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NOTE

Complete sequence of pBFUK1, a carbapenemase-harboring mobilizable plasmid from *Bacteroides fragilis*, and distribution of pBFUK1-like plasmids among carbapenem-resistant *B. fragilis* clinical isolates

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Bacteroides fragilis, among the most common anaerobic pathogens recovered from various infection sites such as intra-abdominal abscesses, is resistant to a broader range of antimicrobials, compared with most other anaerobes.[1–4] In fact, only carbapenems and nitroimidazoles are highly effective for eliminating *B. fragilis*.[4] While, to date, carbapenem resistance in *B. fragilis* still remains rare (approximately 2%),[3–5] a class B metallo-β-lactamase (carbapenemase), encoded by the *cfiA* (also known as *ccrA*) gene, has been identified in *B. fragilis*.[6,7] The CfiA protein confers high level of resistance (MIC, 25–200 μg ml^{-1}) to carbapenems, other β-lactams, and β-lactamase inhibitor combinations.[5,8] In addition, the CfiA expression is determined by the integration, immediately upstream of the *cfiA* gene, of an insertion sequence (IS) element carrying an efficient promoter (for example, IS*613*, *-615*, *-942* and *-1186*).[9–11]

Although the *cfiA* gene is generally located on the *B. fragilis* chromosome, we previously identified for the first time a clinical isolate, the GAI92082 (10–73) strain, in which CfiA is encoded by a mobilizable plasmid—pBFUK1.[12] Nakano *et al.*[13] subsequently identified a second clinical isolate (*B. fragilis* C68c strain) harboring a CfiA-encoding plasmid in size of approximately 6.4 kb. Despite the risk of carbapenem resistance spreading among *B. fragilis* strains via these mobile elements, neither the detailed structure of CfiA-encoding plasmids nor their distribution among carbapenem-resistant *B. fragilis* isolates has been fully investigated.

In this study, we analyzed the complete nucleotide sequence of pBFUK1 to determine whether it contains the *cfiA* promoter and ISs, as well as its possible origin. We also conducted the first distribution analysis of pBFUK1-like plasmids among carbapenem-resistant *B. fragilis* isolates.

We sequenced pBFUK1 that was purified from the *B. fragilis* Tc 30 strain described in our previous report.[12] In total, 101 reads (providing an approximately 3.9-fold coverage across the length of pBFUK1) were sequenced by inverse PCR and primer walking (Supplementary Text S1 and Table S1). Moreover, to investigate the distribution of pBFUK1-like plasmids, we extracted plasmids from two strains (GAI92082 and Tc 30) as positive controls, as well as the following 12 carbapenem-resistant *B. fragilis* strains isolated from patients in Japan from 1987 to 2006: GAI05079, -05184, -06112, -30079, -30144, -92084, -92085, -92087, -92212, -92213, -92214 and -94199 (Supplementary Table S2 and Text S1).[9,12] All strains used in this study are resistant to carbapenem (MIC of imipenem, 16–200 μg ml^{-1}; Supplementary Table S2). Extracted plasmids were separated by electrophoresis in a 0.6% agarose gel. To examine whether these 14 strains contain plasmids with sizes >20 kb, we also performed pulsed-field gel electrophoresis (PFGE; Supplementary Text S1). DNA fragments in the 0.6% mini-gel and PFGE gel were then transferred to a nylon membrane, followed by the Southern blotting analysis to detect seven genes, including *cfiA*; transposase A and B (*tnpA* and *tnpB*); replication protein A (*repA*); and mobilization protein A, B and C (*mobA*, *mobB* and *mobC*). Probes for Southern blotting were synthesized based on the pBFUK1 sequence (Supplementary Text S1 and Table S1). The pBFUK1 sequence and annotation data have been deposited in the DNA Data Bank of Japan database under the accession number AB646744.

The sequenced pBFUK1 plasmid consists of 12 817 nucleotides (average G + C content, 46.3%) and encodes 16 putative open reading frames (ORFs; Table 1 and Supplementary Figure S1). All ORFs

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showed 95–100% amino-acid identity to known proteins identified in the genus *Bacteroides*, *B. fragilis* in particular (Table 1), suggesting that pBFUK1 was most likely derived from and rearranged in the genus *Bacteroides*. In general, plasmids can be classified into three categories according to mobility: mobilizable, conjugative and non-mobilizable.[14] pBFUK1 belongs to the mobilizable plasmid group because it encodes mobilization proteins that are sufficient to allow its mobilization when coresident with a chromosome or a helper plasmid encoding the system for mating-pair formation.[14] Three mobilization proteins in pBFUK1, designated as MobA, -B and -C, showed almost 100% amino-acid identity (with the exception of the MobC carboxyl-terminus) to those in the 4.2 kb pLV22a plasmid from the *B. fragilis* LV22 strain (MbpA, -B and -C, respectively; Supplementary Figure S2).[15,16] As Garcillán-Barcia *et al.*[17] recently reported that pLV22a MbpB (DNA relaxase) belongs to the MOB_P family, we also assigned pBFUK1 MobB to the same family. Like all three-Mbp proteins in pLV22a,[15] three Mob proteins in pBFUK1 may also be essential for mobilization. We previously reported that pBFUK1 is mobilizable into some *B. fragilis* strains;[12] however, its detailed host range is not yet clear. Moreover, Thomas and Hecht[16] recently reported that pLV22a is mobilizable into *Escherichia coli* when coresident with the IncP conjugative plasmid RP4, and therefore, pBFUK1 may also be mobilizable into *E. coli* (or other strains in the genus *Bacteroides*) with a helper factor (for example, IncP plasmid, conjugative transposon and chromosome) that carries genes encoding the system for mating-pair formation.

In the pBFUK1 plasmid, we identified a novel composite transposon (designated as Tn*6186*) in size of approximately 8.5 kb (Table 1 and Supplementary Figure S1). To the best of our knowledge, Tn*6186* in pBFUK1 is the first composite transposon that contains the *cfiA* gene and its promoter region. In addition, the 5075-bp CfiA–ORF9 region in Tn*6186* showed 96.3% nucleotide identity to the corresponding 5170-bp region in the 155-kb contig (GenBank accession no. NZ_ABZX01000055) derived from the *B. fragilis* 3_1_12 strain (Supplementary Figure S3). As the contig of the 3_1_12 strain contains some housekeeping genes and is probably chromosomal, it is possible that the CfiA–ORF9 region in Tn*6186* was also derived from a *B. fragilis* chromosome (Supplementary Figure S3).

Table 1 Details of putative ORFs in pBFUK1 and their closest relationships to previously studied proteins

ORF no.	*Position[a] (bp) (start–stop)*	*Size (aa)[d]*	*Description of homolog (gene)*	*Identity[b] (%)*	*Overlap[c] (aa)[d]*	*Species[d] (GenBank accession no.) of closest homolog by BLASTP*
1	1–1005	334	Replication protein A (*repA*)	100	334/334	Bcl (EGF52541), Bfi (EEX44395)
2	1274–1044	76	Hypothetical protein	100	76/76	Bcc (EDU99871), Bpl (EDY96033)
3	2763–1477	428	Putative transposase A (*tnpA*)	100	428/428	Bfr (BAA95632)
4	2913–3662	249	Metallo-beta-lactamase (*cfiA*)	100	249/249	Bfr (BAC06841, BAC06842, BAC06844, BAC06847, CAH41960, CAQ87581, AEI52430)
5	3889–4215	108	Putative small multidrug resistance protein SugE homolog	100	108/108	Bfr (EFR54930)
6	4911–4225	228	Putative hydrolase	99	228/228	Bfr (EFR54931)
7	5041–5925	294	Hypothetical protein	95	294/294	Bfr (EFR54932)
8	6122–6997	291	Putative lipoprotein	99	291/291	Bfr (EFR54933)
9	7163–7987	274	Hypothetical protein	97	274/276	Bfr (EFR54934)
10	9675–8389	428	Putative transposase B (*tnpB*)	100	428/428	Bfr (BAA95693), Bcc (EDV02813), Bcp (EEF78324), Bsa (ADY36922)
11	10257–9793	154	Mobilization protein C (*mobC*)	97	149/154	Bfr (AAA82754, EFR56176), Bcc (EDU99870), Bce (EEF92091), Beg (EFV27945), Bin (EDV07349), Bpl (EDY96032)
12	11008–10214	264	Mobilization protein B (*mobB*)	100	264/264	Bfr (AAA82753, EFR56175), Bcl (EGF52534), Bpl (EDY96031)
13	11304–11005	99	Mobilization protein A (*mobA*)	100	99/99	Bfr (AAA82752), Bcl (EGF52535), Bin (EDV07351), Bpl (EDY96030)
14	11683–11486	65	Hypothetical protein	100	65/65	Bcl (EGF52536), Bcc (EDU99867), Bce (EEF92089)
15	11969–11694	91	Putative addiction module toxin	100	91/91	Bcl (EGF52537), Bfi (EEX44392), Bcc (EDU99866), Bce (EEF92088), Bin (EDV07353), Bpl (EDY96037)
16	12217–11966	83	Putative addiction module antitoxin	100	83/83	Bcl (EGF52538), Bfi (EEX44393), Bcc (EDU99865), Bce (EEF92087), Beg (EFV27941), Bfr (EFR56174), Bpl (EDY96036)

Abbreviations: aa, amino acids; Bcc, *Bacteroides coprocola*; Bce, *Bacteroides cellulosilyticus*; Bcl, *Bacteroides clarus*; Bcp, *Bacteroides coprophilus*; Beg, *Bacteroides eggerthii*; Bfi, *Bacteroides finegoldii*; Bfr, *Bacteroides fragilis*; Bin, *Bacteroides intestinalis*; Bpl, *Bacteroides plebeius*; Bsa, *Bacteroides salanitronis*; ORF, open reading frame.
[a]Nucleotide position in the pBFUK1 deposited in the DDBJ/EMBL/GenBank databases under the accession no. AB646744.
[b]Amino-acid identity spans the entire gene.
[c]Overlap is indicated as the number of overlapping amino acids/total number of amino acids.
[d]The different species (or strains) listed for each ORF show the same level of homology (the closest relationship).
Accession no. in parenthesis shows the GenBank accession number of each homologous protein sequence.

Tn*6186* contains two previously identified IS elements, IS*613* (1595 bp) and IS*615* (1594 bp) (Supplementary Figures S1 and S4a).[9] We have reported that IS*613* and IS*615* are also located immediately upstream of *cfiA2* and *cfiA4* in the *B. fragilis* GAI92082 and GAI20264 genome, respectively.[9] Both IS elements in pBFUK1 belong to the IS*4* family,[9] which includes another IS element, IS*942*, previously found in some drug-resistant *B. fragilis* strains. The putative transposase genes (designated as *tnpA* and *tnpB*, respectively) in IS*613* and IS*615* of pBFUK1 also show 100% nucleotide identity to those in the GAI92082 and GAI20264 genome, respectively. However, there is only 69.0% nucleotide identity between IS*613* and IS*615* in pBFUK1. As the homology is not very high, we propose that intramolecular homologous recombination between these two ISs in pBFUK1 does not occur at high frequency. Indeed, we have not yet observed any deletion of the six ORFs (*cfiA* to ORF9) from pBFUK1 (data not shown). Moreover, the target-site sequence of the transposase in the known *cfiA*-related IS elements is located at both ends of each IS element,[9,11] whereas the target-site sequence (GGGAA) of the transposase(s) in pBFUK1 is present at both ends of Tn*6186* (downstream of IS*613* IRR and upstream of IS*615* IRL; Supplementary Figure S4a). These findings suggest that Tn*6186* is a composite transposon. Furthermore, two putative transposases encoded by pBFUK1 exhibit 80.6% identity at the amino-acid level. Further functional studies are needed to determine whether one or both of putative transposases can mediate the Tn*6186* transposition.

The *cfiA* gene in pBFUK1 showed 100% nucleotide identity to *cfiA2* in the GAI92082 genome (Supplementary Figure S5). The region upstream of *cfiA* in pBFUK1 includes a putative *cfiA* promoter sequence that not only contains the consensus −7 and −33 motifs[9,11] (Supplementary Figure S4b) but also shows 100% nucleotide identity to that of IS*613* in the GAI92082 genome (Supplementary Figure S5).[9] Although whether the 1.6-kb amplicon (Supplementary Figure S5) that was PCR amplified from the GAI92082 genome[9] is derived from the chromosome itself or from the pBFUK1 remains unclear, we suspect the former (Supplementary Figure S5). Moreover, based on the complete nucleotide identity of this *cfiA* gene and promoter region with those of the GAI92082 genome, we expect that pBFUK1 can express a functionally active CfiA protein. This expectation is also based on our previous study in which not only the carbapenem-sensitive *B. fragilis* TM4000 strain that was electrotransformed with purified pBFUK1 but also the Tc 30 strain showed sufficient imipenem-hydrolytic activity (Supplementary Table S2).[12]

Next, to investigate the distribution of pBFUK1-like plasmids, plasmid preparations from 14 carbapenem-resistant strains (Supplementary Table S2) were subjected to electrophoresis in a 0.6% agarose gel. Although three strains (GAI05079, -92212 and -92214) contained no plasmids, the remaining strains possessed at least 1–3 plasmids with sizes ranging from approximately 2.8 to 12.8 kb (Supplementary Figure S6a). PFGE also showed that none of these 14 strains contained plasmids >20 kb (Supplementary Figure S6d). One plasmid in size of 35–48 kb was detected in the GAI92082 and Tc 30 strains (Supplementary Figures S7a and S7b), and was further verified as the pBFUK1 plasmid that migrated anomalously[18] during PFGE (Supplementary Figure S7). Subsequently, we performed the Southern blotting analysis using probes derived from pBFUK1 to examine the presence of seven genes (*cfiA*, *tnpA*, *tnpB*, *repA*, *mobA*, *mobB* and *mobC*) in plasmids of the 14 resistant strains (Supplementary Figure S6a). The Southern blotting analysis on PFGE gel (Supplementary Figure S6d) was also carried out to examine whether homologs of these seven probe targets are present in the chromosomes of these 14 strains. The representative and overall results of Southern blotting are shown in Supplementary Figure S6 and Table 2, respectively. Isolated from five strains (GAI06112, -92084, -92085, -92213 and -94199), the plasmids in sizes of approximately 4.9 kb contained genes encoding RepA, MobA, -B and -C homologs; however, these plasmids had no *cfiA* or transposase genes. Therefore, these 4.9-kb plasmids, despite being partially similar to pBFUK1, are unlikely to have a role in carbapenem resistance. Overall, we found that CfiA-encoding pBFUK1-like plasmids were not widely distributed among carbapenem-resistant clinical isolates collected in Japan from 1987 to 2006 (Table 2), although we previously reported that pBFUK1 was transferable by mating *in vitro*

Table 2 Location of seven genes encoded by pBFUK1 in different carbapenem-resistant *Bacteroides fragilis* strains

	Summary of positive signals[a] *in Southern blots using seven probes*[b] *(probe length)*						
Strain	*cfiA (625 bp)*	*tnpA (989 bp)*	*tnpB (853 bp)*	*repA (783 bp)*	*mobA (258 bp)*	*mob (525 bp)*	*mobC (401 bp)*
GAI05079	C	–	–	–	–	–	–
GAI05184	C	–	–	–	–	–	–
GAI06112	C	–	–	P (4.9 kb)	P (4.9 kb)	P (4.9 kb)	P (4.9 kb)
GAI30079	C	–	–	–	–	–	–
GAI30144	C	–	–	–	–	–	–
GAI92082	C, P (pBFUK1)	C, P (pBFUK1)	C, P (pBFUK1)	P (pBFUK1)	P (pBFUK1)	P (pBFUK1)	P (pBFUK1)
GAI92084	C	–	–	P (4.9 kb)	P (4.9 kb)	P (4.9 kb)	P (4.9 kb)
GAI92085	C	–	–	P (4.9 kb)	P (4.9 kb)	P (4.9 kb)	P (4.9 kb)
GAI92087	C	–	–	–	–	–	–
GAI92212	C	–	–	–	–	–	–
GAI92213	C	C	C	P (4.9 kb)	P (4.9 kb)	P (4.9 kb)	P (4.9 kb)
GAI92214	–	–	–	–	–	–	–
GAI94199	–	–	–	P (4.9 kb)	P (4.9 kb)	P (4.9 kb)	P (4.9 kb)
Tc 30	P (pBFUK1)	P (pBFUK1)	P (pBFUK1)	P (pBFUK1)	P (pBFUK1)	P (pBFUK1)	P (pBFUK1)

[a]C and P indicate chromosome and plasmid, respectively, of each strain. The pBFUK1 and 4.9 kb in parentheses indicate the kind of plasmid detected by agarose gel electrophoresis (Supplementary Figure S6a). Minus (−) indicates no corresponding genes (no signal).
[b]Details of probes are described in Supplementary Table S1.

between some *B. fragilis* strains.[12] One explanation for this observation may be that the frequent pBFUK1 transfer among strains *in vivo* may require a higher density of cell–cell contact. In spite of the low incidence of CfiA-encoding plasmids, continuous surveillance is necessary to monitor their presence, especially in light of dramatically increased tetracycline and clindamycin resistance in *Bacteroides* species in the past 30 years owing to mobile genetic elements.[19] One should also be careful not to activate a silent *cfiA* gene, because expression of such a gene can be activated dramatically by exposure to low concentrations of carbapenem.[20]

Furthermore, the GAI92214 and GAI94199 strains had no *cfiA*, as determined by the lack of a signal in the Southern blotting using the *cfiA* probe (Supplementary Figure S6e and Table 2). This finding is consistent with the observations that neither PCR amplicons of *cfiA* and IS nor carbapenemase activity were detected in these strains (Supplementary Table S2). However, these strains did show moderate-level carbapenem resistance (MIC of imipenem, $16-25\,\mu g\,ml^{-1}$; Supplementary Table S2), suggesting that other carbapenem resistance mechanisms, such as efflux pumps,[2] may exist in these strains.

The GAI92082 chromosome contained *cfiA* and two transposase genes (*tnpA* and *-B*), in addition to those present on the pBFUK1 plasmid (Supplementary Figures S6, S7a, and Table 2). To our knowledge, this strain is the only strain that carries *cfiA* genes in both its chromosome and plasmid. The *tnpA* probe (in IS*613*) and *tnpB* probe (in IS*615*) were also hybridized with the GAI92213 chromosome (Table 2); however, further work is needed to determine whether the chromosomes of both strains encode all ORFs in Tn*6186*. If the GAI92082 chromosome contains the composite transposon, pBFUK1 Tn*6186* may have been either replicated from or inserted into the GAI92082 chromosome, which was originally carbapenem sensitive.

We discovered a novel composite transposon, including the *cfiA* gene and its promoter, in the pBFUK1 sequence. We believe that our first distribution analysis of pBFUK1-like plasmids will also be a valuable first step toward better understanding of the potential spread of carbapenem resistance via CfiA-encoding plasmids.

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