriction sites of EcoRI followed by the ribosomal binding site in the forward direction and XbaI in the reverse direction. PCR primers are utilized to amplify pVir45 and pVir46 genes as well as the pVir45-46 operon as shown in Table 2. The PCR reactions were performed using the Eppendorf Gradient Thermocycler (Eppendorf, Hamburg, Germany) and the following conditions: initial denaturation cycle of 95 ^{o}C for 5 minutes; 35 cycles at 95 ^{o}C for 30 seconds, 50 ^{o}C for 30 seconds, and 72 ^{o}C for 1 minute; and a final extension cycle of 72 ^{o}C for 10 minutes. The genomic DNA of *C. jejuni* IA 3902 was used as the DNA template for the PCR reactions. After PCR amplification, the toxin-antitoxin gene products were purified with the QIAquick PCR purification kit (Qiagen, Venio, Netherlands). Double digestion of the PCR products and the pBAD30 vector with the restriction enzymes of EcoRI and XbaI (New England Biolabs, Ipswich, MA) were performed at a temperature of 37 ^{o}C for 2 hours and then the reactions were purified with the QIAquick PCR purification kit (Qiagen). The digested gene products were ligated into the pBAD30 vector using the Roche ligation kit (Roche Applied Science, Penzberg, Germany). The ligation reactions were transformed into *E. coli* DH5α competent cells (Invitrogen, Carlsband, CA) and transformants were selected

with ampicillin (100 μg/ml). The correct inserts in the transformants were confirmed by PCR.

Bacterial growth curve. The objective of measuring bacterial growth curve was to determine if the individual toxin gene in the expression vector pBAD30 inhibits the growth of *E. coli* in the presence of an arabinose inducer. The growth curves of *E. coli* DH5α containing an empty control vector, antitoxin, toxin, or the toxin-antitoxin system were determined by making measurement of OD_{600nm} for eight hours after the induction with 0.2% arabinose or glucose in LB broth. Prior to the induction, the bacterial cultures were grown in the Forma Orbital Shaker (Thermo Fisher, Waltham, MA) for approximately 16 hours at 200 rpm at 37 ^{o}C. The cultures were diluted 100x fold in LB broth for two hours to reach the mid-logarithmic (OD_{600} 0.4). After two hours of shaking, 0.2% arabinose or glucose inducer was added to determine if the cloned genes affected the growth of *E. coli* DH5α. After initiating the induction, hourly measurement of OD_{600nm} was conducted for 8 hours. An independent trial of triplicate replicates are used to verify the growth inhibition results obtained from these experimental trials.

Mutagenesis of pVir46 in *C. jejuni*. A kanamycin resistance cassette was inserted into pVir46 located in the pVir plasmid of *C. jejuni* by insertional mutagenesis shown in Figure 1B. This was accomplished by using the primers of pVir46-F1 and pVir46-R1 (Table 1) to amplify a region of approximately 1476 base pair (bp) in the pVir plasmid. The pVir primers were incorporated with the recognition sites of EcoRI and KpnI to the 5 prime end to facilitate the cloning of the PCR product into the pGEM®-T vector (Promega, Fitchburg,

WI). After PCR amplification, the PCR product was purified with the QIAquick PCR purification kit (Qiagen) and ligated into the pGEM®-T vector (Promega) using the Roche ligation kit (Roche Applied Sciences). The ligation reaction was transformed into *E. coli* DH5α (Invitrogen). The transformants with the PCR product inserted in the correct orientation in the pGEM®-T vector (Promega) were confirmed by PCR.

Once the pVir46-containing fragment was cloned into the pGEM®-T vector, inverse PCR using *ex Taq* polymerase (Takara, Shiga, Japan) was conducted to create a deletion of approximately 200 bp within the pVir46 gene. The kanamycin resistance gene (Kan^R) was amplified from the pMW10 plasmid using the *ex Taq* polymerase (Takara) was ligated with the inverse PCR product to make pGEM®-T-46K. The ligation reaction was transformed into *E. coli* DH5α and the transformants were selected with100 μg/ml of ampicillin and 50 μg/ml of kanamycin. The insertion of the kanamycin resistance gene into pVir46 was confirmed by PCR. The plasmid, pGEM®-T-46K, was extracted from *E. coli* DH5α with the Qiagen miniprep kit (Qiagen) and was introduced into *C. jejuni* IA3902 by electroporation. Then the *Campylobacter* cells were plated onto the MH agar plates containing kanamycin (50 μg/ml). Since pGEM®-T-46K was not able to replicate in *Campylobacter*, the selection with kanamycin resulted in the generation of mutants with the resistance gene inserted in pVir46. PCR was used to verify the creation of the pVir46 mutant. A motility assay was performed in triplicate to verify that the mutation did not affect the motility of *C. jejuni* strain IA3902.

Plasmid stability of pVir46 mutant. A plasmid stability assay was conducted to determine if the TA system affects the stability of the pVir plasmid in *C. jejuni*. Since the wild-type pVir plasmid does not carry a selection marker, it was necessary to tag the plasmid to monitor its stability. Thus we used the pVirB10 mutant previously in our laboratory as a control to the pVir46 mutant. The pVirB10 mutant had a kanamycin resistance marker inserted into the VirB10 gene, which inactivated the function (type IV secretion) of virB10, but the TA module was intact. Single colonies of the pVir46 and pVirB10 mutant are picked from MH plates containing kanamycin and inoculated into 5 ml MH broth at 42 ^{o}C for 24 hours. A volume of 100 µl of the previous culture growth is reinoculated in MH broth and continued in this manner for 35 days. A volume of 100 µl samples of pVir46 and pVirB10 mutants are serially diluted and plated on MH. After 48 hours, 100 colonies are randomly selected and inoculated to MH kanamycin plates. The ratio of the colonies grown on the MH kanamycin is compared with the total viable count to determine the percentage of plasmid retained in the population.

Distribution of the toxin-antitoxin genes in different *C. jejuni* strains. A pair of PCR primers was designed to detect the toxin-antitoxin system in various isolates of 124 *C. jejuni* obtained from sheep (58), broiler (18), turkey (23), and humans (25). Total DNA of different *C. jejuni* isolates were obtained by the boiling method. The bacterial cells in 50 µl of water were boiled for approximately 10 minutes and then the supernatant was harvested by a centrifugation step. PCR primers utilized to amplify the TA system and the pVirB10 gene are shown in Table 2. The PCR reaction was prepared with a total volume of 25 µl containing 1 µl of DNA template, 10x *Taq* buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM

$MgCl_2$), 2.5 mM deoxynucleoside triphosphate mixture, 5 pM each primer, and 5 units of *ex Taq* polymerase (Takara). The PCR parameters using the Eppendorf Gradient Thermocycler (Eppendorf) were as follows: an initial denaturation cycle of 95 ^{o}C for 5 minutes; 35 cycles at 95 ^{o}C for 30 seconds, 52 ^{o}C for 30 seconds, and 72 ^{o}C for 1 minute; and a final extension cycle of 72 ^{o}C for 10 minutes. The PCR reactions were examined by 1% agarose gels to determine if products for the target genes were amplified.

Reverse Transcription PCR analysis. Reverse transcription PCR (RT-PCR) was performed to verify the expression of toxin-antitoxin module in *C. jejuni* IA 3902 obtained by using RNeasy minikit (Qiagen). Total RNA was purified from IA3902 using the RNeasy minikit (Qiagen). To prevent false positive results in RT-PCR, the isolated RNA sample was further treated with Ambion DNase I (Applied Biosystems, Foster City, CA) to remove DNA contamination. A Nanodrop microscale spectrophotometer (Thermo Scientific) was used to access the quality and quantity of the RNA. RT-PCR primers were designed to amplify both pVir45 and pVir46 of the TA system and are shown in Table 2. RT-PCR was performed by using SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen). The RT-PCR cycling conditions were the following: initial denaturation cycle of 95 ^{o}C for 5 minutes; 35 cycles at 95 ^{o}C for 30 seconds, 52 ^{o}C for 30 seconds, and 72 ^{o}C for 1 minute; and a final extension cycle of 72 ^{o}C for 10 minutes. In negative control reaction for DNA contamination, the PCR mix contained *Taq* polymerase (Promega) without the RT step.

Real-Time quantitative PCR. Real-time quantitative PCR (qRT-PCR) was employed to determine the rate of transcription of the toxin-antitoxin module in relation to the growth phase of *C. jejuni* IA 3902. Total RNA was isolated from *C. jejuni* grown under microaerophilic conditions at different incubation times (3 to 24 hours) using the RNeasy minikit (Qiagen). The isolated RNA sample was further treated with Ambion DNase I (Applied Biosystems) to remove DNA contamination. qRT-PCR was performed by using the MyiQ iCycler real-time PCR detection system (Bio-Rad, Hercules, CA). A 10-fold dilution series between 2.5 pg and 25 ng were prepared for each RNA sample for qRT-PCR using iScript One-step RT-PCR kit with SYBR Green (Bio-Rad). Primers were designed specifically for the pVir45, pVir46, and 16S rRNA genes by using the Primer3 online interface (http://frodo.wi.mit.edu/primer3/). The primers used for qRT-PCR are listed in Table 2. The gene expression of the TA system was normalized with the reference gene, 16S rRNA. The qRT-PCR was performed as described in our previous publication (23). The change in gene expression of the toxin-antitoxin module was determined by the $2^{-\Delta\Delta CT}$ method (24).

Results

Sequence analysis identifies a putative TA system in *C. jejuni* IA3902. *C. jejuni* IA3902 harbors a pVir plasmid that is similar to the pVir plasmid identified in *C. jejuni* 81-176, but has a few unique genes, such as pVir45 and pVir46. The RASTA-Bacteria analysis indicates that the pVir46 gene has a significant amino acid identity of 19% (E Value – 5e-4) with RelE, a cytotoxic translational repressor of the TA system (34). The function of RelE is

to inhibit translation by targeting ribosomal RNA and triggering hydrolytic cleavage at the mRNA A site (29). RASTA-Bacteria assigned the open reading frames of the pVir45 and pVir46 to previously annotated toxin-antitoxin pairs. The pVir45 and pVir46 genes have a score of 60.73 and 70.75 calculated based on a scoring model adapted from a previous study suggesting that there is a great likelihood that the two genes form a TA system in the pVir plasmid (27).

According to the BLAST search, the pVir45 ORF shows a significant sequence homology to two uncharacterized putative proteins in *C. jejuni* subspecies *doylei* strain ATCC BAA-1458 and *Helicobacter pullorum* MIT 98-5489 with a sequence identity of 96% and 73%, respectively. The lack of sequence identity of pVir45 with known antitoxin sequences suggests that it is evolutionarily distant and diverse from the other antitoxin genes. On the contrary, the BLAST search revealed that the pVir46 gene shows high amino acid identity with RelE. The BLAST search of pVir46 gene has a sequence identity of 85% to an addiction module toxin in the chromosome of *Helicobacter pullorum* MIT 98-5489. The pVir46 gene has 100% sequence identity to an uncharacterized putative protein in *C. jejuni* subspecies *doylei* strain ATCC BAA-1458 (Fig. 2).

Characteristics of the pVir45 and pVir46 genes in *C. jejuni*. pVir45 is 285 bp in length and encodes a putative protein of 94 amino acids, while pVir46 is 276 bp in length and encodes a putative protein of 91 amino acids. The predicted protein molecular mass of pVir45 and pVir46 are 22.9 and 22.6 kDA, respectively. The overlap between the translational start site of pVir46 and the termination stop codon of pVir45 strongly suggests

transcriptional coupling and coexpression of this gene pair. A schematic diagram of the genomic organization of the pVir45 and pVir46 genes and its promoter region is depicted in Figure 3. RT-PCR was used to determine if co-transcription occurs between pVir45 and pVir46. The primers of pVir45int-F and pVir46int-F were designed to amplify an expected product of 260 bp that spans both pVir45 and pVir46. As shown in Figure 4, a RT-PCR product of 260 bp was obtained with the RNA template, but no product was seen in the control reaction without the RT step. This result suggests that pVir45 and pVir46 are expressed in *C. jejuni* and are cotranscribed as a single transcriptional unit.

pVir46 is toxic to *E. coli*. To functionally characterize the TA system, we cloned pVir45 and pVir46 into *E. coli* and measured their toxicity to the *E. coli* host. These *E. coli* constructs contained either an empty pBAD30 vector, a cloned pVir45, pVir46, or both. First, the growth of various *E. coli* constructs was measured on LB plates. In the absence of an inducer or presence of glucose, none of the cloned genes affected *E. coli* growth (Fig. 5). However, when arabinose was added to the plate, the *E. coli* containing the cloned pVir46 gene stopped growth, while the other *E. coli* hosting the constructs grew normally, suggesting that pVir46 is toxic to *E. coli*. To further examine the growth dynamics, we measured the growth rates of the *E. coli* constructs in LB broth. All of the constructs showed similar growth characteristics in the presence of glucose (Fig. 6). However, under the induction by arabinose, the *E. coli* harboring the cloned pVir46 gene showed little growth, while other constructs including those harboring pVir45 or both pVir45 and pVir46 grew normally as the E coli carrying an empty pBAD30 vector (Fig 6). Together, these results indicate that the pVir46 gene is an addiction module toxin that significantly impairs bacterial

growth when cloned and expressed into *E. coli*. The results also suggest pVir45 encodes an antitoxin because co-expression of pVir45 and pVir46 neutralized the toxicity of pVir46.

The TA system affects the stability of pVir in *Campylobacter*. The TA system in pVir of *C. jejuni* IA3902 was inactivated to assess its effect on plasmid stability. The result showed that the control pVir plasmid (with a mutation in pVirB10) was stably maintained during the prolonged passage in MH broth without any antibiotic selective pressures, because 100% of the bacterial colonies contained the plasmid even at the end of the 35 days passage (Fig. 7). On the contrary, the pVir46-negative plasmid, in which the addiction toxin gene was inactivated by insertional mutation, was unstable during the passage in MH broth and was gradually lost. In fact, only 11% of the bacterial cells retained the plasmid after 35 days of passage (Fig. 7). These results clearly showed that this TA system is necessary for the genetic stability of the pVir plasmid in *C. jejuni*.

Detection of the pVir45/46 toxin-antitoxin system in *C. jejuni*. A PCR-based screening method was used to determine the distribution of this TA system in *C. jejuni* isolates from different host species. The primer pair of pVir45-F1 and pVir46-R1 was used to amplify a 260 bp amplicon that spans both pVir45 and pVir46. *C. jejuni* IA3902 was used as a positive control. Among all of the 124 *C. jejuni* isolates examined in this study, only one isolate, L4-F3, from a sheep slaughter showed a positive amplification by the primers. The TA module is not widely distributed in *C. jejuni* isolates.

Growth phase regulated expression of the TA system. Real-time quantitative PCR was conducted to determine the expression of the TA system at different growth phases. The results indicated that the expression levels of pVir45 and pVir46 decreased as the growth time increased (Table 3). The greatest expression of the two genes occurred at the early log phase, while the lowest expression of the genes was observed at the stationary phase as expression was compared to the 3 hour growth phase. The toxin gene expression initially starts with a threefold decrease during log phase (6 hours), then has a sevenfold decrease in the mid log phase (9 hours) and fourfold decrease in the late log phase (12 hours), and finally ends with a tenfold decrease at the stationary phase (24 hours). This data reveals a trend of decreasing expression of the TA system associated with the growth phase.

Discussion

In this study, we characterized a *relBE*-type toxin-antitoxin system in the pVir plasmid of *C. jejuni* IA3902. The pVir45 and pVir46 genes represent the first identified TA stability complex discovered in *C. jejuni* species. The toxicity of pVir46 to bacterial cells was clearly shown by cloning and expression of the pVir45, pVir46, or pVir45-46 genes in *E. coli* DH5α consistent with the pVir45 gene encoding an antitoxin that neutralizes the toxicity of pVir46. Thus the pVir45 and pVir46 genes constitute a functional TA system in *Campylobacter*. We further showed that this TA is essential for the maintenance of the pVir plasmid. Since pVir is a virulence plasmid in *C. jejuni*, retention of pVir may enhance the virulence of *C. jejuni*. Thus the identified TA system indirectly contributes to *Campylobacter* virulence by maintaining the stability of the pVir plasmid.

The multiple sequence alignments suggest that the identified TA system in this study is a member of the *relBE* family. The amino acid sequence conservation in TA systems is not well conserved, but it is clearly evident that the genetic organization is similar to other TA systems. For example, parD and ccD genes have a weak sequence homology with previously annotated TA systems, but they belong to the same family based on similar genetic structure and functional characteristics. Even though there is not tremendous protein sequence similarity in the relBE family, TA systems share similar genomic organization allowing for identification.

The expression of the TA system is growth phase regulated as its gene expression is more active in the logarithmic phase compared to the stationary phase. These experimental results suggest that the *relBE* TA system in *C. jejuni* is down regulated during the stationary phase, possibly indicating that the TA system inhibits its response under unfavorable growth and environmental conditions, but has increased expression in more favorable situations. TA systems are expressed differently or altered under different environmental or nutritional stimuli in response to thermal stress response, oxidative stress, and DNA damage (4, 17).

The data from the cloning and expression of pVir45, pVir46, and pVir45-46 in *E. coli* DH5α exhibited characteristics of a TA system. It is evident that *E. coli* growth was significantly impaired when pVir46 was expressed. Although it is conclusive that the pVir46 gene encodes a bacterial toxin, but it is unknown at this stage if the toxin is bacteriostatic or bactericidal. Additionally, it is unknown how pVir46 inhibits bacterial growth. Since it is a

member of the RelBE family, it is possible that the toxin targets translation, but this possibility remains to be determined in future studies. The plasmid stability test indicates that the TA system is associated with pVir maintenance in *Campylobacter*. This is consistent with findings in other bacteria that TA systems carried in plasmids are associated with plasmid stability.

The distribution of the relBE toxin-antitoxin system is not widely prevalent in the *C. jejuni* population. We employ a PCR screening method to detect the presence of the TA system, however some variation of sequences in the primer binding region would render *Campylobacter* strains with TA negative by our assay (26). The lack of a wide distribution of TA systems in *C. jejuni* could be explained by the speculation that horizontal gene transfer of the TA system to *Campylobacter* is a recent evolutionary event. BLAST searches in different bacterial species indicate that the antitoxin gene is not as conserved as the toxin gene, and this lack of nucleotide conservation could lead to the lack of detection of the TA system in various *C. jejuni* isolates.

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TABLE 1. Bacterial strains and plasmids used in the study

Plasmid or strain	Relevant characteristics	Source or reference
Strains		
C. jejuni		
IA 3902	Originally isolated from the placenta of aborted sheep	(33)
pVir46$^-$	Iowa 3902 derivative; pVir46$^-$::Kan^r	This study
E. coli		
DH5α	F$^-$ϕ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1-hsdR17 (r_k^-, m_k^+) phoA supE44 thi-1 gyrA96 relA1 ɻ$^-$ endA1 recA1 gyrA96 thi hsdR17 (r_k^-, m_k^+) relA1$^-$	Invitrogen
Plasmids		
pBAD30	araBAD promoter-based expression vector, p15a ori, Amp^r	(13)
pBAD30-45	pBAD30 containing full-length pVir45, Amp^r	This study
pBAD30-46	pBAD30 containing full length pVir46, Amp^r	This study
pBAD30-45-46	pBAD30 containing full length pVir45 and pVir46, Amp^r	This study
pGEM®-T	PCR cloning vector, Amp^r	Promega
pMW10	*E. coli- C. jejuni* shuttle vector with promoterless *lacZ* gene, Kan^r	(37)

Additional *C. jejuni* strains from the origins of sheep, broiler, turkey, and clinical isolates are used for PCR screening that are not listed above.

TABLE 2. Oligonucleotide primers used in the study

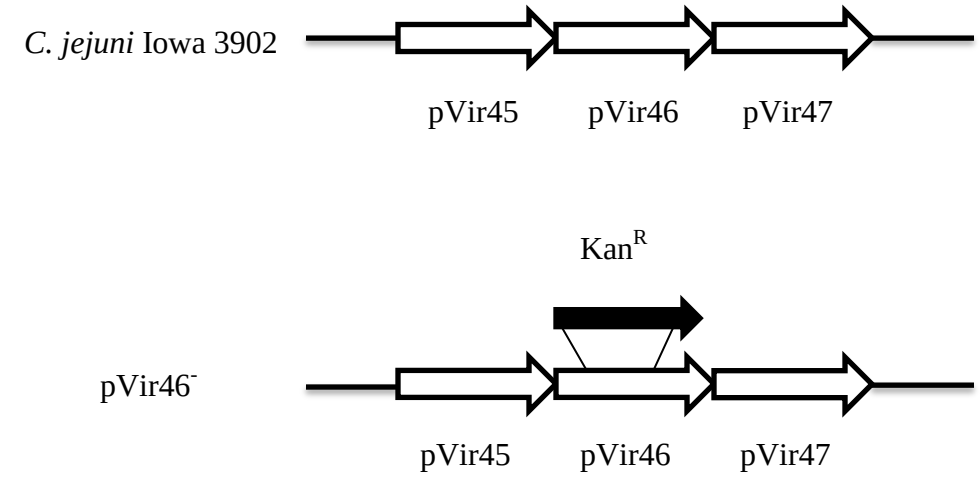
Primer	Primer Sequence*
pVir46f-F1	5'-TAAAGACAATATAAAACAGT-3'
pVir46f-R1	5'-CTATTTGATTGGATTATGTA-3'
pVir46inv-F1	5'-ATA**TTAATTAA**GCTAATTTAGGAAATCACAA-3' *(PacI)*
pVir46inv-R1	5'-ATA**GCTAGC**TTATATCGCAATGAAAAAGA-3' *(NheI)*
aphA-3kan-F	5'-ATA**GCTAGC**ATAGGCAGCGCGCTTATC-3' *(NheI)*
aphA-3kan-R	5'-ATA**TTAATTAA**CCACAATGATAGAACCAACGA-3' *(PacI)*
pVir45-F1	5'-ATA**GAATTC**AAGGAGTAAAAATGTTTGATTATT-3' *(EcoRI)*
pVir45-R1	5-CTT**TCTAGA**AAAGTAACGCTATATTTATTCTGCA-3' *(XbaI)*
pVir46-F1	5'-AGA**GAATTC**TAAGGCTTTAAATGCAGAATAA-3' *(EcoRI)*
pVir46-R1	5'-TAT**TCTAGA**TATATGTTTTTGTTATTTTTAGACT-3' *(XbaI)*
pVir45int-F	5'-AAAAAGCAGATCAAAGAAGA-3'
pVir46int-R	5'-TCCTTTTAGTGCGTGGTCT-3'
pVir45rt-F	5'-AAGCAGATCAAAGAAGACCTGAA-3'
pVir45rt-R	5'-AACGCTTCTTCTACATTGGCATA-3'
pVir46rt-F	5'-GAAAAATACAAAGACCACGCACT-3'
pVir46rt-R	5'-TGCGATAGACTAATAACAAATCAGG-3'
virB10-F	5'-CCGTCTAGATAGAATGATAAGAGCAGGAAG-3'
virB10-R	5'-TAGCTGCAGCAAACCACATATGATTGGTAG-3'

*The restriction sites are underlined and in bold in the primer sequences. The names of the restriction enzymes are in the parenthesis.

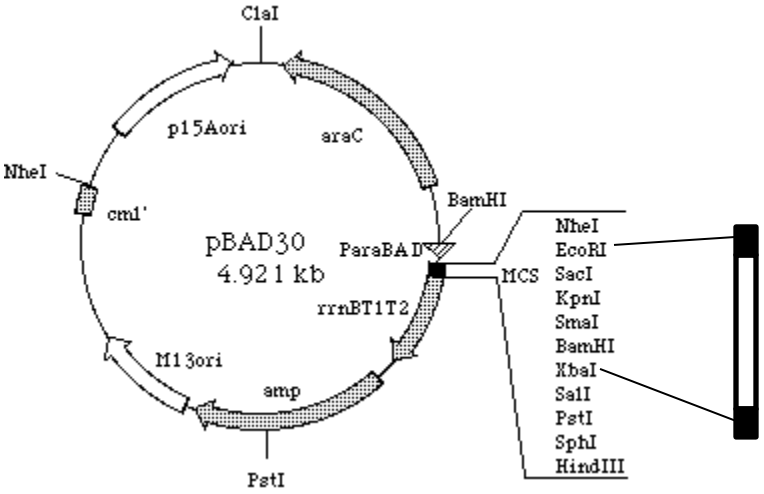
TABLE 3. Growth phase regulated expression of the TA system

Gene Identification	**Description**	**Growth time (Hours)**	**Fold change by Real Time PCR (RT-PCR)**
pVir45	RelB Antitoxin		
		6	-3.29
		9	-6.59
		12	-3.78
		24	-8.46
pVir46	RelE Addiction Module Toxin		
		6	-2.64
		9	-7.31
		12	-4.38
		24	-10.27

(A)



(B)



"pBAD30." *CVector*. Web. 4 Mar 2011. <http://gillnet.lab.nig.ac.jp/~cvector/map/pBAD30.gif>.

FIG. 1. Diagrams depicting the generation of the insertional mutation in *C. jejuni* IA3902 and expression of the TA system in in *E. coli*. (A) A schematic diagram showing the deletion of the pVir46 gene and the insertion of a kanamycin resistance gene in its location in creating the pVir46$^-$ mutant. The boxed arrows indicate the genes while the black arrow represents Kan^R. (B) A diagram showing the cloning and expression of the pVir45, pVir46, and pVir45-46 genes into the multiple cloning site of the pBAD30 plasmid. The restriction sites are shown in black, which are EcoRI and XbaI.

(A)

```
D3FPN5 MFDYSKYENATEKQLIHALTLAEKRAEKLNSQLKENNELFKFLQKKLKNSFNTKKTKKAD 61
A7H3J5 MFDYSKYENATEKQLIHALTLAEKRAEKLNSQLKENNELFKFLQKKLKNSFSTKKTKKAE 61
C5F1A5 MFDYAKYENATQKEIIHALNLTQRKSEKLNQQLKENREIFKFLQKKLKESFSSKKTKK-E 60
       ****:******:*::****.*::::::***.*****.*:**********:**.:***** :

D3FPN5 QRRPELDEAIEDYKNGNVEHYANVEEAFKALNAE 95
A7H3J5 QRRPELDEAIEDYKNGNVEHYANVEEAFKALSAE 95
C5F1A5 KRRPELDEAIRQYENGEVEHYSSVEEAFKALNAE 94
       :*********.:*:**:****:.********.**
```

(B)

```
A7H3J6 MQNKYSVTFSKRFKKDFKKINNNDKKILKKIVNKLANDEVLEEKYKDHALKGNYA----- 56
D3FPN6 MQNKYSVTFSKRFKKDFKKINNNDKKILKKIVNKLANDEVLEEKYKDHALKGNYIGFREC 61
C5F1A4 MQNKYSITFSKQFKKDFKKINKDDKIILKNIVDKLANDETLEAKYKDHALKGNYIGFREC 61
       ******:****:**********::** ***:**:******.** **********

A7H3J6 ------------QKTIKSI------------ 63
D3FPN6 HIKPDLLLVYRKNNDILELYLANLGNHNNIF 92
C5F1A4 HIKPDLLLIYRKRDDILELYLASLGNHNNIF 92
                  .. * .:
```

A7H3J – *Helicobacter pullorum* MIT 98-5489
C5F1A – *C. jejuni* subsp. *doylei*
D3FPN – *C. jejuni* IA 3902

FIG. 2. A multiple sequence alignment constructed by ClustalW to compare the amino acid identity of pVir45 and pVir46 genes of *C. jejuni* IA 3902 with *Helicobacter pullorum* MIT 98-5489 and *C. jejuni* subsp. *doylei* (strain ATCC BAA-1458 / RM4099 / 269.97). The asterisks indicate identical matches of amino acids and dots indicate conservation of similar amino acids below the multiple alignments. The multiple sequence alignments of the antitoxin region (A) and toxin region (B) of *C. jejuni* Iowa 3902 are compared to the other bacterial organisms.

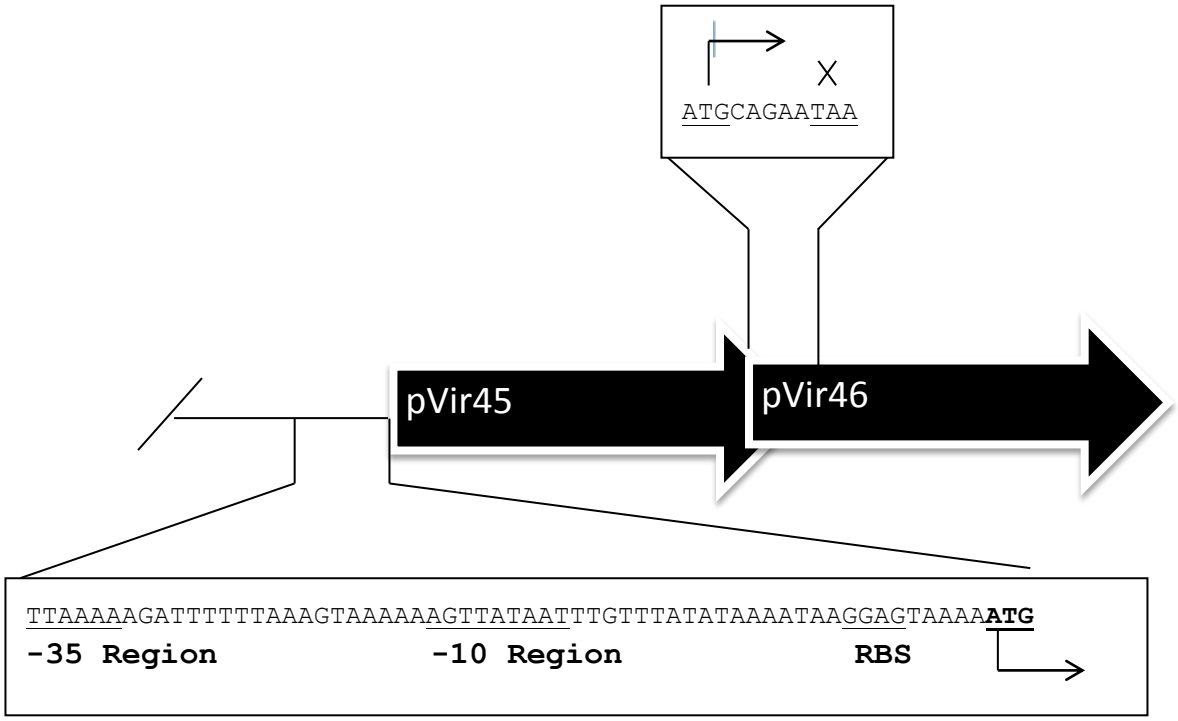


FIG. 3. Genomic organization and sequence features of the toxin-antitoxin system in *C. jejuni* IA3902. The promoter region and the ribosomal binding site are underlined. The two ORFs overlap and the start codon of pVir46 is 5 nucleotides upstream of the stop codon of pVir45.

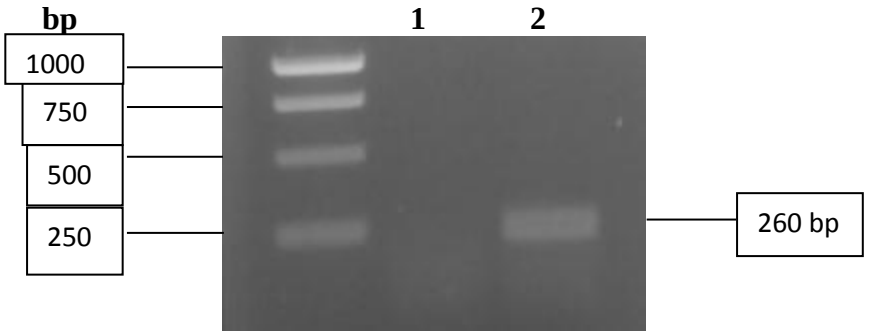


Fig. 4. Expression operon of pVir45 and pVir46 in *C. jejuni* IA3902 as determined by RT-PCR. Primers are designed to amplify a region of 260 bp that spans both genes. Lane 1 is the negative control reaction without the RT step and lane 2 is the reaction with RT.

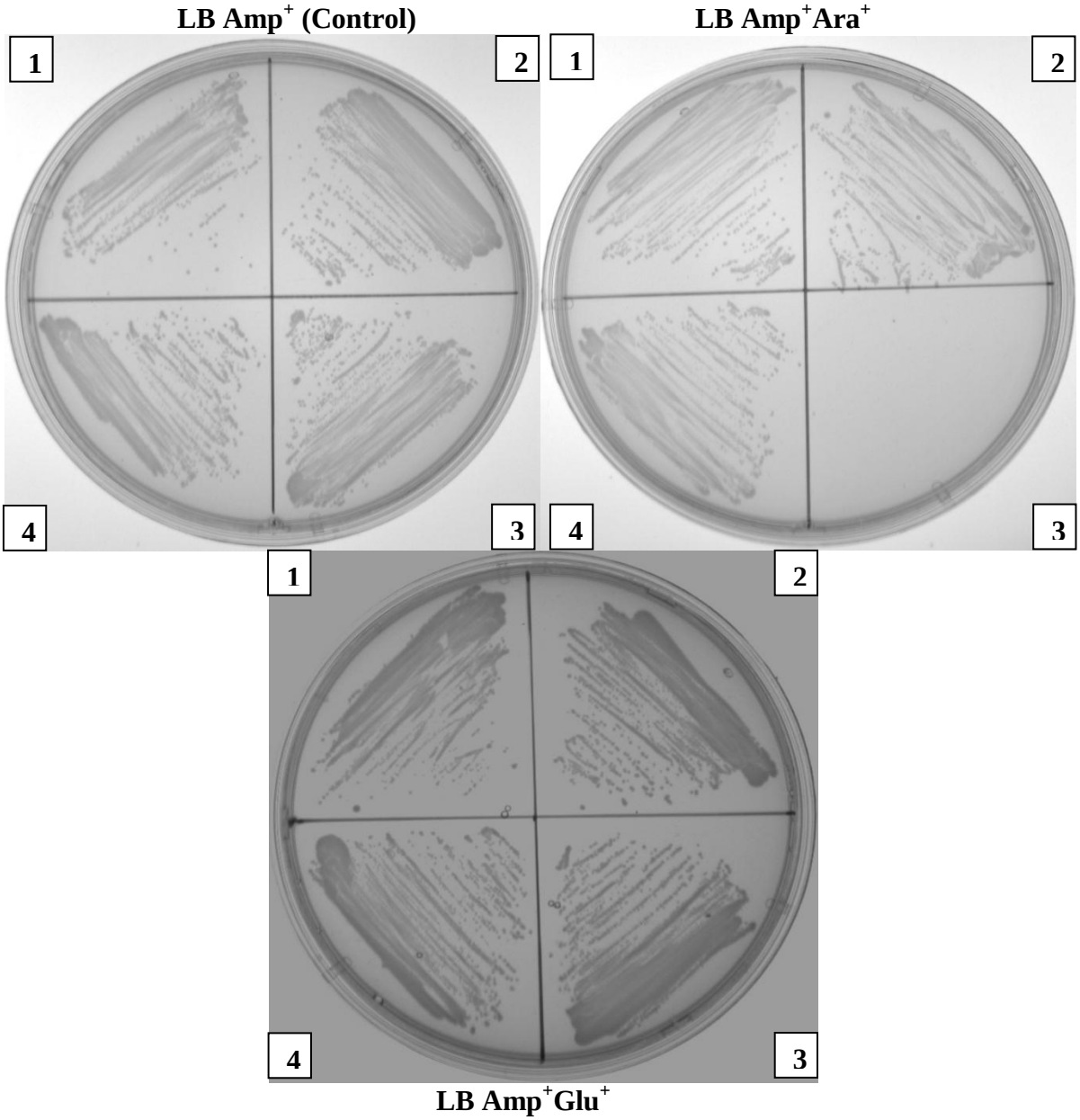
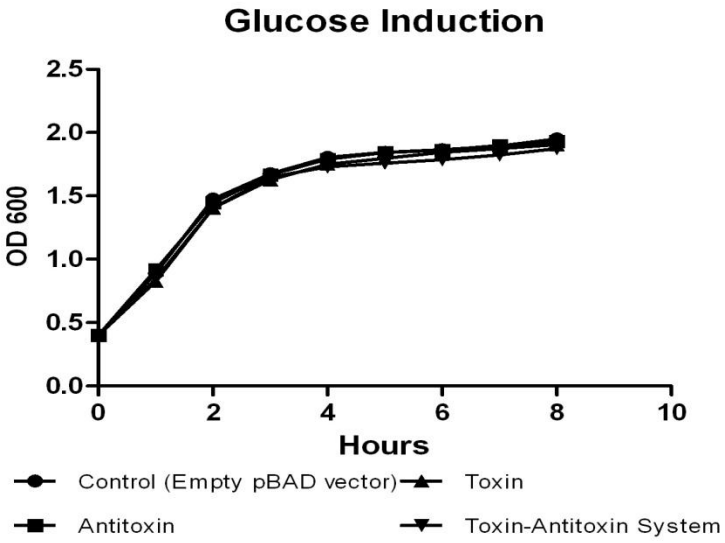


FIG. 5. Growth of *E. coli* DH5α constructs containing an empty vector (1), the antitoxin (2), the toxin (3), and the TA system (4) on LB plates with ampicillin (Amp^+), ampicillin plus arabinose (Amp^+Ara^+), or ampicillin plus glucose (Amp^+Glu^+).

(A)



(B)

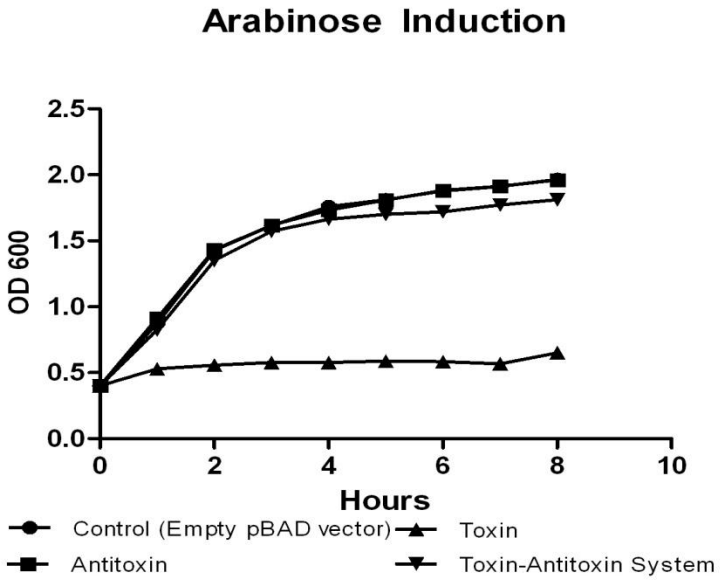


Fig. 6. Growth of various *E. coli* DH5α constructed containing an empty pBAD30 plasmid (control), cloned pVir45 (antitoxin), cloned pVir46 (toxin), and the entire TA system in LB broth added with (A) glucose or (B) arabinose.

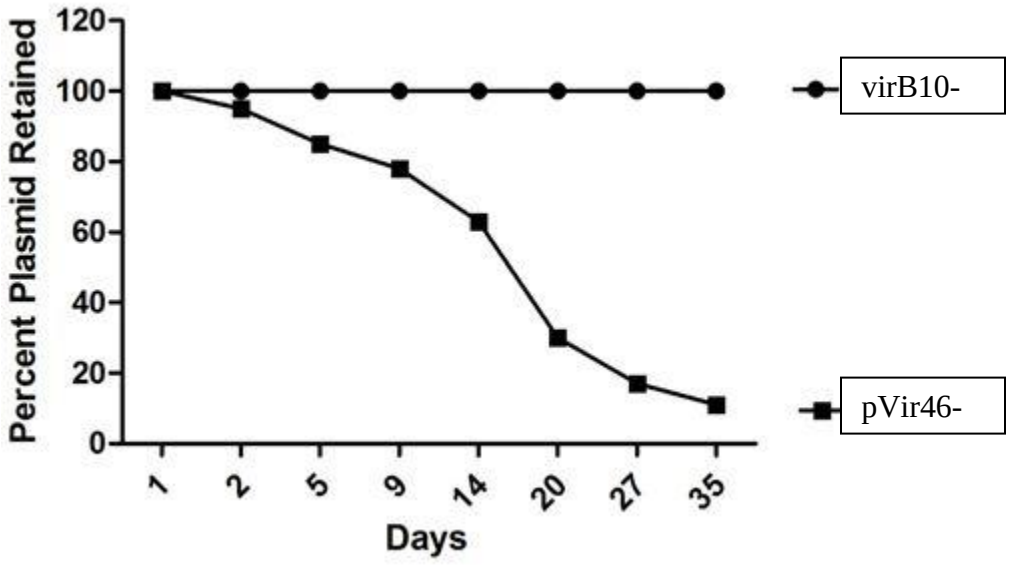


FIG. 7. Plasmid (pVir) stability assay as measured by passage in MH broth. This was done using *C. jejuni IA3902*, in which the pVir plasmid was inserted with an antibiotic resistance marker in either virB10 or pVir46.

CHAPTER 3. General Conclusion

In this experimental study, we identified and characterized a two-gene operon of pVir45 and pVir46 that comprises a TA stability system in the pVir plasmid of *C. jejuni* IA 3902. The genomic organization of pVir45 and pVir46 has a similar structure to TA systems identified in other bacterial organisms. The overlap of the two ORFs suggests coexpression of the gene pair and RT-PCR further confirmed this possibility. Expression of pVir46 alone in *E. coli* inhibited bacterial growth, but this inhibition was relieved when pVir-45 and pVir-46 genes were coexpressed in the same host. This finding indicates that the pVir46 gene encodes an addiction module toxin and pVir45 encodes an antitoxin that neutralizes the toxic effects of pVir46. We further showed that this TA system is necessary for pVir stability in *C. jejuni* because mutation of pVir46 resulted in the loss of the pVir plasmid from the *Campylobacter* strain. Real-time qT-PCR showed that the expression of the TA system is growth phase regulated and its gene expression is highest in the logarithmic growth phase.

These results establish that pVir45 and pVir46 form a functional TA in *C. jejuni*, which represents the first TA system identified in any *Campylobacter* species. The fact that it maintains the pVir plasmid, which is a known virulence factor in *Campylobacter*, suggests that this TA system contributes to the virulence and adaptation of this pathogenic organism in *C. jejuni* IA 3902. Future studies are needed to determine if additional TA systems are present in *Campylobacter*, how the pVir46 toxin produces its toxic effects, and how the expression of this TA system is regulated by growth phases. These studies will contribute to improved understanding of evolution of virulence and pathogenesis in *Campylobacter*.

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