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Original Article

Characterization of mobile genetic elements in multidrug-resistant *Bacteroides fragilis* isolates from different hospitals in the Netherlands

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ABSTRACT

Objectives: Five human clinical multidrug-resistant (MDR) *Bacteroides fragilis* isolates, including resistance to meropenem and metronidazole, were recovered at different hospitals in the Netherlands between 2014 and 2020 and sent to the anaerobic reference laboratory for full characterization.
Methods: Isolates were recovered from a variety of clinical specimens from patients with unrelated backgrounds. Long- and short-read sequencing was performed, followed by a hybrid assembly to study the presence of mobile genetic elements (MGEs) and antimicrobial resistance genes (ARGs).
Results: A *cfxA* gene was present on a transposon (Tn) similar to Tn*4555* in two isolates. In two isolates a novel Tn was present with the *cfxA* gene. Four isolates harbored a *nimE* gene, located on a pBFS01_2 plasmid. One isolate contained a novel plasmid carrying a *nimA* gene with IS1168. The *tetQ* gene was present on novel conjugative transposons (CTns) belonging to the CTn*DOT* family. Two isolates harbored a novel plasmid with *tetQ*. Other ARGs in these isolates, but not on an MGE, were: *cfiA*, *ermF*, *mef(EN2)*, and *sul2*. ARGs harboured differed between isolates and corresponded with the observed phenotypic resistance.
Conclusions: Novel CTns, Tns, and plasmids were encountered in the five MDR *B. fragilis* isolates, complementing our knowledge on MDR and horizontal gene transfer in anaerobic bacteria.

1. Introduction

Bacteroides fragilis is an important member of the human gut microbiome and the most frequently isolated anaerobic bacterium in human infections [1]. Antimicrobial resistance has significantly increased among anaerobic bacteria, especially in *B. fragilis* [2]. Multicentre.

European studies showed an increase in antimicrobial resistance among *B. fragilis* isolates for amoxicillin from 12% in 1988 to 97.4% in 2008, for amoxicillin-clavulanic acid: from 0.4% to 8.7%, and clindamycin: from 7% to 28.5%. Increases in resistance between 1988 and 2008 were also observed for imipenem from 0.5% to 1.2%, and for metronidazole from 0% to 0.5% [3,4]. A study from the UK showed an increase in resistance to meropenem and metronidazole from 0% and 0.7% in 2000 to 1.5% and 2.3% in 2016, respectively [2]. Worldwide, resistance for metronidazole is also increasing, from 0.5% to 6.5% [4,5]. Besides these worrisome observations, recent reports describe multidrug-resistant (MDR) *B. fragilis* isolates, defined as acquired resistance to at least three different categories of antibiotics, with some of these isolates being resistant to metronidazole and carbapenem antibiotics [6–9]. Based on the presence or absence of the *cfiA* gene, encoding a metallo-β-lactamase, *B. fragilis* is divided in two divisions. Isolates not harboring

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the *cfiA* gene belong to division I and isolates with the *cfiA* gene belong to division II [10]. The increase in resistance is primarily due to horizontal gene transfer (HGT) of antibiotic resistance genes (ARGs). Especially when ARGs are biologically linked through the same transfer mechanism, e.g., within species belonging to the same genus, MDR strains can easily originate [11]. Coyne et al. [12] showed that HGT occurs within the human gut between the Bacteroidetes members. Tetracycline resistance in *Bacteroides* species increased from 20 to 30% in the 1970s to 80% in the 1990s, probably facilitated by the *tetQ* gene being located on a conjugative transposon (CTn) [13]. CTns are mobile genetic elements (MGEs), integrated in the chromosome, on which all genes needed for self-transmission are encoded. In addition, a cascade of events occurs, including the transfer of other MGEs, which can ultimately result in a MDR strain. The best characterized CTn in *Bacteroides* is CTn*DOT*, which harbors both the tetQ gene (encoding tetracycline resistance), and the *ermF* gene (encoding resistance to clindamycin). Transfer of this CTn is triggered by low concentrations of tetracycline [14]. Metronidazole is an important antimicrobial agent for treatment of infections in which anaerobic bacteria play a role. Resistance to metronidazole can be caused by several mechanisms, among them production of nitro reductases encoded by *nim* genes [15]. *nim* genes occur both chromosomally as well as on plasmids and can easily be mobilized and transferred [16].

In this study, we performed whole genome sequencing (WGS) on five different MDR division II *B. fragilis* clinical isolates, resistant to both metronidazole and meropenem, from various hospitals in the Netherlands. The presence of CTns, ARGs, and other MGEs harboring ARGs was assessed using bioinformatics tools.

2. Material and methods

2.1. Clinical isolates

In 2018, a metronidazole and carbapenem MDR *B. fragilis* isolate (MDR_L04) was recovered from a post-operative wound culture from an adult patient that received a liver transplant due to acute liver failure. The patient stayed in an African country for 3 months during the year prior to the infection. Next, a search in the national antimicrobial resistance surveillance database (ISIS-AR) at the Dutch Centre for Infectious Diseases, yielded a second MDR isolate [17]. Isolate MDR_E02 was cultured from a blood culture from an adult patient treated for a urogenital tumour with metastases. These two isolates were sent to the central reference laboratory for anaerobic bacteria, where a third isolate was already present. This isolate (MDR_U01) was recovered from an intra-abdominal abscess from a child with a perforated appendicitis complicated by subcutaneous and intra-abdominal abscesses. Following a national signal message on MDR *B. fragilis*, two more isolates were reported and send to the central laboratory. The fourth isolate (MDR_R03) was cultured from a blood culture of an elderly patient with metastatic colon carcinoma who underwent a HIPEC surgery and subsequently developed several complications resulting in persistent sepsis. In the previous year, the patient underwent an appendectomy in a hospital in South-East Asia. The fifth isolate, MDR_H05, was isolated from multiple ascites and intra-abdominal fluid samples from an elderly patient with recurrent diverticulitis. Patients E02 and H05 did not travel abroad in the year prior to the positive cultures with MDR *B. fragilis*. Of patient U01, it is not known if the patient had travelled abroad. All patients were treated in five different hospitals in the Netherlands. No contact between patients or a common source was established. Three patients died due to their underlying condition (E02, L04, H05); one patient died due to persistent sepsis with *B. fragilis* (R03); one patient recovered (U01). Information on antimicrobial treatment received prior to the positive cultures was not available for all patients. An overview is given in Table 1.

2.2. Identification and antimicrobial resistance testing

Identification was performed using Bruker MALDI-TOF MS version 11 database. Production of β-lactamase was determined using the BD BBL cefinase disk (Becton Dickinson, Franklin Lakes, NJ, USA). Antimicrobial susceptibility was determined for amoxicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, meropenem, clindamycin, metronidazole, and tetracycline using the Etest® (bioMérieux, Marcy-l'Étoile, France). All tests were performed according to the manufacturer's recommendations. Resistance was assessed using EUCAST breakpoints (v 11.0; 2021). CLSI breakpoints were used for tetracycline (M100-ED31; 2021), as EUCAST has not defined breakpoints.

2.3. Whole genome sequencing, hybrid assembly and analysis

DNA extraction, followed by WGS, was performed as previously described by Lisotto et al. [18]. Short read quality control and trimming were performed using AfterQC (v0.9.7; https://github.com/OpenGene/AfterQC) [19]. Long read fastQ files were demultiplexed and trimmed from barcode adapters using Porechop (v0.2.3, https://github.com/rrwick/Porechop). Hybrid assembly was performed using Unicycler (v0.4.8 Beta; https://github.com/rrwick/Unicycler) [20]. Genome similarity was determined based on the ANI-value calculated by comparing the obtained genomes with two reference genomes present in NCBI (division I: NC_003228; division II: NZ_CP080295) using an online ANI-calculator (https://www.ezbiocloud.net/tools/ani) [21]. *B. fragilis* multi locus sequence typing (MLST) was performed using pubMLST.org (https://pubMLST.org/organisms/bacteroides-fragilis; accessed 08/02/2023) [22].

2.4. Antimicrobial resistance genes and mobile genetic elements

Annotation of genes was performed using RAST (https://rast.nmpdr.org/). In Seed viewer, a manual search for ARGs was performed (including "beta-lactamase", *cepA*, *cfxA*, *cfiA*, *erm*, *nim*, *tet*, *bexA*, *sul2*, *mef(EN2))* [23]. Genomes were uploaded to ResFinder (V4.1; https://cge.cbs.dtu.dk/services/ResFinder/) and CARD (V3.1.0; https://card.mcmaster.ca/analyze/rgi) for detection of ARGs (accessed May 2022) [24,25]. All detected ARGs were confirmed using blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [26]. More than 30% identity over at least 70% of the protein is considered to be a reliable identification.

CTns, related to the CTn*DOT* family, were located in the genome by performing a manual BLAST against the sequence of CTn*341* (AY512263); the only CTn of which a complete sequence is available in NCBI. Putative CTns present were manually examined for ARGs and compared with each other using the blastn tool, and visualized using SnapGene software (Dotmatics, Windhill, UK; available at https://www.snapgene.com) and Easyfig [27].

3. Results

3.1. Primary identification and phenotypical resistance

All isolates were identified as *B. fragilis* using MALDI-TOF MS. All isolates produced β-lactamase and were resistant to all tested antibiotics, except for isolate MDR_U01, which was susceptible to clindamycin and tetracycline, but susceptible, increased exposure, to amoxicillin-clavulanic acid and meropenem (Table 1). Early 2022, EUCAST changed the breakpoint of meropenem such that all

Table 1
Overview of MDR *B. fragilis* clinical isolates, including clinical background, MIC values, and encountered ARGs and MGEs. Resistant phenotypes are indicated with a bold MIC value as for MGEs harboring ARGs.

	Clinical background		Antimicrobial susceptibility		ARGs and MGEs		
Strain			Antimicrobial agent (breakpoint)	MIC (mg/l)	ARG	Upstream IS element	Origin in WGS
MDR_U01	Year	2014	AC (R > 2)	**>256**	*cfxA*		**Tn*7531*** (OP204847)
	Hospital	UMCG	XL (R > 8)	8			
	Material	Abdominal abscess	PT (R > 16)	**96**			
	Patient history	Perforated appendicitis, complicated by multiple abscesses	MP (R > 8)	3	*cfiA*	IS1186	chromosomal
			CM (R > 4)	3			
			MZ (R > 4)	**64**	*nimE*	ISBf6	**pBFS01_2**
			TC (R ≥ 16)	3	*tetQ*		**CTn*7524*** (OP227216) pBFO17_2
MDR_E02	Year	2019	AC (R > 2)	**>256**	*cfxA*		**Tn*7528*** (OP204844)
	Hospital	Erasmus MC	XL (R > 8)	**32**			
	Material	Blood culture	PT (R > 16)	**>256**			
	Patient history	Urogenital tumour, with translocated metastases	MP (R > 8)	**>32**	*cfiA*	IS613	chromosomal
			CM (R > 4)	**>256**	*ermF*		chromosomal
			MZ (R > 4)	**>256**	*nimE*	ISBf6	**pBFS01_2**
			TC (R ≥ 16)	**>256**	*tetQ*		**pBsp_tetQ** (OP204842)
					sul2		chromosomal pBFO17_2 pFunn3_4
MDR_R03	Year	2018	AC (R > 2)	**>256**	*cfxA*		**Tn*7529*** (OP204845)
	Hospital	Radboud UMC	XL (R > 8)	**>256**			
	Material	Blood culture	PT (R > 16)	**>256**			
	Patient history	Persistent sepsis, after HIPEC surgery for metastatic colon carcinoma	MP (R > 8)	**>32**	*cfiA*	IS613	chromosomal
			CM (R > 4)	**>256**	*ermF*		chromosomal
			MZ (R > 4)	**>256**	*nimE*	ISBf6	**pBFS01_2**
			TC (R ≥ 16)	**>256**	*tetQ*		**pBsp_tetQ** (OP204841)
					sul2		chromosomal pBFO17_2
MDR_L04	Year	2019	AC (R > 2)	**>256**			
	Hospital	LUMC	XL (R > 8)	**>256**			
	Material	Wound	PT (R > 16)	**>256**			
	Patient history	Liver transplant due to acute liver failure	MP (R > 8)	**>32**	*cfiA*	IS613	chromosomal
			CM (R > 4)	**12**	*mef(EN2)*		chromosomal
			MZ (R > 4)	**>256**	*nimA*	IS1168	**pBspL04_2** (OP227220)
			TC (R ≥ 16)	**>256**	*tetQ*		**CTn*752*5** (OP227218)
					tetQ		**CTn*7526*** (OP227219) pBspL04_3 (OP204843) pFunn3_3
MDR_H05	Year	2020	AC (R > 2)	**>256**	*cfxA*		**Tn*7530*** (OP204846)
	Hospital	Haga hospital	XL (R > 8)	**64**			
	Material	Abdominal abscess	PT (R > 16)	**>256**			
	Patient history	Recurrent diverticulitis	MP (R > 8)	**>32**	*cfiA*	IS613	chromosomal
			CM (R > 4)	**>256**	*ermF*	IS1188	chromosomal
			MZ (R > 4)	**>256**	*nimE*	ISBf6	**pBFS01_2**
			TC (R ≥ 16)	**24**	*tetQ*		**CTn*7527*** (OP227217) pBFO17_2 pBcacCL03T12-2

Abbreviations: AC: amoxicillin, XL: amoxicillin-clavulanic acid, PT: piperacillin-tazobactam, MP: meropenem, CM: clindamycin, MZ: metronidazole, TC: tetracycline.

isolates harboring a *cfiA* gene are resistant. Therefore, the MDR_U01 isolate is considered resistant to meropenem.

3.2. Hybrid assembly and analysis

The genomes of MDR_U01 and MDR_L04 were assembled into a complete chromosome of identical size (5.3 Mb). The assembly of MDR_E02 and MDR_R03 resulted in a large chromosomal contig (4.9 Mb and 5.2 Mb, respectively) and smaller chromosomal contigs that formed a chromosome when bridged with Unicycler. The assembled sequence data from MDR_H05 yielded two large chromosomal contigs of 2.8 Mb and 2.0 Mb, which could be bridged in one chromosome using Unicycler. The isolates harbored 2 to 4 plasmids each, of which an overview is presented in Table 1. The genomes varied in size between 5.3 Mb and 5.7 Mb and had a GC-content between 43.3% and 43.6% (Table S1, supplemental data). The ANI-value of the genomes showed a similarity of <88% with division I *B. fragilis* (NC_003228) but >97% with division II *B. fragilis* (NZ_CP080295). Isolates MDR_E02 and MDR_R03 belonged to the same sequence type (ST159). MDR_H05 and MDR_L04 both belonged to a different sequence type, ST35 and ST124, respectively. Isolate MDR_U01 did not have a known sequence type.

3.3. Non-mobile ARGs and MGEs not harboring ARGs

All isolates harbored several MGEs, some containing ARGs. An overview, including Genbank accession numbers, is presented in Table 1, and Table S2 (supplemental data). All isolates harbored a plasmid identical to pBFO17_2, on which no ARG is located, except for isolate MDR_L04. The latter harbored a non-ARG bearing plasmid mostly similar to pBFO17_1 instead of pBFO17_2 (novel plasmid pBspL04_2; Genbank accession number OP227220). Furthermore, we observed other non-ARG bearing plasmids pFunn3_4, pFunn3_3, and pBcacCL03T12_2 in some isolates. Furthermore, ARGs were present which were not located on an MGE. All isolates harbored a *cfiA* gene in their chromosome, with an insertion sequence (IS)-element upstream. This IS-element was either IS1186 or IS613 in two and three isolates, respectively. The *ermF* gene was present in three of the four clindamycin resistant isolates. The latter isolate harbored a *mef(EN2)* gene (Table 1). Isolates MDR_E02 and MDR_R03 both harbored a *sul2* ARG in the genome, which is responsible for sulphonamide resistance.

3.4. The tetQ gene

The *tetQ* gene was present in all isolates. In three isolates (MDR_U01, MDR_L04, and MDR_H05), *tetQ* was located on a CTn belonging to the CTn*DOT* family (Fig. 1). Isolate MDR_L04 harbored two *tetQ* genes located on two different CTns. The CTns varied in size from 52,167 bp to 60,419 bp, with some similar parts. A graphic presentation of the CTns is shown in Fig. 1, in which they are compared with CTn314. CTn7524 (strain MDR_U01; Genbank accession number: OP227216) and CTn7525 (strain MDR_L04; Genbank accession number: OP227218) have an identical insertion of a series of genes between the tetQ and rteC gene, encoding membrane proteins and efflux pumps. BLAST results showed that this series of genes is most similar (98%) to a series of genes present on CTn*GERM1* (Genbank accession number: AJ557257). These two CTns did not harbor the typical *rteA* and *rteB* genes. The presence of an IS-element was observed downstream of the *tetQ* gene in CTn*7525*. The second CTn in MDR_L04, CTn*7526* (Genbank accession number: OP227219), differed from CTn*7525*, genes encoding membrane proteins and efflux pumps were not present. An insertion of an abi-protein involved in bacteriophage resistance was observed downstream of the *rteC* gene. Furthermore, CTn*7526* lacked the genes encoding NTPase and ATPase present in all the other CTns. In both CTns from MDR_L04, there was an insertion of several genes encoding tyrosine type integrases, and an additional gene in the lysozyme gene. The insertion of a transcriptase was observed in the *mobC* gene in the CTn in MDR_H05, CTn*7527* (Genbank accession number: OP227217).

In isolates MDR_E02 and MDR_R03, the *tetQ* gene was not localized on a CTn but on a novel plasmid of 9,932 bp (Fig. 1), assigned pBsp_tetQ (Genbank accession numbers: OP204842; OP204841). Besides the *tetQ* gene, the *rteA* gene and *rteB* gene are also present, but not the *rteC* gene. Furthermore, the plasmid harbors a toxin-antitoxin system. The GC content was 41%, and the read depth was 16.7% and 17.0%, respectively.

3.5. The cfxA gene

The *cfxA* gene was present on the chromosome in all isolates except MDR_L04. The *cfxA* gene was located on a transposon (Tn) of 10,069 bp similar to Tn*4555* in isolates MDR_E02 (Tn*7528;* Genbank accession number: OP204844) and MDR_R03 (Tn7529; Genbank accession number: OP204845), except for an insertion of an IS4-like element family transposase which was present in Tn*4555* but not in our isolates (Fig. 2). The remaining two isolates, MDR_U01 and MDR_H05, harbored a novel Tn on which the *cfxA* gene was located (Fig. 2). Besides hypothetical proteins, a site-specific integrase and a mobilization protein are located on the Tn. Both isolates harbor a nearly identical Tn of, respectively, 9,470 bp (Tn*7531*; Genbank accession number: OP204847) and 9,420 bp (Tn7530; Genbank accession number: OP204846), flanked by direct repeats. The only difference is a gap of 50 bp in Tn*7530,* prior to the direct repeat, which does not affect the translation of genes.

3.6. Nim genes

The most prevalent *nim* gene was *nimE* located on the pBFS01_2 plasmid, previously described by Sydenham et al. [7]. The plasmid was present in four isolates, MDR_U01, MDR_E02, MDR_R03, and MDR_H05. In isolate MDR_L04, a *nimA* gene was found on a novel plasmid pBspL04_3 (Genbank accession number: OP204843; Fig. 3), accompanied by a *repA*, *btgA* and *btgB* gene, and a *yoeB* - *yefM* toxin-antitoxin system. The plasmid was identical to pBACSA03 (Genbank accession number: CP002533) except for the insertion of the *nimA* gene and IS1168 between genes encoding a hypothetical and a BtgB protein. The sequence of pBspL04_3 was 7,610 bp in length with a read depth of 27.9x and a GC% content of 43%.

4. Discussion

Antimicrobial resistance is less studied in anaerobic bacteria than in aerobic bacteria, even though the commensal microbiota in healthy humans consists mainly of anaerobic bacteria and is an important reservoir of ARGs [12]. *B. fragilis* is the species most often isolated from human clinical specimens and the most virulent species [9]. Even though these isolates are often susceptible to antibiotics widely used against anaerobic bacteria, such as metronidazole and carbapenems, resistance against these antibiotics is increasing worldwide [4,5]. In five hospitals throughout the Netherlands, clinically important MDR *B. fragilis* resistant to both meropenem and metronidazole were isolated from patients with no epidemiological link. WGS was performed on these isolates to assess the possible role of MGEs in the spread of ARGs.

Recently, Wallace et al. compared the genomes of different *Bacteroides* species and showed that the isolates belonging to *B. fragilis* division II formed a distinct clade, with ANI-values of

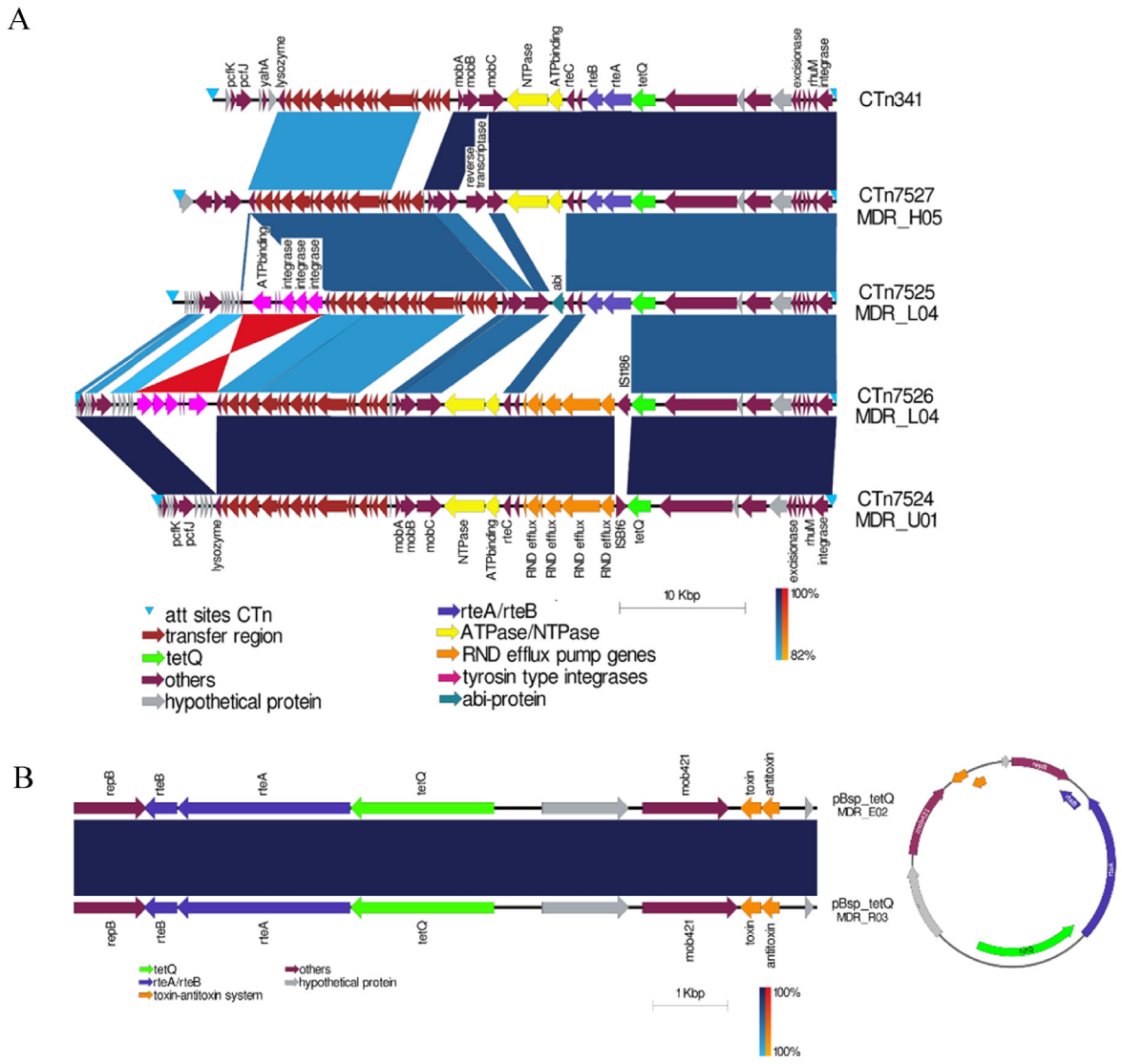

Fig. 1. Comparison of MGEs harboring a *tetQ* gene. A) Comparison of the genes in the CTns encountered in isolates MDR_H05, MDR_L04, and MDR_U01. As reference, we used the sequence of CTn*341*, shown at the top. B) Comparison of the genes on the novel *tetQ* harboring plasmid pBsp_tetQ from isolates MDR_E02 and MDR_R03 and a visualization of the circularized plasmid (https://www.snapgene.com).

85–90% compared to *B. fragilis* division I isolates [28]. Therefore, it can be concluded that they belong to a different genomospecies, as in our study.

All five isolates in this study harbored a *tetQ* gene, even though only four isolates were resistant to tetracycline. Three isolates harbored a CTn in their genome, of which one isolate notably harbored two different CTns. All CTns harbor a *tetQ* gene and are related to CTn*DOT*, as defined by its attachment sites. The excision of CTn*DOT* is regulated by a series of events, triggered by low concentrations of tetracycline and regulated by proteins encoded by the *rteA*, *rteB*, *tetQ* operon [14]. Interestingly, the tetracycline sensitive isolate, MDR_U01, harbored no *rteA* and *rteB* genes on its CTn, similar to CTn*7524*. The latter isolate was tetracycline resistant. Instead, these CTns harbored genes encoding MDR efflux proteins in this region. These are all transcribed in the same direction, indicating that this series of genes are possibly an integron, as previously suggested by Wang et al. [29].

The insertion of genes in other genes was observed in the CTns of our MDR isolates, for example: the insertion of genes encoding integrases into a lysozyme gene, hereby disenabling the production of the encoded protein. The consequences of these events remain unclear.

The two isolates that did not harbor a CTn, harbored a plasmid on which the *tetQ* gene was located. Besides the *tetQ* gene, the *rteA* and *rteB* genes were also present on the plasmid, known to regulate the expression of the *rteC* gene. However, the *rteC* gene, which in CTn*DOT* stimulates the expression of the excision genes, is absent on the plasmid [14]. That *tetQ* can be harbored by a plasmid was also reported by Cao et al. [30]. Since it is unknown what the ratio CTn and plasmid located *tetQ* gene is, it remains unclear what the impact is on the frequency of HGT.

nim genes play a role in metronidazole resistance and can be located on the chromosome or on a plasmid [16]. In all our isolates, the *nim* gene was located on a plasmid. Plasmids harboring *nim* genes are not uncommon in *Bacteroides* isolates. Four isolates harbored a *nimE* gene, with an IS-element upstream, on a plasmid identical to pBFS01_2, previously described by Sydenham et al. [7]. Isolate MDR_L04 harbored a novel plasmid on which *nimA* was located, with an IS1168 upstream of the gene, a combination regularly found in other *Bacteroides* isolates [16]. This plasmid

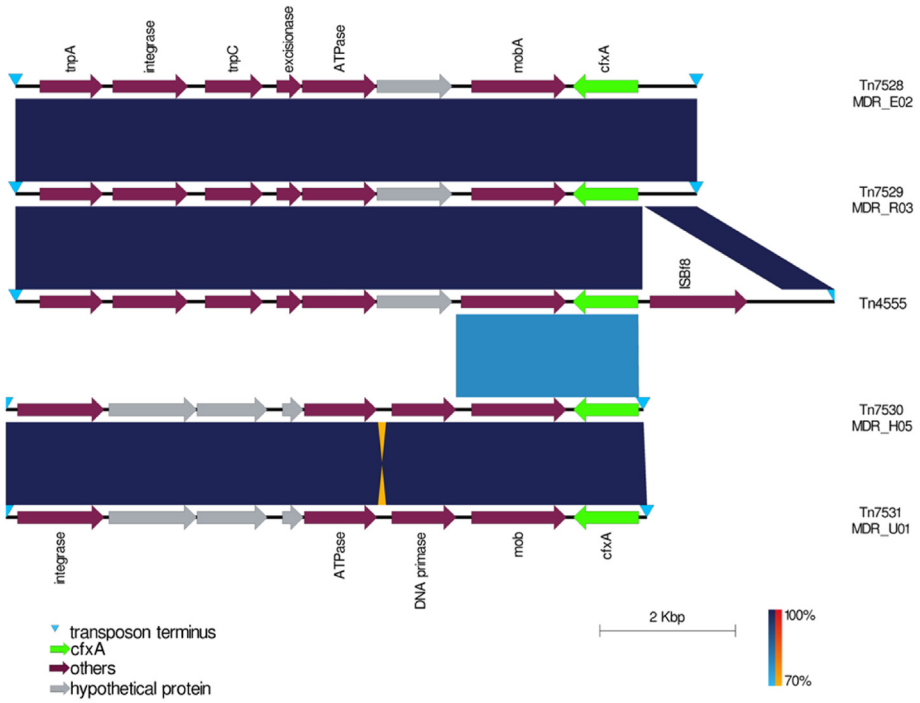

Fig. 2. Comparison of the genes on the Tns encountered in isolates MDR_E02, MDR_R03, MDR_H05, and MDR_U01. As reference, we used the sequence of Tn*4555*, shown in the middle.

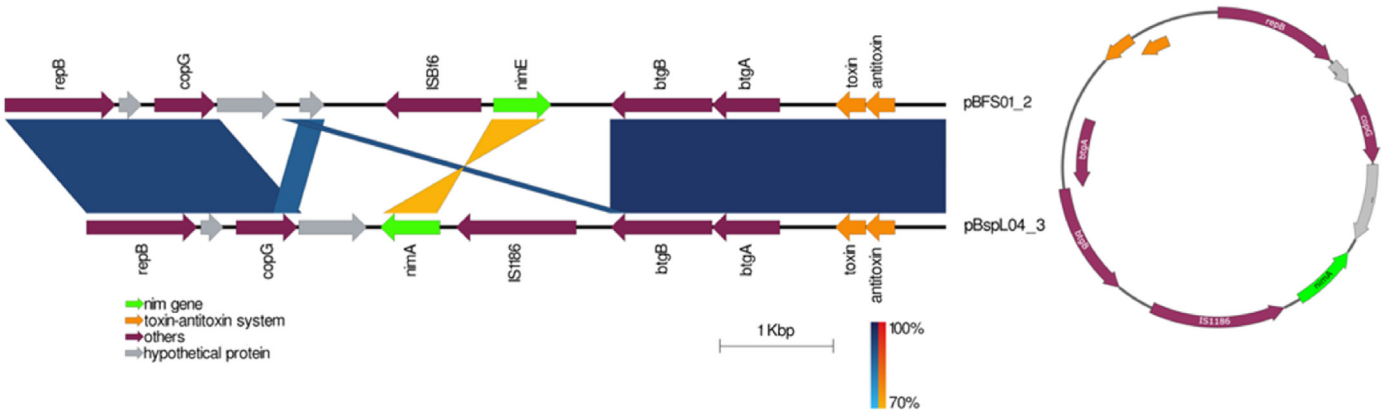

Fig. 3. Comparison of the genes on the novel plasmid pBspL04_3 with the pBFS01_2 plasmid and a visualization of the circularized pBspL04_3 plasmid (https://www.snapgene.com).

resembled plasmids pBACSA03 and pBFS01_2. Besides plasmids harboring ARGs, other plasmids were present in our isolates. The possibility exists that ARGs may integrate in their DNA, as seen in the pBspL04_3 plasmid where *nimA* and IS1168 were inserted into the pBACSA03 plasmid. This enhances the transfer of *nimA* between different bacterial strains.

As described previously, the *cfxA* gene is located in the chromosome on a mobilizable transposon, Tn*4555* [31]. Two isolates harbored a Tn similar to Tn*4555*, while in two other isolates, the *cfxA* gene was located on a Tn not previously described, Tn*7530* and Tn*7531*. The *cfxA* gene present on this novel Tn was associated with a MobA protein. Handal et al. hypothesized that the presence of *mobA* in association with *cfxA* is the minimal necessity for mobility within Bacteroidaceae [32].

The first MDR *B. fragilis* isolate resistant to metronidazole and imipenem was described in 1995 by Turner et al. [8]. In this study, a *B. fragilis* isolate resistant to metronidazole, and reduced susceptible to imipenem and amoxicillin-clavulanic acid, was recovered from a blood culture. After treatment with imipenem, a second isolate was cultured from pleural pus, that had acquired resistance to imipenem and amoxicillin-clavulanic acid in addition to the metronidazole resistance. Urban et al. described the first Hungarian MDR *B. fragilis* isolate in 2015, resistant to several antibiotics [9]. It harbored a range of ARGs, including a plasmid on which a *nimA* gene and IS1168 were located, as in one of our isolates. Hartmeyer et al. reported on the first Scandinavian MDR *B. fragilis* isolate, from a blood culture and abdominal fluid of a patient with colon cancer, resistant to meropenem and metronidazole, harboring both *cfiA* and a *nimD* [33]. Baaity et al. described 11 metronidazole resistant *Bacteroides* isolates from Kuwait, of which one MDR *B. fragilis* isolate was resistant to both metronidazole and imipenem, harboring both a *nimE* and a *cfiA* gene, with ISBf6 and IS613 [34].

Cao et al. described the resistome of *cfiA* harboring *B. fragilis* isolates from both human and domestic animal sources in Hong Kong. They found one human isolate resistant to both metronidazole and imipenem that harbored the pBFS01_2 plasmid carrying *nimE* that was also detected in four of our isolates [30].

The introduction of WGS enabled scientists to extract the genetic composition of CTns and Tns from a bacterial genome. Sydenham et al. assembled short and long reads of six MDR *B. fragilis* isolates, which resulted in their complete genome and eleven putative plasmids [7]. At that moment, only three of these plasmids were previously described. Addition of these WGS and plasmids to commonly used databases aid the reconstruction of possible HGT events.

In this study, we report on five unrelated MDR *B. fragilis* isolates, with mostly different ST's, cultured from clinical specimens from patients with unrelated backgrounds and foreign connection. Multiple novel MGEs were detected, including CTns carrying *tetQ* not previously described, a new Tn harboring the *cfxA* gene, and a new plasmid harboring the *nimA* gene. Furthermore, we found that the *tetQ* gene can also be located on a plasmid. Although the impact of these novel MGEs on the frequency of HGT remains unclear, these findings add to our knowledge about MDR in anaerobic bacteria and show that microbiologists must be alert on antimicrobial resistance in anaerobic bacteria and perform antimicrobial susceptibility testing routinely, especially when a *Bacteroides* spp. is isolated.

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CRediT authorship contribution statement

K.E. Boiten: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualizsation. **E.J. Kuijper:** Conceptualization, Resources, Writing – review & editing. **L. Schuele:** Investigation, Writing – review & editing. **J. van Prehn:** Resources, Writing – review & editing. **L.G.M. Bode:** Resources, Writing – review & editing. **I. Maat:** Resources, Writing – review & editing. **S.A.V. van Asten:** Resources, Writing – review & editing. **D.W. Notermans:** Writing – review & editing. **J.W.A. Rossen:** Writing – review & editing, Supervision. **A.C.M. Veloo:** Conceptualization, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: JWAR has received consulting fees from IDbyDNA, ARES genetics, and Illumina. ACMV has received a grant from the National Institute for Public Health and the Environment. All other authors have none to declare.

Data availability

Sequences of novel mobile genetic elements have been submitted to NCBI and will be available after publication.

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Appendix A. Supplementary data

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