Inter- and intraspecies transfer of a Clostridium difficile conjugative transposon conferring resistance to MLSB.

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Inter and intra-species transfer of a *Clostridium difficile* conjugative transposon conferring resistance to MLS_B

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ABSTRACT

Resistance to MLS_B in *Clostridium difficile* is generally due to *erm*(B) genes. Tn*6194*, a conjugative transposon initially detected in PCR-ribotype 027 isolates is an *erm*(B)-containing element also detected in other relevant *Clostridium difficile* PCR-ribotypes. In this study, the genome of a *C. difficile* PCR-ribotype 001 strain was sequenced and an element with two nucleotidic changes compared to Tn*6194* was detected. This element was transferred by filter-mating assays to recipient strains of *C. difficile* belonging to PCR-ribotype 009 and 027, and to a recipient strain of *Enterococcus faecalis*. Transconjugants were characterised by Southern blotting and genome sequencing, and integration sites in all transconjugants were identified by sspPCR. The element integrated the genome of *C. difficile* at different sites and the genome of *E. faecalis* at a unique site. This study is the first molecular characterisation of a *erm*(B)-containing conjugative transposon in *C. difficile*, and provide an additional evidence of the antibiotic resistance's transmission risk between pathogenic bacteria occupying the same human intestinal niche.

INTRODUCTION

Resistance to the macrolide-lincosamide-streptogramin B (MLS_B) group of antibiotics is the most common phenotype in clinical isolates of *C. difficile* worldwide, and is generally conferred by *erm*(B) genes encoding 23S rRNA methylases (Huang *et al.,* 2009). These genes are located on mobile elements showing an important heterogeneity in their genetic organisation (Farrow *et al.,* 2001; Spigaglia *et al.,* 2005; Spigaglia *et al.,* 2011). The best known of them is Tn*5398* from *C. difficile* 630, a mobilizable non-conjugative element containing two *erm*(B) copies (Farrow *et al.,* 2001).

A putative conjugative transposon belonging to the Tn*916*-like family of transposons and carrying an *erm*(B) gene has been recently detected in *C. difficile* 2007855, a strain belonging to the hyper-virulent PCR-ribotype 027, and designated Tn*6194* (He *et al.,* 2010; He *et al.,* 2012). Conjugative transposons related to Tn*916* were originally detected in *Enterococcus faecalis*, which inhabits the same niche of *C. difficile*. Both species are responsible for nosocomial infections, and horizontal gene transfer between those two species has been previously proven (Jasni *et al.,* 2010).

A recent whole-genome sequencing analysis of *C. difficile* 027/BI/NAP1 isolates demonstrated that Tn*6194* was present in most of the strains belonging to the FQR1 lineage, isolated in the United States and in Asia (He *et al.,* 2012). Moreover, recent studies showed that this transposon is the most frequently found *erm*(B)-containing element in different epidemic PCR-ribotypes circulating in European hospitals (Spigaglia *et al.,* 2011; Wasels *et al.,* 2013), and a transposon almost identical has been detected in the PCR-ribotype 017 strain M68 genome (Brouwer *et al*., 2012)..

In this study, we characterized the element of a *C. difficile* PCR-ribotype 001 clinical isolate and we investigated its intra and inter-species transfer capacities by transferring it to recipient strains of *C. difficile* and *E. faecalis*.

MATERIALS AND METHODS

Bacterial strains, culture conditions and antibiotic susceptibility

All strains used in this study, listed in Table 1, were grown on Brain Heart Infusion (BHI) agar plates or in BHI broth (Oxoid Ltd, Basingstoke, UK) supplemented with 0.5% yeast extract and 0.1% L-cysteine (BHIS). *C. difficile* was grown at 35°C in anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) and *E. faecalis* at 37°C in aerobic conditions. Erythromycin and rifampicin were used at a final concentration of 20 mg/L.

Minimal Inhibitory Concentrations (MICs) were determined using the Etest (AB Biodisk, Solna, Sweden) as already described. The breakpoint used for both erythromycin and clindamycin was 8 mg/L, in accordance with the guidelines established by the Clinical and Laboratory Standards Institute (CLSI, 2007). The breakpoint for rifampicin was 4 mg/L, in accordance with the CLSI interpretive categories approved for *Staphylococcus aureus*, since no values are provided for anaerobes (CLSI, 2008).

Filter-mating assays

Filter-mating experiments were performed as already described (Farrow *et al.,* 2001). *C. difficile* transconjugants were selected anaerobically on plates supplemented with erythromycin and rifampicin, and were confirmed by PCR-ribotyping as already described (Bidet *et al.,* 2009) and PCR detection of *erm*(B) using primers E5 and E6 (Spigaglia & Mastrantonio, 2004). *E. faecalis* transconjugants were selected aerobically on plates supplemented with erythromycin, and confirmed by PCR detection of *erm*(B).

Genomic DNA extraction

Genomic DNA extractions were performed using the NucleoBond® AXG columns and NucleoBond® Buffer Set III (Macherey-Nagel) according to the manufacturer's

instructions, with the following modifications: double volume of lysozyme and proteinase K were used during the cell disruption step and incubation time at 37°C was tripled.

Analysis of *C. difficile* CII7 transposon insertion sites

Southern blotting was carried out by transferring 3 µg of *Hin*dIII-digested genomic DNA from *C. difficile* strains to a Hybond™ N+ membrane (Amersham Biosciences, Buckinghamshire, UK). The *erm*(B)-specific probe was obtained by PCR using primers E5 and E6. Hybridisation and signal detection were carried out as described in the ECL™ Direct Nucleic Acid Labelling and Detection System (Amersham Biosciences).

SspPCR were performed using primers listed in the Supplementary Table 1 on fragments of genomic DNA digested by *Hin*dIII, *Sau*3AI or *Nde*I genomic fragments ligated within the dephosphorylated pUC19 digested with *Hin*dIII, *Bam*HI or *Nde*I. Primers used to confirm insertion sites by PCR are listed in the Supplementary Table 2.

Genome sequencing

Single-end multiplex libraries were created with multiplexed protocol according to the manufacturer's specification; the sequencing was performed on the Illumina HiSeq 2000 platform. The read length was 110-bp for both *C. difficile* CII7 and *C. difficile* CII7xCD37(A). Then reads were assembled de novo into contigs using Velvet (Zerbino & Birney, 2008) (Options: Kmer : 71 and 75 respectively for CII7 and CII7xCD37(A) ; –short ; –fastq ; –unused_reads ; -read_trkg). Finally contigs (minimum size, 500 bases) were reorganized and oriented based on alignment to the finished genome of *C. difficile* strain 630 (CD630) (Sebaihia *et al.,* 2006). Contigs that did not match CD630 were localized at the end to obtain each genome scaffold. The short reads genomic data have been deposited in the European Nucleotide Archive (ENA) under accession number

115 ‘PRJEB1488’ and can be accessed online (http://www.ebi.ac.uk/ena/data/view/
116 PRJEB1488).
117 The nucleotide sequence of the Tn*6194*-like element from *C. difficile* CII7 has been
118 deposited in the ENA database (http://www.ebi.ac.uk/ena/) under accession no.
119 HG475346.

RESULTS

The entire genome of the PCR-ribotype 001 *C. difficile* strain CII7 that has been previously shown to contain an element highly similar to Tn*6194* was sequenced, and a putative conjugative transposon of 28014 bp in length with two nucleotide changes compared to Tn*6194* was detected (Fig. 1). Insertion of a guanine at position 7076 (between positions 3152104 and 3152105 in *C. difficile* 2007855; GenBank accession number FN665654) determines a 207nt-elongation of the CDS coding for a replication initiation factor homologous to *orf20* of Tn*916*. The same insertion is also present in the transposon from the *C. difficile* M68 strain (GenBank accession number FN668375). The replacement of a guanine by a thymine at position 9691 (positions 3149490 in *C. difficile* 2007855) causes the substitution of a methionine by an isoleucine within the sequence of a protein with unknown function. The transposon is located between orthologs of *C. difficile* 630 *CD630_31060* and *CD630_31070* in *C. difficile* CII7, while it is integrated within the coding sequences of the ortholog of *CD630_28310* and *CD630_33170* in strains 2007855 and M68, respectively.

Filter-mating assays were carried out to transfer the transposon from CII7 to *C. difficile* CD37 (MICs = 0.75 mg/L for erythromycin and 3.0 mg/L for clindamycin) and to CD196R, a rifampicin-resistant derivative of *C. difficile* CD196 (MICs = 1.0 mg/L for erythromycin and 2.0 mg/L for clindamycin). In this study, transfer was achieved at a frequency of 1.01 ± 0.36 x 10^{-8} and 1.43 ± 0.87 x 10^{-8} per donor for CD37 and CD196R, respectively. Transconjugants were selected from independent experiments to exclude the possibility of analysing siblings. Filter-mating assays were also performed using *C. difficile* CII7 or each one of the four transconjugants CII7xCD37 as donor, and the MLS_B-susceptible *E. faecalis* JH2-2 strain as recipient (MICs = 0.38 mg/L for erythromycin and 8.0 mg/L for clindamycin). Transfer was achieved at a frequency of 1.74 ± 1.91 10^{-6} per donor. Two

146 independent transconjugants were obtained from each transfer, for a total of 10 *E. faecalis*
147 transconjugants. All transconjugants obtained were highly resistant to both erythromycin
148 and clindamycin (MIC ≥ 256 mg/L), as the donor strain *C. difficile* CII7 was.
149
150 Southern blot analysis showed that the conjugative transposon from CII7 integrated the
151 genome of *C. difficile* CD37 and CD196 at different sites, whereas a unique insertion site
152 in the genome of *E. faecalis* JH2-2 was observed (Fig. 2).
153 The genome of the transconjugant CII7xCD37a was completely sequenced and compared
154 to that of CD37 (GenBank accession number AHJJ01). The transposon was detected
155 within a copy of the 23S rRNA gene, and no other genetic material from strain CII7 was
156 detected in CII7xCD37a. The integration site of the element was also determined in all
157 transconjugants by sspPCR and confirmed by subsequent PCR and Sanger sequencing.
158 In total, seven different insertion sites were detected in the eight *C. difficile*
159 transconjugants examined, whereas a unique insertion site was identified in the ten *E.*
160 *faecalis* transconjugants analysed. All target sites of the element contained a central AG
161 dinucleotide (Table 2).
162 Integration within a copy of the 23S rRNA gene was confirmed in CII7xCD37a and the
163 transposon was also found in a copy of this gene in CII7xCD196Rd, although at a different
164 site. In CII7xCD37d the transposon was located between orthologs of *C. difficile* 630
165 *CD630_31060* and *CD630_31070*, like in the donor *C. difficile* CII7, whereas in
166 CII7xCD196Rc it was detected within the coding sequences of the ortholog of
167 *CD630_28310*, like in *C. difficile* 2007855. Finally, the transposon was integrated at the
168 same position within a copy of the $tRNA^{Arg}$ gene in transconjugants CII7xCD37c and
169 CII7xCD196Rb. In all *E. faecalis* transconjugants, the transposon was always located
170 within a copy of the $tRNA^{Arg}$ gene. Although no genomic data were available about strain
171 JH2-2, it can be hypothesised that, like *E. faecalis* V583 and OG1RF (Genbank accession

number NC_004668 and CP002621, respectively), its genome contains several copies of the $tRNA^{Arg}$ gene. Interestingly, the element always integrated the same locus (Fig. 2). In CII7 and in all transconjugants obtained in this study, the transposon was able to excise from the genome and form a circular intermediate, restoring the original target site (data not shown).

DISCUSSION

Tn*6194* is an *erm*(B)-containing conjugative transposon recently detected in strains belonging to different PCR-ribotypes, worldwide (He *et al.,* 2010; Spigaglia *et al.,* 2011; He *et al.,* 2012; Wasels *et al.,* 2013). In this study, the capacity of this element to be transferred to other strains of *C. difficile* as well as to *E. faecalis* was investigated. While the conjugative transposon Tn*5397* from *C. difficile* 630, which confer resistance to tetracycline, has been successfully transferred to *E. faecalis* JH2-2 (Jasni *et al.,* 2010), this is the first evidence of transfer of MLS_B resistance between these two species, at our knowledge.

There are clear differences of behaviour between conjugative transposons, most likely due to their respective recombination modules and to the recipient strain used. While Tn*916* has a highly preferred insertion site in *C. difficile* CD37, it can integrate the genome of *C. difficile* 630Δ*erm* and R20291 or *E. faecalis* JH2-2 at different sites (Mullany *et al.,* 2012; Wang *et al.,* 2000). Differently, Tn*5397*, which contains the *tndX* gene encoding a serine recombinase, has a preferred integration site in *C. difficile* CD37, two in *C. difficile* R20291, one in *E. faecalis* JH2-2 and enters the genome of *Bacillus subtilis* CU2189 at different sites (Jasni *et al.,* 2010; Mullany *et al.,* 1990). Like Tn*916*, Tn*6194* contains genes encoding for putative tyrosine integrase and excisionase (Brouwer *et al.,* 2012). Our results suggest that there are a limited number of integration sites of this transposon within the genome of *C. difficile* CD37 and CD196, and a highly preferential integration site in *E. faecalis* JH2-2.

Elements containing *erm*(B) genes show an important heterogeneity in their genetic structure. Although it has been extensively studied, Tn*5398* from *C. difficile* 630 has been mainly detected in PCR-ribotype 012 isolates. Unlike this mobilizable non-conjugative element, Tn*6194* contains all modules required for its transfer between cells. These characteristics may explain its diffusion among most of the clinically relevant PCR-

204 ribotypes, underlining the importance of mobile genetic elements in the spread of
205 resistance in the *C. difficile* population.

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319 **Table 1.** Bacterial strains used in this study.

Strain [a]	Characteristics [b]	PCR-ribotype	Source (Reference)
C. difficile			
CII7	Ery^R Cli^R Rif^S	001	(Barbut 2007)
CD37	Ery^S Cli^S Rif^R	009	(Smith 1981)
CD196	Ery^S Cli^S Rif^S	027	(Popoff 1988)
CD196R	Ery^S Cli^S Rif^R	027	this study
CII7xCD37 a, b, c, d (4)	Ery^R Cli^R Rif^R	009	this study
CII7xCD196R a, b, c, d (4)	Ery^R Cli^R Rif^R	027	this study
E. faecalis			
JH2-2	Ery^S Cli^S Rif^R		(Jacob 1974)
CII7xJH2-2 a, b (2)	Ery^R Cli^R Rif^R		this study
[CII7xCD37 A/B/C/D]xJH2-2 a, b (8)	Ery^R Cli^R Rif^R		this study

[a] in brackets, number of replicates
[b] Ery^S: erythromycin susceptible; Ery^R: erythromycin resistant; Cli^S: clindamycin susceptible; Cli^R: clindamycin resistant Rif^S: rifampicin susceptible; Rif^R: rifampicin resistant.

320

321 **Table 2**. Insertion sites of the Tn*6194* and Tn*6194*-like elements in wild-type strains and transconjugants analysed in this study

Strains		Transposon localisation [a]	Insertion site		
C. difficile					
2007855		putative adhesin CDS (*CD630_28310*)	TGTATCTTATGCTTCAGAATTAGG	**AG**	AGAATAGTCAGATTCAAAGTGGTT
M68		*fdhF* (*CD630_33170*)	TTCATCATCTTCAACTTCCATAGG	**AG**	GTCTCCATTCAGCAGCGAACAAGT
CII7		5'UTR of a transcriptional antiterminator (*CD630_31070*)	AATAAATAGTAACTAAGATTTAGG	**AG**	GGTTTGCATTGAACAAAAGATTAT
Transconjugants					
CII7xCD37	a	23S rRNA gene	GAGGAGAGTATCCTAAGGCCAGCG	**AG**	AGAACTGTTGTTAAGGAACTCGGC
	b	5'UTR of *hfQ* (*CD630_19740*)	TTAGAAGTTCAATTTAATCTTGGG	**AG**	GGTACAAATCTAATGAAAAATACA
	c	$tRNA^{Arg}$ gene	CGAACCCCGGACACACGCCTTAGA	**AG**	GGCGTTGCTCTATCCAGCTGAGCT
	d	5'UTR of a transcriptional antiterminator (*CD630_31070*)	AATAAATAGTAACTAAGATTTAGG	**AG**	GGTTTGCATTGAACAAAAGATTAT
CII7xCD196R	a	*eutL* (*CD630_19150*)	TTGACGAAGCAACTAAAGCTTCAG	**AG**	GTTGATGTTGTATATGCAAAATCA
	b	$tRNA^{Arg}$ gene	CGAACCCCGGACACACGCCTTAGA	**AG**	GGCGTTGCTCTATCCAGCTGAGCT
	c	putative adhesin CDS (*CD630_28310*)	TGTATCTTATGCTTCAGAATTAGG	**AG**	AGAATAGTCAGATTCAAAGTGGTT
	d	23S rRNA gene	GGGAAAGGTGAAAAGAACCCCGGG	**AG**	GGGAGTGAAATAGAACCTGAAACC
E. faecalis					
All transconjugants		$tRNA^{Arg}$ gene	TGAACCAGCGACCTACCGCTTAGG	**AG**	GCGGTTGCTCTATCCTACTGAGCT

[a] orthologous CDS in *C. difficile* 630 are indicated in brackets.

Figure 1: Tn*6194*-like element from *C. difficile* CII7.

Grey boxes show homology with Tn*916* from *E. faecalis* DS16 and Tn*5398* from *C. difficile* 630. Genes involved in conjugation and in recombination are shown in dark grey and black, respectively. Genes involved in regulation, accessory or still unknown functions are in white.

Figure 2: Southern blot analysis of the transconjugants obtained in this study.

Detection of *erm*(B) on *Hind*III-digested genomic DNA of donor, recipient and transconjugants obtained after transfer of the transposon from *C. difficile* CII7 to *(A) C. difficile* CD37, *(B) C. difficile* CD196 and *(C) E. faecalis* JH2-2. M: marker (DNA Molecular Weight Marker II, Roche). *(A)* Lane 1: *C. difficile* CII7; lane 2: *C. difficile* CD37; lane 3-6: transconjugants CII7xCD37 a, b, c and d, respectively. *(B)* Lane 1: *C. difficile* CII7; lane 2: *C. difficile* CD196R; lane 3-6: transconjugants CII7xCD196R a, b, c and d, respectively. *(C)* Lane 1: *C. difficile* CII7; lane 2: *E. faecalis* JH2-2; lane 3-4, 5-6, 7-8, 9-10 and 11-12: transconjugants CII7xJH2-2, (CII7xCD37a)xJH2-2, (CII7xCD37b)xJH2-2, (CII7xCD37c)xJH2-2 and (CII7xCD37d)xJH2-2, respectively.