RESEARCH PAPER

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The Ellis Island Effect

A novel mobile element in a multi-drug resistant *Bacteroides fragilis* clinical isolate includes a mosaic of resistance genes from Gram-positive bacteria

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Objectives: *Bacteroides fragilis*, a Gram-negative anaerobic bacterium, is alternately a gut commensal or virulent pathogen and is an important reservoir for horizontal gene transfer (HGT) of bacterial resistance and virulence genes in the human gastrointestinal tract. We identified a unique conjugative transposon (CTn) in a multidrug resistant clinical isolate of *B. fragilis* (BF-HMW615); we named this element CTnHyb because it included a hybrid mosaic of foreign elements. This study reports the characterization of CTnHyb and discusses the potential impact on horizontal spread of resistance genes.

Results: CTnHyb contains several efflux pump genes and several genes that confer or may confer antibiotic resistance to tetracycline, kanamycin, metronidazole and spectinomycin (truncated gene). CTnHyb also contains a mosaic of mobile elements from Gram-positive organisms. CTnHyb is easily transferred from BF-HMW615 (the original isolate) to BF638R (lab strain) and integrated into the BF638R chromosome. The "foreign" (from Gram-positive bacteria) nucleotide sequences within CTnHyb were > 99% preserved indicating that the gene acquisition from the Gram-positive bacteria was very recent.

Conclusion: CTnHyb is a novel CTn residing in a multidrug resistant strain of *B. fragilis*. The global nature and wide phylogenetic reach of HGT means that any gene in any bacterium can potentially be mobilized. Understanding the mechanisms that drive the formation and transfer of these elements and, potentially, ways to limit the transfer are necessary to prevent a devastating spread of resistance elements.

Introduction

The fluidity of the human gut microbiome has been recognized for decades but the recent data explosion from the analysis and sequencing of the gut microbiota is clarifying the vast extent of this gene transfer. Not only are genes regularly transferred among permanent residents of the gut, but organisms such as *Staphylococcus* and *Streptococcus* that do not colonize the intestine can participate in this genetic "swap meet"[1] (when they pass through the gut).

Of the resident gut population, ~99% of the microbiota belongs to two divisions (superkingdoms) of Bacteria–the Bacteroidetes (48%) (including *Bacteroides* species) and the Firmicutes (51%).[2] *Bacteroides* species, the dominant bacterial genus in the human gut are known to harbor many conjugative and mobilizable elements.[3]

Conjugative transposons, also known as integrative conjugative elements (ICEs), are a subset of mobile elements that also include plasmids and transposons.[3] Like transposons, CTns can integrate into diverse sites in the host chromosome. The CTns do not exclude each other as do plasmids, so a strain can accumulate more than one CTn. Furthermore, there is some evidence that the presence of more than one copy of the CTn in the strain results in a stimulation of transposition (transactivation).[4] Theoretically, this implies that as CTns with antibiotic resistance genes accumulate in the environment, the transfer of these genes to other bacteria will also increase and may result in upward spiraling of antibiotic resistance.[5]

We recently investigated a multidrug resistant clinical isolate of *Bacteroides fragilis* (BF-HMW615) isolated from a pediatric appendiceal specimen.[6] BF-HMW615 is resistant to multiple antibiotics, including metronidazole. Our analysis identified a *nimJ* gene which conferred increased MICs to metronidazole when introduced into a susceptible strain[7] and *nimJ* is carried on a novel conjugative transposon. We are now reporting the identification of this novel conjugative transposon, CTnHyb (for

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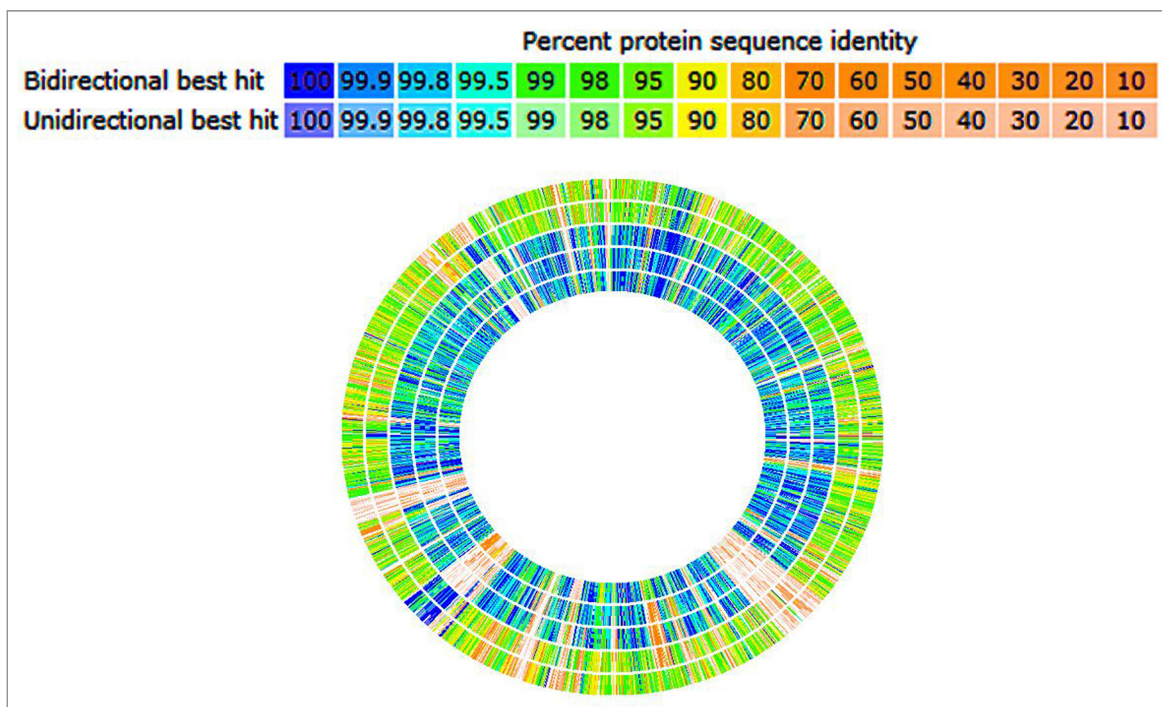


Figure 1. Genomic sequence comparison using the RAST Server (http://rast.nmpdr.org/). HMW 615 is compared with HMW 610, HMW 616, BF 9343, BF638R, and BF YCH46, respectively from perimeter of circle inward; HMW 615 sequence is inferred (not shown). The sequence of HMW 615 has been taken from the published supercontigs and rearranged according to the Mauve prediction. Hits on the comparison organisms are displayed graphically. Percent protein sequence identity is indicated (legend).

"hybrid"), which contains genes from Gram-positive bacteria. CTnHyb is transferable to *B. fragilis 638R* and thus is confirmed as a mobile element. The CTnHyb has (besides from *Bacteroides* spp) exact nucleotide homologs from at least three phylogenetically distinct Gram-positive organisms. The extent of the "hybrid" nature of this CTn, to our knowledge, has not been reported before in *Bacteroides.*

Results

Identification of CTnHyb in BF-HMW615 by comparative genome analysis

Comparative RAST-based genomic analysis[8,9] indicated a few continuous regions of BF-HMW615 chromosome (> 50,000 bp) that contained genes with no homologs in *B. fragilis* ATCC 9343, BF638R, BF Y46H or the multidrug resistant clinical isolates BF-HMW610 and BF-HMW616. The blue lines indicate that BF-HMW615 has homologs and red lines indicate absence of homologs (**Fig. 1**). One of these segments (indicated by red arrows, **Fig. 1**) included the *tetQ* gene as well as multiple *Bacteroides tra* genes (implicated in conjugative transposition). Thus, we considered this a potential CTn. Initial BLAST analysis of this nonhomologous segment in BF-HMW 615 indicated that some of the genes have been horizontally transferred from other species.

Transfer of CTnHyb to *B. fragilis* 638R

We mobilized CTnHyb from BF-HMW615 to BF638R by mating. The new 638R-CTnHyb mutant was selected using tetracycline and rifampicin (BF638R is rifampicin resistant, tetracycline sensitive; BF-HMW615 is rifampicin sensitive, tetracycline resistant). To rule out the small chance of the *tetR/rifR* transconjugant of BF-HMW615 origin, we confirmed the BF638R origin by PCR amplification and partial sequencing of two BF638R genes (BF638R_2089 and BF638R_4382) which are not present in BF-HMW615. There was no evident tetracycline-mediated increase in frequency of CTnHyb.

Determination of the CTnHyb ends and the point of insertion into the BF638R chromosome

We selected six colonies (HMW 874, HMW 875, HMW 876, HMW 877, HMW 878, and HMW 879) from independent mating experiments (of BF638R and BF-HMW615) to determine the insertion point of CTnHyb. The insertion points were determined by the SRP technique as described in Materials and Methods. The isolates HMW 874, HMW 875, HMW 877, and HMW 878 had identical ends and insertion points, whereas HMW 876 and HMW 879 had slightly altered ends and insertion point.

The genome of BF-HMW615 has been sequenced but the published sequence is in supercontigs: the sizes range from 1.1 (largest supercontig) to 1.14 (smallest supercontig). Our analysis indicated that the transferred CTnHyb fragment was partially contained in two supercontigs annotated as 1.1 and 1.3. Our PCR data connecting the supercontigs 1.1 and 1.3 was supported by MAUVE alignment of BF-HMW615 supercontigs with BF638R (**Fig. 2**). Also, an unannotated transposase was identified, through PCR, in the junction sequence of 1.1 and 1.3.

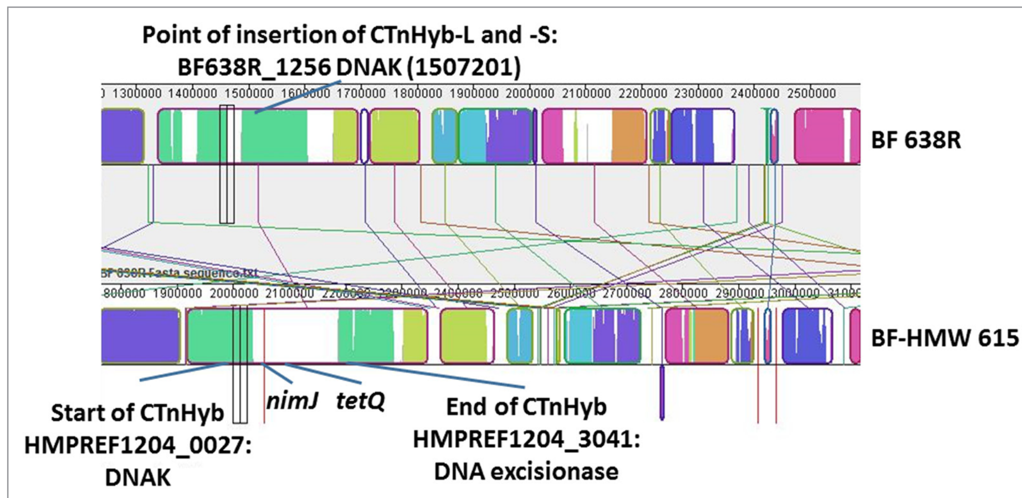


Figure 2. MAUVE-based alignment of BF 638R and BF-HMW615 and schematic indication of CTnHyb insertion into BF 638R. The MAUVE Contig Mover module was used to order (and orient) the BF-HMW615 supercontigs relative to the BF 638 reference genome. Colored block is a region of the genome that is homologous to a similarly colored part of another genome without extensive genomic rearrangement. Regions outside blocks lack detectable homology among the input genomes. Only a small part of the alignment is shown to visualize the insertion; *nimJ* and *tetQ* are indicated for orientation.

The predicted crossovers that would result in the sequences in 638R/CTnHyb isolates is depicted in **Figures 3A and B**, respectively. The left end of CTnHyb is *dnaK2* (HMPREF1204_0027 in reverse complement supercontig 1.1 of BF-HMW615) and the right end is between HMPREF1204_03040 and HMPREF1204_03041 (in contig 1.3 in BF-HMW615). The insertion is in *dnaK2* (BF638R_1256, between 1507197 and 1507222 bp).

Determination of size of CTnHyb

The two transconjugants, HMW 874 and HMW 875, were used to determine the length of CtnHyb. Surprisingly, two different size inserts had been incorporated into the BF638R chromosome. HMW 875 had a larger insert (thus named, CTnHybL) of 131,471 bp and HMW 874 had a smaller insert (thus named, CTnHybS) of 98,099 bp. Sequence analysis indicated that both inserts had the same ends but there were two internal segments missing in the CTnHybS (98099 bp), leading to the transconjugant with the smaller insertion as depicted in **Figure 3C**. Primers were designed across distances of 13327 bp (if the first deletion had not occurred) and 21512 bp (if the second deletion had not occurred), respectively, to detect whether the deletions were present in various isolates. PCR and subsequent sequencing across these regions indicated that the deletions were present not only in HMW874, but were also present in chromosomal DNA of BF-HMW 615 and in HMW875. The amplicons spanning the deletions (861 and 579 bp, respectively) were sequenced to confirm that they represented the deletion region. Thus, it appears that these deletions can occur in BF-HMW 615 as well as in BF638R/CTnHybL indicating the volatile nature of CTnHyb.

Determination of the circular form of CtnHyb

The primers (2783 and 2784) were used in a PCR reaction on BF-HMW615 and the BF638R/CTnHyb transconjugants to determine if we could detect a circular intermediate, which is a critical intermediary step in CTn transfer. The resultant amplicons indicated that there was indeed a junction between the two ends of CTnHyb indicating the presence of circular intermediate in BF-HMW 615. Interestingly, the circular intermediates were also detected in 4 independent isolates of BF638R/CTnHyb which indicated that CTnHyb can continue to make conjugative circle intermediates even after transfer to BF638R.

To visualize this circular intermediate, we constructed a GenBank (gb) file for CTnHyb from: 1) the sequence included within supercontig 1.1, 2) an unannotated transpose downstream of the predicted HMPREF1204_0001, 3) a short sequence obtained by PCR of the supercontig 1.1 and 1.3 junctions, and 4) the sequence contained within supercontig 1.3. The 4 gb files were concatenated using the SeqNinja program (DNASTAR, Inc., Madison, WI). The concatenated file was annotated and viewed using SeqBuilder (DNASTAR, Inc., Madison, WI) (**Fig. 4**). The *nimJ* and *tetQ* genes are indicated, as are the "foreign element" (from Gram-positive bacteria) extending from HMPREF1204_2965 to HMPREF1204_2980 and the predicted crossover point. The inner most circle is the GC% (green is higher than average, purple is lower than average).

CTnHyb contains 144 genes

BLAST analysis of the CTnHyb indicated that it contained CTn specific *tra* genes (*traE*, *traG*, *traJ*, *traK*, *traM* and *tra*N, excisionases, transposases and other DNA-associated proteins, a tetracycline resistance gene (*tetQ*), 3 putative pump system genes coding for efflux pumps (MefA, ABC, and RND type transporters), genes coding for hemagglutinin and thioredoxin (both may be important in virulence), and genes coding for metronidazole, kanamycin and tetracycline resistance. The genes contained within CTnHyb are shown in **Table S2**. RNA-Seq results for all of the BF-HMW 615 genes will be published as part of a larger study comparing the total transcriptome of BF clinical isolates (Husain F, Veeranagouda Y, Wexler HM, unpublished data) but

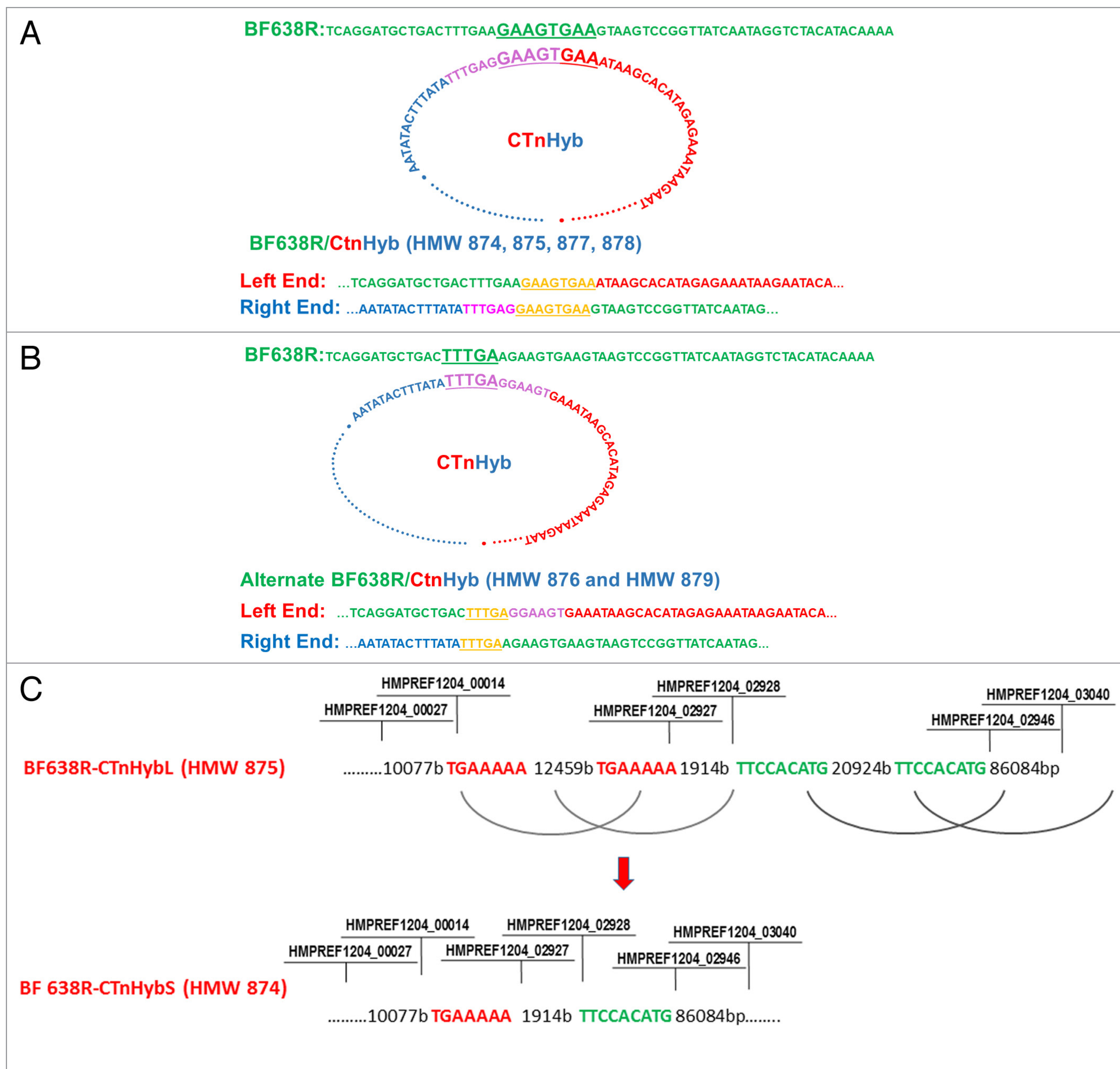


Figure 3. (**A**) Predicted events leading to integration of CTnHyb into the BF638R chromosome to result in BF638R/CTnHyb. The circular form of CTnHyb recombines with BF638R at the underlined sequence (GAAAGTGAA). (**B**) Alternate integration of CTnHyb into the BF638R chromosome. Predicted events leading to integration of CTnHyb into the BF638R chromosome to result in BF638R/CTnHyb found in HMW 876 and HMW 879 . The circular form of CTnHyb recombines with BF638R at the underlined sequence (TTTGA). (**C**) Predicted model of the deletions in CTnHybL leading to CTnHybS. The locus tag labels serve as approximate reference points. The bases are referred to as "bases" or "b". The regions of homology that are predicted to recombine are color coded. The *nimJ* gene is among the genes deleted in the first deletion and metronidazole has a lower MIC for BF638R/CTnHybS than for BF638R/CTnHybL, as expected (**Table 2**).

the RNA-Seq counts for the CTnHyb genes are presented here, as well. The distribution of genes in CTnHyb according to COG (Cluster of Orthologous Gene class) is shown in **Figure 5**.

CtnHyb includes a "foreign segment" containing genes homologous to a variety of Gram-positive bacteria (**Fig. 4**; **Fig. 6A**). HMPREF 1204_02969 (coding for aminoglycoside 3′-phosphotransferase conferring kanamycin resistance) is 100% identical to *Staphylococcus epidermidis* RP62A *aphA* (SEA0010). HMPREF 1204_02965 encodes a MefA type efflux pump and is 100% homologous to *mefA* genes in many *Streptococcus* species. Downstream of these genes is a 6790 bp segment (HMPREF1204_2967 through HMPREF1204_02977) that is homologous to a nucleotide segment in *Eubacterium ventriosum* 27560 (a Gram-positive

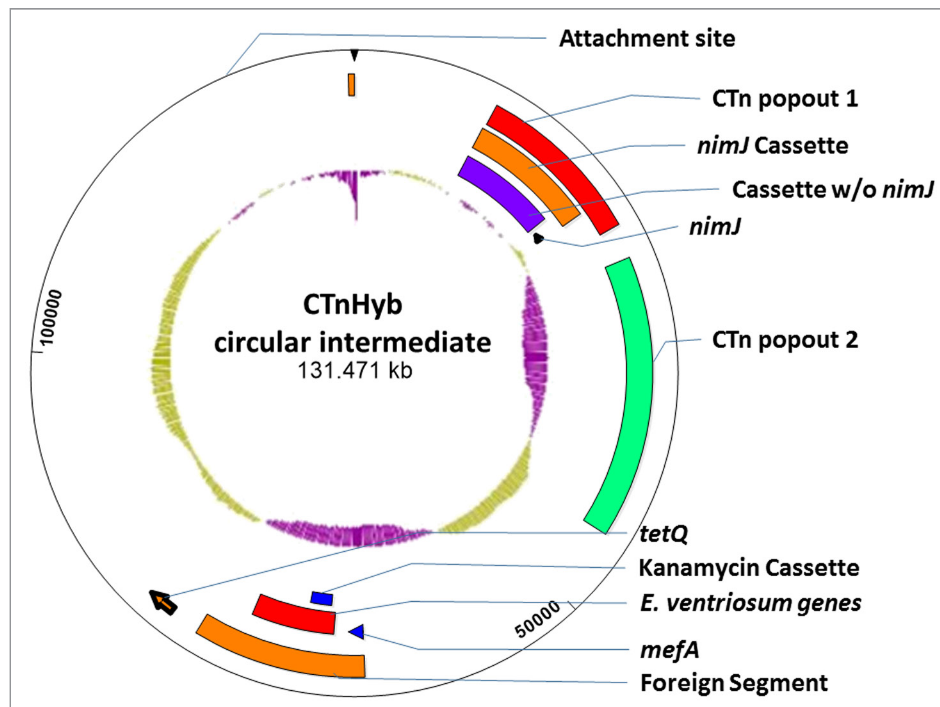


Figure 4. CTnHyb: Circularized form and predicted nested ICEs. Circular intermediates were detected in HMW 615, and in 4 isolates of BF638R/CTnHybL using CTnHyb/BF 638R Junction primers at either end of CTnHyb (Primers 2783 and 2784). The continuous sequence was generated using 4 GenBank files consisting of 1) the known Broad sequence within supercontig 1.1, an unannotated transpose downstream of the predicted HMPREF1204_0001, a short nucleotide segment and the sequence within supercontig 1.3 (we obtained the 2nd and 3rd sequence from manual PCR and sequencing). The GenBank files were concatenated using the SeqNinja program (DNA Star) and the generated sequence was annotated and visualized with the SeqBuilder program (DNA Star). The inner most circle is the GC% (green is higher than average BF GC % and purple is lower than average BF GC %). The predicted attachment site, the *nimJ*, *mefA* and *tetQ* genes and the predicted nested ICEs are indicated on the figure. 1) CTnHyb "Popout 1": (missing in CTnHybS) 12466 bases long, extending from HMPREF1204_00014 to HMPREF1204_00001 of contig 1.1 and the unannotated transposase and HMPREF1204_02924 to HMPREF1204_02926 of contig 1.3); 2) NimJ cassette, 9955 bases, present in 3 copies in BF HMW 615; 3) Cassette (minus *nimJ*) is found in other BF strains; 3) *nimJ* gene; 4) CTnHyb "Popout 2 (missing in CTnHybS): 20,933 bases long; extending from HMPREF1204_02928 to HMPREF1204_02946; 5) The "foreign segment" extends from HMPREF1204_2965 to HMPREF1204_2980; 6) *mefA* (HMPREF 1204_02965, 1232 bp) is a 95% match to *mefA* genes on ICEs from *Staphylococcus, Streptococcus*, and *Enterococcus*[22], and a close *mefA* homolog was also found in CTnGerm[23]; 7) *Eubacterium ventriosum* cassette (7178 bp not counting the kanamycin cassette within); 8) kanamycin cassette (1517 bp); HMPREF1204_2969-HMPREF1204_2971 is 100% match to nucleotide regions on ICEs from *Streptococcus.* However, the transposase (HMPREF 1204_02971) in the "kanamycin cassette" is not homologous to the *Staphylococcus* or *Enterococcus* transposases, but is hom0logous to a transposase from *Blautia hansenii*—a novel genus of Gram-positive, anaerobic, non-sporulating coccobacillus-shaped bacteria that includes several former coccobacillary shaped species of *Clostridia* and *Ruminococcus*.[24] HMPREF 1204_02971 is truncated at a 10 bp palindrome (ACTTCCGCCG, bp 195–204 and 218–227 within HMPREF 1204_2970) which is characteristic of transposase insertions. Bacterial repetitive *extragenic* palindromic sequences are known DNA targets for Insertion Sequence elements.[25] This palindrome, however, is contained within the coding region of HMPREF1204_02971.

gut anaerobe) from EUBVEN_02875 thru EUBVEN_02862 except for a short missing stretch (**Fig. 6B**). The "missing piece" is replaced by a 3617 bp sequence (HMP1204_02969, HMP1204_02970 and HMP1204_02971, GenBank Accession AGXR01000023.1 46489–48006) that is highly conserved in both genome sequences and ICEs in S*taphylococcus, Streptococcus* and *Enterococcus.* In *S. pneumonia,* for example, it is present on mobile elements carrying multidrug resistance determinants[14] and in *Enterococcus faecalis* RE25 it is present on a 50-kb conjugative multidrug resistance plasmid (pRE25)[15] (**Fig. 6B**). We don't know whether the insertion of the 3617 bp segment (containing HMPREF1204_02969, 02970 and 02971) happened before or after the *Eubacterium* segment was transferred into BF-HMW 615. Also, whether the acquisition of the *E. ventriosum* genes is a result of a BF CTnHyb-like element having moved into *Eubacterium* and then back into BF is not clear at this point.

CTnHybL contains a novel metronidazole resistance gene

CTnHyb contains a cassette with a recently reported metronidazole resistance ("*nimJ*") gene, found in two metronidazole resistant clinical BF isolates (BF-HMW 615 and BF-HMW 616).[7] Downstream of *nimJ* is an unannotated transposase of the IS4 class (in opposite orientation) which spans the junction of supercontigs 1.1 and 1.3. It is in reverse orientation to *nimJ* and contains the consensus promoter sequence TAnnTTTG that is found on insertion sequences containing the *cfiA* (imipenemase) gene.[16] *nimJ* (HMPREF1204_00002) is present within CTnHybL but is not present in CTnHybS (see **Fig. 3C**). As expected, BF638R/CTnHybL had increased MICs for metronidazole (1.0–1.5 μg/ml) while BF638R/CTnHybS had MICs

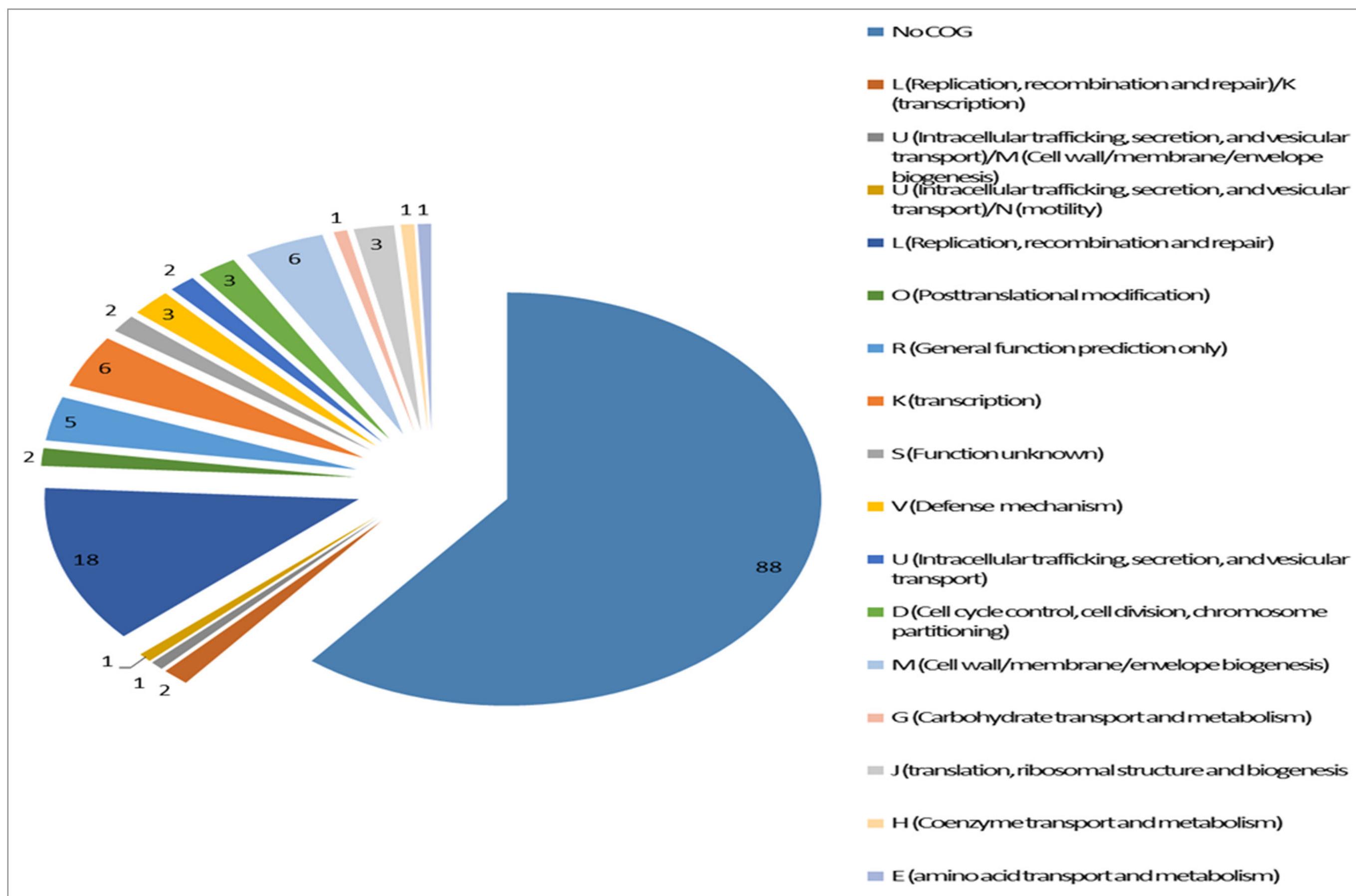


Figure 5. Distribution of genes in CTnHyb according to COG (Cluster of Orthologous Gene class). COG ID numbers and classification were taken from the Integrated Microbial Genomes and Metagenomes (IMG) at the Department of Energy Joint Genome Institute. (http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=FindGenes&page=geneSearch). Eighty-eight genes in CTnHyb do not belong to any COG. Similarly, RAST analysis of the entire BF-HMW 615 genome sequence indicates that 69% of the putative genes are not assigned to any subsystem (a subsystem is a method of categorization that can be thought of as roughly equivalent to a COG). Of the remaining 57 genes in CTnHyb(L), almost one third of the genes (n = 18) are in COG L (replication, recombination and repair) which includes such genes as DNA primases, excionases, integrases and transferases. Another 11 genes are annotated as "viral proteins" and are not assigned to a COG class, even though several are also functionally annotated as excisionase or integrase proteins.

Figure 6 (See opposite page). (**A**) Schematic representation of the "foreign" segment in CTnHyb. White arrows correspond to conserved genes in *Bacteroides* sp strains. See the figure for explanation of other arrows. APH(3'): aminoglycoside 3'-phosphotransferase; ABC: ABC transporter ; Mob: mobilization protein; Spe R: spectinomycin adenylyltransferase; KNTase: nucleotidyltransferase; HMTase: methlytransferase; HDc: Metal dependent phosphohydrolases with conserved "HD" motif; SIS: Sugar Isomerase; ZnCP: Zinc peptidase; BmgA and BmgB: mobilization proteins. (**B**) ACT alignment of area of homology between *Eubacterium ventriosum* and CTnHyb (BF-HMW 615). A segment (7671 bp) of the *E. ventriosum* genome is nearly completely conserved except for a three gene insert that replaces part of EUBVEN_02872, EUBVEN_02873 and part of EUBVEN_02874. In the conserved portions, only 11 nucleotides differed.

Table 1. Minimal inhibitory concentrations of strains and constructs

	MICs (mg/l)[a]		
	Tetracycine	**Erythromycin**	**Metronidazole**
BF 25285	**0.60** ± 0.22	**3.5** ± 0.71	**0.41** ± 0.04
BF 638R	**0.16** ± 0.00	**1** ± 0.00	**0.44** ± 0.08
BF HMW 615	**192.00** ± 0.00	**256** ± 0.00	**12** ± 5.66
BF 638R/CTN HybL	**60.00** ± 5.66	**13** ± 4.24	**1.125** ± 0.18
BF 638R/CTN HybS	**64.00** ± 0.00	**6** ± 0.00	**0.41** ± 0.04

[a]MIC is the average of two technical repeats and two biological repeats.

A

02954 02955 02956 02957 02958 02959 02960 02961 02962 02963 02964

traK traM traN transposase

tra region

02965 02967 02968 02969 02970 02971 02972 02973 02974 02975 02976 02977 02978

mefA Kan R ABC Mob

Regulator Transposase Excisionase Regulator Phage integrase Spe R

02979 02980 02981 02982 02983 02984 02985 02986 02987 02988

HDc Transposase *tetQ* *rteA* RND/ MPF RND/ IMP RND/ OMP SIS

KNTase HMTase

02990 03040 03041 03042 03043 03044 03045 03046 03047 03048

ZnCP Recombinase BmgA

Transposase BmgB

97% protein identity with Streptococcus suis R61

Identical gene match to *Eubacterium ventriosum* ATCC 27560

High level of gene identity (>95%) with *Eubacterium ventriosum* ATCC 27560

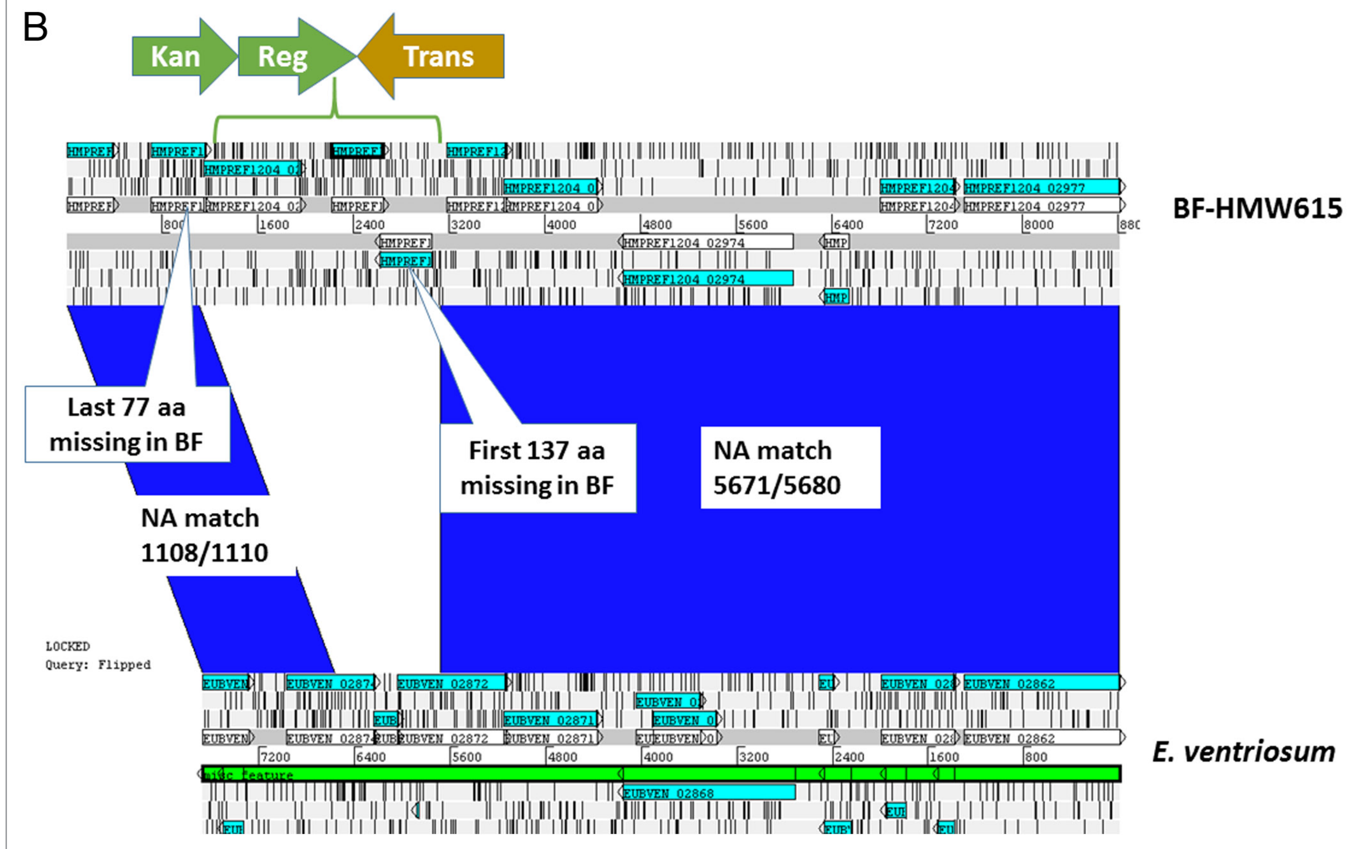
Identical gene match to *Staphylococcus aureus* strain TW20

78% of protein identity with *Eubacterium rectale* ATCC 33656

81% of protein identity with *Staphylococcus aureus*

91% of protein identity with *Enterococcus faecium* TX0133A

91% identity with *Staphylococcus epidermidis* RP62A



in the same range as BF638R (0.38–0.5 μg/ml). Additionally, MICs of erythromycin for BF638R/CTnHybL and BF638R/CTnHybS were increased from those of BF638R (**Table 1**).

CTnHyb contains multiple transposase genes of the IS4 category

In addition to the transposase gene adjacent to *nimJ*, CTnHyb contains two additional transposase genes of the IS4 class that are divergently transcribed from their adjacent genes. HMPREF1204_2983 is adjacent to HMPREF1204_2984 (*tetQ*) and contains the consensus promoter sequence, and HMPREF1204_2964 is another IS4 family transposase adjacent to HMPREF1204_2965 (*mefA*).

Expression of HMPREF1204_02969 (conferring *kan*^{R}) and HMPREF1204_02965 (*mefA*) in *E. coli*

HMPREF1204_2969 (encoding aminoglycoside 3′-phosphotransferase for kanamycin resistance) and HMPREF1204_02965 (*mefA*) were cloned into pSportI and introduced into *E. coli*: HMPREF1204_2969 into *E. coli* AG100 to determine changes in kanamycin MICs and HMPREF1204_02965 into both *E. coli* AG100 and *E. coli* Kam43 (a pump deficient mutant, lacking AcrAB, AcrEF and TolC[17]) to determine erythromycin MIC changes due to *mefA* expression. *E. coli*/pSportI::HMPREF1204_2969 grew in the presence of 40 μg/ml kanamycin, while *E. coli*/pSportI (without the kanamycin gene) did not grow, indicating that HMPREF1204_2969 was fully functional in *E. coli*. HMPREF1204_02965 (*mefA*) did not confer erythromycin resistance in either AG100 or KAM43. The mating of BF-HMW615 and *E. coli* AG100 did result in DNA transfer that yielded *E. coli* that were kanamycin resistant, but the transconjugants were not stable and did not yield further generations on purification.

Discussion

The importance of the gut flora in human health and disease is at the forefront of scientific and public awareness and major efforts are underway to characterize and sequence the gut microbiota as part of the massive Human Microbiome Project (HMP) at the Broad Institute/NIH. The gut bacteria, in terms of total cell and gene numbers in the human[18], "represents a virtual inner organ"[19]. BF is a human gut commensal that only accounts for 2% of the total *Bacteroides* but it is the agent of > 70% of *Bacteroides* infections.[5] As a commensal, it hydrolyzes complex polysaccharides and produces volatile fatty acids used by the host as source of energy[5] and is important in immune development.[20,21] However, it is very virulent when it escapes the gut and has been associated with nearly all types of infections. Additionally, it may be a reservoir of resistance genes that can get passed, by horizontal gene transfer (HGT), to other organisms resident in or passing through the gut.

Horizontal gene transfer among gut microbiota is particularly intense; gut microbes, therefore, may be a major reservoir for antibiotic resistance genes.[22,23] Indeed, the taxonomically different representatives of gut microbiota may share the pool of closely related antimicrobial resistance genes. HGT is also a crucial event in the development of virulence traits.[24]

One of the major elements responsible for HGT are conjugative transposons (CTns). CTns are similar to transposons (that integrate into the host semi-randomly) but also carry the genes necessary for conjugal transfer to other cells. The first complete sequence of the transfer region of a *Bacteroides* conjugative transposon was described in 2001.[25] The many known conjugative transposons and other mobile elements that are present in strains of *Bacteroides* were reviewed recently[3] and include CTnDOT (the most widely studied *Bacteroides* CTn),[26] CTnGerm,[11] CTn341,[27] CTnBST[28] and CTn12256.[29] CTnGerm and CTnBst were reported to carry a variety of genes with high similarity to genes from aerobic bacteria[11,28] and CTn12256 was described as a chimeric transposon composed of two independently active mobile elements.[29] Sequence comparisons between CTnHyb and other *Bacteroides* CTns, including CTnDOT, CTnGerm or CTnBST did not reveal any significant homology.

CTnHyb is unique and noteworthy among the many conjugative transposons and other mobile elements present in strains of *Bacteroides*. First, several genes coding for antibiotic resistance proteins are contained within CTnHyb. The *tetQ* and *nimJ* genes conferred tetracycline and metronidazole resistance, respectively, in BF638R and HMPREF1204_2969 conferred kanamycin resistance in *E.coli*. The encoded efflux pumps including the RND and *mefA* pumps may also contribute to drug resistance. Also, there is a truncated gene for spectinomycin resistance. Second, CTnHyb has a long sequence stretch of homologous sequences from a Gram-positive species (i.e., *Eubacterium*) that includes a mosaic of resistance genes from other aerobic Gram-positive species. The degree of nucleotide conservation (6779/6790 nucleic acids) within the area homologous to *Eubacterium ventriosum* indicates a very recent transfer to BF, otherwise some degree of adaptation to codon usage bias patterns of BF would be expected.[30] The manner in which the various "foreign" elements are arranged within CTnHyb suggests an "Ellis Island" effect where incoming CTns are preferentially drawn to genomic regions that are composed of other mobilizable elements; ICEs are nested within each other (**Fig. 4**) (note: Ellis Island was the gateway for millions of immigrants to the United States from 1892 to 1954). This type of modular transfer within conjugative elements has been described in other bacteria and in one CTn in *Bacteroides* and may be an important mechanism for the accumulation of resistance and virulence genes in the gut and the subsequent development of resistance or pathogenicity islands.[31]

Target site selection is an important characteristic for each transposon and determines dissemination and stability.[32] We detected a circular intermediate of CTnHyb in BF HMW 615 and in HMW615/BF638R transconjugants and predicted two potential crossover points that would yield sequences consistent with the sequences found in the resultant BF638R/CTnHyb transconjugants. In CTnDOT, the most extensively studied *Bacteroides* CTn, a tyrosine recombinase called IntDOT catalyzes integration into, and excision out of, the bacterial host

chromosome.[33] The core (GTANNTTT), are inverted repeat sequences that flank target sites in the chromosome and in CTnDOT, where strand exchange takes place catalyzed by IntDOT. The target sites, *attB*, on the host chromosome consist of a pair of inverted repeat core sites (B and B'). The complementary sites on CTnDOT, attDOT sites, have the core sites D and D'. CTnBST, another *Bacteroides* CTn, appears to integrate more site specifically than CTnDOT, with a 6-amino-acid signature that is associated with the catalytic regions of members of the tyrosine recombinase family.[32] Although the core site sequence is present (~5037 times in BF638R), it is not at the crossover position of CTnHyb.

Transfer of several of the widely studied *Bacteroides* CTns, including CTnDOT, is mediated by low levels of tetracycline and it is believed that the wide use of antibiotics therapeutically and in animal feed (with subsequent contamination of both meat and manure-fertilized crops) influence the introduction of these mobile-element bearing-organisms into the human gut. It is possible that elimination of the inducers (i.e., antibiotic) might be achieved by radically reducing their use. In the case of CTnHyb, however, transfer (from the multidrug resistant clinical isolate to the susceptible lab strain) could be easily achieved without any need for induction by tetracycline so change in tetracycline use may not affect transfer frequency. The global nature and wide phylogenetic pool of the horizontal transfer described in recent years means that any gene in any bacterium can potentially be mobilized and resistance phenotypes can be established in a diverse range of organisms worldwide. Understanding the mechanisms that drive this transfer and ways to limit the transfer are necessary to quell the spread of resistance elements.[26,34-36]

Table 2. Strains and plasmids used in this study

Strains	Description	Phenotypes	Source or reference
B. fragilis			
BF638R	Wild type parental strain	Tet^S, Rif^R	37
HMW 615	Drug resistant clinical isolate	Tet^R, Rif^S	6
HMW 874	638R/CTnHybS	Tet^R, Rif^R	This study
HMW 875	638R/CTnHybL	Tet^R, Met^R, Rif^R	This study
HMW 876	638R/CTnHyb 599	Tet^R, Met^R, Rif^R	This study
HMW 877	638R/CTnHyb 600	Tet^R, Met^R, Rif^R	This study
HMW 878	638R/CTnHyb 604	Tet^R, Met^R, Rif^R	This study
HMW 879	638R/CTnHyb 605	Tet^R, Met^R, Rif^R	This study
E. coli			
AG100	Cloning strain	Amp^S, Kan^S	38
DH5α	Cloning strain	Amp^S, Kan^S	Invitrogen
Kam43	TG 1 Δ*acrAB*, Δ*ydhE*, Δ*tolC*, efflux pump deficient	Amp^S, Kan^S	39
	AG100/CTnHybL	Kan^R (unstable)	This study
	DH5α/pSPORT1::*kan*	Amp^R, Kan^R	This study
	DH5α/pSPORT1::*mefA*	Amp^R	This study
	AG100/pSPORT::*mefA*	Amp^R	This study
	Kam43/pSPORT::*mefA*	Amp^R	This study
Plasmids	pSPORT1		Invitrogen
	pSportI::*kan*		This study
	pSportI::*mefA*		This study

Materials and Methods

Strains and culture conditions

Strains used in this study are listed in **Table 2**. All strains were grown as described[40] using Brain Heart Infusion media supplemented with 15 μg /ml hemin (BHIS) for *Bacteroides* isolates (Anaerobe Systems, Morgan Hill, CA) and Luria Bertani (LB) agar or broth (Sigma) for *Escherichia coli*. The multidrug resistant clinical isolates BF-HMW610, BF-HMW615 and BF-HMW616 have been previously described.[6,41,42] *E. coli* AG100 was used as the host to test for the kanamycin (*kan*) and spectinomycin resistance phenotypes. *E. coli* Kam43 (a pump deficient mutant, lacking AcrAB, AcrEF and TolC[17]) was a kind gift from Dr Tomofuso Tsuchiya (Okayama University, Japan). Ampicillin (50 μg/ml), erythromycin (10 μg/ml), and kanamycin (40 μg/ml) were used for selection as indicated.

Molecular methods

DNA extraction, restriction digestions, gel electrophoresis and analysis were done as previously described.[40] The size and sequence of the transferred CTn was determined by PCR and sequencing of the CTn at regular intervals. Based on BF-HMW 615 sequence, primers were designed to yield 150 to 200 bases products targeting DNA approximately every ~20 KB on either side of the *tetQ* gene (HMPREF1204_02983). Using these primers, the PCR amplification was done with genomic DNA from BF-HMW 615, BF638R, and the selected BF-HMW 615/BF638R transconjugants as templates. The exact boundaries of CTnHyb insertion into BF638R, the insertion points in BF638R, the segments deleted in CTnHybS, and the sequence of the junction of contig 1.1, the unannotated transposase, and contig 1.3 were determined by semi-random priming (SRP)-PCR.[43] The primers used for sequencing the BF638R/CTnHyb junctions and the gaps found within the CTnHyb in one of the isolates are listed in **Table S1**.

Genome sequencing

BF-HMW615 (along with two other multidrug resistant isolates BF-HMW610 and BF-HMW616) were submitted to the Broad Institute and sequenced as part of the Human Microbiome Project, *Bacteroides* group Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/). The Broad sequencing project utilized 454 Whole Genome Shotgun methodology and Newbler (454 Life Sciences) assembly. This sequencing project was supported by the National Institute of

Allergy and Infectious Disease/National Institutes of Health-funded Genome Sequencing Center for Infectious Diseases at the Broad Institute. BF-HMW610, BF-HMW615 and BF-HMW616 have been given the Broad designations HMPREF1203, HMPREF1204, and HMPREF1205, respectively. For the sake of consistency, the BF-HMW 615 genes are referred to by their designation HMPREF1204_, etc.; these are the designations used in GenBank FASTA files of the genome sequences and associated annotations were downloaded from the Broad Institute.

Genomic analysis

The RAST (Rapid Annotation using Subsystem Technology) Annotation Server[8] was used for comparative genome analysis. All sequences submitted to RAST were downloaded from either the Broad Institute (for the clinical isolates) or NCBI (for the reference strains) and submitted to the RAST server.[8,9] Genomes were compared by the sequence comparison feature of the SEED server.[9] Comparisons of known CTns and the BF-HMW 615 genome sequence were analyzed as described earlier.[7] The Double ACT server (http://www.hpa-bioinfotools.org.uk) was used to generate the comparison file with the BLASTN function and a cutoff of 1000–2000. Results were viewed using the ACT viewer.[44] MAUVE alignment software, which is specifically designed for multiple genome alignment in the presence of large-scale evolutionary events such as rearrangement and inversion,[45] was used to help predict the arrangement and orientation of supercontigs in the BF-HMW615 genome. The MAUVE Contig Mover module was used to order and orient the BF-HMW615 contigs relative to the BF638R reference genome.[46] When needed, nucleotide or amino acid sequences were aligned using ClustalW.

Annotation

The sequences in GenBank were annotated by the Broad Institute as part of the genome sequencing but more than 80% of the proteins (122/144) were annotated as "hypothetical." To augment this annotation, we separately annotated all three sequences using the RAST Server, which assigns proteins to subsystems. With the addition of the RAST information, 55 proteins remained annotated as hypothetical. Finally, we submitted the protein sequences to Phyre2-fold recognition analysis[47] and succeeded in obtaining a predicted protein function for all but 17 proteins (38 were annotated at greater than 50% confidence and 25/55 at greater than 90% confidence) (**Table S2**).

Mating of BF-HMW615 with *B. fragilis* 638R

BF638R and BF-HMW615 were grown overnight anaerobically at 37 °C in BHIS. 200 μl of each culture were mixed, centrifuged at 13000 g for 2 min and then reconstituted in 100 μl of BHIS. The mixed cells were then spread on BHI plates (Anaerobe systems, CA, USA) and incubated anaerobically overnight at 37 °C. The growth on the plate was harvested and then resuspended (mating mix) in ~1.5 ml 10% glycerol TSB storage media (Key Scientific Products Inc., TX, USA). A 150 μl aliquot of mating mix was plated on BHIS plates containing tetracycline (1 μg/ml) and rifampicin (10μg/ml). The frequency of the transfer was estimated to be 1×10^{-9} (number of transconjugants [BF638R-CtnHyb]/numbers of donors [BF HMW615]).

Potential tetracycline-mediated increase in mating frequency was measured by three methods with slight alterations to the regular mating procedure. 1) BF-HMW615 was grown overnight in presence of tetracycline (1 μg/ml) prior to regular mating. 2) BF-HMW615 was treated with tetracycline (1 μg/ml) for 1 h at 37 °C. The cells were then washed twice with equal volumes of BHIS and the regular mating procedure with BF638R was followed. 3) Overnight grown cells of BF638R and BF-HMW615 mix (200 μl of each) were each plated on BHIS plates containing a very low concentration of tetracycline (0.001 μg/ml), incubated overnight anaerobically at 37 °C, and then the regular mating procedure was followed.

Mating of BF-HMW615 with *E. coli*

200 μl of overnight cultures of BF-HMW615 and *E. coli* AG100 cells were mixed and plated on BHI plates and incubated overnight at 37 °C under anaerobic conditions. The cells were then harvested and pooled in 1 ml of broth and 50 μl of cells were plated on LB plate containing 40 μg/ml kanamycin and incubated aerobically at 37 °C.

Cloning of resistance genes contained within CTnHyb and introduction into *E. coli*

HMPREF1204_02969 (a gene coding for the aminoglycoside 3′-phosphotransferase that confers kanamycin resistance) and HMPREF1204_02965 (a *mefA*-like efflux transporter gene) were PCR amplified from BF-HMW615, ligated into pSportI (Invitrogen) and introduced into *E. coli DH5*α. Transformants were selected and purified on ampicillin containing LB plates and the presence of the introduced genes were confirmed by PCR and sequencing.

MIC determinations

MICs were determined using E-test technology (BioMerieux) according to the manufacturer's directions. An inoculum of one McFarland unit was used on a Brucella Blood Agar plate (Anaerobe Systems, Morgan Hill, CA).

Transcription of genes contained within CTnHyb genes in BF-HMW615

RNA was prepared from cells using the Qiagen RNAeasy kit (Qiagen, CA, USA) according to manufacturer's directions. The total RNA was enriched for mRNA by removing the majority of rRNA using the Ambion Microbe Express Kit (Life Technologies). cDNA was prepared using the Invitrogen Superscript kit (Life Technologies). cDNA was quantified by RNA-Seq analysis (Otogenetics, Norcross, USA). RNA-Seq files were analyzed using the Lasergene Genomics Suite (DNASTAR, Inc., Madison, USA).

Registration of CTnHyb

CTnHyb was registered in the transposon registry (http://www.ucl.ac.uk/eastman/research/departments/microbial-diseases/tn) as Transposon 6243. The complete sequence and annotation has been submitted to NCBI GenBank (Submission ID 1725586, to be released June 10, 2014).

Conclusion

CTnHyb represents a mechanism for a single *Bacteroides* isolate to become a reservoir for a variety of resistance genes, both from *Bacteroides* and other species; these genes can then

be transferred both to other *Bacteroides* and unrelated bacteria. Identifying the factors that increase CTn accumulation within a strain as well as factors that increase transfer of CTns to other bacteria is critical information that could lead to therapeutic regimens against resistance dissemination.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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