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Characterization of TraM, a Required Bacteroides Fragilis Conjugal Apparatus Protein That Interacts with the B. Fragilis Putative Coupling Protein Bcta and the B. Fragilis Relaxase Protein BmpH

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LOYOLA UNIVERSITY CHICAGO

CHARACTERIZATION OF TraM, A REQUIRED Bacteroides fragilis CONJUGAL APPARATUS PROTEIN THAT INTERACTS WITH THE B. fragilis PUTATIVE COUPLING PROTEIN BctA AND THE B. fragilis RELAXASE PROTEIN BmpH

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BY
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CHICAGO, ILLINOIS

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For my dear deceased brother, Dzung Tri Nguyen
“The larger the island of knowledge, the longer the shoreline of mystery”.

- Unknown
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS........................................................................................................ iii

LIST OF TABLES.................................................................................................................. viii

LIST OF FIGURES............................................................................................................... ix

LIST OF ABBREVIATIONS............................................................................................... xi

ABSTRACT......................................................................................................................... xiv

CHAPTER I: INTRODUCTION.......................................................................................... 1

CHAPTER II: LITERATURE REVIEW.............................................................................. 8
  Antibiotics and Antibiotic Resistance.............................................................................. 8
  *Bacteroides* spp, from Commensals to Pathogens......................................................... 13
    *Bacteroides* spp. as Gut Commensals ......................................................................... 14
    *Bacteroides* spp., Commensals Gone Bad Pathogens............................................... 16
    *Bacteroides* spp. and Antibiotic Resistance ................................................................ 20
  Bacterial Conjugation...................................................................................................... 20
    Initiation (DNA processing)............................................................................................ 22
    Conjugal Apparatus........................................................................................................ 25
    Coupling Protein ........................................................................................................... 30
  *Bacteroides* spp. Conjugation........................................................................................ 33
  Mobile Genetic Elements in *Bacteroides* ...................................................................... 33
  *B. fragilis* LV23, Conjugative Transposon BTF-37, Putative Coupling Protein BctA and the Relaxase BmpH ................................................................. 40
  The Search for Better Genetic Modification Tools in Anaerobes.................................... 41

CHAPTER III: MATERIALS AND EXPERIMENTAL METHODS.................................. 44
  Chemicals and Reagents ............................................................................................... 44
  Bacterial Strains and Plasmids....................................................................................... 44
  Bacterial Media, Growth Conditions and Optical Density Readings.......................... 50
  Antibiotics....................................................................................................................... 51
  Recombinant DNA Techniques...................................................................................... 51
  *B. fragilis* Genomic DNA Preparation......................................................................... 52
TraM is Required for Conjugation in *B. fragilis* and Application of Antisense RNA .......................................................................................................140
Future perspective .......................................................................................................141
Significance.................................................................................................................142
REFERENCES ............................................................................................................... 143
VITA ............................................................................................................................... 162
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Comparison of conjugal apparatus of different <em>A. tumefaciens</em>, <em>E. coli</em> and <em>Bacteroides</em> spp. mating systems</td>
<td>29</td>
</tr>
<tr>
<td>2. Summary of mobile genetic elements found in <em>Bacteroides</em> spp.</td>
<td>33</td>
</tr>
<tr>
<td>3. Bacterial strains used in this study</td>
<td>44</td>
</tr>
<tr>
<td>4. Plasmids used in this study</td>
<td>45</td>
</tr>
<tr>
<td>5. Primers used for RT-PCR and Q-PCR</td>
<td>55</td>
</tr>
<tr>
<td>6. Primers for construction of <em>traM</em>- antisense RNA</td>
<td>73</td>
</tr>
<tr>
<td>7. Primers for generation of site directed mutagenesis in <em>traM</em></td>
<td>79</td>
</tr>
</tbody>
</table>


LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Bacteroides</em> relaxosome and conjugal apparatus structural model, based on studies in <em>E. coli.</em></td>
<td>7</td>
</tr>
<tr>
<td>2. Mechanism of resistance acquisition</td>
<td>12</td>
</tr>
<tr>
<td>3. Proportions of <em>Bacteroides</em> species observed clinically</td>
<td>19</td>
</tr>
<tr>
<td>4. Scheme of the relaxase domain of a relaxase</td>
<td>24</td>
</tr>
<tr>
<td>5. Depicted catalytic activities of a relaxase</td>
<td>24</td>
</tr>
<tr>
<td>6. Biogenesis pathway of the <em>A. tumefaciens</em> VirB/D4 T4SS</td>
<td>28</td>
</tr>
<tr>
<td>7. Conjugation</td>
<td>32</td>
</tr>
<tr>
<td>8. Steps involved in the conjugal transfer of a conjugative transposon</td>
<td>38</td>
</tr>
<tr>
<td>9. pET-DEST42 expression vector map</td>
<td>66</td>
</tr>
<tr>
<td>10. Schematic of the BacterioMatch II two-hybrid system dual reporter construct</td>
<td>69</td>
</tr>
<tr>
<td>11. BctA is required for conjugation mediated by CTn BTF-37 in <em>B. fragilis.</em></td>
<td>85</td>
</tr>
<tr>
<td>12. BTF37 conjugative transposon</td>
<td>88</td>
</tr>
<tr>
<td>13. mRNA expression of 11 genes in the 16kb sequenced region of BTF37 in conditions with and without tetracycline induction</td>
<td>88</td>
</tr>
<tr>
<td>14. Bacterial two-hybrid analysis of interactions between BctA and other BTF37 gene products</td>
<td>92</td>
</tr>
<tr>
<td>15. Bacterial two-hybrid analysis of interaction of ORF7 (TraM) with other BTF-37 gene products</td>
<td>93</td>
</tr>
<tr>
<td>16. Homology of some of <em>Bacteroides</em> conjugative transposons</td>
<td>95</td>
</tr>
</tbody>
</table>
17. Summary of conjugal apparatus mutation studies in CTn341, showing the requirement of TraM for conjugation

18. Q-PCR standard curves of 16s rDNA (A), traM (B) and tetQ (C)

19. \textit{traM} gene expression under conjugation conditions


21. Interaction of $\lambda$cI-TraM with BctA

22. Interaction of $\lambda$cI-TraM with the relaxase BmpH

23. TraM is associated with membrane fractionation

24. \textit{traM}-antisense constructs

25. Effect of \textit{traM}-antisense constructs on conjugation capacity of \textit{B. fragilis} LV23 to recipients \textit{B. fragilis} TM4000 (in blue) or \textit{E. coli} HB101 (in red)

26. The predicted secondary structures of anti-traM AS constructs

27. Relative expression of A) \textit{traM} mRNA, B) ORF8 mRNA, C) bctA mRNA in \textit{B. fragilis} LV23 cells harboring the control plasmid with the cefoxitin promoter alone or that carrying AS1, AS2 or AS3 construct

28. TraM expression in the presence of AS in \textit{B. fragilis} membrane fraction

29. Coiled coil heptad amino acid arrangement of two partner proteins

30. TraM and predicted coiled coil regions

31. Interaction of TraM mutants with BctA

32. Interaction of TraM mutants with BmpH

33. Far western analysis of the interactions between TraM mutants and BctA or BmpH

34. Predicted localization of TraM
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>(A.) (tumefaciens) () &amp; (A.) (tumefaciens) () &amp; (Agrobacterium) (tumefaciens)</td>
<td></td>
</tr>
<tr>
<td>AS</td>
<td>Antisense</td>
</tr>
<tr>
<td>asRNA</td>
<td>Antisense RNA</td>
</tr>
<tr>
<td>3-AT</td>
<td>3-amino-1,2,4-triazole</td>
</tr>
<tr>
<td>(B.) (fragilis) () &amp; (B.) (fragilis) () &amp; (Bacteroides) (fragilis)</td>
<td></td>
</tr>
<tr>
<td>bp</td>
<td>Base pair(s)</td>
</tr>
<tr>
<td>BHIS</td>
<td>3.7% brain heart infusion medium supplemented with 0.0005% hemin and 5 g of yeast extract/liter</td>
</tr>
<tr>
<td>CA</td>
<td>Conjugal apparatus</td>
</tr>
<tr>
<td>Cm</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>CP</td>
<td>Coupling protein</td>
</tr>
<tr>
<td>CTn</td>
<td>Conjugative transposon</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>(E.) (coli) () &amp; (E.) (coli) () &amp; (Escherichia) (coli)</td>
<td></td>
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<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>IHF</td>
<td>Integration host factor</td>
</tr>
<tr>
<td>IM</td>
<td>Inner membrane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiolgalactopyranoid</td>
</tr>
<tr>
<td>IR</td>
<td>Inverted Repeat</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani media</td>
</tr>
<tr>
<td>LV23</td>
<td>B. fragilis clinical isolate</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>M9</td>
<td>Miminal</td>
</tr>
<tr>
<td>mob</td>
<td>Mobilization protein</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>μl</td>
<td>microliter</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MPBS</td>
<td>Modified phosphate buffered saline</td>
</tr>
<tr>
<td>MTn</td>
<td>Mobilizable transposon</td>
</tr>
<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>oriT</td>
<td>Origin of transfer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>Quantitative PCR</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per min</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Sm</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>Sp</td>
<td>Spectinomycin</td>
</tr>
<tr>
<td>T4SS</td>
<td>Type IV secretion system</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate ethylenediaminetetraacetic</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris borate EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>20mM Tris-Cl, 150mM NaCl, pH 7.5</td>
</tr>
<tr>
<td>TBST</td>
<td>20mM Tris-Cl, 150mM NaCl, 1% Tween 20, pH 7.5</td>
</tr>
<tr>
<td>TBSTT</td>
<td>20mM Tris-Cl, 150mM NaCl, 0.05% Tween 20, 0.2% Triton X-100, pH 7.5</td>
</tr>
<tr>
<td>Tc</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>Tet</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TG</td>
<td>2.5mM Tris-Cl, 19.2% Glycine</td>
</tr>
<tr>
<td>TGS</td>
<td>2.5mM Tris-Cl, 19.2% Glycine, 0.01% SDS</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>Tn</td>
<td>Transposon</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/ volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/ volume</td>
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</tbody>
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Since the first antibiotics became widely available 60 years ago, they have been used aggressively. However, bacterial infections continue to be significant clinical problems both in hospital and community settings. Bacterial pathogens have become increasingly resistant to a variety of antibiotics. They can acquire resistance genes, even from distant related genera. Even the common resident (commensal) bacteria of the human colon can carry and transfer antibiotic resistance elements to each other, or to human pathogens. *Bacteroides* spp. organisms, the most predominant commensal bacteria in the human gut, harbor a plethora of these elements, many of which are mobile (transmissible); thus *Bacteroides* spp. are considered to be reservoirs of antibiotic resistance genes. Mobile elements are transferred within and from the *Bacteroides* spp. primarily by conjugation. One important approach to prevent the dissemination of antibiotic resistance is to design drugs to disrupt the conjugation apparatus in the *Bacteroides* spp. However, little is known about the molecular mechanism of this machinery in *Bacteroides* spp.

Previously, our laboratory identified BTF-37, a 37kb conjugative transposon, from the *B. fragilis* clinical isolate LV23. BTF-37 encodes conjugal apparatus proteins and confers mobility to non-mobile plasmids. *BctA*, a highly conserved ORF on BTF-37,
encodes a potential coupling protein, the “gatekeeper” that couples transferring DNA molecules to the membrane-associated conjugal apparatus. Studies from our laboratory have shown that BctA is required for conjugation. One other gene on BTF-37 is orf7, whose product ORF7 is also called TraM since it is a closely related homolog of a previously identified, but unstudied putative conjugal apparatus protein harbored on the transposon CTn341.

In this study, we demonstrated that TraM exhibits characteristics of a required conjugal apparatus protein including 1) sequence conservation with other Bacteroides spp. conjugal apparatus proteins; 2) upregulated expression under conjugation conditions; 3) localization to the bacterial inner membrane; 4) interaction with the putative coupling protein BctA and the relaxase BmpH; and 5) absolute requirement for DNA transfer within and from B. fragilis LV23. Sequence alignment, quantitative PCR, localization studies, protein interaction studies and RNA antisense studies were performed to examine the above characteristics. Moreover, mutagenesis and protein interaction studies revealed that two amino acids (F66 and L123) in two predicted coiled-coil domains of TraM were required for TraM interaction with BctA, suggesting that these two amino acids may be essential for TraM function in mediating DNA transfer in B. fragilis. In addition, we also identified a different amino acid, L123, as being important for the interaction of TraM with the relaxase BmpH. These results suggest that there are likely complex mechanisms involved in the interaction between TraM and BctA and/or BmpH, to facilitate DNA transfer efficiently within and from B. fragilis.
This study represents the first in-depth characterization of a conjugal apparatus protein in *B. fragilis*, and will be useful for future studies aimed at developing interventions to prevent dissemination of antibiotic resistance from *Bacteroides* spp. to other bacteria. Moreover, this is one of very few studies using RNA antisense technology to knock-down target gene expression in anaerobes, and has avoided the known difficulty in genetically manipulating DNA in these organisms.
CHAPTER I
INTRODUCTION

One of the biggest battles that humans have ever faced is the battle against infectious diseases. With rapid technology development, we thought that we could conquer all diseases. However, no matter how technologies develop, microbes mutate themselves to adapt and survive most treatments. In addition, they can also multiply rapidly and transfer their adaptation characteristics to others. It is likely not efficient to simply increase direct approaches to kill microbes. More efforts need to be focused on other directions, specifically those designed to prevent microbial adaptation to treatments, and to prevent the dissemination of adaptation traits amongst microbes.

One challenge that we encounter is the emergence and spread of bacteria that are resistant to a broad range of “first-line” antibiotics (74, 75, 161, 170, 180). These include bacterial infections that contribute most to human diseases: diarrhoeal diseases, respiratory tract infections, meningitis, sexually transmitted infections, and hospital-acquired infections. Some examples include penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus*, multi-resistant salmonellae, and multi-resistant *Mycobacterium tuberculosis*. The consequences are severe. Treatments fail to treat infections caused by resistant bacteria,
resulting in prolonged illness and greater risk of death. Treatment failures also lead to longer periods of infectivity, which increase the numbers of infected people moving in the community. As a consequence, the general population is exposed to the risk of acquiring a resistant strain during infection. Moreover, when infections become resistant to first-line antibiotics, treatment has to be switched to second- or third-line drugs, which are much more expensive and sometimes more toxic. In many countries, this is also unaffordable. As a result, some diseases can no longer be treated in areas where resistance to first-line drugs is widespread. Moreover, bacteria will finally adapt to resist even the latest drugs. Humans may come to a point when no available drug can treat certain diseases. Therefore, alternative approaches to prevent the dissemination of antibiotic resistance elements are smart choices.

Bacteria are particularly efficient at disseminating the effects of resistance, partly because they can transfer their resistance genes to others. Of immense concern, even common resident (commensal) bacteria of the human colon can carry and transfer antibiotic resistance elements to other bacteria, including human pathogens (189). Moreover, the most predominant commensal bacteria of the gut, *Bacteroides* species (spp.), are considered the reservoir of antibiotic resistance elements (151, 152, 161). Nearly 100% of all *Bacteroides* spp. clinical isolates are now resistant to tetracycline (126, 127). Many of them are resistant to β-lactam antibiotics (penicillin, ampicillin, cephalosporins, cefoxitin, cephemycins and carbapenems), metronidazole and the macrolide-lincosamide-streptogramin (MLS) group of antibiotics (erythromycin and clindamycin) (161, 192). All of these resistant traits have been found on transmissible
genetic elements (161). Over the past 3 decades, carriage of the tetracycline resistance
gene, \textit{tetQ}, has increased from about 30\% to nearly 100\% of strains (161). The fact that
these resistance determinants are prevalent and found on transmissible elements in
\textit{Bacteroides} spp., particularly conjugative transposons (161), raised the concern that
\textit{Bacteroides} spp. may act as reservoirs of antibiotic resistance genes, which would then
be transferred to other bacterial species. The primary means for \textit{Bacteroides} spp. to pass
on their resistance genes to other bacteria is "conjugation", whereby the genetic material
carrying antibiotic resistance genes is transferred from one bacterium to another (151,
192). It would be useful if we understood the conjugation machinery of \textit{Bacteroides} spp.,
and designed preventions to disrupt the formation or action of the conjugation process.
This would help prevent the dissemination of antibiotic resistance genes in bacteria.
However, very little is known about the molecular mechanism of conjugation in
\textit{Bacteroides} spp.

To gain deeper understanding of conjugation mechanism in \textit{Bacteroides} spp., this
study focused on characterization of TraM, an important protein required for conjugation
in \textit{Bacteroides fragilis} (\textit{B. fragilis}), the most common anaerobic pathogen isolated in
human bacterial infections (62).

Conjugation involves two major sets of events: initiation (DNA processing) and
conjugal apparatus formation.

DNA processing includes binding, nicking and unwinding of the DNA, independent of conjugal apparatus formation (89, 134). This process occurs via the
relaxosome, a nucleoprotein complex composed of specific proteins (mobilization
proteins) covalently linked to the 5’ end of the DNA molecule to be transferred (133, 135). A relaxase, the major mobilization protein, nicks the DNA to be transferred at its origin of transfer (oriT) (89, 134). This nicked DNA is unwound and transmitted in single stranded copy with 5’-3’ polarity from the donor to the recipient (89). The passage from the donor to the recipient occurs through a specialized membrane-traversing channel called the conjugal apparatus.

The conjugal apparatus (CA) is a multi-protein channel that is assembled across donor and recipient cell membranes during conjugation for DNA transferring (63, 154). Although the formation of the conjugal apparatus is well studied in Agrobacterium tumefaciens Ti plasmids and E. coli F and RP4 plasmids, little is known about its structure and function in the Bacteroides spp. In E. coli and Agrobacterium tumefaciens, this membrane channel is formed by 10-12 proteins (4, 63, 99, 100). In Agrobacterium spp., each of these proteins has been extensively characterized (26, 29). However, the Bacteroides spp. CA in even the best-studied elements is still poorly understood. To date, the only detailed description of CA-encoding genes in Bacteroides spp. is from Smith C.J. et al., who assessed the requirement of each CA gene of the CTn341 isolated from B. vulgatus for DNA conjugation by creating deletion mutants (7).

Previously, our laboratory reported the capture of BTF-37, a 37kb conjugative transposon, from a clinical B. fragilis isolate, LV23 (181). Due to A-T rich regions, only 16kb of BTF-37 was sequenced. This 16kb region carries 11 open reading frames (ORFs). By sequence homology analysis with other Bacteroides known transfer factors, and by RT-PCR studies, these 11 genes correspond to the transfer region of BTF-37 (77).
In this work, RT-PCR studies showed that the transcripts of all of these 11 genes were up-regulated when LV23 cells were stimulated with tetracycline. One of these 11 ORFs, *bctA*, is an ORF highly conserved between different *Bacteroides* spp. transposons. *BctA* encodes a putative coupling protein, the “gatekeeper” that couples transferring DNA molecules to the membrane-associated conjugal apparatus, of BTF-37 CTn (77). We also showed that *BctA* was required for transfer of BTF-37 (77).

*BmpH* is a relaxase encoded by the mobilizable transposon Tn5520 that was also isolated from *B. fragilis* clinical isolate LV23 (182, 183). Studies from our laboratory have shown that *BmpH* is a relaxase protein that is required for the formation and function of the relaxosome, required for the mobility of Tn5520 (182).

We then identified ORF7, a *Bacteroides* spp. *TraM* homologous protein, as an important conjugal apparatus protein. *TraM* is a 393 amino acid protein, and has 92% sequence identity to *TraM* from *B. fragilis* NCTC9343, 61% sequence similarity to *TraM* of *B. fragilis YCH46* and 32% identity to *TraM* of *B. thetaiotaomicron* CTnDOT.

In this study, we demonstrated that *TraM* exhibits characteristics of a required conjugal apparatus protein including 1) sequence conservation with other *Bacteroides* spp. conjugal apparatus proteins; 2) upregulated expression under conjugation conditions; 3) localization to the bacterial inner membrane; 4) interaction with the putative coupling protein *BctA* and the relaxase *BmpH*; and 5) requirement for DNA transfer within and from *B. fragilis* LV23. Sequence alignment, quantitative PCR, localization studies, protein interaction studies and RNA antisense studies were performed to examine the above characteristics. Moreover, mutagenesis and protein interaction studies revealed that
two amino acids (F66 and L123) in two predicted coiled-coil domains of TraM were required for TraM interaction with BctA, suggesting that these two amino acids may be essential for TraM function in mediating DNA transfer in *B. fragilis*. We also identified another amino acid, L123, as being important for the interaction of TraM with the relaxase BmpH.

This study represents the first in depth characterization of a conjugal apparatus protein in *B. fragilis*, which will be useful for future studies aimed at developing interventions to prevent dissemination of antibiotic resistance from *Bacteroides* spp. to other bacteria. Moreover, this is one of very few studies using RNA antisense technology to knock-down target gene expression in anaerobes, avoiding the difficulty in modifying genes in these genera.
Figure 1. *Bacteroides* relaxosome and conjugal apparatus structural model, based on studies in *E. coli*. Adapted from Christie, Nature, 2009 (28).
CHAPTER II
LITERATURE REVIEW

**Antibiotics and Antibiotic Resistance**

Antibiotics are drugs that act to kill bacteria or to inhibit their growth. For thousands of years, many ancient cultures (Chinese, Greek, Egyptians) used plants or molds with antimicrobial properties to treat infections. However, it was not until early in the twentieth century that natural antibiotics produced by microorganisms were discovered. In 1928, the British microbiologist Sir Alexander Fleming made a chance discovery of the first antibiotic, penicillin, from the common bread mold *Penicillium* growing on a culture of *Staphylococcus aureus* (187). By the 1940s, penicillin was available for medical use and was successfully used to treat infections in soldiers during the World War II. Penicillin has since been commonly used to treat a wide range of infections including ulcers, diphtheria, gonorrhea, meningitis, pneumonia, syphilis and tuberculosis (8). Since then, many more antibiotics have been isolated from fungi (molds) and bacteria. One group of bacteria, the *Streptomyces*, produces most of the medically important antibiotics, such as quinolones, spectinomycin, tetracycline, and streptomycin (186). Moreover, with the development of medical chemistry, many semi-synthetic and
novel synthetic antibiotics were also introduced into clinical practice (162). It has been estimated in 2002 that 100,000-200,000 tons per annum, and, a total of more than one million tons of antibiotics have been applied worldwide in human and animals since the 1940s (194).

Antibiotics can be classified based on their mode of action, structure or spectrum of activity. They can be bactericidal (kill susceptible bacteria), or bacteriostatic (inhibit bacterial growth). Most antibiotics target bacterial functions or growth processes. They can target the bacterial cell wall (penicillins, cephalosporins), cell membrane (polymixins), protein synthesis (the aminoglycosides, macrolides, and tetracyclines), and nucleic acid synthesis and function (rifampicin, quinolones) (46). Narrow-spectrum antibiotics target particular types of bacteria, such as Gram-negative or Gram-positive bacteria, while broad-spectrum antibiotics affect a wide range of bacteria.

The extreme efficiency of antibiotics in clearing bacteria led many to believe that infectious diseases would be completely wiped out. However, the golden age of antibiotics did not last long. During the past few decades, many strains of bacteria have evolved and disseminated resistance to antibiotics. Just a few years after the mass introduction of penicillin, i.e., by 1950, 40% of hospital Staphylococcus aureus isolates were penicillin resistant; and by 1960, this had risen to 80% (24). In 1980, it was estimated that 3-5% of Streptococcus pneumoniae were penicillin resistant and 34% of S. pneumoniae samples were resistant to penicillin (41). Antibiotic resistance by other organisms reflects the same trend. Tetracycline was introduced in the 1950s and quickly became one of the most widely prescribed broad-spectrum antibiotics. However,
tetracycline resistance by normal human intestinal flora has exploded from 2% in the 1950s to 80% in the 1990s (161). Kanamycin, an antibiotic used in the 1950s, has become clinically useless due to the abundance of kanamycin resistant bacteria. Dangerously, the emergence of many strains of multidrug resistant bacteria has become a major clinical problem, complicating the treatment of bacterial infections and leading to increased mortality (39, 112, 117, 152). Many strains of *Staphylococcus aureus*, a major cause of deadly infections in hospitals, are already resistant to all antibiotics except the reliable vancomycin. However, over the last few years, vancomycin-resistant strains have also emerged (25). Multidrug resistant strains of *Mycobacterium tuberculosis*, the causative agent of tuberculosis - that kills 1.7 million people annually - are also spreading all over the world (117).

How do we overcome the problem of antibiotic resistance? Obviously, increasing the direct combative approach may not be a good choice as bacteria may quickly evolve to surpass new generations of antibiotics. Even when the use of antibiotics is restricted, the incidence of antibiotic resistance is reduced, but never disappears (53, 55). Therefore, alternative approaches to prevent the dissemination of antibiotics resistant elements are smart choices.

It has been known that antibiotic resistant bacteria have evolved to acquire many mechanisms to resist antibiotics, such as reduction of cell permeability to antibiotics, increase efflux of antibiotics, alteration of the antibiotic target site, enzymatic inactivation of antibiotics, alteration of metabolic synthesis to bypass the inhibited reaction of antibiotics, and overproduction of antibiotic targets (39, 170). These
mechanisms can be acquired through spontaneous mutation. However, in the majority of cases, acquisition from exogenous sources is the primary mechanism by which bacteria obtain genes encoding resistance to antibiotics (39). In these cases, the acquisition of genes encoding antibiotic resistance occurs mainly through horizontal gene transfer mechanisms: transduction (acquisition from a bacteriophage), transformation (acquisition from free DNA from and conjugation (acquisition from another bacterium through a cell-cell contact). Among these methods, conjugation is by far the most widespread, and most efficient. Therefore, a deeper understanding of horizontal gene transfer, especially conjugation mechanisms, may help in designing interventions to prevent antibiotic resistance dissemination in bacteria.
Figure 2: Mechanism of resistance acquisition. DNA encoding an antibiotic resistance gene (red) can be transferred by horizontal gene transfer into a recipient by several paths: cell-to-cell conjugation; transformation by naked DNA (on plasmids or as linear DNA) that is released by dead cells; or phage-mediated transduction. Resistance can also arise
by *de novo* mutation (indicated as a red cross). (Adapted from Andersson *et al.* Nat Rev Microbiol, 2010 (3)).

**Bacteroides spp. from Commensals to Pathogens**

It has been estimated that microbes in our bodies collectively make up to 100 trillion cells, tenfold the number of human cells (96, 197). Moreover, the number of genes in our microbiome is more than 100 times the number of human genes, making us genetically 1% human and 99% bacteria (169, 197). The majority of these normal flora, with over 500 bacterial species, $10^{11}$ organisms per gram of wet weight, reside in the gut, have a profound influence on human physiology and nutrition, and are crucial for human life (110). Of these, 99% are anaerobes (65). The anaerobic *Bacteroides* spp. are the most predominant species, accounting about 30% of all bacteria in the human gut (149, 189).

*Bacteroides* spp. are bile-resistant, non-spore forming, gram-negative, rod-shaped anaerobes that are the most predominant bacterial species in the human colon and are less abundant in the intestines of other animals and in the environment (161, 192). The genus comprises more than 20 species, of which *B. fragilis* is the most frequent isolate from clinical specimens (189). *Bacteroides* sp are passed from mother to child during vaginal birth and thus become part of the human flora in the earliest stages of life (145). The C+G nucleotide composition of *Bacteroides* genome is in the range of 40-48%. Its membranes contain sphingolipids, which is unusual in bacteria.
**Bacteroides spp. as Gut Commensals**

As part of the human gut normal flora, *Bacteroides* spp. play a number of commensal roles, such as providing energy for the host in the form of short fatty acids and sugars, being involved in the recycling of bile acids and aiding in the development of the host immune system (189). They also exhibit unique adaptations to successfully colonize the gut such as the ability to change their cell surface architecture, ability to stimulate host expression of fucosylated glycoproteins and synthesize them, and the ability to tolerate and use oxygen.

*Bacteroides* spp. provide energy for the host in the form of fatty acids and sugars. *Bacteroides* spp. and other intestinal bacteria ferment carbohydrates to produce a pool of volatile fatty acids, predominately acetate, propionate (from succinate), and butyrate, that can be easily reabsorbed through the large intestine and utilized by the host as an energy source (81). Studies show that germ-free rats lacking gut flora need 30% more calories to maintain body mass than normal rats (56). *Bacteroides* can also generate sugars via their glycosylhydrolases; this benefits not just the host but also other organisms that do not have such sugar utilization enzymes. *B. thetaiotaomicron* has an extensive starch utilization system and multiple genes (*sus* genes) that are involved in starch binding and utilization (198). *B. thetaiotaomicron* contains more glycosylhydralases than any sequenced prokaryote and appears to be able to cleave most of the glycosidic bonds found in nature (197). Encoding 172 glycohydrolases and 163 homologs of starch
binding proteins, they can utilize the wide variety of dietary carbohydrates that might be available in the gut (196, 198).

In addition, \textit{Bacteroides} spp. also play a key role in the recycling of bile acids. Before secretion in bile, bile acids are synthesized in the human liver, conjugated to taurine or glycine polar side groups. These conjugated forms help bile assist in absorption of dietary fats in the upper intestine. If bile acids are not used, they are deconjugated by bacteria to re-enter the enterohepatic circulation, return to the liver and re-conjugated for further use (15). Containing many enzymes required for these reactions, including a hydrolase, dehydrogenase, and dehydroxylase, \textit{Bacteroides} spp. are known to play a major role in the deconjugation of bile acids (116).

Moreover, during mammalian development, \textit{Bacteroides} spp. stimulate intestinal angiogenesis and induce local and systemic immune function (80, 82, 114). Intestinal bacteria are important in the development of gut-associated lymphoid tissue (GALT). Without bacterial colonization in the gut, the development of GALT is defective (80). In rabbits, \textit{B. fragilis} and \textit{Bacillus subtilis} promote development of GALT and the pre-immune antibody repertoire (147). In addition, it has been shown that in newborn mice, \textit{B. thetaiotaomicron} promotes angiogenesis and postnatal development (171). The gut epithelium undergoes constant renewal throughout the postnatal period. The appearance of Paneth cells, a lineage of stem cells and a key cellular component of the innate immune system, coincides with initial colonization of the gut and their subsequent differentiation is influenced by the gut microbiota, (6, 20). \textit{B. thetaiotaomicron} can stimulate production of an antimicrobial Paneth cell protein (Ang4) that can kill certain
pathogens (171). Moreover, zwitterionic polysaccharides (ZPS) produced by \textit{B. fragilis} have been shown to be important in the activation of CD4$^+$ T cells and appear to be involved in the development of CD4$^+$ T cells (113).

As the most predominant bacteria residing in the gut, \textit{Bacteroides} spp. have several unique adaptations allowing them to successfully survive in this niche. They can rapidly change their cell surface architecture through the production of an unusually large number of phase-variable capsular polysaccharides (87), an adaptation that appears specific to the intestinal environment (33). This surface-altering capability is most developed in \textit{B. fragilis}, which is more frequently found at the mucosal surface, the site of attack by host defense (88). Also, \textit{Bacteroides fragilis} has been shown to stimulate expression of fucosylated glycoconjugates on the intestinal epithelia of colonized mice (21). \textit{Bacteroides} spp. produce enzymes to harvest fucose from host mucosal glycans and have a rare bacterial pathway to incorporate this exogenous fucose directly into capsular polysaccharides and glycoproteins (35). It is suggested that the ability of \textit{B. fragilis} to synthesize fucosylated glycoproteins is essential for its competitive colonization in the mammalian intestine (32, 35). Finally, the ability of \textit{B. fragilis} to tolerate and use oxygen may partly explain the observation that it is mostly found at the mucosal surface, where oxygen tensions are higher than within the intestinal lumen (12).

\textbf{\textit{Bacteroides} spp., Commensals Gone Bad Pathogens}

As discussed above, \textit{Bacteroides} spp. are normally commensals in the gut. However, when they escape the gut due to surgery or other trauma, they can cause life-
threatening infections such as peritonitis and intra-abdominal sepsis (161, 185, 189). Intra-abdominal sepsis is the most common infection caused by *Bacteroides* spp. Following the disruption of the intestinal wall due to a surgical wound, malignancies, or appendicitis, the gut flora spill out and infect the normally sterile peritoneal cavity. At first, the aerobes, such as *E. coli*, dominate the infection site, reducing the oxidation-reduction potential of the oxygenated tissue. Once sufficient oxygen has been removed, anaerobic *Bacteroides* spp. replicate and predominate during the second, chronic stage of infection (189). *Bacteroides* spp. rarely cause endocarditis, inflammation of the inner layer of the heart, but when it occurs it can be serious with a mortality rate of 21-43% (19). They can also be found in other infections such as skin and soft tissue infections, bacteremia, septic arthritis, brain abscess and meningitis (189). Enterotoxigenic *Bacteroides fragilis* (ETBF), a sub-group of *B. fragilis* also has been implicated in inflammatory bowel disease (IBD) (10, 140) and colon cancer (176).

Although *B. fragilis* accounts for only 1-2% of the human intestinal flora, it is the most commonly isolated anaerobic pathogen, partly due to its virulence factors including surface polysaccharide capsules, outer membrane vesicles, toxins and \( \beta \)-lactamase (139). In addition, the capacity of *B. fragilis* to tolerate nanomolar concentrations of oxygen allows this species to predominate in infections in peritoneal cavity.

*B. fragilis* expresses the most number of distinct capsular polysaccharides (at least eight). The *Bacteroides* capsule has an unusual structure, composed of repeating units of two distinct high molecular weight polysaccharides, each of which contains exposed positively and negatively charged side-chains (179). This zwitterionic motif is critical for
promoting the formation of abscesses (179). Injection of capsules alone has proven sufficient to induce abscess formation in rats (34). Capsule also can resist to complement-mediated killing and to phagocytic uptake and killing (54, 146).

In addition, *B. fragilis* produces numerous outer membrane vesicles (OMVs). These vesicles have been shown to have haemagglutin function and sialidase activity. Neuraminidase activity in OMVs was correlated to virulence (42). Moreover, OMVs can carry endotoxins to target cells.

Moreover, *B. fragilis* may secrete two toxins: endotoxin (LPS) and *B. fragilis* enterotoxin (named BFT or fragilysin). BFT is a 20kD zinc-dependent metalloprotease, secreted by enterotoxigenic *B. fragilis*. ETBF causes acute inflammatory diarrheal disease in children and adults (157). BFT mediates proteolytic cleavage of the extracellular domain of the adherent protein, E-cadherin, which is a cell-surface protein of epithelial cells (195). Higher ETBF colonization levels have also been found in individuals with colon cancer relative to others without colon cancer, suggesting that EBTF may be involved in development of colon cancer (176).

Most *Bacteroides* strains express constitutive β-lactamase activity. β-lactamase enzyme is extra-cellular, and thus is capable of diffusing within an abscess or other site of infection. Production of extra-cellular β-lactamases has been shown to protect other organisms in the vicinity during a mixed infection (129).
Figure 3. Proportions of *Bacteroides* species observed from clinical isolations.

Bacteroides spp. and Antibiotic Resistance

Many Bacteroides spp. are resistant to aminoglycosides (gentamicin, kanamycin, streptomycin), tetracycline (nearly 85% of clinical isolates), β-lactam antibiotics (penicillin, ampicillin, cephalosporins, cefoxitin, cephemycins and carbapenems), metronidazole and the macrolide-lincosamide-streptogramin (MLS) group of antibiotics (erythromycin and clindamycin) (161, 192). All of these resistance traits have been found on transmissible genetic elements (161). Over the past 3 decades, carriage of the tetracycline resistance gene, tetQ, has increased from about 30% to more than 80% in clinical strains (161). The fact that these resistance determinants are prevalent and found on transmissible elements in Bacteroides spp., particularly conjugative transposons (161), is of particular concern. The concern is not only that opportunistic infections caused by Bacteroides spp. may become untreatable, but also that Bacteroides spp., as reservoirs of antibiotic resistance genes, may then transfer them to other bacterial species.

The mechanism responsible for the dissemination of genetic elements in Bacteroides spp. is conjugation, one of the most important mechanisms of horizontal gene transfer in prokaryotes. However, the molecular structure and mechanism of this process is poorly understood in Bacteroides spp.

Bacterial Conjugation
Conjugation, a subset of the bacterial type IV secretion system (T4SS), is defined as the unidirectional transfer of single-stranded DNA molecule from a bacterial donor cell to a recipient cell, in a process requiring cell-to-cell contact (193). During conjugation, one copy of the DNA strand is transferred to, and replicated in, the recipient cell. The parent DNA is retained and replicated in the donor cell. Transfer DNA molecules, which can be either plasmids or transposons, are of two types: conjugative and mobilizable. Conjugative plasmids and transposons are self-transmissible elements. They encode all of the components necessary for transfer. Mobilizable plasmids and transposons are non-self-transmissible elements. Their transfers require the assistance of a co-resident conjugative transfer element. Conjugative elements tend to be large (>30kb), while mobilizable elements are small (<15kb) (50). Conjugative plasmids tend to have low copy number, while mobilizable plasmids tend to have high copy number (50).

All transfer elements contain a cis-acting origin of transfer (oriT), where transfer is initiated. The oriTs are specific sequences, about 30 to 500 bp, significantly located adjacent to the transfer initiation genes known as mobilization (“mob”) genes to form a compact mobilization region (89). A common feature of the oriT is the presence of inverted repeats adjacent to the sequence where nicking of DNA occurs (132). The nick (nic) site, a short stretch of about 10 nucleotides is the site for recognition by a relaxase, an enzyme required for the nicking of the transfer DNA in the initiation process.

Conjugation involves two major sets of events: Initiation (DNA processing) and conjugal apparatus formation.
Initiation (DNA processing)

DNA processing includes binding, nicking and unwinding of the DNA, independent of conjugal apparatus formation (89, 134). This process occurs via the relaxosome, a nucleoprotein complex composed of specific proteins (mobilization proteins), one of which (relaxase) is covalently linked to the 5’ end of the DNA molecule to be transferred (133, 135). The relaxase, the major mobilization protein, nicks the DNA to be transferred in a site- and strand-specific manner at its origin of transfer (oriT) (89, 134), and then covalently associates with the 5’-end of the nicked DNA via a phosphotyrosyl linkage. This nicked DNA is unwound and transmitted in single stranded copy with 5’-3’ polarity from the donor to the recipient (89). Single-stranded copies in both the donor and the recipient are then re-circularized and made double stranded (89). The passage from the donor to the recipient occurs through a specialized membrane traversing channel called the conjugal apparatus.

Relaxase proteins, the major mobilization proteins of the relaxosomes, are large and usually contain two or more proteins domains. The relaxase domain is always located at the N-terminus of the protein (50). At the C terminus, a DNA helicase, DNA primase or other domain of unknown function is almost always found (50). Crystal structures of some relaxases have been established, including, the relaxase domain of TraI from F plasmid with and without a bound DNA substrate (90, 91), the relaxase domain of TrwC from plasmid R388 with bound DNA (13, 66) and the relaxase domain of MobA from IncQ plasmid R1162 (121). In many cases, the relaxase domain contains at least three
conserved protein motifs. Motifs I contains the active site tyrosine, which creates a single-stranded 5’ DNA nick through a trans-esterification reaction similar to type I topoisomerase (134). This reaction involves the nucleophilic attack on the DNA-phosphate backbone by the tyrosine’s hydroxyl group, resulting in a reversible covalent phosphodiester bond (23, 89). Motif II might be responsible for the recognition and noncovalent binding of the relaxase with the end of the trailing region 3’ of the nic site. Motif III is histidine-rich and is called HUH (His-hyrophobic residue-His) or HHH (His-His-His). This motif may facilitate the cleavage reaction (trans-esterification) by abstracting a proton from the terminal tyrosine hydroxyl, allowing the oxygen to act as a nucleophile (89). The termination of strand transfer occurs via a second cleavage reaction, releasing a single-stranded DNA molecule in the recipient cell (89).

Most relaxases require assistance from accessory proteins to bend and change the conformation of the DNA to facilitate relaxase binding. The RP4 E. coli plasmid encoded relaxosome requires three proteins (two cognate proteins and a host encoded integration host factor-IHF) for correct conformational DNA bending, which helps the relaxase to easily bind and nick the DNA (23). Similarly, relaxase activity of the TrwC relaxase of the R388 plasmid system also requires assistance from TrwA (120). In the F plasmid system, TraY also enhances the relaxase/helicase activity of TraI (108).
Figure 4. Scheme of the relaxase domain of a relaxase. The three conserved motifs are represented by red (I), purple (II) or orange (III) boxes.

Figure 5. Depicted catalytic activities of a relaxase. (1) The relaxase with relaxase domain in blue and helicase domain in red recognizes an extruded cruciform at oriT. (2) The binding results in DNA melting and cleavage of the T-strand. (3) The end 3’ to nic remains covalently bound to the catalytic tyrosine while the protein moves in the 5’ to 3’ direction unwinding the DNA. The uncleaved strand serves as a template for complementary strand synthesis (orange) by host DNA polymerase. (4) The relaxase reaches the regenerated oriT DNA and again recognizes the specific nic sequence. (5) A second cleavage occurs followed by a strand-transfer reaction that (6) produces a
circularized T-strand DNA (in blue) that will be transferred to the recipient cell. Adapted from Guasch et al., Nature structural biology, 2003 (66).

**Conjugal Apparatus**

The second process in conjugation is the formation of the conjugal mating apparatus. The conjugal apparatus (CA) is a multi-protein channel that is assembled across donor and recipient cell membranes during conjugation for DNA transferring (63, 154). In addition, there is often a pilus or other surface filament or proteins(s) associated with the core complex to facilitate adhesion and contact between two cells (30, 31).

Although the formation of the conjugal apparatus is well studied in *Agrobacterium tumefaciens* Ti plasmids and *E.coli* F, RP4 and R388 plasmids, little is known about its structure and function in *Bacteroides* spp. In *E. coli* and *A. tumefaciens*, this membrane channel is formed by 10-12 proteins (4, 63, 99, 100). In *E. coli* RP4 plasmid system, the mating channel is composed of 10 mating pair gene products, a TraF pilin support protein and the coupling protein TraG (63, 71). In *E. coli* F plasmid system, that channel is composed of 11 proteins including the coupling protein TraD (93). In *A. tumefaciens*, each of these 12 proteins named VirB1 to VirB11 and VirD4 has been extensively characterized (26, 29). Recently, a cryo-electron microscopy (cryo-EM) structure of the core complex of the conjugal apparatus encoded by the *E. coli* conjugative plasmid pKM101 showed that the CA complex is 108 Angstrom wide and high and spans from the inner to outer membranes (48). However, in *Bacteroides* spp. CA in even the best studied element is still poorly understood. To date, the only detailed
description of CA-encoding genes in *Bacteroides* spp. is from the laboratory of Smith C.J. et al., who assessed the requirement of each CA gene of the CTn341 isolated from *B. vulgatus* for DNA conjugation by creating deletion mutants (7).

Studies from T4SS in *A. tumefaciens* VirB/D4 system suggested four stages required for the formation of the CA (30).

- **Stage I: Formation of the core complex.** In this stage, a stable structure, termed a core complex, composed of several highly conserved proteins, assembles across the cell envelope. In *A. tumefaciens*, the core complex is composed of five highly conserved proteins. The existence of this core complex is demonstrated by interactions among these proteins (30). There is indirect evidence that this core substructure alone confers function. During conjugation between *A. tumefaciens* donor and recipient cells, synthesis of core subunits in the recipient cells stimulates acquisition of DNA by several orders of magnitude (14, 102). Similarly, in *Helicobacter pylori*, homologs of *A. tumefaciens* core complex assemble as a competence system (79).

- **Stage II: Recruitment of pilus-associated proteins.** In the stage II reaction, the core complex recruits subunits required for trans-membrane pilus assembly. In *A. tumefaciens*, the production of the stage II subunits stimulates DNA transfer to a greater extent than does synthesis only of the core components (102).

- **Stage III: Recruitment of an ATPase to the inner membrane.** It is proposed that the ATPase stimulates formation of trans-envelope structures composed of an
inner membrane platform, a polymer that extends across the periplasm, and an outer membrane pore complex (104).

- **Stage IV: Modifications to yield the pilus or secretion channel.** In this stage, the pilus may undergo depolymerization to facilitate close cell-to-cell contact. The mating apparatus may also undergo conformational changes to become fully functional (30).
Figure 6. Biogenesis pathway of the *A. tumefaciens* VirB/D4 T4SS. A four-stage assembly pathway is presented. Stage I: assembly of the core complex; Stage II: Recruitment of pilus associated components (B2, B3, B5). Stage III: Recruitment of ATPase. Stage IV: Formation of T-pilus or DNA secretion channel. Adapted from Christie *et al.*, Annu. Rev. Microbiol., 2005.
Table 1. Comparison of conjugal apparatus of different *A. tumefaciens*, *E. coli* and *Bacteroides* spp. mating systems.

<table>
<thead>
<tr>
<th>Mating system</th>
<th>Number of CA proteins&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Energetic components&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Core channel components</th>
<th>Pilus components</th>
<th>Specific for own cognate relaxosome</th>
</tr>
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<tbody>
<tr>
<td><em>A. tumefaciens</em> VirB/D system (2)</td>
<td>12</td>
<td>VirD4 (CP), VirB11, VirB4</td>
<td>VirB3, VirB8, VirB10, VirB6, VirB7, VirB9, VirB1</td>
<td>VirB2, VirB5</td>
<td>Yes (73)</td>
</tr>
<tr>
<td><em>E. coli</em> F factor (93)</td>
<td>11</td>
<td>TraD (CP), TraC</td>
<td>TraL, TraE, TraK, TraB, TraV, TraG, TraW, TraC</td>
<td>TraA</td>
<td>Yes (155)</td>
</tr>
<tr>
<td><em>B. thetaiomicron</em> CTnDOT (161)</td>
<td>17 putative gene products</td>
<td>Putative CP: TraG (OrfG)</td>
<td>N/A</td>
<td>N/A</td>
<td>No</td>
</tr>
<tr>
<td><em>B. fragilis</em> BTF37 (181)</td>
<td>N/A</td>
<td>Putative CP: BctA (77)</td>
<td>ORF7 (TraM), ORF8 (TraN)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>N/A</td>
<td>No</td>
</tr>
<tr>
<td><em>B. vulgatus</em> CTn341 (7)</td>
<td>17 putative gene products</td>
<td>Putative CP: TraG (OrfG)</td>
<td>N/A</td>
<td>N/A</td>
<td>No</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Number of CA proteins includes energetic components, core channel components and pilus components.<sup>b</sup>: Energetic components include coupling protein and other ATPase proteins. <sup>c</sup>: This study.
**Coupling Protein**

One of the CA’s important components is the coupling protein (CP). CPs are present in all conjugative plasmids or transposons. No CP is encoded in any mobilizable elements. The CP is unique to conjugation and is considered the first point of contact that the relaxosome and/or transfer DNA makes with the CA. The best characterized CPs are protein TrwB of plasmid R388 (IncW group), TraD of F plasmids (IncF group), TraG of RP4 plasmids (IncP group), and VirD4 of *A. tumefaciens* Ti plasmids (61). These proteins share the following characteristics:

1. CPs are not involved in DNA processing events or in pilus production. They are needed after pilus assembly and contact formation. For example, *traD* mutants of plasmid F prevent DNA transport to the recipient cell, but not its initiation in the donor cell (86).

2. CPs are composed of transmembrane α-helices in their N-terminal region that mediate anchoring to the inner membrane. Indeed, they are integral inner membrane proteins (103, 119, 131). They also typically have a main cytoplasmic C-terminal domain (38, 63, 94, 103). Thus, their location is the link between a cytoplasmic system and the membrane complex CA.

3. CPs have a nucleotide binding motif, and in fact, can bind both single- and double-stranded DNA nonspecifically, suggesting a specific role in DNA transfer (119).
4. A CP has a Walker box domain (an ATP hydrolysis motif), and some other cytoplasmic domains that interact with the relaxosome (141). No ATPase activity has been reported so far, but the presence of Walker box motifs suggest that CPs use ATP hydrolysis as an energy source to work as motors. It is speculated that when CP interacts with the relaxosome, the Walker-box mediates ATP hydrolysis to provide energy to “pump” the relaxosome through the CA and into the recipient cell (104, 175). With this role, CPs are considered “gatekeepers” of conjugation. The *A. tumefaciens* CA has two of these CP “gatekeepers”, VirD4 and its required partner VirB4, both of which have nucleotide binding activity (44, 115, 141). However, in *E. coli*, only one CP has been described for each conjugative system.

5. CPs are often multimeric proteins. The 3D crystal structure of the soluble cytoplasmic domain of TrwB, the CP of the IncW plasmid R388, shows that it is a hexameric protein, resembles a ring helicases (60).

Most importantly, in many transfer systems, including those elaborated by the *E. coli* F plasmids and *A. tumefaciens* Ti plasmids, their CPs are highly selective for the respective relaxosome to be transferred (73, 155). However, in *B. fragilis*, the putative CPs seem not selective for the relaxosome. Many different conjugative transposons and mobile elements even from different origins can be transferred from *B. fragilis* to bacteria from other genus, such as *E. coli*. This is one reason explaining why *Bacteroides* spp. are considered as reservoirs for transferring antibiotic resistance elements to other bacteria.
Figure 7. Conjugation. Conjugation systems represent a large subfamily of the T4SSs and are used by bacteria in the process of the conjugative transfer of DNA from donor to recipient cells. A) cell-to-cell contact usually by the retraction of the pilus-like structures (B). C. ssDNA of the mobile genetic element is transferred from the donor to recipient bacteria with the help of the relaxase. D. Complementary DNA strands are synthesized in both cells and the former recipient becomes a new potential donor of the mobile DNA. Adapted from Juhas et al., Microreview, 2008 (85).
Bacteroides spp. Conjugation

Mobile Genetic Elements in Bacteroides

Bacteroides spp. harbor many conjugative and mobilizable elements, which can be transposons or plasmids. A significant proportion of these carries antibiotic resistance genes and is critically important in the spread of antibiotic resistance genes.

Table 2: Summary of mobile genetic elements found in Bacteroides spp.

<table>
<thead>
<tr>
<th>I. Conjugative elements</th>
<th>II. Mobilizable elements</th>
</tr>
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<tbody>
<tr>
<td>Self-transmissible</td>
<td>Need help for transfer</td>
</tr>
<tr>
<td>Process DNA for transfer</td>
<td>Process DNA for transfer</td>
</tr>
<tr>
<td>Encode mating channel (CA) for completely autonomous transfer</td>
<td>Depend on mating channel formed by co-resident conjugative elements</td>
</tr>
<tr>
<td>Many carry many antibiotic resistance genes</td>
<td></td>
</tr>
<tr>
<td>I.a. Conjugative plasmids:</td>
<td>II. a. Mobilizable plasmids:</td>
</tr>
<tr>
<td>Have oriT and trans-acting mobilization gene</td>
<td></td>
</tr>
<tr>
<td>Can replicate independently</td>
<td></td>
</tr>
<tr>
<td>Some can integrate into the recipient chromosome</td>
<td></td>
</tr>
<tr>
<td>Examples: pBF4 (159), pBI136 (164)</td>
<td>Examples: pBTM10 (168)</td>
</tr>
<tr>
<td>I. b. Conjugative transposons:</td>
<td>II. b. Mobilizable transposons:</td>
</tr>
</tbody>
</table>
Located on chromosome

<table>
<thead>
<tr>
<th>Do not replicate independently</th>
</tr>
</thead>
<tbody>
<tr>
<td>52 kb to 150 kb</td>
</tr>
<tr>
<td>Much smaller</td>
</tr>
</tbody>
</table>

Are referred to as Tet elements since most of them carry the tetracycline resistance gene tet*Q*.

Examples: BTF-37 (181), CTnDOT (16), CTnERL (17), Tcr Emr DOT (97), Tcr Emr 7853 (127), CTnBST (70), CTnGERM1 (184), CTn86 (22) and CTn9343 (22).

Examples: Tn4399 (78), Tn5520 (183), cLV25 (9), NBU1, NBU2 (98), Tn4555 (166).

**Plasmids.** Plasmids are very common in *Bacteroides* spp. and are found in 20 to 50% of strains (189). Almost all plasmids can replicate as independent elements in host cells, and some can integrate into the host chromosome (153). Many plasmids have ori*T* and a *trans*-acting mobilization gene, which allow them to be transferred by conjugation (189).

Genes resistant to many antibiotics have been found in plasmids in *Bacteroides* spp. Genes resistant to metronidazole, chloramphenicol, carbapnems, clindamycin and erythromycin have been found in mobile plasmids in *Bacteroides* spp. worldwide. Resistance genes *nimA* - *nimF*, encode metronidazole resistance, have been identified on transferrable plasmids and observed in several cases worldwide (106). The *cfiA* gene,
conferring resistance to carbapenems, has also been found on a plasmid in clinical isolates (124).

To date, two conjugative plasmids have been identified: the *B. fragilis* 41 kb pBF4 plasmid and *B. ovatus* 80.6 kb pBI136 plasmid. Sequence analysis of the transfer regions of these plasmids have been limited due to A-T rich sequence properties. Only one gene, *bctA*, encoding a putative 110 kD protein that localizes to the membrane, has been characterized as being required in mating process (123). There are also other mobilizable plasmids that can only be transferred via mating channel formed by other co-resident conjugative elements, such as *B. fragilis* BFTM10 (168).

**Transposons.** Transposons, both mobilizable and conjugative, are located on the chromosome and do not replicate independently. They excise from and integrate into chromosomal DNA and are copied along with the chromosomal DNA.

Mobilizable transposons, like mobilizable plasmids, cannot self-transfer but can transfer from bacterium to bacterium in the presence of the helper element (189). Mobilizable transposons are much smaller than conjugative transposons and carry genes required for excision, mobilization and integration. However, they do not encode the conjugal apparatus components and have to rely their transfer on other co-resident conjugal elements like conjugative transposons. Some identified mobilizable transposons are *B. fragilis* 3.9 kb Tn4399 (78), *B. fragilis* 4.69 kb Tn5520 (183), *B. fragilis* 15.3 kb cLV25 (9), *B. uniformis* 10.3 kb NBU1, *B. uniformis* 11.1 kb NBU2 (98) and *B. vulgatus* 12.5 kb Tn4555 (166).
Conjugative transposons (CTn) are frequently found in *Bacteroides* spp. More than 80% of *Bacteroides* strains contain at least one conjugative transposon (161). Several conjugative transposons or tetracycline resistance factor (Tcr) ranging from 52 kb to 150 kb have been identified in *Bacteroides* spp., including *B. fragilis* BTF-37 (37 kb) (181), *B. thetaiotaomicron* CTnDOT (65 kb) (16), *B. thetaiotaomicron* CTnERL (52 kb) (17), *B. thetaiotaomicron* Tcr Emr DOT (70 kb) (97), *B. thetaiotaomicron* Tcr Emr 7853 (70 kb) (127), *B. thetaiotaomicron* CTnBST (100 kb) (70), *B. thetaiotaomicron* CTnGERM1 (75 kb) (184), *B. vulgatus* CTn341 (52 kb) (7), *B. fragilis* CTn86 (57 kb) (22) and *B. fragilis* CTn9343 (64 kb) (22). Of these, CTnDOT (65 kb) from *B. thetaiotaomicron* is the best described. CTns are also referred to as Tet elements since most, but not all, carry the tetracycline resistance gene *tetQ* (153, 158). Tet elements also carry the *rteABC* gene cluster involved in the regulation of Tet element conjugal transfer (160, 173). *rteA* and *rteB* genes encode a tetracycline inducible two-component regulatory system, which controls *rteC* expression (173). As a result, very low (sub inhibitory) levels of tetracycline or its analogs with brief exposures can markedly elevate conjugal transfer of Tet elements and other co-resident factors 1,000-to 10,000-fold (144, 160). Many *B. fragilis* conjugative transposons also carry erythromycin resistance genes such as *ermF* (CTnDOT) (190), *ermB* (CTnBST) (188) or *ermG* (CTnGERM1) (184).

Conjugative transposons are mainly responsible for the spread of tetracycline and erythromycin resistance in clinical isolates of *Bacteroides* spp (161). They are not only responsible for the transfer of antibiotic resistance genes on itself but also for the transfer of antibiotic resistance genes on other mobilizable elements. They are able to stimulate
the excision and transfer of mobilizable transposons. The exact mechanism by which conjugative transposons stimulate the excision and transfer of mobilizable transposons is unknown. However, it is known that RteA and RteB encoded by genes located within the central regulatory region of the CTnDOT/ERL family of conjugative transposons are essential for the excision and mobilization of the NBUs mobilizable plasmids (172).
Figure 8: Steps involved in the conjugal transfer of a conjugative transposon (192).

The integrated conjugative transposon (rectangle) excises from the chromosome of the donor to form a covalently closed circular transfer intermediate in which the left and right ends of the conjugative transposons are joined. A single-stranded nick is subsequently made at the origin of transfer (oriT, black circle) in the circular intermediate, and the nicked strand is presumably to be transferred from donor to recipient by a process similar to conjugal transfer of plasmid DNA. In the donor and recipient, the single-stranded copy of the conjugative transposon is replicated, yielding a double-stranded form of the
conjugative transposon which then integrates in the donor and recipient chromosomes, respectively. Adapted from Whittle et al., Cell Mol Life Sci, 2002.
**B. fragilis LV23, Conjugative Transposon BTF-37, Putative Coupling Protein**

*BctA and the Relaxase BmpH*

Previously, our laboratory reported the capture of BTF-37, a 37kb conjugative transposon, from a clinical *B. fragilis* isolate, LV23 (181). BTF-37 alone is capable of facilitating transfer of DNA not only within the Bacteroides, but also from *B. fragilis* to *E. coli* (181). Due to A-T rich regions, only 16kb of BTF-37 was sequenced. This 16kb region carries 11 ORFs. By sequence homology analysis with other known *Bacteroides* transfer factors, and by RT-PCT studies, these 11 genes likely correspond to the transfer region of BTF-37 (77). Of these 11 ORFs, bctA is an ORF highly conserved between different *Bacteroides* transposons.

*bctA* encodes a putative coupling protein of BTF-37 CTn (77). It is identical to the putative coupling protein BctA of *B. fragilis* conjugative plasmid pBF4, 98% identical to that of the *B. fragilis* NCTC9343, 74% to the *B. fragilis* YCH46 strain from Japan, and 48% to a BctA homolog from the *B. fragilis* CTnDOT conjugative transposon (77). Computer analysis also reveals that BctA has two Walker-box-sequences as a characteristic of a coupling protein. We also showed that BctA is a conjugal apparatus protein required for transfer of BTF-37 (77). It has signal sequence required for membrane localization, its expression is upregulated under conjugation conditions and purified BctA migrated as a tetramer under non-denaturing PAGE.

BmpH is a relaxase encoded by the 4.6 kb mobilizable transposon Tn5520 that was also isolated from *B. fragilis* clinical isolate LV23 (182, 183). Tn5520 carries only two genes, that encode an integrase (*bipH*) and a mobilization protein (*bmpH*)
respectively. Studies from our laboratory have shown that BmpH is a relaxase protein that is required for the formation and function of the relaxosome, required for the mobility of Tn5520 (182). BmpH alone is sufficient and required for DNA transfer of Tn5520. It has been shown to be multifunctional, performing the specific recognition, binding, and nicking of the oriT DNA (182).

**The Search for Better Genetic Modification Tools in Anaerobes**

To investigate protein function in a bacterial strain, it is important to generate a knock-out (KO) strain to determine if a target protein is unable to perform a predicted function. In *B. fragilis* and many other anaerobes like *Clostridium* spp., a traditional method is to use a suicide vector to deliver a disrupted construct of the target gene from *E. coli* to the recipient strain (32, 36). This method is based on integration of the altered construct into the chromosome followed by screening for spontaneous resolution of the diploid to yield the desired product (deletion of the target gene in the chromosome). However, this method is time-consuming and problematic due to stringent recombination barriers encountered in these genera (150). An alternative approach uses resistance to trimethoprim, encoded by a chromosomal *thyA* mutation in *B. fragilis*, to select for resolution of diploids where the suicide plasmid carries a functional copy of *thyA* (11). Although this method helps resolve diploids, the strain to be engineered must be made
trimethoprim resistant and all resulting strains contain a \(thyA\) mutation, which may have undesirable consequences for further studies.

A recent study shows a new approach to delete a target gene in \(B.\) \textit{fragilis} NCTC 9343 by using the I-SceI meganuclease to mediate double strand breakage, allowing resolution of diploid (137). In essence, a suicide plasmid (pEP185.2), containing an I-SceI recognition site, and sequences homologous to chromosomal DNA flanking an \textit{ermF} cassette and replacing the gene to be deleted is introduced into \(B.\) \textit{fragilis} by conjugation, and transconjugations are selected for resistance to erythromycin (137). Then, another plasmid expressing I-SceI enzyme under the control of the fucose-inducible promoter \(P_{\text{fucR}}\) is transformed into the \(B.\) \textit{fragilis} transconjugant. Under inducible condition, I-SceI is expressed, induces breakage of double strand DNA at the I-SceI recognition site, allowing resolution of the diploid to generate either the deletion or wild-type genotypes by homologous recombination. However, this method has its own drawbacks. First, it requires transformation to introduce the plasmid expressing I-SceI into \(B.\) \textit{fragilis} cells, which is known to be difficult to work with. Second, the suicide plasmid pEP185.2 is tetracycline and erythromycin resistant, which is not useful for the selection of this plasmid in clinical strains already tetracycline and erythromycin resistant like LV23. Moreover, the presence of \textit{ermF} in the suicide vector may allow plasmid incorporation into \textit{ermF} in the \(B.\) \textit{fragilis} chromosome, not into the desired target gene.

Although not widely investigated in prokaryotic systems, antisense RNA (asRNA) is a potent and flexible tool for manipulating microbial genetic. AsRNAs have been successfully used to suppress the expression of bacterial proteins in several studies,
especially in the clostridia (118, 125, 138, 143, 177, 178), where very similar difficulties are encountered in generating knock-out mutants. Thus, although not has been tested in *B. fragilis* this approach seems to be applicable in *B. fragilis* studies.
CHAPTER III
MATERIALS AND EXPERIMENTAL METHODS

Chemicals and Reagents

Chemicals and reagents used in this study were purchased from Sigma-Aldrich, Inc. (St. Louis, MO), Fisher Scientific (Pittsburgh, PA) and Thermo Scientific (Waltham, MA) unless otherwise mentioned.

Bacterial Strains and Plasmids

Bacterial Strains and plasmids used in this study are listed in Table 3 and 4.

Table 3. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristic(s)</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli HB101</td>
<td>Sm$^R$</td>
<td>(18), Invitrogen</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>recA1, endA1</td>
<td>(64), Invitrogen</td>
</tr>
<tr>
<td>E. coli XL-1Blue</td>
<td>recA1, endA1, Kn$^R$</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>E. coli XL-1Blue MRF</td>
<td>Tc$^R$, Δ(mcrA)183 Δ(mcrCB-</td>
<td>Agilent Technologies</td>
</tr>
</tbody>
</table>
hsdSMR-mrr)173 endA1 supE44

thi-1 recA1

Bacteriomatch II KnR Agilent Technologies

Reported Cells

Bacteriomatch II KnR Agilent Technologies

Validation Reporter

Competent Cells

E. coli BL-21AI araB::T7RNAP, TcR Invitrogen

B. fragilis LV23 TcR Clinical isolate

B. fragilis TM4000 RfR (163)

B. fragilis TM4000 Hecht’s laboratory

BTF-37

a: SmR, KnR, TcR, RfR indicate resistance to streptomycin, kanamycin, tetracycline and rifampicin, respectively.

<table>
<thead>
<tr>
<th>Table 4. Plasmids used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid</td>
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</tr>
<tr>
<td>BTF37</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>pGAT400ΔBglII</td>
</tr>
<tr>
<td>Vector</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>RK231</td>
</tr>
<tr>
<td>pQE30Xa</td>
</tr>
<tr>
<td>pA8-Xa</td>
</tr>
<tr>
<td>p17-Xa</td>
</tr>
<tr>
<td>pDEST42</td>
</tr>
<tr>
<td>p42M</td>
</tr>
<tr>
<td>p24M</td>
</tr>
<tr>
<td>p24bmpH</td>
</tr>
<tr>
<td>pFD351</td>
</tr>
</tbody>
</table>

*(Bacteroides sp.)*
pFD288 Shuttle vector, Erm<sup>R</sup> (Cc<sup>R</sup> in <i>B. fragilis</i>), Sp<sup>R</sup>, (<i>Bacteroides</i> sp.)

pFD288P <i>pfxA</i> cloned in pFD288 This study

pFD288MAS1 <i>traM</i>-antisense AS1 cloned in pFD288P

pFD288MAS2 <i>traM</i>-antisense AS2 cloned in pFD288P

pFD288MAS3 <i>traM</i>-antisense AS3 cloned in pFD288P

pBT Bait vector for bacterial two hybrid experiment Agilent Technologies

pTRG Target vector for bacterial two hybrid experiment Agilent Technologies

pBT-LGF2 Positive control bait vector for bacterial two hybrid experiment Agilent Technologies

pTRG-Gal11 Positive control target vector for bacterial two hybrid experiment Agilent Technologies

pBT-M (pBT-ORF7) Full-length <i>traM</i> cloned in bait vector, pBT This study

pBT-BctA Full-length <i>bctA</i> cloned in bait vector, pBT This study
<table>
<thead>
<tr>
<th>Construct</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBT-BmpH</td>
<td>Full-length <em>bmpH</em> cloned in bait vector, pBT</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-ORF5</td>
<td>Full-length <em>orf5</em> cloned in bait vector, pBT</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-ORF6</td>
<td>Full-length <em>orf6</em> cloned in bait vector, pBT</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-ORF8</td>
<td>Full-length <em>orf8</em> cloned in bait vector, pBT</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-M-F66A</td>
<td><em>traM</em> mutation F66A in pBT vector</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-M-F66G</td>
<td><em>traM</em> mutation F66G in pBT vector</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-M-F66R</td>
<td><em>traM</em> mutation F66R in pBT vector</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-M-L69A</td>
<td><em>traM</em> mutation L69A in pBT vector</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-M-L69G</td>
<td><em>traM</em> mutation L69G in pBT vector</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-M-L69R</td>
<td><em>traM</em> mutation L69R in pBT vector</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-M-L123A</td>
<td><em>traM</em> mutation L123A in pBT vector</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-M-L123G</td>
<td><em>traM</em> mutation L123G in pBT vector</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-M-L123S</td>
<td><em>traM</em> mutation L123S in pBT vector</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-M-L123R</td>
<td><em>traM</em> mutation M123R in pBT vector</td>
<td>This study</td>
</tr>
</tbody>
</table>
pBT-M-M154A  \textit{traM} mutation M154A in pBT \hspace{1cm} This study

pBT-M-M154G  \textit{traM} mutation M154G in pBT \hspace{1cm} This study

pBT-M-M154R  \textit{traM} mutation M154R in pBT \hspace{1cm} This study

pBT-M-M154C  \textit{traM} mutation M154C in pBT \hspace{1cm} This study

pTRG-M  Full-length \textit{traM} cloned in target \hspace{1cm} This study

pTRG-BctA  Full-length \textit{bctA} cloned in target \hspace{1cm} This study

pTRG-BmpH  Full-length \textit{bmpH} cloned in target \hspace{1cm} This study

pTRG-L123S  \textit{traM} mutation L123S in pTRG \hspace{1cm} This study

pTRG-M154C  \textit{traM} mutation M154C in pTRG \hspace{1cm} This study

pBT-BctAtrunc  Truncated \textit{bctA}, lacking the N-terminal signaling sequence (32
amino acids), in pBT vector.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTRG-BctAtrunc</td>
<td>Truncated $bctA$, lacking the N-terminal signaling sequence (32 amino acids), in pTRG vector.</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-TraG</td>
<td>TraG of <em>E. coli</em> RP4 plasmid in pTRG vector</td>
<td>This study</td>
</tr>
<tr>
<td>pBT-TetQ</td>
<td><em>B. fragilis</em> LV23 $tetQ$ in pBT vector</td>
<td>This study</td>
</tr>
<tr>
<td>pTRG-TetQ</td>
<td><em>B. fragilis</em> LV23 $tetQ$ in pTRG vector</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Bacterial Media, Growth Conditions and Optical Density Readings**

*Escherichia coli* cells were grown in Luria-Bertani (LB) medium (1% w/v tryptone (Sigma), 0.5% w/v yeast extract (Sigma), 1% sodium chloride). *Bacteroides* spp. were grown in BHIS medium (3.7% brain heart infusion medium supplemented with 0.0005% hemin and 5 g of yeast extract/liter) in a Coy anaerobic chamber (5% CO$_2$, 10% H$_2$, and 85% N$_2$). Agar (Difco) was added to broth at 1.5% (w/v) for plating. Media were sterilized by autoclaving for 30 mins at 15 pounds per square inch prior to addition of antibiotics.

*E. coli* growth was measured using a Spectronic Genesys 10UV Spectrophotometer (Thermo Scientific Inc., Waltham, MA) by taking absorbance readings at a wavelength of 600nm. *B. fragilis* growth was measured at a reading
wavelength of 660nm. 1 ml of bacterial culture was measured and compared to cell free medium as the blank in disposable plastic cuvettes (VWR, West Chester, PA).

**Antibiotics**

All antibiotics were supplied by Sigma-Aldrich, Inc. Antibiotic concentrations used for the selection of strains and plasmids included the following: ampicillin, 200 μg/ml; clindamycin, 12 μg/ml; streptomycin, 50 μg/ml; spectinomycin, 50 μg/ml; rifampin, 25 μg/ml; kanamycin, 25 μg/ml; gentamicin 25 μg/ml; tetracycline, 13.5 (for *E. coli*) or 5 μg/ml (for *Bacteroides* spp.).

Antibiotic stocks were prepared at 10X concentrations and stored in 1 ml aliquots at -20°C. Ampicillin, spectinomycin, streptomycin or clindamycin powder was dissolved in Milli-Q ultrapure water (Milipore Co.) by vortexing. Chloramphenicol was dissolved in 100% ethanol by vortexing. Tetracycline was dissolved in 50% ethanol (v/v) by vortexing. All antibiotics were filtered sterilized through 0.25 μm syringe filters (Pall Corporation, East Hill, NY) attached to 5 ml syringes.

**Recombinant DNA Techniques.**
Plasmid DNA was prepared by affinity purification (Qiagen, Chatsworth, CA). Restriction endonucleases and DNA ligase were purchased from New England Biolabs (Beverly, MA). PCR was performed using a commercially available kit that contained the AmpliTaq-Gold DNA polymerase (Applied Biosystems, Foster City, CA). DNA sequencing was performed using an ABI377 sequencer at the DNA sequencing facility of the University of Florida, Gainesville, FL.

**B. fragilis Genomic DNA Preparation**

*B. fragilis* genomic DNA was purified from 5 ml overnight cultures by using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer’s instructions with some modifications. Briefly, 5 ml of overnight culture was harvested and centrifuged at 7,500 rpm for 10 mins. Cell pellets were then resuspended in 900 μl ATL lysis buffer. 100 μl of proteinase K was added to the lysis and mixed by vortexing. Lysis was then incubated at 55°C for 15 mins with vortexing occasionally during incubation and was vortexed for 15 secs. 1 ml of AL buffer was added to the sample with vortex, incubated for 10 mins at 70°C. Then, 1 ml of HPLC purified Ethanol (Sigma) was added and vortexed, and the lysate added to a DNeasy Mini Spin column, centrifuged at 8,000 rpm for 1 min to discard flow through, and then harvested in new 2 ml collection tubes. 500 μl of the first wash solution AW1 was then added, and the column centrifuged for 1 min at 8,000 rpm. The column was then placed in another new 2 ml collection tube, 500 μl of
the second wash solution AW2 added, and centrifuged for 3 mins at 14,000 rpm to dry the DNeasy membrane. The column was then placed in a clear 1.5 ml microcentrifuge tube, 200 μl of elution buffer AE added, incubated at room temperature for 1 min, and centrifuged for 1 min at 8,000 rpm to elute.

**B. fragilis RNA Purification, RT-PCR and Quantitative PCR**

*B. fragilis* overnight cultures were sub-cultured in pre-reduced BHIS liquid media under anaerobic conditions from an overnight (ON) culture with the ratio of 1/50. When the OD$_{660}$ = 1.2 (about 1.5 hrs after culture), the culture was stimulated with tetracycline to a final concentration of 1 μg/ml. About 4 hrs after tetracycline induction, when bacteria reached mid-exponential phase, OD$_{660}$ = 0.6, 0.5 ml of each culture was collected for RNA purification.

RNA for RT-PCR was purified using Qiagen RNeasy mini kit and protocol. For RT-PCR of BTF-37 11 ORFs, Biorad RT-PCR kit was used for RT-PCR reactions. The RNA was tested for DNA contamination by checking RT-PCR products from reactions with or without reverse transcriptase. The transcript production from each of 11 ORFs was investigated by RT-PCR with specific primers from 250 ng purified RNA. RT-PCR from 16S was used as the control and for normalization. RT-PCR from *tetQ* was used as a positive control. RT parameters: $25^\circ$C: 5 min, $42^\circ$C: 30 min, $85^\circ$C: 5 min. cDNA was diluted to 1:5 then diluted DNA was used for PCR reaction. PCR parameters: $95^\circ$C: 5
min, 25 cycles of (95°C: 15sec, 51°C: 30sec, 70°C: 30sec), 72°C: 7 min. RT-PCR products were examined on 1% agarose gels. The concentration of DNA was measured by densitometry in an imaging system FluochemTM 8900, Alpha Innotech, and was normalized according to the density of 16S rDNA transcripts.

RNA for Q-PCR was purified using Ambion MICROBExpress™ Bacterial mRNA Enrichment Kit (Applied Biosystem, Carlsbad, CA), following the manufacturer instructions. However, due to easy contamination of DNA in the *B. fragilis* RNA samples, RNA containing columns were digested three times with DNAse before elution to collect pure RNA. The RNA was tested for DNA contamination by checking on agarose gel RT-PCR products from reactions with or without reverse transcriptase.

Quantitative PCR reactions were performed using SYBR labeling technique, using 5 Prime reagent kits (5 Prime, Gaithersburg, MD) and Eppendorf Realplex Q-PCR (Eppendorf, Hamburg, Germany) machine.

Q-PCR data was analyzed using the ΔΔCt method (77):

E = Efficiency; ref = reference gene; gene = test gene

Relative expression = (E gene) x ΔCt gene (Tc−-Tc+)

Adjusted relative expression (REadj) = RE (unknown) x 1/RE (ref).

Reference gene: 16S rDNA for expression of tram in *B. fragilis* LV23 with or without conjugation condition induction; *tetQ* for expression of tram in *B. fragilis* LV23 strains in the presence of different antisense constructs.
<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF1 Fwd</td>
<td>TTG CTT TGC ACC ATG ATT TT</td>
</tr>
<tr>
<td>ORF1 Rev</td>
<td>GGG CGT GAA GAA CTT GTA GC</td>
</tr>
<tr>
<td>ORF2 Fwd</td>
<td>GCC AAT GAA GCA AAA ATT CC</td>
</tr>
<tr>
<td>ORF2 Rev</td>
<td>TGC ATA TTG CGA GAA GGT G</td>
</tr>
<tr>
<td>ORF4 Fwd</td>
<td>TTG TCG GGA CAG ATG TAA ACC</td>
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<tr>
<td>ORF4 Rev</td>
<td>CCC CGG AGC TAA TGT TTG TA</td>
</tr>
<tr>
<td>ORF5 Fwd</td>
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<tr>
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<td>ORF6 Rev</td>
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<tr>
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<td>ORF8 Rev</td>
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<td>ORF10 Fwd</td>
<td>TGC CGC AAT AAT GAA CTT TG</td>
</tr>
<tr>
<td>ORF10 Rev</td>
<td>AAG TGC GTT CCT CCA ATG AT</td>
</tr>
<tr>
<td>ORF11 Fwd</td>
<td>AGA GCA GCA GCA GTA GGA AA</td>
</tr>
</tbody>
</table>
DNA and RNA concentrations were measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Preparation of Competent *E. coli* cells and Transformations

Preparation and storage of competent *E. coli* cells to be used for transformation was adapted as described previously (128). A fresh, single colony of *E. coli* cells was grown in 5 ml of LB media overnight. 500 μl of the overnight culture was diluted 1:100 in 50 ml of competent cell growth media (LB medium supplemented with 10 mM magnesium sulfate (Mg$_2$SO$_4$) and 0.2% (w/v) glucose). Cells were grown to mid-log phase to an optical density reading of about 0.6 at wavelength of 600nm. Cells were then incubated on ice for 10 mins, and centrifuged at 3000 rpm for 10 mins at 4°C in the
Beckman Allegra X-15R bench top centrifuge to pellet the cells. Cell pellets were then resuspended in 500 μl of cold competent cell growth media. 2.5 ml of cold competent cell storage (LB medium supplemented with 36% glycerol, 12% (w/v) polyethylene glycol (PEG) and 12 mM MgSO₄) was added to the cells. 200 μl aliquots of cells were transferred into pre-chilled microcentrifuge tubes and stored at -80°C.

Ligation reactions of a sub-cloning procedure or plasmids or a mixture of plasmids for a bacterial two hybrid experiment were introduced into E. coli competent cells by transformation. A desired amount of DNA (10 ng of plasmid, or 20 μl of a ligation reaction, or a mixture of 50 μg DNA of both the bait and the target plasmids in a bacteria two hybrid experiment) were pipetted into a polypropylene tube and chilled on ice. 100 μl of competent cells were thawed on ice and transferred to the tube with DNA. The cells were incubated with the DNA on ice for 30 mins, heat shocked at 42°C for 30 secs and then chilled on ice for 2 mins. 900 μl of LB medium was added to the cells and they were grown at 37°C shaking at 250 rpm for 1 h. 100 μl of the transformation culture was plated onto a selective medium plate. The remaining 900 μl of cells were centrifuged at 3500 rpm for 5 mins in the Beckman centrifuge. The pellet was gently resuspended in 100 ml of LB medium and plated onto selective medium plate. Plates were incubated at 37°C overnight to promote colony growth.

Special growth conditions were applied for transformations for bacterial two hybrid assays. This is described in the bacterial two hybrid assay section.
Non-denaturing Protein Purifications

To prepare for Far-Western experiments, non-denatured BctA and BmpH proteins were purified from pA8-Xa and p17Xa expression vectors, respectively, in XL1-Blue cells. Briefly, 25 ml of LB medium was inoculated with 500 μl of overnight culture cells of XL1-Blue pA8-Xa or XL1-Blue p17Xa, and grown at 37°C with shaking at 250 rpm. For induction of BctA from pA8Xa expression vector in XL1-Blue cells, 1 mM final concentration of IPTG was added to the culture at mid-logarithmic phase (OD_{600nm}=0.5). For induction of BmpH from pDEST42BmpH vector, a 0.2% (w/v) final concentration of L-arabinose was added to the culture at mid-logarithmic phase. After induction, cell cultures were incubated at 30°C, overnight with shaking. After incubation, cells were harvested by centrifugation at 3500 rpm for 30 min by using a Beckman Coulter Allegra X-15R bench top centrifuge. Cell pellets were then frozen overnight at -20°C. After being thawed, cell pellets were resuspended in 15 ml native lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 1.5% Tween-20, pH 7.5), added 600 μl of proteinase inhibitor cocktail (Roche, Indianapolis, IN). The cell suspension was gently rocked at room temperature for 2 hrs and then sonicated on ice at 55% for 6 x 30 secs pulse with 30 secs rest in between. Lysates were cleared by centrifugation at 2850 g, 4°C for 30 mins and then applied to TALON cobalt affinity column (Clontech, Mountain View, CA) pre-equilibrated with 2 x 1 ml lysis buffer and 1 x 0.5 ml lysis buffer plus 0.5 ml wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 15 mM imidazole, pH 7.5). Columns were then washed 3 x 1 ml of
wash buffer. Immobilized His-tagged proteins were eluted in 3 x 550 μl fractions (50 mM NaH₂PO₄, 300 mM NaCl, and 200 mM imidazole, pH 7.5)

**Protein Preparation from *B. fragilis* Cultures**

To collect proteins from *B. fragilis* cultures, overnight cultures were subcultured in 1:50 dilution to 45ml of pre-reduced BHIS medium, grown anaerobically at 37°C until mid-logarithm phase (OD₆₆₀nm=0.5). Cells were then harvested by centrifugation at 3500 rpm, 4°C for 30 mins in a Beckman Allegra X-15R centrifuge. Pellets were then resuspended in 3.5 ml PBS, protease inhibitor added (Roche, Indianapolis, IN), sonicated for 4x 15 secs with 15 secs rest in between at 45% power, then centrifuged at 11,000 rpm, 4°C for 1 hr using Beckman Coulter TLA 100 Ultracentrifuge with a TLA100.3 rotor. Supernates containing total proteins were collected and stored at -20°C overnight. The next day, supernates were thawed on ice, centrifuged at 70,000 rpm, 4°C, for 2 hrs using Beckman Coulter TLA 100 Ultracentrifuge with a TLA100.3 rotor, and cytoplasmic proteins collected from supernatant liquids. Pellets were washed with 3 ml PBS and centrifuge at 70,000 rpm, 4°C, for 2 hrs, after which, those supernatant fluids were discarded. Pellets containing membrane proteins were resuspended in 600 μl PBS, sonicated 4x 15 secs with 15 second rest in between, at 45%.

**Protein Quantification**
In most cases, the concentrations of total proteins was measured using the Bicinchoninic Acid (BCA)™ Protein Assay Kit (Pierce), according to the manufacturer’s instructions. A series of dilutions of known concentration of bovine serum albumin (BSA) was prepared and assayed together with the unknown protein and then the protein concentration of the each unknown sample was determined in μg/ml based on the standard curve.

Purified His-BctA, His-BmpH and TraM-His in elution buffer containing 200 mM imidazole were quantified by Coomassie Plus the Better Bradford Kit (Pierce, Rockford, IL).

**Agarose Gel Electrophoresis**

The DNA or PCR products were separated by agarose gel electrophoresis with an agarose concentration of about 1%. The agarose (Sigma-Aldrich) gel was prepared in 1X TAE buffer (40 mM Tris-acatate, 1 mM EDTA). After the gel was run for a required time, the DNA was visualized by staining the gel with 0.5 μg/ml of ethidium bromide and images were taken using Alphalmager automated CCD camera, AlphaInnotech- Cell Biosciences, San Leandro, CA.
Polyacrylamide Gel Electrophoresis

Proteins samples were boiled in Laemmli sample buffer (BioRad, Hercules, CA) containing 5% β-mercaptoethanol in a 1:1 ratio for 10 mins. Samples were then loaded on Tris-CL polyacrylamide gel in 1 x TGS (2.5 mM Tris-Cl, 19.2 mM Cycledine, 0.01% SDS) (BioRad, Hercules, CA). Electrophoresis was carried out at constant 200 volts for various times depending on the gel percentage.

Gels were washed for 10 secs in dH2O and then stained with GelCode Blue Stain reagent (Thermo Scientific Inc., Waltham, MA) for 60 mins, and destained with dH2O.

Immunoblot Experiments

Proteins were visualized by western blotting as described previously (199). Electrophoresis was performed as stated above and gel contents transferred overnight onto 0.45 μM nitrocellulose membrane at 50 volts in 1 x TG buffer (2.5 mM Tris-Cl, 19.2% Glycine) at 4°C with stirring in a Trans-Blot cell (BioRad, Hercules, CA). For primary antibodies lacking the horse-radish peroxidase label, blots were developed using a Roche Western Blotting Kit (Roche, Indianapolis, IN). In short, blots were blocked with shaking for at least 80 mins at room temperature or overnight at 4°C, using 1% blocker in 1X TBS and then incubated for 60 mins with primary antibodies in 0.5% blocker in TBS, at room temperature. Anti-λcI antibodies and anti-TraM antibodies were
used at the dilution of 1: 1,000 and 1: 200, respectively. Blots were then washed 3 x 10 mins with TBST (50 mM Tris-Cl, 150 mM NaCl, pH 7.5 and 0.1% Tween 20). Then, blots were incubated for 30 mins with secondary antibodies, anti rabbit and anti mouse IgG antibodies, washed 4 x 15 mins with TBST. Blots were developed for 1 min using a mixture of 1 part solution A and 100-1000 part solution B, and exposed to CL-XPosure film (Pierce, Rockfort, IL).

When using horse-radish peroxidase-labeled (HRP) antibodies like RGS-His and 6x His antibodies (Qiagen, Chatsworth, CA and Clontech, Mountain View, CA), the western blots were blocked in 1% Casein in TBS (Pierce, Rockford, IL) with 0.1% Tween- 20, washed with TBST. RGS-His and 6x His antibodies were incubated at dilution of 1: 25,000 and 1: 10,000, respectively, for 60 mins. No secondary antibody was required. Blots were developed for 5 mins using North2South Chemiluminescent substrate for HRP (Pierce, Rockfort, IL), exposed to CL-XPosure film (Pierce, Rockfort, IL).

**Far-Western Blotting Experiments**

Far-Western experiments for cell lysates overlayed with purified, non-denatured BetA or BmpH was performed using a previously published protocol (174). Briefly, the crude extracts containing λcI-TraM were electrophoresed using SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked of free binding
sites by incubation with blocking solution 1% Casein TBS for 2 hrs. Then, the membrane was incubated with 10 μg purified His-BctA or 30- 50 μg His-BmpH proteins in 15 ml 1% Casein TBS for 30 mins at room temperature. This incubation allowed BctA or BmpH to bind to its potential partners. After washing with TBS T/T (TBS with 0.2% Triton X-100, 0.05% Tween 20), the membrane was incubated with antibodies for His tag of BctA or BmpH western blot procedure was performed. A negative control Far-western blot was incubated with anti-His antibodies but not with purified proteins.

**Anti-TraM Antiserum Generation**

To generate TraM antibodies, TraM was subcloned into the pDEST42 expression vector, so that TraM was tagged with V5 and 6xhistidine at its C terminal. Full-length TraM was subcloned into pENTR vector by using NdeI (forward direction) and SacI (reverse direction) restriction enzymes. pENTR-M and pET-DEST42 were recombined, according to the manufacture’s instruction to obtain expression clone p42M. p42M was checked by enzyme digestion and sequencing. The expression vector p42M was transformed into *E. coli* BL21AI cells.

TraM-6His was purified from *E. coli* BL21AI cells by using denatured lysis, affinity purification and electro elution methods. Briefly, 25 ml of LB medium was inoculated with 500 μl of overnight culture cells of BL21-AI p42M, grown at 37°C with shaking at 250 rpm. 1 mM final concentration of IPTG were added to the culture at mid-logarithm phase (OD_{600nm}=0.5). After induction, cell cultures were grown for 2.5 hrs further. Cells were harvested by centrifugation at 3500 rpm for 30 min by using a
Beckman Coulter Allegra X-15R centrifuge. Cell pellets were then freezed overnight at -20°C. After being thawed, cell pellets were resuspended in 15 ml denatured lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 8 M urea, pH 7.5), and 600 μl of proteinase inhibitor cocktail (Roche, Indianapolis, IN). The cell suspension was gently rocked at room temperature for 2 hrs and then sonicated on ice at 55% for 6 x 30 secs pulse with 30 secs rest in between. Lysates were cleared by centrifugation at 2850 g, 4°C for 30 mins and then applied to TALON cobalt affinity column (Clontech, Mountain View, CA) pre-equilibrated with 2 x 1ml lysis buffer and 1 x 0.5 ml lysis buffer plus 0.5 ml wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 15 mM imidazole, pH 7.5). Column was then washed 3 x 1 ml of wash buffer. Immobilized His-tagged proteins were eluted in 3 x 550 μl fractions (50 mM NaH₂PO₄, 300 mM NaCl, and 200 mM imidazole, pH 7.5). All affinity purified TraM was applied in a prep PAGE gel and electrophoresed. 500 μl of proteins was loaded in the large lane, while 50 μl was loaded in a small lane to serve as a reference. The reference lane was cut out of the gel and was stained with GelCode Blue Stain Reagent (Pierce, Rockfort, IL). Meanwhile, the prep lane was submerged in a small amount of TGS buffer and kept at 4°C. After staining, the reference gel was used as a reference to align the size of TraM in the prep gel. Polyacrylamide gel at TraM expected size was cut out of the prep gel, cut into small pieces and subjected for electro elution.

Protein elution was performed using a Bio-Rad electro eluter 422 (BioRad, Hercules, CA), according to manufacturer’s instruction. Briefly, membrane caps were soaked in TGS, protein elution buffer, for at least 1 hr at 60°C and was placed into the bottom of silicone adaptor, filled with TGS, then slided onto the bottom of the elution
glass tube with frit. Each tube was filled with TGS and placed gel slices. Protein was eluted at 8-10 mA/glass tube constant current for 6-8 hrs. After the elution was completed, proteins were collected from the membrane caps. Proteins were also checked by western blot to confirm purification.

About 5 mg of purified TraM in TGS were submitted to Alpha Diagnostic International (San Antonio, TX) for custom antibodies production. Antiseria were generated by injected antigen (TraM) to two rabbits five times with 14 days intervals. Immune bleeds (antisera) were collected at week 7 and week 9. Final antiserum was collected at the end of the procedure (63 days). Antisera were tested by western blot. Final bleeds from both rabbits were mixed and subjected for affinity purification. 5 mg of purified TraM in TGS was dialysed to PBS plus 0.01% SDS, and was applied for preparation of affinity column. Antibodies collected from affinity purification were tested using ELISA and western blot for purification.
Figure 9. pET-DEST42 expression vector map (Invitrogen)
Bacterial Two Hybrid Studies

Bacterial two-hybrid screens were performed using the Stratagene BacterioMatchII two hybrid system (Agilent Technologies, La Jolla, CA), according to the manufacture’s instruction with some modifications. Briefly, bait genes \((bctA, bmpH, traM\) or \(traM\) mutants) were constructed in fusion with the full-length bacteriophage \(\lambda\) repressor protein \((\lambda\text{cI})\) in the bait vector, pBT. Expressions of bait proteins were tested on western blots using anti-\(\lambda\text{cI}\) or anti-TraM antibodies. Target genes \((bctA, bmpH, traM (orf7), traM\) mutants, \(orf5, orf6\), etc.) were fused to the N-terminal domain of the \(\alpha\)-subunit of RNA polymerase in the target vector, pTRG. The bait is pulled to the \(\lambda\) operator sequence upstream of the reporter through the DNA binding domain of \(\lambda\text{cI}\). When the bait and target interact, they recruit and stabilize the binding of RNA polymerase to at the \(lacZ\) promoter, activating the transcription of the \(HIS3\) and \(aadA\) \((\text{Str}^r)\) genes. \(HIS3\) encodes a component of the histidine biosynthetic pathway that complements a \(hisB\) mutation in the reporter strain. The \(HIS3\) gene product is produced from the reporter gene cassette at low levels in the absence of transcription activation, allowing the reporter strain to grow on minimal medium lacking histidine. The compound 3-amino-1,2,4-triazole (3-AT) acts as a competitive inhibitor of the \(HIS3\) gene product. In the presence of 5mM 3-AT, the reporter strain is unable to grow on media lacking histidine. When the reporter strain is co-transformed with hybrid bait and target proteins that interact, the RNA polymerase is recruited to the promoter, activates the transcription of \(HIS3\) and allows the cells to grow on selection media containing 5mM 3-AT.
50 ng of each of the bait and target plasmids were co-transformed, and grown in LB rich media for 90 mins. Cells were then washed 3 times by centrifugation at 2000 g with 1 ml of M9 His-dropout broth (histidine drop out amino acid supplement in M9 media additive broth). M9 His-dropout broth: 1X M9 salt (Clontech, Mountain View, CA), 0.4% glucose, 0.2 mM andenine HCl, 1X His dropout amino acid supplement (Clontech, Mountain View, CA), 1 mM MgSO4, 1 mM Thiamine HCl, 10 μM ZnSO4, 100 μM CaCl2, 50 μM IPTG. Then, cells were grown in M9 His-dropout broth for 2.5 hrs before being plated on non-selection plates prepared from M9 His-dropout broth and 0.17% agar, containing antibiotic selection for both plasmids (25 μg/ml chloramphenicol and 12.5 μg/ml tetracycline). Cells were allowed to grow on non-selection plates at 37°C, overnight. Cells were then replica-plated on selection plates containing 25 μg/ml chloramphenicol, 12.5 μg/ml tetracycline 5mM 3-AT, 37°C, overnight. The next day, cells were replica-plated on double selection plates containing 25 μg/ml chloramphenicol, 12.5 μg/ml tetracycline, 5 mM 3-AT and 12.5 μg/ml streptomycin. The percentage of cells grown on double selection plates was calculated by dividing the number of cells on the double selection plate and the number of cells on the non-selection plate.
Figure 10: Schematic of the BacterioMatch II two-hybrid system dual reporter construct (adapted from Agilent Technologies).
Bacterial Sub-cellular Fractionation Experiments

To determine if TraM is associated with the membrane, a cellular fractionation was carried out as previously described (77). Briefly, *traM* was cloned into pDEST42 vectors so that TraM is tagged with 6xHis at its C terminus. *E. coli* harboring TraM- x6 His-V5 construct was harvested and lysed under non-denaturing conditions using phosphate-buffered saline (PBS) and sonication. Sonicates were then centrifuged at 1300 X g to separate crude bacterial debris from total lysate. After addition of protease inhibitor cocktail (Roche, Indianapolis, IN), clear lysates were centrifuged at ultra-high speed, 265,000 X g for 2.5 hrs to separate total membrane in the pellet and cytoplasmic proteins in the supernatant (77). The pellets containing membrane proteins were then further centrifuged at high speed in PBS and were sonicated to solubilize membrane proteins. Proteins were quantitated and an equal amount of proteins were electrophoresed on SDS-PAGE gel, then, transferred to nitrocellulose membranes and subjected to Western blotting using anti-His antibodies coupled to horseradish peroxidase RGS-His antibodies (Qiagen, Chatsworth, CA).

To determine if TraM was associated with the inner membrane, cellular fractionation experiments to separate the inner and outer membrane fractions of *E. coli* BL21AI p42M cells were performed as previously described (52). Saturated bacterial cultures were diluted 1/50 in 25 ml LB medium. When cell density reached an OD$_{595nm}$ of 0.5 - 0.6, cells were induced with 1 ml of 20 % L-arabinose for 2 h. The culture was harvested, centrifuged at 3500 rpm in Beckman X-15R centrifuge for 30 min 4°C,
washed with phosphate buffer saline (PBS) and resuspended in 1 ml of 50 mM Tris (pH-7)-20 % sucrose with protease inhibitors. Cells were treated with 40 μl of 0.25 M EDTA-0.25 mg of lysozyme/ ml (final concentration of 10 mM and 10 μg/ ml, respectively), for 10 min at room temperature. The periplasmic fraction was isolated from the rest of the cells by centrifugation at 8,000 X g for 10 min. The pellet was resuspended in 1 ml of sonication buffer (10 mM Tris-HCl pH 7 and proteases inhibitors) and sonicated three times for 15 secs each time at (Fisher sonicator, 55% power). Unbroken cells were removed by centrifugation at 16,000 g for 2 min, and the clear lysate supernatant containing cytoplasmic proteins and inner and outer membranes was removed and centrifuged for 1 hr at 31,000 X rpm to pellet the membranes (Beckman Coulter TLA 100 Ultracentrifuge with a TLA100.3 rotor). The supernatant containing the cytoplasmic fraction was removed; the membrane pellet was washed with sonication buffer, resuspended in 0.1 ml sonication buffer with 0.5% N-lauroylsarcosine, which selectively solubilizes the inner membrane, and centrifuged at 31,000 X rpm for 1 hr. The supernatant containing the inner membrane fraction was then removed. The outer membrane pellet was washed with sonication buffer with 0.5% N-lauroylsarcosine. The final pellet was suspended in 0.2 ml of sonication buffer with 0.5% N-lauroylsarcosine and 0.1% sodium dodecyl sulfate (SDS). The proteins in each fraction were quantitated by BCA method and measured by Nanodrop 1000 (Thermo Scientific). Proteins were added with SDS sample buffer with β-mercaptoethanol, boiled for 10 min, and equal amounts of proteins from each fraction was electrophoresed using SDS-PAGE, transferred to nitrocellulose membranes and subjected to immuno-blotting using anti-His
antibodies coupled to horseradish peroxidase (Clontech, Mountain View, CA). A control Western blot was probed with anti-OmpA antiserum that detects a specific outer membrane protein, OmpA. Another control Western blot was probed with anti-DnaK antiserum that detects a specific cytoplasmic protein, DnaK.

**Construction of traM antisense RNAs**

To knock-down TraM expression in *B. fragilis* LV23, an antisense approach was applied. A shuttle vector was constructed so that it carried a *traM* asRNA fragment downstream of *cfxA* promoter (P*cfxA*). The pFD288 shuttle vector (167) was used for this construction, and the desired vector was called pFD288MAS1, 2 or 3. Figure 25 shows the strategy for construction of this shuttle vector. First, cefoxitin promoter (P*cfxA*) was amplified from the shuttle vector pFD351 (136), and then subcloned in pFD288, using PstI and BamHI restriction sites. Then, three different *traM*-antisense fragments were amplified from *traM* in LV23 genomic DNA, subcloned in pFD288P plasmid in opposite direction to P*cfxA*, using PstI and EcoRI restriction sites. AS1 is complementary to a region of 316 nucleotides, covering a putative Shide Dalgano sequence of *traM* and the first 166 nucleotides of *traM* sequence. AS2 has 179 nucleotides, complementary to a similar segment like AS1 but shorter, also complementary to the first 166 nucleotide of *traM* sequence. AS3 is complementary to 120 nucleotides of *traM* sequence from nucleotide 102 to nucleotide 221. AS1, 2 and 3 were subcloned in pFD288P in the
opposite orientation to that of \( P_{cfxA} \), so that, when the plasmid was transcribed, AS1, 2 and 3 RNA would be transcribed in the opposite direction, resulting in mRNA species that were antisense to \( traM \) mRNA.

Table 6: Primers for construction of \( traM \)-antisense RNA\(^a\).

<table>
<thead>
<tr>
<th>No</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EcoRI-10074F(^b)</td>
<td>ATCA GAATTCCCAATGCAAGTTTTTCAACGA</td>
</tr>
<tr>
<td>2</td>
<td>EcoRI-10211F</td>
<td>ATCA GAATTCTGGGAAATTAACTCCAGAAAGACA</td>
</tr>
<tr>
<td>3</td>
<td>BamHI-10389R(^c)</td>
<td>CTCA GGATCCCTGCTGGGGCAGAGTTTATC</td>
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<tr>
<td>4</td>
<td>EcoRI-M102F</td>
<td>ATCA GAATTCTGCCCCAGCAGAAGAAACTA</td>
</tr>
<tr>
<td>5</td>
<td>BamHI-M221R</td>
<td>CTCA GGATCCTTGAAGCTCCTTTGAGTGATTC</td>
</tr>
<tr>
<td>6</td>
<td>PstI-Pcfx-F</td>
<td>ATCACTGCAAGCCATGTATATTTATTTATTTGGTTTGACGAG</td>
</tr>
<tr>
<td>7</td>
<td>BamHI-Pcfx-R</td>
<td>CTCA GGATCCAAAATCAGTTCTTTAGCGATTAC</td>
</tr>
</tbody>
</table>

\(^a\): AS1 was amplified using primers EcoRI-10074F and BamHI-10389R (the numbers appear in these primer names indicate the start nucleotide of the primers in BTF-37 contig). AS2 was amplified using primers EcoRI-10211F and BamHI-10389R. AS3 was
amplified using primers EcoRI-M102F and BamHI-M221R (the numbers appear in these primer names indicate the start nucleotide of the primers in TraM). Red nucleotides show restriction enzyme sequences. \(^{b}\): F: forward primer. \(^{c}\): R: Reverse primer.

pFD288MAS1, 2, and 3 were transformed into the conjugation-competent \textit{E. coli} cells HB101-RK231 (the RK231 plasmid provides necessary products for DNA transfer by conjugation). One control was the transformation of the construct pFD288P that carries just the promoter \textit{PcfxA}. Another control was the transformation of pFD351 shuttle vector that carries the whole \textit{cfxA} gene instead of TraM asRNA fragment. The presence of the vectors in the cells was also confirmed by PCR.

A mating experiment of HB101-RK231-pFD288MAS and \textit{B. fragilis} strain LV23 to select for transconjugants \textit{Tc}\(^{R}\) (select for LV23) and \textit{Cc}\(^{R}\) (select for pFD288MAS) was carried out.

The requirement of TraM for conjugation was tested by performing quantitative conjugation assays from the donor \textit{Bacteroides} LV23-TraM AS to the recipients \textit{E. coli} HB101 or \textit{B. fragilis} TM4000. Transconjugants were selected by ampicillin and streptomycin resistance. Conjugation frequency was calculated relative to the number of donors.
Conjugation Experiments

E. coli to B. fragilis mating

Mating experiments to transfer traM-antisense carrying plasmids from E. coli HB101 RK231 to B. fragilis LV23 were performed as previously described (67). The E. coli donor strains were grown overnight aerated in LB media containing selective antibiotics 25 μg/ ml kanamycin for RK231 and 50 μg/ ml spectinomycin for pFD288P or pFD288MAS plasmids. The B. fragilis LV23 recipient strain was grown overnight in BHIS broth (brain heart infusion broth supplemented with hemin) with 5 μg/ ml tetracycline for LV23 strain selection. 500 μl of the recipient overnight culture was diluted into 25 ml pre-reduced BHIS (1:50 ratio) and grown to mid-logarithmic phase (OD$_{660}$nm = 0.6) in anaerobic chamber. About 1 hr and 30 mins after the subcultures of B. fragilis recipients, the E. coli donor was subcultured from the overnight culture to LB media in 1:50 ratio, and was grown to early-logarithm phase (OD$_{600}$nm = 0.4). The donor and recipient cultures were mixed in 1:1, 1:5 and 1:10 ratios, in which, the donor was used at 100 μl. The mixtures were centrifuged in 1.5 ml tubes for 2 mins at 3600 rpm in Eppendorf 5415R centrifuge. Supernatants were discarded. Pellets were resuspended in 50 μl BHIS and plated onto the centers of plain BHIS plates, which were allowed to dry out and incubated aerobically overnight at 37°C. 100 μl of each culture (donor and recipient) was plated onto BHIS plates supplemented with clindamycin and gentamycin and was incubated anaerobically at 37°C as negative controls. After overnight incubation, the mixture cells of donor and recipient were scraped from BHIS plates and plated on
pre-reduced BHIS plates supplemented with tetracycline for selection for LV23 strain, clindamycin for selection of the shuttle vectors and gentamycin for clearance of *E. coli* cells, and were grown anaerobically at 37°C for 24 to 48 hrs. Transconjugants were collected and transconjugant plasmid DNA was prepared and checked by restriction enzyme analysis.

**B. fragilis to E. coli quantitative mating**

Quantitative *B. fragilis* to *E. coli* filter mating was performed as previously described (183). *B. fragilis* strains containing pFD288MAS shuttle vectors or other control plasmids were used as donors. *E. coli* HB101 cells were the recipients. Stationary-phase cultures of the donors were used to inoculate fresh BHIS medium at a 1:50 dilution under anaerobic conditions. Subcultures were induced with 1 μg of tetracycline/ml after 1.25 hr, and, were grown further for 3 to 4 hrs (optical density at 660 nm reached 0.6). The recipient *E. coli* cells were subcultured at a 1:100 dilution, 2 hrs after subcultures of the *B. fragilis* donors. Recipients were grown aerobically until optical density at 600 nm was about 0.55. Then, 2.5 ml of donor were applied to 0.45-μm-pore-size Nalgene filters, which were vacuumed to remove medium, then washed with 10 ml of sterile modified phosphate buffered saline (MPBS) (80 mM Na₂HPO₄, 2 mM NaH₂PO₄, 1.45 mM NaCl, pH 6.9), then, mixed with 2.5 ml of the recipient cells, vacuumed to remove medium. Nalgene filters containing mixture of cells were aseptically transferred to BHIS agar plates using a scalpel fitted with a disposable blade, and incubated anaerobically overnight. Following incubation, filters were placed in 5 ml
of MPBS and vortexed vigorously for 15 secs to loosen cells and suitable dilutions were plated on selective BHIS media (streptomycin and spectinomycin for selection of HB101 and plasmid pFD288, respectively) and were allowed to grow aerobically. Conjugation frequency was calculated by dividing the number of transconjugants obtained by the total number of viable donor cells. Viable donor cells were measured by plating serial dilutions of the donor strains onto selective medium.

**B. fragilis to B. fragilis quantitative mating**

Quantitative *B. fragilis* to *B. fragilis* filter mating was performed as previously described (183). *B. fragilis* strains containing pFD288MAS shuttle vectors or other control plasmids were used as donors. *B. fragilis* TM4000 was the recipients. Stationary-phase cultures of the donors and recipient were used to inoculate fresh BHIS medium at a 1:50 dilution under anaerobic conditions. Subcultures of the donors were induced with 1 μg of tetracycline/ml after 1.25 hr, and were grown further for 3 to 4 hrs (optical density at 660 nm reached 0.6). Then, mating experiments on Nalgene filters were carried as described in mating experiments from *B. fragilis* to *E. coli*. Nalgene filters containing mixture of cells were aseptically transferred to BHIS agar plates and incubated anaerobically overnight. Following incubation, filters were placed in 5 ml of MPBS and vortexed vigorously for 15 secs to loosen cells and suitable dilutions were plated on selective BHIS media (clindamycin for selection of the shuttle vectors and rifampicin for selection of the recipients). Conjugation frequency was calculated by dividing the number
of transconjugants obtained by the total number of viable donor cells. Viable donor cells were measured by plating serial dilutions of the donor strains onto selective medium.

**Site Directed Mutagenesis**

Site directed mutations in *traM* were generated using Stratagene’s QuikChange Site-Directed Mutagenesis Kit, according to the manufacturer’s instruction. The oligonucleotide primers containing the designed mutation were designed complementary to opposite strands of the vector encoded TraM. Extension of the primers created a mutated plasmid containing staggered nicks. After PCR, the product was treated with DpnI enzyme. The DpnI endonuclease is specific for methylated and hemimethylated DNA and is used to digest parental DNA plasmid and select for mutation-containing newly synthesized DNA. The nicked vector DNA containing the desired mutations was then transformed into XL-1 Blue supercompetent cells. The cells repair the nick in the mutated plasmid. The mutated plasmids were sequenced to confirm mutations.
Table 7: Primers for generation of site directed mutagenesis in *traM*

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
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CHAPTER IV
EXPERIMENTAL RESULTS

**BctA is Essential for Conjugation from *B. fragilis* LV23**

Previously, our laboratory reported the capture of BTF-37, a 37kb conjugative transposon, from a clinical *B. fragilis* isolate, LV23 (181). BTF-37 alone is capable of facilitating transfer of DNA not only in *B. fragilis* but also in *E. coli* (181). Due to many repeat A-T rich regions which hinders the sequencing process, only 16kb of BTF-37 was sequenced. This 16kb region carries 11 ORFs. By sequence homology analysis with other *Bacteroides* known transfer factors, and by RT-PCT studies, these 11 genes likely correspond to the transfer region of BTF-37 (77). Of these 11 ORFs, *bctA* is an ORF highly conserved between different *Bacteroides* transposons.

*BctA* encodes a putative coupling protein of BTF-37 CTn (77). It is identical to the putative coupling protein BctA of *B. fragilis* conjugative plasmid pBF4, 98% identical to that of the *B. fragilis* NCTC9343, 74% to the *B. fragilis* YCH46 strain from Japan, and 48% to a BctA homolog from the *B. fragilis* CTnDOT conjugative transposon (77). Computer analysis also reveals that BctA has two Walker-box-sequences, ATP-hydrolysis motifs, as characteristic of a coupling protein. Previous studies from our
laboratory also shown that BctA has a signal sequence required for membrane localization and that it is indeed associated with the membrane. Moreover, BctA expression is upregulated under conjugation conditions and purified BctA migrates as a tetramer under non-denaturing PAGE (77).

To determine if BctA is required for conjugation in *B. fragilis* LV23, we generated a *bctA* null mutant. The traditional method is to introduce into a *B. fragilis* parent strain a suicide vector (pFD516) carrying a disrupted form of *bctA* gene (the middle region of *bctA* gene is disrupted by a *cfxA* gene). Previous work from our laboratory has shown it was very difficult to generate such a null mutant in the parental LV23 strain. Therefore, a *bctA* null mutant was generated from the parental TM4000BTF-37 strain. TM4000 is a transfer deficient strain. TM4000BTF-37 is a DNA transferrable strain carrying BTF-37 CTn generated by introduction of pBTF-37 into TM4000. The scheme for the selection of the TM4000BTF37*bctA* transconjugants was depicted in figure 11A. Figure 11B shows evidence of the presence of the disrupted *bctA* gene in the chromosomes of transconjugants, as seen by the PCR products of the amplifications of the right arm of the disrupted *bctA* gene, a region of about 1.6kb covering a *cfxA* gene insertion and the right flanking region of *bctA*. This evidence confirmed that the TM4000BTF37*bctA* transconjugants are true null mutants. First, this amplification cannot be from the original suicide vector because this suicide vector cannot replicate in *B. fragilis*, thus it should not present in the transconjugatns after several passages. Second, if the construct can insert into somewhere into the chromosome, we expect to be able to amplify not just the disrupted form of *bctA* but also
its original gene. Third, if bctA was not truly deleted due to the non-resolution of the suicide vector, a large portion of the suicide plasmid is still present in the chromosome, then a short species of 1.6kb PCR product should have not been detected.

To test for the transfer frequency of TM4000BTF37bctA null mutant, a shuttle plasmid pGAT400 was introduced into each test strain by conjugation from E. coli HB101 donor, and then this pGAT400 plasmid was used as a transferrable DNA to be measured in quantitative conjugation experiments. Quantitative conjugation experiments were carried out from donors TM4000BTF37bctA-GAT400 to the transfer deficient strain TM4000. Positive controls were mating experiments from donors LV23GAT400 and TM4000BTF37-GAT400 to TM4000. A negative control was the mating from TM4000-GAT400 to TM4000. Figure 11C shows that BctA is indeed required for the conjugation of BTF-37 CTn in B. fragilis. bctA KO strains were totally defective in DNA transfer activity.

A complementation assay was carried out to test if the re-introduction of BctA into BctA KO strain could recover DNA transfer activity. A shuttle plasmid (pFD288-BctA) carrying bctA was introduced into TM4000BTF37BctAGAT400. However, the complementation assay did not work, most likely because BctA was not successfully expressed in pFD288 shuttle vector. This may due to two reasons. First, the construction of bctA into pFD288 did not include any promoter region for successful expression of BctA. A reconstruction of pFD288-BctA including an upstream region of bctA, which most likely carries an original bctA promoter region, may solve this issue. Another solution is to use a promoter known to work well in B. fragilis to drive the expression of
bctA, such as cefoxitin promoter PcfxA. Second, the fact that pFD288 is a low copy shuttle vector obviously affects the level of expression of BctA. The use of a better shuttle vector system may help solving this problem. The lack of an anti-BctA antibody also hindered the verification of BctA expression.

However, the lack of a supporting complementation assay data does not preclude the likelihood that BctA is truly essential for conjugation mediated by BTF-37 in B. fragilis.
Figure 11. BctA is required for conjugation mediated by CTn BTF-37 in *B. fragilis*.

A) Strategy to generate a *bctA* null mutant in *B. fragilis* TM4000BTF-37. B) Evidence for the presence of *bctA* disrupted construct in transconjugant chromosomes. C) *bctA* null mutants (TM4000BTF37bctA #5 and #8) abolishes DNA transfer capacity of BTF-37 as measured by transfer frequency of GAT400 plasmid.
BTF37 Genes are Involved In Conjugation Process in B. fragilis LV23

Although the formation of the conjugal apparatus is well studied in other conjugative systems such as A. tumefaciens Ti plasmid and E. coli F and RP4 plasmids, little is known about its structure and function in Bacteroides spp. (181). BTF-37, a conjugative transposon isolated from the B. fragilis clinical isolate LV23, harbors genes encoding conjugal apparatus proteins and confers mobility on non-mobile plasmids (181). To identify important conjugal apparatus components, we investigated the expression of BTF-37 genes under conjugation conditions, i.e. when Bacteroides cells are stimulated with a low level of tetracycline (≤ 1 µg/mL). Under these conditions, the frequency of conjugal transfer has been shown to be elevated 1,000-10,000 fold (160, 173), and is likely directly related to the increased expression of proteins that assemble into the conjugal apparatus. Thus, we hypothesized that if transcript production from a BTF-37 gene was up-regulated under tetracycline induction conditions, that gene product may be involved in the DNA transfer process.

Using semi-quantitative RT-PCR, we found that expressions of all 11 ORFs in the known 16 kb sequence of BTF-37 were up-regulated when B. fragilis cells were exposed to tetracycline. After normalization with 16S rDNA expression, the transcripts of the 11 ORFs were up-regulated 1.2 to 2.1-fold. traM-orf7 transcription was elevated 1.5 fold, similar to that previously observed for bctA (77).
We have previously reported that $bctA$ expression is elevated to a maximum of 2.5-3 fold using real time RT-PCR and spot densitometry, respectively (77). These low (but consistently reproducible) levels of transcript up-regulation are likely due to the gene products being membrane-associated or integral membrane proteins; thus they cannot be highly over-expressed without deleterious effects to the cells. Further, actual DNA transfer events from donors to recipient bacteria occur very quickly (likely in secs), and only one conjugal apparatus is finally formed to connect the donor with the recipient [40](181). Therefore, these small increases in gene expression are likely sufficient for the production of a functional conjugal apparatus. Other laboratories have also reported similarly low increases in conjugal apparatus gene expression that correlate with increased DNA transfer (C. Jeffrey Smith, personal communication). We therefore conclude from these results that this up-regulation of conjugal apparatus gene expression from 1.2-2.1 fold indicated that all 11ORFs of BTF-37 are involved in the conjugation process.
Figure 12. BTF37 conjugative transposon

A.

![Gene expression diagram](image)

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Figure 13: mRNA expression of 11 genes in the 16 kb sequenced region of BTF37 in conditions with and without tetracycline induction. A) a representative 1% agarose gel profile. +: Tc induction; -: no Tc induction; B) Ratio of gene expression in Tc induction condition vs no Tc, normalized to the expression of 16S transcripts.
Identification of BctA and TraM Interaction Partners

The function of a coupling protein is to couple the relaxosome complex to be transferred though the conjugal apparatus to the recipient. The coupling protein localizes at the base of the conjugal apparatus and interacts with both the relaxosome complex and the conjugal apparatus (29, 59, 61). In \textit{B. fragilis}, such interaction partners of the putative coupling protein BctA have not been identified. In \textit{E. coli} and \textit{A. tumefaciens}, interactions of many mobilization proteins and conjugal apparatus components with the coupling protein were initially identified by two-hybrid system and then confirmed by biochemical experiments (5, 57, 69, 105, 174). Thus, to identify BTF-37 conjugal apparatus proteins interacting with the coupling protein, similar approaches were employed.

First, Bacteriomatch II two-hybrid system (Stratagene-Agilent Technologies, (43)) was employed to screen for gene products that interact with the putative coupling protein BctA. Briefly, individual BTF37 ORFs were cloned into the “prey” vector, pTRG; \textit{bctA} was cloned into the “bait” vector, pBT. The reporter cells (XL-1 Blue MFR Kan\textsuperscript{R}) were co-transformed with 50 ng each of bait and prey plasmids, phenotypically expressed transformant genes in His\textsuperscript{−} minimal media, plated on nonselective plates for 24hrs then replica plated on selective media containing 5mM 3AT, and then on dual selective plates containing 5mM 3AT and streptomycin. All plates had 25mM chloramphenicol and 12.5\textmu M tetracycline throughout to select for bait and prey plasmids.
The strength of interaction was calculated as the percentage of the co-transformed colonies that grown on the double selection plates. This was calculated by dividing the number of colonies on double selection plate for the number of colonies that grown on the non-selection plate. BctA was also examined for its stable protein production (Figure 14A). Our results of the bacterial two hybrid experiment in Figure 14B indicate that BctA interacts with ORF7 and ORF8 and that the interaction with ORF7 is stronger than with ORF8. From sequence homology analysis, ORF7 and ORF8 are homologous to TraM and TraN in other *Bacteroides* spp. CTn’s, respectively.

Of particular interest, BTF-37 ORF7 (TraM) is a 393 amino acid protein, and has 92% sequence identity to TraM in *B. fragilis* NCTC9343, 61% to TraM of *B. fragilis* YCH46, 32% to TraM of *B. thetaiotaomicron* CTnDOT and 28% to *B. vulgatus* CTn341. It is predicted to be a membrane protein by the TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and DAS (http://www.sbc.su.se/~miklos/DAS/maindas.html) membrane predicted programs (37). Moreover, deletion studies from CTn341 shown that TraM is required for conjugation (7), indicating that TraM is an important component of the conjugal apparatus in *Bacteroides* spp. Thus, we decided to focus our study on ORF7 (TraM).

Another two-hybrid screen was performed, in which, *traM* was used as the bait and other genes were constructed in the target vector. Our results further confirmed that TraM interacts with BctA (both BctA full-length and N-terminal truncation). Interestingly, TraM interacts with BmpH and ORF8 (TraN). A positive control for BmpH interaction was the interaction between *E. coli* TraG and BmpH. Work from our
laboratory has previously shown that TraG of *E. coli* RP4 plasmid system interacts with the *B. fragilis* relaxase BmpH of the Tn5520 mobilizable plasmid.

This was the first time a two-hybrid approach was used to study the interactions of *Bacteroides* spp. conjugal apparatus component proteins with a putative coupling protein as well as a relaxase. These data indicate that TraM interacts with BctA, ORF8 (TraN) and BmpH, of which, the interaction of TraM with BctA is strongest. Thus, TraM is likely a conjugal apparatus component protein at the base of the CA and interacts with the putative coupling protein BctA. This *in vivo* interaction between TraM and BctA is the first demonstration of an interaction of the CP with a CA component protein in *Bacteroides* spp. This is also the first demonstration of a CA component protein in *Bacteroides* spp. interacting with a relaxase. In *E. coli* F plasmid conjugative system, the relaxase forms a complex with some other partners such as IHF, TraM and TraY but they are mostly cytoplasmic proteins and not involved in the CA (142). Thus, if this interaction is true, it is an interesting finding, supporting the idea that the conjugation molecular mechanism in *Bacteroides* spp. is distinct from that of *E. coli*. 
Figure 14: Bacterial two-hybrid analysis of interactions between BctA and other BTF37 gene products (data from a representative experiment).  

**A.** Western blot shows full-length BctA in fusion with \( \lambda \)-cI in the bait vector. Cells: reporter cells (XL-1 Blue MFR Kan\(^R\)), pBT: reporter cells expressing the bait vector alone, pBT-BctA: reporter cells expressing BctA in bait vector.  

**B.** Interactions of BctA with other BTF-37 gene products. Individual BTF37 ORFs were cloned into the “prey” vector; \( bctA \) was cloned into the “bait” vector. The reporter cells (XL-1 Blue MFR Kan\(^R\)) were co-transformed with 50 ng each of bait and prey plasmids, phenotypically expressed transformant genes in His\(^+\) minimal media, plated on nonselective plates for 24hrs then replica plated on selective media containing 5mM 3AT, and then on dual selective plates containing 5mM 3AT and streptomycin. All plates had 25mM chloramphenicol and 12.5\( \mu \text{M} \) tetracycline throughout to select for bait and prey plasmids.
Figure 15: Bacterial two-hybrid analysis of interaction of ORF7 (TraM) with other BTF-37 gene products (data from a representative experiment). A. Western blot shows full-length ORF7 (TraM) in fusion with λ-cI (a total MW of about 72kD) expressed in the bait vector. Cells: reporter cells (XL-1 Blue MFR Kan\textsuperscript{R}), pBT: reporter cells expressing the bait vector alone, pBT-ORF7: reporter cells expressing ORF7 (TraM) in the bait vector. B. Interactions of ORF7 (TraM) with other BTF-37 gene products. BctA truncation is a construct that does not have the N terminal cleavage signal of BctA.
Hypothesis

Of all the putative conjugal apparatus genes encoded by BTF-37, we were interested in ORF7 (TraM) because our bacterial two-hybrid data showed that TraM strongly interacted with the putative coupling protein BctA and that it also interacted with the relaxase BmpH. Computer analysis of our BTF-37 TraM showed that it harbors characteristics of a required conjugal apparatus protein. First, this 393 amino acid protein exhibits sequence conservation with other Bacteroides spp. conjugal apparatus proteins, i.e., 92% sequence identity to TraM in B. fragilis NCTC9343, 61% to TraM of B. fragilis YCH46, 32% to TraM of B. thetaiotaomicron CTnDOT and of B. vulgatus CTn341. Second, TraM was predicted to be associated with the membrane, suggesting its function as a member of the transverse-membrane mating channel. Third, previous study shown that our TraM homolog in B. vulgatus CTn341 is required for conjugation, indicating that our BTF-37 TraM may also be important for conjugation in B. fragilis (7). Moreover, our preliminary data indicated that TraM interacts with both the putative coupling protein BctA and the relaxase BmpH, suggesting a special role of TraM in DNA transfer process in B. fragilis.

We, therefore, hypothesized that TraM is an important conjugal apparatus protein of the conjugative transposon BTF-37. If TraM is an important member of the Bacteroides spp. conjugal apparatus, then its function must be elucidated in more detail. In the studies described below, we thus demonstrated that 1) TraM expression is indeed
upregulated under conjugation conditions; 2) TraM associated with the inner-membrane and 3) TraM is required for DNA transfer process in *B. fragilis*. Moreover, we also investigated the interaction of TraM with its important putative partners, the putative coupling protein BctA and the relaxase BmpH.

**Figure 16.** Homology of some of *Bacteroides* conjugative transposons.
Figure 17. Summary of conjugal apparatus mutation studies in CTn341, showing the requirement of TraM for conjugation. Adapted from Bacic, *J. Bacteriol*, 2005 (7).
TraM Expression is Upregulated under Conjugation Conditions

TraM is a member of the putative conjugal apparatus operon of the conjugative transposon BTF-37. If TraM is truly a conjugal apparatus protein, its expression most likely is up-regulated under conjugation condition. In fact, our reverse transcriptase PCR study showed that traM expression, along with other genes of the BTF-37 contig was upregulated when B. fragilis conjugation condition was induced by tetracycline. Thus, we began our in-depth study of TraM by more accurately quantitating its gene expression levels under conjugation conditions. We used SYBR-Green based fluorescence for detection and quantification of traM expression by fully-quantitative real-time PCR (Q-PCR). The expression of both the 16S rDNA and tetQ genes was used as controls. Q-PCR data was analyzed using the ΔΔCt method (77). tetQ was used as a positive control, since it has been previously observed that tetQ transcription is up-regulated ~10 fold upon tetracycline exposure (77).

Q-PCR revealed that traM gene expression was up-regulated ~2-fold under conjugation (induction) conditions; thus further strengthening our hypothesis that TraM is required for Bacteroides spp. conjugation.
Figure 18. Q-PCR standard curves of 16s rDNA (A), traM (B) and tetQ (C).

Figure 19. *traM* gene expression under conjugation conditions. Adjusted relative expression values were calculated from mean ΔCt values of three replicate Q-PCR reactions.
**TraM Interacts with Both The Coupling Protein BctA and The Relaxase BmpH**

As presented above, we identified by bacterial two-hybrid analysis that TraM strongly interacts with both the coupling protein BctA and the relaxase BmpH. In fact, by performing more quantitative study, we further confirmed that BctA and BmpH interacts with TraM in *E. coli* bacterial two hybrid system. Bacterial two-hybrid experiments were carried out as described in the experimental methods. The strength of interactions were calculated as the percentage of the positive colonies showing the interaction of the bait and the target proteins on the double selection plates with the original number of colonies grew on the non-selective plates. All experiments were repeated in triplicate. As can be seen from figure 20, TraM interaction with BctA is stronger than that with BmpH. Moreover, when TraM was expressed in the bait vector, BctA in the target vector, the strength of interaction of TraM with BctA is not as strong as that of BctA and TraM (when BctA was in the bait and TraM in the target vector). This was probably due to the different level of expression of proteins in the bait and the target. We could evaluate the level of protein expression in the bait vector by performing a western blot using anti-λcI antibodies, antibodies against the fusion domain of the bait. However, we cannot evaluate the level of production of the target due to the lack of necessary antibodies. However, TraM and BctA either in the bait-target or target-bait relationships still show strong interactions with each other.
This was the first time a two-hybrid approach was used to study the interaction between *Bacteroides* spp. conjugal apparatus component proteins with a putative coupling protein and a relaxase. These interactions indicate that TraM is likely a conjugal apparatus component protein at the base of the conjugal apparatus and interacts with the putative coupling protein BctA. This *in vivo* interaction between TraM and BctA is the first demonstration of an interaction of a coupling protein with a CA component protein in *Bacteroides* spp. This is also the first demonstration of a CA component protein in *Bacteroides* spp. interacting with a relaxase. It is an interesting finding, supporting the idea that the conjugation molecular mechanism in *Bacteroides* spp. is distinguished from that of *E. coli*. 
Figure 20: Bacterial two-hybrid analysis of A) Interaction of TraM with BctA, B) Interaction of TraM with BmpH.
A more direct approach to support our bacterial two-hybrid protein interaction studies is one based on co-immunoprecipitation analyses. However, due to problems with compatibility and selection of available expression vectors, Far-Western experiments instead of co-immunoprecipitation analysis were performed to investigate the interaction of TraM with BctA and BmpH in vitro. Briefly, 6His-BctA and 6His-BmpH were expressed in Xl-1 Blue and BL-21 AI cells, respectively. Then the proteins were purified using native purification lysis buffer and TALON cobalt affinity column (histidine affinity column). Native proteins were eluted with elution buffer containing 200 mM imidazole. The crude extract expressing λcI-TraM from pBT-M vector and control cells (Xl-1Blue cells and XL1-Blue cells carrying p-BT vector alone) were electrophoresed on SDS-PAGE gel, then were transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with purified His-BctA or His-BmpH proteins, then with anti-RGS His antibodies, antibodies specific for histidine tag of BctA or BmpH. After that, western blot was performed as normal. A negative control Far-western blot was incubated with anti-His antibodies but not with purified proteins to show non-specific binding of antibodies. Figure 21 showed the presence of His-BctA at the right MW of λ-cI TraM (~70kD) when purified His-BctA overlayed on membrane harbouring TraM. This further confirmed that BctA interacted with TraM.
**Figure 21: Interaction of λcI-TraM with BctA.** 25 μl of control cell lysates (1: XL-1 Blue cells, 2: XL-1 Blue cells carrying control vector pBT) and of Xl-1 Blue cells expressing λcI-TraM were electrophoresed and processed for: blot A: western detection of TraM, blot B: Far western analysis overlaid with native 6His-BctA, blot C: antibody Far western control blot. Arrow indicates full-length λcI-TraM at 70 kD.
Moreover, a far western experiment was also carried out to confirm that BmpH interacted with TraM. Because we encountered difficulty in detecting interaction of BmpH and TraM in a far western experiment, we tried to obtain a better extraction of TraM by utilizing different lysis methods. In figure 22, lane 3 and 4 are cell lysates expressing TraM but with two different lysis methods, 3) using Sarkorsyl detergent and 4) using 1.5% Tween. Overlaid BmpH showed a stronger band interacting with TraM in lane 4 than lane 3, most likely due to the different lysis conditions. Although this result was observed only one time, our result still shows that BmpH interacts with TraM in biochemical assay, supporting the result in the bacterial two hybrid assay. This was further confirmed by our far western experiments for interaction of BmpH with TraM mutant L123S, which will be discussed later. This hard-to-produced data may be because of the following reasons: 1) purified BmpH is hard to maintain a good native structure for far-western experiments and 2) the interaction of TraM with BmpH is weaker than that with BctA, and therefore, is harder to be detected on a far western experiment. BmpH has high pI of 9.5, has large stretches of positively charge amino acids and is multifunctional. Thus, as a result, BmpH may non-specifically aggregate in self and non-self associations, hindering the interaction with TraM on the membrane in a far western experiment.
Figure 22: Interaction of λcl-TraM with the relaxase BmpH. 25μl of control cell lysates (1: XL-1 Blue cells, 2: XL-1 Blue cells carrying control vector pBT) and of Xl-1 Blue cells expressing λcl-TraM with two different lysis methods with Sarkosyl detergent (3) and 0.5 % Tween 20 (4) were electrophoresed and processed for: A: Far western analysis overlaid with 30 μg native 6-His-BmpH/ 15 ml far western incubation solution, blot B: antibody Far western control blot.
**TraM is Associated with The Inner Membrane**

The conjugal apparatus must be a membrane-traversing channel so that DNA can pass through from the donor to the recipient bacteria. In fact, studies in *E. coli* and *A. tumefaciens* indicate that most of conjugal apparatus proteins are membrane associated (63, 100). Computational analysis from TMHMM and DAS transmembrane predicted servers indicates that TraM is a membrane protein and aa14-30 from the N terminal of TraM are in the membrane spanning region. Moreover, if our BTF-37 TraM truly directly interacts with the two key proteins of the conjugation, the coupling protein BctA and the relaxase BmpH, it is most likely that TraM is associated with the membrane. In addition, mutational analysis of CTn341 (another *Bacteroides sp* CTn) show that TraM is required for *Bacteroides* conjugal transfer (7). Thus, TraM is most likely a membrane associated protein required for *B. fragilis* conjugation; and the interaction between TraM and BmpH may be required for TraM function in the CA. Moreover, because the potential coupling protein BctA is expected to be associated with the inner membrane, TraM is also expected to be in the inner membrane because it interacts with both BctA and the relaxase BmpH.

First, a simple cell fractionation was applied to determine if TraM is located in the membrane or cytoplasmic fraction. In this experiment, cells expressing TraM was separated into cytoplasmic fraction and membrane fraction by using high speed centrifugation. We have observed that TraM was strongly associated with the membrane.
fraction and it was not detected in the cytoplasmic fraction. Interestingly, a band above TraM was observed at about 55kD. This is likely full-length TraM that was completely processed for signal-sequence cleavage. Although TraM has no computationally predicted signal sequences, there is still a high possibility that TraM has a signal sequence as it is a membrane protein. We have observed this for other *Bacteroides* spp. CA proteins as well (BctA (77)).

To further examine if TraM is associated with the inner membrane, another cellular fractionation assay was performed to separate inner and outer membrane fractions, using high speed centrifugation, sonication and 0.5% N-lauroylsarcosine (as described in the experimental methods).

Figure 23 shows membrane fractionation of TraM-(6) His. As seen in the blot probed with OmpA antibodies, the membrane fractionation is still not totally clean, as indicated by the presence of some outer membrane protein OmpA in both inner membrane fraction (IM) and outer membrane fraction (OM). However, the majority of TraM was shown in the IM fraction. This infers that TraM most likely associates with the inner membrane. This localization of TraM is speculated to support the interaction of TraM with BctA at the base of the CA and also with the relaxosome via the relaxase BmpH from the cytosol.
Figure 23. **TraM is associated with membrane fractionation.** P: Periplasmic fraction, C: Cytoplasmic; IM: Inner membrane; OM: Outer membrane. 12μg of total proteins/lane.

A) SDS-PAGE gel stained with Coomassie blue. B) blot probed with 6HIS antiserum for detection of TraM-6His-V5. C) blot probed with antibodies for DnaK cytoplasmic protein. D) blot probed with antibodies for OmpA outer membrane protein.
TraM is Required for Conjugation Within and From *B. fragilis* LV23

Mutational analysis of CTn341 (another *Bacteroides* sp CTn) showed that TraM is required for *Bacteroides* conjugal transfer (7). Moreover, if BTF-37 TraM truly directly interacts with the two key proteins of the conjugation, the coupling protein BctA and the relaxase BmpH, then it is most likely that TraM is essential for *Bacteroides* conjugation. Thus, TraM is most likely essential for *B. fragilis* conjugation and the interaction between TraM and BmpH may be required for TraM function in the CA.

To determine if TraM is required for *B. fragilis* conjugation, it is important to generate a *traM* null mutant to study if this strain unable to transfer. A traditional method is to use a suicide vector to deliver a TraM disrupted construct from *E. coli* to *B. fragilis*. However, this method is difficult and time-consuming due to stringent recombination barriers encountered in *B. fragilis*. Thus, we decided to suppress the expression of *traM* in *B. fragilis* by using an antisense RNA (asRNA) approach. AsRNAs have been successfully used to suppress the expression of bacterial proteins in several studies, especially in the clostridia (118, 125, 138, 143, 177, 178), where very similar difficulties are encountered in generating KO mutants. A similar approach was employed to generate a *traM* mutant that decreases in the expression of TraM.

A shuttle vector carrying an asRNA fragment covering the translation initiation site of TraM was generated from the shuttle vector pFD288 (167). The transcription of this asRNA gene should be driven by a strong promoter in *B. fragilis*. However, the
promoter present in the shuttle vector pFD288 originated from *E. coli* and does not work in *B. fragilis*. Moreover, in *Bacteroides* spp., little is known about promoter properties; and it seems that gene promoters in *Bacteroides* spp. do not possess similar consensus sequences like those of *E. coli*. It has been shown that when the cefoxitin resistance (*cfxA*) gene was introduced into *B. fragilis* along with an upstream region of 146 nucleotides, the gene product was strongly expressed for cefoxitin antibiotic selection (166). Thus, this 146-nucleotide upstream region of *cfxA* may contain a strong promoter region for this gene. Thus, the shuttle vector was constructed so that it would carry a TraM asRNA fragment downstream of *cfxA* promoter (*P_{cfxA})*. And the vectors we generated were called pFD288MAS1, 2 and 3.

**Figure 24. traM-antisense constructs.** *P_{cfxA}*: cefoxitin A promoter. Cc\(^{R}\): clindamycin resistance (for selection in *B. fragilis*). Sp\(^{R}\): spectinomycin resistance (for selection in *E. coli*).
Figure 25. Effect of traM-antisense constructs on conjugation capacity of *B. fragilis* LV23 to recipients *B. fragilis* TM4000 (in blue) or *E. coli* HB101 (in red). The frequency of the control *B. fragilis* LV23-pFD288P was set at 100% for comparison with test constructs. Other controls pFD288 and pFD351 (pFD288 carrying the whole cefoxitin gene) did not show significant effect on conjugation.
Quantitative conjugation experiments from *B. fragilis* to *B. fragilis* and from *B. fragilis* to *E. coli* were carried out to determine if TraM is required for conjugation from *B. fragilis*. Experiments were processed using filters as described in the experimental methods. Briefly, in the quantitative conjugation experiments from *B. fragilis* to *B. fragilis*, *B. fragilis* strains containing pFD288MAS shuttle vectors or other control plasmids were used as donors and *B. fragilis* TM4000 was the recipients. The donor and the recipients were mixed together on Nalgene filters on working bench. Nalgene filters containing mixture of cells were aseptically transferred to BHIS agar plates and incubated anaerobically overnight. Following incubation, filters were placed in MPBS solution and vortexed vigorously to loosen cells and suitable dilutions were plated on selective BHIS media (clindamycin for selection of the shuttle vectors in *B. fragilis* and rifampicin for selection of the recipients TM4000). Conjugation frequency was calculated by dividing the number of transconjugants obtained by the total number of viable donor cells. Viable donor cells were measured by plating serial dilutions of the donor strains onto selective medium. Similarly, in the quantitative conjugation experiments from *B. fragilis* to *E. coli*, *B. fragilis* strains containing pFD288MAS shuttle vectors or other control plasmids were used as donors and *E. coli* HB101 cells were the recipients. Nalgene filters containing mixture of donor and recipient cells were also aseptically transferred to BHIS agar plates and incubated anaerobically overnight. After incubation, cells were loosened from the filter in MPBS solution and suitable dilutions were plated on selective BHIS media (streptomycin and spectinomycin for selection of HB101 and plasmid pFD288, respectively). These selective plates were allowed to grow aerobically. Conjugation
frequencies were also calculated as described with the quantitative conjugation experiment from \textit{B. fragilis} to \textit{B. fragilis}.

Figure 25 shows the results of the quantitative conjugation experiments. Our data show that two constructs AS2 and AS3 caused significant impairment to the conjugation capacity of \textit{B. fragilis} LV23. AS2-harboring cells exhibited almost 100\% reduction in conjugation frequency. AS3 showed up to 75\% and 82\% conjugation reduction when LV23 was mated with \textit{B. fragilis} TM4000 and \textit{E. coli} HB101 recipients, respectively. These results clearly indicated that TraM was required for conjugation. The AS1 construct did not reduce conjugation in \textit{B. fragilis} TM4000 but did so in \textit{E. coli} HB101 (up to 100\%). This maybe due to several reasons: First, this maybe because AS1 is the longest RNA fragment (316nt) with the highest required free energy, $\Delta G = -30.7$ kcal/mol (figure 26). This high free energy may cause AS1 secondary structure to be very stable and can not relax to bind to and form a complex with \textit{traM} mRNA. On the other hand, AS2 and AS3 have much lower free energy ($\Delta G = -9.9$ kcal/mol and $\Delta G = -18.4$ kcal/mol, respectively) (Figure 16). These lower free energy values may allow AS2 and AS3 secondary structure to be more flexible to relax and bind to the target \textit{traM} mRNA. Importantly, a TraM-irrelevant control did not show significant alteration in conjugation frequency, indicating that the effects of AS2 and AS3 on LV23 conjugation proficiency were indeed TraM-specific. Second, the different effects of AS1 on conjugation from \textit{B. fragilis} to \textit{B. fragilis} and to \textit{E. coli} may indicate the proficiency of conjugation from \textit{B. fragilis} to \textit{B. fragilis} is distinct from that to \textit{E. coli}. Our western blot in Figure 28 showed that AS1 did suppressed TraM expression in comparison to the control. However, this
suppression is not as strong as that caused by AS2 and AS3 (Figure 28). This data might suggest that a certain level of TraM expression is sufficient for conjugation to occur from \textit{B. fragilis} to \textit{B. fragilis}, however, it may not sufficient for conjugation to occur from \textit{B. fragilis} to \textit{E. coli}.

This is the first time an antisense approach has been used to study protein function in \textit{Bacteroides} spp. Because the antisense constructs were designed to cover the ribosomal binding site of TraM, it’s most likely that they bind to the mRNA of \textit{traM} and prevent translation of this protein. However, there is also a possibility that the AS bind to \textit{traM} mRNA and direct these transcripts to degradation process.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{antitraM.png}
\caption{The predicted secondary structures of anti-traM AS constructs. The predicted structures were obtained by using the Vienna RNAfold engine (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).}
\end{figure}
Although the quantitative conjugation data showed that TraM was required for conjugation, it is needed to determine if these AS constructs were truly specific for \textit{traM} inhibition. If TraM-AS RNAs were indeed gene-specific, then TraM protein production would be significantly reduced in the presence of \textit{traM} AS molecule(s). Moreover, if the AS molecules affected TraM expression at the transcriptional level, then both \textit{traM} mRNA transcripts and TraM protein production would be suppressed. But if the AS RNAs affected only TraM translation, then \textit{traM} mRNA transcripts would not be affected while protein production would be greatly reduced. Thus, to determine if these AS constructs were truly specific for \textit{traM} inhibition we quantitated the production of \textit{traM} mRNA transcripts as well as the TraM protein level.

\textit{Quantitative PCR (Q-PCR) experiments to estimate the level of \textit{traM} transcripts in the presence of different antisense molecules.}

The control for these studies was the \textit{B. fragilis} LV23 strain that harbored the shuttle vector containing only the cefoxitin A (\textit{cfxA}) promoter (the promoter constructed into this shuttle vector to drive the transcription of the antisense constructs). In these Q-PCR studies, because there was no realiable reference gene to be used in \textit{B. fragilis}, even 16S rRNA, the relative expression of \textit{tetQ} was used as the reference. However because \textit{tetQ} is highly upregulated in conjugation conditions, it is possible that \textit{tetQ} did not equally upregulated at the same level in different cell cultures, resulting in higher variation in the results (Figure 27).
Q-PCR showed that the effect of ASs on \textit{traM} is independent from those on ORF8 and/or BctA expression (Figure 27). AS1, the AS that did not impair conjugation suppressed some \textit{traM} and \textit{bctA} transcripts but those effects probably were not significant enough to affect conjugation. AS2, the most effective AS seemed significantly suppress \textit{traM} transcripts but not those of ORF8 and \textit{bctA}. Our data showed that AS2 significantly have upregulated effect on the transcript level of ORF8. However, this effect may not be real, as discussed above, the use of tetQ as the reference gene for this Q-PCR experiment may result in high variations among different samples. If the effect of AS2 on ORF8 was real, an evaluation of the protein level of ORF8 is necessary to confirm this data. AS3, the AS that suppressed conjugation up to 82% did not seem to have any effect on transcription level. However, we do not conclude that AS3 may have effect on translation level of \textit{traM} because complex mechanisms may be involved in the regulation of the expression of TraM.

Our results show that \textit{traM} expression is indeed specifically down regulated in the presence of AS2, and that this results in a corresponding reduction on conjugation frequency. TraM is thus required for \textit{B. fragilis} conjugation. Further, we also conclude that the use of an antisense-RNA based option to knock-down gene expression in \textit{Bacteroides fragilis} is a viable approach, and that the nature of the construct (sequence and size) is important for consistent and interpretable results. With the use of appropriate controls as described above (multiple target gene AS constructs, non-target gene AS construct, and quantitation of gene expression of genes surrounding the target gene) the contribution of TraM to \textit{B. fragilis} conjugation could be rigorously assessed.
Figure 27: Relative expression of A) traM mRNA, B) ORF8 mRNA, C) bctA mRNA in B. fragilis LV23 cells harboring the control plasmid with the cefoxitin promoter alone or that carrying AS1, AS2 or AS3 construct.
*TraM protein expression in the presence of different antisense molecules.*

To determine the protein expression level of TraM in the presence of different antisense molecules, we have successfully generated specific antibodies for TraM.

**Figure 28: TraM expression in the presence of AS in *B. fragilis* membrane fraction.**

About 20 μg of total proteins of *B. fragilis* membrane fractions were loaded into each lane. TM: proteins from *B. fragilis* conjugation deficient TM4000 strain; P: the control *B. fragilis* LV 23 strain that expression the control vector pFD288P; AS1, AS2 and AS3: LV23 strain expressing AS1, AS2 and AS3, respectively.
Figure 28 shows expression of TraM in the membrane fraction of *B. fragilis* LV23 in the presence of different AS constructs. α-OpmA antibodies were used for the control detection of a housekeeping protein in the membrane, OmpA. Our data shows that TraM is strongly associated in the membrane fraction of *B. fragilis*, confirming previous findings in *E. coli*. Moreover, our data also confirmed that the protein expression level of TraM was indeed significantly reduced in the presence of AS2 and AS3, explaining the impairment of DNA transfer in the presence of these antisenses in the quantitative conjugation experiments.

In short, these results show that TraM expression is indeed specifically down regulated in the presence of AS2 and AS3, and that these results correspond to the reduction in conjugation frequency. TraM is thus required for *B. fragilis* conjugation.

**Identification of TraM’s Regions Required for Interactions with BctA and BmpH**

Computer analysis of the TraM sequence revealed that some amino acids had good probability (P=>50%) of forming coiled-coil structures under physiological conditions – these were amino acids 47-75; 106-134; and 150-162 (COILS program (109)). Coiled-coil domains are structural motifs commonly involved in protein-protein interactions (109). Computer analyses of both BctA and BmpH (our putative coupling protein and DNA relaxase, respectively) also showed several predicted coiled-coil structures. Thus, we hypothesized that one or more predicted coiled-coil regions of TraM
may aid in its interaction with one or more predicted coiled-coil domains of BctA and/or BmpH. If this is true, then a TraM mutant protein with a disrupted coiled-coil domain(s) should be impaired in its interaction with BctA and/or BmpH, indicating that the predicted coiled-coil regions may indeed be required for the interaction. Published data strongly support the contention above. A predicted coiled-coil region of an \textit{E. coli} conjugation protein TrhB was shown to be essential for its interaction with the coupling protein TraG (57). Further, a coiled-coil domain of the \textit{Coprinus cinereus} Rad50 protein was required for formation of complexes with the exonuclease Mre11; point mutations as well as short insertions within the coiled-coil domains of Rad50 reduced or abolished function \textit{in vivo} (1, 83). For these studies (and those proposed below), the specific protein structure conferred by the coiled-coil was exploited to study its interaction-conferring ability. A coiled-coil region is a heptad repeat of amino acids labeled “a,b,c,d,e,f,g,”, of which positions a and d are hydrophobic and form the helix interface that mediates interaction with other proteins (Fig 1) (109). Mutagenesis of the amino acid at position(s) a or d can disrupt the coiled-coil, and thus alter its interaction with another protein. One elegant example of this particular approach is the published study identifying the requirement of two amino acids at position a and d of a coiled coil domain of the \textit{Neurospora} sp protein FRQ for its dimerization (27). In this study, alteration of the hydrophobic amino acid Leucine at position a or d to a hydrophilic amino acid Arginine, weakened or abolished the intra-subunit interaction(27). Another example is the study that identified the requirement of amino acids at position a or d within the coiled-coil domain of the \textit{E. coli} protein EspA for its oligomerization (40). Similar site directed
mutagenesis approaches were used to identify interacting regions within a coiled-coil domain required for oligomerization of the *Enterococcus faecalis* DivIVA protein (148), and dimerization of the *E. coli* MukB protein, (101).

Based on the published studies above, we hypothesized that if coiled-coil domains were indeed required for TraM interaction with BctA/ BmpH, and if hydrophobic amino acids at position a or d of the heptad were mutated to hydrophilic amino acids, the coiled-coil structure would be disrupted, leading to an impairment of protein-protein interaction(s). Usually, to determine if a coiled-coil structure is disrupted, circular dichroism (CD) and melting temperature experiments are performed to measure the helicity and stability of the protein (101). Since our current infrastructure does not allow us to perform such experiments, we proposed to use site-directed mutagenesis of multiple amino acids at position a or d of a predicted TraM coiled-coil domain, followed by bacterial two-hybrid screens and far-western analyses to determine if those specific TraM amino acids were required for its interaction with BctA/BmpH.

**Figure 29. Coiled coil heptad amino acid arrangement of two partner proteins.**
To test if an amino acid (aa) at position a or d of the three predicted coiled-coil regions of TraM is required for its interaction with BctA or BmpH, we used site directed mutagenesis to alter that hydrophobic amino acid to a more hydrophilic aa, in an effort to disrupt the coiled-coil structure. Potential coiled-coil domains are designated A, B and C (figure 30). There are 22 amino acids at positions a and d in these three predicted coiled-coil regions of TraM. The COILS program was used to identify amino acids, which when mutated, would cause the most significant reduction in the probability of forming coiled-coils. Thus, amino acid at position F66, L69 (region A), L123 (region B) and M154 (region C) were selected for mutagenesis studies. If an amino acid of the predicted coiled-coil region is required for the interaction, its mutants should show different interaction strengths with BctA and/or BmpH. Mutation to a stronger hydrophobic amino acid is expected to strengthen the interaction of TraM with its partner protein. Conversely, mutation to a weaker hydrophobic or hydrophilic amino acid is expected to
impair the interaction(s). The interactions of most important mutants were further biochemically confirmed by Far western analyses. The expression of TraM mutants were also tested by western blot, showing that they were stably expressed.

**TraM's amino acid F66 and L123 are required for interaction with BctA**

Figure 31 shows the bacterial two-hybrid analysis of the interactions of different TraM mutants with BctA. The control was the interaction of the wild type TraM with BctA. Western blots were performed to confirm that all of the mutants were made and stable. A western blot showing the expression of the most significant mutants was shown in Figure 33, blot 3. The multiple bands observed in this blot are degradation products of TraM, which were commonly observed when TraM was expressed under induction conditions. As can be seen from Figure 31, mutations of amino acid (aa) F66 in region A and amino acid L123 in region B significantly impaired the interaction of TraM with BctA, while other mutations did not. As expected, alterations to amino acids of increased hydrophilicity resulted in greater impairment of protein-protein interactions. These results thus strongly suggest that F66 and L123 are involved in the interaction of TraM with BctA, and that the chosen amino acids in region C are not.

Moreover, Far Western analyses of mutants F66R, L123R and L123S also revealed significantly reduced interactions between TraM and BctA (Figure 33). Together with the bacterial two-hybrid data presented above, there is now sufficient evidence confirming that amino acid F66 and L123 are indeed involved in the interaction
between TraM and BctA, and that TraM, via interaction with the coupling protein BctA, is required for DNA transfer from *B. fragilis* to other bacteria.

**TraM’s amino acid L123 is required for interaction with BmpH**

Bacterial two hybrid analyses of TraM mutants and the relaxase BmpH as can be seen from Figure 32 revealed that the specific TraM amino acids chosen for mutagenesis (except one – L123S) above did not affect/alter the interaction between the two proteins. Interestingly, the L123S mutant showed an increase up to 50% in its interaction strength with BmpH compared to that of the white type TraM. This significant alteration suggests that L123 may also be involved in the interaction between TraM and BmpH, but in a manner that facilitates protein contact.

Far western experiments also confirmed the strong interaction of mutant L123S with the relaxase BmpH. The interactions of TraM and other mutants (F66R and L123R) with BmpH are not detectable in a far western experiment. This may be due to two reasons. First, the interaction between TraM and BmpH may not be as strong as that with BctA. Second, under the purification conditions used, BmpH molecules may not be fully non-denatured, hindering its interactions with other proteins on the membrane. Even though the amount of purified BmpH used for overlaying the blot was increased from 10 μg to 30 μg, it was still not sufficient to detect the interaction of BmpH with TraM.

The above data strongly suggest that L123 may also be involved in the interaction between TraM and BmpH. The same amino acid may be a target for competition between two interacting partners, the coupling protein BctA and the relaxase BmpH. If this is true,
there may be a regulatory mechanism(s) to control the strength of interaction of TraM with either BctA or BmpH when needed. Indeed, the lowered strength of interaction seen with wild-type TraM and BmpH as opposed to L123S-BmpH may actually be required for rapid DNA transfer, where interacting complexes may need to be generated and dis-assembled in a dynamic fashion.
Figure 31: Bacterial two hybrid analysis of interactions of TraM mutants with BctA.
Mutants tested for region A were: F66A, F66G, F66R, L69A, L69G and L69R. In region B, tested mutants were: L123A, L123G, L123R and L123S. In region C: tested mutants were: M154A, M154G and M154R.

Figure 32: Bacterial two hybrid analysis of interactions of TraM mutants with BmpH.
Figure 33: Far western analysis of the interactions between λ-cI-TraM mutants and BctA or BmpH. Cells: XL-1 Blue cell lysate. pBT: cell lysate of XL-1 Blue cells that carried pBT vector alone. Wild type TraM and other mutants were expressed in pBT vector in XL-1 Blue cells. Blot 1: Overlaid with 10 μg of natively purified 6His-BctA, Blot 2: overlaid with 30 μg of natively 6His-BmpH, 3: Detection of λ-cI-TraM with α-TraM antibodies, 4: Non-specific control blot with α-His antibodies. The multiple bands seen in blot 3 are degradation products of TraM, which were commonly observed under induction condition.
CHAPTER V
DISCUSSION

**BctA is Essential for Conjugation from *B. fragilis* LV23**

Although many conjugative mobile elements have been identified in *Bacteroides* spp., none of the core components of the transfer regions of these elements have been characterized in detail except BctA of BTF-37 (77). BctA of BTF-37 and its homolog TraG (OrfG) in other *Bacteroides* conjugative transposon systems (*B. thetaiotaomicron* CTnDOT, CTnERL and CTnXBU422) have long been predicted to be coupling proteins of *Bacteroides* CA (150). All of the above proteins exhibit characteristics of coupling proteins: they have Walker box (ATPase) motifs, a DNA binding motif, they are associated with the membrane when expressed in *E. coli* (BctA of BTF-37 (77) and TraG of CTnDOT (191)) and their expressions upregulated under conjugation conditions (BctA of BTF-37 (77)). Furthermore, BctA has been shown to be a tetramer (77), which would allow it to form a ring structure at the base of the membrane like other described coupling proteins (TrwB of R388 system (58), TraD of F system (72), TraG of RP4 system (156), and VirB4 of Ti system (115)). However, there were still not enough data to confirm that BctA and its TraG homologs were indeed *bona fide* coupling proteins in *Bacteroides* spp.
conjugation systems, since their ATPase activity was not determined, and gene knock-outs were not available. In this study, we further showed that the putative coupling protein BctA was essential for conjugation mediated by the conjugative transposon BTF-37. This is another important piece of evidence to further indicate the role of a coupling protein of BctA in *B. fragilis*. Experiments to investigate the requirement of BctA Walker A and B motifs and their ATPase activities for conjugation need to be further carried out.

Moreover, it is known that the CP acts as a “gate-keeper” of the CA system. One of its important roles is to recognize and interact with the cognate relaxosome. This is the first step for the transfer of the relaxosome through the CA. In many conjugative systems including the *E. coli* F plasmid and *A. tumefaciens* Ti plasmid, the coupling protein is highly selective for the respective cognate relaxosome (73, 155). CPs of other *E. coli* systems like RP4 and R388 (though less selective) still only interact with their own cognate relaxosomes and closely related plasmids (73). In contrast, *Bacteroides* conjugative transposons are known for non-selective or permissive transfer of DNA. DNA from different bacterial origin (from *E. coli* or from other *Bacteroides* species) can be easily transferred by *Bacteroides* CTns. Meanwhile, our data show that when *bctA* was deleted, no transfer could occur. This result indicates that BctA is a key component to determine the permissive characteristic of *B. fragilis* CA for different types of transfer DNA (180). Although the precise molecular mechanism of relaxosome recognition by the CP has not been fully defined, our result still indicates that *Bacteroides* CP is unique and different from that of *E. coli* and *A. tumefaciens*. This non-specificity feature is the key
for the easy and promiscuous dissemination of mobile elements carrying antibiotic resistance genes from \textit{B. fragilis} to other bacteria.

**BTF37 Genes are Involved In Conjugation Process in \textit{B. fragilis} LV23**

To investigate the involvement of all ORFs in the known 16 kb region of BTF-37 contig, we examined the transcriptional expression of these ORFs under conjugation conditions by RT-PCR. Our results reveal that all 11 genes are up-regulated under conjugation induction conditions, suggesting that all known BTF-37 contig genes are involved in the conjugation process. They are most likely predicted CA proteins. These ORFs show similarity to respective putative conjugative genes in other \textit{Bacteroides} conjugative systems, such as \textit{B. thetaiotaomicron} CTnDOT and \textit{B. vulgatus} CTn34. However, no homology has been found for these genes with conjugation genes of \textit{E. coli} or \textit{A. tumefaciens}. In contrast, conjugation systems in \textit{E. coli}, \textit{A. tumefaciens} and even in some other gram negative species always exhibit some homologous components (2). Thus, the fact that \textit{B. fragilis} BTF-37 CA components are not homologous to that of other genera further strengthens the idea about the unique and distinguishing nature of \textit{Bacteroides} conjugation system. It therefore becomes all the more necessary to fully understand the structure and molecular mechanism of \textit{Bacteroides} conjugation. Only a full understanding of the \textit{Bacteroides} conjugation process can help design suitable
interventions to inhibit the dissemination of antibiotic resistance genes from *Bacteroides* spp. to other bacteria.

**TraM and TraN are Identified as Components of *B. fragilis* CA**

As a “gate-keeper” of the DNA transfer process, the CP does not just interact with the relaxosome but also with some components of the CA. In *A. tumefaciens*, the CP VirB4 has been shown to interact with other ATPases like VirD4, VirB11 and other non-ATPase components of the CA like VirB8 and VirB3 (47). In *E. coli* R27 plasmid, a F-type system, the CP TraG_H has been shown to have interactions with TrhB_H, another multimeric protein that may form a ring structure to extend the pore of the coupling protein into the periplasmic space (57). This CP also interacts with other CA components like TrhE_H and TrhI_H (92).

In the search for CA components that interact with the CP BctA, we have found that BctA interacts with two gene products, ORF7 (TraM) and ORF8 (TraN). In bacterial two-hybrid analyses, BctA interacts most strongly with TraM. The interaction with TraN is weaker. This is an early step in understanding CA protein contacts in *B. fragilis*. If TraM and TraN truly interact with BctA, then they are most likely localized in the membrane, close to BctA as well as to the base of the CA, and may be important components of the CA. In fact, we demonstrated that TraM indeed interacts with BctA and it is localized to the inner membrane. Further biochemical studies are required to determine if TraN truly interacts with BctA.
Moreover, further studies are required to find other components of CA, to determine how they interact, and how are they are regulated to ensure complex function of the CA. These components might be other ORFs of the 16 kb BTF-37 contig and interact with TraM or TraN. They may be unkown ORFs in the unkown sequence of BTF37. A bacterial two hybrid analysis for the interaction of BctA with *B. fragilis* LV23 genomic library will help identifying all possible interacting partners of BetA. Moreover, a similar genomic library screening can also be carried out to identify all possible interacting partners of TraM or TraN. In these experiments, a genomic library of *B. fragilis* LV23 can be generated by purifying the strain genomic RNA and randomly amplifying this genomic RNA with short, random nucleotide primers. This genomic library can be randomly constructed into the target vector of the bacterial two hybrid system and then a normal bacterial two hybrid analysis will be carried out. Any positive colonies that show interaction with the bait (BctA, TraM or TraN) will be collected and the putative gene that interacts with the bait will be sequenced by using specific primers for the target vector.

An alternative approach to identify other components of the CA is to amplify the whole BTF-37 CTn and generate a random library of BTF-37 genes by random PCR, then perform a bacterial two hybrid library screening. It is now possible to amplify this 37 kb fragment by using specific polymerase kits for long fragment amplification from Biorad. Another method is to use transposon insertion to generate a random library of BTF37 CTn.
**TraM Localization, Interactions and Function in *B. fragilis* CA**

Our bacterial two-hybrid analysis and Far Western experiments show that TraM interacts with both the CP BctA and the relaxase BmpH; this is the first report of an interaction between a CA component with a CP in *Bacteroides* spp. This is also the first demonstration of an interaction of a CA protein other than a CP with the relaxosome (via interaction with the relaxase).

In the *A. tumefaciens* Ti plasmid system, *E. coli* RP4 and F plasmid systems and in other species, the relaxase may complex with other partners, but they are not involved in the CA (89). For example, in the F plasmid system, the relaxase TraI interacts with partners like IHF, TraY and TraM but none of them are core components of the CA (142). In contrast, in *A. tumefaciens*, the relaxase VirD2 has not been found to have interaction(s) with any other component of the CA other than with the CP (29). Thus, the demonstration of the interaction of TraM, a component of the CA, with BmpH, a relaxase, is a new finding, supporting the idea that the molecular mechanism of conjugation in *Bacteroides* spp. is distinct from that of *E. coli* and other species.

In addition, the relaxosome of *Bacteroides* spp. in general requires far fewer numbers of proteins for DNA processing than *E. coli* (95, 130). There are two examples in which *Bacteroides* spp. relaxosomes have only one DNA processing protein. They are BmpH of Tn5520 and MobA of Tn4555 (165, 182). Therefore, it is highly possible that
TraM may support the direction of relaxosome complex through the mating channel by interacting with the relaxase BmpH.

The triad nature of the interactions of TraM with BctA and/or BmpH also suggests that TraM may act as a helper for both BmpH and BctA to facilitate movement of the relaxosome through the CA channel. In fact, the finding that amino acids F66 and L123 in TraM two predicted coiled-coil domains are required for interaction with BctA indicate that TraM interaction with BctA may be important for conjugation. Moreover, these two amino acids may be essential for TraM function in mediating DNA transfer in *B. fragilis*. Further examination of the requirement of these amino acids for conjugation in *B. fragilis* is required to test these hypotheses. To test these hypotheses, first, a *B. fragilis* *traM* null mutant must be generated. Then, different TraM mutants should be introduced for expression into the *traM* null mutant. Finally, quantitative conjugation experiments will be carried out to investigate the impacts of TraM mutants on conjugation proficiency of *B. fragilis*. If it is true that amino acid F66 and L123 are required for TraM interaction with BctA and if this interaction is important for DNA transfer, then we expect to observe a significant defect in the DNA transfer capacity of *B. fragilis* in the presence of TraM mutants. In addition, it is important to determine the regions on BctA and BmpH that are required for interaction with TraM. This can be performed by using a mutator strain such as *E. coli* XL-1 Red to generate libraries of BctA and BmpH mutants then analyse the interactions of the mutants with TraM by using a bacterial two hybrid library screening. The mutants that show significant defect on
interaction of BctA/ BmpH with TraM will also be further investigated for their requirements for DNA transfer in *B. fragilis*.

A recent cryo-EM structure of the core conjugation complex from *E. coli* pKM101 shows that the CA core complex spans from the inner to the outer membranes, and is formed by the different components TraN/VirB7, TraO/VirB9 and TraF/VirB10 (48). The inner layer of the T4SS in *A. tumefaciens* was speculated to be composed of VirB6 and VirB8 because these proteins interact directly with the transfer T-DNA (47). Thus, in the *B. fragilis* conjugation system, and along with the interaction with the relaxase BmpH and localization to the inner membrane, TraM may also be a component of the inner ring complex like VirB6 and VirB8. An investigation by using cryo-EM or X-ray crystallography approaches to examine the 3D structure of TraM will reveal this speculation. Moreover, to investigation TraM possible oligomerization nature, a molecular mass determination of TraM can be performed as previously described (77). In this experiment, TraM will be purified under non-denature condition and then electrophoresed under native condition. A Ferguson plot for analysis of migration ratios of TraM possible species will be performed to calculate their molecular weights.

Moreover, the finding that the same amino acid L123 is required for TraM interaction with both BctA and BmpH indicates a possible regulatory mechanism for interaction of TraM with BctA or BmpH to facilitate DNA transfer efficiency in *B. fragilis*. A very complicated regulatory system of many different CA proteins has been defined in IncF plasmid (including F, R100 and pRK100) conjugation (49). In *Bacteroides* spp., the regulatory cascade *tetQ-rteA-rteB* and *rteC* is responsible for
controlling the excision of CTnDOT (122). It was thought that RteC also regulates tra gene expression. However, recently, it has been demonstrated that the expression of CTnDOT transfer genes is activated by the excision proteins, independent of RteC (84). All of these reported regulatory systems suggest that the regulatory mechanism of conjugation is complicated and that there may be more new mechanisms that need to be explored.

Thus, we propose multiple models for a controlled regulatory system in which TraM switches interactions between BmpH and/to BctA (Figure 34). In the first model, model A, TraM is anchored in the inner membrane and interacts with BctA in normal conformation. When the relaxasome heads to the CA, TraM may change conformation to interact with BmpH first, assist the relaxasome to interact with BctA to translocate through the CA. On the other hand, a second model, model B, would be one in which TraM is a part of the inner ring of the CA and interacts with BctA in its normal conformation after the CA is formed to maintain the CA ring structure. However, when the relaxosome passes through the gate (BctA), TraM may change conformation, allowing interaction with BmpH to assist the movement of the relaxosome. It is also possible that multi copies of TraM may present in the actual CA structure. A 3D crystal structure of TraM, BctA and BmpH will solve this question.

To distinguish the above proposed models, several approaches can be employed. First, a 3D crystal structure of the whole CA complex with the presence of TraM, BctA and BmpH may show the exact localization of TraM in this triad relationship. Second, a time-line cryo-EM or X-ray crystallography experiment can be carried out to freeze the
movements of the relaxosome and the conformation changes of TraM. This experiment will show whose protein, BctA or TraM will contact with the relaxosome first. Third, the DNA to be transferred can be radio-label and then the contact of the DNA with CA components can be captured with time-line procedure and DNA binding assay such as mobile shift gel electrophoresis to determine which protein will contact with the relaxosome first (via interaction with BmpH or the DNA).
**Figure 34. Predicted localization of TraM, model A.** The red arrow shows the direction where the relaxosome directed by BmpH will pass through during conjugation. **Model A:** TraM is localized in the inner membrane and interacts with BctA in normal conformation. When the relaxasome heads to the CA, TraM may change conformation to interact with BmpH, assist the relaxasome to interact with BctA to translocate through the CA. **Model B:** TraM, as a member of the inner ring of the CA, interacts with BctA in normal conformation to maintain the CA ring structure. When the relaxosome passes through the gate (after contacting with BctA), TraM may change conformation, allowing interaction with BmpH to assist the movement of the relaxosome through the CA. Adapted from Christie, Nature, 2009 (28).
Furthermore, the finding that the AS1 construct showed different effects on conjugation frequencies from *B. fragilis* to *B. fragilis* and to *E. coli*, suggesting that the required conjugation machine for transfer to *B. fragilis* may be different for *E. coli*. The fact that AS1 can suppress the transfer to *E. coli* up to 100% in comparison to the control, while does not have effect on the transfer to *B. fragilis*, indicates that a certain level of TraM production may be enough for transfer to occur from *B. fragilis* to *B. fragilis*. But a much higher level of TraM production may be required for transfer from *B. fragilis* to *E. coli*. This speculation may make sense as more energy may be required for conjugation to occur to a different genus. An experiment in which TraM expression level can be controlled by an induced agent may help verify this speculation. In this experiment, a *B. fragilis* *traM* null mutant must be generated. Then, TraM will be introduced into *B. fragilis* via an expression vector, in which TraM expression can be controlled by an induced agent. Under non-induction condition, there should be a leaky expression of a small amount of TraM. However, when the induced agent is used, TraM expression is upregulated. Quantitative conjugation of different TraM production levels will be carried out to determine the required level of TraM for conjugation to occur to *B. fragilis* and to *E. coli*.

In short, further investigation is required to obtain more in-depth knowledge of the requirements of amino acids F66 and L123, and the possible regulatory relationship of the interactions of TraM with BctA and BmpH for DNA transfer in *B. fragilis*. A further mutagenesis study is required to identify the region of BctA and BmpH required
for the interaction with TraM. Moreover, these mutants need to be examined by quantitative conjugation in *B. fragilis* to confirm the requirement of these interactions for DNA transfer in *B. fragilis*. Due to difficulty in genetically modifying *B. fragilis* chromosomal genes, work in this dissertation did not attempt such experiments. However, in the future, when we have the right tools, it would be informative for *B. fragilis* conjugation studies to pursue this direction.

**TraM is Required for Conjugation in *B. fragilis* and Application of Antisense RNA**

By using antisense RNA tools, we further confirmed the requirement of TraM for DNA transfer within *B. fragilis* and from *B. fragilis* to *E. coli*. This is the first time an antisense RNA approach has been applied in *B. fragilis* to knock down expression of a target gene to investigate its function. This method helped overcome the difficulty encountered in generating a traditional knock-out mutant in *Bacteroides* spp. Previously, antisense RNAs also have been successfully used to suppress the expression of bacterial proteins in several studies, especially in the clostridia (118, 125, 138, 143, 177, 178), where very similar difficulties are encountered in generating knock-out mutants. This technology proved to be efficient and will be useful for future study in anaerobes.
**Future perspective**

As the most prominent group of bacteria residing in the gut and as those that also harbor a plethora of transmissible genetic elements carrying many antibiotic resistance genes, *Bacteroides* spp. have long been considered as a reservoir for the dissemination of antibiotic resistance traits to other bacteria. With the alarming rise and spread of antibiotic resistance to even new generation of antibiotics, the need to prevent the dissemination of antibiotic resistance has become more urgent. Because conjugation is the major means for bacteria, especially *Bacteroides* spp., to disseminate antibiotic resistance genes to other bacteria, effective intervention targets this process is an appropriate approach. Currently, many groups are exploring non-antibiotic-based methodologies to prevent conjugation-based DNA transfer (45, 51, 107, 111, 180). Different ways to inhibit the conjugative relaxase have been tested in laboratories. Antibody libraries against the relaxase TrwC of conjugative plasmid R388 can be used to block relaxase activity within recipient cells (51). Other studies report being able to disrupt the conjugation process by using specific inhibitors to the conjugative relaxase of the F plasmid (107). Interestingly, a short palindromic repeat (CRISPR) RNA interference can also limit gene transfer – this has been tested in Staphylococci by targeting relaxase genes (111). Overall, the search for better antibiotic resistance
interventions targeting conjugation is promising. Our study provides a better understanding of the *B. fragilis* conjugation system. This will be useful for the design of appropriate drugs to intervene with the conjugation process in *Bacteroides* spp., and ultimately inhibit the spread of antibiotic resistance genes from this genus to other bacteria.

**Significance**

In conclusion, this study represents the first in-depth characterization of a conjugal apparatus protein, TraM, in *B. fragilis*, which will be useful for future studies aimed at developing interventions to prevent dissemination of antibiotic resistance from *Bacteroides* spp. to other bacteria. This is the first demonstration of interactions of a CA protein with a CP and a relaxase in *B. fragilis*. Moreover, this is one of very few studies using RNA antisense technology to knock-down target gene expression in anaerobes, avoiding the difficulties encountered in modifying genes in these genera.
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VITA

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