Mobile genetic elements in the genus *Bacteroides*, and their mechanism(s) of dissemination

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Abbreviations: CA, conjugation apparatus; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; CTn, conjugative transposon; ETBF, enterotoxigenic *Bacteroides fragilis*; IBD, inflammatory bowel disease; IHF, Integrative Host Factor; MIC, minimum inhibitory concentration; MLS, macrolide-lincosamide-streptogramin; Mob, mobilization; oriT, origin of transfer

Bacteroides spp organisms, the predominant commensal bacteria in the human gut have become increasingly resistant to many antibiotics. They are now also considered to be reservoirs of antibiotic resistance genes due to their capacity to harbor and disseminate these genes via mobile transmissible elements that occur in bewildering variety. Gene dissemination occurs within and from *Bacteroides* spp primarily by conjugation, the molecular mechanisms of which are still poorly understood in the genus, even though the need to prevent this dissemination is urgent. One current avenue of research is thus focused on interventions that use non-antibiotic methodologies to prevent conjugation-based DNA transfer.

Introduction

It has been estimated that the human microflora collectively number up to 100 trillion cells, 10-fold the number of human cells.^{1,2} The majority of these normal flora reside in the gastrointestinal tract, and comprise over 500 bacterial species that exert a profound influence on human physiology.³ Of these bacteria, 99% are anaerobes.⁴ Anaerobic bacteroidetes are the predominant class, accounting about 30% of all bacteria in the human gut.^{5,6}

Bacteroides spp are bile-resistant, non-spore-forming, gram-negative, rod-shaped anaerobes that are a dominant bacterial genus in the human colon and are less abundant in the intestines of other animals and in the environment.^{7,8} *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.⁹ The C+G nucleotide composition of the *Bacteroides* genome is in the

range of 40–48%. Its membranes contain sphingolipids, which are unusual in bacteria.

As an integral part of the normal human gut flora, *Bacteroides* spp play a number of roles, such as providing energy for the host in the form of short-chain fatty acids and sugars, recycling bile acids, and aiding in the development of the host immune system.⁶ They also exhibit unique adaptations to successfully colonize the gut such as the ability to change their cell surface architecture,¹⁰ ability to stimulate host expression of fucosylated glycoproteins as well as synthesize them,¹¹ and the ability to tolerate and use oxygen.¹²

However, when *Bacteroides* spp escape the gut due to surgery, trauma or disease, they can cause life-threatening infections such as peritonitis and intra-abdominal sepsis.^{6,8,13} *Bacteroides* spp rarely cause endocarditis, inflammation of the inner layer of the heart, but when it does occur, it can be serious with a mortality rate of 21–43%,¹⁴ and increase hospital stays by up to 15 days.¹⁵ These organisms can also be associated with other infections such as those of the skin, soft tissue, joints (septic arthritis), and brain (abscesses and meningitis).⁶ Among more than 20 species of the genus, *B. fragilis* is most frequently isolated from clinical specimens, followed by *B. ovatus* and *B. thetaiotaomicron*.^{6,16,17} Enterotoxigenic *Bacteroides fragilis* (ETBF; a sub-group of *B. fragilis*) has also been implicated in inflammatory bowel disease (IBD)^{18,19} and colon cancer.²⁰ Recently, a 5 y study in Japan revealed that *Bacteroides* bacteremia was the primary cause of colorectal carcinoma in a group of patients.²¹ *Bacteroides* spp are thus among the most commonly isolated anaerobic pathogens,¹⁹ and it is now appreciated that this is likely due to the expression of diverse virulence factors including surface polysaccharide capsules, outer membrane vesicles, toxins and β -lactamases.²² In addition, the capacity of *B. fragilis* to tolerate nanomolar concentrations of oxygen allows this species to predominate in infections of the peritoneal cavity.

Antimicrobial resistance in the *Bacteroides* further complicates the clinical picture. Many *Bacteroides* spp are resistant to aminoglycosides (gentamicin, kanamycin, streptomycin), tetracycline

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Figure 1. Schematic highlighting the differences between conjugative and mobilizable elements. In the former, both DNA processing as well as mating apparatus functions are encoded, whereas, in the latter, only DNA-processing functions are elaborated.

(nearly 85% of clinical isolates), β -lactam antibiotics (penicillin, ampicillin, cephalosporins, cefoxitin, cephamycins and carbapenems), metronidazole and the macrolide-lincosamide-streptogramin (MLS) group of antibiotics (erythromycin and clindamycin).^{7,8} Of concern, all of these resistance traits have been found on transmissible genetic elements obviously contributing to resistance gene dissemination.⁸ Over the past decades, carriage of the tetracycline resistance gene, *tetQ*, has increased from about 30% to more than 80% in clinical strains.⁸ Specifically, over the past 10 y, resistance of *Bacteroides* spp has increased dramatically worldwide, especially to commonly prescribed antibiotics such as clindamycin and the cephalosporins.^{15,23-26} *Bacteroides* spp resistance to fluoroquinolones has also increased from 1.5–12% during the past few years.^{27,28} Metronidazole-resistant strains of *B. fragilis* have been reported in many countries, including Brazil,²⁹ India,³⁰ United States,³¹ Hungary³² and Poland,³³ and some Indian strains show very high levels of resistance (MIC 512 mg/L).³⁴ Therefore, anaerobic infections have reemerged as a serious health threat, and *Bacteroides* spp are now recognized as important pathogens.

Mobile Genetic Elements in the Bacteroides

Bacteroides spp harbor many conjugative and mobilizable elements (**Table 1**). Conjugative elements are autonomous and self-transferable, i.e., they encode all functions for DNA transfer, including those resulting in nucleic acid processing (DNA transfer initiation) as well as conjugation channel/portal assembly and DNA translocation (mating apparatus formation). Mobilizable elements encode only DNA processing functions (**Fig. 1**), and

use (or “hijack”) the mating apparatus of co-resident conjugative element(s) to transfer to recipient bacteria. Interestingly, in the *Bacteroides*, a wide variety of mobilizable elements appear to be able to translocate through the same mating apparatus encoded by a single conjugative element. A significant proportion of *Bacteroides* mobile elements also harbor antibiotic resistance genes, and are thus responsible for the widespread dissemination of those genes. Both conjugative and mobilizable elements can be transposons or plasmids.

Plasmids. Plasmids are very common in *Bacteroides* spp and are found in 20–50% of strains.⁶ Plasmids can replicate as independent elements in the host bacteria, and some can integrate into the host genome.³⁵ Many plasmids also have an origin of transfer (*oriT*) and a *trans*-acting mobilization gene, which allow them to be transferred by conjugation.

Antibiotic resistance genes have been found on plasmids in *Bacteroides* spp. Genes whose products confer resistance to metronidazole, chloramphenicol, carbapenems, clindamycin and erythromycin have been found on mobile plasmids from *Bacteroides* spp clinical isolates worldwide. Resistance genes *nimA-nimF*, encoding metronidazole resistance, have also been identified on transferrable plasmids and recovered worldwide.³⁶ The *cfiA* gene, conferring resistance to carbapenems, has also been found in a plasmid in clinical isolates.³⁷

Conjugative and mobilizable plasmids. To date, two conjugative plasmids have been identified: the *B. fragilis* 41kb pBF4 plasmid and *B. ovatus* 80.6kb pBI136 plasmid. DNA sequencing of the transfer regions of these plasmids has been limited due to A-T rich tracts that confound assembly and analyses. Only one gene, *bctA*, encoding a 110kD protein that localizes to the

Table 1. Mobile genetic elements found in *Bacteroides* spp.

Conjugative Elements	Mobilizable Elements
Self-transmissible	Not self-transmissible
Encode DNA-processing functions	Encode DNA-processing functions
Encode conjugation apparatus (CA) or mating channel for completely autonomous transfer	Do not encode CA. Transfer is dependent on CA formed by co-resident conjugative element(s)
Almost always harbor antibiotic resistance genes	May harbor antibiotic resistance genes
Conjugative plasmids	Mobilizable plasmids
Have <i>oriT</i> and <i>trans</i> -acting mobilization gene(s)	Have <i>oriT</i> and <i>trans</i> -acting mobilization gene(s)
Can replicate independently	Can replicate independently
May integrate into the recipient chromosome	May integrate into the recipient chromosome
Examples: pBF4, ¹²⁵ pBI136 ¹²⁶	Examples: pBFTM10 ³⁹
Conjugative transposons	Mobilizable transposons
Located on chromosome	Located on chromosome
Do not replicate extra-chromosomally	Do not replicate extra-chromosomally
52 kb to 150 kb	~4 kb to 15 kb
Also referred to as "Tet elements" since most carry the tetracycline resistance gene <i>tetQ</i>	
Examples: BTF-37, ⁵⁴ CTnDOT, ⁵⁵ CTnERL, ⁵⁶ Tcr Emr DOT, ⁵⁷ Tcr Emr 7853, ⁵⁸ CTnBST, ⁵⁹ CTnGERM1, ⁶⁰ CTn86 ⁶² and CTn9343 ⁶²	Examples: Tn4399, ⁴⁵ Tn5520, ⁴⁶ cLV25, ⁴⁷ NBU1, NBU2 ⁴⁸ and Tn4555 ⁴⁹

Table 2. Comparison of conjugation apparatus components of *A. tumefaciens*, *E. coli* and *Bacteroides* spp. mating systems.

Mating system	Number of CA proteins ^a	Energetic components ^b	Core channel components	Pilus components	Specific for own cognate relaxosome
<i>A. tumefaciens</i> VirB/D system ¹²⁷	12	VirD4 (CP), VirB11, VirB4	VirB3, VirB8, VirB10, VirB6, VirB7, VirB9, VirB1	VirB2, VirB5	Yes ¹¹⁹
<i>E. coli</i> RP4 plasmid ⁹⁵	12	TraG (CP)	TrbB, TrbC, TrbD, TrbE, TrbF, TrbG, TrbH, TrbI, TrbJ, TrbL	TraF	Yes (to closely related plasmids) ¹¹⁹
<i>E. coli</i> F factor ¹⁰³	11	TraD (CP), TraC	TraL, TraE, TraK, TraB, TraV, TraG, TraW, TrbC	TraA	Yes ¹²⁰
<i>B. thetaimicron</i> CTnDOT ⁸	17 putative gene products	Putative CP: TraG (OrfG)	N/A	N/A	No
<i>B. fragilis</i> BTF37 ⁵⁴	N/A	Putative CP: BctA ¹²⁸	ORF7 (TraM), ORF8 (TraN) ^c	N/A	No
<i>B. vulgatus</i> CTn341 ⁶¹	17 putative gene products	Putative CP: TraG (OrfG)	N/A	N/A	No

^aNumber of CA proteins includes energy-providing components, core channel components and pilus components. ^bEnergetic components include coupling protein and other ATPase proteins. ^cData from the Hecht and Vedantam laboratories.

membrane, has been identified to be required in mating process.³⁸ Multiple mobilizable plasmids have been identified, that can only be transferred via the mating channel formed by other co-resided conjugative elements; these include *B. fragilis* pLV22a and pBFTM10.³⁹

Cryptic plasmids. *Bacteroides* spp are also known for harboring small molecular weight cryptic plasmids at high frequency (50%).⁴⁰ These small plasmids are characterized in size classes: class I, 2.7 kb, class IIA, 4.2 kb, class IIB, 5.0kb, class IIC, 7.9 kb and class III, 5.6 kb.^{40,41} The majority of small plasmids are found in class I, IIA and III. Class IIB and IIC are rare among both normal flora and clinical isolates.⁴⁰ These small molecular

weight plasmids are called cryptic because, beside basic plasmid maintenance functions like replication, mobilization and in some cases, stability, they do not encode for any other obviously biological useful traits such as antibiotic resistance or production of virulence-associated proteins. For example, pBI143, a 2.7 kb plasmid, and pB8–51, a 4.2 kb plasmid, have been found to encode only replication and mobilization functions.^{42–44} pBF35, a representative of the most frequent class III plasmids, and originating in Hungary, was also found to encode only replication, mobilization and stability functions.⁴¹

At first glance, it may appear that cryptic plasmids have no clinical importance because they do not carry resistance genes.

However, they can have important clinical effects since they are capable of acquiring one or more antibiotic resistance genes and becoming mobile via integration with other conjugative or mobilizable transposons (or even other plasmids), resulting in further dissemination of antibiotic resistance genes. In addition, the abundance and diversity of these cryptic plasmids is cause for concern.

Transposons. Transposons, both mobilizable and conjugative, are most often located on the bacterial genome, do not replicate independently, and are copied along with the chromosomal DNA.

Mobilizable transposons. Mobilizable transposons, like mobilizable plasmids, cannot transfer autonomously, but can be disseminated from donor bacteria via a co-resident “helper” element.⁶ They use the mating apparatus of a co-resident conjugative element such as a conjugative plasmid or transposon for transfer to a recipient cell. Mobilizable transposons are invariably smaller than conjugative transposons and carry genes whose products are required for excision, DNA processing and integration of the element. However, they do not encode conjugal apparatus components and have to depend on co-resident conjugal elements like conjugative transposons. Some well-characterized mobilizable transposons in the bacteroidetes are the *B. fragilis* 9.6kb Tn4399,⁴⁵ *B. fragilis* 4.69kb Tn5520,⁴⁶ *B. fragilis* 15.3kb cLV25,⁴⁷ *B. uniformis* 10.3kb NBU1, *B. uniformis* 11.1kb NBU248 and *B. vulgatus* 12.5kb Tn4555.⁴⁹ Tn4399 requires the gene products encoded by *mocA* (a predicted relaxase *MocA*), and *mocB*, for mobilization.⁵⁰ During transposition, Tn4399 creates a 3-bp target site repeat and inserts an extra 5bp between the right inverted repeat and the target site repeat.⁵¹ However, other known mobilizable transposons require just one gene, encoding a relaxase, for mobilization. These are Tn5520 (BmpH^{46,52}), Tn4555 (MobA⁵³), NBU1 (*MobN1*), and NBU2 (*MobN2*⁴⁸). To date, the BmpH protein, encoded by the smallest known mobilizable transposon Tn5520, is the best characterized *B. fragilis* relaxase.⁵²

Conjugative transposons. Conjugative transposons (CTn's) are frequently found in *Bacteroides* spp. More than 80% of *Bacteroides* strains contain at least one conjugative transposon.⁸ Conjugative transposons in *Bacteroides* are often called “tetracycline resistance factors,” and many of them can be stimulated to transfer via tetracycline exposure. They range in size from 52kb - 150kb, and include *B. fragilis* BTF-37 (37kb),⁵⁴ *B. thetaiotaomicron* CTnDOT (65kb),⁵⁵ *B. thetaiotaomicron* CTnERL (52kb),⁵⁶

Figure 2. Schematic representing the major events occurring during conjugative DNA transfer. Cell membranes separating donor and recipient bacteria are depicted as solid black lines. The transferring element (plasmid-like in this example) is shown harboring an origin of conjugative transfer (*oriT*) as well as a mobilization (MOB) protein-encoding segment (Mob genes). As described in the text, a single-stranded DNA molecule is generated during the conjugation process, and translocated to the mating apparatus, of which one member is the coupling protein.

B. thetaiotaomicron TcrEmrDOT (70kb),⁵⁷ *B. thetaiotaomicron* TcrEmr7853 (70kb),⁵⁸ *B. thetaiotaomicron* CTnBST (100kb),⁵⁹ *B. thetaiotaomicron* CTnGERM1 (75kb),⁶⁰ *B. vulgatus* CTn341 (52kb),⁶¹ *B. fragilis* CTn86 (57kb)⁶² and *B. fragilis* CTn9343 (64kb).⁶² Of these, CTnDOT (65kb) from *B. thetaiotaomicron* is the best described. CTn's have also been referred to as “Tet elements” since most, but not all, carry a tetracycline resistance gene (usually *tetQ*).^{35,63} Many CTn's also carry the *rteABC* gene cluster, whose products are involved in the regulation of conjugal transfer.^{64,65} *rteA* and *rteB* genes encode a tetracycline inducible two-component regulatory system, which controls *rteC* expression.⁶⁵ *rteC*, in turn, controls expression of genes required for excision of transmissible elements. Transcription of *tetQ*, *rteA* and *rteB* is constitutive but translation of these genes is elevated during exposure to tetracycline due to a translational attenuation mechanism. As a result, very low (sub-inhibitory) levels of tetracycline or its analogs can markedly elevate conjugal transfer of Tet elements and other co-resident factors by 1,000-to 10,000-fold even upon brief exposure.^{64,66} It should be noted that this induction of transfer, while notable in some elements, occurs alongside the constitutive transfer events that are hallmarks of all these elements, and that have likely been occurring for millenia. Many *B. fragilis* conjugative transposons also carry erythromycin resistance genes such as *ermF* (cTnDOT),⁶⁷ *ermB* (cTnBST)⁶⁸ or *ermG* (cTnGERM1).⁶⁰

Conjugative transposons are mainly responsible for the spread of tetracycline and erythromycin resistance in clinical isolates of

Bacteroides spp.⁸ They are not only responsible for the dissemination of the antibiotic resistance genes which they themselves carry, but also for the transfer of antibiotic resistance genes harbored by co-resident mobilizable elements, via stimulation of the excision and transfer of those mobile elements. RteA and RteB encoded within the central regulatory region of the CTnDOT/ERL family of conjugative transposons regulate the excision and mobilization of the NBU mobilizable plasmids.⁶⁹

To date, CTnDOT (65kb) from *B. thetaiotaomicron* is the best studied conjugative transposon. Since it was first recovered from a patient with a *Bacteroides* infection,⁸ CTnDOT has served as a model to study the transfer mechanism of conjugative transposons in *Bacteroides* spp. It contains an excision region, a central regulatory region and a transfer region, and its excision, integration and regulation have been extensively studied. Excision is the first step in CTnDOT transfer from the chromosome, and results in the formation of a non-replicating circular intermediate.⁷ An operon containing genes required for excision (*orf2c*, *orf2d*, and *exc*) was identified and is regulated at the transcriptional level by the tetracycline-inducible regulatory proteins RteA, RteB and RteC.^{65,70-72} Integration is the final step when the CTnDOT intermediate recombines with the recipient bacterial genome. The integration reaction requires IntDOT, a CTnDOT-encoded protein that is a member of the tyrosine recombinase family, as well as an uncharacterized *Bacteroides* host factor.^{70,73,74} Interestingly, and somewhat surprisingly, the excision proteins Orf2C, Orf2d and Exc also appear to be involved in integration.⁷⁰ However, little is known about the organization and regulation of the transfer genes responsible for the formation of the conjugation apparatus that allows the transfer of co-resident mobilizable elements. Expression of the transfer genes (*traA* to *traQ*) is activated directly by the excision proteins, and independently of RteC.⁷⁵

Chimeric transposons. Due to their ability to integrate into DNA, it should not be surprising to find chimeric transposons, i.e., a transposon integrated into another transposon. However, to date, there are only two chimeric transposons in which a CTn inserted into another CTn, have been reported, a CTn in gram-positive bacteria, the streptococcal Tn5253⁷⁶; and a CTn in gram-negative bacteria, the *Bacteroides* CTn12256.⁷⁷ Although the 188kb CTn12256 was isolated from a clinical isolate in 1977, only recently has its chimeric nature been studied in more detail.⁷⁸ By cloning parts of CTn12256 into fosmids and performing a PCR walking survey, Wang, et al. found that the element was a chimera formed by the integration of a CTnDOT type element (CTnDOT2) into another CTn, CTn3Bf, that is most similar to a putative CTn found in *B. fragilis* YCH46.⁷⁸ It is interesting that although CTnDOT2 has 98% identity to CTnDOT, the transfer of the chimeric transposon CTn12256 is not dependent on tetracycline stimulation like CTnDOT and most other *Bacteroides* sp transposons. Because the *traG* homolog on CTnBf3, but not the *traG* of CTnDOT2, was essential for transfer, it was thought that CTnBf3 possibly dominant in the transfer of CTn12256. However, that is not the case because CTnBf3 cannot transfer independently of CTnDOT2 due to a large deletion near the integration site of CTnDOT2. This suggests that the unstudied

Orf BF2884 encoded by this deleted region may present an important transfer or mobilization gene.

How are Mobile Elements Transferred Within and from *Bacteroides* spp?

The primary 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 mechanism(s) of this process is poorly understood in *Bacteroides* spp.

Conjugation, a subtype of the bacterial Type IV secretion system (T4SS), is defined as the uni-directional transfer of a single-stranded DNA molecule from a bacterial donor cell to a recipient cell, in a process requiring cell-to-cell contact.⁷⁹ 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. Transferred DNA molecules, which can be either plasmids or transposons, are of two types: conjugative and mobilizable. As described above, conjugative plasmids and transposons are autonomously- or self-transmissible elements, encoding all 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).⁸⁰

All transfer elements contain a *cis*-acting origin of transfer (*oriT*) sequence where transfer is initiated. *oriTs* are specific sequences, about 30–500bp in length, most often located adjacent to the transfer initiation genes known as mobilization (“Mob”) genes, and forming a compact mobilization region.⁸¹ A common feature of the *oriT* is the presence of inverted repeats juxtaposed near a DNA sequence that is nicked during the transfer process.⁸² The nick (*nic*) site, a short stretch of about 10 nucleotides, is the site for recognition by the relaxase, required for the DNA transfer process.

Conjugation involves two major sets of events: Initiation (DNA processing) and conjugal apparatus formation as described below, and diagrammed in **Figure 2**.

Initiation (DNA processing). DNA processing includes binding, nicking and unwinding of the DNA, and these reactions are independent of conjugal apparatus formation.^{81,83} Processing occurs via the relaxosome, a nucleoprotein complex composed of specific proteins (mobilization proteins), one of which is the relaxase.^{84,85} This critical mobilization protein nicks the DNA to be transferred in a site- and strand- specific manner at the origin of transfer (*oriT*),^{81,83} and then covalently associates with the 5'-end of the nicked DNA via a phosphotyrosyl linkage. The nicked DNA is further unwound from the parent molecule, and transmitted in single-stranded fashion with 5'-3' polarity from the donor to the recipient.⁸¹ Single-stranded copies in both the donor and the recipient are then re-circularized and restored to the double-stranded form.⁸¹ The passage from the donor to the recipient occurs through a specialized membrane-traversing channel called the conjugal apparatus (discussed below).

Relaxase proteins, the major mobilization proteins of the relaxosomes, are usually multifunctional, and thus contain two or more protein domains. The nicking domain is always located at the N-terminus of the protein.⁸⁰ At the C-terminus, a DNA helicase, DNA primase or other domain of unknown function is almost always found.⁸⁰ Crystal structures of some relaxases have been obtained, including that of the F plasmid TraI nicking domain, with and without a bound DNA substrate,^{86,87} the nicking domain of TrwC from plasmid R388 with bound DNA^{88,89} and that of MobA from Inc.Q plasmid R1162.⁹⁰ In many cases, the nicking domain itself contains at least three conserved protein motifs. Motif I contains the active site tyrosine, which creates a single-stranded 5' DNA nick through a trans-esterification reaction similar to that of type I topoisomerase.⁸³ This reaction involves the nucleophilic attack on the DNA-phosphate backbone by the hydroxyl group of the tyrosine, resulting in a reversible covalent phosphodiester bond.^{81,91} Motif II is likely responsible for the recognition and noncovalent binding of the relaxase with the end of the trailing region 3' to the *nic* site. Motif III is histidine-rich and is called HUH (His-hydrophobic residue-His) or HHH (His-His-His). This motif likely facilitates the cleavage reaction (trans-esterification) by abstracting a proton from the terminal tyrosine hydroxyl, allowing the oxygen moiety to act as a nucleophile.⁸¹ The termination of strand-transfer occurs via a second cleavage reaction, releasing a single-stranded DNA molecule in the recipient cell.⁸¹

Most relaxases require the activity of accessory proteins to alter the conformation of the DNA to facilitate relaxase binding. The *E. coli* plasmid RP4-encoded relaxosome requires three proteins (two cognate proteins and the host encoded integration host factor, IHF) to achieve the conformation required for the relaxase to bind and nick the DNA.⁹¹ Similarly, relaxase activity of the TrwC relaxase of the R388 plasmid system also requires assistance from TrwA.⁹² In the F plasmid system, TraY enhances the relaxase/helicase activity of TraI.⁹³ In *Bacteroides* spp, to date, the mobilization region of CTn341⁹⁴ and Tn5520⁵² have been elaborated in detail. In CTn341 system, activity of the relaxase mobB required the accessory protein mobA.⁹⁴ However, in Tn5520, the relaxase BmpH is the first relaxase in *Bacteroides* spp reported to not require any accessory protein.⁵²

Conjugation apparatus formation. The second major process in conjugation is the formation of the conjugal- or mating apparatus. The conjugal apparatus (CA) is a multi-protein channel that is assembled across donor and recipient cell membranes during conjugation, through which the DNA strand is transferred.^{95,96} A pilus or other surface filament or proteins(s) may also be produced to facilitate adhesion and contact between two cells.^{97,98}

Although the formation of the conjugal apparatus has been 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 (**Table 2**).^{95,99–101} In the *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.^{95,102} In *E. coli* F plasmid system, the channel is composed of 11 proteins including the

coupling protein TraD.¹⁰³ In *A. tumefaciens*, each of 12 proteins named VirB1 to VirB11 and VirD4 has been extensively characterized.^{104,105} Recently, a cryo-electron microscopic structure of the core complex of the conjugal apparatus encoded by the *E. coli* conjugative plasmid pKM101 showed that the CA complex is 108Å wide and spanned the inner and outer membranes.¹⁰⁶ However, in *Bacteroides* spp the nature and function of the 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 a study of the *B. vulgatus* CTn341 element, where the requirement of each CA gene was assessed via the generation of gene mutants.⁶¹

Specificity (coupling protein). One of the CA's most important components is the coupling protein (CP). CPs appear to be encoded by all conjugative plasmids and transposons, but not by mobilizable elements. The CP is unique to conjugation and is considered to be the first point of contact that the relaxosome and/or tDNA makes with the CA. The best characterized CPs are TrwB of plasmid R388 (Inc.W group), TraD of F plasmids (Inc.F group), TraG of RP4 plasmids (Inc.P group), and VirD4 of *A. tumefaciens* Ti plasmids.¹⁰⁷ These proteins share the following characteristics: (1) they 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.^{108–110} They also typically have a cytoplasmic C-terminal domain.^{95,108,111,112} Thus, their location is the link between a cytoplasmic system and the membrane complex. (2) CPs have a nucleotide binding motif, and can bind both single- and double-stranded DNA, suggesting a specific role in DNA transfer.¹⁰⁹ (3) A CP has a Walker box domain, and cytoplasmic domains that interact with the relaxosome.¹¹³ The presence of Walker box motifs suggest that they use ATP hydrolysis as an energy source. 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.^{114,115} With this role, CPs are considered to be “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.^{113,116,117} However, in *E. coli*, one CP has been described for each conjugative system. (4) CPs are often multimeric proteins. The crystal structure of the soluble cytoplasmic domain of TrwB, the CP of the Inc.W plasmid R388, shows that it is a hexameric protein, resembles a ring helicase.¹¹⁸

Most importantly, and in many transfer systems, including those elaborated by the *E. coli* F plasmids and *A. tumefaciens* Ti plasmids, CPs are highly selective for the cognate relaxosome.^{119,120} Thus, a CA will only allow transfer of the plasmid that encodes it, as well as co-resident plasmids belonging to the same incompatibility group. All other plasmids and mobile elements are strictly excluded. However, and interestingly, in *B. fragilis*, putative CPs do not appear to harbor this selectivity. Conjugative transposons facilitate their own transfer, as well as that of all co-resident mobile elements, and “incompatibility” does not seem to play any role in the selection of molecules destined for dissemination. Thus, mobile elements can be transferred within, and from

B. fragilis even to bacteria from other genera. This promiscuous transfer may be one significant driving force behind *Bacteroides* spp as reservoirs of transferable antibiotic-resistance conferring elements.

Combating Antibiotic Resistance by Targeting Bacterial Conjugation

With the alarming rise and spread of antibiotic resistance to even new generations of antibiotics, the need to prevent the dissemination of antibiotic resistance is more urgent than ever before. Obviously, it is biologically impractical to use approaches that will eradicate all *Bacteroides* spp organisms, antibiotic-resistant or not. Since conjugation is the primary means by which *Bacteroides* spp. disseminate antibiotic resistance (and other) genes, interventions that target this process present an attractive approach to preventing unwanted horizontal DNA transfer—and only in the subset of organisms that are competent to do so. Currently, multiple groups are exploring non-antibiotic-based methodologies to prevent conjugation-based DNA transfer.^{15,121-124} Different methodologies to inhibit the conjugative relaxase have been tested. Antibody libraries against the TrwC relaxase of conjugative plasmid R388 have been used to block its nicking activity within recipient cells.¹²² Other studies report being able to disrupt the conjugation process by using specific inhibitors to the F plasmid relaxase.¹²¹ Interestingly, a short palindromic repeat (CRISPR) focusing on RNA interference can also limit gene transfer—this has been tested in staphylococci by targeting relaxase genes.¹²³

Thus, one approach in the search for interventions to mitigate antibiotic resistance gene spread will likely focus on conjugation system-based targets.

Conclusion

Bacteroides spp, the most prominent group of bacteria in the gut, can be both beneficial symbionts, as well as dangerous opportunistic pathogens. Arguably, one of the most significant traits that characterizes the *Bacteroides* as important clinical pathogens is their capacity to harbor a plethora of transmissible genetic elements carrying many antibiotic resistance genes. Since these genes are efficiently disseminated and acquired, *Bacteroides* spp are now considered to be reservoirs of these antibiotic resistance traits. Despite some of the advances described in this review, it should be emphasized that the majority of the mechanistic aspects of conjugative transfer—the pre-eminent process that drives the spread of antibiotic resistance in the genus—still remain to be elucidated. In-depth studies on *Bacteroides* spp conjugational components and mechanisms will thus provide insights to designing effective interventions that might focus on conjugation-system targets to prevent antibiotic resistance gene transfer.

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