TRANSCRIPTIONAL REGULATION OF MOBILIZATION AND TRANSFER OF THE *BACTEROIDES* CONJUGATIVE TRANSPOSON CTNDOT

BY

JILLIAN WATERS

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Microbiology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

Doctoral Committee:

Professor Abigail A. Salyers, Chair
Professor Peter Orlean
Associate Professor Joanna L. Shisler
Assistant Professor Carin K. Vanderpool

ABSTRACT

Bacteroides spp. are a primary inhabitant of the human colon, and play an important role in the health of the human host as *Bacteroides* are important for nutrient acquisition, the breakdown of starches that would otherwise go undigested, and the management of relevant pathogens such as *C. difficile.* Although generally considered a commensal, *Bacteroides* can become an opportunistic pathogen if it should escape the colon. *Bacteroides* is the most commonly isolated causative agent of anaerobic infections, and these infections are rather difficult to treat due to the prevalence of antibiotic resistance within this genus. The prevalence of resistance determinants in *Bacteroides* is often due to mobile genetic elements. One such element is a conjugative transposon (sometimes referred to as an integrative conjugative element, or ICE) called CTnDOT, which is a 65 kb ICE that encodes resistance to the antibiotics erythromycin and tetracycline. A notable feature of CTnDOT is that excision from the donor chromosome and conjugative transfer are coordinately regulated upon exposure of donor cells to tetracycline (Tc). While no transfer is detected in the absence of Tc, upon Tc induction a regulatory cascade ultimately stimulates synthesis of the excision proteins, which are required for excision of CTnDOT from the chromosome. These proteins also have a regulatory role, in that they are required for the transcriptional activation of the 13 kb *tra* operon that encodes the mating apparatus. The work presented in this dissertation has characterized a negative regulator, RteR, that appears to prevent conjugative transfer of CTnDOT in the absence of Tc by possibly initiating the formation of an intrinsic terminator within *traB,* thus truncating the transcript so there is no

substrate for translation, and hence no proteins are formed to assemble the mating apparatus. For the first time, we also describe the regulation of the CTnDOT mobilization region. These three genes that encode the relaxases and coupling protein required for mobilization are organized in an approximately 4 kb operon that is regulated upon Tc induction. The excision proteins Xis2d and Exc are required for enhancement of *mob* transcription upon exposure to Tc. This differs from the neighboring divergently transcribed *tra* operon, which does not require Exc, but also requires Xis2d and in addition Xis2c. A negative regulator is preventing *mob* transcription in the absence of Tc, and we currently predict that a gene encoded downstream of *intDOT, orf2,* is the *mob* transcriptional repressor. Taken together, the work described in this dissertation has further shed light on the intricate regulation governing the conjugative transfer of CTnDOT.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

This is an exciting time to be a microbiologist, as we are finally appreciating just how paramount microbes are to human health. We have known that microbes cause debilitating disease for quite some time, so it is not the sinister villains of the microbial world that I am referring to. Instead, I am referring to the microbes that have established themselves as commensal and even symbiotic residents on or within their human host. This consortium of indigenous microbes is collectively referred to as the human microbiome, as coined by the late Joshua Lederberg (85). The concept of a core group of microbes inhabiting humans is not entirely new, as it has been understood since the late 1970's that the human body contains 10 times as many microbial cells compared to human cells (101). However, it wasn't until the advancement of sequencing techniques that characterizing the human microbiome could develop to fruition, starting with the commencement of the human microbiome project a little over 10 years ago (85).

Now that methods have been established to characterize the native microbiota within individuals, there are an exciting number of studies looking at the various ways that the human microbiome shapes the health of the human host, especially with respect to the microbiota of the gastrointestinal tract. The colon is considered to be the most densely populated region with respect to the human microbiota. There are approximately 10^{13} to 10^{14} microbial cells in the colon (primarily bacteria) representing between 150-800 species. The main phyla associated with the gut microbiota are the Firmicutes, Bacteroidetes, Actinobacteria, and the Proteobacteria (12, 78).

Perturbations of the gut microbiome that alter the microbial composition are considered to cause a wide range of diseases. In fact, Antonie von Leeuwenhoek noticed after a bout of diarrhea that there were different "little animals" observed compared to his normal stool (29). With the advent of the human microbiome project, we are now understanding that the composition of the intestinal flora can influence the following diseases: obesity (52–54, 127), inflammatory bowel disease (5, 71, 87), type 1 and type 2 diabetes (37, 51, 72), allergies (16, 118), and colorectal cancer (60, 110, 132). This is likely just the beginning of the laundry list of diseases that are associated with perturbations of this rich microbial community.

Another concept that has emerged from the human microbiome is the concept of a hologenome. The hologenome, then considers our genome not only for what is encoded in the host DNA, but also incorporates the gene products of the microbiome, which are estimated to outnumber the host genetic material 100-fold (45, 90, 147). This concept of looking at the total genetic content of the microbiome, rather than individual organisms is greatly relevant, as the rapid horizontal gene transfer (HGT) between microbes in this dense environment provides a rather flexible gene pool (45, 50, 78).

The assortment of mobile genetic elements and bacteriophage that contribute to this flexible gene pool within the gut are referred to as the "mobile metagenome". Although the role of the mobile metagenome within the gut has yet to be extensively explored, some findings are suggesting that perhaps the mobile metagenome may confer advantages for the survival and persistence of microbes within the gut, as well as influencing host-microbe interactions (45, 78).

Many mobile genetic elements are associated with resistance to a wide range of antibiotics (2, 74, 122). Although antibiotics are considered to disrupt the composition of the gut microbiome, an interesting observation has been made where erythromycin appears to cause no detectable disruption (45). The most likely explanation for this observation is that conjugative elements such as conjugative transposons are responsible. The persistence of erythromycin resistance, as well as tetracycline resistance in *Bacteroides* has been well documented for quite some time. Sequence analysis of various tetracycline resistance encoding genes (*tet*) from diverse organisms has demonstrated a very high degree of similarity, again suggesting extensive HGT. Shoemaker *et al* demonstrated that approximately 80% of *Bacteroides* isolates are resistant to tetracycline, and that this resistance is linked to conjugative transposons (105).

So what does the human gut microbiota have to do with transcriptional regulation of CTnDOT? As stated previously, *Bacteroides* is one of the more abundant genera of the human colon. This genus of bacteria is widely recognized as being host to a wealth of mobile elements (109). Further, evidence has demonstrated that one of the more prominent elements, CTnDOT, has an effect on the genes encoded on the *Bacteroides* chromosome (68). My gut instinct (yes, that pun was intended) is that in the future we will find that mobile elements such as CTnDOT shape the interactions of *Bacteroides* with both the host and other members of the intestinal microbiota. For now though, I will tell you a story in this dissertation of how we have come to better understand the transcriptional regulation of CTnDOT genes that govern conjugative transfer of this element. In order to better understand this story, in this

first chapter I will provide you with a basic understanding of *Bacteroides,* followed by a description of mobile genetic elements within this genus before delving in to more specifics with respect to CTnDOT. I will end this introduction with a brief explanation of the body of work I will describe in later chapters of this dissertation.

1.1 Clinical significance of *Bacteroides* spp.

Bacteroides spp. are Gram-negative obligate anaerobes that primarily colonize the human gastrointestinal tract, and are one of the more abundant members of the bacterial community within the colon. Previous cultivation-based studies had identified *Bacteroides* as abundant, suggesting that *Bacteroides* accounted for roughly 25-30% of the microbiota (69, 93, 101). However, more recent sequence-based studies have demonstrated that the Bacteroidetes phylum can account for upwards of 40% of the human gut microbiome (6, 27, 32).

The abundance of *Bacteroides* within the gut community appears to be very important to human health, as the proportion of Bacteroidetes within the colon has been implicated in relevant diseases such as obesity (12, 49, 52–54, 126, 127) and type 1 diabetes (72). Although the research on diabetes is still very new, for both obesity and diabetes having a decreased level of *Bacteroides* relative to the *Firmicutes* is correlated with these diseases. The abundance of *Bacteroides* is also being investigated as a factor that may contribute to inflammatory bowel disease (IBD), which includes the two main subtypes Crohn's disease and ulcerative colitis. IBD is a result of immune system intolerance to the gut microflora, which manifests as a chronic inflammation of the gastrointestinal tract. Associated with IBD is also a

dysbiosis of the bacterial community in the GI tract, which may be a result of decreased metabolic functions of the microbiome (33, 65, 71). Other studies are starting to tease out how the relative levels of the dominant gut genera change with respect to Crohn's disease, a subtype of IBD. Although the studies looking at Crohn's specifically are still in the beginning phases, some are seeing that there is a loss of diversity within the microbiome, and there is perhaps an increase in *Bacteroides* associated with Crohn's disease (5, 84, 87). Although more research is required to elucidate the role of *Bacteroides* in IBD, it is evident that the presence of this genus within the gut is significant.

Bacteroides is also important for the acquisition of nutrients for the host, by aiding in the biosynthesis of vitamins and breaking down complex polysaccharides that would otherwise go undigested by the human host (43, 59, 145, 148). We can attribute much of this role to the fact that *Bacteroides* actually has the largest glycobiome among any of the sequenced bacteria, having over 200 gene products involved in the hydrolysis of glycosidic bonds (12, 98). In fact, the short chain fatty acids produced by carbohydrate metabolism are also a significant source of caloric energy for the host (137). The role of *Bacteroides* with respect to nutrient acquisition is so paramount that malnutrition can result if there is a deficit of *Bacteroides* in the colon (79).

The benefits do not stop there, as *Bacteroides* spp. are also medically important in that they can aid the host by preventing the establishment of relevant pathogens such as *Clostridium difficile* and *Helicobacter hepaticus* (65, 92, 124, 137). In addition, the presence of *Bacteroides* in the colon may also protect against

autoimmune central nervous system (CNS) demyelinating disease, such as multiple sclerosis (76, 77).

Despite the presence of *Bacteroides* in the lower gastrointestinal tract offering a wealth of advantages, *Bacteroides* can also have a more deleterious effect on the health of the host, occurring if *Bacteroides* somehow escapes the confines of the colon. This can occur through disease, but most commonly results when the lining of the colon has been compromised due to surgical trauma (137). When *Bacteroides* escapes the colon, opportunistic infections arise, some of which can be life threatening. The most common infection is intra-abdominal sepsis, but other types of infection include appendicitis, and necrotizing soft tissue infections. *Bacteroides* spp. are actually the most commonly isolated organism from anaerobic infections. Although rare, other possible infections due to *Bacteroides* include endocarditis, meningitis, and septic arthritis (137). These infections are becoming increasingly difficult to treat due to the prevalence of antibiotic resistance among members of this genus, which makes the risk of a *Bacteroides* infection a serious threat (74). For example, *Bacteroides* spp. are naturally resistant to aminoglycosides because they are lacking an oxygen dependent electron transport chain, and are also resistant to the β-lactam antibiotics due to a chromosomally encoded β-lactamase (11, 19, 89). However, much of the antibiotic resistance within *Bacteroides* is acquired through mobile genetic elements that carry the genes encoding resistance to various antibiotics, such as the macrolide-lincomide-streptogramin (MLS) group of antibiotics and the broad-spectrum tetracycline group (88).

1.2 Mobile genetic elements

Mobile genetic elements are segments of DNA that are most often not required for the life cycle of the host bacterium, but serve as a "flexible gene pool" that confers advantageous traits such as virulence or antibiotic resistance by transferring from one organism to another (121). Further compounding the risk associated with this flexible gene pool within the human colon, is that bacteria within this environment have a 25-fold increase in the likelihood for genetic exchange compared to either terrestrial or aquatic environments (108). This elevated transfer of genetic material is quite frightening with respect to antibiotic resistance. *Bacteroides* possesses multiple elements such as plasmids, transposons, conjugative transposon, and bacteriophage. With the exception of bacteriophage, all of these mobile elements have demonstrated an important role in the transmission of antibiotic resistance within *Bacteroides* (26, 130, 137).

Plasmids

Plasmids function as extra-chromosomal elements that replicate autonomously, often having multiple copies per cell (113). Generally, most plasmids do not encode functions that are essential to the bacterial host, but rather present a phenotype that confers some sort of beneficial trait such as virulence factors or antibiotic resistance (21). *Bacteroides* has a number of plasmids ranging in size from 2.7 to >80 kb that are present in approximately 20-50 % of strains (73, 74, 117, 130).

There are a number of prevalent low molecular weight plasmids in *Bacteroides* that can be classified in to three major groups by size: 2.7, 4.2, and 5.6 kb,

which respectively correlate to classes I through III (13, 112). These plasmids appear to be geographically distributed and are frequently found in clinical isolates. However, these low weight plasmids are cryptic, meaning there is no phenotype associated with these elements (111, 112, 117). One reason these plasmids may be so prevalent is that these cryptic elements all possess an *oriT*, rendering them capable of transfer when they are co-resident with another element such as a conjugative plasmid or conjugative transposon (109).

Many plasmids in *Bacteroides* are associated with antibiotic resistance phenotypes. The first of these plasmids described in *Bacteroides* were those encoding resistance to the MLS class of antibiotics. These plasmids, pBF4, pBFTM10, and pBI156 range in size from 15-80 kb, but what they have in common is that the MLS resistance gene *ermF* is present on all three (109). For each of these plasmids the *ermF* region originated from the insertion of a composite transposon containing IS4351 (138). Further aiding in their spread throughout this genus is that pBFTM10 is mobilizable, and pBF4 and pBI156 are both capable of self transfer (70, 109).

Another group of commonly isolated plasmids among *Bacteroides* are those encoding metronidazole resistance, with the resistance genes *nimA-F*. The best characterized of these plasmids are pIP417, pIP419, and pIP421. These plasmids, such as the others described thus far, are also capable of conjugative transfer. Another feature of interest with these plasmids is that the transcription of the *nim* genes is induced by an insertion sequence (IS) element (41). The spread of these plasmids is worrisome because metronidazole was a key antibiotic used for the treatment of *Bacteroides* infections because it was one of the few antibiotics to which

this genus was susceptible. Transmission appears to be occurring frequently in nature since metronidazole resistant strains are often detected from clinical samples (86, 123, 137).

Conjugative transposons

Conjugative transposons (CTns) are mobile elements that normally reside within the chromosome, and account for the majority of antibiotic resistance transfer within *Bacteroides* (83, 139). CTns range in size between 18 and 500 kb in size, while the majority of CTns average roughly 50 to 80 kb in size. These elements are rather large because they encode the entire suite of gene products necessary to facilitate conjugative transfer (104, 140). Interestingly, these elements contain remnants of transposons, plasmids, and bacteriophage, and thus behave in many ways like each of these other mobile elements. CTns are transposon-like in that they are maintained within the chromosome, yet can excise from the chromosome and integrate elsewhere. However, the mechanism of excision and integration more closely resembles that of bacteriophage rather than classic transposons. In fact, the integrase and excision proteins themselves are quite similar to those from bacteriophage. Lastly, conjugative transposons are plasmid-like in that they form a covalently closed circular intermediate; however, unlike plasmids CTns do not replicate autonomously (96).

The first conjugative transposons identified were in the Gram-positive bacteria, with Tn916 from *Enterococcus faecalis* and Tn5253 from *Streptococcus pneumoniae* (which is Tn916-like) being the first discovered (9, 34, 106, 140). Soon

after the discovery of Tn916, many other Tn916-like elements were described in *E. faecalis* and other *Streptococcus* spp. such as *S. pyogenes* and *S. agalactia.* These Tn916-like elements encode resistance to tetracycline, which is the most common resistance marker carried on conjugative transposons (109). The first conjugative transposon described outside of the Gram-positive organisms is the *Bacteroides* conjugative CTnDOT, which will be described in further detail below (35).

Another notable feature of conjugative transposons is their ability to mobilize other elements. For example, many co-resident plasmids may be mobilized when a conjugative transposon is in *trans,* as the CTn provides the gene products encoding the mating apparatus and mobilization proteins. However, the *Bacteroides* CTns are unique in that they can mobilize elements when in *cis,* which has not been observed in other genera. This observation suggests that the *Bacteroides* CTns have a greater capacity to mobilize other elements (95).

In order for transfer of the conjugative transposon to occur, the element first excises from the chromosome to form a circular intermediate. A single stranded copy is then transferred through the mating pore to a recipient cell, after which the copy becomes double stranded before integrating into the chromosome of the recipient (140). It is important to note that a copy of the CTn is retained in both the donor and recipient cells (Figure 1.1). Because the element resides within the chromosome, it can also be transferred vertically to progeny cells, in addition to the CTn being horizontally transferred. This is important, because in the case of antibiotic resistance determinants being present on CTns, these elements are not only transferred readily but are also very stably maintained. This is a great cause for

concern, as this is leading to a prevalence of antibiotic resistance with *Bacteroides*, which can result in great difficulty when treating infections. Further, it is possible that *Bacteroides* can act as a reservoir of antibiotic resistance determinants by disseminating these genes to other organisms even outside of the *Bacteroides* genus and possibly transferring these elements to organisms that are transiently passing through the gut (11, 91, 139). All in all, the spread of these conjugative transposons may pose a serious threat to the future ability to treat infections with antibiotics. For example, tetracycline used to be a frontline antibiotic used for the treatment of *Bacteroides* infections due to its broad spectrum, ease of use, low incidence of side effects, and relatively low cost (115). After the debut of tetracycline in the 1950's, few isolates were resistant. By the 1970's 20-30% of isolates were tetracycline resistant, and by the 1990's over 80% of *Bacteroides* isolates from clinical and community samples were resistant to tetracycline (97). In fact, resistance to tetracycline has become so commonplace that strains are often not tested for susceptibility, and are rather assumed to be resistant (94, 105). Of the strains that were resistant to tetracycline, every strain tested carried a conjugative transposon (96).

1.3 The *Bacteroides* conjugative transposon CTnDOT

As the title of this dissertation implies, the goal of my work has been to further understand the transcriptional regulation that ultimately governs conjugative transfer of CTnDOT. The focus for the rest of this chapter will be to better understand the nuances of CTnDOT. I will first provide a general overview of

CTnDOT, and an introduction to the regulatory cascade that initiates transfer, which is immediately followed by a more detailed explanation of the genes involved in this cascade. I will then outline the regulatory targets that constitute the bulk of my work outlined in this dissertation: the transfer and mobilization regions of CTnDOT. I will then describe the negative regulation of CTnDOT transfer, and I will finish by outlining the specific questions posed in this dissertation that aim to better understand the regulation of CTnDOT.

The most common conjugative transposon within *Bacteroides* is CTnDOT, in addition to a number of CTnDOT-like elements (105). CTnDOT is a 65 kb conjugative transposon that carries genes encoding resistance to the antibiotics erythromycin (*ermF*) and tetracycline (*tetQ*). One of the characteristics that makes CTnDOT such a fascinating element is that exposure to low levels of tetracycline actually stimulates the excision and conjugative transfer of CTnDOT (23, 44, 67, 114, 141). Upon tetracycline induction a cascade of transcriptional regulation is initiated, and translation of the *tetQ-rteA-rteB* operon ensues. RteB next activates the transcription of *rteC,* which in turn activates transcription of the *xis2c-2d-exc* operon. Xis2c, Xis2D, and Exc activate transcription of the *tra* and *mob* operons, which then results in CTnDOT conjugative transfer. However, when no tetracycline is present there is no detectable CTnDOT transfer. A summary of this regulatory cascade is depicted in Figure 1.2. A more detailed description of the genes involved and the steps in this regulatory pathway is found below.

An operon that encodes the ribosomal protection gene that confers resistance to tetracycline, *tetQ-rteA-rteB,* is regulated by tetracycline induction through a

translational attenuation mechanism. That is, although the *tetQ* operon is transcribed constitutively, it is only upon exposure to tetracycline that translation occurs (75, 115, 133, 134). Homologue searches suggest that RteA and RteB comprise a two component regulatory system, where RteA is the signal recognition protein and RteB is the response regulator (116). At this point it is not yet clear what signal RteA is sensing, but findings suggest that it is not tetracycline (96). Upon Tc induction, RteB activates the transcription of *rteC,* encoding another transcriptional activator (67, 81, 133). Together, *tetQ-rteA-rteB-rteC* comprises what is often referred to as the central regulatory region of CTnDOT.

To continue the regulatory cascade upon tetracycline induction, RteC then activates the transcription of the excision operon, which contains x*is2c, xis2d, orf3,* and *exc* (67, 81, 141). As the name of the operon suggests, these genes are involved in the excision of CTnDOT from the donor chromosome. Both Xis2c and Xis2d (formerly known as Orf2c and Orf2d) are small basic DNA-binding proteins, with Xis2c having homology to phage Lambda xis (119). Xis2d is homologous to the *E. coli* excisionase TorI, and to the Tn916 excisionase (1, 31, 48). Exc, which has homology to a class III DNA topoisomerase, is also required for CTnDOT excision (24, 47, 120). Although *orf3* is a part of this operon that encodes proteins necessary for excision, Orf3 is not required (24). The CTnDOT encoded tyrosine recombinase IntDOT is also required for excision, in addition to a chromosomally encoded *Bacteroides* host factor, which is typical of most transposition systems (47, 57, 104, 143). Interestingly, Xis2C, Xis2D, and Exc are not only essential for the excision of CTnDOT, but they also play a regulatory role by acting as transcriptional activators of the *tra*

and *mob* operons. As with excision, Orf3 does not appear to play a role in the transcriptional activation of the *tra* operon.

The transfer genes

The transfer (*tra*) genes are assembled in a 13 kb operon containing 17 protein encoding genes, *traA* through *traQ*. These Tra proteins are required to assemble the mating bridge, a type IV secretion like apparatus that is required for conjugative transfer. These *tra* genes have a much higher GC content (48-60%) relative to the *Bacteroides* chromosome (approx. 42%), which suggests that the *tra* genes originated from another genus of bacteria (17). However, only 4 of the 17 *tra* genes are homologous to characterized genes from other transfer systems. The CTnDOT protein TraG was the best match to any other known transfer proteins. The most similar homologue is the *Bacteroides* protein BctA (49% identity), a membrane bound putative ATPase which is present on the conjugative plasmid pBF4 (70) and the *B. uniformis* conjugative transposon BTF-37 (42). Western analysis of $TraG_{CTnDOT}$ has demonstrated that it is associated with both inner and outer membrane fractions, which is consistent with the involvement of TraG in formation of the mating pair (18). TraG is also homologous to the VirB4 ATPase from the *Agrobacterium* Ti plasmid (15, 28), in addition to TraC from the *E. coli* F plasmid, which is required for assembly of the F pilus (102, 103).

TraK from CTnDOT appears to be very distantly related to VirB8 and TrbL. VirB8 is an inner membrane Ti plasmid protein that is thought to function as a nucleating factor in assembly of the type IV secretion system complex (107).

Although the function of TrbL is not known, it has been demonstrated that TrbL is essential for Ti plasmid transfer, RP4 plasmid transfer, as well as transfer of the *R. leguminosarum* plasmid pRL1JI (40, 56, 142).

The CTnDOT protein TraJ shares very limited similarity with other known conjugation proteins such as the F plasmid protein TraG, which is an inner membrane protein thought to play a role in stabilization or interaction of the relaxosome with the DNA transport complex (7, 58, 99). Analysis of the TraJ sequence also reveals a Bac_flav_CT_J superfamily conserved domain, which is distantly related to Virb6 and TrbL of the *Agrobacterium* Ti plasmid (63). VirB6 is predicted to play a role in the biogenesis of the T-pilus (46), where as TrbL, a homologue of the F plasmid protein TraG, is considered likely to play a role in stabilization of the mating pair and was demonstrated to be required for conjugative transfer of the Ti plasmid (56, 63).

The last of the CTnDOT *tra* proteins with known transfer homologues is TraN, which has been annotated as a protein involved in stabilization of mating pair formation (61–63). TraN shares limited similarity with the Ti protein VirB9, which aids in stabilization of the mating pair (14). Western blotting analysis of $TraN_{CTnDOT}$ has demonstrated that this protein is associated with the outer membrane, which is consistent with TraN being a component of the mating bridge (18).

Although no studies have been performed on the CTnDOT Tra proteins to confirm a specific role in conjugative transfer, some work has been done to determine which of the Tra proteins are essential for CTnDOT conjugative transfer. Insertion deletion analysis was performed in CTnERL, a *Bacteroides* conjugative

transposon that for the most part is similar to CTnDOT but lacks the *ermF* region, allowing erythromycin to be used as a selectable marker for the insertions. The insertions contained a maltose inducible promoter (P_{susA}) placed downstream of *ermF,* thereby preventing the possibility of polar effects from the insertion (18). It should also be noted that although the majority of the *tra* genes have greater than 90% identity, the 5' end of *traA* through the 3' end of *traD* are poorly conserved, with sequence identity less than 35% (17). Bonheyo *et al* (2001) focused on the conserved region downstream of *traD* for the insertion analysis. No insertions were generated in *traC, -E, -F,* or *traL* because these open reading frame were too small to construct reliable insertions. They were also unsuccessful in constructing an insertion in *traK.* All insertion mutants, *traD, -G, -H, -I, -J, -M, -N, -O, -P, -Q,* were tested for the ability to transfer CTnERL to an *E. coli* recipient. The wild type transfer frequency of CTnERL is approximately 10^{-5} to 10^{-6} transconjugants per recipient, and in the cases of *traG, -I, -J,* and *traM* the insertions were sufficient to abolish CTnERL transfer, which suggested these proteins were essential for conjugative transfer. Mutations in *traH* and *traN* resulted in a reduction of CTnERL transfer frequency by two orders of magnitude to a rate of about 10^{-8} transconjugants/recipient. A surprising finding from this study was that some insertions actually resulted in an increase of transfer activity. Disruption of *traO* resulted in an increase to 10^{-4}, while insertions in *traP* and *traQ* gave an increase to 10^{-3} transconjugants per recipient. Unfortunately, sequence analysis of TraO and TraQ does not reveal a putative function for these proteins, which makes it difficult to predict what role they play in conjugation. TraP does have a domain that falls within the DnaG DNA primase

superfamily, but it is only for the N terminus of TraP. Studies from a CTnDOT-like conjugative transposon CTn341 also determined that $TraP_{341}$ was not required for CTn341 transfer, yet $TraP_{341}$ was required for the mobilization of an unlinked plasmid (10). It should also be noted that with every disruption the ability of CTnERL to excise from the chromosome was assayed to ensure that faulty excision was not to blame for changes in the CTnERL transfer frequency, and was instead a result of disruption of the mating apparatus or mating pair formation (18).

As stated previously, the regulatory cascade that initiates with tetracycline induction ultimately results in the upregulation of a number of genes. The excision proteins Xis2c, Xis2d, and Exc not only increase transcription of the *tra* operon, but they also appear to enhance transcription of the *mob* genes.

The mobilization genes

The mobilization (*mob*) genes are a nearly 4 kb operon containing 3 genes, *mobA, -B,* and *-,C* that facilitate CTnDOT conjugative transfer, and are divergently transcribed from the *tra* operon. There are approximately 80 bp between the two promoters, prompting us to hypothesize that the binding of the excision proteins to mediate transcriptional activation of the *tra* operon might also affect the regulation of the divergently transcribed *mob* genes. Cloning of the *mob* genes, along with the CTnDOT *tra* operon and *oriT* onto a vector was sufficient to form a plasmid (pLYL72) that was capable of self-transfer, suggesting that this region comprises the bulk of the transfer machinery (55).

Blastp searches of MobA do not reveal any homologues of known transfer proteins (3). MobA is only annotated as a hypothetical protein, but it does appear to be well conserved throughout the Bacteroidetes phylum. A structural homologue search revealed more insight as to how MobA may function in transfer. Phyre^2 analysis suggested with 94.1 % confidence that the N terminal fragment of MobA is structurally homologous to the NikA protein from the plasmid R64. NikA forms a relaxation complex with another protein, NikB, that together nick the R64 OriT and initiate conjugative transfer (36, 48, 146). The ribbon-helix-helix motif in MobA is part of a family that is found in conjugative systems that participates in nicking reactions by facilitating access of the relaxase to the nick site, the *oriT* (129). No function for the C terminus of MobA was predicted, as Phyre^2 only generated structural homologues with a poor confidence score for this region (<27%).

MobB is also well conserved within the Bacteroidetes phylum. Although it is simply annotated as a putative mobilization protein, MobB does possess a relaxase domain (61–63). A Phyre^2 structural homologue search reveals homology of the N terminus to the relaxase MobA (98.9% confidence) from the *P. aeruginosa* mobilizable plasmid R1162. The N terminus of $MobA_{R1162}$ nicks the plasmid DNA, and is required for transfer of R1162 (66, 82, 144). No function for the C terminus of MobB was predicted, as Phyre^2 structural homologues for this region had less than a 15% confidence score.

MobC is also conserved in the Bacteroidetes phylum, and is annotated as a TraG/VirD4 superfamily protein that contains an ATP binding domain. VirD4 is a cytoplasmic ATPase that aids in delivery of DNA to the mating pore and is required

for conjugative transfer (25, 131). Phyre[2] structural homologue analysis supports this. With 100% confidence, MobC is predicted to be homologous to the ATPase domain of RecA. MobC is also a structural homologue of FtsK (98.5% confidence), which functions to translocate DNA through the division septum during cell division using ATP hydrolysis (8, 48).

Recent work from C. Jeff Smith's lab has begun to characterize the *mob* region of a CTnDOT-like conjugative transposon CTn341, which is 93% identical at the nucleotide level to the CTnDOT *mob* region (4). Null mutations were made in all three of the *mob* genes, and in the case of $mobA_{341}$ and $mobB_{341}$, transfer of CTn341 was lost. What was interesting, however, was that the transfer of co-resident elements was not affected by these null mutations. Even more surprising was that a null mutation in $mobC_{341}$ had no affect on the transfer frequency of CTn341. This finding was not expected, as the coupling protein is generally required in other type IV secretion systems. Three independent $mobC_{341}$ deletions had the same phenotype in that there was no defect in CTn341 transfer. The *B. thetaiotaomicron* chromosome does have four *mobC* homologues, so it is possible that one of the chromosomal copies is compensating for the CTn341 *mobC* deletion, thus resulting in wild type conjugative transfer (83).

Peed *et al* (2010) also began a preliminary characterization of the transcriptional regulation of the CTn341 *mob* region. The *mob* genes, which are divergently transcribed from the *tra* region, are organized in an operon that is induced by exposure to tetracycline and requires RteA and RteB (83). As I will

describe later in this dissertation, their findings are only the tip of the iceberg with respect to the regulation of this operon.

Negative regulation of CTnDOT conjugative transfer

As stated previously, the cascade of events that is initiated upon tetracycline induction is complex and involves many operons. However, tetracycline induction does not merely act as a "molecular switch" to turn on this regulatory cascade that activates conjugative transfer of CTnDOT. Instead, the regulation of CTnDOT has another layer of complexity. The operons encoding the gene products necessary to facilitate conjugative transfer, namely the transfer (*tra*) operon and the mobilization (*mob*) operon, are actually transcribed constitutively with respect to tetracycline induction yet become regulated when in the presence of the entire CTnDOT element (118; J.L. Waters, unpublished). For the *tra* operon, the negative regulator (RteR) is actually transcribed constitutively, so repression is somehow overridden upon tetracycline induction. We are still elucidating how the *mob* genes are down-regulated in the absence of tetracycline.

The negative regulator that targets the transfer (*tra*) operon is a non-coding small RNA, RteR, which will be discussed further in Chapter 2 of this dissertation (135). Small RNAs are gaining much recognition due to their important role as global regulators within bacteria, regulating important cellular processes such as iron homeostasis, stationary phase transition, and sugar phosphate stress (38, 64, 128). These small RNAs are most often around 50-100 nt in length, and although there are exceptions, the majority of these regulatory small RNAs are non-coding

(39). Not only can sRNAs mediate positive and negative regulation, but also there are a wide variety of sRNA regulatory mechanisms.

The majority of sRNAs that have been characterized act as negative regulators. One method of regulation is when the sRNA acts to destabilize the target mRNA, which results in a shorter mRNA half-life (39). This regulation can occur when the sRNA binds to a target mRNA to form an imperfect base-pairing between the two transcripts, which is generally mediated by the hexameric RNA chaperone Hfq (30, 80, 100, 136). Ultimately the sRNA-target mRNA duplex is degraded via the degradosome, which in *E. coli* consists of RNase E, PNPase, enolase, and the RNA dependent helicase RhlB (20, 22, 125). However, we have reason to believe that RteR does not work through an Hfq dependent mechanism. First, no Hfq homologue has been identified in the Bacteroidetes phylum. In addition, if there is a *Bacteroides* equivalent of the degradosome, it may differ from the degradosome described in *E. coli* because there does not appear to be an RNase E homologue in *Bacteroides*.

As stated earlier, the *mob* genes are also regulated in a similar fashion to the *tra* operon. The *mob* operon is constitutively transcribed when independent of CTnDOT. However, when an intact copy of CTnDOT is also present, there is no detectable *mob* product without tetracycline present, and upon tetracycline induction there is more *mob* mRNA relative to the constitutively transcribed levels. As I will illustrate in Chapter 3, it appears that the excision proteins Xis2c, Xis2d, and Exc are responsible for the up-regulation of *mob* transcription upon tetracycline induction. At this time, we are not confident of what is acting as the negative regulator of the *mob* region, but it appears that RteR is not responsible.

1.4 Questions addressed in this dissertation

The overarching question posed in this dissertation is: what factors are regulating the transcription of genes required for conjugative transfer of CTnDOT? As stated previously, we know that ultimately conjugative transfer is not detected in the absence of tetracycline, but upon exposure to tetracycline transfer of CTnDOT occurs. A fair amount of work has been done to characterize the transfer operon, which is a 13 kb operon containing genes *traA*-*traQ.* The culmination of these studies has demonstrated that the *tra* promoter is constitutively transcribed, and that the excision proteins Xis2c, Xis2d, and Exc are responsible for the up-regulation of *tra* transcription. However, as stated previously, when the entire CTnDOT element is present the *tra* operon is not transcribed in the absence of tetracycline. Preliminary evidence suggested a 500 bp region that contained a putative regulatory RNA was sufficient to mediate this inhibition (44, 141). As I will describe in Chapter 2 of this dissertation, my first objective was to confirm that this was in fact a small RNA that was inhibiting CTnDOT conjugative transfer. I also characterized RteR by identifying the 5' and 3' ends of the transcript, in addition to locating the *rteR* promoter. The next objective was to address how RteR was inhibiting transfer by identifying not only the actual target of RteR, but also by proposing a possible mechanism describing how RteR may be interacting with the target to prevent conjugative transfer of CTnDOT. The summation of this portion of my work suggests that RteR may be interacting with a segment of the polycistronic transcript within *traB* to initiate the formation of an intrinsic terminator, thus halting the elongation of the *tra* mRNA.

Another story that I will describe further in Chapter 3 describes the tetracycline-dependent regulation of the mobilization (*mob*) region, which encodes three proteins required for conjugative transfer. Much of the focus in previous research was on the transfer region, yet little if any work was performed to characterize these *mob* genes. Recently, Peed and colleagues started to tease out a regulatory mechanism for the *mob* genes on another CTnDOT-like element, CTn341. Their work demonstrated that tetracycline induces transcription of this operon, and that RteA and RteB are required for this regulation (83). Because of the striking similarity between CTn341 and CTnDOT, we have every reason to believe that CTnDOT is regulated in the same manner. RteA and RteB are required for much of the tetracycline dependent regulation of CTnDOT; however, their role is generally more indirect as they are simply required for the transcriptional activation of downstream products. This led us to investigate what downstream products are responsible for the regulation of the *mob* operon.

Lastly, Chapter 4 will provide a summation of my work with some concluding remarks, and a description of further questions to be answered. The regulatory mechanisms of CTnDOT have proven to be quite fascinating, and I am sure we still have much to learn from this unique element.

1.5 References

1. **Abbani M, Iwahara M, Clubb RT**. 2005. The structure of the excisionase (Xis) protein from conjugative transposon Tn916 provides insights into the regulation of heterobivalent tyrosine recombinases. J. Mol. Biol. **347**:11–25.

2. **Ahangarzadeh Rezaee M, Langarizadeh N, Aghazadeh M**. 2012. First report of class 1 and class 2 integrons in multidrug-resistant Klebsiella pneumoniae isolates from northwest Iran. Jpn. J. Infect. Dis. **65**:256–259.

3. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ**. 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.

4. **Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ**. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389–3402.

5. **Andoh A, Kuzuoka H, Tsujikawa T, Nakamura S, Hirai F, Suzuki Y, Matsui T, Fujiyama Y, Matsumoto T**. 2012. Multicenter analysis of fecal microbiota profiles in Japanese patients with Crohn's disease. Journal of gastroenterology. **47**: 1298-1307.

6. **Arumugam M, Raes J, Pelletier E, Paslier DL, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto J-M, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K, Leclerc M, Levenez F, Manichanh C, Nielsen HB, Nielsen T, Pons N, Poulain J, Qin J, Sicheritz-Ponten T, Tims S, Torrents D, Ugarte E, Zoetendal EG, Wang J, Guarner F, Pedersen O, Vos WM de, Brunak S, Doré J, Members) MC (additional, Weissenbach J, Ehrlich SD, Bork P**. 2011. Enterotypes of the human gut microbiome. Nature **473**:174–180.

7. **Audette GF, Manchak J, Beatty P, Klimke WA, Frost LS**. 2007. Entry exclusion in F-like plasmids requires intact TraG in the donor that recognizes its cognate TraS in the recipient. Microbiology **153**:442–451.

8. **Aussel L, Barre FX, Aroyo M, Stasiak A, Stasiak AZ, Sherratt D**. 2002. FtsK Is a DNA motor protein that activates chromosome dimer resolution by switching the catalytic state of the XerC and XerD recombinases. Cell **108**:195–205.

9. **Ayoubi P, Kilic AO, Vijayakumar MN**. 1991. Tn5253, the pneumococcal omega (cat tet) BM6001 element, is a composite structure of two conjugative transposons, Tn5251 and Tn5252. J. Bacteriol. **173**:1617–1622.

10. **Bacic M, Parker AC, Stagg J, Whitley HP, Wells WG, Jacob LA, Smith CJ**. 2005. Genetic and structural analysis of the Bacteroides conjugative transposon CTn341. J. Bacteriol. **187**:2858–2869.

11. **Bacic MK, Smith CJ**. 2008. Laboratory maintenance and cultivation of bacteroides species. Curr Protoc Microbiol **Chapter 13**:Unit 13C.1.

12. **Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI**. 2005. Host-bacterial mutualism in the human intestine. Science **307**:1915–1920.

13. **Bawdon RE, Crane LR, Palchaudhuri S**. 1982. Antibiotic resistance in anaerobic bacteria: molecular biology and clinical aspects. Rev. Infect. Dis. **4**:1075–1095.

14. **Beaupré CE, Bohne J, Dale EM, Binns AN**. 1997. Interactions between VirB9 and VirB10 membrane proteins involved in movement of DNA from Agrobacterium tumefaciens into plant cells. J. Bacteriol. **179**:78–89.

15. **Berger BR, Christie PJ**. 1993. The Agrobacterium tumefaciens virB4 gene product is an essential virulence protein requiring an intact nucleoside triphosphate-binding domain. J. Bacteriol. **175**:1723–1734.

16. **Bisgaard H, Li N, Bonnelykke K, Chawes BLK, Skov T, Paludan-Müller G, Stokholm J, Smith B, Krogfelt KA**. 2011. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. J. Allergy Clin. Immunol. **128**:646–652.e1–5.

17. **Bonheyo G, Graham D, Shoemaker NB, Salyers AA**. 2001. Transfer region of a bacteroides conjugative transposon, CTnDOT. Plasmid **45**:41–51.

18. **Bonheyo GT, Hund BD, Shoemaker NB, Salyers AA**. 2001. Transfer region of a Bacteroides conjugative transposon contains regulatory as well as structural genes. Plasmid **46**:202–209.

19. **Bryan LE, Kowand SK, Van Den Elzen HM**. 1979. Mechanism of aminoglycoside antibiotic resistance in anaerobic bacteria: Clostridium perfringens and Bacteroides fragilis. Antimicrob. Agents Chemother. **15**:7–13.

20. **Burger A, Whiteley C, Boshoff A**. 2011. Current perspectives of the <i>Escherichia coli</i> RNA degradosome. Biotechnology Letters **33**:2337–2350.

21. **Carattoli A**. 2011. Plasmids in Gram negatives: Molecular typing of resistance plasmids. International Journal of Medical Microbiology **301**:654–658.

22. **Carpousis AJ**. 2007. The RNA degradosome of Escherichia coli: an mRNA-degrading machine assembled on RNase E. Annu. Rev. Microbiol. **61**:71–87.

23. **Cheng Q, Paszkiet BJ, Shoemaker NB, Gardner JF, Salyers AA**. 2000. Integration and excision of a Bacteroides conjugative transposon, CTnDOT. J. Bacteriol. **182**:4035–4043.

24. **Cheng Q, Sutanto Y, Shoemaker NB, Gardner JF, Salyers AA**. 2001. Identification of genes required for excision of CTnDOT, a Bacteroides conjugative transposon. Mol. Microbiol. **41**:625–632.

25. **Christie PJ, Atmakuri K, Krishnamoorthy V, Jakubowski S, Cascales E**. 2005. Biogenesis, architecture, and function of bacterial type IV secretion systems. Annu. Rev. Microbiol. **59**:451–485.

26. **Comstock LE, Coyne MJ**. 2003. Bacteroides thetaiotaomicron: a dynamic, niche-adapted human symbiont. Bioessays **25**:926–929.

27. **Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R**. 2009. Bacterial community variation in human body habitats across space and time. Science **326**:1694–1697.

28. **Dang TA, Christie PJ**. 1997. The VirB4 ATPase of Agrobacterium tumefaciens is a cytoplasmic membrane protein exposed at the periplasmic surface. J. Bacteriol. **179**:453–462.

29. **de Vos WM, de Vos EA**. 2012. Role of the intestinal microbiome in health and disease: from correlation to causation. Nutrition Reviews **70**:S45–S56.

30. **Desnoyers G, Massé E**. 2012. Noncanonical repression of translation initiation through small RNA recruitment of the RNA chaperone Hfq. Genes Dev. **26**:726–739.

31. **Elantak L, Ansaldi M, Guerlesquin F, Méjean V, Morelli X**. 2005. Structural and genetic analyses reveal a key role in prophage excision for the TorI response regulator inhibitor. J. Biol. Chem. **280**:36802–36808.

32. **Faust K, Sathirapongsasuti JF, Izard J, Segata N, Gevers D, Raes J, Huttenhower C**. 2012. Microbial Co-occurrence Relationships in the Human Microbiome. PLoS Comput Biol **8**:e1002606.

33. **Fava F, Danese S**. 2011. Intestinal microbiota in inflammatory bowel disease: friend of foe? World J. Gastroenterol. **17**:557–566.

34. **Franke AE, Clewell DB**. 1981. Evidence for a chromosome-borne resistance transposon (Tn916) in Streptococcus faecalis that is capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bacteriol. **145**:494–502.

35. **Franklin TJ, Snow GA**. 2005. Biochemistry and Molecular Biology of Antimicrobial Drug Action. Springer.

36. **Furuya N, Komano T**. 1995. Specific binding of the NikA protein to one arm of 17-base-pair inverted repeat sequences within the oriT region of plasmid R64. J. Bacteriol. **177**:46–51.

37. **Giongo A, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, Drew JC, Ilonen J, Knip M, Hyöty H, Veijola R, Simell T, Simell O, Neu J, Wasserfall CH, Schatz D, Atkinson MA, Triplett EW**. 2011. Toward defining the autoimmune microbiome for type 1 diabetes. ISME J **5**:82–91.

38. **Gottesman S**. 2004. Small RNAs shed some light. Cell **118**:1–2.

39. **Gottesman S, Storz G**. 2011. Bacterial small RNA regulators: versatile roles and rapidly evolving variations. Cold Spring Harb Perspect Biol **3**.

40. **Haase J, Lurz R, Grahn AM, Bamford DH, Lanka E**. 1995. Bacterial conjugation mediated by plasmid RP4: RSF1010 mobilization, donor-specific phage propagation, and pilus production require the same Tra2 core components of a proposed DNA transport complex. J. Bacteriol. **177**:4779–4791.

41. **Haggoud A, Trinh S, Moumni M, Reysset G**. 1995. Genetic Analysis of the Minimal Replicon of Plasmid pIP417 and Comparison with the Other Encoding 5-Nitroimidazole Resistance Plasmids fromBacteroidesspp. Plasmid **34**:132–143.

42. **Hecht DW, Kos IM, Knopf SE, Vedantam G**. 2007. Characterization of BctA, a mating apparatus protein required for transfer of the Bacteroides fragilis conjugal element BTF-37. Research in Microbiology **158**:600–607.

43. **Hooper LV, Midtvedt T, Gordon JI**. 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu. Rev. Nutr. **22**:283–307.

44. **Jeters RT, Wang G-R, Moon K, Shoemaker NB, Salyers AA**. 2009. Tetracycline-associated transcriptional regulation of transfer genes of the Bacteroides conjugative transposon CTnDOT. J. Bacteriol. **191**:6374–6382.

45. **Jones BV, Marchesi JR**. 2007. Accessing the mobile metagenome of the human gut microbiota. Mol. BioSyst. **3**:749–758.

46. **Judd PK, Mahli D, Das A**. 2005. Molecular characterization of the Agrobacterium tumefaciens DNA transfer protein VirB6. Microbiology **151**:3483–3492.

47. **Keeton CM, Gardner JF**. 2012. Roles of Exc protein and DNA homology in the CTnDOT excision reaction. J. Bacteriol. **194**:3368–3376.

48. **Kelley LA, Sternberg MJE**. 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc **4**:363–371.

49. **Koren O, Spor A, Felin J, Fåk F, Stombaugh J, Tremaroli V, Behre CJ, Knight R, Fagerberg B, Ley RE, Bäckhed F**. 2011. Human oral, gut, and plaque microbiota in patients with atherosclerosis. Proc. Natl. Acad. Sci. U.S.A. **108 Suppl 1**:4592–4598.

50. **Kurokawa K, Itoh T, Kuwahara T, Oshima K, Toh H, Toyoda A, Takami H, Morita H, Sharma VK, Srivastava TP, Taylor TD, Noguchi H, Mori H, Ogura Y, Ehrlich DS, Itoh K, Takagi T, Sakaki Y, Hayashi T, Hattori M**. 2007. Comparative Metagenomics Revealed Commonly Enriched Gene Sets in Human Gut Microbiomes. DNA Res **14**:169–181.

51. **Larsen N, Vogensen FK, van den Berg FWJ, Nielsen DS, Andreasen AS, Pedersen BK, Al-Soud WA, Sørensen SJ, Hansen LH, Jakobsen M**. 2010. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. PLoS ONE **5**:e9085.

52. **Ley RE**. 2010. Obesity and the human microbiome. Curr. Opin. Gastroenterol. **26**:5–11.

53. **Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI**. 2005. Obesity alters gut microbial ecology. Proc. Natl. Acad. Sci. U.S.A. **102**:11070–11075.

54. **Ley RE, Turnbaugh PJ, Klein S, Gordon JI**. 2006. Microbial ecology: human gut microbes associated with obesity. Nature **444**:1022–1023.

55. **Li LY, Shoemaker NB, Salyers AA**. 1995. Location and characteristics of the transfer region of a Bacteroides conjugative transposon and regulation of transfer genes. J. Bacteriol. **177**:4992–4999.

56. **Li P-L, Hwang I, Miyagi H, True H, Farrand SK**. 1999. Essential Components of the Ti Plasmid trb System, a Type IV Macromolecular Transporter. J Bacteriol **181**:5033–5041.

57. **Malanowska K, Salyers AA, Gardner JF**. 2006. Characterization of a conjugative transposon integrase, IntDOT. Mol. Microbiol. **60**:1228–1240.

58. **Manning PA, Morelli G, Achtman M**. 1981. traG protein of the F sex factor of Escherichia coli K-12 and its role in conjugation. Proc. Natl. Acad. Sci. U.S.A. **78**:7487–7491.

59. **Manson JM, Rauch M, Gilmore MS**. 2008. The commensal microbiology of the gastrointestinal tract. Adv. Exp. Med. Biol. **635**:15–28.

60. **Marchesi JR, Dutilh BE, Hall N, Peters WHM, Roelofs R, Boleij A, Tjalsma H**. 2011. Towards the human colorectal cancer microbiome. PLoS ONE **6**:e20447.

61. **Marchler-Bauer A, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, Gwadz M, He S, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Liebert CA, Liu C, Lu F, Lu S, Marchler GH, Mullokandov M, Song JS, Tasneem A, Thanki N, Yamashita RA, Zhang D, Zhang N, Bryant SH**. 2009. CDD: specific functional annotation with the Conserved Domain Database. Nucleic Acids Res. **37**:D205–210.

62. **Marchler-Bauer A, Bryant SH**. 2004. CD-Search: protein domain annotations on the fly. Nucleic Acids Res. **32**:W327–331.

63. **Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Lu F, Marchler GH, Mullokandov M, Omelchenko MV, Robertson CL, Song JS, Thanki N, Yamashita RA, Zhang D, Zhang N, Zheng C, Bryant SH**. 2011. CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res. **39**:D225–229.

64. **Massé E, Vanderpool CK, Gottesman S**. 2005. Effect of RyhB small RNA on global iron use in Escherichia coli. J. Bacteriol. **187**:6962–6971.

65. **Mazmanian SK, Round JL, Kasper DL**. 2008. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature **453**:620–625.

66. **Meyer R, Hinds M, Brasch M**. 1982. Properties of R1162, a broad-host-range, high-copy-number plasmid. J. Bacteriol. **150**:552–562.

67. **Moon K, Shoemaker NB, Gardner JF, Salyers AA**. 2005. Regulation of excision genes of the Bacteroides conjugative transposon CTnDOT. J. Bacteriol. **187**:5732–5741.

68. **Moon K, Sonnenburg J, Salyers AA**. 2007. Unexpected effect of a Bacteroides conjugative transposon, CTnDOT, on chromosomal gene expression in its bacterial host. Mol. Microbiol. **64**:1562–1571.

69. **Moore WE, Holdeman LV**. 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl Microbiol **27**:961–979.

70. **Morgan RM, Macrina FL**. 1997. bctA: a novel pBF4 gene necessary for conjugal transfer in Bacteroides spp. Microbiology (Reading, Engl.) **143 (Pt 7)**:2155–2165.

71. **Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, Reyes JA, Shah SA, LeLeiko N, Snapper SB, Bousvaros A, Korzenik J, Sands BE, Xavier RJ, Huttenhower C**. 2012. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biology **13**:R79.

72. **Musso G, Gambino R, Cassader M**. 2011. Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes. Annu. Rev. Med. **62**:361–380.

73. **Nagy E, Dányi E, Földes J**. 1990. Plasmid analysis of clinical isolates of Bacteroides fragilis group strains. Acta Microbiol. Hung. **37**:367–373.

74. **Nguyen M, Vedantam G**. 2011. Mobile genetic elements in the genus Bacteroides, and their mechanism(s) of dissemination. Mobile genetic elements **1**:187–196.

75. **Nikolich MP, Shoemaker NB, Salyers AA**. 1992. A Bacteroides tetracycline resistance gene represents a new class of ribosome protection tetracycline resistance. Antimicrob. Agents Chemother. **36**:1005–1012.

76. **Ochoa-Repáraz J, Mielcarz DW, Wang Y, Begum-Haque S, Dasgupta S, Kasper DL, Kasper LH**. 2010. A polysaccharide from the human commensal Bacteroides fragilis protects against CNS demyelinating disease. Mucosal Immunology **3**:487–495.

77. **Ochoa-Repáraz J, Mielcarz DW, Ditrio LE, Burroughs AR, Begum-Haque S, Dasgupta S, Kasper DL, Kasper LH**. 2010. Central nervous system demyelinating disease protection by the human commensal Bacteroides fragilis depends on polysaccharide A expression. J. Immunol. **185**:4101–4108.

78. **Ogilvie LA, Firouzmand S, Jones BV**. 2012. Evolutionary, ecological and biotechnological perspectives on plasmids resident in the human gut mobile metagenome. Bioeng Bugs **3**:13–31.

79. **Ottman N, Smidt H, de Vos WM, Belzer C**. 2012. The function of our microbiota: who is out there and what do they do? Front Cell Infect Microbiol **2**.

80. **Panja S, Woodson SA**. 2012. Hfq proximity and orientation controls RNA annealing. Nucleic Acids Res. **40**:8690–8697.

81. **Park J, Salyers AA**. 2011. Characterization of the Bacteroides CTnDOT regulatory protein RteC. J. Bacteriol. **193**:91–97.

82. **Parker C, Meyer RJ**. 2007. The R1162 relaxase/primase contains two, type IV transport signals that require the small plasmid protein MobB. Molecular Microbiology **66**:252–261.

83. **Peed L, Parker AC, Smith CJ**. 2010. Genetic and functional analyses of the mob operon on conjugative transposon CTn341 from Bacteroides spp. J. Bacteriol. **192**:4643–4650.

84. **Peterson DA, Turnbaugh PJ**. 2010. A Microbe-Dependent Viral Key to Crohn's Box. Sci Transl Med **2**:43ps39–43ps39.

85. **Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, Bonazzi V, McEwen JE, Wetterstrand KA, Deal C, Baker CC, Francesco VD, Howcroft TK, Karp RW, Lunsford RD, Wellington CR, Belachew T, Wright M, Giblin C, David H, Mills M, Salomon R, Mullins C, Akolkar B, Begg L, Davis C, Grandison L, Humble M, Khalsa J, Little AR, Peavy H, Pontzer C, Portnoy M, Sayre MH, Starke-Reed P, Zakhari S, Read J, Watson B, Guyer M**. 2009. The NIH Human Microbiome Project. Genome Res. **19**:2317–2323.

86. **Pumbwe L, Wareham DW, Aduse-Opoku J, Brazier JS, Wexler HM**. 2007. Genetic analysis of mechanisms of multidrug resistance in a clinical isolate of Bacteroides fragilis. Clinical Microbiology and Infection **13**:183–189.

87. **Rausch P, Rehman A, Künzel S, Häsler R, Ott SJ, Schreiber S, Rosenstiel P, Franke A, Baines JF**. 2011. Colonic mucosa-associated microbiota is influenced by an interaction of Crohn disease and FUT2 (Secretor) genotype. Proc. Natl. Acad. Sci. U.S.A. **108**:19030–19035.

88. **Roberts MC**. 2003. Acquired tetracycline and/or macrolide-lincosamides-streptogramin resistance in anaerobes. Anaerobe **9**:63–69.

89. **Rogers MB, Parker AC, Smith CJ**. 1993. Cloning and characterization of the endogenous cephalosporinase gene, cepA, from Bacteroides fragilis reveals a new subgroup of Ambler class A beta-lactamases. Antimicrob. Agents Chemother. **37**:2391–2400.

90. **Rosenberg E, Koren O, Reshef L, Efrony R, Zilber-Rosenberg I**. 2007. The role of microorganisms in coral health, disease and evolution. Nat. Rev. Microbiol. **5**:355–362.

91. **Rotimi VO, Duerden BI, Hafiz S**. 1981. Transferable plasmid-mediated antibiotic resistance in Bacteroides. J. Med. Microbiol. **14**:359–370.

92. **Round JL, Mazmanian SK**. 2009. The gut microbiota shapes intestinal immune responses during health and disease. Nature Reviews Immunology **9**:313–323.

93. **Salyers AA**. 1984. Bacteroides of the human lower intestinal tract. Annu. Rev. Microbiol. **38**:293–313.

94. **Salyers AA, Shoemaker NB**. 1995. Conjugative transposons: the force behind the spread of antibiotic resistance genes among Bacteroides clinical isolates. Anaerobe **1**:143–150.

95. **Salyers AA, Shoemaker NB, Li LY**. 1995. In the driver's seat: the Bacteroides conjugative transposons and the elements they mobilize. J. Bacteriol. **177**:5727–5731.

96. **Salyers AA, Shoemaker NB, Stevens AM, Li LY**. 1995. Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. Microbiol. Rev. **59**:579–590.

97. **Salyers AA, Gupta A, Wang Y**. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol. **12**:412–416.

98. **Samuel BS, Gordon JI**. 2006. A humanized gnotobiotic mouse model of host-archaeal-bacterial mutualism. Proc. Natl. Acad. Sci. U.S.A. **103**:10011–10016.

99. **Santini JM, Stanisich VA**. 1998. Both the fipA Gene of pKM101 and thepifC Gene of F Inhibit Conjugal Transfer of RP1 by an Effect on traG. J. Bacteriol. **180**:4093–4101.

100. **Sauer E, Schmidt S, Weichenrieder O**. 2012. Small RNA binding to the lateral surface of Hfq hexamers and structural rearrangements upon mRNA target recognition. Proc. Natl. Acad. Sci. U.S.A. **109**:9396–9401.

101. **Savage DC**. 1977. Microbial Ecology of the Gastrointestinal Tract. Annual Review of Microbiology **31**:107–133.

102. **Schandel KA, Maneewannakul S, Vonder Haar RA, Ippen-Ihler K, Webster RE**. 1990. Nucleotide sequence of the F plasmid gene, traC, and identification of its product. Gene **96**:137–140.

103. **Schandel KA, Muller MM, Webster RE**. 1992. Localization of TraC, a protein involved in assembly of the F conjugative pilus. J. Bacteriol. **174**:3800–3806.

104. **Scott JR, Churchward GG**. 1995. Conjugative Transposition. Annual Review of Microbiology **49**:367–397.

105. **Shoemaker NB, Vlamakis H, Hayes K, Salyers AA**. 2001. Evidence for extensive resistance gene transfer among Bacteroides spp. and among Bacteroides and other genera in the human colon. Appl. Environ. Microbiol. **67**:561–568.

106. **Shoemaker NB, Smith MD, Guild WR**. 1980. DNase-resistant transfer of chromosomal cat and tet insertions by filter mating in pneumococcus. Plasmid **3**:80–87.

107. **Sivanesan D, Baron C**. 2011. The Dimer Interface of Agrobacterium tumefaciens VirB8 Is Important for Type IV Secretion System Function, Stability, and Association of VirB2 with the Core Complex. J. Bacteriol. **193**:2097–2106.

108. **Smillie CS, Smith MB, Friedman J, Cordero OX, David LA, Alm EJ**. 2011. Ecology drives a global network of gene exchange connecting the human microbiome. Nature **480**:241–244.

109. **Smith CJ, Tribble GD, Bayley DP**. 1998. Genetic elements of Bacteroides species: a moving story. Plasmid **40**:12–29.

110. **Sobhani I, Tap J, Roudot-Thoraval F, Roperch JP, Letulle S, Langella P, Corthier G, Tran Van Nhieu J, Furet JP**. 2011. Microbial dysbiosis in colorectal cancer (CRC) patients. PLoS ONE **6**:e16393.

111. **Soki J, Szöke I, Nagy E**. 1999. Characterisation of a 5.5-kb cryptic plasmid present in different isolates of Bacteroides spp. originating from Hungary. J. Med. Microbiol. **48**:25–31.

112. **Sóki J, Wareham DW, Rátkai C, Aduse-Opoku J, Urbán E, Nagy E**. 2010. Prevalence, nucleotide sequence and expression studies of two proteins of a 5.6 kb, Class III, Bacteroides plasmid frequently found in clinical isolates from European countries. Plasmid **63**:86–97.

113. **Solar G del, Giraldo R, Ruiz-Echevarría MJ, Espinosa M, Díaz-Orejas R**. 1998. Replication and Control of Circular Bacterial Plasmids. Microbiol. Mol. Biol. Rev. **62**:434–464.

114. **Song B, Wang G-R, Shoemaker NB, Salyers AA**. 2009. An unexpected effect of tetracycline concentration: growth phase-associated excision of the Bacteroides mobilizable transposon NBU1. J. Bacteriol. **191**:1078–1082.

115. **Speer BS, Shoemaker NB, Salyers AA**. 1992. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. Clin. Microbiol. Rev. **5**:387–399.

116. **Stevens AM, Sanders JM, Shoemaker NB, Salyers AA**. 1992. Genes involved in production of plasmidlike forms by a Bacteroides conjugal chromosomal element share amino acid homology with two-component regulatory systems. J. Bacteriol. **174**:2935–2942.

117. **Stiffler PW, Keller R, Traub N**. 1974. Isolation and characterization of several cryptic plasmids from clinical isolates of Bacteroides fragilis. J. Infect. Dis. **130**:544–548.

118. **Stsepetova J, Sepp E, Julge K, Vaughan E, Mikelsaar M, de Vos WM**. 2007. Molecularly assessed shifts of Bifidobacterium ssp. and less diverse microbial communities are characteristic of 5-year-old allergic children. FEMS Immunol. Med. Microbiol. **51**:260–269.

119. **Sutanto Y, DiChiara JM, Shoemaker NB, Gardner JF, Salyers AA**. 2004. Factors required in vitro for excision of the Bacteroides conjugative transposon, CTnDOT. Plasmid **52**:119–130.

120. **Sutanto Y, Shoemaker NB, Gardner JF, Salyers AA**. 2002. Characterization of Exc, a novel protein required for the excision of Bacteroides conjugative transposon. Mol. Microbiol. **46**:1239–1246.

121. **Top EM, Springael D**. 2003. The role of mobile genetic elements in bacterial adaptation to xenobiotic organic compounds. Current Opinion in Biotechnology **14**:262–269.

122. **Tremblay C-L, Letellier A, Quessy S, Daignault D, Archambault M**. 2012. Antibiotic-Resistant Enterococcus faecalis in Abattoir Pigs and Plasmid Colocalization and Cotransfer of tet(M) and erm(B) Genes. J. Food Prot. **75**:1595–1602.

123. **Trinh S, Haggoud A, Reysset G**. 1996. Conjugal transfer of the 5-nitroimidazole resistance plasmid pIP417 from Bacteroides vulgatus BV-17: characterization and nucleotide sequence analysis of the mobilization region. J. Bacteriol. **178**:6671–6676.

124. **Troy EB, Kasper DL**. 2010. Beneficial effects of Bacteroides fragilis polysaccharides on the immune system. Front. Biosci. **15**:25–34.

125. **Tsai Y-C, Du D, Domínguez-Malfavón L, Dimastrogiovanni D, Cross J, Callaghan AJ, García-Mena J, Luisi BF**. 2012. Recognition of the 70S ribosome and polysome by the RNA degradosome in Escherichia coli. Nucl. Acids Res. **40**: 10417-10431.

126. **Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI**. 2009. A core gut microbiome in obese and lean twins. Nature **457**:480–484.

127. **Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI**. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature **444**:1027–1031.

128. **Vanderpool CK, Gottesman S**. 2007. The novel transcription factor SgrR coordinates the response to glucose-phosphate stress. J. Bacteriol. **189**:2238–2248.

129. **Varsaki A, Moncalián G, Garcillán-Barcia M del P, Drainas C, de la Cruz F**. 2009. Analysis of ColE1 MbeC unveils an extended ribbon-helix-helix family of nicking accessory proteins. J. Bacteriol. **191**:1446–1455.

130. **Wallace BL, Bradley JE, Rogolsky M**. 1981. Plasmid analyses in clinical isolates of Bacteroides fragilis and other Bacteroides species. J. Clin. Microbiol. **14**:383–388.

131. **Walldén K, Williams R, Yan J, Lian PW, Wang L, Thalassinos K, Orlova EV, Waksman G**. 2012. Structure of the VirB4 ATPase, alone and bound to the core complex of a type IV secretion system. Proc Natl Acad Sci U S A **109**:11348–11353.

132. **Wang T, Cai G, Qiu Y, Fei N, Zhang M, Pang X, Jia W, Cai S, Zhao L**. 2012. Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. ISME J **6**:320–329.

133. **Wang Y, Rotman ER, Shoemaker NB, Salyers AA**. 2005. Translational control of tetracycline resistance and conjugation in the Bacteroides conjugative transposon CTnDOT. J. Bacteriol. **187**:2673–2680.

134. **Wang Y, Shoemaker NB, Salyers AA**. 2004. Regulation of a Bacteroides operon that controls excision and transfer of the conjugative transposon CTnDOT. J. Bacteriol. **186**:2548–2557.

135. **Waters JL, Salyers AA**. 2012. The Small RNA RteR Inhibits Transfer of the Bacteroides Conjugative Transposon CTnDOT. J. Bacteriol. **194**:5228–5236.

136. **Waters LS, Storz G**. 2009. Regulatory RNAs in bacteria. Cell **136**:615–628.

137. **Wexler HM**. 2007. Bacteroides: the good, the bad, and the nitty-gritty. Clin. Microbiol. Rev. **20**:593–621.

138. **Whittle G, Hund BD, Shoemaker NB, Salyers AA**. 2001. Characterization of the 13-kilobase ermF region of the Bacteroides conjugative transposon CTnDOT. Appl. Environ. Microbiol. **67**:3488–3495.

139. **Whittle G, Shoemaker NB, Salyers AA**. 2002. The role of Bacteroides conjugative transposons in the dissemination of antibiotic resistance genes. Cell. Mol. Life Sci. **59**:2044–2054.

140. **Whittle G, Salyers AA**. 2002. Bacterial Transposons—An Increasingly Diverse Group of Elements in Streips, UN, Yasbin, RE (eds.), Modern Microbial Genetics. John Wiley & Sons, Inc. p. 385–427.

141. **Whittle G, Shoemaker NB, Salyers AA**. 2002. Characterization of genes involved in modulation of conjugal transfer of the Bacteroides conjugative transposon CTnDOT. J. Bacteriol. **184**:3839–3847.

142. **Wilkinson A, Danino V, Wisniewski-Dyé F, Lithgow JK, Downie JA**. 2002. N-Acyl-Homoserine Lactone Inhibition of Rhizobial Growth Is Mediated by Two Quorum-Sensing Genes That Regulate Plasmid Transfer. J. Bacteriol. **184**:4510–4519.

143. **Wood MM, DiChiara JM, Yoneji S, Gardner JF**. 2010. CTnDOT Integrase Interactions with Attachment Site DNA and Control of Directionality of the Recombination Reaction. J. Bacteriol. **192**:3934–3943.

144. **Xia S, Robertus JD**. 2009. Effect of divalent ions on the minimal relaxase domain of MobA. Arch. Biochem. Biophys. **488**:42–47.

145. **Xu J, Bjursell MK, Himrod J, Deng S, Carmichael LK, Chiang HC, Hooper LV, Gordon JI**. 2003. A genomic view of the human-Bacteroides thetaiotaomicron symbiosis. Science **299**:2074–2076.

146. **Yoshida H, Furuya N, Lin Y-J, Güntert P, Komano T, Kainosho M**. 2008. Structural basis of the role of the NikA ribbon-helix-helix domain in initiating bacterial conjugation. J. Mol. Biol. **384**:690–701.

147. **Zilber-Rosenberg I, Rosenberg E**. 2008. Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. FEMS Microbiol. Rev. **32**:723–735.

148. **Zocco MA, Ainora ME, Gasbarrini G, Gasbarrini A**. 2007. Bacteroides thetaiotaomicron in the gut: molecular aspects of their interaction. Dig Liver Dis **39**:707–712.

1.6 Figures

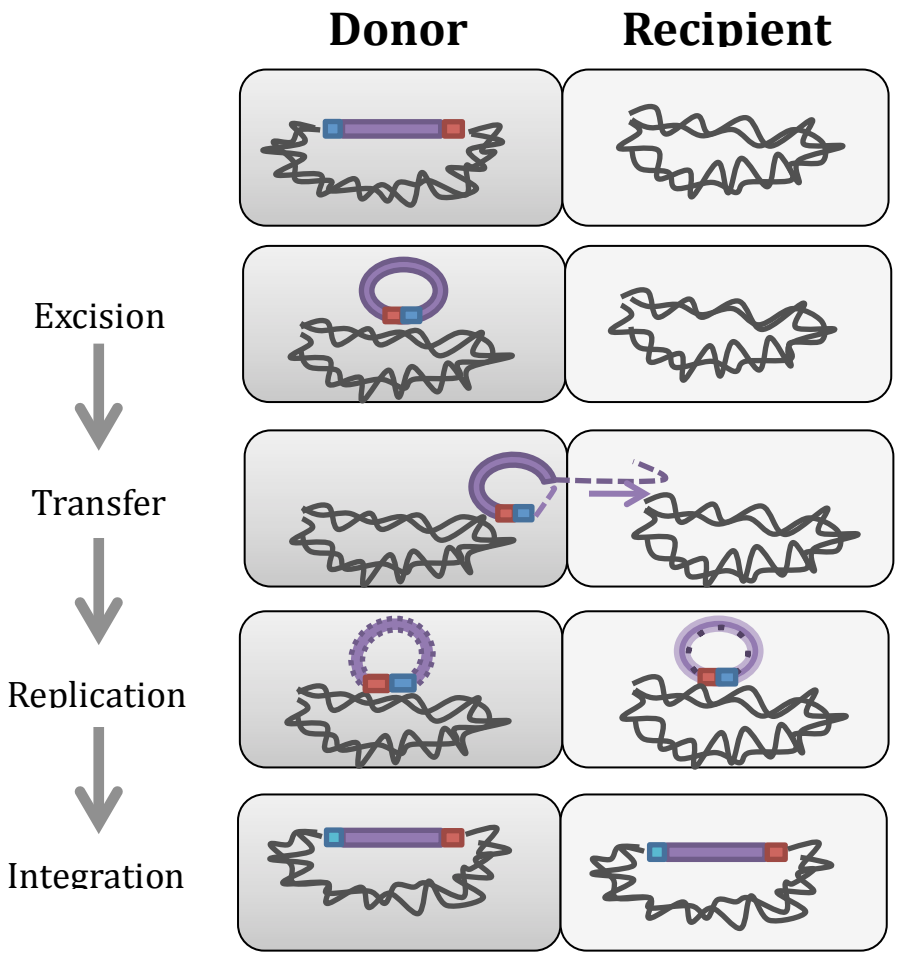


Figure 1.1: Conjugative transfer of CTnDOT. CTnDOT is a 65 kb conjugative transposon present in *Bacteroides* spp. that encodes resistance to the antibiotics erythromycin and tetracycline. The conjugative transfer of CTnDOT is actually stimulated by tetracycline induction. The first step in conjugative transfer is that CTnDOT must first excise from the host chromosome to form a circular intermediate. CTnDOT is then nicked at the OriT after which CTnDOT is then replicated, and made double-stranded, before integrating in to the chromosome of both the donor and recipient.

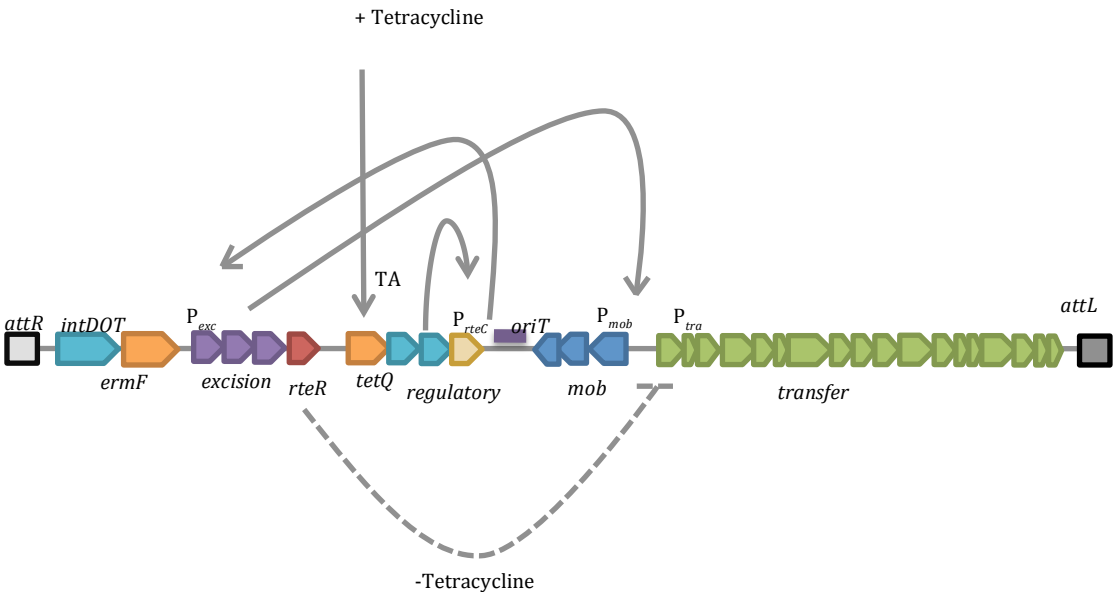


Figure 1.2: Organization and tetracycline dependent regulation of CTnDOT. The regulatory events that occur upon tetracycline (Tc) induction are shown in solid gray lines. The *tetQ-rteA-rteB* operon is regulated by a translational attenuation (TA) mechanism where after Tc exposure (step 1), translation occurs which ultimately allows RteB to activate the transcription of RteC (step 2). RteC then activates transcription of the excision operon (step 3). The proteins encoded by the excision operon not only excise CTnDOT from the chromosome, but also upregulate transcription of the transfer genes as well as the *mob* operon (step 4). In the absence of Tc, RteR mediates inhibition within the transfer operon to prevent conjugative transfer of CTnDOT (step 5, gray dotted line).

CHAPTER 2: THE SMALL RNA RTER INHIBITS TRANSFER OF THE *BACTEROIDES* CONJUGATIVE TRANSPOSON CTNDOT[1]

2.1 Abstract

CTnDOT is a 65 kb conjugative transposon present in *Bacteroides* spp. that confers resistance to erythromycin (*ermF*) and tetracycline (*tetQ*). An interesting feature of CTnDOT is that both excision from the chromosome and transfer of CTnDOT are stimulated by exposure to tetracycline. However, when no tetracycline is present, transfer of CTnDOT is not detectable. Previous studies suggested that a region containing a small RNA, RteR, appeared to be mediating repression of CTnDOT transfer; however, virtually nothing was known about RteR. We have demonstrated that RteR is a 90 nt transcript that is not further processed. RteR inhibits conjugative transfer of CTnDOT by targeting the transfer region, a 13 kb operon that encodes the *tra* genes required to assemble the mating apparatus. We report here that RteR is interacting with the region downstream of *traA*. Levels of the downstream *tra* mRNA are dramatically reduced when RteR is present. Further, RteR does not appear to decrease the half-life of the *tra* mRNA transcript, suggesting that RteR is not binding to the transcript to initiate ribonuclease dependent decay, similar to other *trans*-acting small RNAs. We predict that RteR may be acting to enhance termination of the *tra* operon within *traB*, which could account for the decreased abundance of *tra* transcript downstream of *traA* and explain why the *tra*

[1] This chapter appeared in its entirety in the Journal of Bacteriology and is referred to later in this dissertation as "Waters, JL and AA Salyers". Waters JL, Salyers AA. 2012 . The small RNA RteR inhibits transfer of the *Bacteroides* conjugative transposon CTnDOT. **194**(19): 5228-36. This article is reprinted with the permission of the publisher and is available from http://www.ncbi.nlm.nih.gov/pubmed and using PMID:22821972.

mRNA has the same half-life whether or not RteR is present. RteR is the only small RNA that has been characterized so far within the Bacteroidetes phylum.

2.2 Introduction

Bacteroides are Gram-negative obligate anaerobes that are a major component of the human intestinal microbiota (5, 11). *Bacteroides* can act as reservoirs of antibiotic resistance genes within the gastrointestinal tract, by transferring these resistance genes to other organisms via mobile genetic elements such as conjugative transposons (13, 18, 19, 26). One such example is CTnDOT, a 65 kb conjugative transposon that confers resistance to the antibiotics erythromycin and tetracycline.

Exposure to low levels of tetracycline stimulates both the excision of CTnDOT from the chromosome and conjugative transfer of CTnDOT (13, 20, 21). Upon tetracycline induction, translation of the *tetQ-rteA-rteB* operon is allowed to continue. *TetQ* encodes a ribosomal protection type of tetracycline resistance; *rteA* and *rteB* encode a two-component signal transduction system, with RteA recognizing an external signal, and RteB being the response modulator (21). It is not known what signal RteA is sensing, although it is not tetracycline (14). RteB then activates transcription of RteC, which then activates transcription of the excision operon, containing *xis2c-xis2d-exc.* The proteins encoded by this operon are not only important for excision and integration of CTnDOT, but they also serve a regulatory role by activating transcription of the transfer (*tra*) operon (7).

In the absence of tetracycline, however, there is no detectable transfer of CTnDOT (28). Previous studies demonstrated that a 500 bp region containing a small RNA, RteR, was sufficient for mediating repression of the self-transmissible plasmid pLYL72 (9) when there is no tetracycline present (7, 28). Western blotting and subcloning analysis demonstrated that the target of RteR was within the 13 kb *tra* operon (28).

In this study, we have identified the 5´ and 3´ ends of RteR using rapid amplification of cDNA ends (RACE) analysis, and we have demonstrated that RteR is a 90 nt non-coding RNA that is not further processed into a smaller transcript. We have also identified the promoter of *rteR* and localized *rteR* to the region immediately downstream of the excision gene *exc.* Interestingly, *rteR* is transcribed constitutively with respect to tetracycline exposure, unlike the other CTnDOT regulatory genes that have proven to be expressed only after tetracycline induction.

We report here that we have narrowed the target of RteR to an approximately 1 kb region between *traA* and *traC,* and have demonstrated that downstream of *traA,* there is little mRNA detectable in the presence of RteR. We also show that the half-life of the *tra* mRNA is not affected by the presence of RteR. These observations suggest that RteR may be acting to initiate premature transcription termination early in the *tra* operon, thus resulting in inhibition of CTnDOT conjugative transfer.

2.3 Materials and methods

Strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this study are summarized in Table 1. Unless otherwise noted, all *Bacteroides* strains were initially cultured in chopped meat broth (Remel), and then transferred to anaerobic trypticase-yeast extract-glucose (TYG) media (16) for overnight growth with antibiotics when appropriate. Sub-culturing of *Bacteroides* strains was also done in TYG liquid media, and all *Bacteroides* culturing was performed anaerobically at 37 oC. *E. coli* was grown aerobically in Luria-Bertani (LB) broth at 37 oC, with antibiotics when appropriate. Unless otherwise noted, antibiotic concentrations used for culturing were as follows: Ampicillin 100 μg/mL; cefoxitin 10 μg/mL; chloramphenicol 10 μg/mL; erythromycin 10 μg/mL; gentamycin 200 μg/mL; kanamycin 100 μg/mL; rifampicin 2 μg/mL; streptomycin 100 μg/mL; tetracycline 1 μg/mL.

Bacterial mating assays

E. coli strain HB101 containing the IncPα plasmid RPI was used to mobilize plasmids from an *E. coli* donor strain to *Bacteroides* recipients, as described previously (16).

For assays measuring the transfer frequency of pLYL72 from a *Bacteroides* donor to the recipient *E. coli* strain HB101, the procedure was performed as described previously (9). The transfer frequency is expressed as the ratio of transconjugants per recipient.

Site directed mutagenesis

Site directed mutagenesis was performed using the Stratagene Quikchange XL II kit (Agilent Genomics). To create the mutation in the putative RteR promoter, pJW305 plasmid DNA was used as a template, with primers SDM 5740 top and SDM 5740 bottom. To create the substitutions within the sequence of *rteR* that changed nucleotides 49-52 from GGAU to ACCA, plasmid pJW305 was used with primers SDM RteR 49-52 top and bottom. All primers used in this study are summarized in Table 2. The PCR-generated products were then treated with DpnI at 37 ^{o}C for 1 hour, and subsequently transformed into chemically competent *E. coli* DH5α. The resulting plasmid was mobilized into *Bacteroides* strain BT4001containing pLYL72 via a triparental mating as described above.

PCR amplification and sequencing of RteR from other CTnDOT-like elements

A 500 bp region containing the 3' end of *exc* and *rteR* from various strains containing CTnDOT-like conjugative transposons was amplified using primers JLW10F and JLW11R (Table2). These PCR products were then cloned into a commercial cloning vector per the manufacturer's protocol (Promega) and submitted for sequencing at the University of Illinois Core Sequencing Facility.

In addition, BLAST searches were performed to determine whether other *exc-rteR* sequences could be found in the nucleotide databases. All sequences were then aligned using ClustalW2 analysis (6, 8) to compare sequence similarity among RteR homologues. Secondary structure predictions of RteR homologues were also performed using MFOLD (29).

RNA isolation

Bacteroides strains were grown in anaerobic TYG medium to an OD650 of 0.4 – 0.6. RNA was then isolated using the hot phenol method as described previously (1). RNA concentrations were quantified using a nanodrop spectrophotometer.

Rapid amplification of cDNA ends (RACE) analysis

5´ RACE analysis of RteR was performed as previously outlined (23) using the *rteR* specific primer JLW11R. RNA was isolated from strain BT4001 ΩQAB pJW305. Cells were grown anaerobically in TYG to an OD_{650} of 0.4 – 0.6.

3´ RACE analysis of RteR was performed as previously outlined (2) using the *rteR* specific primer JLW12F. RNA was isolated from BT4001 ΩQAB pJW305, grown anaerobically in TYG to an OD_{650} of 0.4 – 0.6.

Northern analysis

Approximately 2 µg of total RNA was electrophoresed on an 8% acrylamide gel (Sequagel, ureagel-8) at 100 V for 1 hour. A radiolabeled Decade marker (Ambion) was used as a size standard. RNA was electro-transferred to a Nytran + membrane (Whatman) at 200 mA for 1 hour, followed by UV cross-linking at 1200 µJoules X 1000. Pre-hybridization was performed at 42 ^{o}C for at least 2 hours in Ultrahyb solution (Ambion). A 200 bp RteR-specific probe was synthesized using the Maxiscript T7 kit (Ambion), using the primers JLW12F and T7JLW22R. Following pre-hybridization the membrane was incubated with the probe overnight at 42 ^{o}C.

The membrane was then washed for 30 minutes at 42 °C in 2X SSC + 0.1% SDS, followed by another 30 minute wash at 42 °C in 0.1X SSC + 0.1% SDS.

Qualitative RT-PCR

Bacteroides strains were grown in anaerobic TYG medium to an OD_{650} of 0.4 – 0.6, and RNA was isolated as described above. RNA samples were then treated with Ambion turbo DNase, and quantified using a nanodrop spectrophotometer. RT-PCR was performed using the Access RT-PCR system (Promega). Reactions that omitted reverse transcriptase were tested to ensure that all genomic DNA was adequately removed. Samples were electrophoresed on a 1.5% agarose gel for analysis.

RT-qPCR

Bacteroides strains were grown in anaerobic TYG medium to an OD_{650} of 0.4 – 0.6, and RNA was isolated as described above. RNA samples were then treated with Ambion turbo DNase, and RT-PCR was subsequently performed to ensure complete removal of genomic DNA. cDNA was synthesized using Superscript III reverse transcriptase as outlined in the manufacturer's protocol (Invitrogen). qPCR was performed on a Realplex2 Mastercycler (Eppendorf), using the Ssofast EvaGreen as a signal reporter (BioRad). Unless otherwise noted, *rpoD* was used as a reference marker. The final concentrations of reaction components are: 1 µM primers, 5 µL cDNA, and 10 µL Ssofast EvaGreen Supermix (BioRad). The reaction conditions were as follows: initial denaturation 2 minutes at 98.0 °C, amplification and quantification at 98.0 °C, 5 sec, 55 °C 10 sec for 40 cycles. A melting curve was performed at 95.0 °C

for 15 sec, followed by 55.0 ^{o}C for 15 sec with subsequent heating to 95.0 ^{o}C over the course of 20 minutes with continuous fluorescence measurement, followed by a final incubation at 95.0 ^{o}C for 15 sec. Each measurement was performed in triplicate, and relative quantification was performed using the equation RQ=1/ECT, with E representing amplification efficiency and CT the threshold cycle (12).

Tra mRNA half-life assays

To further narrow the target of RteR within the *tra* region, quantitative RT-PCR was implemented to determine where changes in transcript levels were occurring. *Bacteroides* strain BT4100 with pLYL72 and pAFD1 (empty vector) or pJW305 (+ RteR) were grown up to an OD650 of ~0.5. Aliquots of cultures were then taken 0, 5, and 10 minutes post rifampicin (400 µg/mL) addition. RNA was isolated as described previously, and subsequently treated with Ambion turbo DNase. cDNA was then synthesized using Superscript III reverse transcriptase (Invitrogen) as described by the manufacturer's guidelines. RT-qPCR was then performed to detect the relative abundance of transcript over time, as described above. Quantification was performed by normalizing the amount of transcript remaining post rifampicin induction, relative to time 0 which was set at 100%.

In order to confirm that the concentration of rifampicin used was sufficient to inhibit RNA polymerase, a set of cultures with no added rifampicin was also taken at the same time points. Qualitative RT-PCR was performed, and a similar amount of transcript was detected after 30 minutes in the cells without rifampicin treatment; however, very little transcript was detected in cells treated with rifampicin. This

observation suggested that the quantity of rifampicin used was sufficient for RNA polymerase inhibition.

2.4 Results

RteR inhibits conjugative transfer of the self-transmissible plasmid pLYL72

The transfer (*tra*) operon, mobilization (*mob*) operon, and OriT of CTnDOT were previously cloned into a vector, resulting in the 35 kb plasmid pLYL72 that transferred constitutively at a rate of approximately 10^{-5} to 10^{-6} transconjugants per recipient (9). However, when CTnDOT regulatory genes were provided in *trans*, transfer of pLYL72 was then regulated upon tetracycline induction (28). Previous studies demonstrated that a 500 bp region containing *rteR* was sufficient for reducing pLYL72 transfer frequency to levels less than 10^{-8} transconjugants per recipient. To further localize the region containing *rteR*, various sub-clones were produced to generate 5´ and/or 3´ truncations of this 500 bp region. The resulting sub-clones were placed in *trans* to pLYL72 to determine whether the truncated region was still sufficient for inhibition of pLYL72 conjugative transfer. Using these sub-clones, we were able to define the region containing *rteR on* a 200 bp fragment (Fig. 2.1).

These results suggest that RteR is not controlling conjugation via the excision operon containing *xis2c, xis2d,* and exc, because RteR can still inhibit conjugative transfer of pLYL72 without the excision operon present. After localizing the region containing RteR that is required for pLYL72 transfer regulation, we confirmed that no open reading frames are present, thus suggesting that RteR is a non-coding RNA.

The rteR *promoter flanks the 3´ end of* exc*, and produces a 90 nt RteR transcript that is constitutively transcribed*

Previous results suggested that *rteR* is transcribed constitutively (7), unlike many other CTnDOT regulatory genes that are transcribed and/or translated only upon tetracycline induction (28). However, that preliminary study had localized *rteR* using primer extension to a region that lay within the 3´ end of *exc* (Fig. 2.1), which we report here is not correct. The results of subcloning experiments conflicted with the previously proposed transcription start site of *rteR.* It was possible then that RteR was not the transcript detected in the previous Northern analysis that indicated constitutive expression of *rteR*. To further examine *rteR* expression, we repeated the Northern analysis using a probe specific to the recently localized 200 bp fragment that was sufficient for inhibition of pLYL72 conjugative transfer. Northern blotting analysis was performed using RNA harvested from *B. thetaiotaomicron* strain BT4104 grown either in the absence or presence of tetracycline (1 μg/mL). As shown in Figure 2.2, the relative abundance of RteR was the same regardless of whether or not tetracycline was present in the growth medium. This result confirmed that *rteR* is constitutively transcribed with respect to tetracycline exposure.

The fact that *rteR* is constitutively expressed suggests that *rteR* is not part of the operon containing *xis2c-xis2d-orf3-exc,* which is only transcribed upon tetracycline stimulation. Instead, *rteR* appears to be transcribed from an independent promoter. Sequence analysis revealed what appeared to be a consensus *Bacteroides* promoter (3, 10) that flanked the 3´ end of *exc,* which was in the vicinity

of the expected *rteR* promoter (Fig. 2.3). To confirm that this was in fact the promoter directing transcription of *rteR*, site directed mutations were made in the putative -7 portion that would change the TTTG of the conserved TnTAnTTTG to an AAAC. Mutations like this have been previously determined to abolish activity of other *Bacteroides* promoters (3, 10). Northern analysis was performed to see whether the RteR transcript was detectable in this mutant, BT4001 pLYL72 pJW310. RteR was not detected (Fig. 2.2) even after prolonged exposure (data not shown), suggesting that this mutation was sufficient to completely abolish activity from what we have now identified as the *rteR* promoter.

To further demonstrate that this mutation abolished activity of the *rteR* promoter, we also placed this mutation in *trans* with the self-transmissible plasmid pLYL72, to determine whether this mutant was still capable of repressing pLYL72 conjugative transfer. The transfer frequency of pLYL72 with pJW310 in *trans* was similar to that of the constitutive levels of transfer, further demonstrating that an intact copy of RteR was no longer present to mediate inhibition of conjugative transfer. Together, these results confirm that we have identified the *rteR* promoter.

Both 5′ and 3′ rapid amplification of cDNA ends (RACE) were performed to definitively identify the extent of the RteR sequence. 5′ RACE was performed not only to map the 5′ end of RteR, but also to identify whether RteR is the primary transcription product or if RteR undergoes internal 5′ processing. The primary transcription start site was mapped to the G nucleotide located 7 bp downstream of the -7 element of the *rteR* promoter, which is a common transcription start site relative to other characterized *Bacteroides* promoters (3). Further, 5′ RACE also

indicated that RteR does not undergo processing at the 5′ end of the transcript, as it has a 5′ triphosphate present. The 3′ end of RteR, as shown in Figure 2.4, is 90 nt downstream of the transcription start site. The secondary structure of RteR, predicted using MFOLD (29), is shown in Figure 2.5A.

The rteR *region is present on other CTnDOT-like elements, and both the primary sequence and predicted secondary structure are well conserved*

We wanted to determine whether RteR was an sRNA regulator that is specific to CTnDOT, or whether RteR was present on other *Bacteroides* conjugative transposons (CTns). Not only were we able to find *rteR* in other *Bacteroides* spp., but we observed that *rteR* was present on other conjugative transposons and was also present in other members of the Bacteroidetes phylum. What was even more surprising is that *rteR* is very well conserved, with most homologues having 98% or greater identity, and many being 100% identical. We were able to find, however, a few sequences that were only about 73% identical to *rteR* present on CTnDOT. An alignment of these sequences was performed using ClustalW2 analysis (6, 8), and is shown in Figure 2.5. An interesting observation was that despite the substitutions in these *rteR* sequences, the predicted secondary structure remained similar. Any of the nucleotide substitutions within RteR was either in a single stranded region of RteR, or resulted in a substitution where the substitution could still base-pair with the adjacent nucleotide in the predicted secondary structure (data not shown).

The secondary structure of RteR appears to be important for regulatory function

As previously mentioned, *rteR* is very well conserved, and these homologues all share nearly identical predicted secondary structures. This observation led us to suggest that perhaps it is the secondary structure of RteR that is important for RteR-mediated negative regulation of conjugative transfer. In the first mutant we constructed, we made site directed mutations in 4 nucleotides (pJW312, Fig. 2.6B) that changed the predicted secondary structure of RteR in a way that would form a stem loop in what is predicted to be a single stranded bulge (Fig. 2.6A). When pJW312 was placed in *trans* to pLYL72, conjugative transfer was no longer inhibited, demonstrating that this mutation was sufficient to abolish the regulatory function of RteR.

We also created a deletion mutant that contained an internal deletion within *rteR*, yet also contained the excision operon containing *xis2C, xis2D,* and *exc,* to ascertain if there is an indirect interaction between RteR and the excision proteins. Based on our results demonstrating that a 4 nt substitution could abolish RteR-mediated repression, we expected that a 30 nt deletion (pJW318, Fig. 2.6A and 2.6B) within *rteR* would also be sufficient to abolish activity. However, this mutant that deletes approximately 33% of RteR was still able to inhibit conjugative transfer of pLYL72. Observing that pJW318 is still active may actually reveal more about the previous mutant pJW312. The deletion in pJW318 actually removes the nucleotides that had site directed mutations in pJW312. This suggests that perhaps the mutations in pJW312 did not change critical nucleotides needed for base pairing, because these nucleotides are not even present in pJW318. It suggests then that

perhaps the site directed mutations in pJW312 altered the secondary structure in a way that abolished the ability of RteR to interact with the target (Fig. 2.6A).

The level of tra *mRNA transcript downstream of* traA *is dramatically decreased when RteR is present*

If RteR was preventing transcription entirely, as suggested in a previous study (7), no *tra* mRNA should be detected at other points of the *tra* transcript. We tested this using qualitative RT-PCR (data not shown) and demonstrated that there is in fact detectable transcript throughout the entire length of the *tra* operon. In the case of *traA*, a similar intensity band was seen whether RteR was present or not, which suggested that in this portion of the operon, RteR had no effect. Beyond *traA,* however, the relative level of transcript rapidly decreased. RT-qPCR was performed to confirm our qualitative RT-PCR results. A similar relative level of transcript was observed in the *traA* portion; however, immediately downstream of *traA* the relative percentage of transcript was dramatically reduced (Fig. 2.7). This observation demonstrates that RteR is not preventing transcription as previously suggested, and the target of RteR is downstream of *traA.* We predict that the target of RteR may in fact be in the region between *traA* and *traC*.

The half-life of tra mRNA is not affected by the presence of RteR

The half-life of the *tra* mRNA transcript was measured to better ascertain how RteR might be working to inhibit conjugative transfer. One possibility is that RteR exhibits an effect on the stability of the *tra* transcript. If the *tra* mRNA half-life

decreases when RteR is present, it would suggest that RteR is working to recruit ribonucleases to the RteR-*tra* mRNA duplex. This type of interaction is often mediated by binding of the sRNA to the target mRNA transcript via a chaperone protein such as Hfq (22). The complex is then targeted for ribonuclease-mediated decay, resulting in a decreased half-life of the target mRNA (25).

The half-life of pLYL72 *tra* mRNA was measured in both the presence and absence of RteR. At *traA, traB-C,* and *traD,* the half-life of the *tra* transcript was approximately 1.63 minutes, whether or not RteR was present, demonstrating that RteR is not acting to destabilize the *tra* mRNA transcript by means of ribonuclease-dependent decay (Fig. 2.8).

RteR has no effect on excision from the chromosome, and does not regulate the mob genes

Many steps are required for transfer of conjugative transposons such as excision from the chromosome, nicking of the OriT, and assembly of the mating bridge (27). To investigate the effect of RteR on excision from the chromosome, we introduced *rteR* on a plasmid into a strain containing CTnERL. CTnERL is essentially identical to CTnDOT but lacks the *ermF* region. We used this strain rather than a strain containing CTnDOT, so that we could use erythromycin as a selectable marker for the plasmid containing *rteR,* pJW305. Although CTnERL already contains a single copy of *rteR*, by placing *rteR* on a plasmid in *trans* we could determine whether there was a reduction of the excised element associated with increasing the copy number of RteR. Using RT-PCR, we found that the copy number of RteR did not affect the

amount of excision from the chromosome, suggesting that RteR does not affect conjugative transfer via any regulatory effect on excision (Figure 2.9).

To assess whether RteR affect the CTnDOT mobilization region, we used RT-PCR to determine if RteR had any regulatory effect on expression of the *mob* genes. We observed similar levels of *mobA, mobB,* and *mobC* transcript whether RteR was present or not, confirming that the *mob* genes are not a target of RteR (Figure 2.10). These findings were also confirmed using RT-qPCR, which demonstrated that *mob* expression was not affected by the presence of *rteR* in *trans.*

2.5 Discussion

CTnDOT has proved to be an interesting mobile genetic element, with a complex regulatory system where stimulation of excision and transfer is dependent upon low levels of tetracycline (21). A small RNA, RteR, was previously suggested as a possible negative regulator that is responsible for inhibition of conjugative transfer in the absence of tetracycline (7, 28). Previous studies had indicated that RteR was an approximately 100 nt sRNA (7), but had only localized RteR to a 500 bp region (28). We report here that the region required for mediating repression of conjugative transfer contains a 90 nt non-coding RNA, RteR. We have identified both the 5′ and 3′ ends of the RteR transcript, and we have also confirmed that the promoter is located at the 3′ end of *exc.*

RteR was previously reported to act within the 13 kb transfer (*tra*) operon (7, 28). Previous studies suggested that RteR was preventing transcription of the entire *tra* operon (7), but we demonstrate here that this is not the case. The previous study

had taken a similar approach as we present here, detecting the *tra* transcript with and without RteR present. However, that study only investigated *traG,* assuming that because the *tra* genes are organized in an operon any effect at *traG* would be representative of the entire *tra* operon. Their results demonstrated that there was barely any *traG* transcript detectable when RteR is present, leading to the conclusion that RteR was preventing transcription of the entire operon. We report that RteR is acting to prevent efficient transcription downstream of *traA*, but *traA* itself is still transcribed.

Our observation that there is a significant decrease in the amount of transcript downstream of *traA*, coupled with the finding that RteR does not result in a decreased half-life of the *tra* mRNA suggests that RteR may be enhancing premature transcription termination. To support this finding we analyzed the *tra* sequence downstream of *traA* and found a region within *traB* that has the potential to form an intrinsic terminator. If this terminator structure within *traB* is what is forming to mediate premature transcription termination, we propose that RteR may be binding the elongating *tra* mRNA, thus altering the *tra* secondary structure. This alteration then results in the formation of this intrinsic terminator. The result would be to abolish transcription of the remaining *tra* operon, and ultimately lead to inhibition of CTnDOT conjugative transfer.

Upstream of this predicted terminator is a region that is complementary to RteR. Importantly, the complementary region of RteR is within the 8 nt single stranded loop and is also one of the only regions of RteR that was conserved in all of the homologues identified. This could explain why the mutant pJW312 (Fig. 2.6A and

2.6B) can no longer repress pLYL72 transfer, because the site directed mutations resulted in this region becoming double stranded, so it may not have been able to bind the *traB* transcript efficiently. Conversely, our mutant pJW318 (Fig. 2.6A and 2.6B) may still be able to bind to *traB* because there is still a largely single stranded loop that has the potential to interact with *traB* despite the change in a part of the single stranded loop.

Our results do not elucidate how CTnDOT is able to overcome RteR-mediated negative regulation upon tetracycline induction. We posit that perhaps the activation of the *tra* operon by Xis2c, Xis2d, and Exc could override the effects of RteR by overwhelming the RteR copy number. Another possibility is that upon tetracycline induction a protein may be produced to sequester RteR, thereby overriding any negative regulation by RteR.

We have demonstrated that *rteR* is not only present in *Bacteroides* spp., but is also present in other members of the Bacteroidetes phylum. This finding is consistent with the observation that CTnDOT is readily transferred within the phylum. CTnDOT-like elements have been observed in close relatives of *Bacteroides* such as *Prevotella, Porphyromonas, Tannerella,* and *Cytophaga* spp.. Our database search revealed that *rteR* is also present on a related conjugative transposon, CTn341, and is found in *B. fragilis* strain YCH46 that contains a CTnERL-like element in the chromosome (24). We also report that we were able to PCR amplify and sequence *rteR* from other *Bacteroides* strains harboring the following conjugative transposons: CTnERL, CTn12256, and CTnV479. RteR is the only sRNA thus far

described in this phylum. RteR is also the first regulatory sRNA to be found on a conjugative transposon.

2.6 Acknowledgements

We are grateful to Carin Vanderpool for thoughtful discussion, and to Nadja Shoemaker for reading of this manuscript. We also thank Jennifer Rice and Divya Balasubramanian for comments and advice with RNA techniques. We thank Erika Bongen for assistance with the database search for RteR homologues.

This work was supported by grant AI 22383 from the National Institutes of Health.

2.7 References

1. **Aiba H, Adhya S, de Crombrugghe B**. 1981. Evidence for two functional gal promoters in intact Escherichia coli cells. J. Biol. Chem. **256**:11905–11910.

2. **Argaman L, Hershberg R, Vogel J, Bejerano G, Wagner EG, Margalit H, Altuvia S**. 2001. Novel small RNA-encoding genes in the intergenic regions of Escherichia coli. Curr. Biol. **11**:941–950.

3. **Bayley DP, Rocha ER, Smith CJ**. 2000. Analysis of cepA and other Bacteroides fragilis genes reveals a unique promoter structure. FEMS Microbiol. Lett. **193**:149–154.

4. **Boyer HW, Roulland-Dussoix D**. 1969. A complementation analysis of the restriction and modification of DNA in Escherichia coli. J. Mol. Biol. **41**:459–472.

5. **Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R**. 2009. Bacterial community variation in human body habitats across space and time. Science **326**:1694–1697.

6. **Goujon M, McWilliam H, Li W, Valentin F, Squizzato S, Paern J, Lopez R**. 2010. A new bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Res. **38**:W695–699.

7. **Jeters RT, Wang G-R, Moon K, Shoemaker NB, Salyers AA**. 2009. Tetracycline-associated transcriptional regulation of transfer genes of the Bacteroides conjugative transposon CTnDOT. J. Bacteriol. **191**:6374–6382.

8. **Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG**. 2007. Clustal W and Clustal X version 2.0. Bioinformatics **23**:2947–2948.

9. **Li LY, Shoemaker NB, Salyers AA**. 1995. Location and characteristics of the transfer region of a Bacteroides conjugative transposon and regulation of transfer genes. J. Bacteriol. **177**:4992–4999.

10. **Mastropaolo MD, Thorson ML, Stevens AM**. 2009. Comparison of Bacteroides thetaiotaomicron and Escherichia coli 16S rRNA gene expression signals. Microbiology (Reading, Engl.) **155**:2683–2693.

11. **Moore WE, Holdeman LV**. 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl Microbiol **27**:961–979.

12. **Rieu I, Powers SJ**. 2009. Real-time quantitative RT-PCR: design, calculations, and statistics. Plant Cell **21**:1031–1033.

13. **Salyers AA, Shoemaker NB**. 1997. Conjugative transposons. Genet. Eng. (N.Y.) **19**:89–100.

14. **Salyers AA, Shoemaker NB, Stevens AM, Li LY**. 1995. Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. Microbiol. Rev. **59**:579–590.

15. **Salyers AA, Gupta A, Wang Y**. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol. **12**:412–416.

16. **Shoemaker NB, Getty C, Guthrie EP, Salyers AA**. 1986. Regions in Bacteroides plasmids pBFTM10 and pB8-51 that allow Escherichia coli-Bacteroides shuttle vectors to be mobilized by IncP plasmids and by a conjugative Bacteroides tetracycline resistance element. J. Bacteriol. **166**:959–965.

17. **Shoemaker NB, Salyers AA**. 1987. Facilitated transfer of IncP beta R751 derivatives from the chromosome of Bacteroides uniformis to Escherichia coli recipients by a conjugative Bacteroides tetracycline resistance element. J. Bacteriol. **169**:3160–3167.

18. **Shoemaker NB, Salyers AA**. 1988. Tetracycline-dependent appearance of plasmidlike forms in Bacteroides uniformis 0061 mediated by conjugal Bacteroides tetracycline resistance elements. J. Bacteriol. **170**:1651–1657.

19. **Shoemaker NB, Vlamakis H, Hayes K, Salyers AA**. 2001. Evidence for extensive resistance gene transfer among Bacteroides spp. and among Bacteroides and other genera in the human colon. Appl. Environ. Microbiol. **67**:561–568.

20. **Shoemaker NB, Wang GR, Stevens AM, Salyers AA**. 1993. Excision, transfer, and integration of NBU1, a mobilizable site-selective insertion element. J. Bacteriol. **175**:6578–6587.

21. **Stevens AM, Shoemaker NB, Li LY, Salyers AA**. 1993. Tetracycline regulation of genes on Bacteroides conjugative transposons. J. Bacteriol. **175**:6134–6141.

22. **Storz G, Opdyke JA, Zhang A**. 2004. Controlling mRNA stability and translation with small, noncoding RNAs. Curr. Opin. Microbiol. **7**:140–144.

23. **Vogel J, Gerhart E, Wagner H**. 2008. Approaches to Identify Novel Non-messenger RNAs in Bacteria and to Investigate their Biological Functions: RNA Mining, p. 595–613. *In* Hartmann, RK, Bindereif, A, Schön, A, Westhof, E (eds.), Handbook of RNA Biochemistry. Wiley-VCH Verlag GmbH.

24. **Wang G-R, Shoemaker NB, Jeters RT, Salyers AA**. 2011. CTn12256, a chimeric Bacteroides conjugative transposon that consists of two independently active mobile elements. Plasmid **66**:93–105.

25. **Waters LS, Storz G**. 2009. Regulatory RNAs in bacteria. Cell **136**:615–628.

26. **Whittle G, Shoemaker NB, Salyers AA**. 2002. The role of Bacteroides conjugative transposons in the dissemination of antibiotic resistance genes. Cell. Mol. Life Sci. **59**:2044–2054.

27. **Whittle G, Salyers AA**. 2002. Bacterial Transposons—An Increasingly Diverse Group of Elements in Streips, UN, Yasbin, RE (eds.), Modern Microbial Genetics. John Wiley & Sons, Inc. p. 385–427.

28. **Whittle G, Shoemaker NB, Salyers AA**. 2002. Characterization of genes involved in modulation of conjugal transfer of the Bacteroides conjugative transposon CTnDOT. J. Bacteriol. **184**:3839–3847.

29. **Zuker M**. 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. **31**:3406–3415.

2.8 Tables and figures

Table 2.1: Bacterial Strains and Plasmids

Strain or plasmid	Relevant phenotype[a]	Description and/or reference
B. thetaiotaomicron 5482		
BT4001	(Rif^R)	Spontaneous RifR mutant *B. thetaiotaomicron*
BT4001 ΩQAB	(Rif^R ,Tc^R)	BT4001 with a site specific insertion of *tetQ-rteA-rte* in the chromosome (28)
BT4100	(Thy-, Tp^R)	A spontaneous thymidine auxotroph mutant of *Bacteroides* type strain BT5482 (17)
BT4100 ΩQABC	(Thy-, Tp^R, Tc^R)	BT4100 with a site specific insertion of the regulator region *tetQ-rteA-rteB-rteC*.
BT4104	(Thy-, Tp^R, Tc^R)	BT4100 with CTnERL integrated into the chromosom (20)
E. coli		
DH5aMCR	RecA	Gibco BRL
HB101	RecA, Str^R	*E. coli* strain used for study of transfer frequency (4)
HB101 (RP1)	RecA, Str^R	HB101 containing the IncPα plasmid RP1 (15)
plasmids		
pAFD1	Ap^R (Em^R)	*E. coli-Bacteroides* shuttle vector containing *ermF*
pGW59	Ap^R (Cf^R)	1.4 kb fragment containing C terminal end of *exc* (28
pGW87	Ap^R (Cf^R)	A 780 bp fragment containing RteR (28)
pLYL05	Ap^R (Cf^R)	*E. coli-Bacteroides* shuttle vector (L.Y. Li, unpublishe data)
pJW100	Ap^R (Cf^R)	450 bp PCR fragment of the RteR region cloned into pLYL05 (this study)
pJW101	Ap^R (Cf^R)	A 370 bp PCR fragment containing RteR cloned into pLYL05 (this study)
pJW102	Ap^R (Cf^R)	A 465 bp PCR fragment of the region containing RteR cloned into pLYL05 (this study)
pJW305	Ap^R (Em^R)	A 283 bp fragment containing RteR cloned into pAFD (this study)
pJW306	Ap^R (Em^R)	A 374 bp fragment containing the RteR region clone into pAFD1 (this study)
pJW310	Ap^R (Em^R)	A 465 bp PCR fragment containing RteR with site direted mutation in putative promoter changing TTT to AAAC (this study)
pJW312	Ap^R (Em^R)	pJW305 with site directed mutations that change positions 49-52 of *rteR* from GGAT to ACCA (this study)
pJW318	Ap^R (Em^R)	Contains *xis2c, xis2d, exc,* and a 30 nt internal deletio within *rteR* (This study)
pLYL72	Kn^R, Cm^R (Cm^R)	Self transmissible plasmid containing *mob* genes*, tra* operon, and OriT from CTnDOT (9)

[a] *Bacteroides* phenotypes are shown in parentheses, whereas *E. coli* phenotypes are shown without parentheses. Abbreviations are Ap, ampicillin; Cf, cefoxitin; Cm, chloramphenicol; Em, erythromycin; Kn, kanamycin; Rif, rifampicin; Str, streptomycin; Tc, tetracycline; Thy, thymidine auxotroph; Tp, trimethoprim.

Table 2.2: Primers used in this study

Primer Name	5´ to 3´ Sequence
JLW10F	gtacacacgtgagataacctccga
JLW11R	tatccctccgagtgccaccactaaat
JLW12F	ttggatttagtggtggcactcgga
RpoD 467 fwd	acctgattcaacgggagacgaca
RpoD 548 rev	acgctgtatggtggattcgtcagt
RteR promoter SDM bottom	aacaattcattactctgaacaagtgggtttgttaatactatttcttccgtcctgaaaatt
RteR promoter SDM top	aattttcaggacggaagaaatagtattaacaaacccacttgttcagagtaatgaattgtt
Tra D-E 5480 fwd	cacacccaagtccgtggaaacaaa
Tra D-E 5561 rev	ttgtcctttgtcgggcggttgata
TraA 3250 fwd	tggaggacgggcattacaaagtga
TraA 3486 rev	tgtctgcgatgatgggtgtgaaga
TraB 4188 fwd	ttcttgacaacgtgctgacc
TraC 4423 rev	cgggaacatcacaggtcttt
TraC 4468 fwd	tcggaaatcaaaacacgaca
TraC 4641 rev	ggcgttttcaatctgttcca
TraD 4812 fwd	accttgcatagtgaagcctcggat
TraD 4954 rev	tcttcttccgacagctcaatgcct
TraE 5629 fwd	aatcctctctgcggcactcatcat
TraE 5811 rev	aatttcccgtacactttcacgccc
TraG 6218 fwd	cccgtctgttcaaacgacaacgaa
TraG 6495 rev	agcgttccttgatgaaccagtcct
TraI 9433 fwd	tcagtgaacaaccttgtccgggat
TraI 9661 rev	tcatggagagcgtggtgatgttca
TraK 11024 fwd	agatctatgtgctggacaacggca
TraK 11441 rev	tttgggttatcgtccgaccgtgat
TraN 13612 fwd	tcaatgtgaaaatacgccgccgaac
TraN 13739 rev	ttcgctgcccagctctttcagata
T7JLW22R	gaaattaatacgactcactatagggataattaacgggataatcggttgga

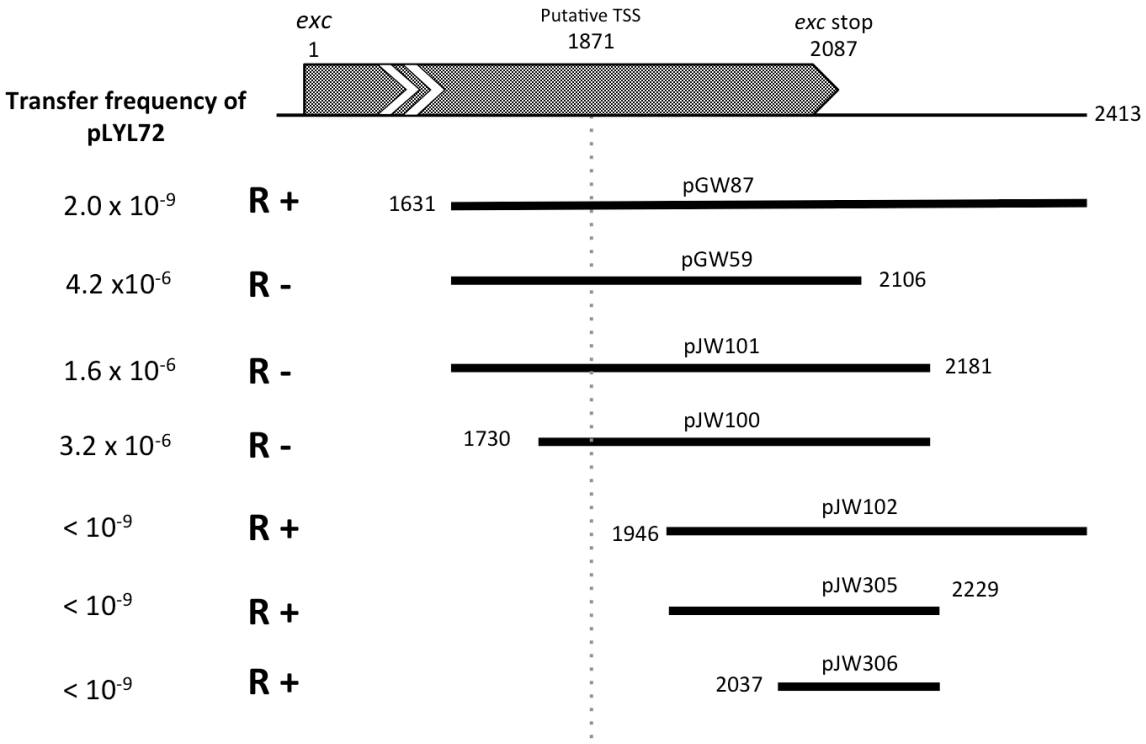


Figure 2.1: Localization of the region containing *rteR* that is required for inhibition of pLYL72 conjugative transfer. A putative transcription start site (TSS) was identified in a previous study, yet this was not consistent with other subcloning results (7). We constructed more subclones to further minimize the region containing *rteR* that is sufficient for pLYL72 transfer inhibition. The transfer frequency of pLYL72, expressed as transconjugants per recipient, is shown to the left of each plasmid construct. Plasmids labeled R+ were able to inhibit conjugative transfer of pLYL72, suggesting an intact copy of RteR was present. Plasmids labeled R- were no longer able to mediate regulation of pLYL72 transfer.

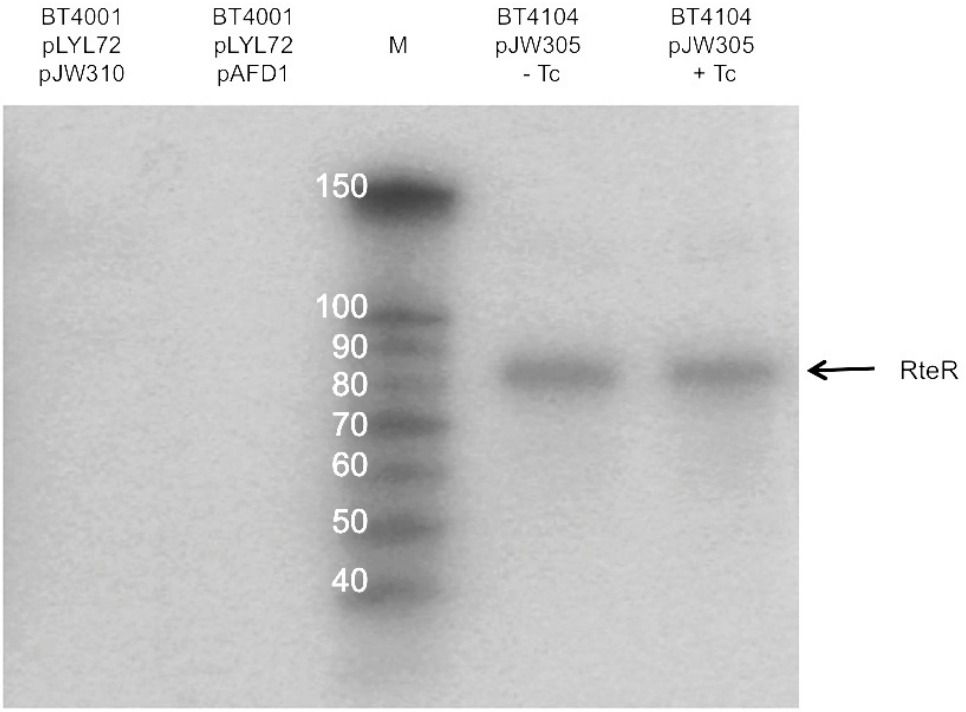


Figure 2.2: Northern analysis of RteR. For Northern analysis of the RteR transcript, a radiolabeled probe was created that was specific to the 200 bp fragment demonstrated to inhibit conjugative transfer of pLYL72. Approximately 2 μg of RNA were loaded per well. Size standards used (M) are shown in the center, with sizes of fragments shown. No RteR was detectable in the empty vector control, BT4001 pLYL72 pAFD1, or in a strain containing mutations that abolish transcription from the *rteR* promoter (BT4001 pLYL72 pJW310). Similar levels of RteR were detected from BT4104 pJW305 whether or not tetracycline (Tc) was present, suggesting that *rteR* is constitutively transcribed.

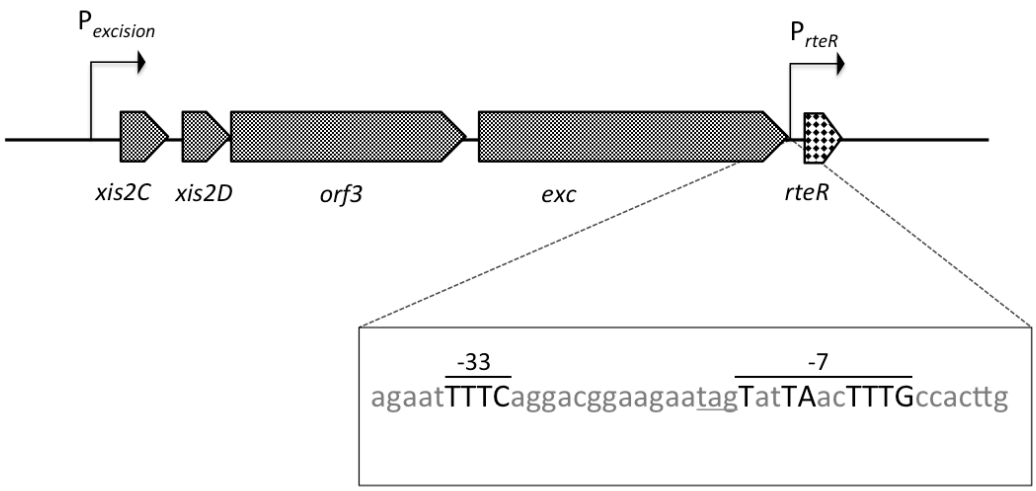


Figure 2.3: The *rteR* promoter flanks the 3´ end of *exc.* A putative promoter was identified near the 3´ end of *exc.* The -7 and -33 regions of the *rteR* promoter are shown in uppercase black text, with the relevant regions of the promoter noted above. The stop codon of *exc* is underlined. Gray arrows represent the genes that are part of the excision operon, whereas *rteR*, which is a negative regulator, is shown as a dotted arrow.

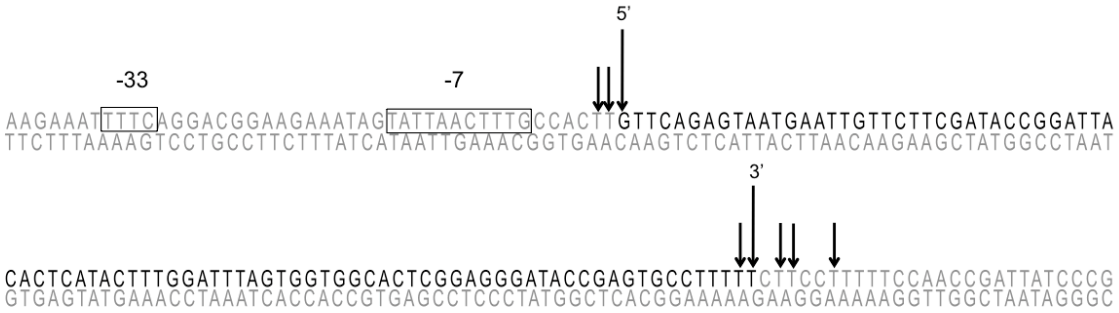


Figure 2.4: RACE analysis to identify the 5´ and 3´ ends of RteR . Rapid amplification of cDNA ends (RACE) was performed to confirm the ends of the RteR transcript. The 5´ end of RteR was mapped to the G nucleotide as indicated by the larger arrow, since this end was identified in 10 out of 15 sequences. Other possible transcription start sites were detectable, as indicated by the shorter arrows at the 5´ end. The 3´ end is indicated by a large black arrow, and was detected in 8 out of 12 samples. The other 3´ ends that were detected are indicated by short black arrows. The nucleotides that we have identified as part of the RteR sequence are shown in black text. The *rteR* promoter identified in this study is boxed, with labels designating the -7 and -33 regions.

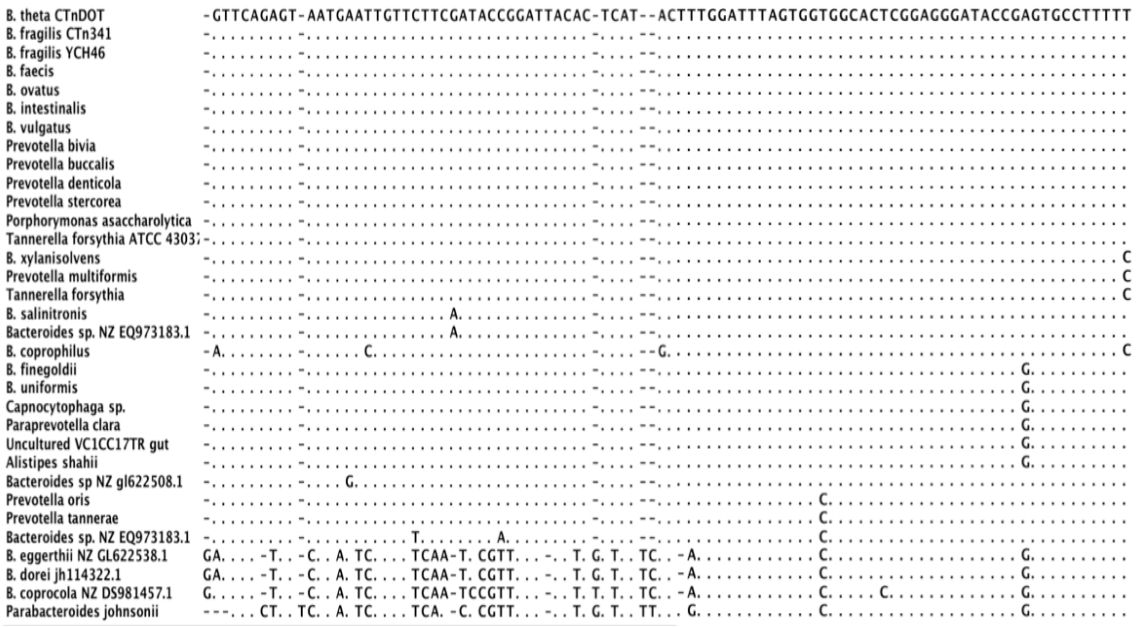


Figure 2.5: A database search reveals that *rteR* homologues are well conserved. ClustalW2 analysis (6, 8) was performed to align sequences of *rteR* homologues identified using basic local alignment search tool (BLAST) analysis. This analysis demonstrates that *rteR* is present among other members of the Bacteroidetes phylum. The sequence of *rteR* is shown in the top row for comparison. If the sequence is a match to *rteR*, the nucleotide is labeled as a dot, whereas any substitutions are shown with the corresponding change in nucleotide. Many homologues had 100% sequence identity. Of the homologues that did have substitutions in the *rteR* sequence, most had approximately 97% or greater sequence identity, but some sequences had as little as 73% identity. All of these homologues have nearly identical secondary structures that were predicted using MFOLD analysis (29).

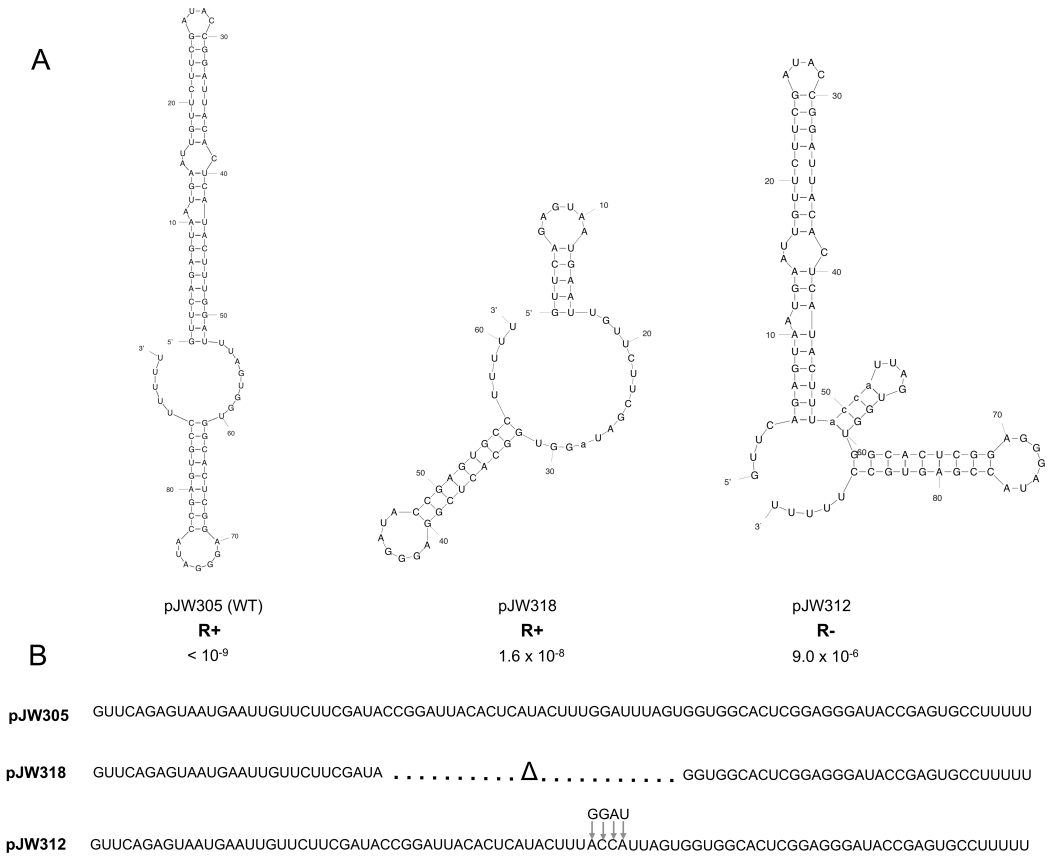


Figure 2.6: The secondary structure of RteR appears to be important for RteR-mediated repression of conjugative transfer. A. The secondary structures of RteR clones were predicted using MFOLD analysis (29). Beneath each structure of RteR is the transfer frequency of pLYL72 when provided in trans, expressed as the number of transconjugants per recipient. Clones that were able to repress transfer of pLYL72 are labeled as R+, and are labeled as R- if the clone could no longer regulate pLYL72 transfer. B. The changes in the primary nucleotide sequence of each of these mutants are shown, relative to wild type RteR.

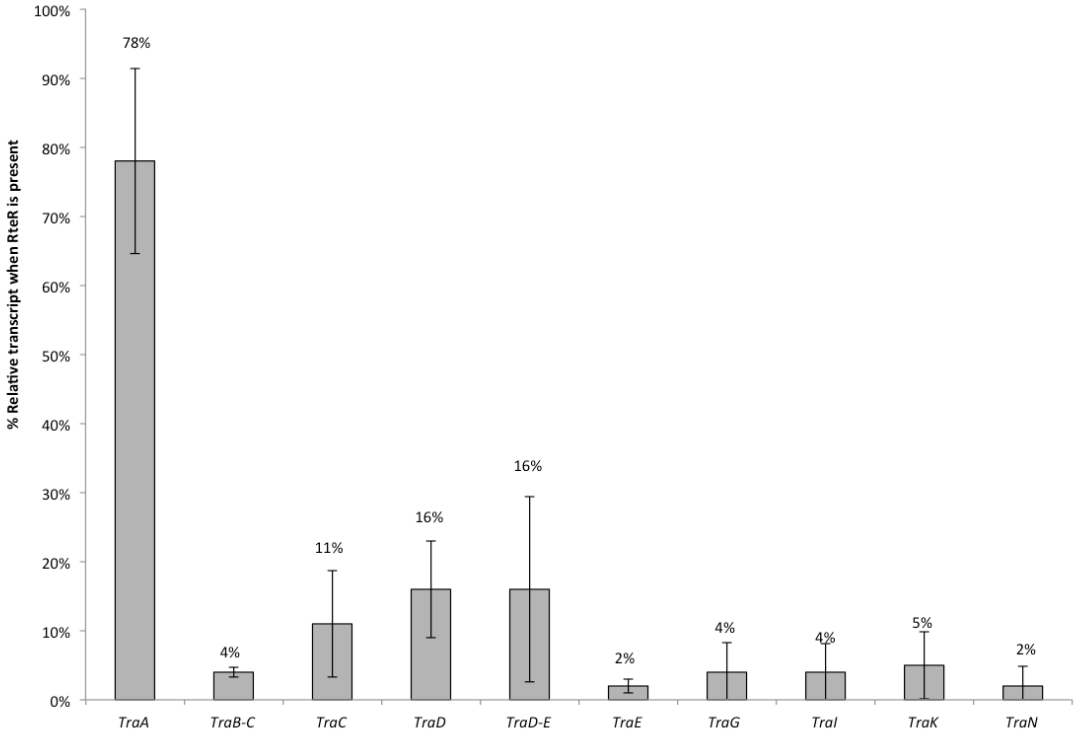


Figure 2.7: RT-qPCR analysis to measure the relative levels of *tra* mRNA in the presence of RteR. RT-qPCR was performed to assess the levels of *tra* transcript that are detected when RteR was present, relative to the amount of transcript without RteR. Samples containing RteR were normalized against samples without RteR to obtain the relative percentage of transcript remaining in the presence of RteR, which is shown above each primer set. The percentage shown for each primer set is an average value of relative transcript present.

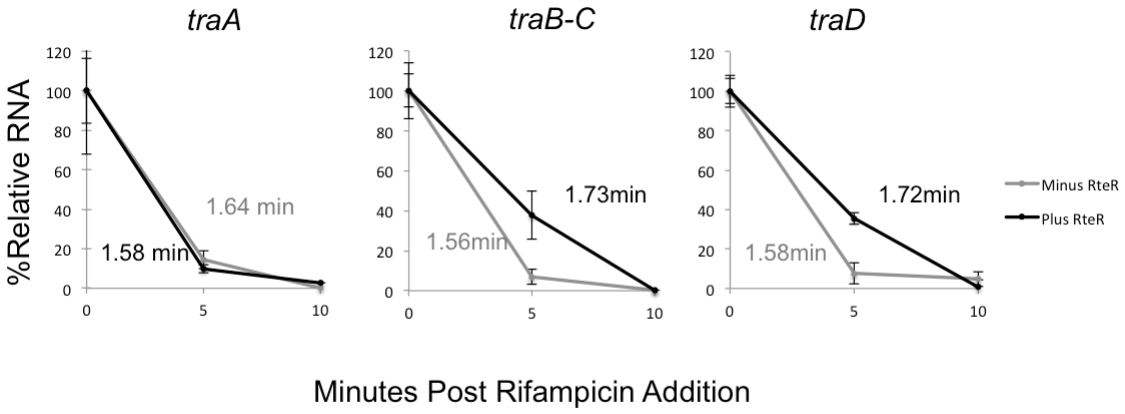


Figure 2.8: The half-life of the *tra* mRNA is similar whether or not RteR is present. A rifampicin chase assay was performed to assess the stability of the *tra* mRNA transcript in both the presence and absence of RteR. 400 µg/mL of rifampicin were added to mid-log phase *Bacteroides* cultures and RNA samples were taken at 0, 5, and 10 minutes post rifampicin addition. Relative RNA levels were then measured using RT-qPCR, and were quantified by normalizing the abundance of RNA remaining over time relative to time 0. Primer sets were used to detect the message at *traA, traB-C,* and *traD.* At all points of the transcript, the half-life was approximately 1.6 minutes whether or not RteR was present, suggesting that RteR does not destabilize the *tra* mRNA transcript. For each primer set, the calculated half-life of the *tra* transcript with RteR present is shown in black text, and the minus RteR half-life is in grey text.

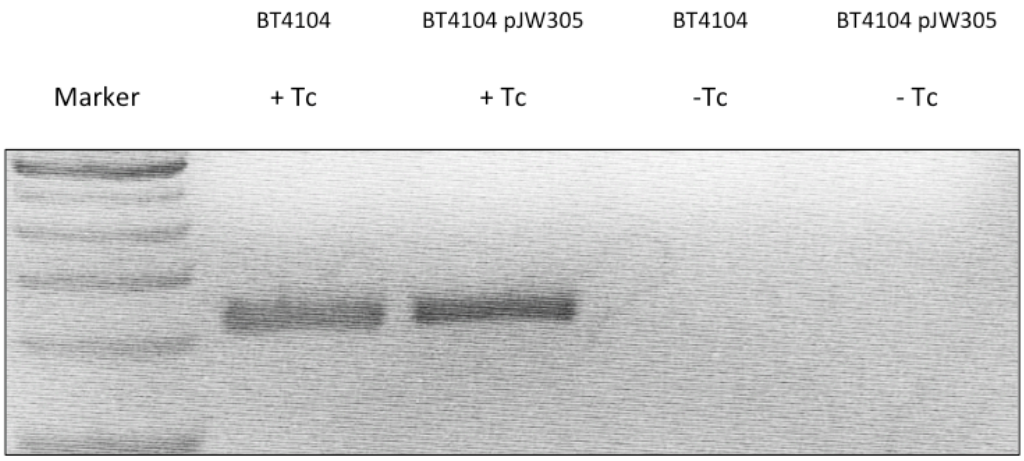


Figure 2.9: An increase of RteR does not affect excision of CTnERL.

To determine whether RteR has an affect on excision from the chromosome, RT-PCR was performed detecting the joined ends of the CTnERL circular intermediate (*attERL*). RNA was isolated from a strain containing a single copy of RteR on the chromosome within CTnERL (BT4104), as well as from the same strain that also contained RteR on a vector with 8-10 copies per cell (BT4104 pJW305). *AttERL* is detected in similar quantites independent of the copy number of RteR when cells are exposed to tetracycline (Tc), suggesting that RteR does not affect excision. No *attERL* is detected in the absence of Tc, because the excision proteins that are required for the formation of *attERL* are only expressed upon Tc induction.

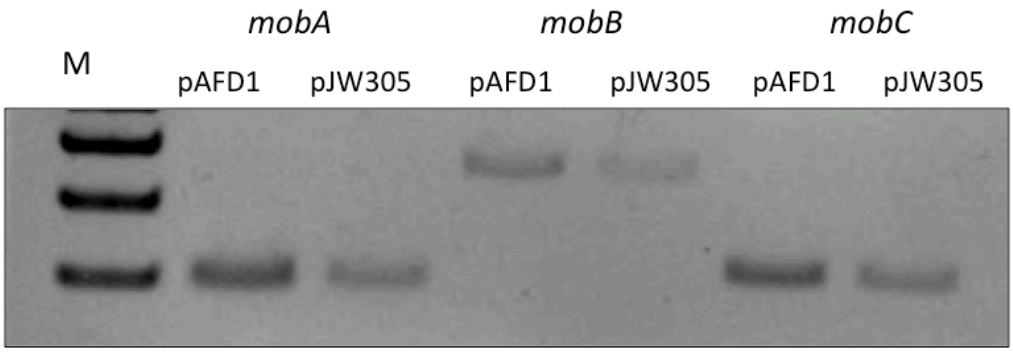


Figure 2.10: The presence of RteR does not affect transcription of the CTnDOT mobilization region. To determine whether the mobilization genes, *mobA, -B,* and –*C,* are a target of RteR, we performed RT-PCR to see whether providing RteR in *trans* had an effect on *mob* expression. RNA was isolated from BT4001 pLYL72 containing an empty vector, pAFD1, or pJW305 that has a single copy of *rteR.* Similar quantities of each of the *mob* genes were detected whether or not *rteR* was present, suggesting that RteR does not affect *mob* transcription. These observations were also confirmed using RT-qPCR.

CHAPTER 3: IDENTIFYING AN HFQ HOMOLOGUE IN *BACTEROIDES THETAIOTAOMICRON*

Small RNAs (sRNAs) are very important elements that are responsible for the regulation of a variety of cell functions such as cellular stress responses, conjugation, plasmid replication, and motility [4, 11–14]. The majority of *trans*-acting small RNAs mediate their regulatory function by binding to the target mRNA, and thereby recruiting the degradosome which results in degradation of a target mRNA::sRNA duplex [2, 3]. This binding and subsequent formation of an sRNA-target mRNA duplex is generally facilitated by the chaperone protein Hfq [13]. There are many examples where a deletion of Hfq results in the loss of sRNA mediated regulation, thus indicating an important role for Hfq in riboregulation [8].

While characterizing RteR, a sRNA encoded on CTnDOT that inhibits conjugative transfer (described further in Chapter 2), we wanted to address whether Hfq was important for RteR-mediated regulation. However, no Hfq or Hfq-like homologue was annotated in the *B. thetaiotaomicron* 5482 (BT5482) genome, nor was there evidence of Hfq present in any other *Bacteroides* spp. [15]. This observation was not a surprise though, as many of the open reading frames in the *Bacteroides* genome are often classified as hypothetical proteins, and are later assigned functions through experimental evidence.

Nonetheless, we wanted to see if we could identify putative Hfq homologues present in *Bacteroides* that could be facilitating the interaction of RteR with the target *tra* mRNA. The logic behind such an exploratory project was that if we were able to

identify any Hfq-like proteins, this would further shed light on RteR-mediated negative regulation. Further, the identification and characterization of potential homologues would have been the basis for another publication.

The majority of research performed with the intent of characterizing Hfq has been performed within the Proteobacteria, especially in *E. coli*, the genetic workhorse of bacteria. Phylogenetically, *E. coli* and *B. thetaiotaomicron* are quite distinct, which can make identifying homologues between these two genera rather difficult [1]. Although Hfq is well conserved among bacteria, an attempt to find an Hfq homologue by BLASTp analysis of the *Bacteroides* genomes using Hfq amino acid sequences did not generate any relevant hits [10]. Shortly after our quest to hunt down an Hfq homologue began, another group identified an Hfq homologue in *Borrelia burgdorferi,* the causative agent of Lyme disease. The publication of this work was quite exciting for us because this Hfq-like protein (Bb0268) was vastly different compared to the canonical Hfq homologues that have been previously described. Interestingly, Hfq from *E. coli* was able to complement a *Borrelia* Hfq deletion mutant despite the fact that they appear to be nothing alike [8]. The remarkable differences between these two Hfq proteins would then hopefully open the door for identifying a homologue within *Bacteroides*. Further, *Borrelia,* which is within the Spirochaetes phylum, is more closely related to the Bacteroidetes than the Proteobacteria [1].

Similarly, a study characterizing a unique Hfq homologue in *Sinorhizobium meliloti* also provided another scaffold for mining the *Bacteroides* genome. The *S. meliloti* Hfq, Smc01113, had very little similarity to Hfq from *E.* coli, but functional studies suggested this protein was a true homologue of Hfq [9]. Although

Sinorhizobium is less related to *Bacteroides* than *Borrelia,* the novelty of this Hfq homologue increased our likelihood of finding potential homologues within the BT5482 genome.

When we first started searching for homologues, a close match to *E. coli* Hfq was identified in *Bacteroides cappilosus.* Surprisingly though, no homologues of the *B. cappilosus* Hfq-like protein were found in any of the other *Bacteroides* genomes. Closer inspection of *B. cappilosus* revealed that this organism shares 76% 16s identity with *B. thetaiotaomicron.* After further investigation, I found that *B. cappilosus,* despite being called *Bacteroides,* is actually described as a Firmicute in the NCBI database.

A BLASTp search comparing the *B. burgdorferi* homologue or *S. meliloti* homologue against the *B. thetaiotaomicron* genome yielded matches with poor coverage, limited identity, and high E-value scores, which gave us little confidence that we were identifying true homologues of Hfq. We did not give up, however, and instead moved to a method of further identifying possible Hfq-like proteins in *Bacteroides* by searching for structural homologues using Phyre [6]. By making functional predictions based on predicted secondary structural homologues, we could then narrow down our list of homologues and parse out those that should warrant further investigation.

I first used to Phyre to see what predictions would be made with Bb0268 and Smc01113, as these proteins did not have primary sequence homology to other known Hfq homologues. The Phyre prediction for *Borrelia* Bb0268 was a neurotransmitter-gated ion channel pore; however, the estimated precision was

rather low (25%) which suggested it was not accurate. The structural prediction for the *S. meliloti* Hfq homologue Smc01113 suggested with great precision (100%) that this protein is a Zincin-like metalloprotease. Although quite different from Hfq, we identified a *Bacteroides* protein Bt3283, which has some similarity at the primary sequence level to Smc01113. Interestingly, Phyre also generated the same structural prediction for Bt3283, suggesting this as the likely first candidate that could function as Hfq within *Bacteroides.* Smc01113 is a 168 AA protein with a pI of 4.2. Similarly, BT3283 is 137 AA with a pI of 4.7. Although not exact, the similarities between Smc01113 and BT3283 warrants further investigation of BT3283 is a putative Hfq homologue. A pairwise alignment of these two proteins is shown in Figure 3.1, along with the Phyre predicted structures.

The next candidate homologues identified in *B. thetaiotaomicron*, were identified through a combination of BLASTp searches and Phyre structural predictions. BT0784 and BT4380 are predicted with high precision (100% and 85% respectively) to be involved in RNA binding. Last, BT0740 came up as a potential homologue, but is predicted to be involved in electron transport based on Phyre predictions. A ClustalW alignment of these *B. thetaiotaomicron* putative homologues is shown along with the known Hfq homologues from *E. coli, B. burgdorferi,* and *S. meliloti* (Figure 3.2). There is little identity among these homologues, which is neither surprising nor entirely discouraging. Although not conclusive, we have narrowed down some proteins that could potentially be Hfq homologues in *B. thetaiotaomicron,* with BT3283 being the most likely homologue based on our preliminary search.

A list of the remaining *Bacteroides* proteins that came up in our search is shown in Table 3.1. Those that are colored in bold print were hits that came up multiple times or were in the rough ballpark size of other Hfq homologues (roughly 80-250 AA).

3.1 References

1. **Boussau B., L. Guéguen, and M. Gouy**. 2008. Accounting for horizontal gene transfers explains conflicting hypotheses regarding the position of aquificales in the phylogeny of Bacteria. BMC Evolutionary Biology **8**:272.

2. **Burger A., C. Whiteley, and A. Boshoff**. 2011. Current perspectives of the Escherichia coli RNA degradosome. Biotechnol. Lett. **33**:2337–2350.

3. **Carpousis A. J.** 2007. The RNA degradosome of Escherichia coli: an mRNA-degrading machine assembled on RNase E. Annu. Rev. Microbiol. **61**:71–87.

4. **Gottesman S., C. A. McCullen, M. Guillier, C. K. Vanderpool, N. Majdalani, J. Benhammou, K. M. Thompson, P. C. FitzGerald, N. A. Sowa, and D. J. FitzGerald**. 2006. Small RNA regulators and the bacterial response to stress. Cold Spring Harb. Symp. Quant. Biol. **71**:1–11.

5. **Goujon M., H. McWilliam, W. Li, F. Valentin, S. Squizzato, J. Paern, and R. Lopez**. 2010. A new bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Res. **38**:W695–699.

6. **Kelley L. A., and M. J. E. Sternberg**. 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc **4**:363–371.

7. **Larkin M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins**. 2007. Clustal W and Clustal X version 2.0. Bioinformatics **23**:2947–2948.

8. **Lybecker M. C., C. A. Abel, A. L. Feig, and D. S. Samuels**. 2010. Identification and function of the RNA chaperone Hfq in the Lyme disease spirochete Borrelia burgdorferi. Mol. Microbiol. **78**:622–635.

9. **Pandey S. P., B. K. Minesinger, J. Kumar, and G. C. Walker**. 2011. A highly conserved protein of unknown function in Sinorhizobium meliloti affects sRNA regulation similar to Hfq. Nucleic Acids Res. **39**:4691–4708.

10. **Sun X., I. Zhulin, and R. M. Wartell**. 2002. Predicted structure and phyletic distribution of the RNA-binding protein Hfq. Nucleic Acids Res. **30**:3662–3671.

11. **Tomita H., and D. B. Clewell**. 2000. A pAD1-encoded small RNA molecule, mD, negatively regulates Enterococcus faecalis pheromone response by enhancing transcription termination. J. Bacteriol. **182**:1062–1073.

12. **Vanderpool C. K., and S. Gottesman**. 2004. Involvement of a novel transcriptional activator and small RNA in post-transcriptional regulation of the glucose phosphoenolpyruvate phosphotransferase system. Mol. Microbiol. **54**:1076–1089.

13. **Waters L. S., and G. Storz**. 2009. Regulatory RNAs in bacteria. Cell **136**:615–628.

14. **Wong J. J. W., J. Lu, and J. N. M. Glover**. 2012. Relaxosome function and conjugation regulation in F-like plasmids - a structural biology perspective. Mol. Microbiol. **85**:602–617.

15. **Xu J., M. K. Bjursell, J. Himrod, S. Deng, L. K. Carmichael, H. C. Chiang, L. V. Hooper, and J. I. Gordon**. 2003. A genomic view of the human-Bacteroides thetaiotaomicron symbiosis. Science **299**:2074–2076.

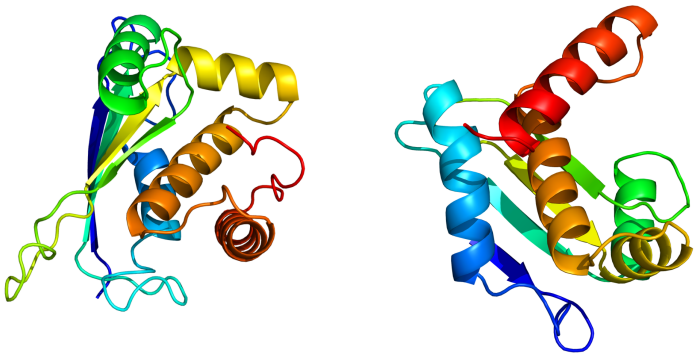
3.2 Tables and figures

A.

```
S_meliloti_Smc01113     MTALDIQISVEAGDWPPEDELQSFCERVLEAAADFLAREENQPLPAQAAELSLVFTDDQS
BT_3283                 ---MAVTYQTEGVKMP-DIKKRETTEWIKNVAASYGKR---------LGEIAYIFCSDEK
                           : :  ..*. . * : : :.  * : :.**.:  *          .*:: :* .*:.

S_meliloti_Smc01113     IRAINAEWRGQDKATNVLSFPAFPVTPGRMPGPMLGDIVVAHETLRREAAELEKPFDAHL
BT_3283                 ILEVNRQYLQHDYYTDIITFDYCQGDR------LSGDLFISLDTIRTNAEQFGAAYDDEL
                        *  :* ::  :*  *::::*             : **:.:: :*:* :* ::  .:* .*

S_meliloti_Smc01113     THLLVHGFLHLFGYDHIEDDEAERMEGLETRILARLGLSDPYGDQPPH
BT_3283                 HRVIIHGILHLCGINDKGPGEREIMEEAENKALAMR-----------
                         ::::**:*** * :.   .* * **  *.: **
```



B.

Figure 3.1: BT3283 has limited sequence homology to the *S. meliloti* Hfq homologue Smc01113, and Phyre secondary structure predictions suggest structural homology. A. A pairwise alignment of the *S. meliloti* Hfq-like protein Smc01113 and putative *Bacteroides* homologue BT3283 was generated using ClustalW. The pairwise alignment generated a score of 20%, which is calculated by the number of identities between the two proteins divided by the length of the sequence. B. Phyre structural predictions suggest that both Smc01113 (Left) and BT3283 (right) are Zincin-like metalloproteases. Smc01113 is reported to affect sRNA regulation similar to Hfq, suggesting this is a true Hfq homologue [9]. Both models shown are >93% of residues modeled at >90% confidence.

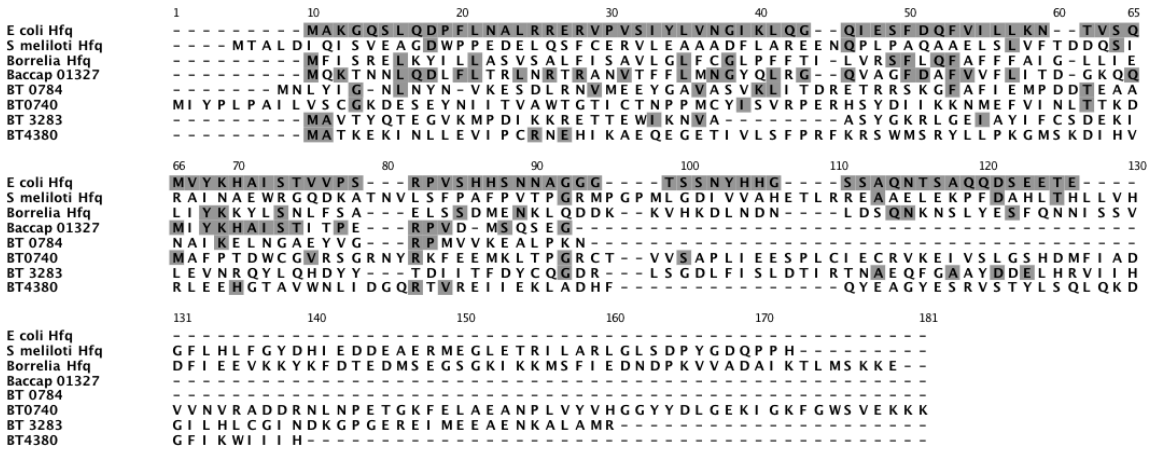


Figure 3.2: Putative *B. thetaiotaomicron* Hfq homologues do not resemble Hfq from *E. coli*, but neither do Hfq known Hfq homologues from *S. meliloti* and *B. burgderfori.* Our top four candidates of *Bacteroides* homologues were aligned with known Hfq homologues from *E. coli, B. burgdorferi,* and *S. meliloti.* Alignments were generated using ClustalW [5, 7], and sequences where the amino acid is an exact match to the *E. coli* Hfq are colored in gray.

Table 3.1: Low confidence putative Hfq homologues identified using BLASTp

BT5482 Prot. No		Acc. No. of Homologue	Genus of Homologue
BT	46	CAH35519.1	*Burkholderia*
BT	58	YP_002731196.1	*Persephonella*
BT	124	NP_243231.1	*Bacillus*
BT	158	NP_212402.1	*Borrelia*
BT	**206**	**YP_001681128.1**	***Heliobacterium***
BT	**206**	**YP_429973.1**	***Moorela***
BT	**206**	**YP_075575.1**	***symbiobacterium***
BT	224	YP_003505574.1	*Denitrovibrio*
BT	298	NP_243231.1	*Bacillus*
BT	342	baccap_01327	*B. cappilosusilosus*
BT	362	YP_429973.1	*Moorela*
BT	383	NP_243231.1	*Bacillus*
BT	383	YP_002121914.1	*Hydrogenobaculum*
BT	450	PDB 1Q1S	*Staphylococcus*
BT	470	YP_003505574.1	*Denitrovibrio*
BT	502	NP_243231.1	*Bacillus*
BT	504	NP_243231.1	*Bacillus*
BT	504	YP_075575.1	*symbiobacterium*
BT	**554**	**YP_003827750.1**	***Acetohalobium***
BT	**554**	**baccap_01327**	***B. cappilosus***
BT	**554**	**NP_243231.1**	***Bacillus***
BT	**554**	**YP_429973.1**	***Moorela***
BT	**566**	**NP_243231.1**	***Bacillus***
BT	**566**	**YP_001681128.1**	***Heliobacterium***
BT	**566**	**ZP_05405115.1**	***Mitsuokela***
BT	570	NP_212402.1	*Borrelia*
BT	634	NP_212402.1	*Borrelia*
BT	664	NP_243231.1	*Bacillus*
BT	695	NP_243231.1	*Bacillus*
BT	823	NP_243231.1	*Bacillus*
BT	866	NP_243231.1	*Bacillus*
BT	867	NP_243231.1	*Bacillus*
BT	1019	NP_243231.1	*Bacillus*
BT	**1020**	**baccap_01327**	***B. cappilosus***
BT	**1020**	**NP_243231.1**	***Bacillus***
BT	**1020**	**YP_001681128.1**	***Heliobacterium***
BT	**1020**	**YP_429973.1**	***Moorela***
BT	**1020**	**YP_075575.1**	***symbiobacterium***
BT	1029	baccap_01327	*B. cappilosus*
BT	1033	YP_429973.1	*Moorela*
BT	1162	NP_243231.1	*Bacillus*
BT	1223	YP_075575.1	*symbiobacterium*
BT	1268	ACE63256.1	*E. coli*
BT	1281	YP_075575.1	*symbiobacterium*
BT	1289	NP_243231.1	*Bacillus*
BT	1362	NP_243231.1	*Bacillus*

Table 3.1 (cont.)

BT5482 Prot. No.		Acc. No. of Homologue	Genus of Homologue
BT	1381	ZP_05405115.1	*Mitsuokela*
BT	1445	NP_243231.1	*Bacillus*
BT	1476	NP_243231.1	*Bacillus*
BT	1553	CAH35519.1	*Burkholderia*
BT	1600	NP_212402.1	*Borrelia*
BT	1628	YP_001681128.1	*Heliobacterium*
BT	1649	YP_002121914.1	*Hydrogenobaculum*
BT	1649	CAH35519.1	*Burkholderia*
BT	1751	NP_243231.1	*Bacillus*
BT	1768	NP_243231.1	*Bacillus*
BT	1772	NP_243231.1	*Bacillus*
BT	1781	YP_001681128.1	*Heliobacterium*
BT	1809	YP_075575.1	*symbiobacterium*
BT	1837	NP_243231.1	*Bacillus*
BT	1855	PDB 1Q1S	*S. Aureus*
BT	1934	NP_243231.1	*Bacillus*
BT	1934	YP_075575.1	*symbiobacterium*
BT	1936	CAH35519.1	*Burkholderia*
BT	1993	NP_243231.1	*Bacillus*
BT	2000	NP_243231.1	*Bacillus*
BT	2000	YP_429973.1	*Moorela*
BT	2050	YP_429973.1	*Moorela*
BT	**2069**	**NP_243231.1**	***Bacillus***
BT	**2069**	**YP_075575.1**	***symbiobacterium***
BT	**2069**	**ZP_05405115.1**	***Mitsuokela***
BT	2073	NP_243231.1	*Bacillus*
BT	2073	YP_075575.1	*symbiobacterium*
BT	2082	ZP_05405115.1	*Mitsuokela*
BT	2087	ZP_05405115.1	*Mitsuokela*
BT	2107	NP_243231.1	*Bacillus*
BT	2167	PDB 1Q1S	*S. Aureus*
BT	2189	ZP_02035730.1	*B. cappilosus*
BT	2194	CAH35519.1	*Burkholderia*
BT	2215	NP_212402.1	*Borrelia*
BT	2217	NP_243231.1	*Bacillus*
BT	2219	NP_243231.1	*Bacillus*
BT	2244	YP_429973.1	*Moorela*
BT	2326	ACE63256.1	*E. coli*
BT	2417	NP_243231.1	*Bacillus*
BT	**2425**	**YP_003827750.1**	***acetohalobium***
BT	**2425**	**baccap_01327**	***B. cappilosus***
BT	**2425**	**NP_243231.1**	***Bacillus***
BT	**2425**	**YP_001681128.1**	***Heliobacterium***
BT	**2425**	**YP_429973.1**	***Moorela***
BT	**2425**	**YP_003577953.1**	***Rhodobacter***
BT	**2425**	**YP_075575.1**	***symbiobacterium***

Table 3.1 (cont.)

BT5482 Prot. No.		Acc. No. of homologue	Genus of homologue
BT	**2425**	**ZP_05405115.1**	***Mitsuokela***
BT	2425	YP_003505574.1	*Denitrovibrio*
BT	2432	YP_002121914.1	*Hydrogenobaculum*
BT	2537	YP_429973.1	*Moorela*
BT	2576	YP_075575.1	*symbiobacterium*
BT	2590	NP_243231.1	*Bacillus*
BT	2656	CAH35519.1	*Burkholderia*
BT	**2729**	**baccap_01327**	***B. cappilosus***
BT	**2729**	**NP_243231.1**	***Bacillus***
BT	**2729**	**YP_001681128.1**	***Heliobacterium***
BT	2796	NP_243231.1	*Bacillus*
BT	2818	ZP_05405115.1	*Mitsuokela*
BT	2895	YP_075575.1	*symbiobacterium*
BT	2898	YP_075575.1	*symbiobacterium*
BT	2917	YP_003505574.1	*Denitrovibrio*
BT	2943	ACE63256.1	*E. coli*
BT	2965	NP_243231.1	*Bacillus*
BT	2999	YP_429973.1	*Moorela*
BT	3035	ACE63256.1	*E. coli*
BT	3050	NP_243231.1	*Bacillus*
BT	3051	YP_001681128.1	*Heliobacterium*
BT	**3067**	**ZP_02035730.1**	***B. cappilosus***
BT	**3067**	**NP_243231.1**	***Bacillus***
BT	**3067**	**YP_003577953.1**	***Rhodobacter***
BT	3076	NP_243231.1	*Bacillus*
BT	3102	ACE63256.1	*E. coli*
BT	3102	AAT39524.1	*V. harveyi*
BT	3102	CAH35519.1	*Burkholderia*
BT	3121	YP_003505574.1	*Denitrovibrio*
BT	3167	CAH35519.1	*Burkholderia*
BT	3169	ZP_05405115.1	*Mitsuokela*
BT	**3192**	**YP_003827750.1**	***acetohalobium***
BT	**3192**	**ZP_02035730.1**	***B. cappilosus***
BT	**3192**	**NP_243231.1**	***Bacillus***
BT	**3192**	**ZP_06883448.1**	***Clostridium***
BT	**3192**	**YP_001681128.1**	***Heliobacterium***
BT	**3192**	**YP_003577953.1**	***Rhodobacter***
BT	3192	YP_003505574.1	*Denitrovibrio*
BT	3192	CAH35519.1	*Burkholderia*
BT	3253	NP_243231.1	*Bacillus*
BT	3292	YP_003827750.1	*acetohalobium*
BT	3292	YP_001681128.1	*Heliobacterium*
BT	3299	NP_243231.1	*Bacillus*
BT	3299	YP_075575.1	*symbiobacterium*
BT	3304	YP_429973.1	*Moorela*
BT	3327	NP_243231.1	*Bacillus*

Table 3.1 (cont.)

BT5482 Prot. No.		Acc. No. of homologue	Genus of homologue
BT	3327	YP_429973.1	*Moorela*
BT	3347	YP_003505574.1	*Denitrovibrio*
BT	3355	NP_243231.1	*Bacillus*
BT	3440	YP_075575.1	*symbiobacterium*
BT	3440	ZP_05405115.1	*Mitsuokela*
BT	3482	PDB 1Q1S	*S. Aureus*
BT	3592	YP_002121914.1	*Hydrogenobaculum*
BT	3613	ACE63256.1	*E. coli*
BT	3630	ZP_04584326.1	*sulfurihydrogenibium*
BT	3633	NP_243231.1	*Bacillus*
BT	3648	YP_075575.1	*symbiobacterium*
BT	3664	NP_243231.1	*Bacillus*
BT	3680	CAH35519.1	*Burkholderia*
BT	3695	CAH35519.1	*Burkholderia*
BT	3725	NP_243231.1	*Bacillus*
BT	3739	NP_212402.1	*Borrelia*
BT	3744	ZP_05405115.1	*Mitsuokela*
BT	3809	NP_243231.1	*Bacillus*
BT	3903	NP_212402.1	*Borrelia*
BT	3938	CAH35519.1	*Burkholderia*
BT	3951	NP_243231.1	*Bacillus*
BT	3975	NP_243231.1	*Bacillus*
BT	4069	NP_243231.1	*Bacillus*
BT	4119	ACE63256.1	*E. coli*
BT	4124	NP_243231.1	*Bacillus*
BT	4124	YP_001681128.1	*Heliobacterium*
BT	4135	NP_243231.1	*Bacillus*
BT	4166	YP_003505574.1	*Denitrovibrio*
BT	4168	YP_003505574.1	*Denitrovibrio*
BT	4170	YP_001681128.1	*Heliobacterium*
BT	4170	YP_075575.1	*symbiobacterium*
BT	4181	YP_429973.1	*Moorela*
BT	4191	NP_243231.1	*Bacillus*
BT	4193	baccap_01327	*B. cappilosus*
BT	4201	YP_075575.1	*symbiobacterium*
BT	4296	NP_243231.1	*Bacillus*
BT	4302	NP_212402.1	*Borrelia*
BT	4397	ZP_05405115.1	*Mitsuokela*
BT	4412	CAH35519.1	*Burkholderia*
BT	4469	NP_243231.1	*Bacillus*
BT	4484	NP_212402.1	*Borrelia*
BT	4535	YP_002121914.1	*Hydrogenobaculum*
BT	4548	NP_212402.1	*Borrelia*
BT	4613	NP_243231.1	*Bacillus*
BT	4650	NP_212402.1	*Borrelia*
BT	4684	NP_243231.1	*Bacillus*
BT	4684	YP_075575.1	*Symbiobacterium*

Table 3.1 (cont.)

BT5482 Prot. No.		Acc. No. of homologue	Genus of homologue
BT	4738	ZP_04584326.1	*Sulfurihydrogenibium*
BT	4744	NP_243231.1	*Bacillus*
BT	4745	YP_002121914.1	*Hydrogenobaculum*
BT	murE	ZP_05405115.1	*Mitsuokela*
BT	p548214	YP_003505574.1	*Denitrovibrio*

CHAPTER 4: TETRACYCLINE RELATED TRANSCRIPTIONAL REGULATION OF THE CTNDOT MOBILIZATION REGION

4.1 Abstract

CTnDOT is a 65 kb conjugative transposon (CTn) in *Bacteroides* spp. that confers resistance to the antibiotics erythromycin and tetracycline (Tc). Conjugative transfer of CTnDOT is regulated upon exposure to Tc. In the absence of Tc, no transfer is detectable. However, upon Tc induction a cascade of regulatory events results in the conjugative transfer of CTnDOT. Previous studies addressing regulation of CTnDOT conjugative transfer focused primarily on the 13 kb transfer (*tra*) operon, which encodes the proteins required for assembly of the mating apparatus. We report here that the *mob* operon that encodes the relaxase and coupling proteins required for mobilization of CTnDOT, which is divergently transcribed from the *tra* region, is regulated at the transcriptional level upon Tc induction. The excision proteins, Xis2d and Exc are required for the up-regulation of *mob* transcription upon Tc induction, yet a deletion of *xis2c* has no effect. Although a deletion of *exc* reduces *mob* transcription, there is no defect in pLYL72 transfer, suggesting that the abundance of *mob* transcript does not directly affect transfer frequency. A repressor of *mob* transcription is also encoded on CTnDOT, and preliminary evidence suggests that Orf2 may be a negative regulator of the *mob* operon.

4.2 Introduction

Bacteroides spp. are Gram-negative obligate anaerobes that are primarily found within the lower part of the human gastrointestinal tract. *Bacteroides* are an abundant member of the gut microbiota, and play an important role within this environment because they aid in processes such as nutrient acquisition and pathogen exclusion (6, 18, 26). However, *Bacteroides* can be detrimental to the host if they escape the colon, causing opportunistic infections that may be difficult to treat. These infections may not respond well to antibiotic therapy due to the increasing prevalence of antibiotic resistance within *Bacteroides,* which is generally due to the dissemination of mobile genetic elements within the colon (14, 26, 28). One common mobile element is CTnDOT, a 65 kb conjugative transposon that encodes resistance to the antibiotics erythromycin (*ermF*) and tetracycline (*tetQ*).

An interesting feature of CTnDOT is that excision and conjugative transfer of CTnDOT are dependent upon tetracycline (Tc) exposure (19, 22, 23). In the absence of Tc, there is no detectable transfer of CTnDOT (30). Upon exposure to low levels of Tc (< 1 μg/mL), a regulatory cascade initiates through the *tetQ-rteA-rteB* operon, which is regulated by a translational attenuation mechanism. RteB activates the transcription of *rteC,* and RteC in turn activates transcription of the excision operon that contains *xis2c, xis2d, orf3* and *exc* (15, 23). Xis2c, -2d, and Exc not only function to excise CTnDOT from the chromosome, but Xis2c and -2d also activate transcription of the *tra* operon, which encodes the mating apparatus (7, 8). We have yet to identify any regulatory or structural role for Orf3. An overview of the steps involved in the regulation of CTnDOT conjugative transfer is summarized in Figure 1.

Many studies have described transcriptional regulation of both excision and transfer of CTnDOT (7, 9, 13, 15), but thus far we have yet to characterize a very important set of genes involved in CTnDOT conjugative transfer: the mobilization region. The CTnDOT *mob* genes encode proteins that are involved in formation of the relaxosome that is required to nick the *oriT* and for coupling the CTnDOT circular intermediate to the mating bridge (16).

We report here that the *mob* genes of CTnDOT are part of a 4 kb operon that is divergently transcribed from the transfer (*tra*) operon. Like the *tra* operon, the *mob* genes are also regulated at the transcriptional level by Tc induction. CTnDOT *mob* mRNA is only detectable from the element in the chromosome when cells have been exposed to Tc. However, if detected from the self-transmissible plasmid pLYL72, a transcript was seen whether or not Tc was present, demonstrating that the *mob* operon is actually transcribed constitutively with respect to Tc exposure. This observation suggests that CTnDOT encodes transcriptional activators that are enhancing the level of transcription upon Tc induction, and that a repressor of *mob* transcription is also present on CTnDOT that serves to prevent *mob* transcription in the absence of Tc. Xis2d and Exc are required for enhancement of *mob* transcription upon Tc induction. Xis2c, which is required for activation of the neighboring *tra* genes, does not appear to play a role in *mob* activation because a deletion of *xis2c* shows no detectable defect in *mob* transcription. This observation was surprising to us given the close proximity between the *mob* and *tra* promoters. Nonetheless, this further demonstrates the complex nature of coordinating excision and transfer of CTnDOT. Preliminary evidence suggests that the repressor of *mob* expression when

no Tc is present may be *orf2.* Although *orf2* is annotated as a hypothetical protein, structural homologue predictions suggest that Orf2 is a winged-helix DNA binding protein (10).

Not only did we characterize the transcriptional regulation of the *mob* operon, but we also wanted to confirm the location of the *oriT,* which is the DNA site nicked by the relaxases to initiate mobilization of CTnDOT. We had previously identified an *oriT* that was downstream of *mobC;* however, the *oriT* on a CTnDOT-like element CTn341 is in fact upstream of *mobA* (16). The upstream *mobA* regions are approximately 94% identical, which prompted us to evaluate whether this region on CTnDOT also contained an *oriT.* We report that the upstream *mobA* region from CTnDOT does contain an *oriT,* similar to CTn341. However, the CTnDOT-like element CTnERL, which is remarkably similar to CTnDOT with the exception that CTnERL is lacking the 13 kb *ermF* region, does not recognize the upstream *mobA oriT* (1, 27). CTnDOT does possess an additional *oriT* that is located in a 900 bp region downstream of *mobC,* and CTnERL was able to recognize that *oriT* region. It is unique that CTnDOT harbors two *oriT* regions, as most elements have a single *oriT.*

Last, we report that the enhancement of *mob* transcription upon Tc induction does not appear to directly correspond with frequency of conjugative transfer. A deletion of *exc*, which is required for *mob* transcriptional enhancement but has no role in *tra* regulation, shows no defect in the transfer frequency of pLYL72. Further, we show that the *mob* genes are transcribed at a higher level on CTnDOT compared to CTnERL, yet these two CTns possess the same frequency of conjugative transfer.

4.3 Materials and methods

Bacterial strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this study are summarized in Table 4.1. Unless otherwise noted, all *Bacteroides* strains were initially cultured in chopped meat broth (Remel), and then transferred to anaerobic trypticase-yeast extract-glucose (TYG) medium (20) for overnight growth with antibiotics when appropriate. Sub-culturing of *Bacteroides* strains was also done in TYG liquid medium, and all *Bacteroides* culturing was performed anaerobically at 37 ^{o}C. *E. coli* was grown aerobically in Luria-Bertani (LB) broth at 37 ^{o}C, with antibiotics when appropriate. Unless otherwise noted, antibiotic concentrations used for culturing were as follows: Ampicillin 100 μg/mL; cefoxitin 10 μg/mL; chloramphenicol 10 μg/mL; erythromycin 10 μg/mL; gentamycin 200 μg/mL; kanamycin 100 μg/mL; rifampicin 2 μg/mL; streptomycin 100 μg/mL; tetracycline 1 μg/mL.

Bacterial mating assays

E. coli strain HB101 containing the IncPα plasmid RPI was used to mobilize plasmids from an *E. coli* donor strain to *Bacteroides* recipients, as described previously (20).

For mating out assays measuring the transfer frequency of pLYL72 from a *Bacteroides* donor to recipient *E. coli* (HB101), the procedure was performed as described previously (12). The transfer frequency of pLYL72 is represented as the ratio of transconjugants per recipient.

RNA isolation

Bacteroides strains were grown in anaerobic TYG medium to mid-log phase, which corresponds to an OD_{650} of 0.3 – 0.5. RNA was then isolated using the QIAgen RNeasy kit. Removal of contaminating genomic DNA was performed using the Ambion turbo DNA-free reagents, and were subsequently tested to confirm that no DNA was remaining by performing qualitative RT-PCR reactions omitting the reverse transcriptase. RNA concentrations were measured using a nanodrop 2000c spectrophotometer (Thermo Scientific).

Qualitative RT-PCR

RT-PCR was performed using the Access RT-PCR system (Promega). Reactions that omitted reverse transcriptase were tested to ensure that all genomic DNA was adequately removed. Samples were electrophoresed on a 1.4-1.5% agarose gel for analysis. Amplification of *rpoD* was used as a control to ensure that equal concentrations of RNA were present in each sample tested. Sequences of the primers used for qualitative RT-PCR are shown in Table 4.2.

RT-qPCR

Synthesis of cDNA was performed using the BioRad iScript kit per the manufacturer's protocol, with the addition of 200 ng random pentadecamers (Integrated DNA Technologies) per reaction. Approximately 1 μg of DNase treated RNA was used for each reaction. Quantitative PCR was then performed on a Realplex2 Mastercycler (Eppendorf), using BioRad Ssofast EvaGreen as a signal

reporter. Amplification of *mobB* from various strains was used as a marker to measure the effect on transcription of various CTnDOT regions in *trans* to the *mob* operon. Amplification of *rpoD* was used as a reference marker to normalize for variations in the quantity of RNA template, as *rpoD* is a single copy gene and the expression of *rpoD* is not altered by Tc induction (13). The sequences of primers used are listed in Table 4.2. The final concentrations of reaction components are: 1 µM primers, 5 µL cDNA, and 10 µL Ssofast EvaGreen Supermix (BioRad). The reaction conditions were as follows: initial denaturation 2 minutes at 98.0 ^{o}C, amplification and quantification at 98.0 ^{o}C, 5 sec, 55 ^{o}C 10 sec for 40 cycles. A melting curve was performed at 95.0 ^{o}C for 15 sec, followed by 55.0 ^{o}C for 15 sec with subsequent heating to 95.0 ^{o}C over the course of 20 minutes with continuous fluorescence measurement, followed by a final incubation at 95.0 ^{o}C for 15 sec. Each measurement was performed in triplicate, and relative quantification was performed using the Pfaffl equation $N=2^{-\Delta\Delta Ct}$ where N is the relative fold difference. The ΔC_t represents the C_t (cycle threshold) value difference within a sample between the target gene (*mob*) and the reference marker (*rpoD*). The $\Delta\Delta C_t$ then represents the difference in ΔC_t values between tetracycline induced and non-induced samples (17).

Localization of the oriT regions recognized by CTnDOT and CTnERL

A previous study from our lab demonstrated that CTnERL could mobilize a fragment of DNA from CTnDOT that contained an approximately 900 bp region between *rteC* and *mobC*, which led us to believe that the *oriT* of CTnDOT was within this region (12). However, recent work by Peed and colleagues confirmed that the

oriT sequence of CTn341 is in fact located upstream of *mobA* (16). Due to the close relatedness of CTn341 and CTnDOT, this prompted us to re-evaluate the location of the CTnDOT *oriT* region.

A 900 bp region containing the intergenic region between *rteC* and *mobC,* was PCR amplified using primers GRW54 fwd and rev (Table 4.2) from BT4007 and cloned into pLYL7*oriT*$_{(RK2)}$, which is mobilizable by the IncP plasmid RP4, but cannot be mobilized by *Bacteroides.* The resulting plasmid, pGRW54, was then introduced into both BT4007 and BT4004, and mating assays were performed to see whether any of these strains could recognize the putative *oriT* region, and thus mobilize pGRW54.

Similarly, a 200 bp region containing the region upstream of *mobA* was PCR amplified from BT4007 using primers GRW55 fwd and rev (Table 4.2). The resulting product was then sub-cloned into pLYL7*oriT*$_{(RK2)}$, and the resulting plasmid pGRW55 was introduced into BT4007 and BT4004 via a triparental mating, as described above. Mating assays were then performed to determine whether CTnERL (BT4004) and/or CTnDOT (BT4007) could mobilize pGRW55. All mating assays were performed with the addition of tetracycline (1 μg/mL) to the donor growth medium, as the presence of tetracycline is required for the RteC-dependent transcriptional activation of the excision operon (13, 15).

4.4 Results

The CTnDOT mob genes are regulated by tetracycline induction and are assembled in an operon

A recent study from a CTnDOT-like element, CTn341, revealed that the CTn341 *mob* genes comprise an operon that is induced at the transcriptional level by tetracycline (Tc) exposure. To determine if the CTnDOT *mob* genes are regulated in response to tetracycline exposure we performed qualitative RT-PCR detecting *mobA, -B,* and *-C* from BT4007, a *B. thetaiotaomicron* strain that contains a single copy of CTnDOT integrated in to the chromosome (Table 4.1). BT4007 cultures were grown in the absence of Tc or with low levels of Tc (1 μg/mL). As shown in Figure 4.2, *mobA, -B,* and *-C* transcripts are only detectable when cells are exposed to Tc, demonstrating that the CTnDOT *mob* genes are in fact regulated by Tc exposure.

To establish whether the *mob* genes are assembled in an operon we tested for transcriptional linking, where the intergenic regions of *mobA-B* and *mobB-C* were amplified using qualitative RT-PCR. If these genes are transcriptionally linked, and thus organized in an operon, we would expect to detect these intergenic transcripts. Conversely, if each *mob* gene were transcribed from an independent promoter, no transcript pertaining to the intergenic region would be detected. An amplification product was detected for both the *mobA-B* and *mobB-C* transcript, which suggests that the *mob* genes are in an operon (Fig. 4.3). Further supporting these findings is Peed and colleagues' recent demonstration that the *mob* genes of a CTnDOT-like element, CTn341, are also an operon that is regulated by Tc induction (16).

CTnDOT contains two OriT *regions that are located upstream of* mobA *and downstream of* mobC

Prior studies of CTnDOT suggested that the *oriT* of CTnDOT was within the approximately 1 kb region that is between *rteC* and *mobC.* However, a recent study characterizing the Mob proteins on a related element CTn341, suggested that the *oriT* of CTn341 was actually just upstream of *mobA.* The previous studies that localized the CTnDOT *oriT* used sub-cloned regions in *trans* to another element, CTnERL, which is also considered to be virtually identical to CTnDOT with the exception that CTnERL lacks the 13kb *ermF* region (27, 29). So it was possible then that in attempting to localize the CTnDOT *oriT,* we were wrong about the similarities between CTnERL and CTnDOT, and were thus incorrect about inferring the location of the CTnDOT *oriT.*

A 900 bp region located between *rteC* and *mobC,* previously reported to contain the CTnDOT *oriT,* was cloned onto a vector that cannot be mobilized from *Bacteroides*. The resulting plasmid, pGRW54, was then introduced into a *Bacteroides* strain that contained either CTnDOT (BT4007) or CTnERL (BT4004) integrated into the chromosome. Mating assays were performed from both of these *Bacteroides* strains, to see if pGRW54 was able to transfer to an *E. coli* recipient (HB101). If transconjugants were detected, this would suggest that an *oriT* region was present on pGRW54 and could be mobilized by the conjugative transposon present in the donor *Bacteroides* strain. Plasmid pGRW54 was able to transfer from both BT4004 and BT4007, which demonstrates that this *oriT* region downstream of *mobC* is mobilized by both CTnERL and CTnDOT (Table 4.3).

A similar experiment was done where instead a 200 bp region upstream of *mobA,* that is identical to the CTn341 *oriT* region, was cloned into a vector that is not mobilizable in *Bacteroides*. Transfer of the resulting plasmid pGRW55 was only detected from BT4007 and not from BT4004, thus suggesting that an *oriT* was present upstream of *mobA* that is mobilizable by CTnDOT yet not by CTnERL (Table 4.3).

Due to this observation that the *oriT* recognition differs between CTnDOT and CTnERL, we wanted to also investigate whether the CTnERL *mob* genes are also regulated at the transcriptional level in response to Tc induction. We performed both qualitative and quantitative RT-PCR to detect the *mob* genes from CTnERL (BT4014) and CTnDOT (BT4007). The *mob* genes from both CTnERL and CTnDOT were regulated by exposure to Tc (Figure 4.4A). However, much more transcript was observed upon Tc induction from CTnDOT relative to CTnERL. CTnERL showed an approximately 55-fold induction in detectable *mob* transcript upon Tc induction, whereas CTnDOT had an over 130-fold increase in transcript (Figure 4.4B). Despite the difference in *mob* transcription, both elements are reported to transfer at similar rates, suggesting that the level of *mob* expression does not directly affect the frequency of conjugative transfer (4, 29).

Proteins encoded within the excision operon act as transcriptional activators of the mob operon upon Tc induction

After observing that the *mob* genes are only detected upon exposure to Tc, we predicted that proteins encoded within the excision operon may be acting as regulators of the *mob* operon. We have previously reported that when the excision

operon is provided in *trans* to the self-transmissible plasmid pLYL72 , there is transcriptional activation of the *tra* genes as well as enhanced pLYL72 transfer (7, 9, 30). Because of the close proximity between the *tra* promoter and the putative *mob* promoter, we predicted that the interactions that regulate the *tra* operon could also be mediating *mob* transcriptional activation. Using both qualitative and quantitative RT-PCR, we first amplified the *mob* genes from BT4001 ΩQABC pLYL72 pHopp1 both with Tc (1µg/mL) and without Tc. As shown in Figure 4.5, an increase in *mob* transcript is observed upon Tc induction, which suggests that proteins encoded within the excision operon can act as transcriptional activators of the *mob* operon.

We then wanted to see if we could determine which excision proteins were necessary for enhancement of *mob* transcription. Various sub-clones of the excision region were provided in *trans* to pLYL72 to detect the regulation of *mob.* As shown in Figure 4.5, a deletion of *orf3* (pGW45) showed no detectable effect on the enhancement of *mob* transcription. However, a deletion of either Xis2d or Exc drops the detectable level of *mob* transcript to the constitutive minus Tc levels, suggesting that Xis2d and Exc are required for the up-regulation of *mob* transcription. A deletion of Xis2c had no noticeable effect on the *mob* transcriptional activation. This was unexpected, as we predicted that a deletion of Xis2c would greatly diminish *mob* activation based on the observation that this same deletion drops the activity of a *traA::uidA* fusion to approximately 5% activity (9). Further, a deletion of Exc has no effect on the *traA::uidA* fusion, yet brings *mob* activation down to constitutive minus Tc levels. These observations suggest that the transcriptional regulation of the *mob* and *tra* operons are not as similar as we had predicted based on their close proximity.

A deletion of Exc does not diminish the transfer frequency of pLYL72

Previous studies suggested that Exc was required for the enhanced transfer of pLYL72. This conclusion was based on the observation that when pGW46 (*xis2c, -2d, exc, rteR, orf4a*) was in *trans* to pLYL72, transfer was enhanced approximately 100 to 1000-fold upon Tc induction. This resulted in a transfer frequency of approximately 10^{-2} to 10^{-3} transconjugants per recipient in the presence of Tc, and no transfer was detected in the absence of Tc due to RteR-mediated inhibition of transfer. However, a clone that deleted *exc* and the region downstream (pKS04) resulted in constitutive transfer with respect to Tc induction at a rate of approximately 10^{-2} to 10^{-3} transconjugants per recipient. Transfer was now detectable in the absence of Tc due to the deletion of *rteR,* and this finding suggested that a deletion of *exc* resulted in a loss of the enhancement effect upon the addition of Tc to the growth medium (Table 4.4).

More recently, a study that further investigated excision protein-mediated activation of the CTnDOT *tra* region suggested that although Xis2c and Xis2d were required for sufficient activation of a *traA::uidA* fusion, a deletion of *exc* did not significantly effect the GUS activation (9). Our results have demonstrated that a deletion of *exc* results in a loss of *mob* transcriptional activation, so it was possible that this defect in pLYL72 transfer was due to a reduction in *mob* expression.

Mating assays were performed to detect the transfer frequency of pLYL72 from BT4001 ΩQABC containing various excision operon deletions on a second plasmid provided in *trans* to pLYL72. When the intact excision operon (pGW45) or a plasmid containing a deletion of *orf 3* was present (pGW46), both enhancement and

repression of pLYL72 transfer were observed with and without the addition of Tc, respectively. When we investigated the ability of pGRW53ΔExc to regulate pLYL72 transfer, the transfer frequency was similar to a strain containing the fully intact excision operon, where both enhancement and repression were observed in response to Tc induction. This observation suggests that Exc is not required for enhanced transfer of pLYL72.

A deletion of *xis2c* (pGRW53Δ2c) resulted in a loss of enhancement, and pLYL72 transferred at a rate similar to the empty vector control upon Tc induction where transconjugants were detected at a frequency of approximately 10^{-5} to 10^{-6} transconjugants per recipient. However, a deletion of *xis2d* (pGRW53Δ2d) resulted in no pLYL72 transfer even with the induction of Tc. Similarly, a deletion of both *xis2c* and *xis2d* (pYS41) results in no detectable pLYL72 transfer, likely due to the requirement for Xis2d. For each of the plasmids described above, no transconjugants were detectable without Tc added to the growth medium due to RteR-mediated negative regulation.

We also tested the effect of the CTnDOT integrase, IntDOT, on the transfer of pLYL72. We hypothesized that IntDOT could be a candidate for the negative regulator of *mob* transcription in the absence of Tc since the integrase of tn916 is thought to bind the *oriT* in an effort to prevent premature conjugative transfer (5). Our results demonstrated that the *mob* genes are transcribed constitutively from pLYL72 when *intDOT* is in *trans* (Figure 4.6). RT-qPCR confirmed that the *mob* genes are transcribed constitutively from this strain (data not shown). Interestingly though, no transfer of pLYL72 was detected with or without Tc induction. This suggests that

IntDOT may play a role in preventing premature conjugative transfer, but IntDOT is not doing so by preventing transcription of the *mob* operon.

4.5 Discussion

Coordinating the events of CTnDOT excision, mobilization, transfer, and integration results in a mobile element that is very tightly regulated. Such coordination is crucial, as it is important to delay the initiation of mobilization and transfer function until CTnDOT has properly excised from the chromosome. If transfer were to happen prematurely, an Hfr-like transfer would be the consequence, which can be rather costly to the donor cell. Many studies have detailed the coordinated regulation of excision and transfer of CTnDOT (9, 13, 15, 25), while this study is the first to characterize the transcriptional regulation of CTnDOT mobilization functions.

We have shown that the *mob* genes of CTnDOT are part of an operon that is transcribed upon induction with low levels of the antibiotic tetracycline (Tc), while no transcript is detectable in the absence of Tc. This tetracycline dependent *mob* regulation is similar to observations made with respect to a related conjugative transposon, CTn341. This group reported that the *rteA* and *rteB* genes were required in *trans* for transcriptional activation of the mob_{CTn341} operon, a fact that prompted us to further investigate the regulation of the CTnDOT *mob* operon (16).

From what we know about the CTnDOT regulatory cascade initiated upon Tc induction (Figure 4.1), RteA and RteB are required for the activation of a regulatory protein RteC, which acts as a transcriptional activator of the excision operon (13, 15, 23, 24). We predicted then, that perhaps the genes encoded in the excision region

could be acting as transcriptional activators of the *mob* genes. We report here that the excision proteins Xis2d and Exc are required for the activation of *mob* transcription upon Tc induction (Figure 4.5). Although Exc is required for enhancement of *mob* expression, a deletion of *exc* has no obvious effect on pLYL72 transfer (Table 4.4). This suggests that there is no direct correlation between the level of *mob* expression and the frequency of conjugative transfer. This is further supported by the observation that although there is a large difference in *mob* expression between CTnERL and CTnDOT, they are reported to transfer at similar rates (Figure 4.5 and Table 4.3).

However, a deletion of *xis2c* has no detectable effect on *mob* expression decreases the transfer of pLYL72 to a rate of 10^{-5} to 10^{-6} transconjugants per recipient. A deletion of *xis2d* on the other hand, results in no detectable transfer of pLYL72 upon Tc induction. Previous studies have demonstrated that a deletion of *xis2c* or *xis2d* reduces the induction of a *traA::uidA* fusion, but the different affects these deletions have on pLYL72 conjugative transfer demonstrates that a deletion of *xis2d* bears a greater consequence than *xis2c.* This suggests that perhaps there is a greater requirement of Xis2d for transcriptional regulation, and that maybe Xis2c is acting as more of an accessory protein that facilitates Xis2d-mediated regulation.

Our findings have also demonstrated that a negative regulator is preventing transcription of the *mob* operon when no Tc is present. The *mob* genes are transcribed constitutively from the self-transmissible plasmid pLYL72, yet a transcript is only detected from CTnDOT upon Tc induction. We investigated the possibility of the CTnDOT integrase, IntDOT, as a potential repressor, as the integrase

from Tn916 can bind the *oriT* which is thought to prevent premature conjugative transfer (5). RT-PCR demonstrated that the *mob* genes were transcribed constitutively when *intDOT* was provided in *trans*; however, no pLYL72 transfer was detectable (data not shown). Although IntDOT is not acting as the negative regulator of the *mob* operon, this finding suggests that IntDOT plays a regulatory role in preventing conjugative transfer.

In summary, our findings have added another layer of complexity to the already intricate regulation of CTnDOT. These observations further support the importance of highly coordinated regulation of excision and conjugative transfer. It is advantageous to initiate these steps as soon as possible, so antibiotic resistance determinants can readily disseminate throughout the population, but not prematurely to the disadvantage of the donor cell.

4.6 Acknowledgements

We thank Nadja Shoemaker for thoughtful discussion. This work was supported by grant AI 22383 from the National Institutes of Health.

4.7 References

1. **Bonheyo G, Graham D, Shoemaker NB, and Salyers AA**. 2001. Transfer region of a bacteroides conjugative transposon, CTnDOT. Plasmid 45(1):41–51.

2. **Boyer HW and Roulland-Dussoix D**. 1969. A complementation analysis of the restriction and modification of DNA in Escherichia coli. J. Mol. Biol. 41(3):459–472.

3. **Cheng Q, Sutanto Y, Shoemaker NB, Gardner JF, and Salyers AA**. 2001. Identification of genes required for excision of CTnDOT, a Bacteroides conjugative transposon. Mol. Microbiol. 41(3):625–632.

4. **Franco AA, Cheng RK, Chung G-T, Wu S, Oh H-B, and Sears CL**. 1999. Molecular Evolution of the Pathogenicity Island of Enterotoxigenic Bacteroides fragilis Strains. J. Bacteriol. 181(21):6623–6633.

5. **Hinerfeld D and Churchward G**. 2001. Specific binding of integrase to the origin of transfer (oriT) of the conjugative transposon Tn916. J. Bacteriol. 183(9):2947–2951.

6. **Hooper LV, Midtvedt T, and Gordon JI**. 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu. Rev. Nutr. 22:283–307.

7. **Jeters RT, Wang G-R, Moon K, Shoemaker NB, and Salyers AA**. 2009. Tetracycline-associated transcriptional regulation of transfer genes of the Bacteroides conjugative transposon CTnDOT. J. Bacteriol. 191(20):6374–6382.

8. **Keeton CM and Gardner JF**. 2012. Roles of Exc protein and DNA homology in the CTnDOT excision reaction. J. Bacteriol. 194(13):3368–3376.

9. **Keeton CM, Park J, Wang G-R, Hopp CM, Shoemaker NB, Gardner JF, and Salyers AA**. 2013. The excision proteins of CTnDOT positively regulate the transfer operon. Plasmid 69(2):172–179.

10. **Kelley LA and Sternberg MJE**. 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc 4(3):363–371.

11. **Li LY, Shoemaker NB, and Salyers AA**. 1993. Characterization of the mobilization region of a Bacteroides insertion element (NBU1) that is excised and transferred by Bacteroides conjugative transposons. J. Bacteriol. 175(20):6588–6598.

12. **Li LY, Shoemaker NB, and Salyers AA**. 1995. Location and characteristics of the transfer region of a Bacteroides conjugative transposon and regulation of transfer genes. J. Bacteriol. 177(17):4992–4999.

13. **Moon K, Shoemaker NB, Gardner JF, and Salyers AA**. 2005. Regulation of excision genes of the Bacteroides conjugative transposon CTnDOT. J. Bacteriol. 187(16):5732–5741.

14. **Nguyen M and Vedantam G**. 2011. Mobile genetic elements in the genus Bacteroides, and their mechanism(s) of dissemination. Mobile genetic elements 1(3):187–196.

15. **Park J and Salyers AA**. 2011. Characterization of the Bacteroides CTnDOT regulatory protein RteC. J. Bacteriol. 193(1):91–97.

16. **Peed L, Parker AC, and Smith CJ**. 2010. Genetic and functional analyses of the mob operon on conjugative transposon CTn341 from Bacteroides spp. J. Bacteriol. 192(18):4643–4650.

17. **Pfaffl MW**. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29(9):e45.

18. **Round JL and Mazmanian SK**. 2009. The gut microbiota shapes intestinal immune responses during health and disease. Nature Reviews Immunology 9(5):313–323.

19. **Salyers AA and Shoemaker NB**. 1997. Conjugative transposons. Genet. Eng. (N.Y.) 19:89–100.

20. **Shoemaker NB, Getty C, Guthrie EP, and Salyers AA**. 1986. Regions in Bacteroides plasmids pBFTM10 and pB8-51 that allow Escherichia coli-Bacteroides shuttle vectors to be mobilized by IncP plasmids and by a conjugative Bacteroides tetracycline resistance element. J. Bacteriol. 166(3):959–965.

21. **Shoemaker NB and Salyers AA**. 1987. Facilitated transfer of IncP beta R751 derivatives from the chromosome of Bacteroides uniformis to Escherichia coli recipients by a conjugative Bacteroides tetracycline resistance element. J. Bacteriol. 169(7):3160–3167.

22. **Shoemaker NB, Wang GR, Stevens AM, and Salyers AA**. 1993. Excision, transfer, and integration of NBU1, a mobilizable site-selective insertion element. J. Bacteriol. 175(20):6578–6587.

23. **Stevens AM, Shoemaker NB, Li LY, and Salyers AA**. 1993. Tetracycline regulation of genes on Bacteroides conjugative transposons. J. Bacteriol. 175(19):6134–6141.

24. **Wang Y, Shoemaker NB, and Salyers AA**. 2004. Regulation of a Bacteroides operon that controls excision and transfer of the conjugative transposon CTnDOT. J. Bacteriol. 186(9):2548–2557.

25. **Waters JL and Salyers AA**. 2012. The Small RNA RteR Inhibits Transfer of the Bacteroides Conjugative Transposon CTnDOT. J. Bacteriol. 194(19):5228–5236.

26. **Wexler HM**. 2007. Bacteroides: the good, the bad, and the nitty-gritty. Clin. Microbiol. Rev. 20(4):593–621.

27. **Whittle G, Hund BD, Shoemaker NB, and Salyers AA**. 2001. Characterization of the 13-kilobase ermF region of the Bacteroides conjugative transposon CTnDOT. Appl. Environ. Microbiol. 67(8):3488–3495.

28. **Whittle G, Shoemaker NB, and Salyers AA**. 2002. The role of Bacteroides conjugative transposons in the dissemination of antibiotic resistance genes. Cell. Mol. Life Sci. 59(12):2044–2054.

29. **Whittle G, Hamburger N, Shoemaker NB, and Salyers AA**. 2006. A bacteroides conjugative transposon, CTnERL, can transfer a portion of itself by conjugation without excising from the chromosome. J. Bacteriol. 188(3):1169–1174.

30. **Whittle G, Shoemaker NB, and Salyers AA**. 2002. Characterization of genes involved in modulation of conjugal transfer of the Bacteroides conjugative transposon CTnDOT. J. Bacteriol. 184(14):3839–3847.

4.8 Tables and figures

Table 4.1: Bacterial Strains and Plasmids

Strain or plasmid	Relative phenotype[a]	Description and/or reference
B. thetaiotaomicron		
BT4001	(Rif^R)	Spontaneous Rif^R mutant *B. thetaiotaomicron*
BT4001 ΩQABC	(Thy^-, Tp^R, Tc^R)	BT4100 with a site specific insertion of the regulatory region *tetQ-rteA-rteB-rteC* (30)
BT4001 ΩQABC Ω2.6pGERM	(Rif^R Tc^R)	BT4001 ΩQABC containing an insertion of pDJE2.3, which contains *intDOT,* integrated into the chromosome (3)
BT4004	(Rif^R, Em^R)	BT4001 containing CTnERL integrated in to the chromosome
BT4007	(Rif^R Tc^R, Em^R)	BT4001 containing CTnDOT integrated in to the chromsome
BT4104	(Thy^-, Tp^R, Tc^R)	BT4100 containing a chromosomal copy of CTnERL (21)
E. coli		
DH5aMCR	RecA	Gibco BRL
HB101	RecA, Str^R	*E. coli* strain used as a conjugative transfer recipient (2)
HB101 RP1	RecA, Str^R	HB101 containing the IncPα plasmid RP1 (20)
Plasmids		
pAFD1	Ap^R (Em^R)	*E. coli-Bacteroides* shuttle vector containing *ermF* (A.M. Stevens, unpublished)
pGRW53Δ2C	Ap^R (Em^R)	pGW46 insert containing a114 AA in-frame deletion of *xis2c,* ligated in to pAFD1 (9)
pGRW53Δ2D	Ap^R (Em^R)	pGW46 insert containing an inframe deletion of *xis2d* from AA 3 through the end of *orf3,* ligated in to pAFD1 (9)
pGRW53ΔExc	Ap^R (Em^R)	pGW46 insert containing a 670 AA in-frame deletion of the C terminal end of Exc, ligated in to pAFD1 (9)
pGRW54	Ap^R (Cf^R)	A 900 bp fragment containing the region between *rteC* and *mobC* from CTnDOT, cloned into pLYL7*oriT*$_{RK2}$ (this study)
pGRW55	Ap^R (Cf^R)	A 200 bp fragment containing the putative CTnDOT *oriT* region upstream of *mobA*, cloned into pLYL7*oriT*$_{(RK2)}$ (this study)
pGW45	Ap^R (Cf^R)	A 1.6 kb fragment containing *orf2b, xis2c, -2d, exc, orf2e,* and *rteR* cloned into pLYL05 (30)
pGW46	Ap^R (Cf^R)	A 1.3 kb fragment containing *xis2c, -2d, -exc, orf2e,* and *rteR* cloned into pLYL05 (30)
pHopp1	Ap^R (Em^R)	pAFD1 containing *xis2c, xis2d, orf3, exc,* and *rteR* (9)
pLYL05	Ap^R (Cf^R)	*E. coli-Bacteroides* shuttle vector (L.Y. Li, unpublished data)
pLYL72	Kn^R, Cm^R (Cm^R)	Self transmissible plasmid containing *mob* genes, *tra* operon, and OriT from CTnDOT (12)
pLYL7*oriT*$_{RK2}$	Ap^R (Cf^R)	*E. coli* – *Bacteroides* shuttle vector that is mobilizable in *E. coli* by the IncP plasmid RP4, but is not mobilizable in *Bacteroides* (11)
pYS41	Ap^R (Cf^R)	A 1.5 kb fragment ligated in to pLYL05 containing *orf2a, -2b, exc, orf2e,* and *rteR* (Y. Sutanto, unpublished).

[a]*Bacteroides* phenotypes are shown in parentheses, whereas *E. coli* phenotypes are shown without parentheses. Abbreviations are Ap, ampicillin; Cf, cefoxitin; Cm, chloramphenicol; Em, erythromycin; Kn, kanamycin; Rif, rifampicin; Str, streptomycin; Tc, tetracycline; Thy, thymidine auxotroph; Tp, trimethoprim.

Table 4.2: Primers used in this study

Primer Name	5′ to 3′ Sequence
Cloning	
GRW54 fwd	gatgactgcagggaatcacaat
GRW54 rev	tcttgataagagctctagaagtcatt
GRW55 fwd	tgaaaggtatctgcagtccgt
GRW55 rev	ggatcgagctcgggatttct
RT-PCR	
mobA fwd	tcaaggtgctgaaggtggacaaga
mobA rev	tctgacggctcagtttcacgagtt
mobB fwd	tttccgtgctgcaaggcacttatg
mobB rev	atgaaagcctcttcctcggcatct
mobC fwd	tgaaccgccgctacgaggatattt
mobC rev	tcgggattgttgatgtccagcgta
mobA-B fwd	tggactactacaccaagctatcgg
mobA-B rev	tttccagatactcgcgggc
mobB-C fwd	acaaccgccgataccgttatccat
mobB-C rev	ccctttcgtacccagacaggaca
RpoD 67 fwd	tttaatctgacgaacgcgctcacg
RpoD 548 rev	acgctgtatggtggattcgtcagt
RT-qPCR	
mobA fwd	acaaccgtttcctcgccatgtttg
mobA rev	tcttgtccaccttcagcaccttga
mobA-B fwd	aactcgtgaaactgagccgtcaga
mobA-B rev	tgtagatgcggttggtggtgagaa
mobB fwd	acaaccgccgataccgttatccat
mobB rev	atgaaagcctcttcctcggcatct
RpoD 467 fwd	acctgattcaacgggagacgaca
RpoD 548 rev	acgctgtatggtggattcgtcagt

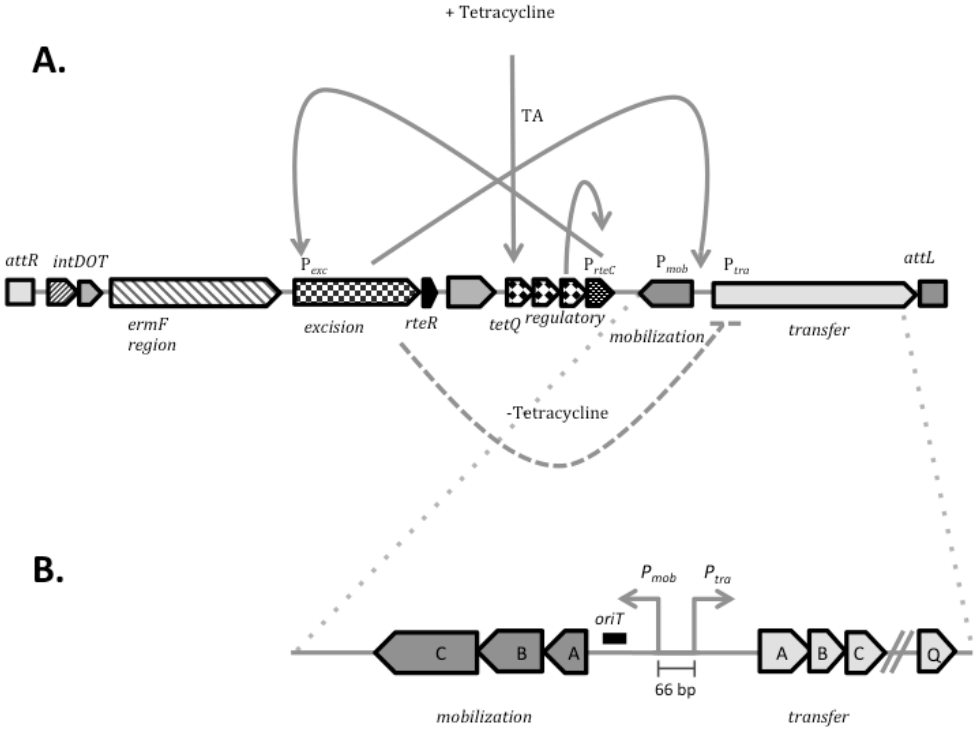


Figure 4.1: Tetracycline dependent regulation of CTnDOT transfer. A. Upon tetracyilne (Tc) induction translation is initiated through the *tetQ-rteQ-rteB* operon. RteB activates the transcription of *rteC,* which activates transcription of the excision operon. The proteins encoded in the excision operon then activate transcription of the transfer operon, thereby inducing conjugative transfer of CTnDOT. In the absence of Tc, no transfer is detectable, which in part is due to RteR initiating premature transcription termination in the *tra* operon. B. The *mob* genes are divergently transcribed from the *tra* operon. There are approximately 66 bp between the *mob* and *tra* promoters. The *oriT* is downstream of the putative *mob* promoter.

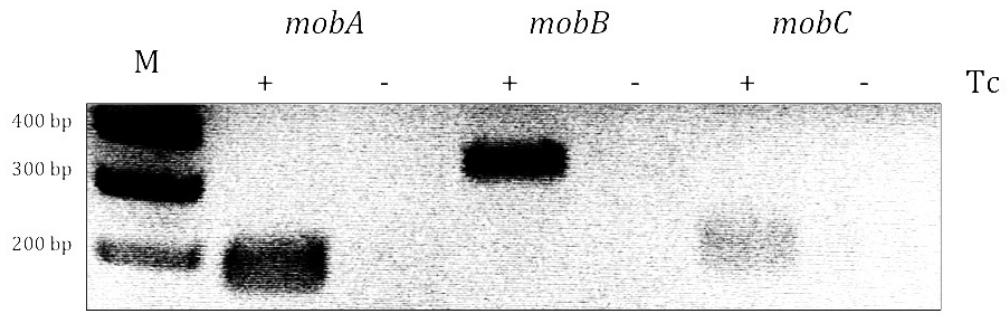


Figure 4.2: The *mob* genes are only detected from CTnDOT upon Tc induction.

To establish whether the transcription of the *mob* genes is regulated by Tc induction, RT-PCR was performed detecting *mobA, mobB,* and *mobC* from BT4007, which contains a copy of CTnDOT integrated in to the chromosome. RT-PCR was performed on BT4007 RNA samples from cells that were non-induced and induced (1 μg/mL) tetracycline (Tc). An amplicon pertaining to each of the *mob* genes was only detected upon Tc induction, thus confirming that the transcription of the *mob* genes is dependent upon exposure of cells to Tc.

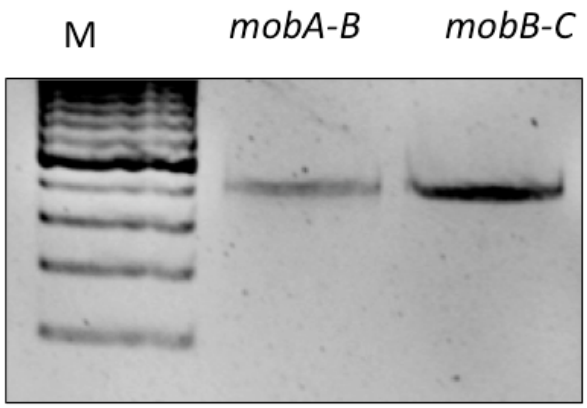


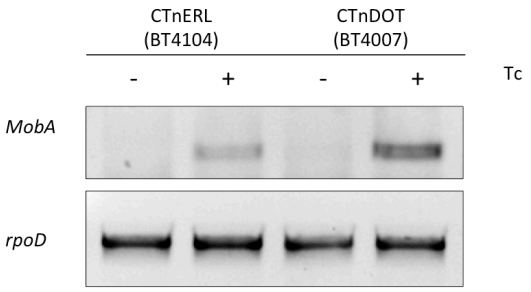
Figure 4.3: The CTnDOT *mob* genes are transcriptionally linked. To address whether the *mob* genes form a polycistronic transcript, RT-PCR was performed using primers that amplified the regions of the *mobA-B* and *mobB-C* intergenic junctions from BT4001 pLYL72 pGW45 induced with tetracycline (1 μg/mL). Amplicons for both of these primer sets were observed, thus indicating the *mob* genes are transcriptionally linked and organized as an operon.

Table 4.3 Transfer of putative *oriT* from CTnERL vs. CTnDOT

Donor Strain	Transfer frequency [a]
BT4004 pGW54	$1.3 \pm 0.5 \times 10^{-6}$
BT4007 pGRW54	$6.3 \pm 2.2 \times 10^{-4}$
BT4004 pGRW55	Not Detectable
BT4007pGRW55	6.7×10^{-3}

[a] The transfer frequency is represented as the ratio of transconjugants per recipient cell.

A.



B.

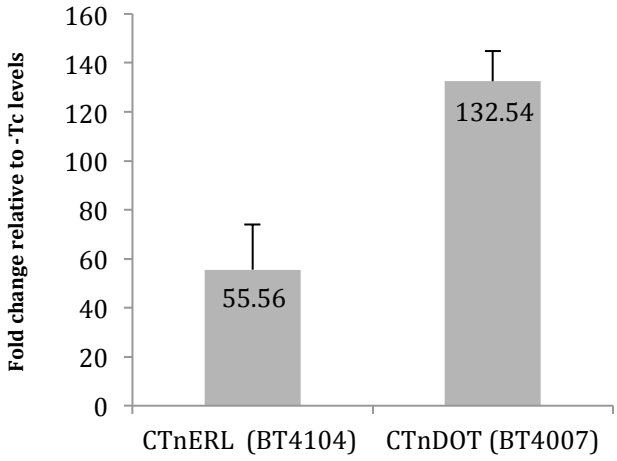


Figure 4.4: More *mob* transcript is detectable from CTnDOT compared to CTnERL. A. Qualitative RT-PCR was performed using primers to detect *mobA* from a strain containing CTnERL (BT4104) and CTnDOT (BT4007) both with and without Tc induction. No transcript is detectable in the absence of Tc, yet upon exposure to Tc a *mob* transcript is then detected. However, more *mobA* transcript is seen in cells containing CTnDOT compared to CTnERL. *RpoD* is a reference marker used to ensure equivalent RNA concentrations between samples. B. RT-qPCR was performed using primers to detect *mobB.* RNA content was normalized to the reference marker *rpoD,* and the $\Delta\Delta C_T$ was calculated comparing induced (+ Tc) to non-induced (- Tc) levels.

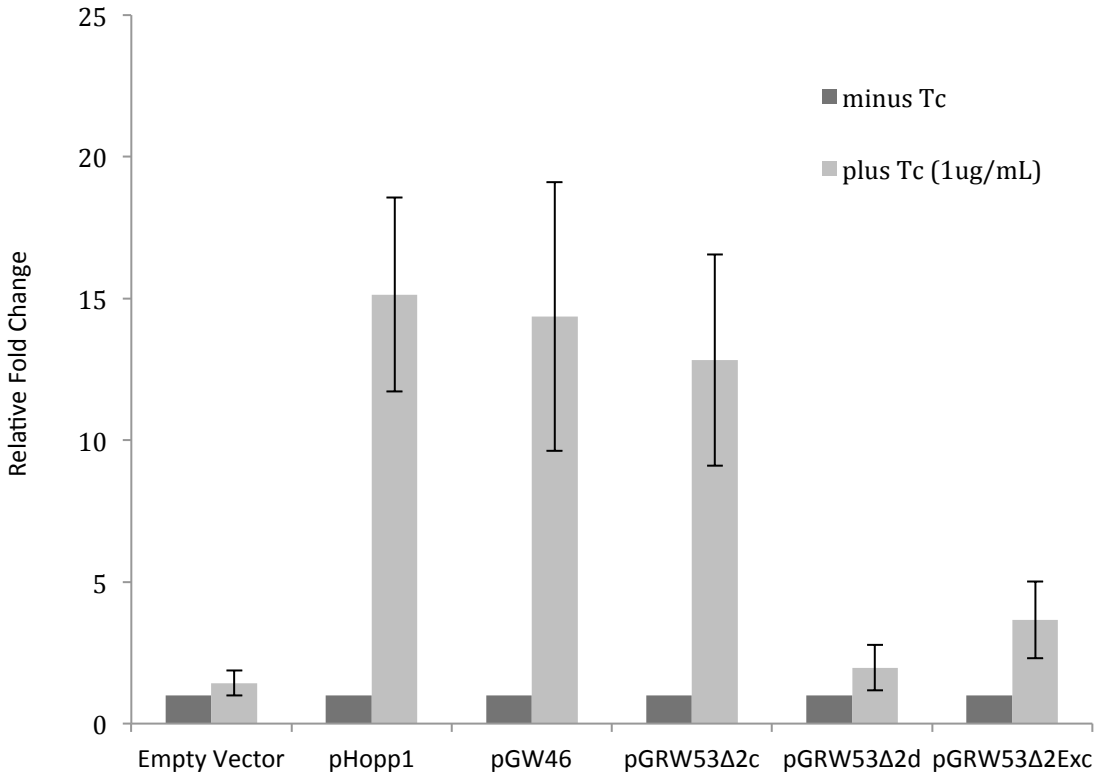


Figure 4.5: The *mob* genes are transcribed constitutively from pLYL72, and the excision proteins Xis2d and Exc are required for sufficient enhancement of transcription upon Tc induction. RT-qPCR was performed to localize potential regulators of *mob* transcription, shown are the relative fold changes in transcription measuring *mobB,* relative to the minus tetracycline levels. Fold changes were calculated using the Pfaffl $\Delta\Delta C_T$ equation, and samples were normalized using the reference marker *rpoD* (17). The above observations were also confirmed using qualitative RT-PCR (not shown). The *mob* genes are transcribed constitutively from pLYL72, and the excision operon in *trans* is sufficient for transcriptional activation. A deletion of *xis2C* appears to have no deleterious effect on *mob* transcription, where as a deletion in either *xis2d* or *exc* drops transcription to the constitutive empty vector levels.

Table 4.4: Effect of excision protein deletions on the transfer of pLYL72

Donor strain BT4001 ΩQABC pLYL72 plus:	Transfer frequency of pLYL72[a]		Regulation of Transfer[b]	Source or reference
	+ Tc	- Tc		
pLYL05	10^{-5}-10^{-6}	10^{-5}-10^{-6}	E-, R-	(28)
pGW46	5.5×10^{-3}	$< 10^{-9}$	E+, R+	this study
pGRW53Δ2c	1.8×10^{-4}	$< 10^{-9}$	E-, R+	this study
pGRW53Δ2d	$< 10^{-9}$	$< 10^{-9}$	R +, R +	this study
pGRW53ΔExc	1.2×10^{-3}	$< 10^{-9}$	E+, R+	this study
pYS41	$< 10^{-9}$	$< 10^{-9}$	R +, R +	this study
Ω2.6pGERM	$< 10^{-9}$	$< 10^{-9}$	R +, R +	this study

[a] Transfer frequency of pLYL72 is expressed as the average ratio of transconjugants per recipient cell. Where ranges are shown, the value accounts for the variation observed among three separate mating experiments.
[b] E, enhancer; R, repressor; +, present; -, absent.

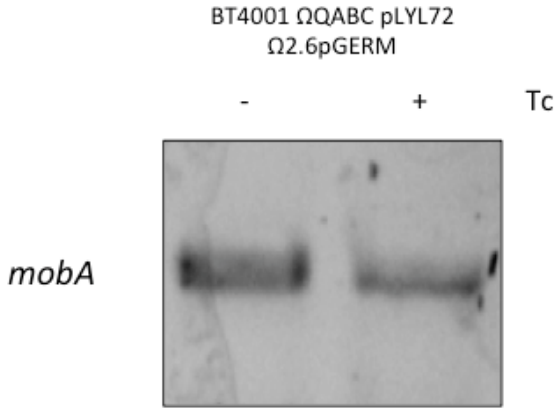


Figure 4.6: The *mob* genes are transcribed constitutively when *intDOT* is present. Qualitative RT-PCR was performed detecting *mobA* from strain BT4001 ΩQABC pLYL72 Ω2.6pGERM, which contains a copy of *intDOT* integrated in to the chromosome. *Mob* transcript was detectable whether or not Tc was present, which suggests that IntDOT is not preventing transcription of the *mob* operon.

CHAPTER 5: CONCLUDING REMARKS AND FUTURE STUDIES

CTnDOT has proven to be a very engaging subject of study, as CTnDOT is a very complex mobile element that whose genes are very tightly regulated. In the absence of tetracycline (Tc) no transfer is detected, yet upon exposure to low levels of Tc (less than 1 μg/mL) transfer is stimulated. It is not a far stretch to conceptualize the need for regulated transfer, as it is easy to imagine that there is a fitness cost to the donor cell for transfer of the element. However, the regulation of transfer has proven to be rather complex, with many proteins having dual functions with respect to conjugative transfer of CTnDOT.

The work I have presented in this dissertation has further described regulation of CTnDOT conjugative transfer, both positive and negative, at the transcriptional level. The small RNA RteR proved to be rather elusive. Members of our laboratory have previously attempted to investigate this regulatory RNA with the aim of better characterizing how RteR was inhibiting transfer of the self-transmissible plasmid pLYL72 (contains an 18 kb region of CTnDOT including the *mob* operon, *tra* operon, and *oriT*). The summation of my work described in Chapter 2 confirmed that RteR is a 90 nt transcript and is targeting within the *tra* region, as previously suspected. However, I was able to clear up a misconception, and that is that RteR is actually downstream of the excision protein encoding gene *exc,* rather than within the 3' end of *exc* as previously suggested. Further, I have shown that RteR is not preventing *tra* transcription as we thought previously, but rather I have

provided evidence that RteR is likely initiating premature transcription termination within *traB.*

One question that arose while working with RteR, was to determine if an Hfq homologue was aiding in facilitating the interaction of RteR with the elongating *tra* message. While no Hfq-like homologue has been annotated in the *B. thetaiotaomicron* genome, this is not surprising as *B. thetaiotaomicron* is phylogenetically distant from the more heavily studied Proteobacteria. We suspected then, that it was very likely a functionally equivalent Hfq homologue could be present. I attempted a brief bioinformatics-based approach to identify possible Hfq candidates, which I have described in Chapter 3 of this dissertation.

While investigating RteR-mediated negative regulation of conjugative transfer, I wanted to see if the 4 kb mobilization region of CTnDOT was also a target of RteR, as this region is also present on the self-transmissible plasmid pLYL72. I saw Tc dependent transcriptional regulation, where *mob* transcripts were only detectable from CTnDOT when cells were exposed to Tc, and a second project was thus initiated. Given the close proximity between the *mob* genes and the *tra* operon (Figure 5.1) we expected that the transcriptional regulation would be quite similar. Another student, Crystal Hopp, has been studying the binding of the excision proteins upstream of the *tra* promoter, and her current predictions are that they are bending the DNA in such a way as to enhance *tra* transcription upon Tc induction. However, the work I have described in Chapter 4 of this dissertation has demonstrated there are in fact different players involved in the transcriptional regulation of the *mob* operon.

We have confirmed that the excision proteins Xis2d and Exc are required for the enhancement of pLYL72 conjugative transfer. Without either of these genes in *trans,* the *mob* expression is similar to empty vector levels. A deletion of Xis2c on the other hand, has no detectable effect on the transcription of the *mob* genes. This differs from the *tra* operon, in that both Xis2c and Xis2d are required for the activation of a *traA::uidA* fusion, and a deletion of Exc shows similar levels of GUS activity relative to the intact excision operon (1). In addition, a negative regulator encoded on CTnDOT is preventing transcription of the *mob* operon in the absence of Tc. We have confirmed that this repressor is not RteR, and localization studies have suggested that a hypothetical protein Orf2, which is immediately downstream of IntDOT, is likely acting as the negative regulator. Phyre structural homologue predictions suggest that Orf2, a 120 AA protein, is a winged helix DNA binding protein that shares structural homology with known transcriptional regulators such as the cyanobacterial SmtB and HlyU from *Vibiro vulnificus* (2–4).

Although the work described in this dissertation has led us in further understanding the complex regulation of CTnDOT conjugative transfer, there are still some gray areas in our knowledge of CTnDOT transcriptional regulation. The biggest questions that still remain have to do more with the negative regulation of CTnDOT. First, what is the mechanism of RteR? Second, what is the mechanism of the negative regulator of the *mob* region? Each of these questions could readily warrant at least one publication, and taken together, could possibly make a thesis project for a graduate student.

5.1 Confirming the 3' end of the *tra* transcript in the presence of RteR

As stated previously, we hypothesize that RteR is ultimately preventing conjugative transfer of CTnDOT by binding the elongating *tra* mRNA within *traB*, and inducing a conformational change in the secondary structure of the *tra* message that results in the formation of an intrinsic terminator. Our evidence for this mechanism is that RT-PCR (qualitative and quantitative) demonstrates that a similar amount of *traA* transcript is detected whether or not RteR is present; however, downstream of *traA* and through the remainder of the *tra* operon the relative amount of transcript is dramatically reduced in the presence of RteR. This suggests that something is happening at the transcriptional level. This hypothesis is further supported by the fact that the presence RteR does not appear to result in destabilization of the *tra* mRNA, as the half-life is equivalent with and without *rteR* in *trans* (5). Unfortunately, this is our only form of experimental evidence to support this hypothesis. A preliminary bioinformatics analysis does aid this hypothesis in that there are two sequences upstream of the intrinsic terminator that are complementary to the single stranded loop of RteR. These sequences are located approximately 20 bp and 200 bp upstream of the predicted terminator structure.

To gather further support for this mechanism, we attempted 3' RACE on two separate occasions. The logic was that if RteR were truly terminating the *tra* message, we would detect a 3' end that corresponds to the predicted terminator structure. If we were wrong, we would then detect a different 3' end or perhaps visualize an end corresponding to the full-length *tra* transcript. Alas, our attempts

failed, as no 3' end was ever detected. It is possible, given the many steps that are involved in RACE analysis, that the integrity of the RNA transcript was compromised. 3' RACE analysis could again be repeated, with the aim of detecting any 3' end of the *tra* message to either support or disprove this hypothesis.

An alternative would be to attempt Northern-blotting analysis to detect the *tra* message. Because the full-length *tra* message is over 13kb, it is a bit too large to visualize. However, if we are correct about premature transcription termination the terminated transcript is roughly 1.4 kb, which would be in the detectable size range to visualize on a Northern blot. Observing a 1.4 kb transcript would further support a premature transcription termination mechanism.

5.2 Further confirmation of the RteR binding site in *traB*

As mentioned in the previous section, we need more evidence that explains just how RteR is inhibiting conjugative transfer of CTnDOT. Although all signs are pointing to the initiation of transcriptional termination, there is still a possibility we are wrong. As described previously, confirming a 3' end of the *tra* message that corresponds to the predicted intrinsic terminator and/or a 1.4 kb transcript detected would further support the hypothesis of premature termination. More conclusive evidence of the RteR mechanism would be to confirm how RteR is interacting with the *tra* mRNA. I predict that RteR is mediating contact with the *tra* transcript through the single stranded 8 nt loop that is located in the middle of the transcript. My logic for this is that site directed mutations that are predicted to change the secondary structure in a way that closes that loop are sufficient to

abolish RteR-mediated negative regulation of conjugative transfer. Second, a complementary region to this loop is present within *traB.* This interaction could be tested by generating site directed mutations that target the 8 nt loop by changing the primary sequence but are not predicted to alter the secondary structure.

If these mutations do result in a loss of RteR regulation, which is expected, I would then make compensatory mutations in the putative RteR binding site(s) within the *tra* message. In the 5' end of *traB* is a sequence that is complementary to 6 of the 8 nt in the single stranded central loop of RteR. The first possible binding site is approximately 20 bp upstream of the predicted intrinsic terminator that is forming in *traB.* Ideally, these compensatory mutations would be made within the context of the entire *tra* operon, however working with a 35 kb plasmid for site directed mutagenesis is not feasible. Another alternative would be to sub-clone a region containing *traB* for site directed mutagenesis and then re-construct an intact *tra* operon on pLYL72 containing this mutation. A third possibility, and perhaps a more feasible option to study the effect of compensatory *tra* mutations, would be to construct a *traC::uidA* fusion. If this fusion has sufficient GUS activity with respect to tetracycline induction, this region would be much easier to manipulate with site directed mutagenesis, and thus further testing where RteR is binding within the *tra* message.

5.3 Confirming the mechanism Xis2d and Exc mediated transcriptional activation of the *mob* operon

Although footprinting of the region between the *mob* and *tra* promoter has confirmed that Xis2D is binding here (C. Hopp, unpublished), at this point we cannot be certain whether this binding interaction is facilitating only the activation of *tra* expression or if this binding is also facilitating the enhancement of *mob* transcription.

Utilization of a *mobA::uidA* fusion will unfortunately not work, as I previously constructed such a fusion (pJW602) and observed that background GUS activity from the chromosome was similar to that of pJW602, which renders this *mob* fusion unusable in *Bacteroides.* I would be hesitant to move this fusion to *E. coli* because previous GUS fusions have shown altered activity in *E. coli* compared to *Bacteroides* (J. Park, unpublished data). This difference is likely attributable to the difference between *E. coli* and *Bacteroides* promoter sequences.

Another possibility then would be to construct a *mob* fusion using a different reporter gene. Although we have not tested this in our own lab, another group has reported the use of the xylosidase/arabinosidase gene *XA* in *Bacteroides ovatus* as a reporter gene. Further, this group also reports the use of this fusion in *B. thetaiotaomicron,* and no background activity was detectable in empty vector controls (6). These observations suggest that the use of *XA* as a reporter fusion is a good alternative for future studies in *B. thetaiotaomicron.*

5.4 References

1. **Keeton CM, Park J, Wang G-R, Hopp CM, Shoemaker NB, Gardner JF, Salyers AA**. 2013. The excision proteins of CTnDOT positively regulate the transfer operon. Plasmid **69**:172–179.

2. **Kelley LA, Sternberg MJE**. 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc **4**:363–371.

3. **Liu M, Rose M, Crosa JH**. 2011. Homodimerization and binding of specific domains to the target DNA are essential requirements for HlyU to regulate expression of the virulence gene rtxA1, encoding the repeat-in-toxin protein in the human pathogen Vibrio vulnificus. J. Bacteriol. **193**:6895–6901.

4. **Morita EH, Wakamatsu M, Hayashi H**. 2001. Studies on the molecular mechanism of the interactions between the cyanobacterial transcription factor, SmtB, and its recognition DNA sequences. Nucleic Acids Res. Suppl. 251–252.

5. **Waters JL, Salyers AA**. 2012. The Small RNA RteR Inhibits Transfer of the Bacteroides Conjugative Transposon CTnDOT. J. Bacteriol. **194**:5228–5236.

6. **Whitehead TR**. 1997. Development of a bifunctional xylosidase/arabinosidase gene as a reporter gene for the gram-negative anaerobes Bacteroides and Porphyromonas, and Escherichia coli. Curr. Microbiol. **35**:282–286.