

Evidence of Extensive DNA Transfer between *Bacteroidales* Species within the Human Gut

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ABSTRACT The genome sequences of intestinal *Bacteroidales* strains reveal evidence of extensive horizontal gene transfer. *In vitro* studies of *Bacteroides* and other bacteria have addressed mechanisms of conjugative transfer and some phenotypic outcomes of these DNA acquisitions in the recipient, such as the acquisition of antibiotic resistance. However, few studies have addressed the horizontal transfer of genetic elements between bacterial species coresident in natural microbial communities, especially microbial ecosystems of humans. Here, we examine the genomes of *Bacteroidales* species from two human adults to identify genetic elements that were likely transferred among these *Bacteroidales* while they were coresident in the intestine. Using seven coresident *Bacteroidales* species from one individual and eight from another, we identified five large chromosomal regions, each present in a minimum of three of the coresident strains at near 100% DNA identity. These five regions are not found in any other sequenced *Bacteroidetes* genome at this level of identity and are likely all integrative conjugative elements (ICEs). Such highly similar and unique regions occur in only 0.4% of phylogenetically representative mock communities, providing strong evidence that these five regions were transferred between coresident strains in these subjects. In addition to the requisite proteins necessary for transfer, these elements encode proteins predicted to increase fitness, including orphan DNA methylases that may alter gene expression, fimbriae synthesis proteins that may facilitate attachment and the utilization of new substrates, putative secreted antimicrobial molecules, and a predicted type VI secretion system (T6SS), which may confer a competitive ecological advantage to these strains in their complex microbial ecosystem.

IMPORTANCE By analyzing *Bacteroidales* strains coresident in the gut microbiota of two human adults, we provide strong evidence for extensive interspecies and interfamily transfer of integrative conjugative elements within the intestinal microbiota of individual humans. In the recipient strain, we show that the conjugative elements themselves can be modified by the transposition of insertion sequences and retroelements from the recipient's genome, with subsequent transfer of these modified elements to other members of the microbiota. These data suggest that the genomes of our gut bacteria are substantially modified by other, coresident members of the ecosystem, resulting in highly personalized *Bacteroidales* strains likely unique to that individual. The genetic content of these ICEs suggests that their transfer from successful adapted members of an ecosystem confers beneficial properties to the recipient, increasing its fitness and allowing it to better compete within its particular personalized gut microbial ecosystem.

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The human intestine harbors a very dense microbial ecosystem containing approximately 10^{11} to 10^{12} bacteria per g of colonic content. The species within this community are diverse; however, most of the numerically dominant species are contained within two bacterial taxonomic groups, the Gram-positive phylum *Firmicutes* and the Gram-negative order *Bacteroidales* (1, 2). There are more than 25 different human gut *Bacteroidales* species, many colonizing this ecosystem simultaneously at high density (3, 4). Coresident gut *Bacteroidales* form ecological networks to utilize dietary polysaccharides (5), with mutualistic interactions likely occurring between these members. Therefore, the presence in the human intestinal microbiota of different *Bacteroidales* species/

strains, each with different phenotypes and fitness properties, may increase the fitness of the *Bacteroidales* community as a whole.

Many important molecules of the gut *Bacteroidales*, such as those involved in microbial interactions with the host, other microbes, and dietary or abiotic substances, are not encoded by conserved genes of a species. These include the immunomodulatory polysaccharide molecule PSA of *Bacteroides fragilis* strain NCTC 9343, the genes for which are contained in less than one-third of *B. fragilis* strains (6), the *B. fragilis* enterotoxin (7) implicated in colon cancer (8), glycoside hydrolases and polysaccharide lyases (5) that allow these bacteria to harvest dietary and host glycans (9, 10), and secreted antimicrobial molecules (M. Chatzidaki-

TABLE 1 Composition of natural *Bacteroidales* communities and identification of highly similar regions in strains coresident in a gut microbial ecosystem

Microbial ecosystem, organism ^a	No. of contigs	Genome size (bp)	CL02 region (size [bp]) ^b :		CL03 region (size [bp]) ^b :				CRISPR/Cas system ^c
			1 (24,866)	2 (116,095)	3 (17,607)	4 (60,734)	5 (42,545)	6 (44,124)	
CL02									
<i>B. cellulosilyticus</i> CL02T12C19	25	7,678,000	✓	✓					Type I
<i>B. dorei</i> CL02T12C06	21	5,997,310	✓	✓					Type I
<i>B. nordii</i> CL02T12C05	10	5,707,590							None
<i>B. ovatus</i> CL02T12C04	15	7,880,760							None
<i>B. salyersiae</i> CL02T12C01	7	5,781,840	✓	✓					Type I, III
<i>P. goldsteinitii</i> CL02T12C30	14	6,690,360							Type I
<i>P. johnsonii</i> CL02T12C29	6	4,613,500		✓					None
CL03									
<i>B. caccae</i> CL03T12C61	6	5,479,120			✓				None
<i>B. dorei</i> CL03T12C01	20	5,387,250							Type I
<i>B. fragilis</i> CL03T12C07	7	5,214,030				✓			Type II, III
<i>B. ovatus</i> CL03T12C18	19	6,972,150				+/-	+/-	✓	None
<i>B. uniformis</i> CL03T12C37	14	4,890,740			✓			✓	Type II
<i>B. xylanisolvens</i> CL03T12C04	13	6,056,100				✓		✓	None
<i>P. distasonis</i> CL03T12C09	5	5,055,860				✓			Type I, II
<i>P. merdae</i> CL03T12C32	13	4,918,050			✓			✓	None

^a All species belong to *Bacteroides* or *Parabacteroides*.

^b ✓, the region is present in the organism; +/-, a large, yet partial segment of the region was identified at >99.9%.

^c Type(s) of CRISPR/Cas systems present in the organism.

Livanis, M. Coyne, and L. Comstock, submitted for publication) predicted to limit local competition.

Many genes contributing to strain diversity are contained in regions likely acquired by horizontal gene transfer (HGT). The genomes of gut *Bacteroidales* strains show evidence of DNA acquisitions from phage (11), conjugative plasmids (12–14), and conjugative transposons (15, 16). In *Bacteroides* species, conjugative plasmids and conjugative transposons have been studied intensely for decades because of the importance of these mobile elements in transferring antibiotic resistance genes (12–14, 17, 18). *Bacteroidales* conjugative transposons fall within the classification of integrative and conjugative elements (ICEs), and as such, they encode the gene products necessary for conjugative transfer, including the mating apparatus, integrases, excisionases, and proteins that regulate transfer (reviewed in references 18 and 19). In order for conjugative transfer to occur, an ICE must excise from the chromosome and form a nonreplicative covalently closed circular intermediate. It is thought that a single strand of the element is then transferred through a mating apparatus to the recipient, with the single strands in both the donor and recipient then being replicated and the element subsequently being (re)integrated into the donor and recipient genomes. Due to the number of genes necessary for these processes, conjugative transposons are relatively large, with those described in *Bacteroides* averaging approximately 50 to 80 kb (18).

As mating aggregates are necessary for the transfer of conjugative elements, these processes should be favored in dense microbial ecosystems. The human gut is an ideal environment for such conjugative transfers due to its high density of related *Bacteroidales* species. Most studies of the transfer of mobile genetic elements (MGEs) of gut bacteria have been performed *in vitro* or with experimental *in vivo* systems (20–22). Data regarding transfer within the natural human gut ecosystem are lacking, especially regarding the extent of transfer that occurs within an individual human's microbiota. One study provided strong evidence for the transfer of an 8.9-kb conjugative plasmid among four coresident

Bacteroidales species in the gut microbiota of a human girl (23). This small plasmid contained genes and elements necessary for replication and mobilization, such as *repA*, *mobA*, *mobB*, and *oriT*, but not genes required for the mating apparatus. Due to the importance of MGEs in supplying closely related strains/species with genes that may allow them to rapidly adapt to an ecosystem (reviewed in reference 24) and to understand the nature of these genetic transfers within an individual's microbiota and how these genomes are modified by interaction with other members of the ecosystem, we studied coresident *Bacteroidales* species for evidence of HGT. We provide evidence for the interspecies and interfamily transfer of large genetic elements within the gut microbial ecosystem of two healthy humans. We show that these MGEs meet the definition of ICEs or conjugative transposons and carry genes predicted to increase the fitness of the recipient.

RESULTS

Analysis of coresident *Bacteroidales* strains for evidence of intraecosystem DNA transfer. Seven strains of different species colonizing subject CL02 and eight strains of different species colonizing subject CL03 were included in the analyses, with each community including both *Bacteroides* and *Parabacteroides* species (Table 1). Within the gut microbiota of each individual, these strains were each present at >10⁸ CFU/g (3). The genomes comprising each of these communities were compared to one another at the DNA level using BLAST. To identify DNA regions with the best likelihood of intraecosystem transfer, we limited the search to identify regions that existed in at least three of the *Bacteroidales* strains of an individual. Moreover, these segments were required to be at least 10 kb in length and have at least 99.9% DNA identity between strains. These criteria were intentionally conservative to avoid detecting small regions coincidentally common between strains without necessarily indicating recent transfer. Each of these 15 genomes were finished to the draft level, wherein a supercontig or scaffold is assembled by linking smaller contigs, often separated by long stretches of Ns representing unassigned or am-

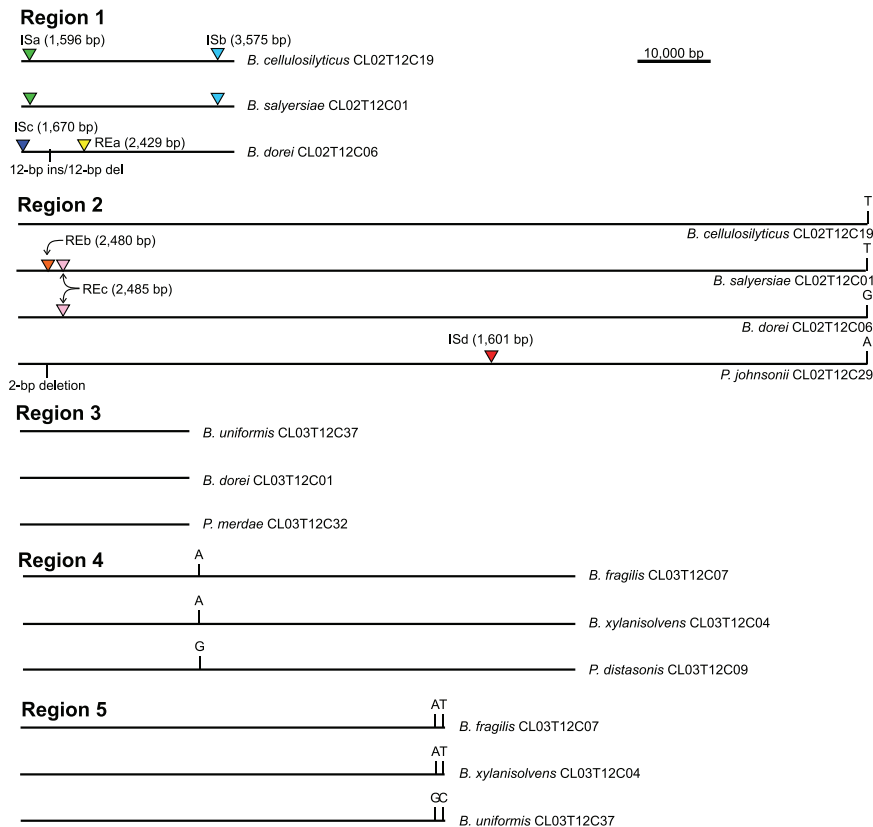


FIG 1 Comparisons of regions 1 to 5 in the three or four genomes containing these MGEs. Differences between strains for each region following sequencing to resolve Ns are shown. The remaining SNPs displayed were not tested by sequencing and represent the original genome sequence for each isolate. The positions of IS and RE in regions 1 and 2 are shown with the corresponding sizes of these elements.

biguous residues. As these Ns cause BLAST to split potentially contiguous hits into multiple returns, the BLAST files were parsed and the results were consolidated and counted as one region if there were gaps of $\leq 5,000$ bp or if the coordinates overlapped. These consolidations revealed six large regions of DNA, referred to herein as regions 1 through 6, two from the CL02 community and four from the CL03 community (Table 1). In general, each of the regions was nearly 100% identical between the identified strains, with the exception of a few single-nucleotide polymorphisms (SNPs), insertion sequences (IS), and/or retroelements (RE) in some regions, as detailed below.

Region 1 was detected in the CL02 community in *Bacteroides cellulosilyticus*, *Bacteroides salyersiae*, and *Bacteroides dorei*. There were several areas where the sequences from these three genomes diverged and were not identified as contiguous aligning segments in our initial analyses, largely due to assembler-introduced Ns. We PCR amplified and sequenced all regions containing Ns (see Table S1 in the supplemental material). These complete sequences revealed that regions 1 from *B. cellulosilyticus* and *B. salyersiae* are 100% identical over their entire 24,866-bp length (Fig. 1), whereas the *B. dorei* genome differed from the other two by a 12-bp insertion and 12-bp deletion and the presence of IS and RE (Fig. 1). The *B. cellulosilyticus* and *B. salyersiae* genomes contain two IS, referred to here as ISa and ISb, which are absent in *B. dorei*, and *B. dorei* contains a different IS and an RE, referred to here as ISc and REa, both of which are absent in the other two genomes (Fig. 1 and 2). Details of these IS and RE are

contained in Table S2. The patterns of these IS and RE suggest that this region initially lacked these elements and was modified by preexisting copies from the genome of a recipient/donor. In fact, each of the strains containing these IS and RE have, in most cases, numerous other copies of these IS and RE in other locations in their genome (Table S2).

Region 2 is very large (116,095 bp) and is present in four of the seven isolates of the CL02 community, *B. cellulosilyticus*, *B. dorei*, *B. salyersiae*, and *Parabacteroides johnsonii*. Segments containing assembler-introduced Ns were PCR amplified and sequenced (see Table S1 in the supplemental material). These data revealed that regions 2 are identical among these four strains except for an IS element (ISd), present only in *P. johnsonii*, and two RE, REb, present only in *B. salyersiae*, and REc, present in both *B. salyersiae* and *B. dorei* (Fig. 2; Table S2).

The three regions from the CL03 community contained no assembler-introduced Ns and no IS element differences between strains. The first of these (region 3) is 17,607 bp and is present in CL03 community members *Bacteroides uniformis*, *B. dorei*, and *Parabacteroides merdae* at 100% identity (Fig. 1 and 2).

Region 4 is 60,734 bp and is present in the genomes of CL03 members *B. fragilis*, *Bacteroides xylanisolvans*, and *Parabacteroides distasonis*. The sequences of these three regions agree perfectly, with the exception of one SNP. The first 44,008 bp of this sequence was also present at 100% identity in the *Bacteroides ovatus* CL03 genome, at the end of scaffold 1.10, and the remaining 16,726 bp was found in the middle of scaffold 1.3. The disconti-

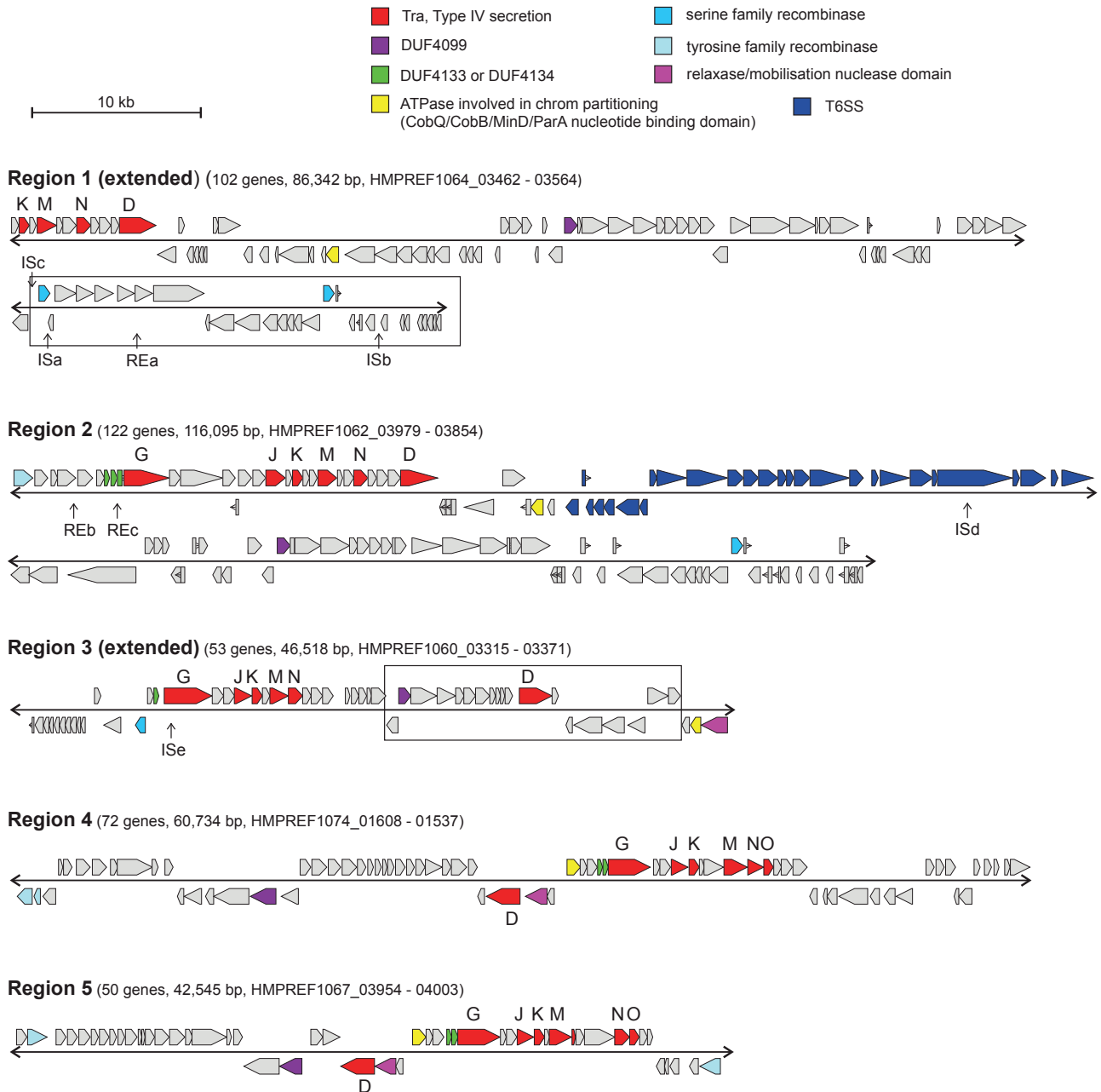


FIG 2 Open reading frame (ORF) maps of regions 1 to 5. Regions are oriented so that the majority of the *tra* genes (red) read left to right. The letter above the red genes indicates the particular *tra* gene. An open reading frame map, excluding variable IS and RE, is shown for each region, with the locations of IS and RE indicated. Genes encoding selective orthologous proteins present in each region are color coded as indicated above. Genes comprising the type VI secretion system (T6SS) of region 2 are shown (blue). The 24,866-bp region 1 (boxed) and the 17,607-bp region 3 (boxed) are extended to show the likely extent of the MGEs that were transferred between strains.

nity of region 4 in this strain may be the result of an error in the assembly of this genome sequence.

Region 5 is 42,545 bp and is present in CL03 community members *B. fragilis*, *B. xylanisolvens*, and *B. uniformis*. The regions 5 are 100% identical between the three genomes, with the exception of two SNPs at the very end of the region in *B. uniformis* (Fig. 1). The second half of this region (28,967 bp) was also detected in the *B. ovatus* genome assembly, residing in the middle of scaffold 1.3.

Region 6 is 44,124 bp and is present in CL03 community mem-

bers *B. ovatus*, *B. xylanisolvens*, and *P. merdae*. This region was not further analyzed due to its presence at 100% identity in numerous noncommunity members (see below).

Presence of highly identical regions in other *Bacteroidales* strains. The possibility existed that these DNA segments represented very promiscuous MGEs and that their presence in these isolates was coincidental and not related to the fact that they were coresident. If so, BLAST analysis of these regions against the database of all draft and completed *Bacteroidetes* genomes should

reveal other strains not present in these natural ecosystems that have similarly sized regions also identical at $\geq 99.9\%$. For each of these six regions, BLAST analyses were performed with each of the regions with all IS and RE removed to allow the best chance to return a similarly conserved region. The results of these BLAST analyses revealed that only one of the six regions had $\geq 99.9\%$ identity to another ≥ 10 -kb segment from other *Bacteroidales* strains not associated with these natural communities (Table 2). CL03 region 6, which is 44,124 bp in length, is present at 100% identity in numerous other *Bacteroidales* strains. In contrast, no other sequenced *Bacteroidetes* genomes contained regions of ≥ 10 kb that matched regions 1 to 5, even at 99.90% identity, whereas the identified regions in coresident strains are 99.99 to 100% identical to each other, even prior to resolving the Ns (Table 2). These data provide strong evidence that regions 1 to 5 were transferred between coresident strains of the CL02 or CL03 ecosystems, but the BLAST data do not support the intraecosystem transfer of region 6.

Analysis of highly similar regions within the genomes of mock communities of *Bacteroidales*. To estimate the frequency with which one might expect to find such long and nearly identical DNA segments (i.e., ≥ 10 kb and $\geq 99.9\%$ identity in three strains) among bacteria that were not coresident, we performed a similar BLAST search using 1,000 eight-member mock communities of *Bacteroidales* assembled from a set of 84 *Bacteroides* and *Parabacteroides* genome sequences of similar quality (see Materials and Methods; see Table S3 in the supplemental material). Genomes were pseudorandomly assigned to each mock community such that no collection contained two genomes of the same species and each microbiota contained at least one but not more than two *Parabacteroides* genomes. Each collection was further restrained by limiting it to contain no more than one genome of each of the CL02, CL03, and CL09 strains, as these groups each represent strains collected from three different subjects (3).

The mock-community BLAST analysis revealed only three unique segments of qualifying DNA that were ≥ 10 kb, $\geq 99.9\%$ identical, and shared by 3 strains within a mock community but not by any other genomes in the BLAST comparison database (Table 3; see Table S4 in the supplemental material). The first of these regions is 12,502 bp and is contained in the same three *Bacteroides* strains that were present in both mock community 59 and mock community 609, the second is 13,248 bp and is present in two *Bacteroides* and a *P. merdae* genome of one mock community, and the third region is 30,598 bp and was contained in three *Bacteroides* genomes from one mock community.

Therefore, in the two natural communities CL02 and CL03, five unique qualifying regions were retrieved with no other matches in the database at 99.9% or greater (mean of 2.5 regions per community), whereas only four such regions (including one unique region found in two different communities) were retrieved from similar analyses of 1,000 communities of non-coresident strains (mean of 0.004 regions per community). Moreover, many of the qualifying DNA segments detected in the real communities were larger than the segments detected in the mock communities. Therefore, the likelihood of detecting such highly similar and unique regions in a set of *Bacteroidales* strains that are coresident is 625 times higher than the likelihood of detecting such a region among non-coresident strains, providing strong evidence that the five identified regions from the CL02 and CL03 ecosystems were

transferred between strains while coresident in the gut microbiota of these humans.

Genetic content of the five transferred regions. Conjugative transposons or ICEs contain genes encoding all the functions for their transfer, including the machinery for the conjugative mating apparatus, which in Gram-negative bacteria largely occurs by type IV secretion systems (T4SS) (19). Regions 2, 4, and 5 each contain numerous genes encoding Tra proteins of T4SS machinery, including TraD, -G, -J, -K, -L, -M, and -N. These *tra* genes from each region have a similar genetic architecture, displaying a modular unit of functionally related genes, characteristic of ICEs (19). Regions 1 and 3 are likely contained on larger MGEs but were truncated in our analyses due to assembly scaffold breaks in at least one of the three qualifying genomes. For region 3, the scaffold from *P. merdae* extended beyond the defined region, and several smaller scaffolds from both the *B. uniformis* and *B. dorei* genomes aligned at 100% identity with the larger *P. merdae* sequence with relatively small gaps or overlaps, indicating that the true size of the transferred element is likely ~ 47 kb (Fig. 3). All of the same *tra* genes were contained in this extended region (Fig. 2), suggesting that this MGE is also an ICE. Region 1 also continued upstream for an additional 61.5 kb at near 100% identity in two of three genomes (Fig. 3). Alignment of this extended region with the *B. cellulosilyticus* sequence indicated that the genome was likely misassembled in this area. However, for the two genomes that continued, the same *tra* genes were identified (Fig. 2). Therefore, three of the five identified regions meet the definition of an ICE, with regions 1 and 3 also likely part of a larger ICE that was truncated in our analysis due to incomplete or incorrect assembly of the genome sequences.

These ICEs also contained other common genes, such as those encoding single-stranded-DNA-binding proteins, relaxases, ParBs, excisionases, TOPRIM-like proteins, ATPases similar to those involved in chromosomal partitioning, and proteins with DUF4133, DUF4134, and DUF4099 (Fig. 2, Table 4). Each of these regions also contains at least one gene with predicted site-specific recombinase activity, likely involved in integration of the element (Fig. 2, Table 4).

As ICEs must excise from the donor genome in order to transfer to a recipient, some encode a toxin-antitoxin pair to ensure that they are not lost in the donor strain prior to replication and reintegration (25). Regions 1 to 5 each encode identifiable toxin-antitoxin or immunity proteins, likely for element maintenance (Table 4; see Table S5 in the supplemental material). In addition, each of these five regions encodes a predicted antirestriction protein, frequently contained on a conjugative element, which facilitates maintenance of the ICE in the recipient prior to its modification.

Genes that may contribute to fitness. Each region also contains numerous genes unrelated to transfer and maintenance of the ICE. The majority of these genes encode hypothetical proteins of unknown function (see Table S5 in the supplemental material); however, many encode products with putative functions that suggest that they could contribute to fitness. Region 1 encodes genes likely involved in fimbria synthesis. Similar FimA orthologs in the oral *Bacteroidales* species *Porphyromonas gingivalis* allow this organism to attach to host cells (reviewed in reference 26). In these gut *Bacteroidales*, these fimbriae may expand the niche of these organisms, allowing them to attach to other host, microbial, or dietary particle surfaces in the gut.

TABLE 2 BLAST output of regions 1 to 6 against the database^a

BLAST target ^b	% Identity ^c	Alignment length	No. of:		Query		Target		Accession no.
			MM ^d	Gaps	Start	End	Start	End	
Query—CL02 region 1									
<i>B. salyersiae</i> CL02T12C01	100.00	22,005	0	0	1,234	23,238	1,381,606	1,403,610	NZ_JH724307.1
<i>B. cellulossilyticus</i> CL02T12C19	100.00	22,005	0	0	1,234	23,238	5,321	27,325	NZ_JH724088.1
<i>B. dorei</i> CL02T12C06	99.99	17,671	1	0	7,196	24,866	530,799	548,469	NZ_JH724135.1
<i>B. eggerthii</i> DSM 20697	99.78	24,878	25	10	1	24,866	622,697	647,557	NZ_DS995509.1
<i>B. plebeius</i> DSM 17135	97.99	10,412	200	9	10,543	20,946	174,332	184,742	NZ_DS990131.1
<i>B. fragilis</i> 3_1_12	95.05	13,605	615	45	11,286	24,866	1,817,695	1,804,125	NZ_EQ973213.1
Query—CL02 region 2									
<i>B. dorei</i> CL02T12C06	100.00	109,844	2	1	6,257	116,095	1,017,432	907,589	NZ_JH724134.1
<i>B. salyersiae</i> CL02T12C01	100.00	109,844	4	1	6,257	116,095	567,507	677,350	NZ_JH724309.1
<i>P. johnsonii</i> CL02T12C29	100.00	55,262	0	2	1	55,262	109,000	164,259	NZ_JH976468.1
<i>B. cellulossilyticus</i> CL02T12C19	100.00	53,650	2	1	62,451	116,095	178,109	231,758	NZ_JH976468.1
<i>B. cellulossilyticus</i> CL02T12C19	100.00	59,402	2	0	1	59,042	290,506	231,465	NZ_JH724088.1
<i>B. cellulossilyticus</i> CL02T12C19	99.99	13,339	1	0	59,002	72,340	231,364	218,026	NZ_JH724088.1
<i>B. cellulossilyticus</i> CL02T12C19	100.00	12,303	0	0	75,233	87,535	214,881	202,579	NZ_JH724088.1
<i>B. cellulossilyticus</i> CL02T12C19	100.00	28,560	1	0	87,536	116,095	202,478	173,919	NZ_JH724088.1
<i>B. ovatus</i> CL02T12C04	99.55	58,086	263	27	58,035	116,095	5,697	63,752	NZ_JH724231.1
<i>Bacteroides</i> sp. strain 3_2_5	98.71	33,023	426	41	2	33,010	2,030,884	2,063,856	NZ_JH636044.1
<i>Bacteroides</i> sp. strain 3_2_5	97.79	24,597	543	9	34,628	59,221	2,065,264	2,089,854	NZ_JH636044.1
Query—CL03 region 3									
<i>B. uniformis</i> CL03T12C37	100.00	17,607	0	0	1	17,607	96	17,702	NZ_JH724271.1
<i>P. merdae</i> CL03T12C32	100.00	17,607	0	0	1	17,607	142,174	159,780	NZ_JH976456.1
<i>B. dorei</i> CL03T12C01	100.00	17,607	0	0	1	17,607	17,607	1	NZ_JH724164.1
<i>B. eggerthii</i> 1_2_48FAA	98.53	17,614	245	7	1	17,607	30,388	47,994	NZ_AKBX01000010.1
<i>B. plebeius</i> DSM 17135	98.48	17,615	250	13	2	17,607	30,516	48,121	NZ_DS990120.2
<i>B. intestinalis</i> DSM 17393	98.50	16,772	236	12	2	16,766	17,245	34,007	NZ_ABJL02000003.1
Query—CL03 region 4									
<i>B. fragilis</i> CL03T12C07	100.00	60,734	0	0	1	60,734	285,831	346,564	NZ_JH724182.1
<i>P. distasonis</i> CL03T12C09	100.00	60,734	0	0	1	60,734	2,432,090	2,492,823	NZ_JH976495.1
<i>B. xylanisolvens</i> CL03T12C04	100.00	60,734	2	0	1	60,734	2,000,696	1,939,963	NZ_JH724294.1
<i>B. ovatus</i> CL03T12C18	100.00	44,008	2	0	1	44,008	31,399	75,406	NZ_JH724250.1
<i>B. fragilis</i> NCTC 9343	100.00	16,726	0	0	44,008	60,733	215,190	231,915	NZ_JH724243.1
<i>B. fragilis</i> NCTC 9343	99.20	38,365	289	17	22,378	60,733	2,040,415	2,078,771	NC_003228.3
<i>B. fragilis</i> NCTC 9343	99.63	15,410	55	2	1,801	17,209	2,017,133	2,032,541	NC_003228.3
<i>B. helcogenes</i> P 36-108	99.60	15,423	58	3	1,801	17,221	230,238	245,659	NC_014933.1
<i>B. uniformis</i> ATCC 8492	95.50	16,906	652	53	30,502	47,366	215,548	198,710	NZ_DS362247.1
Query—CL03 region 5									
<i>B. xylanisolvens</i> CL03T12C04	100.00	42,545	0	0	1	42,545	1,171,697	1,214,241	NZ_JH724294.1
<i>B. fragilis</i> CL03T12C07	100.00	42,545	0	0	1	42,545	457,382	414,838	NZ_JH724184.1
<i>B. uniformis</i> CL03T12C37	100.00	42,545	2	0	1	42,545	725,544	768,088	NZ_JH724268.1
<i>B. ovatus</i> CL03T12C18	100.00	28,967	1	0	13,578	42,544	205,601	176,635	NZ_JH724243.1
<i>Bacteroides</i> sp. strain 3_1_23	96.50	18,468	561	50	16,314	34,740	2,449,865	2,431,442	NZ_GG774949.1
<i>B. finegoldii</i> DSM 17565	96.60	17,611	497	57	17,192	34,740	29,060	46,630	NZ_GG688325.1
<i>B. salyersiae</i> DSM 18765	97.03	16,978	442	35	17,790	34,740	554,600	537,659	NZ_KB905466.1
Query—CL03 region 6									
<i>B. xylanisolvens</i> CL03T12C04	100.00	44,124	0	0	1	44,124	388,361	344,238	NZ_JH724296.1
<i>P. merdae</i> CL03T12C32	100.00	26,817	0	0	1	26,817	204,214	231,030	NZ_JH976457.1
<i>B. ovatus</i> CL03T12C18	100.00	16,701	0	0	27,424	44,124	236,576	253,276	NZ_JH976457.1
<i>B. ovatus</i> CL03T12C18	100.00	12,583	0	0	1	12,583	530,345	542,927	NZ_JH724241.1
<i>B. ovatus</i> CL03T12C18	100.00	23,711	1	0	12,584	36,294	545,391	569,101	NZ_JH724241.1
<i>B. eggerthii</i> DSM 20697	100.00	44,124	0	0	1	44,124	159,910	204,033	NZ_DS995511.1
<i>P. merdae</i> CL09T00C40	100.00	44,124	0	0	1	44,124	372,514	416,637	NZ_JH976526.1
<i>Bacteroides</i> sp. strain 3_1_19	100.00	44,124	0	0	1	44,124	180,923	225,046	NZ_GG774763.1
<i>Bacteroides</i> sp. strain D22	100.00	44,124	0	0	1	44,124	56,078	100,201	NZ_GG774819.1
<i>Alistipes</i> sp. strain HGB5	100.00	44,124	0	0	1	44,124	66,384	110,507	NZ_AENZ01000040.1
<i>Alistipes onderdonkii</i> DSM 19147	100.00	44,124	1	0	1	44,124	55,386	11,263	NZ_KB894552.1
<i>B. intestinalis</i> DSM 17393	100.00	44,124	0	1	1	44,124	456,857	412,735	NZ_ABJL02000006.1
<i>B. stercoris</i> ATCC 43183	100.00	44,124	2	0	1	44,124	103,168	59,045	NZ_DS499672.1
<i>P. merdae</i> ATCC 43184	100.00	44,124	1	1	1	44,124	73,390	117,512	NZ_DS264518.1
<i>B. fragilis</i> YCH46 DNA	100.00	44,124	1	1	1	44,124	163,822	119,700	NC_006347.1

^a All variant IS and RE were removed from query sequences. Boldface indicates strains from a natural ecosystem.^b All species belong to *Bacteroides* or *Parabacteroides*, unless otherwise indicated.^c % Identity was rounded to the closest hundredth of a percent.^d MM, mismatches.

TABLE 3 BLAST output of three unique regions from the mock communities against the database^a

BLAST query (accession no.:position), target ^b	% Identity ^c	Alignment length	No. of:		Query		Target		Accession no.
			MM ^d	Gaps	Start	End	Start	End	
Query— <i>B. stercoris</i> ATCC 43183 (NZ_DS499676.1:176961–207558)									
<i>B. stercoris</i> ATCC 43183	100.00	30,598	0	0	1	30,598	176,961	207,558	NZ_DS499676.1
<i>B. vulgatus</i> PC510	99.96	30,599	10	2	1	30,598	30,597	1	NZ_ADKO01000036.1
<i>B. uniformis</i> ATCC 8492	99.95	30,602	9	3	1	30,598	176,746	207,345	NZ_DS362245.1
<i>B. cellulosilyticus</i> CL02T12C19	99.80	13,767	23	3	1	13,764	624,314	610,549	NZ_JH724089.1
<i>B. vulgatus</i> ATCC 8482	99.66	25,496	76	10	1	25,491	2,046,625	2,021,136	NC_009614.1
<i>P. merdae</i> ATCC 43184	99.61	25,500	83	12	1	25,491	117,639	92,147	NZ_DS264524.1
Query— <i>B. fragilis</i> HMW 616 (NZ_JH815527.1:1–13248)									
<i>B. fragilis</i> HMW 616	100.00	13,248	0	0	1	13,248	1	13,248	NZ_JH815527.1
<i>B. fragilis</i> HMW 616	100.00	13,248	0	0	1	13,248	80,750	67,503	NZ_JH815526.1
<i>P. merdae</i> ATCC 43184	99.99	13,248	1	0	1	13,248	356,073	342,826	NZ_DS264540.1
<i>Bacteroides</i> sp. strain 4_3_47FAA	99.99	13,248	1	0	1	13,248	561,549	548,302	NZ_JH114362.1
<i>B. coprocola</i> DSM 17136	89.09	8,440	800	87	4,875	13,248	8,644	17,028	NZ_DS981488.1
<i>B. plebeius</i> DSM 17135	89.09	8,440	800	87	4,875	13,248	241,574	249,958	NZ_DS990119.1
<i>B. fingoldii</i> CL09T03C10	86.88	5,349	638	46	7,935	13,248	82,421	77,102	NZ_JH951901.1
Query— <i>B. faecis</i> MAJ27 (NZ_AGDG01000049.1:1–12502)									
<i>B. faecis</i> MAJ27	100.00	12,502	0	0	1	12,502	1	12,502	NZ_AGDG01000049.1
<i>B. plebeius</i> DSM 17135	99.98	12,502	2	0	1	12,502	28,019	15,518	NZ_DS990120.2
<i>B. intestinalis</i> DSM 17393	99.98	12,502	2	1	1	12,502	14,748	2,248	NZ_ABJL02000003.1
<i>Bacteroides</i> sp. strain D22	99.87	12,502	0	4	1	12,502	35,210	22,725	NZ_GG774809.1
<i>P. merdae</i> CL03T12C32	98.71	8,731	112	1	603	9,333	139,124	130,395	NZ_JH976456.1
<i>Bacteroides</i> sp. strain 9_1_42FAA	98.67	9,241	120	1	2,512	11,752	25,552	34,789	NZ_EQ973174.1

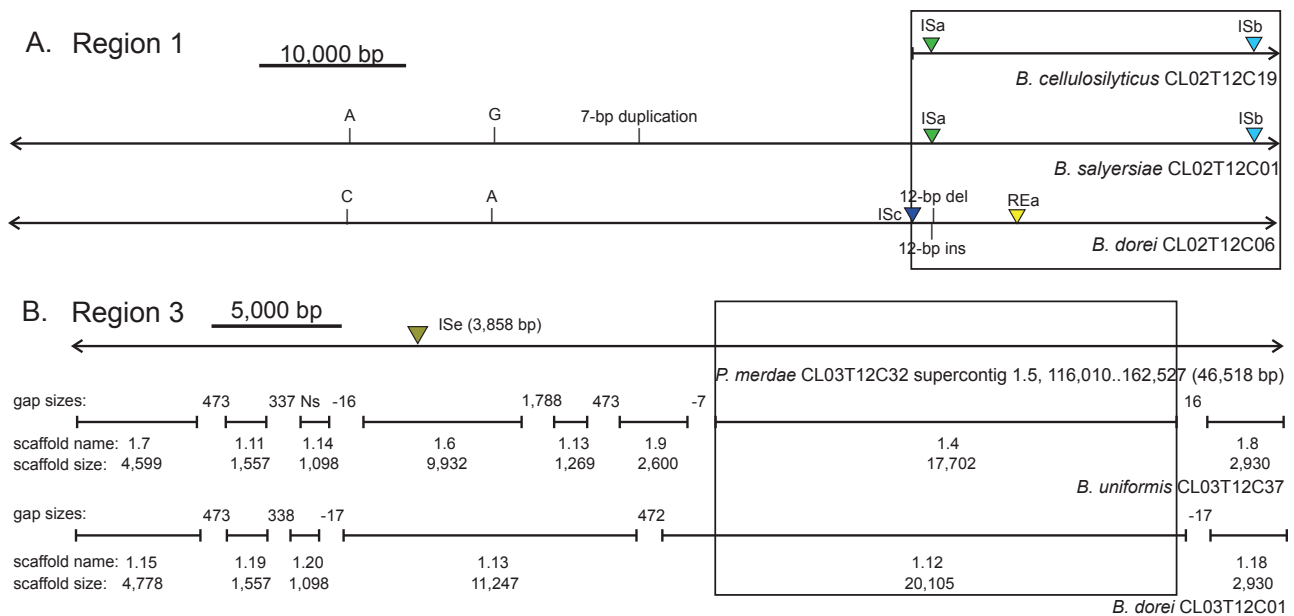
^a Boldface indicates strains from a natural ecosystem.^b All species belong to *Bacteroides* or *Parabacteroides*.^c % Identity was rounded to the closest hundredth of a percent.^d MM, mismatches.

FIG 3 Likely extent of the MGEs containing regions 1 and 3. Boxed regions are the extent of regions 1 and 3 identified by the indicated BLAST criteria. (A) Expansion of region 1 in two of the three genomes. (B) Expansion of region 3 based on smaller matching scaffolds in each of the two genomes that are noncontiguous with the region from *P. merdae*.

TABLE 4 Numbers of various products encoded by the five intracommunity-transferred regions

Putative category	Putative assignment/function of gene products	No. of products in:				
		CL02 region:		CL03 region:		
		1	2	3	4	5
Conjugative transfer machinery	TraD (coupling protein)	1	1	1	1	1
	TraG		1	1	1	1
	TraJ		1	1	1	1
	TraK	1	1	1	1	1
	TraM	1	1	1	1	1
	TraN	1	1	1	1	1
	TraO				1	1
Recombinases	Serine site-specific recombinases	2	1	1		
	Tyrosine site-specific recombinases/integrases		1		2	2
Element transfer/partitioning/segregation	TOPRIM-like, DUF3991	2	1	1		
	TOPRIM primase		1		1	1
	Excisionase		1		1	
	Single-stranded-DNA-binding protein family	3	2			
	ATPases—chromosome partitioning/CobQ/CobB/MinD/ParA nucleotide binding	1	1	1	1	1
	PRTRC system ParB family	1	1		1	
	Chromosome segregation protein SMC	1	1			
Other common proteins/domains	Relaxase/mobilization nuclease			1	1	1
	RibD C-terminal domain, dihydrofolate reductase		1		1	1
	DUF4099	1	1	1	1	1
	DUF4133		1		1	1
	DUF4134			1	1	1
	DUF3408			1	1	1
	PH domain protein	1	1	1		
Transcriptional regulation/DNA binding	RteC family				1	1
	TetR family		1	1		1
	Other transcriptional regulator	2			1	1
	Other helix-turn-helix domain DNA-binding proteins	1		1	4	1
Selfish genes/element survival	Putative toxin	1	1	1	2	1
	Putative antitoxin /immunity protein	1	2	1	4	1
	Anti-restriction protein	1	1	1	1	1
Potential fitness genes	DNA methylase	1	3			
	Fimbria synthesis	2				
	MACPF domain containing			1		
	M23 peptidase family	2	3	1		
	Type VI secretion system (T6SS)		✓ ^a			

^a ✓, the region is present in the organism.

Region 2 encodes three putative orphan DNA methyltransferases not associated with a cognate restriction enzyme. DNA methyltransferases enable genomewide epigenetic modifications which have been shown to have diverse outcomes, including transcriptional regulation, cell cycle control, and regulation of conjugal transfer (27, 28). Therefore, these newly acquired genes may have significant effects on recipient fitness.

There are also genes in these regions that may contribute to competitive ecological interactions. Regions 2 and 3 contain a total of four predicted M23 peptidases (Table 4; see Table S5 in the supplemental material) that hydrolyze peptidoglycan and have various physiological functions, including bacteriocin activity (29). In addition, region 3 encodes a protein with a membrane attack/perforin (MACPF) domain found in proteins widely distributed in *Bacteroidetes* species, one of which we have shown to have secreted antimicrobial activity targeting heterologous strains (*M. Chatzidaki-Livanis et al.*, submitted for publication).

The most notable feature of these regions is a large cluster of genes in region 2 encoding characteristic type VI secretion system (T6SS) proteins (Fig. 2 and Fig. 4; see Table S6 in the supplemental

material). Type VI secretion systems are widely distributed among *Proteobacteria* but have not previously been reported in *Bacteroidetes*. T6SSs translocate toxic effector proteins into neighboring cells in a contact-dependent manner, killing sensitive cells (reviewed in references 30 and 31). T6SS loci are very diverse, and certain hallmark T6SS proteins exhibit little pairwise identity in sequence-sequence comparison. Thus, the identification of these core proteins often relies on the presence of certain motifs (sequence-profile comparisons) or on remote homologies detectable by profile-profile comparisons or structural similarities.

Such profile-profile analyses (32, 33) reveal that this locus encodes numerous proteins encoded by T6SS loci, including TssI (VgrG) and TssD (Hcp), two proteins that comprise the T6SS cell-puncturing structure, the contractile sheath proteins TssB and TssC, the phage baseplate-like protein TssE, and the TssH (ClpV) ATPase, thought to be involved in recycling of TssB and TssC.

The locus also encodes proteins identified as TssF, TssG, and TssK, T6SS proteins whose function is less well understood, and a large transmembrane protein with both a GTP-ATP binding do-

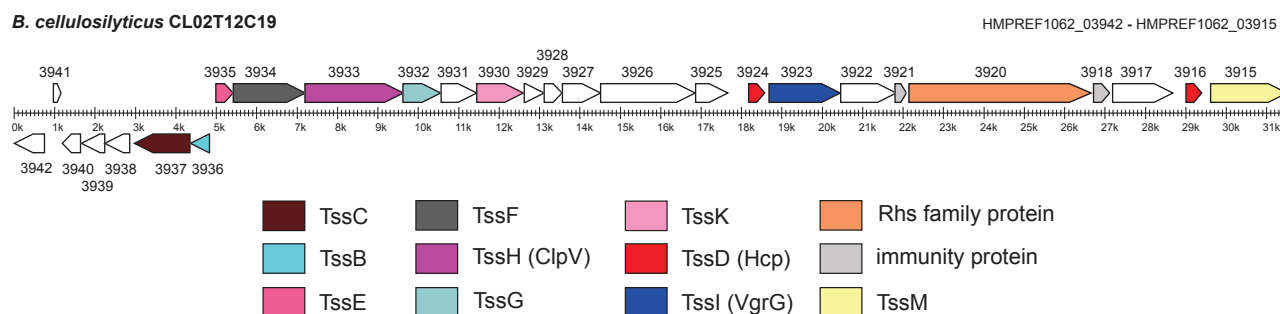


FIG 4 ORF map of portion of region 2, encoding a putative T6SS. Genes encoding proteins characteristic of or commonly associated with T6SS are color coded as indicated below. These designations are based on the analyses as outlined in Table S6 in the supplemental material. The putative functions of all gene products encoded by the genes shown here are included in Table S6.

main and a P-loop ATPase domain, both of which are structural features of TssM, a protein involved in anchoring the T6SS apparatus to the cell wall. Additionally, this locus encodes an Rhs protein with a deaminase domain and two putative immunity proteins, features that are also found associated with T6SS loci. As TssM (34), TssK (35), TssG, and TssF are associated with T6SS but not phage, it is unlikely that this region is an integrated phage. Although T6SS loci have been predicted to be transferred between strains by HGT, this is the first description of a putative T6SS locus likely being transferred on a conjugative element between strains within a natural human ecosystem.

DISCUSSION

By analyzing the genomes of *Bacteroidales* strains cocolonizing the guts of two humans, we provide evidence that as much as 140 kb of DNA has been exchanged within several strains in the microbiota of two individuals and suggest that ICE elements are likely responsible for this transfer. These transfers were not limited to *Bacteroides* species; they also included *Parabacteroides* species. *Bacteroides* are contained within the family *Bacteroidaceae* and *Parabacteroides* within the family *Porphyromonadaceae*, and as such, the *Parabacteroides* are more phylogenetically related to the oral pathogens *Porphyromonas gingivalis* and *Tannerella forsythia* than to the *Bacteroides* genus. However, the *Parabacteroides* have many phenotypes that are more in common with the *Bacteroides* than with the oral *Porphyromonadaceae*. A few notable phenotypes include the synthesis of multiple phase-variable capsular polysaccharides (36) and the production of the enzyme Fkp, which allows these bacteria to incorporate salvaged fucose from the gut environment into their glycans (37). The data from the current study reveal the tremendous capacity for species of these different families to share numerous phenotypes encoded by these ICEs. In fact, these data show that a *Bacteroides* strain and a *Parabacteroides* strain living together in the same human gut share many features that are not shared with other, non-coresident members of the same genus/species.

These genomic comparisons document the continued evolution of these ICEs, which are subject to continued bombardment with IS and RE elements, likely from the recipient's genome. These modifications result in highly personalized genomes that are likely unique to each human. These data also reveal the extent to which our *Bacteroidales* strains are likely altered by the other members of our gut microbial community.

In this retrospective study, we cannot determine which of these

strains may have been the donor of the ICE and which the recipients. However, due to the presence of particular IS or RE in an ICE of one or two strains but not all, some predictions can be made. For example, ISa and ISb are each present in the exact same locations of region 1 for both *B. cellulosilyticus* and *B. salyersiae*, but both are absent in *B. dorei* (Fig. 1). Therefore, it is unlikely that *B. dorei* received this ICE from either *B. cellulosilyticus* or *B. salyersiae*. In addition, as both *B. cellulosilyticus* and *B. salyersiae* each contain other copies of both of these IS in their genomes, these elements were likely transferred from one member's chromosomal copy to the ICE and then transferred to the other strain. In the recipient, the IS present on the ICE then could have served as the donor for transposition into other areas of its chromosome. The data clearly demonstrate that ICEs are efficient vehicles for the transfer of IS and RE between coresident strains (38).

Although ICEs are selfish elements and contain numerous genes dedicated to their transmission and maintenance, the carriage of fitness-conferring genes would increase the chance that the recipient of an ICE is maintained in the ecosystem. Indeed, elements transferred by HGT are known to encode fitness-conferring traits (24), the most obvious being genes encoding antibiotic resistance. In this way, HGT is a means to allow for rapid adaptation of new members into specific adapted communities (39).

In analyzing the contents of these five genetic elements, we can speculate as to the influence on fitness of the transfer and acquisition of these ICEs. The predicted T6SS encoded by region 2 and the putative antimicrobial molecules encoded by regions 2 and 3 are examples of transfers/acquisitions that may be advantageous to both the donor and recipient. The recipient is now endowed with machinery that may allow it to promote antagonistic interactions to limit competition, and the donor may benefit in that the recipient can now deploy this energetically costly defensive machinery and share the burden of protecting the ecosystem from invasion. In *Pseudomonas aeruginosa*, a T6SS was shown to be assembled in response to mating pair formation by a T4SS of *Escherichia coli*, and therefore, it functions to prevent conjugal DNA transfer by killing the attempting donor strain (40). This response is postulated to block the acquisition of parasitic foreign DNA. It will be interesting to determine whether the *Bacteroidales* species that acquired the T6SS are now unable to receive additional T4SS-mediated DNA transfers and, if so, whether it is an advantage or disadvantage for these strains in the human gut ecosystem.

The identification of these intracommunity-transferred ICEs will allow for more in-depth analyses to address ecological interactions between these strains and other *Bacteroidales* strains of these natural communities that do not contain these elements. Because the majority of the genes on the five identified elements encode proteins of unknown function, there are potentially numerous advantages that these regions could confer to a recipient in its interactions with the host and other community members. As these strains represent the evolutionary winners at the time of their isolation, it is unlikely that these ICEs conferred an overall fitness disadvantage to the recipients. The isolation of additional *Bacteroidales* strains from these same subjects will allow us to determine whether strains containing these ICEs have been maintained over time and/or whether the ICEs have since been transferred to the remaining *Bacteroidales* members of these communities.

MATERIALS AND METHODS

Strains and genome sequences. The 15 CL02 and CL03 *Bacteroidales* strains of this study were isolated from human feces, as described previously (3), as part of a study approved by the Partners Human Research Committee IRB that complied with all relevant federal guidelines and institutional policies. The genome sequencing of these strains was performed at the Broad Institute as part of the Human Microbiome Project (41). These sequences were deposited in GenBank and are identified by their project accession numbers, as follows: *Bacteroides caccae*, CL03T12C61 and PRJNA64801; *B. cellulosilyticus*, CL02T12C19 and PRJNA64803; *B. dorei*, CL02T12C06 and PRJNA64807; *B. dorei*, CL03T12C01 and PRJNA64809; *B. fragilis*, CL03T12C07 and PRJNA64813; *Bacteroides nordii*, CL02T12C05 and PRJNA64823; *B. ovatus*, CL02T12C04 and PRJNA64825; *B. ovatus*, CL03T12C18 and PRJNA64827; *B. salyersiae*, CL02T12C01 and PRJNA64829; *B. uniformis*, CL03T12C37 and PRJNA64835; *B. xylanisolvens*, CL03T12C04 and PRJNA64839; *P. distasonis*, CL03T12C09 and PRJNA64883; *Parabacteroides goldsteinii*, CL02T12C30 and PRJNA64887; *P. johnsonii*, CL02T12C29 and PRJNA64889; and *P. merdae*, CL03T12C32 and PRJNA64891.

Intracommunity genome comparisons. The genomes comprising each of the mock or natural communities were compared to one another at the DNA level using BLAST. All hits of $\geq 10,000$ bp that shared $\geq 99.9\%$ identity were retained, with redundancy due to reciprocal hits eliminated. The BLAST files were parsed to detect instances in which a particular query scaffold returned multiple qualifying segments (≥ 10 kb at $\geq 99.9\%$ identity) against a particular target scaffold. These results were consolidated and counted as one qualifying hit if the gaps between the query sequence coordinates were $\leq 5,000$ bp or if the query coordinates overlapped. If the same segment of query DNA produced multiple qualifying returns from different scaffolds of the same target genome, this was also counted as one hit.

Once consolidated, the BLAST results were further parsed for contiguous query sequences producing qualifying matches against two or more target genomes within a community. The overlapping relationship between these BLAST hits was analyzed to calculate the longest contiguous stretch of query DNA present in the target genomes under examination, and the query DNA thus defined was extracted from the proper scaffolds of the query genome.

Analysis of segments found in the natural communities. Sequences flanking the ≥ 10 -kb, $\geq 99.9\%$ identity segments present in three or more genomes of either of the two natural communities and that returned no qualifying hits from the comparison database were compared to identify areas where the sequences diverged. Once the ends of each region were established, the DNA sequences were recovered from all participating genomes and aligned using Clustal W2 (42). Areas where the multiple sequence alignment disagreed (for example, due to stretches of unaligned

sequence from one or more genomes or from Ns inserted during genome sequence assembly, SNPs, etc.) were examined by PCR and/or sequencing (see Table S1 in the supplemental material). The sequencing-corrected and/or PCR-confirmed DNA sequences were realigned, and several relatively short stretches of unaligned DNA present in a subset of the genomes due to the presence of IS or RE were removed. The sequences were translated using Prodigal version 2.6 trained on the appropriate full genome (43).

Selection of genomes for mock-community analysis. 156 genomes identified by NCBI as *Bacteroides* or *Parabacteroides* species were retrieved from the RefSeq repository. Genomes from species originating from non-human sources (e.g., *Bacteroides salanitronis*, acquired from a chicken cecum, or *Bacteroides helcogenes*, acquired from pig feces) were eliminated from the collection. Five duplicate genomes were also removed (*B. dorei* CL02T00C15, *B. uniformis* CL03T00C23, and *B. fragilis* strains CL03T00C08, CL05T00C42, and CL07T00C01 each correspond to a CL0xT12Cxx strain isolated at a different time point from the same subject). The genome sequences of the T00 and the T12 isolates are nearly identical, and including them would have introduced unnecessary duplication. Individual databases prepared for each of the remaining genomes were queried via BLAST with a set of 16S ribosomal DNA sequences acquired from the Ribosomal Database Project (RDP), release 11.1 (44), representing the *Bacteroides* or *Parabacteroides* type strains. The highest-scoring segment pair resulting from each BLAST search was extracted from the target genome and examined further. Genomes with extracted segments of $< 1,000$ bp were excluded, and the remaining segments were used as queries against the RDP database to confirm the species assigned to the genome or assign a species designation to a genome annotated only to the genus level. Genomes whose species identification by this method was ambiguous or appeared incorrect were eliminated from the local collection. Ultimately, 84 genomes representing 26 *Bacteroides* and *Parabacteroides* species were retained.

Presence of DNA regions in noncommunity members. A collection of genomes was retrieved from NCBI to evaluate whether a qualifying DNA segment was unique to the community in which it was found. All DNAs contained in the RefSeq collection classified by NCBI as belonging to taxonomy ID 976 (phylum *Bacteroidetes*) that did not arise from metagenomic or environmental samples and were not also members of taxonomy ID 32644 (unclassified, e.g., unspecified or unidentified samples) were retrieved as FASTA files via the Web. This collection was further processed locally to remove entries whose sequences consisted entirely of rRNA genes and project info files. Scaffolds comprising genomes known to be duplicates were also removed.

Each qualifying segment of DNA found to exist in three or more genomes of a community was compared via BLAST to this comparison database. Only hits from outside the mock community were considered. The comparison database BLAST results were examined to enumerate the number of qualifying hits (≥ 10 kb at $\geq 99.9\%$ identity) returned. Multiple qualifying returns originating from the same target genome were scored as a single hit.

Annotation of genes residing on regions 1 to 5. The utilities of the HMMER suite version 3.1b1 (45) were compiled under Cygwin (version 1.7.27; <http://www.cygwin.com>), and hmmpress was used to convert the Pfam-A data files (version 27) (46) to binaries. Each of the protein sequences from the Prodigal-translated sequences was scanned under Cygwin for matches to the Pfam-A set of motifs using hmmscan, with the sequence and domain E value cutoffs each set to 1.0.

The position-specific score matrix (PSSM) files from NCBI's Conserved Domain Database (CDD, version 3.10) (47) were sorted by source database (Entrez models, SMART version 6.0, TIGRFAM version 13.0, COG and KOG, and LOAD). The PSSM files corresponding to NCBI's Protein Clusters database were further separated into curated prokaryotic and nonprokaryotic groups based on the naming convention of the PSSM files (48). Each of these groupings of PSSM files was compiled separately into RPS-BLAST databases using the NCBI makeprofiledb utility with

default settings. Protein sequences derived from the conserved sequences were scanned for conserved motifs using the NCBI rpsblast utility. The results of these motif scans and those of the Pfam-A scans were collected for each protein and used to inform the annotation (see Table S5 in the supplemental material).

The segment encoding the predicted T6SS detected in region 2 was more extensively analyzed using the HHpred server (<http://toolkit.tuebingen.mpg.de/hhpred>) (32). The use of HMM-HMM profile comparisons and comparisons to structured proteins contained in the Protein Data Bank (PDB; <http://www.rcsb.org/pdb>) (49) allowed the detection of remote homologs not detectable by sequence-sequence or sequence-profile analyses.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01305-14/-/DCSupplemental>.

Table S1, DOCX file, 0.1 MB.

Table S2, DOCX file, 0.1 MB.

Table S3, DOCX file, 0.1 MB.

Table S4, DOCX file, 0.1 MB.

Table S5, XLSX file, 0.1 MB.

Table S6, XLSX file, 0.1 MB.

ACKNOWLEDGMENTS

The authors declare no competing financial interests.

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